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ANTIOXIDANT, ANTI-INFLAMMATORY, AND BIOLOGICAL PROPERTIES OF HYDROALCOHOLIC EXTRACTS FROM *CYDONIA OBLONGA* AND MELA ROSA MARCHIGIANA PULP CALLUS

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ABBREVIATIONS

AAPH, 2,2′-azobis-(2-amidinopropane) hydrochloride

ABTS, 2,2’-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid)

ATP, Adenosine triphosphate

Co, *Cydonia oblonga*

Ct, Cycle-threshold

DCF, 2',dichlorofluorescein

DCF-DA, 2',7'-Dichlorofluorescein-diacetate

DEXA, Dexamethasone

DM, Diabetes Mellitus

DMAPP, Dimethylallyl diphosphate

DMEM, Dulbecco’s Modified Eagle’s Medium

DMSO, Dimethyl sulfoxide

DPPH, 2,2-diphenyl-1-picrylhydrazyl

DSA, DPPH radical scavenging activity

dw, dry weight

EMA, European Medicines Agency

EPA, Environmental Protection Agency

ET, Electron Transfer

FDA, Food and Drug Administration

GA, Gallic Acid

GAEq, Gallic Acid Equivalent

GC, Green Chemistry

HAT, Hydrogen Atom Transfer

HO-1, Heme Oxygenase-1

ICAM, Intercellular Adhesion Molecule

IL-1, Interleukin-1

IL-1β, Interleukin-1 beta

IL-6, Interleukin-6

IkBα, Inhibitor kappa B-alpha

iNOS, inducible Nitric Oxide Synthase

IPP, Isopentenyl diphosphate

LPS, Lipopolysaccharides

MRM, Mela Rosa Marchigiana

MS, Murashige and Skoog

MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide

NADES, Natural Deep Eutectic Solvents

ND1, NADH Dehydrogenase subunit 1

NF-κB, Nuclear Factor kappa B

NIH, National Institutes of Health

NO, Nitric Oxide

Nrf2, Nuclear factor erythroid 2-related factor 2

ORAC, Oxygen Radical Absorbance Capacity

ON, Overnight

PBS, Phosphate Buffered Saline

pNP, p-nitrophenol

pNDG, p-nitrophenyl-α-D-glucopyranoside

qRT-PCR, quantitative Real-Time Polymerase Chain Reaction

RT, Room Temperature

RNS, Reactive Nitrogen Species

ROS, Reactive Oxygen Species

SD, Standard Deviation

SEM, Standard Error of the Mean

SMs, Secondary Metabolites

T1D, Type 1 Diabetes

T2D, Type 2 Diabetes

TBE, Tris Borate EDTA

TBHP, Tert-butyl hydroperoxide

TEAC, Trolox Equivalent Antioxidant Capacity

TEq, Trolox Equivalent

TNF-α, Tumor Necrosis Factor-alpha

TROLOX, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

UVC, Ultraviolet-C

WHO, World Health Organization

WST-8, Water-Soluble Tetrazolium 8

ABSTRACT

In this doctoral thesis, we investigated some biological activities and protective potentialities of hydroalcoholic extracts derived from the fruit pulp calluses of two apple varieties, namely *Cydonia oblonga* and Mela Rosa Marchigiana. Callus culture, an innovative method for generating secondary metabolites (SMs) from plant material, served as the basis for this investigation.

Antioxidant activities were assessed using chemical methods such as DPPH, ABTS, and ORAC assays. Additionally, the evaluation involved quantifying the production of reactive oxygen species (ROS) in the hydrogen peroxide and tert-butyl hydroperoxide (TBHP)-injured human keratinocyte (HaCaT) cell line.

The functional activities of the callus extracts were explored in murine macrophage (RAW 264.7) and HaCaT cell lines. Specifically, anti-inflammatory activity was examined in lipopolysaccharides (LPS)-treated RAW 264.7 cells through the Griess test. In LPS-treated HaCaT human keratinocytes, the study focused on the expression of genes associated with the oxidative stress and the inflammatory process, including Heme Oxygenase-1 (HO-1), inducible Nitric Oxide Synthase (iNOS), Interleukin-6 (IL-6), Interleukin-1 beta (IL-1β), inhibitor kappa B-alpha (ikBα) and Intercellular Adhesion Molecule (ICAM).

With Mela Rosa Marchigiana callus extract it was applied a transition toward a system that mimics an *in vitro* gastrointestinal digestion for a more accurate and complex representation of physiological dynamics.

In addition to the aforementioned activities, extracts from *Cydonia oblonga* and Mela Rosa Marchigiana calluses exhibited inhibitory effects on the activity of α-glucosidase, α-amylase, and lipase enzymes.

The results presented in this thesis indicate that both *Cydonia oblonga* and Mela Rosa Marchigiana callus hydroalcoholic extracts possess anti-inflammatory, antioxidant activities both tested in cell-free and cell-based models and can downregulate the activity of digestive key enzymes involved in carbohydrate and lipid metabolism.

These findings suggest their potential application in delaying and preventing acute or chronic diseases associated with aging, a physiological state in which oxidation and inflammation play a primary role.

Finally, this work holds significance for the future development of a nutraceutical approach to managing hyperglycemia and dyslipidemia.

The present thesis is based on the following papers:

I. De Bellis, R., Chiarantini, L., Potenza, L., Gorassini, A., Verardo, G., De Marco, R., Benayada, L., Stocchi, V., Albertini, M. C., Fraternale, D. (2022). High production of secondary metabolites and biological activities of Cydonia oblonga Mill. Pulp fruit callus. *Journal of Functional Foods*, Volume 94.

II. Gubitosa, F., Fraternale, D., Benayada, L., De Bellis, R., Chiarantini, L., Albertini, M. C., Potenza, L. (2023). Cydonia oblonga Mill. Pulp Callus Inhibits Oxidative Stress and Inflammation in Injured Cells. *Antioxidants,* 12,1076.

III. Gubitosa, F., Benayada, L., Fraternale, D., De Bellis, R., Carloni, S., Potenza, L., Chiarantini, L., Gorassini, A., Verardo, G., Roselli, C., Valentini, L., Gobbi, P., Balduini, W., Ventura, N., Giannaccini, F., Kass, G.E.N., Colomba, M., Albertini, M.C. (2024). Long-lasting bioactive compounds production from *Malus x domestica* Borkh var. Mela Rosa Marchigiana pulp callus culture with antioxidant activity: in vitro, ex vivo and in vivo validation. *Journal of Functional Foods,* Volume 114.

IV. Gubitosa, F., Fraternale, D., Benayada, L., De Bellis, R., Gorassini, A., Saltarelli, R., Donati Zeppa, S., Potenza, L. (2024) Callus cultures from the pulp of *Malus domestica* cv Mela Annurca Campana: first chemical characterization and biological properties investigation. *Submitted.*

V. Gómez-Urios, C., Benayada, L., Esteve, M.J., Blesa, J., Frigola, A., Lopez-Malo, D. (2024). Phenolic Compound Recovery and NADES Recycling from Extracts of Orange By-Products. *Manuscript to be submitted.*

1. INTRODUCTION

1.1 Callus culture from apple pulp as a new technique for secondary metabolites production

1.1.1. Plant secondary metabolites

During the past few years, the pharmaceutical sector has become increasingly interested in studying natural substances, and their derived, with aiming to develop and discover new drugs. Among these phytochemicals, we mainly find "secondary metabolites", a term first described by the biochemist Albrecht Kossel in 1891. Since then, it has been used to define a wide variety of natural compounds that play several roles, both physiological and biochemical, in plant life. They do display a broad array of bioactivities and are continuously produced within plants. These compounds can be specific to plant taxonomy, and their concentration in plants depends precisely on their development state and the type of tissue[1–3]. Moreover, SMs aren´t involved directly in development, growth, and reproduction processes but act as signaling molecules, and are responsible for protecting plants from various external threats: stress conditions (biotic and abiotic), ultraviolet radiations, herbivores, pathogens, and diseases[4–6].

SMs are also found in fruits as natural pigments that are extremely useful in photosynthesis and pollination. These pigments include anthocyanidins (red, purple, and blue); carotenoids (yellow, orange, and red); and flavonoids (yellow) and are characterized by their bioactivity since they confer various health benefits, for instance anticancer, anti-inflammation, antioxidant, and delaying aging[7,8].

There are many classes of SMs; the most widely used classification divides them into four groups: alkaloids, polyphenols, terpenes, and saponins.

- Alkaloids: are nitrogen-containing SMs with a low molecular weight[6]. Their structure consists of one or more rings of carbon atoms with nitrogen atom(s) inside. Nitrogenous bases are products of amino acid biosynthesis and are the basis of the potent bioactivity of alkaloids. Alkaloids are produced by plants (particularly by angiosperms), fungi, bacteria, and animals[2]. More than 20000 have been identified, of which around 600 are biologically active. There are several types of alkaloids, up to seven, depending on their amino acid precursors (phenylalanine, tyrosine, and tryptophan). Alkaloid production in plants is influenced by genetics, but not only, since environmental factors (temperature,

UV rays, soil nutrients) contribute also. Stressful conditions such as drought and high temperatures lead to the accumulation of bioactive alkaloids and their precursors in plants[3,9].

- Polyphenols: are nitrogen-free chemical compounds, they have an aromatic ring attached to one or more hydroxyl groups (-OH). They are the most widespread SMs in vascular plants. More than 8000 polyphenols have been discovered, half of them being flavonoids and the other half consisting of simple phenolics, lignans, stilbenes, coumarins, tannins, and quinones[3,6]. All these phytochemicals originate from two precursors: shikimic acid and phenylalanine[10].

  Phenolic compounds are distributed heterogeneously throughout the cell and in the plant in general: for example, a higher amount of phenolics is found in the outer parts of the plant, soluble components are in plant cell vacuoles while insoluble ones are at the cellular walls. This heterogeneity is due to various factors, including the degree of ripeness (during the harvest), environmental factors, processing methods (like cooking), and storage. Ripening has a strong impact on polyphenol concentrations and ratios; it stimulates the production of anthocyanin (a member of flavonoids) for instance, and decreases phenolic acid concentration. The biological characteristics of polyphenols vary significantly from one another, and their health effects depend not only on the quantity consumed but also on their bioavailability[4]. They are responsible for the color, taste, and nutritional properties of plant-based food (like fruits, vegetables, legumes, tea…) and they prevent cancers, neurodegenerative and cardiovascular diseases[6].

- Terpenes: also called isoprenoids, are a large class of nitrogen-free and naturally occurring SMs. They have been found in various living organisms including, plants, algae, bacteria, and animals. More than 50,000 different types of terpenes with various functional groups have been discovered[5,11]. The name “terpene” originates from turpentine, from which the first chemicals of the class were isolated. The chemical skeleton of terpenes is constituted of isoprene $(C_5H_8)_n$ with n the number of the joined isoprene units. It is called the C5 rule or isoprene rule, and it was formulated by Otto Wallach in the 20th century. However, this rule has a loophole since it does not take into consideration the origin of terpene biosynthesis, therefore it was renamed, by Leopold Ruzicka in 1930, the "biogenetic isoprene rule"[6,12]. Isoprenoids have two precursors,

isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) which in turn are synthesized via two different pathways. Terpenes have cytotoxic properties against various organisms (animals, insects, fungi, and bacteria) and have been used as medicines in case of infectious diseases (e.g. artemisinin), as nutraceuticals (e.g. coenzyme Q10), as pigments (e.g. β-carotene), as well as fragrances (e.g. santalol)[5,11].

- Saponins: are nitrogen-free bioactive compounds, mainly produced by plants, both wild and cultivated. They can be found mostly in wild plants as steroids with glycosides or as polycyclic triterpenes in cultivated crops[5,6,13]. The word "saponin" originates from the Latin root "sapo", which means soap, as saponin molecules emulsify in contact with water, just like soap does. Several studies demonstrated the various biological properties of saponins, but the most important are those concerning their anti-viral activity. We have all witnessed what happened in 2020; the emergence of the COVID-19 pandemic that seriously threatened human beings and caused millions of deaths. Quick and efficient measures could have made a difference, and this necessarily involves the development of effective therapies and drugs. Saponins interact with viral envelope proteins and destroy the coat, in this way, they prevent the binding of the virus to the host cells[14]. Bioactive compounds, including saponins, have promising effects since they can be used as therapy -as previously discussed-, as a complement to standard treatments, as well as adjuvant for vaccines[15].

1.1.2. Vegetal callus culture and its application yields

The development of novel biotechnological processes that enable the production of secondary metabolites on a big scale, at low cost, and with very few drawbacks is one of the top concerns of researchers. Callus culture is considered an alternative, efficient, and controlled approach to producing *in vitro* bioactive compounds regardless of the season and geographical position. Additionally, there is a minor environmental impact, a lower carbon footprint, no need for hazardous agents (such as pesticides or herbicides), and, most crucially, less water use, especially during times of global warming[16,17].

The term “callus” derives from *Callum*, a Latin word that means hard and tough (**Figure 1**). Callus culture has attracted interest for years now. Indeed, in 1902, the concept of tissue culture was first proposed by the botanist Gottlieb Haberlandt, who set out to cultivate plant cells (commonly known as callus) isolated from higher plants in a suitable nutrient medium, resulting

in the regrowth of a new plant[16,18,19]. In fact, via his experiments, he established the principles underlying plant tissue culture.

The early investigations led to embryo and root cultures. In the twenty-first century, molecular biology and agricultural biotechnology studies have given cell cultures significant importance. In fact, the *in vitro* plant tissue culture, or the axenic culture of cells is an essential tool for both fundamental and applied research, as well as for commercial use[20].

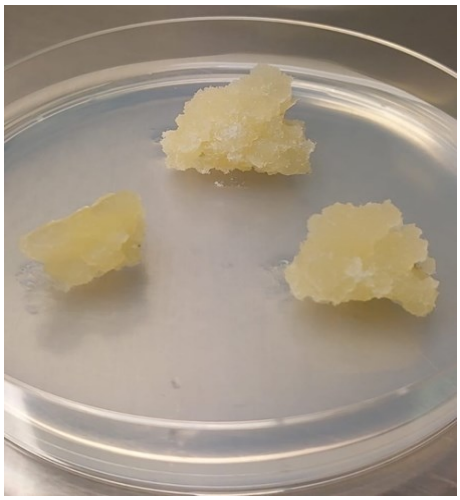

Figure 1. Example of Mela Rosa Marchigiana calluses (Photo taken at the Botanical Garden Laboratory, University of Urbino).

Plants respond to several biotic and abiotic stressors (such as pathogen infection or wounding) by developing calluses, which are unorganized cell masses. In the early stages of botany, the term "callus" was used to describe the rapid cell proliferation and the accumulation of a plant polysaccharide called "callose" localized in the wound. Callus is now a word more extensively used to refer to a mass of disordered and amorphous cells. Moreover, this mass of cells can originate from a unique, differentiated cell. Many callus cells are totipotent. Thus, it has been demonstrated that there are various types of calluses classified according to their macroscopic features or, more precisely, according to the visibility or not of an organ regeneration: the ones without any discernible organ regeneration are friable, while the others can be embryonic, rooty, or shooty, based on the organ they subsequently generate. Basically, calluses are categorized based on how differentiated their cells are[19].

The composition of culture media influences the formation of calluses, and the production of secondary metabolites, and is, therefore, one of the most influential factors. Murashige and Skoog (MS) mediums are commonly used for plant cell culture, but with the growing interest

in the artificial *in vitro* generation of callus, several culture media have been developed like modified SM, B5, etc[17]. These media contain several elements, including plant growth factors such as auxin and cytokinin, two plant hormones that double as inductors and regulators, as their ratio has been shown to establish the degree of differentiation and dedifferentiation of calluses. In fact, on one hand, a high auxin-to-cytokinin ratio promotes root regeneration, while on the other a high cytokinin-to-auxin proportion induces shoot growth[19].

Various SMs (such as rosmarinic acid and shikonin) produced from plant cell culture were commercialized between 1980 and 1990. This innovative production method constitutes a consequent advantage compared to the conventional agricultural one (production with whole plants) since the environmental factors have no impact, neither microbial diseases nor other threats[16,21].

In the laboratory of the University of Urbino Botanical Garden, an innovative method (registered patent no. 102020000012466) of callus culture has been developed from the ripe pulp of the Nordic variety of apple (*Malus x domestica* var. Red Sentinel). In 2020, the MARTHA (Marchigian Apple Related To Healthy Aging) project was created, and aimed at the application of the patented method to enhance the production of high quantities of secondary metabolites through the *in vitro* culture of callus, starting from a local variety of apple called Mela Rosa Marchigiana, with the prospect of realizing a nutritional formulation in the future. This innovative callus culture strategy could be an alternative method of "Green Chemistry" in producing great amounts of phytocomplexes independently of external conditions and more ecologically[22].

Since the dawn of time, plants have been used in everyday life. They were used not only for nutritional purposes but also for therapy. With the development of industrialization, they gave way to chemically synthesized molecules and processed products, which, over time and via research, most often turned out to be harmful to health. Therefore, after several product bans and restrictions, consumer complaints, and even legal actions, manufacturers shifted to sustainable and natural-based products.

Over the past ten years, a wave of plant cell culture technology has emerged in response to the growing trend of producing food and cosmetics naturally[16]. Nowadays, 11% of essential medicines are plant-based. Numerous natural compounds derived from plants serve as key ingredients in cosmetic formulations. It has been demonstrated that bioactive metabolites contained in plant extracts have esthetic and health benefits, or in other words, have cosmetic and pharmaceutical applications; thus, the products developed from these extracts can be

categorized as “cosmeceuticals”. Both cosmetics and pharmaceutics, taken separately or combined, must comply with specific standards because consumers want safe and natural products. The global demand continues to increase as the world's population rises quickly, leading to the extinction of medicinal plants[23]. This demand also concerns food products since numerous studies have described the difficulty of feeding the world's population in adequate quantity and quality[16]. The environment has, in fact, become a major preoccupation for people with a moral compass and manufacturers concerned about consumer health, and since the 1990s two American chemists, Paul Anastas, and John Warner, came in and introduced a newly designed concept, the concept of “Green Chemistry” (GC)[24]. Through this notion, they directed the chemists toward sustainability and reducing their environmental impact. Green chemistry touches multiple industrial sectors, including cosmetics, pharmaceuticals, energy, and agriculture but not only that, it has also had a huge impact on the environment (which was the principal aim), education, and society. The World Health Organization (WHO), European Medicines Agency (EMA), and Food and Drug Administration (FDA) are a few examples of worldwide regulatory agencies that could considerably encourage the adoption of environmentally friendly and sustainable practices by including environmental considerations in good manufacturing practices[25].

In a few words, green chemistry is the creation, development, and use of chemicals and procedures aiming to reduce or suppress the generation or the introduction of any dangerous substance in the biosphere[26]. However, it is governed by twelve principles summarized in one simple mnemonic: PRODUCTIVELY (**Figure 2**)[27]. Among these rules, the 5th is particularly challenging since solvents are massively wasted at the end of different processes. Conventional solvents are generally toxic and flammable, in addition to being soluble and volatile, therefore able to pollute air and water, as well as contaminate lands. Moreover, they are harmful to workers and cause serious incidents. Currently, interest in alternative solvents has grown considerably, since they are recyclable, inexpensive, less dangerous, and simple to use, with a preference for environmentally benign ones. Water, for example, is the most abundant element on earth, and can be considered a harmless universal solvent. It offers some advantages, since through its hydrophobic effect, water can enhance reaction rates. However, there is one major disadvantage to using water as a solvent: the risk of water pollution, which can need a lot of energy to clean up and purify[24,25].

Otherwise, promising green alternative solvents called Natural Deep Eutectic Solvents (NADES) became a new trend for plant extraction.

Figure 2. Twelve principles of Green Chemistry written in the form of a mnemonic: PRODUCTIVELY[27].

1.2 Two important apple varieties used as starting material

1.2.1 *Cydonia oblonga*

Cydonia oblonga Miller (from now on *Co*), more commonly known as quince, is a small plant that belongs to the Rosaceae family, and its season lasts 3 months starting from September[28,29]. This shrub has a height that doesn't exceed 8 m and 5 m in width. Quince fruit has a yellow color and is characterized by not being consumed fresh given its hard texture and astringent taste (**Figure 3**). Consequently, the fruit is processed into jellies, jams, and liquors. More recently, other products have been developed, such as convective-dried quince and freeze-dried quince slices[29–31].

In the food industry, *Co* fruits are used for their pectin content, known to protect the colon from damage[30]. Traditionally, they have also been used to treat respiratory diseases (like bronchitis). In addition, *Co* fruits constitute a source of bioactive compounds, including polyphenols, terpenoids, and vitamin C[28]. Phytochemical content depends on numerous factors such as soil components and climatic conditions[29].

Figure 3. *Cydonia oblonga* apples[29].

1.2.2 Mela Rosa Marchigiana

Mela Rosa Marchigiana (from now on MRM) usually called "Mela Rosa dei Monti Sibillini" is an old variety of apples dating from the Roman period belonging to the Rosaceae family. This apple is cultivated in the Sibillini mountains of the Marche region of Italy, between 400 and 900 m altitude. This variety of apple is particular; indeed, it is characterized by uncommon pomological traits (**Figure 4**): flattened shape, small size, green peel with shades ranging from pink to red, and specific organoleptic traits such as an intense aroma and an acidic/sweet taste[32,33]. This apple is also distinguished by its shelf life and its nutritional value. Indeed, overlooked, and old apples contain more phytonutrients in comparison with commercial ones despite being less attractive.

Figure 4. Mela Rosa Marchigiana apples[33].

Thanks to regional and local authorities, MRM apples have recently regained attention. This variety of apples received Slow Food recognition and has been the subject of scientific investigations focusing on its potential health advantages.

Hence, the identification and valorization of overlooked species are essential to preserve the ecosystem's biodiversity and avoid the germplasm's extinction[32,34].

There are some studies[30–32] that have been carried out on *Co* and MRM fruits and several biological properties have been reported (**Table 1)**.

Fruits	Biological activities
Cydonia oblonga	Anti-bacterial, antioxidant, anti-allergic, anti-inflammatory, genoprotective, wound-healing, anti-diabetic[30]
	Anti-cancer, anti-viral[31]
Mela Rosa Marchigiana	Antioxidant, enzyme inhibition[32]

Table 1. Summary of the main and most recognized biological activities attributed to *Co* and MRM fruits.

The presence of various phytochemicals in these fruits makes them particularly interesting. *Cydonia oblonga* fruits, for example, are considered an inexpensive and natural source of secondary metabolites; particularly polyphenols like quercetin and caffeoylquinic acid, the major phenolic acid found in peel and pulp. The therapeutic potential of *Co* is credited to its strong antioxidant properties arising from these phytochemicals. Moreover, a study has shown that quince polysaccharides were able to inhibit tyrosine phosphatase activity demonstrating its capacity to treat Type 2 Diabetes (T2D) and obesity. Different parts of *Co* (fruits, seeds, leaves, and roots) have been used for the cure of many ailments. It has been demonstrated that a quince leaf extract prevents rabbits, put under a high-cholesterol diet, from atherosclerosis and renal injury[30,35].

The common proverb “an apple a day keeps the doctor away” refers to the health-promoting properties of its various phytochemicals. Apples consumption has been associated with the prevention of various diseases including metabolic and cardiovascular ones, oxidative stress, as well as some cancers[33]. It has been demonstrated that Mela Rosa Marchigiana apples possess antioxidant and enzyme inhibition activities due to their bioactive compounds but there's still a lack of information on all the biological activities of these old varieties, consequently, further studies are needed[32,33].

1.3 The importance of oxidative stress and inflammation in physiological processes

1.3.1 Oxidative stress and reactive oxygen species

Who would have thought that one of the most important elements could at the same time be the most harmful? Oxygen is a paradoxical element that provides energy for our body's cells but also leads to oxidation reactions that produce free radicals and prove to be harmful.

Mitochondria is the organelle that receives most of the oxygen molecules we breathe, which then converts it, together with other nutrients into adenosine triphosphate (ATP), which will further be used by the cells. Although this process may seem simple, in some cases not all oxygen molecules are metabolized correctly resulting in the production of Reactive Oxygen Species (ROS) like the superoxide anion (O2•-). The superoxide anion can impair the cell and its components; it damages the biomacromolecules (proteins, nucleic acids -DNA and RNA-, lipids, and carbohydrates) and the mitochondria. Moreover, O2•- can be transformed into

hydrogen peroxide (H_2O_2) and hydroxyl (·OH), other reactive species. ROS includes both radical and non-radical oxygen derivatives. But what is a radical? A radical is an atom or a molecule with unpaired electrons located at the outermost bonding molecular orbital, which results in their high instability[36,37]. Radicals have also advantages since they are involved in two body's essential functions: phagocytosis and eicosanoid formation (responsible for renal function, hemostasis, and regulation of gastric secretion)[37]. Oxidative stress occurs when oxygen radicals are excessively produced in the cells, and the balance between oxidant and antioxidant molecules is disturbed. At this point, the internal defensive mechanisms constituted of antioxidants (the most ubiquitous being glutathione) and oxygen radical scavenging enzymes (i.e. peroxidase, catalase, and superoxide dismutase) are no longer able to manage and control this balance and consequently can lead to damages such as genotoxicity, cytotoxicity and in the worst case, to carcinogenesis (proliferation of mutated cells)[38,39].

Given the context and argument of the thesis, it is important to discuss and define the term "dietary oxidative stress". In 1995, Lavender first used this term which was taken up, 5 years later, by the Food and Nutrition Board of the Institute of Medicine, National Academy of Sciences and defined as a substance found in food that can considerably lower the negative effects of ROS and reactive nitrogen species (RNS) in the human body's physiological state. The nutritional oxidative stress is caused by a high oxidative load or an insufficient dietary intake/a reduced availability of antioxidants like polyphenols (e.g., from tea, or cocoa), carotenoids, and vitamin C, leading to an impairment of the antioxidant system. A subtype of dietary oxidative stress known as postprandial oxidative stress, results from prolonged post-prandial hyperglycemia and/or hyperlipidemia, and is correlated with increased risks of diseases such as obesity, diabetes, and atherosclerosis[40]. The free radicals overproduction caused by hyperglycemia can hasten the onset and development of diabetes and every potential complication of the illness such as retinopathy (eventually resulting in blindness), neuropathy, cardiovascular diseases, and embryopathy. Reduction in antioxidant defense mechanisms along with an increased generation of free radicals can damage cellular organelles and thus, lead to the development of insulin resistance caused by glucose oxidation for example. Therefore, it is necessary to evaluate the interactions among free radicals, hyperglycemia, and antioxidants to expand the boundaries of diabetes management research[41].

1.3.2 Inflammation

Inflammation is a fundamental pathophysiological response that the body activates in reaction to harmful stimuli, such as infections, injuries, or tissue damage. It is a complex and highly regulated process involving a cascade of events mediated by various signaling molecules and immune cells.

Some anti-inflammatory treatments have been discontinued because of serious side effects. Historically, individuals have incorporated potent anti-inflammatory plants into their diets and pharmaceutical practices, and bioactive molecules derived from these plants could represent valuable reservoirs of anti-inflammatory drugs[42].

Secondary metabolites often derived from plants, exhibit diverse bioactive properties that can influence inflammatory processes. Many studies suggest that certain secondary metabolites possess anti-inflammatory effects, potentially modulating signaling pathways and immune responses associated with inflammation. Polyphenols, alkaloids, and terpenoids are among the secondary metabolites that have demonstrated anti-inflammatory properties. Saponins exhibited also anti-inflammatory activity across various rodents (mice and rats) experimental inflammation models[42].

Understanding the complex mechanisms underlying inflammation pathways and their interactions with bioactive compounds could provide insights into the development of novel therapeutic approaches for managing conditions related to immune responses and tissue damage. Research in this area explores the potential of natural products rich in secondary metabolites as sources for anti-inflammatory agents, contributing to the broader field of pharmacognosy and drug discovery.

1.4 Modulation of digestive enzymes is an important process for human health

The global use of natural products in drug discovery is on the rise, and there is a growing emphasis on supporting traditional healthcare systems. Approximately 80% of the global population relies on plant-based traditional health remedies due to their affordability and safety. Over the recent decades, researchers have focused on identifying bioactive compounds derived from plants with potential applications in the treatment of a range of diseases including diabetes. There is a growing global epidemic of diabetes, and according to the World Health Organization

(WHO), 422 million people around the world are affected by this illness[43]. Diabetes, also called Diabetes Mellitus (DM), is a chronic metabolic disease characterized by hyperglycemia (high blood glucose levels). Diabetes can be classified into various forms, but the main two forms are Type 1 Diabetes (T1D) and Type 2 Diabetes. T2D is the most common one and represents 90–95% of cases of diabetes. Several factors, including hyperinsulinemia, insulin resistance, and decreased insulin-mediated glucose absorption make more difficult the management and treatment of T2D. To prevent the latter, postprandial glucose control is crucial because, over time, it will result in major side effects like renal failure, hypertension, and cardiovascular disease. To control this disease, it is essential to keep a healthy lifestyle, and this involves a controlled diet, frequent physical activity, and the use of antidiabetic medications. Unfortunately, the existing antidiabetic medications (i.e. acarbose and miglitol) are linked to severe side effects (like abdominal cramping, diarrhea, and vomiting); therefore, there is a requirement to identify novel and natural therapeutic agents characterized by high efficacy and minimal adverse effects. One of the adopted therapeutic strategies is to inhibit α-glucosidases and α-amylases, enzymes responsible for hydrolyzing dietary carbohydrates, therefore causing hyperglycemia. Consequently, the inhibitors of these enzymes delay and block the digestion and absorption of glucose[43,44].

Alpha-amylase (EC 3.2.1.1) or 1,4-α-d-glucan-glucanohydrolase, is an endoglucanase that catalyzes the cleavage of α-1,4-glycosidic bonds of one of the most important food carbohydrates, starch, and related polysaccharides to generate oligosaccharides (such as glycogen, amylopectin, and amylose). These endoglucanases are secreted by salivary glands and pancreas in mammals. α-amylase inhibitors contribute to preventing and treating obesity, and T2D since they regulate plasma blood glucose levels[45].

Alpha-glucosidase (EC 3.2.1.20) is an exo-type carbohydrolase located on the intestinal brush border that hydrolyzes oligosaccharides and polysaccharides into monomers that are subsequently absorbed (**Figure 5a**). Inhibition of α-glucosidase in the digestive tract slows down the digestion of carbohydrates, consequently, a reduced amount of glucose is absorbed, and the postprandial blood glucose and insulin levels decrease (**Figure 5b**)[44].

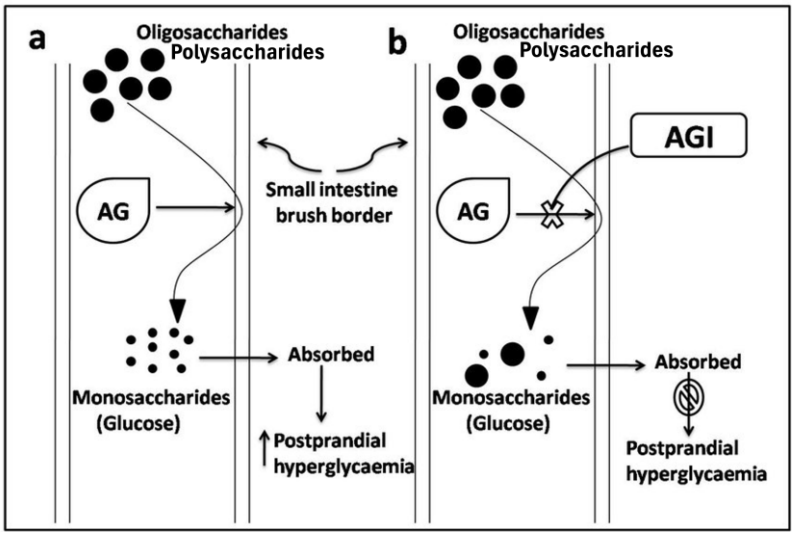

Figure 5. Therapeutic effects of alpha-glucosidase inhibitors. (**a**) shows the cleavage of oligosaccharides and polysaccharides by alpha-glucosidase into monosaccharides. (**b**) shows the mechanism action of alpha-glucosidase inhibitor that results in a decreased production and absorption of monosaccharides. AG: alpha-glucosidase, AGI: alpha-glucosidase inhibitor[44].

One of the main risk factors for T2D is obesity. It's a complicated condition that can be largely avoided. Along with overweight, they affect a third of the world's population.

By 2030, 20% of adults worldwide are predicted to be obese, and 38% of them will be overweight[46]. The National Institutes of Health (NIH) assembled a group of specialists to develop clinical recommendations for diagnosing, assessing, and treating overweight or obese individuals. These guidelines maintain that the cornerstones of treatment, and as for diabetes, are dietary therapy, and increased physical activity. Pharmacologic drugs approved for long-term therapy (i.e. orlistat), have been acknowledged as potentially helpful elements to nutrition and exercise in a certain number of patients. Orlistat was authorized in March 1999 to control obesity, weight loss, and weight maintenance, and is the first available anti-obesity medication that doesn't interfere with the central nervous system balance. In addition to being specific and irreversible, it is a potent inhibitor of gastric and pancreatic lipases. Its mechanism of action is described by the fact that it prevents pancreatic lipase from hydrolyzing dietary fat -which is present in the form of triglycerides- into monoglycerides and free fatty acids, resulting in the fecal elimination of non-digested triglycerides. Therefore, orlistat-induced lipase inhibition reduces the amount of absorbed dietary fat, which increases the caloric deficit[47]. However, this agent has side effects at the gastrointestinal level such as fecal incontinence and diarrhea.

Therefore, the production of safer lipase inhibitors from natural sources would be highly recommended[48].

Lipases (E.C.3.1.1.3) or triacylglycerol acyl hydrolases, are enzymes that act on carboxylic ester bonds. They catalyze the hydrolysis of lipids with the subsequent release of free fatty acids, and different forms of glycerol (such as diacylglycerols, and monoacylglycerols). The pancreas and stomach are the usual sites of production for lipase enzymes. Consequently, they play a key role in digestion processes. Moreover, lipases possess both industrial and physiological importance, in fact, several functional foods are developed with their use[49,50].

2. AIM OF THE STUDY

This study aimed to investigate some biological activities and protective potentialities of hydroalcoholic extracts derived from the fruit pulp calluses of two apple varieties, namely *Cydonia oblonga* and Mela Rosa Marchigiana.

Our experimental setting has been designed in order to study antioxidant and anti-inflammatory activities, genoprotective, and digestive enzymes inhibition properties.

The Results section is subdivided into *Cydonia oblonga* and Mela Rosa Marchigiana topics, and in each subject, cell-free and cell-based assays are presented.

3. MATERIALS AND METHODS

GENERAL NOTES

Most of the procedures performed in this thesis are already reported and published in De Bellis *et al.*[51] and Gubitosa *et al.*[52] with some slight modifications where indicated.

In all the experiments, the concentration of *Co* and MRM calluses extracts were 30 mg dry weight (dw) /mL double-distilled water and 50 mg dry weight /mL 70% ethanol (v/v), respectively.

3.1 Plant material and callus culture

Pulp apples calluses were cultured and received from the research group of Prof. D. Fraternale of the University of Urbino (Botanical Garden). Detailed procedures are described in De Bellis *et al*.[51] and in Verardo *et al*.[22].

3.2 Preparation of pulp callus extract from *Cydonia oblonga* and Mela Rosa Marchigiana

Briefly, freeze-dried pulp calluses of each variety was ground using a potter's type homogenizer and extracted with 70 % (v/v) ethanol/double distilled water. The homogenate was stirred overnight (ON) at 4°C and after that centrifugated at 13,000 rpm for 45 min. The supernatant was filtered with a 0.22 μm filter (Millipore, Molsheim, France), lyophilized using a Speed Vac Concentrator (Thermo Fisher Scientific, MA, USA), resuspended with their respective solvents; (*Co* in double-distilled water and MRM in 70 % (v/v) ethanol), centrifugated, and aliquoted. The aliquots of both lyophilized samples (*Cydonia oblonga* and Mela Rosa Marchigiana) were stored at - 80°C and resuspended before use at a concentration of 30 mg dry weight /mL and 50 mg dw/mL, respectively.

3.3 Cell-free assays

3.3.1 Total phenols and Vitamin C content determination

The quantification of total polyphenols followed the methodology outlined by Zhang *et al.*[53]. In brief, the *Co* and MRM callus extracts and MRM digesta, underwent dilution at ratios of 1:10, 1:20, and 1:40 v/v in double-distilled water. The assay was conducted in a 96-well flat-

bottom microtiter plate. To each well, 100 µL of Folin-Ciocalteu reagent was added, along with 10 µL of Gallic Acid (GA) at a 1:10 v/v ratio, or the callus extract at various dilutions. The plate was incubated for 5 min in darkness at room temperature (RT). Subsequently, 140 µL of Na_2CO_3 (7.5% w/v) was added to each well. The plate underwent further incubation at RT in the dark for 90 – 120 min. The absorbance at 630 nm was measured using a Microplate Reader (Bio-Rad, CA, USA) and a standard calibration curve was constructed utilizing gallic acid at varying concentrations (0.5 – 0.11 mg/mL). The total polyphenols content is expressed as milligrams of Gallic Acid Equivalent (GAEq) / gram of dry weight of callus extract. Double-distilled water or 70 % ethanol or digestion juice were used as blanks.

For the estimation of vitamin C content in the *Co* callus extract, the Folin-Ciocalteu reagent was employed under acidic conditions (pH range 1.0 – 7.0) following the procedure outlined by Jagota & Dani[54]. Specifically, 200 µL of *Co* callus extract was mixed with 800 µL of 10% v/v trichloroacetic acid, stirred, and cooled on ice for 5 minutes. The solution was then centrifuged for 5 minutes at 14,000 rpm, and 500 µL of the supernatant was combined with 500 µL H_2O and 100 µL of Folin-Ciocalteu reagent. The mixture was stirred and left on ice in darkness for 10 minutes. Double-distilled water served as the blank. A standard calibration curve was established using an Ascorbic Acid solution in water (5 – 45 µg/mL), and the absorbance was measured at 760 nm using a UV Beckman spectrophotometer (CA, USA). Results are expressed as Acid Ascorbic Equivalent (AAEq) / g of callus extract.

3.3.2 Antioxidant assays

- **DPPH**

The antioxidant activity of calluses extracts was determined using the stable free radical DPPH• (2,2-diphenyl-1-picrylhydrazyl). In brief, 850 µL of a freshly prepared 100 µM DPPH• ethanol solution was added to 150 µL of *Co*, MRM callus extract or MRM digesta. The mixture was incubated for 30 min in the dark at room temperature and the absorbance decrease was measured at 517 nm (UVIKON 930 UV/VIS spectrophotometer). Double-distilled water or 70 % ethanol or digestion juice were used as blanks and Trolox as a reference compound in the calibration curve. The DPPH radical scavenging activity (DSA) was measured as a reduction in DPPH absorbance by applying the following equation:

DPPH scavenging activity (% DSA) = [(A_{517nm} of blank - A_{517nm} of sample)/ A_{517nm} of blank] ×100.

The values have also been expressed as concentration of Trolox Equivalent (TEq)/ g of callus. The EC_{50}, representing the concentration of extract necessary to achieve 50% free radical scavenging activity, was then determined.

- **ABTS**

Antioxidant activity against ABTS• [2,2,-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] radical was performed as described by De Bellis *et al.*[51] with some modifications.

In summary, the reaction mixture was prepared by combining a 7 mM ABTS solution with 2.45 mM potassium persulphate, followed by incubation in the dark at RT for 12 – 16 hours to generate the ABTS• radical. Subsequently, the solution was diluted with ethanol to achieve an absorbance of 0.80 ± 0.05 at 734 nm prior to use. Aliquots of *Co* or MRM callus extracts or MRM digesta at various concentrations were introduced into 1 mL of the ABTS ethanolic solution and incubated in darkness at RT for 6 minutes. Absorbance was then quantified at 734 nm using a UV Beckman spectrophotometer (CA, USA), with double-distilled water or 70 % ethanol or digestion juice serving as the blank. The ABTS radical scavenging activity (ABTS SA) was determined using the following equation:

ABTS scavenging activity (% ABTS SA) = [(A_{734nm} of blank – A_{734nm} of sample)/ A_{734nm} of blank] × 100.

The Trolox Equivalent Antioxidant Capacity (TEAC) was also calculated and expressed as Trolox Equivalent concentration.

- **ORAC**

The ORAC (oxygen radical absorbance capacity) assay was conducted as described by De Bellis *et al.*[51] using a Fluostar Optima Plate reader fluorimeter (BMG Labtech, Offenburg, Germany). Briefly 200 µL of 0.096 µM fluorescein sodium salt in 0.075 M Na-phosphate buffer (pH 7.0) was mixed to 20 µL of *Co* or MRM callus extracts or MRM digesta or Trolox or 0.075 M Na-phosphate buffer (pH 7.0) as blank. The reaction was initiated with 40 µL of 0.33 M of 2,2′-azobis-(2-amidinopropane) hydrochloride (AAPH). Fluorescence was read at 485 nm excitation and 520 nm emission until complete extinction. In each test, the Trolox calibration curve was made with a concentration ranging from 50 to 500 µM in 0.075 M Na-phosphate buffer (pH 7.0). ORAC values of the tested samples are reported as concentration of Trolox Equivalents (TE)/g of callus extract.

3.3.3 Nicking assay of *Cydonia oblonga* and Mela Rosa Marchigiana callus extracts

- **Genoprotective activity of *Co* callus extract**

The genoprotective effect of *Co* callus extract was investigated on HaCaT cells DNA damaged by Ultraviolet-C (UVC, 280–100 nm) exposure for 1–15 min. UVC irradiation was performed using an Ultraviolet Crosslinker UVC oven (Amersham Life Science, UK). *Co* callus extract concentrations of 0.14, 0.28, and 0.56 mg dw/mL were used. The electrophoresis analysis was conducted on 0.8% agarose gel and the quantitative analysis employed the Delta Cycle-threshold (Ct) method after Real-Time PCR Syber Green (qRT-PCR) in a 20 µL volume with triplicate DNA samples. The qRT-PCR reaction included 1 µL of each DNA, 0.3 µM of each NADH Dehydrogenase subunit 1 (ND1) primer
(ND1: F- 5′- ACGCCATAAAACTCTTCACCAAAG − 3′; ND1: R- 5′– TAGTAGAAGAGCGATGGTGAGAGCTA − 3′) (Accession MZ457933.1), Power up Syber Green Master Mix (Thermo Fisher Scientific, MA, USA), and a Quant Studio1 thermal cycler (Thermo Fisher Scientific, MA, USA). The qRT-PCR thermal conditions comprised an initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min.

- **Genoprotective activity of MRM callus extract**

The Nicking assay was conducted according to Sadat Asadi *et al.*[55] with some modifications. A mixture of 125 ng/µL pGEM-T DNA, the oxidative system (0.33 mM Fe_2SO_4, 5 mM H_2O_2), and 0.62 mM EDTA was prepared in the presence or absence of various MRM callus extract concentrations (ranging from 150 µg/mL to 2.3 µg/mL), in a total volume of 20 µL of Phosphate Buffered Saline (PBS). Additionally, a control sample (CTRL) with DNA, EDTA, and PBS was included. Reaction mixtures were incubated for 30 min at 37°C. DNA samples were loaded onto a 1% Agarose/Tris Borate EDTA (TBE) gel, stained with 1% Midori Green Advance DNA staining (Resnova, Genzano di Roma, Italy), and visualized under UV light using a Gel Doc 2000 (Bio-Rad, Italy). Densitometric analysis for quantification was performed using Quantity One Software 4.01 (BioRad, Italy). The retention of covalently closed circular DNA strands (%) was calculated using the following formula:
Retention % = (Intensity of supercoiled DNA of sample / Intensity of supercoiled DNA of control) × 100.

3.4 Cell-based assays

3.4.1 Cell cultures

Two cell lines were used in the cell studies: murine RAW 264.7 macrophages and immortalized human keratinocytes, HaCaT cells. Both lines were acquired from the European Collection of Cell Cultures (Salisbury, UK) and CLS-Cell Lines Service GmbH (Eppelheim, Germany), respectively. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, Milan, Italy) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 mM non-essential amino acids, 1 mM pyruvate, streptomycin (100 µg/mL), penicillin (100 U/mL), 1% (w/v) L glutamine, and maintained in a humidified (95% humidity) incubator at 37°C with 5% CO_2. The growth medium was replaced every 2-3 days until about 80% of the confluence was reached. All cell-based assays were carried out after 24 h period of cell adhesion.

3.4.2 Cell viability assays

Cell viability was determined using two colorimetric assays, Water-Soluble Tetrazolium (WST-8) and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assays.

- **WST-8 assay:**

Cells were seeded into 96-well plates (5×10^3/well) in 100 µL complete DMEM, and incubated for 24 h at 37 °C. Afterward, the medium was removed, and 100 µL of fresh DMEM containing a known concentration of the callus was added and incubated for a further 24 h. After the overnight treatment, 10 µL of WST-8 salt was added to each well of the plate, and cell metabolic activity was evaluated. Using a plate reader (BioRad Laboratories, Hercules, CA, USA), absorbance was measured at 450 nm for a maximum of 4 hours at 37°C. Cell viability results were expressed as a percentage of the control and three independent experiments were performed.

- **MTT assay**

Following removal of the medium, cells were washed with PBS and each well was treated with a 100 µl di MTT 0.2 mg/mL solution for 1 hour at 37°C. After the incubation, the MTT solution was removed, and 100 µl Dimethyl sulfoxide (DMSO) was added to dissolve the generated formazan salt. The quantity of formazan salt was determined by measuring absorbance at 570

nm using a microplate reader. Cell viability was expressed as a percentage compared to the control and calculated using the formula:
% Cell viability = (OD test / OD control) × 100, where OD represents optical density.

3.4.3 Quantification of ROS production by DCF-DA

Intracellular reactive oxygen species (ROS) were determined using the cell-permeable fluorogenic probe 2′,7′ -dichlorofluorescein diacetate (DCF-DA). HaCaT cells were seeded (1×10^4/100 µL DMEM) in 96-well plates for 48 h at 37°C, washed with PBS (PBS = 8 g/L NaCl, 1.44 g/L Na_2HPO_4, 0.2 g/L KH_2PO_4, 0.2 g/L KCl, pH 7.4), and treated overnight (with *Co* or MRM) at 37°C with different concentrations of samples in DMEM without fetal bovine serum and antibiotics. After the treatment, cells were washed with PBS, and 10 µM DCF-DA was added for 30 min in the dark at 37°C. The DCF-DA was removed using PBS and basal levels of ROS production were determined by FLUOstar OPTIMA fluorescence microplate reader (BMG LABTECH) (emission wavelength 520 nm and excitation wavelength 485 nm). A total of 0.1 mM H_2O_2 or 0.3 mM tert-butyl hydroperoxide (TBHP) was added for 30 min (for *Cydonia oblonga* callus extract experiment) and only 0.1 mM H_2O_2 for 60 min (for MRM callus extract experiment) was added. Afterward, ROS release was determined for a second time.

3.4.4 LPS-induced inflammation in RAW 264.7 cells and Griess assay

The release of nitric oxide (NO) by RAW 264.7 cells stimulated with bacterial lipopolysaccharide (LPS, Sigma-Aldrich, Milan, Italy) is considered a marker of the inflammatory process. The extracellular NO concentration was measured by a UV-visible spectrophotometer. The nitric oxide content was calculated from a nitrite standard curve (calcium nitrite 6.25 – 100 µM) prepared in the fresh culture medium.
RAW 264.7 cells, in the logarithmic phase, were seeded (3×10^4 cells/100 µL) and cultured in 96-well plates for 24 h. Then the experiment was divided into four groups: a control group (CTRL), LPS-treated group, LPS + dexamethasone (DEXA, an anti-inflammatory compound used as a positive control), and LPS + callus extracts (*Co* or MRM) at various concentrations. The treatments were performed for 24 h at 37°C. LPS was used at a concentration of 1 µg/mL and DEXA at 0.0039 mg/mL for *Co* experiments and 10 µM DEXA for MRM experiments. After overnight treatments, aliquots of 50 µl of supernatants were incubated with 50 µl of Griess reagent (40 mg/mL) for 10 min in the dark at RT. The absorbance was measured at 570 nm employing a plate reader (BioRad Laboratories, Hercules, CA, USA).

To eliminate the potential association between the decrease in NO levels and a reduction in cell viability, we performed the WST-8 viability assay on the same plate.

3.4.5 LPS-induced inflammation and H_2O_2-induced oxidative stress in HaCaT cells

Inflammation was induced by 1 μg/mL LPS treatment while oxidative stress was induced by 100 μM H_2O_2. HaCaT cells, in the logarithmic phase, were seeded (12×10^5 cells/well) on 6-well plates and kept in a 5% CO_2 incubator for 24 hours at 37°C. Afterward, cells were co-treated overnight with LPS in the presence or absence of 1.78 mg/mL *Co* callus extract or H_2O_2 in the presence or absence of the 17.88 μg/mL MRM callus extract. Then, the medium was removed, adherent cells were washed with PBS and detached with trypsin, then they were collected and stored in 350 μL of lysis buffer RLT (RNeasy mini kit Qiagen, Milan, Italy) at −80°C until RNA extraction.

3.4.6 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted employing the RNeasy mini kit (Qiagen, Milan, Italy), and subsequently converted to cDNA using PrimerScript™ RT Master Mix (Takara), and finally processed by qRT-PCR to evaluate the expression levels of iNOS, IL-6, IL-1β, ikBα, and ICAM, and HO-1 genes. Their expression levels were normalized with the housekeeping gene 36B4. The quantification was performed in a total volume of 20 μL: 2 μL of each cDNA (corresponding to 25 ng of total RNA),10 μL of Power up Sybr Green Master Mix, 0.6 μL of forward and reverse primers (10 μM), and 6.8 μL of nuclease-free water. qRT-PCR was performed using a QuantStudio 1 qRT-PCR System thermal cycler (Thermo Fisher) and the analysis was conducted using the Pfaffl method. The qRT-PCR thermal conditions included an initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min.

The nucleotide sequences of the primers are summarized in **Table 2**.

	Sequence	Accession Number
HO-1	F-5′-CCAGCAACAAAGTGCAAGA-3′ R-5′ TCACATGGCATAAAGCCCT-3′	NM_002133
iNOS	F-5′-TGACCATCATGGACCACCAC-3′ R-5′-ACCAGCCAAATCCAGTCTGC-3′	NM_000625.4
IL6	F-5′-GGTACATCCTCGACGGCATCT-3′ R-5′-GTGCCTCTTTGCTGCTTTCAC-3′	XM_005249745.6
IL-1β	F-5′-AAAGAAGAAGATGGAAAAGCGATT-3′ R-5′-GGGAACTGGGCAGACTCAAATTC-3′	XM_047444175
IkBα	F-5′-GCTGCTGATGTCAATGCTCA-3′ R-5′-ACACCAGGTCAGGATTTTGC-3′	NM_020529.3
ICAM	F-5′-CCTTCCTCACCGTGTACTGG-3′ R-5′-AGCGTAGGGTAAGGTTCTTGC-3′	NM_000201
36B4	F-5′-CGACCTGGAAGTCCAACTAC-3′ R-5′-ATCTGCTGCATCTGCTTG-3′	NM_053275.4

Table 2. Employed primers for qRT-PCR analysis.

3.5 Digestive enzyme inhibition

The procedures were performed according to De Bellis *et al.*[51]. Due to the natural color of the callus extracts, a set of blanks (without the enzymes) was set up and resulting absorbance was subtracted from the sample absorbance.

- **α-Glucosidase inhibition**

The enzymatic activity of *Saccharomyces cerevisiae* α-glucosidase (EC 3.2.1.20) was assessed at 405 nm by monitoring the liberation of pNP (p-nitrophenol) from the substrate pNDG (p-nitrophenyl-α-D-glucopyranoside). Acarbose, a pharmacological inhibitor, served as a positive control. In a 96-well microplate, a mixture comprising 50 µL of increasing concentrations of *Co* or MRM callus extracts, or Acarbose (1 and 2 mM), and 100 µL of α-glucosidase (0.2 U/mL) in 100 mM Na-phosphate buffer at pH 6.9 was dispensed. The microplate underwent incubation for 10 minutes at 37°C. Subsequently, 50 µL of 5 mM pNDG was added to each well, and the absorbance was measured at 405 nm using a Microplate Reader (Bio-Rad, CA, USA). Each experiment was performed in quadruplicate, and the α-glucosidase activity was determined using the formula:

α-glucosidase activity (%) = [(A_{405nm} sample - A_{405nm} blank) / (A_{405nm} control - A_{405nm} blank)] × 100.

- **α-Amylase inhibition**

Equal volumes (100 µL) of 1% (w/v) starch solution, porcine pancreas-derived α-amylase (EC 3.2.1.1) at a concentration of 0.5 mg/mL (in 100 mM Na-Phosphate buffer at pH 6.9), and increasing concentrations of *Co* or MRM callus extracts, or Acarbose (1 and 2 mM) were combined in each tube and incubated for 10 minutes at 25°C. The α-amylase reaction was terminated by the addition of 200 µL of dinitrosalicylate reagent (comprising 1% w/v 3,5-dinitro salicylic acid, 0.05% w/v sodium sulfite, 1% w/v sodium hydroxide, and 0.25% v/v phenol). The mixture was boiled for 5 minutes and then allowed to cool at room temperature. Subsequently, 100 µL was extracted from each tube and transferred to a 96-well plate, where 100 µL of double-distilled water was added to each well. Absorbance was measured at 540 nm using a Microplate Reader (Bio-Rad, CA, USA). Each incubation was performed in quadruplicate, and the α-amylase activity was computed as follows:

α-amylase activity (%) = [(A_{540nm} sample - A_{540nm} blank) / (A_{540nm} control - A_{540nm} blank)] x 100.

- **Lipase inhibition**

To evaluate the inhibitory effect on lipase activity, 100 µL of porcine pancreas lipase (EC 3.1.1.3) at a concentration of 1 mg/mL in 0.5 M TRIS HCl + 0.01 M $CaCl_2$, pH 7.5, was combined with 50 µL of callus extracts at increasing concentrations or Orlistat (5 µg) as a positive control. The total volume was adjusted to 0.9 mL by supplementing TRIS HCl buffer, and the mixture was incubated at 37°C for 10 minutes. Subsequently, 100 µL of 4-nitrophenyl butyrate at a concentration of 10 mM was added to each tube. The release of 2,4-dinitrophenol in the reaction was quantified at 410 nm using a UV–visible spectrophotometer (Beckman CA, USA). The change in absorbance over the 10-minute incubation period was recorded. Each data point was obtained in quadruplicate, and the lipase inhibition rate was calculated using the following formula:

Lipase activity (%) = [(A_{410nm} sample - $A_{410\ nm}$ blank) / (A_{410nm} control - A_{410nm} blank)] × 100.

3.6 *In vitro* gastrointestinal digestion procedure

The concentration of MRM callus extract was 50 mg dry weight /mL. The *in vitro* digestion was carried out according to Oomen *et al.*[56], Versantvoort *et al.*[57], and Desideri *et al.*[58] with few modifications. This procedure aims to mimic the gastrointestinal tract while respecting human physiological conditions such as the temperature, the pH, the incubation time in each

compartment, and the chemical constituents of digestive juices[57]. The digestive fluids were prepared artificially in the laboratory (**Table 3**) and then maintained in a thermostatic room at 37 ± 2°C which is important to preserve the enzyme's structural and functional properties. The falcon tube containing the MRM callus sample was constantly rotating head-over-heels at 55 rpm[59].

Constituents **Solution and concentration (g/L)**	**Saliva** pH = 6.5 ± 0.2 **(mL)**	**Gastric juice** pH = 1.07 ± 0.07 **(mL)**	**Duodenal juice** pH = 7.8 ± 0.2 **(mL)**	**Bile** pH = 8.0 ± 0.2 **(mL)**
NaCl (175.3)	1.7	15.7	40	30
KCl (89.6)	10	9.2	6.3	4.2
KSCN (20.0)	10			
NaH_2PO_4 (88.8)	10	3.0		
Na_2SO_4 (57.0)	10			
KH_2PO_4 (8.0)			10	
$NaHCO_3$ (84.7)			40	68.2
NaOH (40.0)	1.8			
$CaCl_2$ (22.2)		18	9.0	10
$MgCl_2$ (5.0)			10	
NH_4Cl (30.6)		10		
HCl (440.3)		8.3	0.5	0.5
Urea (25.0)	8	3.4	4.0	10
Glucose (65.0)		10		
Glucuronic acid (2.0)		10		
Glucosamine hydrochloride (33.0)		10		
Solids	**(mg)**	**(g)**	**(g)**	**(g)**
Mucin	50	3		
α-Amylase	145			
Uric acid	15			
BSA		1	1	1.8
Pepsin		1		
Pancreatin			3	
Lipase			0.5	
Bile				6

Table 3. Constituents and composition of 1L of digestive juices[59].

The digestion procedure was done in the thermostatic room (37°C) and occurs in 3 phases according to Desideri *et al.*[58,59] with some modifications:

- Phase 1: 2.5 g of freeze-dried MRM callus sample was mixed with 3.8 mL of artificial saliva, incubated, and rotated for 5 min.

- Phase 2: 7.5 mL of artificial gastric juice was added to the mixture, incubated, and rotated for 2 h.
- Phase 3: 7.5 mL of artificial duodenal juice and 3.8 mL of artificial bile were added simultaneously to the suspension resulting from the second phase, incubated, and rotated for another 2 h.

After that, the digestion falcon tube was placed in ice to stop digestion process and centrifuged for 7 - 8 min at 6000 rpm to separate the surnatant (digested juices) from the pellet (digested matrix/sample).

The pellet was then discarded, the supernatant preserved to be filtered, lyophilized, and used for experiments. The same procedure was applied to obtain a blank sample, containing the digestive juices without MRM extract.

3.7 Statistical analyses

Statistical analyses were conducted utilizing GraphPad Prism 9 Software. The data derived from our experiments were presented as either the mean ± Standard Deviation (SD) or Standard Error of the Mean (SEM). Statistical analysis was conducted using One-way ANOVA, followed by Dunnett's or Unpaired Student's t-test. The differences between samples were considered statistically significant if p values were < 0.05.

4. RESULTS AND DISCUSSION

○ *Cydonia oblonga*

4.1 Partial chemical characterization and *in vitro* antioxidant activity of *Co* callus extract

All experiments have been conducted on a hydroalcoholic extract from *Cydonia oblonga* pulp callus, resuspended in a concentration of 30 mg dry weight / mL double-distilled water.
In order to analyze the biological activities of an extract, it is important to have a preliminary overview of the composition in phytocomplexes. For this reason, the total contents of phenols, and Vitamin C in the hydroalcoholic extracts of *Cydonia oblonga* calluses (*Co* callus extract) were measured. The data were obtained as reported in the Materials and Methods 3.3.1 section and results are presented in **Table 4**. Gallic acid was used as a reference substance for total phenols content, while ascorbic acid was the reference substance for determination of Vitamin C content.

Total Phenols (mg GAEq/g)	10.8 ± 0.08
Vitamin C (mg AAEq/g)	0.27 ± 0.006

Table 4. Total Phenols and Vitamin C content of *Cydonia oblonga* callus extract. Data are expressed as the mean ± Standard deviation (SD) of at least three replicates. GAEq = Gallic Acid Equivalence, Gallic Acid was used as a reference substance. AAEq = Ascorbic Acid Equivalence, Ascorbic Acid was used as a reference substance.

The results obtained and showed in **Table 4** represent the overall phenol compounds content and complement the HPLC-DAD-ESI-MS^n analysis of the *Co* callus extract phenol content already published[51] and obtained from Udine's research group. In this investigation, the quantities of specific phenolic compounds within our extract are assessed (**Table 5**).

Compounds	mg/g
5-CQA	0.72 ± 0.04
5-p-CoQA	0.12 ± 0.03
(-)-Epicatechin	0.02 ± 0.005
3-CQA	0.01 ± 0.004
Procyanidin B2	0.09 ± 0.002
4-p-CoQA	0.06 ± 0.003
(+)-Catechin	0.06 ± 0.002
Quercetin-3-O-glucoside	0.01 ± 0.001
Procyanidin B type dimmer	0.01 ± 0.001
Procyanidin B type dimmer	0.01 ± 0.001
Total	**0.91 ± 0.09**

Table 5. Specific phenolic compounds content of *Cydonia oblonga* callus extract obtained from lyophilized callus culture, identified and quantified using HPLC-DAD-ESI-MS^n. Data are expressed as the mean ± SD of three replicates[51].

Considering the good quantities of total phenols and the bioactive secondary metabolites content present in the *Co* callus extract, especially 5-O-caffeoylquinic acid (5-CQA) (an important class of chlorogenic acid), we evaluated potential antioxidant activity through various methods. Chemical antioxidant assays conducted *in vitro* can generally be categorized into two main types: those reliant on electron transfer (ET) and those centered around hydrogen atom transfer (HAT)[60]. Assays based on single electron transfer, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 2,2′-azinobis [3-ethylbenzothiazoline-6-sulfonic acid] (ABTS), gauge the release or transfer of an electron to a free radical, thereby transforming it into an anion. On the other hand, HAT reactions, such as ORAC, assess the antioxidant's ability to neutralize free radicals by releasing a hydrogen atom[61].

ABTS assay is widely recognized as a method for evaluating overall antioxidant activity and finds application across diverse samples including food and body fluids: generally, it shows a positive correlation and is strongly correlated with DPPH and ORAC results[60].

The DPPH assay is widely adopted as a prominent *in vitro* chemical antioxidant test, primarily due to its high sensitivity, technical simplicity, rapidity, accuracy, reproducibility, reliability, and the absence of the need for any special sample pre-treatment.

The ABTS assay, also known as the ABTS radical assay, is extensively employed for assessing the antioxidant activities of constituents in foods and beverages, owing to its versatility in both aqueous and lipid phases[62].

The ORAC method assesses the capability of antioxidants in the sample to safeguard the target from oxidative damage[63]. It has proven effective in assessing antioxidant capacity in various food items such as fruits, vegetables, and herbal mixtures as well as in biological systems[64,65].

An additional strength of the ORAC method lies in its ability to accommodate different free radical sources. This approach to assessment involves considering both the inhibition time and the extent of inhibition of free radical activity induced by antioxidants. Furthermore, the ORAC assay has several advantages such as the automation for high throughput, enhancing efficiency, and the adaptability to diverse sample matrices.

In **Table 6** we report the DPPH values expressed as DPPH Scavenging Activity (% DSA), ABTS values as ABTS Scavenging Activity (% ABTS SA) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) Equivalent Antioxidant Capacity (TEAC), while ORAC values as concentration of Trolox Equivalent (TEq). For DPPH and ABTS scavenging activity, EC_{50}, representing the concentration of extract necessary to achieve 50% free radical scavenging activity, was reported.

DPPH (% DSA)	73.8 ± 0.1	**EC_{50}** (µg/mL) = 300 ± 0.06
ABTS (% ABTS-SA)	16.1 ± 0.7	**EC_{50}** (µg/mL) = 19.3 ± 0.02
TEAC (mmol TEq/g)	15.9 ± 1.9	
ORAC (µmol TEq/g)	141.9 ± 2.8	

Table 6. Antioxidant activity of *Co* callus extract. Data are expressed as mean ± SD of three replicates.

The antioxidant capacity observed, is most likely due to the presence of polyphenols, and vitamin C in *Co* callus extract[66]. *Cydonia oblonga* callus extract is, infact, a phytocomplex, therefore, the observed biological activities are attributed to the synergy between various bioactive compounds present in the extract. The latter is particularly bioactive since it contains polyphenols and some triterpenic acids[51]. Moreover, several studies have shown that these

compounds have not only antioxidant but also other numerous properties including hypoglycemic, anti-inflammatory, and anti-cancer[67–69].

4.2 *In vitro* genoprotection ability of *Co* callus extract (nicking assay)

The genome of HaCaT cells was used to evaluate the effect of *Cydonia oblonga* callus extract on ultraviolet-C irradiated DNA (Materials and Methods 3.3.3 section). UVC is a widely used method for inducing DNA damage. HaCaT cell DNA was exposed to UVC at 0, 1, 5, 10, and 15 min, and the irradiation caused partial or complete DNA degradation. Genomic DNA of HaCaT cells appears as a smear in UVC-exposed samples, less evident in the samples with *Co* extract treatment (280 µg/mL). As reported in **Figure 6**, at 280 µg/mL, *Co* callus extract has genoprotective effects since it provides partial protection against UVC ray damage.

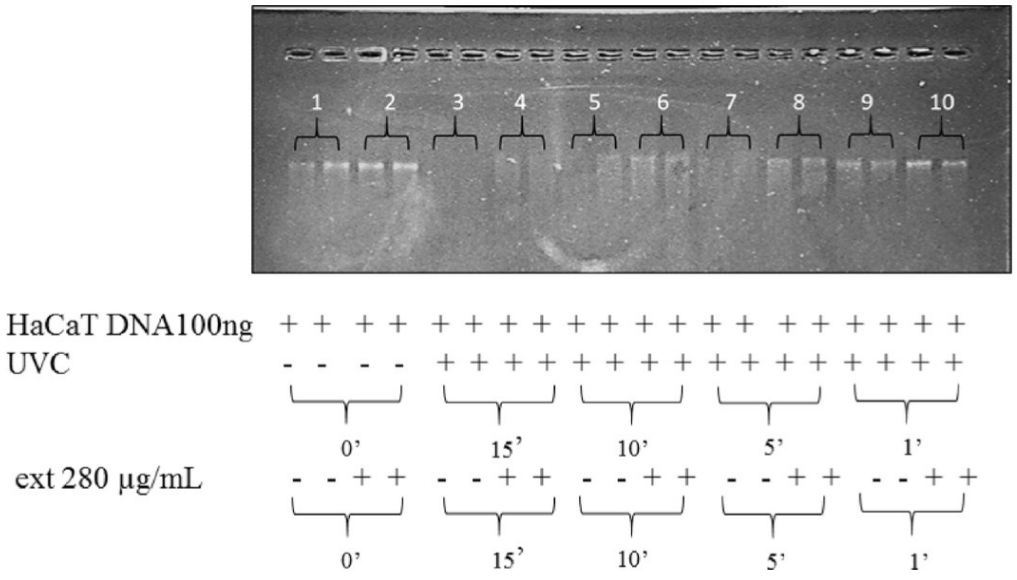

Figure 6. Protective capacity of *Co* callus extract on UVC damaged HaCat cell DNA. Each treatment was performed in duplicate. Samples were treated as following: **1**: HaCaT 100 ng, **2**: HaCaT 100 ng + *Co* ext 280 µg/mL, **3**: HaCaT 100 ng + UVC 15 min, **4**: HaCaT 100 ng + UVC 15 min + *Co* ext 280 µg/mL, **5**: HaCaT 100 ng + UVC 10 min, **6**: HaCaT 100 ng + UVC 10 min + *Co* ext 280 µg/mL, **7**: HaCaT 100 ng + UVC 5 min, **8**: HaCaT 100 ng + UVC 5 min + *Co* ext 280 µg/mL, **9**: HaCaT 100 ng + UVC 1 min, **10**: HaCaT 100 ng + UVC 1 min + *Co* ext 280 µg/mL.

In order to estimate the amount of DNA loss after UVC exposure, a quantitative Real-Time PCR of the previously treated samples was performed: a 100 bp region from the NADH Dehydrogenase subunit 1 was amplified.

Figure 7 suggests that *Co* callus extract (280 µg/mL) has a statistically significant genoprotective effect during a UVC exposure of 15 min (corresponding to 30,000 µJ/ cm^2). In fact, DNA damage decreases in tandem with the reduction in UVC exposure time, and the genoprotective effect of the extract is maintained although the results are not statistically significant.

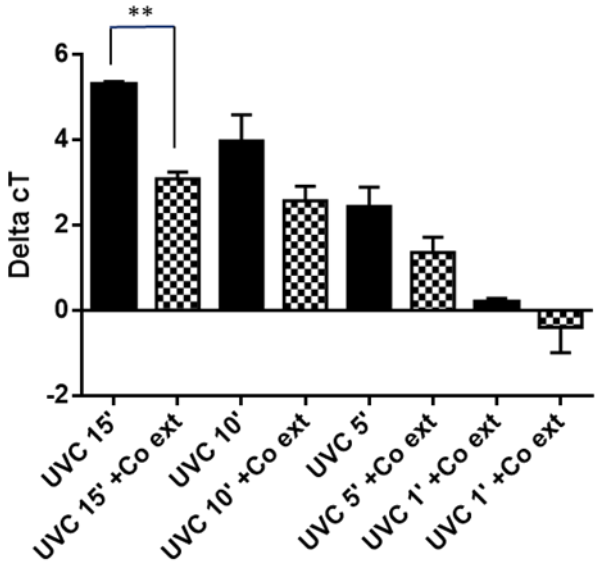

Figure 7. Quantitation of genoprotective effect of *Co* callus extract by qRT-PCR. The results are expressed as mean ± SD of three independent experiments. cT of control DNA (non-treated) was subtracted to obtain a delta. (** $p < 0.01$; Unpaired Student's t-test was performed between UVC-only treated samples and UVC + 280 µg/mL *Co* callus extract-treated samples).

4.3 Anti-inflammatory and antioxidant effects of *Co* callus extract in cell-based models

RAW 264.7 (murine macrophages) and HaCaT cell (keratinocytes) lines were used for preliminary studies of *Co* callus extract and cell viability assay (WST-8) was first performed in order to determine a range of concentrations that does not damage the cells. Data showed that the tested concentrations (0.11 – 3.56 mg/mL) were not cytotoxic and that *Co* callus extract concentrations of 3.56 mg/mL and 1.78 mg/mL increased cell viability of RAW 264.7 and HaCaT cells, respectively (**Figure 8**).

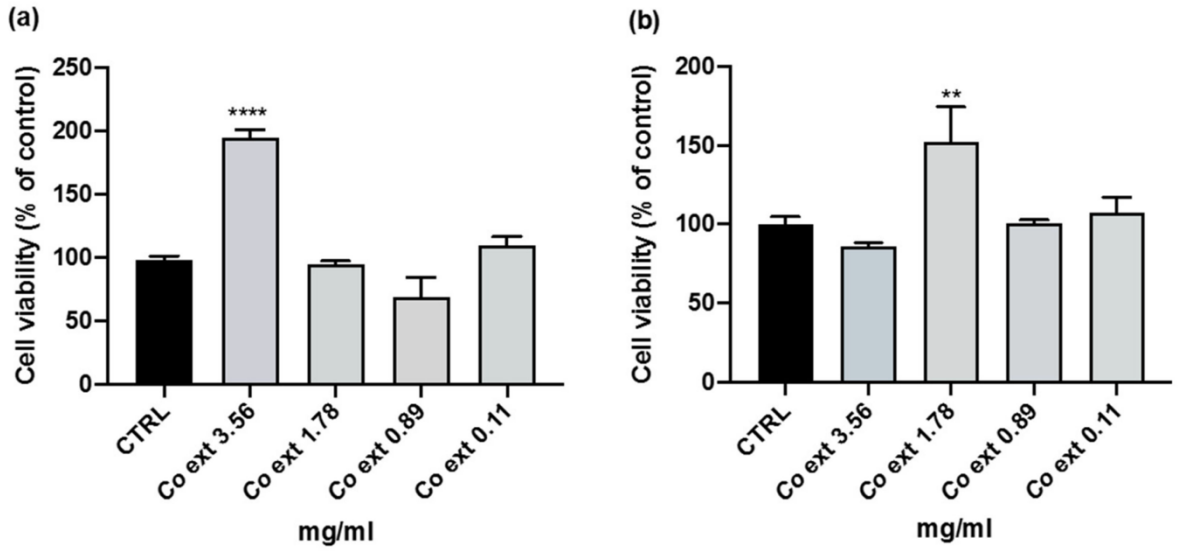

Figure 8. Effect of *Co* callus extract on Raw 264.7 and HaCaT cell viability. Various concentrations of *Co* callus extract (0 – 4 mg/mL) were used to treat both cell lines - Raw 264.7 **(a)** and HaCaT **(b)** - during 24 h and cell viability was evaluated by WST-8 assay. Results are presented in comparison to CTRL (non-treated cells) and are expressed as means ± SEM of three independent experiments. (** $p < 0.01$, **** $p < 0.0001$; ANOVA followed by Dunnett's multiple comparison test was performed).

4.3.1 Anti-inflammatory activity of *Co* callus extract on RAW 264.7 and HaCaT cells

As previously seen, our *Co* callus extract is rich in secondary metabolites and these bioactive compounds are known to have several biological activities such as anti-inflammatory. For this purpose, we decided to assess the anti-inflammatory activity of *Cydonia oblonga* callus extract on both macrophage and keratinocyte cell lines in a model of lipopolysaccharides (LPS) damage. It has been, in fact, demonstrated that nitric oxide released by LPS-stimulated macrophages is a marker for both acute and chronic inflammation.

Macrophages are essential in inflammation processes since they defend the body from external pathogens via a phagocytosis mechanism. They release various inflammatory mediators such as NO, tumor necrosis factor-alpha (TNF-α), and IL-1. LPS-stimulated macrophages produce iNOS, an enzyme that catalyzes the oxidative deamination of L-arginine to produce NO. The excessive NO generation may have adverse effects like septic shock and inflammatory diseases[70,71].

Regarding our experimental protocol, firstly, we performed a Griess assay, on RAW 264.7 cells, to evaluate the NO release after a treatment with bacterial lipopolysaccharides; the anti-inflammatory molecule dexamethasone (DEXA) was used as positive control (Materials and

Methods 3.4.4 section). From the results reported in **Figure 9.a**, the co-treatment overnight with 0.001 mg/mL of LPS and *Co* callus extract at 0.89; 1.78; and 3.56 mg/mL, significantly reduces NO release.

To rule out the possibility that NO reduction resulted from a decrease in RAW 264.7 cell viability following LPS treatment, a cell viability assay (WST-8) was performed. **Figure 9.b** shows that except for LPS-only treated cells, co-treated (LPS with DEXA or *Co* callus extract) cells are comparable to untreated ones (CTRL).

In conclusion, our results demonstrate that *Cydonia oblonga* callus extract has no cytotoxic effect on RAW 264.7 cells and possesses a potent anti-inflammatory property.

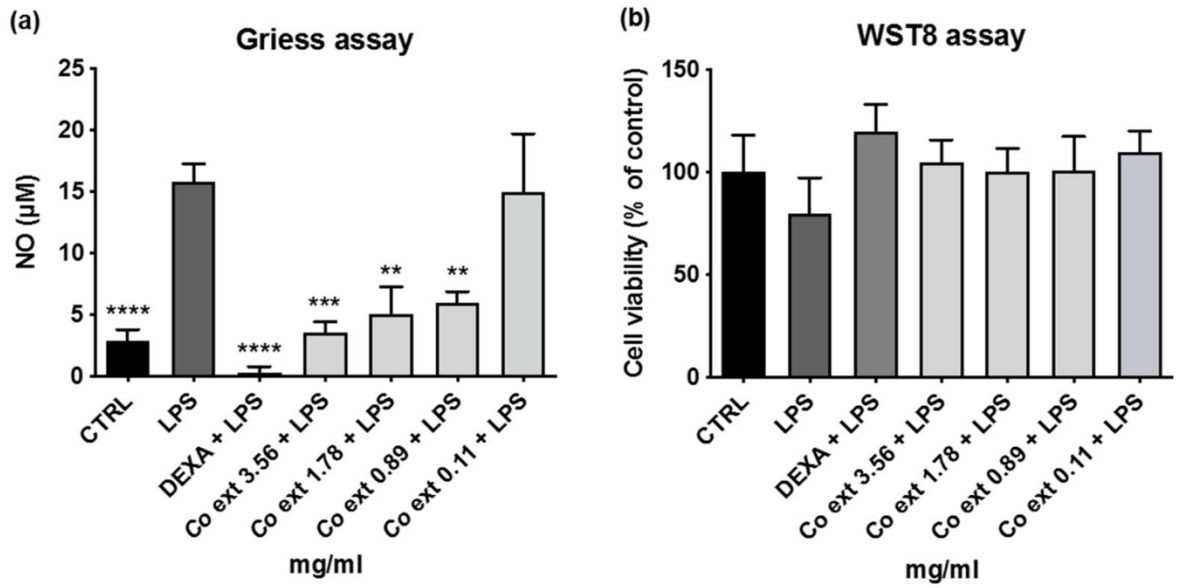

Figure 9. Evaluation of extracellular NO release and cell viability of RAW 264.7 cells after LPS, *Co* callus extract, and DEXA treatment. **(a)** RAW 264.7 cells were co-treated overnight with 0.001 mg/mL LPS either in the absence or presence of *Co* extract at different concentrations or 0.0039 mg/mL DEXA. CTRL: untreated cells. The results are expressed as NO reduction compared to LPS-treated cells (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ANOVA followed by Dunnett's multiple comparison test was performed). **(b)** Cell viability assay on Raw 264.7 cells co-treated with LPS and *Co* callus extract (WST-8). The results are expressed as % of CTRL ± SEM of three independent measurements (ANOVA followed by Dunnett's multiple comparison test was performed; No statistical differences were found).

Keratinocytes (HaCaT) play a role in epidermis homeostasis; they constitute a barrier against external threats. These cells can produce proinflammatory mediators such as chemokines and cytokines and therefore can participate to the skin wound healing process[72]. As it is important to maintain the barrier function of the skin, we assessed the anti-inflammatory activity of *Cydonia oblonga* callus extract on HaCaT cells considering our upcoming research to assess the extract's potential in the management of skin-related diseases. Through qRT-PCR, we

evaluated the expression of inflammation-related genes (iNOS, IL-6, IL-1β, ikBα, and ICAM) after the overnight co-treatment with 0.001 mg/mL LPS in the presence or absence of *Co* callus extract (Materials and Methods 3.4.5 and 3.4.6 section). Data showed that these genes were downregulated by the co-treatment with 0.001 mg/mL of LPS and 1.78 mg/mL of *Co* callus extract (**Figure 10**). This downregulation suggests that the extract acts through the Nuclear Factor kappa B (NF-κB) signaling pathway.

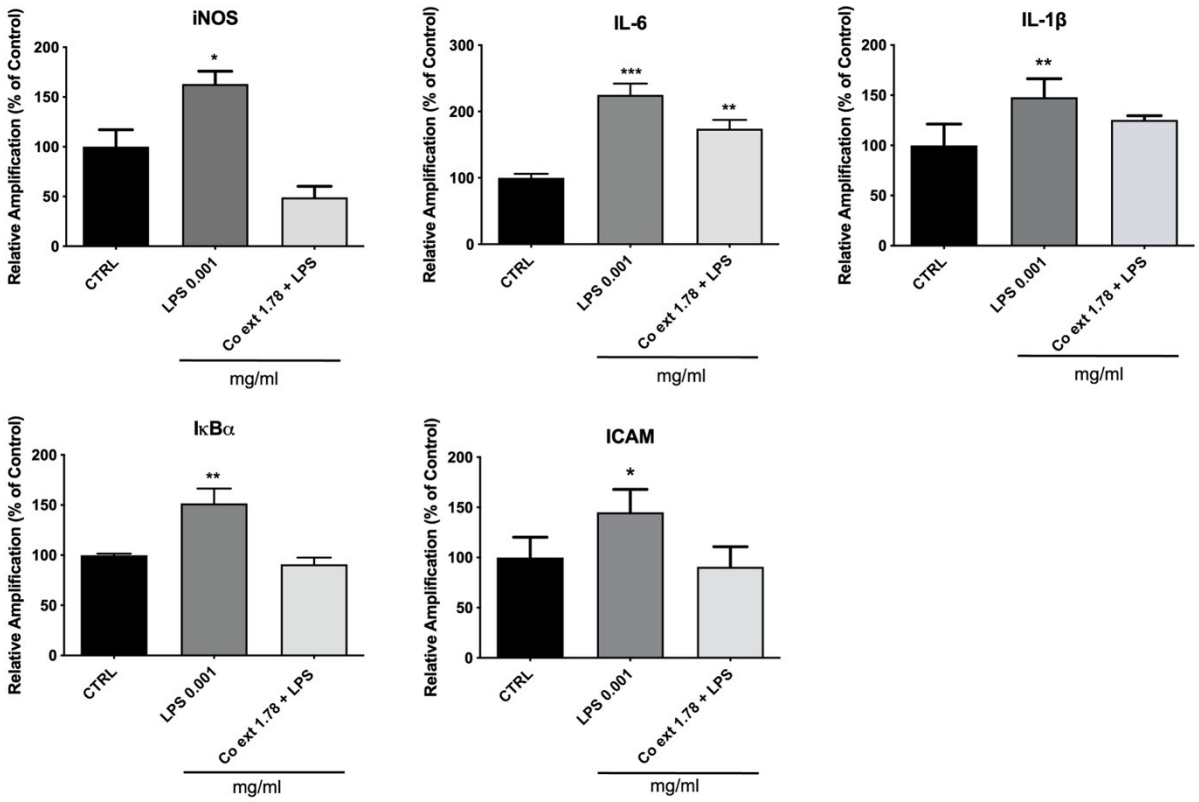

Figure 10. Anti-inflammatory effect of *Co* callus extract on LPS-treated HaCaT cells. Cells were co-treated overnight with LPS 0.001 mg/mL in either the presence or absence of 1.78 mg/mL *Co* extract. *Cydonia oblonga* callus extract significantly decreases the expression of the genes upregulated by LPS. Results are presented in comparison to CTRL and are expressed as the means ± SEM of three independent experiments. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ANOVA followed Dunnett's multiple comparison test was performed).

All experiments conducted on inflammation support the good anti-inflammatory activity of our extract. They further indicate that this activity is expressed through different pathways both genetic and biochemical.

4.3.2 Antioxidant activity of *Co* callus extract on HaCaT cells

Due to the high concentration of secondary metabolites in *Cydonia oblonga* callus extract and considering the positive impact of these substances on human health, the antioxidant capacity of the extract was also investigated on cellular models. The antioxidant activity was already tested in cell-free assays, but the following experiment will enable us to corroborate or complete the results.

The assay was performed on HaCaT cells using a cell-permeable fluorogenic probe 2',7'-Dichlorofluorescein-diacetate (DCF-DA) for the detection of oxidative stress (Materials and Methods 3.4.3 section). In the presence of ROS, DCF-DA transforms into the highly fluorescent compound, 2',dichlorofluorescein (DCF). Intracellular ROS production was induced using tert-butyl hydroperoxide (TBHP) and hydrogen peroxide (H_2O_2), two treatments widely used in oxidative stress studies.

The results obtained, show overnight pre-treatment with *Co* callus extract remarkably and significantly decreases ROS production at the basal levels in comparison to control (CTRL) and in the absence of oxidative stimulus (**Figure 11.a**). As reported in **Figure 11.b,c**, both treatments -TBHP and H_2O_2- of 30 min induced ROS production, which was subsequently significantly reduced by the extract at all the tested concentrations (0.22 – 1.78 mg/mL). These findings supported the antioxidant activity of *Co* callus extract, which had been previously noted in cell-free models and evaluated by the DPPH, ABTS, and ORAC tests.

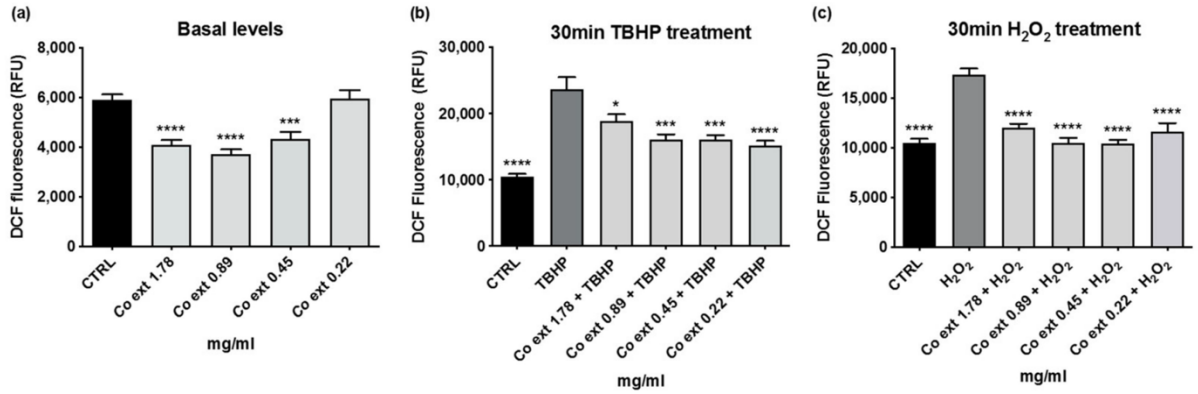

Figure 11. Antioxidant effect of *Co* callus extract in HaCaT cells. The intracellular ROS production was evaluated using the DCF-DA assay in cells treated with different concentrations of *Co* callus extract. ROS production was observed **a)** after overnight treatment with 0 (=CTRL); 1.78; 0.89; 0.45; 0.22 mg/mL *Co* callus extract without any oxidative challenge; **b)** after 30 min treatment with 0.3 mM TBHP; **c)** after 30 min treatment with 0.1 mM H_2O_2. Data represent the mean ± SEM of at least three independent experiments. (* $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$; ANOVA followed Dunnett's multiple comparison test was performed compared to CTRL **(a)**, TBHP **(b)**, H_2O_2 **(c)**.

In conclusion, the *Co* callus extract rich in polyphenols is a good antioxidant that can prevent or slow down free radicals' oxidation. Through many processes, including inhibition of enzymes implicated in the generation of reactive oxygen species, and upregulation or protection of antioxidant mechanisms, it can scavenge various ROS.

4.4 Inhibitory activity of *Co* callus extract on digestive enzymes

Polyphenols are a class of bioactive compounds particularly important to human nutrition. Recently, they have been recognized as key phytochemicals to prevent and treat Type 2 Diabetes. Their activity is associated to the inhibition of carbohydrates hydrolyzing enzymes (such as α-glucosidase and α-amylase), the repair of insulin secretory processes in pancreatic cells, and the restoration of antioxidant mechanisms[73]. Moreover, polyphenols have been shown to inhibit digestive enzymes involved in the hydrolysis of lipids, lipases. In fact, anti-obesity treatments typically target pancreatic lipase. Its inhibition can reduce blood fat levels since it hydrolyzes about 70% of dietary fats[74].

For this reason, we wanted to test the inhibition activity of *Co* callus extract at different concentrations (125 – 1000 µg/mL) on these three enzymes (Materials and Methods 3.5 section). Acarbose was used as a positive control for α-glucosidase and α-amylase, and Orlistat for lipase (**Figure 12**).

The results show that *Cydonia oblonga* callus extract inhibited α-glucosidase activity *in vitro* in a concentration-dependent manner (**Figure 12.a)**; in fact, α-glucosidase activity significantly decreases as extract concentration increases. At a concentration of 250 µg/mL, *Co* callus extract, decreased the enzyme's activity by about 62 % (IC_{50} of 250 ± 2 µg/mL).

In **Figure 12.b,** the *Co* callus extract demonstrates a moderate inhibition of α-amylase activity of about 20 %, not dose-dependent to the extract concentration (IC_{50} undeterminable). A modest inhibitory activity against α-amylase is important because is suggested to avoid the bacteria in the colon, fermenting undigested polysaccharides, which causes diarrhea and flatulence[75].

As previously reported by Oboh *et al.*[41], chlorogenic acid is able to inhibit α-amylase and α-glucosidase. Obtained results by De Bellis *et al.*[51], showed that the major phenolic compound present in *Co* callus extract is 5-O-caffeoylquinic acid (5-CQA), an important class of chlorogenic acid. Consequently, all these results suggest a potential hypoglycemic activity of the *Cydonia oblonga* callus extract, making it a promising potential cure for postprandial hyperglycemia.

Finally, we decided to assess the capacity of our phytochemical-rich extract to regulate the lipolytic enzyme, lipase. **Figure 12.c** shows that *Cydonia oblonga* callus extract moderately inhibited lipase activity *in vitro* in a concentration-dependent manner (IC_{50} of 1.99 ± 0.005 mg/mL). Orlistat-induced lipase inhibition reduces the amount of absorbed dietary fat, which increases the caloric deficit. Unfortunately, this synthetic agent has adverse reactions and the development of a natural anti-lipase inhibitor as an anti-obesity drug is more beneficial[47].

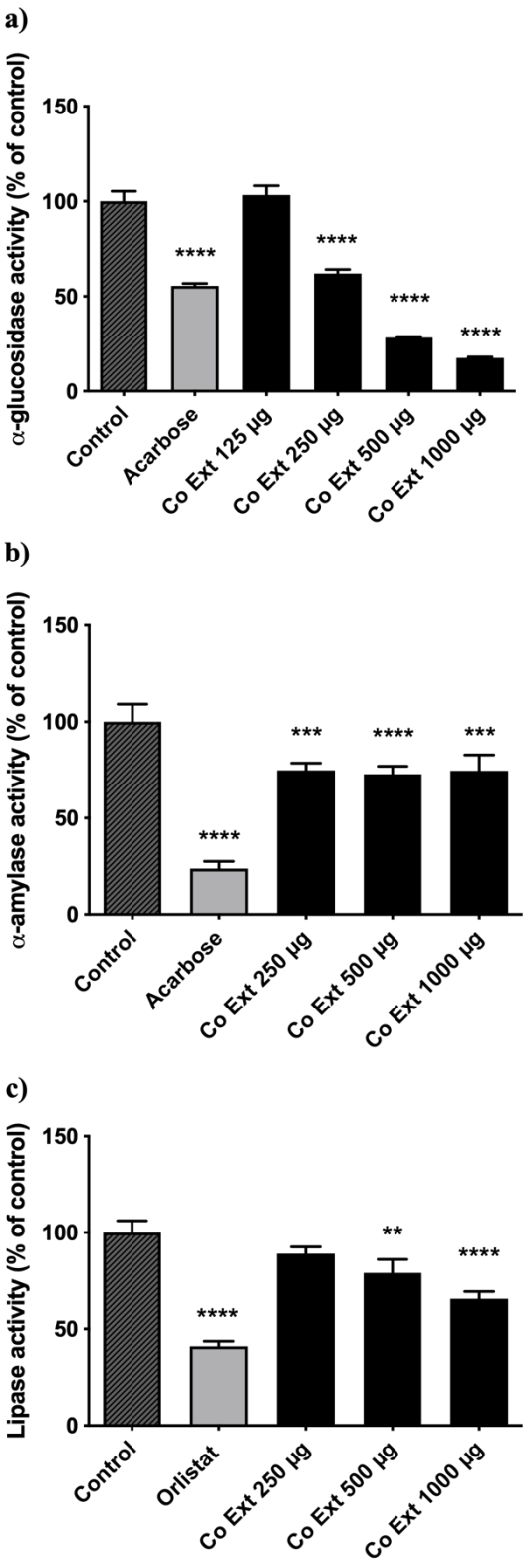

Figure 12. Inhibitory activity of *Co* callus extract on digestive enzymes. a) α-glucosidase; **b)** α-amylase and **c)** Lipase activities are expressed as % of the control that represents the 100% activity of the enzymes. 1 mM Acarbose was used as a reference compound for **a)** and **b)**; 5 µg Orlistat was used as a reference compound for **c)**. Data are the mean ± SD of four independent experiments. (** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ANOVA followed Dunnett's multiple comparison test was performed).

○ Mela Rosa Marchigiana

4.5 *In vitro* gastrointestinal digestion, partial chemical characterization, and *in vitro* antioxidant activity of MRM callus extract

In this second part of the Results, we focused our research on a hydroalcoholic extract from the calluses obtained from Mela Rosa Marchigiana (MRM) apple pulp (of Materials and Methods 3.2 section) in order to get an overview of the biological activities of this sample. All experiments have been conducted with MRM pulp callus extract, suspended in a concentration of 50 mg dry weight / mL 70 % ethanol (v/v).

As previously reported by Verardo *et al.*[22], the MRM callus is characterized by various secondary metabolites, mostly tormentic, maslinic, corosolic, annurcoic acid, and ursolic acid in a concentration of 29; 9.6; 6.8; 3.5; and 1.7 µg/mg callus, respectively. The presence of high amounts of triterpenic acids has prompted us to favor the resuspension of this extract in 70% ethanol (v/v) rather than water. Previous studies have shown that the first three triterpenic acids (tormentic, maslinic, and corosolic acid) have biological properties such as anti-diabetic, anti-inflammatory, and antioxidant activity simultaneously[68,69,76], while annurcoic acid (a bioavailable form of ursolic acid) is characterized by an antioxidant activity[32,77].

With MRM callus extract, we decided to attempt a transition toward a system that mimics an *in vitro* gastrointestinal digestion. The shift to systems simulating *in vitro* digestion represents a further step toward a more accurate and complex representation of physiological dynamics. The simulation of *in vitro* digestion could be essential for analyzing how chemical compounds interact and transform during the human digestive process. This transition is particularly significant in pharmaceutical and food domains, where a detailed understanding of the bioactivity of substances is essential for the development of effective drugs and the production of functional foods[58]. This advanced approach paves the way for new discoveries and practical applications in critical sectors such as medicine and nutrition, contributing to the progress of science and the improvement of quality of life.

The *in vitro* gastrointestinal digestion was performed as described in Materials and Methods 3.6 section; the obtained material (MRM digesta) was compared to undigested sample (MRM callus extract), and the total phenol content (**Table 7**) and antioxidant activities (**Figure 13**) of

both samples (undigested and digested) were assessed. The digestive juices have been also tested and considered as a blank for MRM digesta samples and results are expressed in Gallic Acid Equivalent concentrations (GAEq).

As regards to phenols content data showed that, the amount of phenols after *in vitro* gastrointestinal digestion is conserved, attesting that the digestive process does not alter the assayed molecules. In fact, there are no statically significant differences between the two values of MRM callus extract and digesta's total phenols concentration.

	mg GAEq/g
MRM Callus Extract	1.19 ± 0.17
MRM Digesta	1.21 ± 0.01

Table 7. Total phenol content of undigested (MRM callus extract) and digested (MRM digesta) samples. GAEq= Gallic Acid Equivalent. The two values are not statistically different (Unpaired Student's t-test was performed).

In order to evaluate the potential antioxidant activity of MRM callus extract, and to compare its biological activity before and after the *in vitro* gastrointestinal digestion, DPPH, ABTS and ORAC methods were conducted as described in details in 3.3.2 Materials and Methods section and discussed in 4.1 Results section.

Table 8 reports the scavenging activity of MRM callus extract through DPPH and ABTS assays and the EC_{50} values of each one. In particular, MRM callus extract showed a DPPH scavenging activity percentage (% DSA) of 45 ± 2.1 (EC_{50} = 20.3 ± 1.4 mg/mL) while the MRM digesta presented a % DSA of 44 ± 0.7 (EC_{50} = 16.3 ± 5.4 mg/mL).

The ABTS scavenging activity percentage (% ABTS SA) of MRM callus extract was 28 ± 1.5 (EC_{50} = 0.23 ± 0.02 mg/mL) while the MRM digesta presented a % ABTS SA of 38 ± 0.04 (EC_{50} = 0.17 ± 0.03 mg/mL). Statistical analyses were performed to compare the resultant values from MRM callus extract and MRM digesta, revealing no significant differences for the EC_{50} values. This underscores the preservation of antioxidant activity even after the *in vitro* digestion process (**Table 8**).

	MRM Callus Extract	MRM Digesta
DPPH (% DSA)	45 ± 2.1	44 ± 0.7
DPPH EC_{50} (mg/mL)	20.3 ± 1.4	16 ± 5.4
ABTS (%ABTS SA)	28 ± 1.5	38 ± 0.04 *
ABTS EC_{50} (mg/mL)	0.23 ± 0.02	0.17 ± 0.03

Table 8. Scavenging activity of undigested (MRM callus extract) and digested (MRM digesta) samples. No significant differences were found between MRM callus extract and MRM digesta of each series except for % ABTS SA (* $p < 0.05$; Unpaired Student's t-test was performed).

These samples were also subjected to ORAC test to compare their capacity to safeguard a target from oxidative damage and in this case, MRM callus extract resulted comparable to MRM digesta (51.6 ± 4.6 vs 47.9 ± 5.0 μmol Trolox Equivalent (TEq), respectively) with no significant statistical differences. Trolox, a water-soluble derivative of vitamin E, is used as a reference substance.

In **Figure 13,** data regarding DPPH, ABTS and ORAC assays are reported as Trolox Equivalent (TEq) concentration, so the outcomes of the three test results can be better compared.

In conclusion, the results showed that the antioxidant capacity of Mela Rosa Marchigiana callus extract is maintained after the *in vitro* gastrointestinal digestion with no significant statistical differences between the two samples. This can be explained by the fact that the phytocomplex, present in the extract and responsible for these biological activities, has probably not been modified during the process and remain stable.

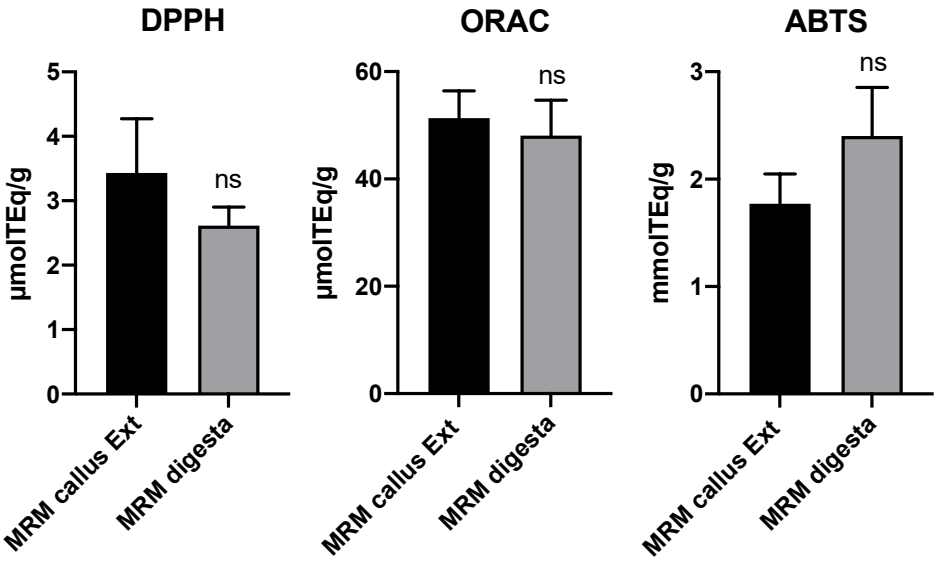

Figure 13. Comparison between antioxidant activity of undigested (MRM callus extract) and digested (MRM digesta) samples by DPPH, ORAC, and ABTS assays. Data are expressed as the concentration of Trolox Equivalent (TEq) / gram of callus extract. (Unpaired Student's t-test was performed; ns: non significant).

Since the antioxidant activity of the two samples remains comparable, and considering that, the presence of digestive juice would have introduced interference, we chose to proceed the following studies using MRM callus extract sample.

4.6 *In vitro* genoprotection activity of MRM callus extract (nicking assay)

The cellular DNA is consistently subjected to oxidative stress and free radicals, such as hydroxyl radicals (·OH). Free radicals can be generated through both cellular metabolism and external aging factors and have the potential to cause oxidative DNA damage. Consequently, there has been significant interest in discovering defense mechanisms that inhibit or delay this oxidative DNA damage. Hydroxyl radicals are generated through the Fenton reaction induced by H_2O_2 and Fe^{2+}. Based on the Fenton reaction (that involves Fe_2SO_4 and H_2O_2), and the generation of hydroxyl free radicals from intracellular iron, the DNA nicking assay mimics *in vivo* the physiological conditions. In this experiment, the formation of hydroxyl radicals induces the plasmid DNA's initial covalently closed circular (CCC or Form I) to change to both open circular (OC or Form II), and linear (L or Form III) forms, each presenting various

electrophoresis gel mobility characteristics (CCC being the fastest form and OC the slowest one)[55].

In previous research, Mela Rosa Marchigiana callus extract, at concentrations ranging from 0.2 mg/mL to 130 mg/mL, was demonstrated to have a genoprotective effect on oxidatively damaged plasmid DNA[78]. Therefore, we decided to reiterate the process with a less concentrated MRM callus extract (from 150 to 2.3 µg/mL) (**Figure 14**). In this experiment, we created a condition where the plasmid DNA in the form of CCC (CTRL) is degraded and converted to the OC form (oxidant system) and then protected by adding MRM callus extract (**Figure 14.a**). **Figure 14.b** shows that MRM callus extract at range concentrations of 150 – 4.7 µg/mL significantly reduces DNA damage.

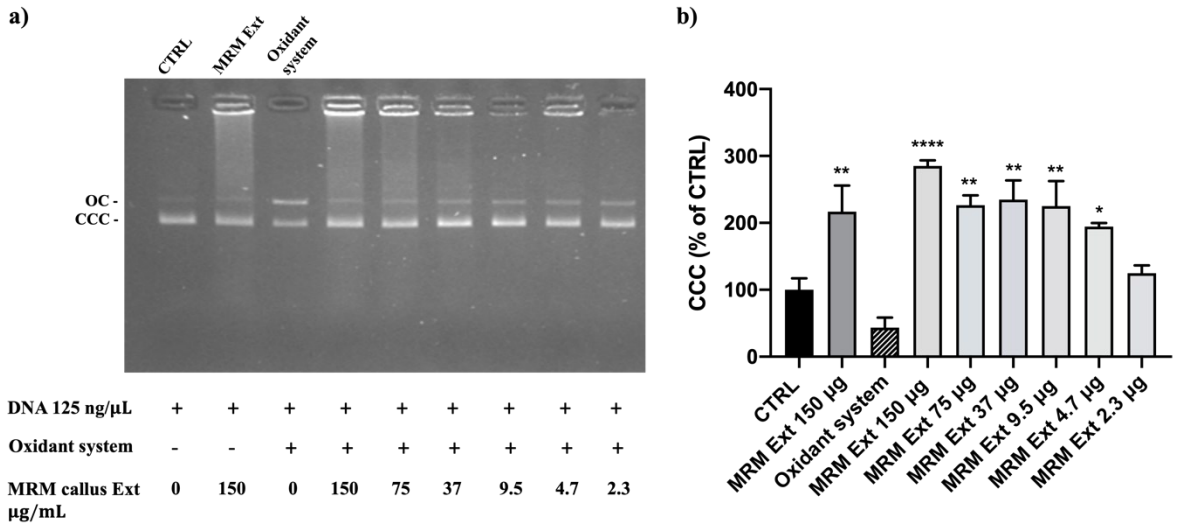

Figure 14. Genoprotective activity of MRM callus extract against DNA strand cleavage using the DNA nicking assay. a) Agarose gel of the pGEM plasmid after incubation with the oxidant system in the presence of various concentrations of MRM callus extract. **b)** Data represent the quantification of gels obtained by the assay and are expressed as the percentage ratio between the volume of the DNA band in CCC form after incubation with the oxidant system and the DNA band volume without oxidant (CTRL). Data represent the mean ± SEM of three independent measurements. (* $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$; ANOVA followed Dunnett's multiple comparison test was performed).

4.7 Antioxidant and anti-inflammatory activities of MRM callus extract on cell-based models

As a preliminary study on a cell model, we exposed HaCaT cells to different concentrations of Mela Rosa Marchigiana callus extract (0.36 – 143 µg/mL) for 24 h and evaluated the cellular

response to this extract performing a cell viability assay, the WST-8 assay (Materials and Methods 3.4.2 section). **Figure 15** shows that none of the tested concentrations were cytotoxic.

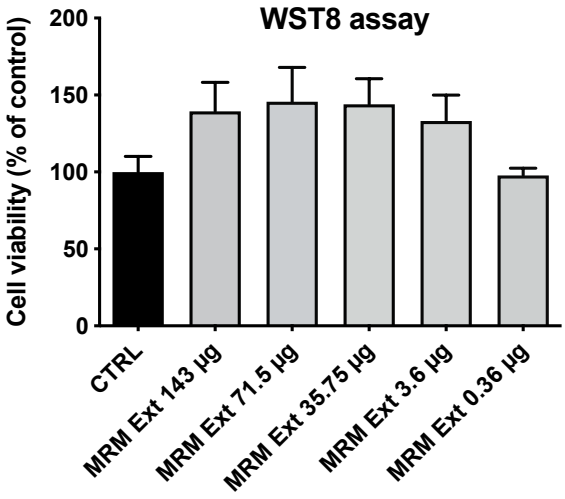

Figure 15. Effects of MRM callus extract on HaCaT cell viability. No statistical differences were found between the samples (ANOVA followed Dunnett's multiple comparison test was performed).

4.7.1 Antioxidant activity of MRM callus extract on HaCaT cells

As mentioned before, Mela Rosa Marchigiana is part of the overlooked species, and it has been demonstrated that this apple possesses numerous biological activities due to its phytochemical content. In fact, its fruits are rich in polyphenols like hydroxycinnamic acids and flavonols, as well as triterpenic acids like ursolic and annurcoic acids. In various studies using animal models of renal ischemia/reperfusion injury; carbon tetrachloride-induced hepatotoxicity; MRM extracts have demonstrated anti-inflammatory and oxidative stress properties[79].

The study of its properties on cell models is very important since it can provide us with additional information on the biological processes that characterize this apple.

Previous results shown in **Figure 15**, determined the non-cytotoxic range of MRM callus extract concentration (143 - 0.36 µg/mL) and, for this reason, all further experiment are carried out within the tested concentration range.

The antioxidant activity of MRM callus extract was assessed on HaCaT cells, performing the DCF-DA assay to evaluate the intracellular ROS levels as already described in Materials and Methods 3.4.3 section and discussed in 4.3.2 Results section. This evaluation was performed both before (basal level) and after the oxidative stress (**Figure 16**). Data show that ROS generation resulted unvariable in the basal levels, represented by the only presence of MRM

extract in different increasing concentrations, and without oxidative damage (**Figure 16.a**). When the oxidative stress was induced by H_2O_2, Mela Rosa Marchigiana callus extract significantly attenuated the ROS production (**Figure 16.b**). These results confirmed the antioxidant activity of the extract in the concentration range of 143 to 2.24 µg/mL, in HaCaT cell model.

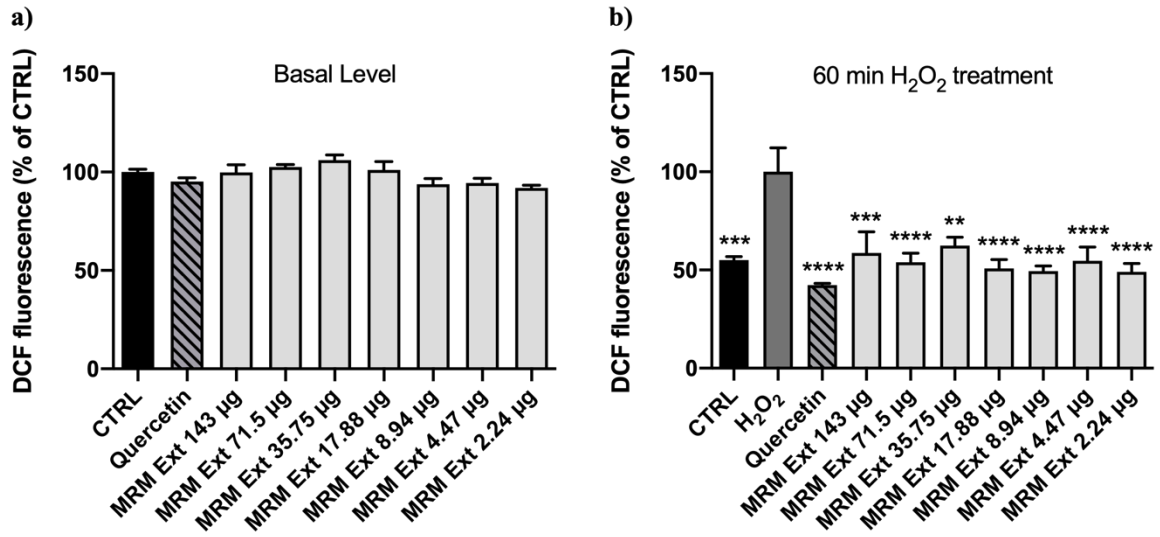

Figure 16. ROS levels in HaCaT cells. a) ROS levels after overnight incubation with increasing amounts of MRM callus extract (CTRL = without MRM) (ANOVA followed Dunnett's multiple comparison test was performed; No statistical differences were found). **b)** further introduction of 100 µM H_2O_2 for 60 min. Results are presented in comparison to the H_2O_2-treated sample. 50 µM Quercetin was used as a reference compound. Data represent the mean ± SEM of at least three independent measurements. (** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ANOVA followed Dunnett's multiple comparison test was performed).

The heme oxygenase-1 (HO-1) is an important enzyme in the heme degradation pathway but is also considered the most sensitive marker of cellular oxidative stress. HO-1 is the inducible isoform of HO and possesses binding sites for several transcription factors including the nuclear factor erythroid 2-related factor 2 (Nrf2), consequently, it is considered as an Nrf2-regulated gene. The induction of HO-1 is a key cellular defense mechanism against oxidative stress, inflammation, and various pathological conditions[80].

In order to assess the antioxidant activity of MRM callus extract on a HaCaT cell model, a qRT-PCR for HO-1 was carried out (Materials and Methods 3.4.5 and 3.4.6 sections). HaCaT cells were co-treated overnight with 100 µM H_2O_2 in the presence or absence of 17.88 µg/mL MRM callus extract (**Figure 17**). The results show that H_2O_2 increases the gene expression of HO-1, whereas the overnight pre-treatment with MRM callus extract reduces its gene expression.

These data strengthen and confirm once again the antioxidant activity of Mela Rosa Marchigiana.

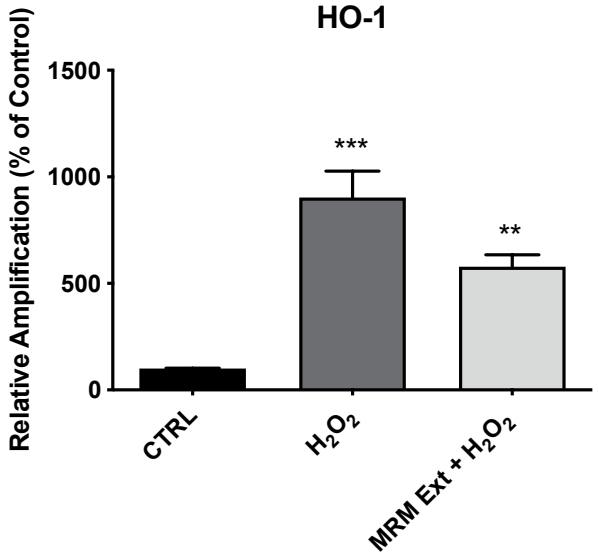

Figure 17. Heme oxygenase-1 gene expression in HaCaT cells treated with H_2O_2 and MRM callus extract. HaCaT cells were co-treated overnight with 100 µM H_2O_2 in the presence or absence of 17.88 µg/mL MRM callus extract. Results are presented in comparison to untreated control cells (CTRL) and are expressed as the mean ± SEM of at least three independent measurements. (** $p < 0.01$ *** $p < 0.001$; ANOVA followed Dunnett's multiple comparison test was performed).

Likewise, Yousefi-Manesh *et al.*[79] have demonstrated that a pre-treatment with MRM apple pulp extract reduces the pro-inflammatory and oxidative stress markers (TNF-α, IL-1β, and malondialdehyde) levels. Moreover, natural polyphenolic molecules have been shown to exhibit anti-apoptotic properties, thus decreasing neuronal cell death after a cerebral ischemic stroke through a series of events including the reduction of reactive oxygen release, and the upregulation of anti-oxidative stress genes like Nrf2 and HO-1[79].

4.7.2 Anti-inflammatory activity of MRM callus extract on RAW 264.7 cells

To further complete our study on the biological properties of MRM callus extract, we assessed its potential anti-inflammatory activity on RAW 264.7 cells and performed the Griess test, which measures in murine macrophages the release of nitric oxide (NO), induced by bacterial lipopolysaccharides (Materials and Methods 3.4.4 section). From the results reported (**Figure 18.a**), the administration of 1 µg/mL of LPS led to NO production while NO could not be

quantified in untreated control cells (CTRL). When Raw 264.7 cells were co-treated overnight with different concentration of MRM callus extract (150 – 2.34 µg/mL) and LPS, a significant reduction of LPS-induced NO release was observed at both 150 and 75 µg/mL concentrations, while lower concentrations are not statistically significant.

To ensure that this reduction in NO production is secondary to the anti-inflammatory effect of MRM callus extract rather than cell death, we performed a viability test (MTT assay). **Figure 18.b** shows that LPS treatment has not altered cell viability and the previously observed reduction in NO release is not caused by cell death but is a consequence of the protective and anti-inflammatory effect of the extract.

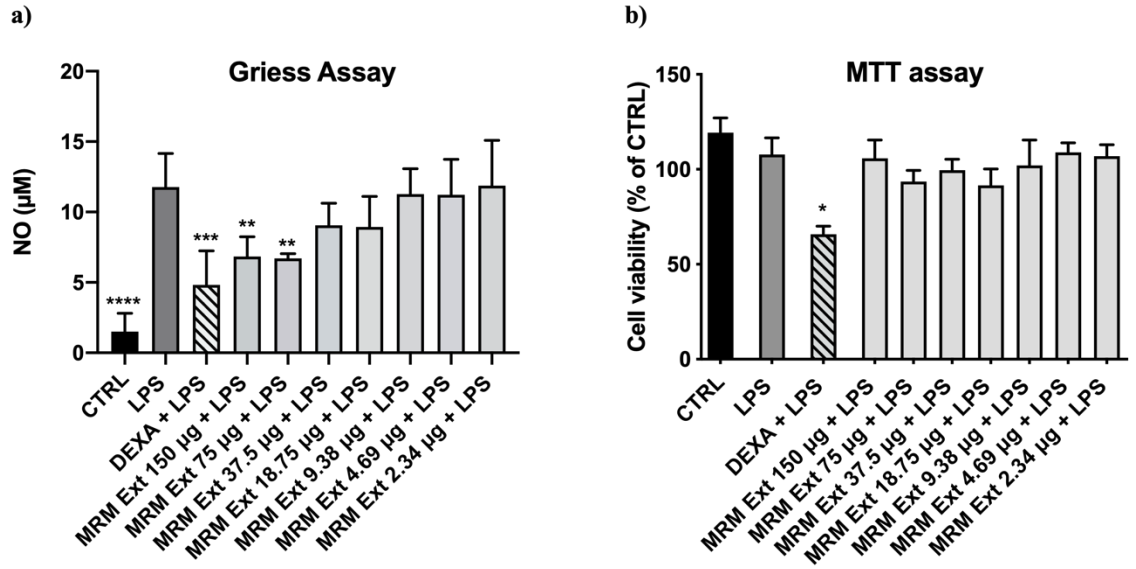

Figure 18. Anti-inflammatory activity of MRM callus extract. a) Anti-inflammatory activity of MRM on Raw 264.7 cells. The results are presented as Nitric Oxide reduction compared to the cells treated with 1 µg/mL lipopolysaccharides (LPS). 10 µM Dexamethasone (DEXA) was used as an anti-inflammatory reference compound. The data show the means ± SEM of all samples compared to LPS (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ANOVA followed Dunnett's multiple comparison test was performed). **b)** Cell viability on LPS-MRM treated Raw 264.7 cells. The results are expressed as % of CTRL ± SEM of three independent measurements (* $p < 0.05$; ANOVA followed by Dunnett's multiple comparison test was performed).

Furthermore, Yousefi-Manesh *et al.*[79] demonstrated that triterpenic and ursolic acids have been shown to decrease lipid peroxidation and the size of infarts as well as to inhibit the expression of Toll-Like Receptor 4 and NF-κB following the injury, exhibiting, thus, anti-inflammatory properties. In addition, the anti-inflammatory capacity of apple-extracted polyphenols was also demonstrated in animal models of inflammatory bowel disease and colitis[81]. Besides, it has

been demonstrated that an animal model of tongue carcinogenesis could be treated by reducing the expression of TNF-α, using whole fruit extract[81].

4.8 Inhibitory activity of MRM callus extract on digestive enzymes

As already discussed in this thesis, there is an increasing interest, among researchers and healthcare professionals, in the comprehension of the relationship between fruit and vegetable consumption and health improvement. Apples are an important source of phytonutrients that have been studied worldwide for their potential to prevent various diseases such as diabetes, cancer, and pulmonary conditions[82]. As previously reported, phytochemicals' activity is also related to the inhibition of digestive enzymes (Results 4.4 section).

Consequently, we assessed the inhibition activity of MRM callus extract on the three enzymes, already stated: α-glucosidase, α-amylase, and lipase. **Figure 19** shows that the extract inhibits the activity of the enzymes in a concentration-dependent manner. Acarbose was used as a positive control for α-glucosidase and α-amylase, while orlistat for lipase.

The obtained results show an enzymatic activity inhibition of 36% for α-glucosidase (2.5 mg/mL MRM extract - **Figure 19.a**), 76% for α-amylase (2.5 mg/mL MRM extract - **Figure 19.b**) and 43% for lipase (1.25 mg/mL MRM extract - **Figure 19.c**). The IC_{50} values are 2.98 ± 0.24, 1.77 ± 0.15, and 2.06 ± 0.31 mg/mL, respectively.

Similarly, a study by Nkuimi Wandjou *et al.*[32] proved that a polyphenol-rich extract from whole apples of the Mela Rosa Marchigiana variety (named in the paper Mela Rosa dei Monti Sibillini), inhibits both enzymes, α-glucosidase (in a dose-dependent manner) and lipase.

Moreover, Nkuimi Wandjou *et al.*[32] demonstrated that secondary metabolites, like polyphenols and triterpenes, can modulate or inhibit physiological enzymes activity as in the case, for example, of catechin and epicatechin that inhibit α-glucosidase and lipase as well as ursolic acid that possesses an inhibitory effect on lipase.

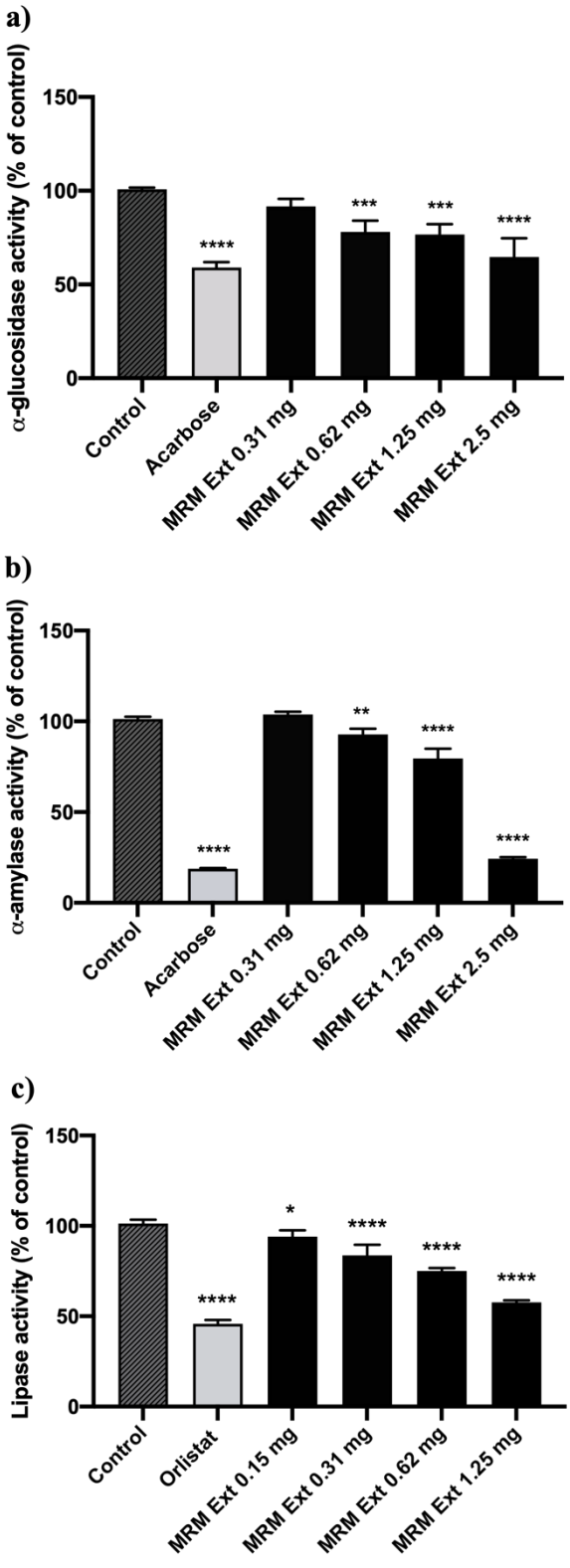

Figure 19. Inhibitory activity of MRM callus extract on digestive enzymes. a) α-glucosidase; **b)** α-amylase and **c)** Lipase activities are expressed as % of the control that represents the 100% activity of the enzymes. 2 mM Acarbose was used as a reference compound for **a)** and **b)**; 5 μg Orlistat was used as a reference compound for **c)**. Data represent the mean ± SD of three independent experiments. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$; ANOVA followed Dunnett's multiple comparison test was performed).

5. CONCLUSIONS

Callus cultures serve as an enticing and sustainable *in vitro* technique, conducted under aseptic conditions. This method can provide an abundant source of bioactive, high-value secondary metabolites, unaffected by external variables like soil composition, environmental conditions, growth period, season, or geographical location. Harnessing the potential of *in vitro* callus culture for secondary metabolite production mitigates the risk associated with redirecting arable land and crops away from food production.

A method of hydroalcoholic extraction from *Cydonia oblonga* and Mela Rosa Marchigiana calluses was set up, and the extracts were tested in different models (cell-free and cell-based) to investigate the antioxidant and anti-inflammatory properties, the genoprotective activities, and the capacities to inhibit the digestive enzymes α-glucosidase, α-amylase, and lipase.

Our findings highlight that *Co* and MRM callus extracts have a remarkable capacity for producing significant quantities of pentacyclic triterpenes and phenol compounds, imparting potent antioxidant and genoprotective effects. The antioxidant efficacy of *Co* and MRM extracts was evidenced by classic antioxidant chemical assays (DPPH, ABTS, and ORAC), and especially by the mitigation of reactive oxygen species levels in oxidatively stressed keratinocytes. An approach of *in vitro* gastrointestinal digestion was also applied to MRM callus extract and no modification in antioxidant results was noted, demonstrating that the involved molecules undergo no substantial changes.

The outcomes of this investigation enhance our understanding of the positive attributes associated with the callus extract of *Co* and MRM in cellular models. In fact, we have substantiated their anti-inflammatory and antioxidant capabilities, notably observed in both macrophage (RAW 264.7) and keratinocyte (HaCaT) cells, irrespective of the specific stimulus employed. In immune cells, a pronounced reduction in nitric oxide levels was discerned, alongside a significant decrease in the gene expression of pro-inflammatory mediators, including iNOS, IL-6, IL-1β, ikBα, and ICAM, in epidermal cells.

Both the extracts have a good capacity to inhibit digestive enzymes, and in most cases, in a dose-dependent manner. This finding is consistent with what is reported in the literature about the ability of some phytocompounds such as polyphenols and triterpenes to modulate the activity of α-glucosidase, α-amylase, and lipase. The inhibition of digestive enzymes, as evidenced by our results, could present a promising avenue for managing hyperglycemia and dyslipidemia. It is however crucial to acknowledge that the limitations inherent in our

experimental approach are associated with the specificities of the cell model system and with the lack of investigation of the mechanisms underlying the highlighted effects.

In conclusion, *Co* and MRM callus extracts should become excellent candidates for future application and medical research. First, callus can serve versatile purposes, including applications in food, feed, and the production of bioproducts for the pharmaceutical industry, and also through the application of green chemistry and green extraction solvents.

Moreover, by modulating the digestion and subsequent absorption of dietary carbohydrates and lipids, *in vitro* callus culture could offer a complementary approach to address these health concerns.

Co and MRM pulp callus extract and/or its active compounds might therefore be valuable candidates for inclusion in a nutraceutical formulation designed to promote healthy aging or prevent oxidative stress-related pathologies, including but not limited to chronic diseases.

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