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Vegetal by-products of Sicilian industry as high healthy molecules: Extraction and biochemical characterization

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TABLE OF CONTENTS

INTRODUCTION

1. Circular economy

In the last years, the increase in food loss and waste production is representing a serious problem all over the world. The European Union and its countries are collaborating to achieve Sustainable Development Goal 12.3, which aims to reduce food loss and waste level by 2030. According to the State of Food and Agriculture (SFA) report of 2019, the percentage of food lost after harvest and during the transport, storage, and processing stages is about 14% globally, which would amount to approximately 1.3 billion tons for year of food lost or wasted (*The State of Food and Agriculture 2019*). Agro-industrial waste and by-products not only impact the economy but also contribute significantly to environmental pollution associated with their disposal.

Although residues can have a different destiny, such as disposal, or be employed in producing animal feed, biofuel, biomaterials, and bio-fertilizers, it is necessary to consider that the production of waste from food processing is considerably increased in recent decades, also in relationship with the demographic growth. Thus, given today's global context, which underscores the necessity for the sustainable use of agri-food by-products and waste, can plant-based by-products and residues be reused so much so they represent a valid added value?

The most represented by-products and residues from vegetables and fruit (about 38%) include the non-edible parts, such as peelings, stems, seeds, shells, bran, and trimmings residues which are produced after extraction of oil and juice (Bharat Helkar and Sahoo, 2016). Moreover, often their disposal is very dangerous for the ecosystem. Therefore, a change in the linear economy, meant as “take, make, dispose of”, is strictly necessary for recycling what should be disposed of.

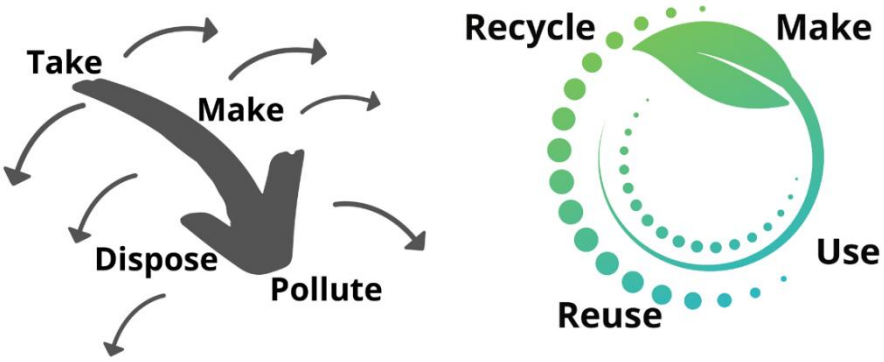


Figure 1. From linear to circular economy.

The new model of the circular economy could be the change that serves to take advantage of the discards. In this model, the recycling and reuse of food waste is a fundamental principle, reducing the environmental impact to a minimum value. Recently, several studies have highlighted the "second life" of by-products and waste due to their bioactive compounds, such as protein, dietary fibre, lipids, and phytochemicals, which are still present (Coman et al., 2020; De Laurentiis et al., 2018). Fruit pomace, such as that from apples and berries, has been suggested as a functional ingredient in bakery and dairy product formulations to boost their natural antioxidant and dietary fiber content. Additionally, the incorporation of natural pigments and volatile compounds can improve the sensory attributes of the final products (Majerska et al., 2019). Moreover, consuming dietary fiber has been linked to lower risks of conditions like diabetes, obesity, hypertension, and gastrointestinal disorders (Zhang et al., 2022).

The studies provide evidence of the progress in food sustainability, focusing on the value of by-products as a source of healthy molecules, and promoting the idea that by-products can have a second useful use. Until today, the scientific community and also companies are investigating new strategies for the recycling of by-products; specifically, to extract the bioactive components that are often underestimated (Ben-Othman et al., 2020) or for applications related to food, pharmaceutical, or cosmetic industries (Osorio et al., 2021). Pectines from apple pomace can be employed in pharmaceutical industries as a vehicle for drug delivery, but also in food industries as gelling agents, for example for jam and desserts (Borah et al., 2023).

2. Major Bioactive Secondary Metabolites Produced by Plants

Phytochemicals belong to the class of secondary metabolites, synthesized by different tissues of plants such as roots, leaves, flowers, and seeds through several metabolic pathways, like pentose phosphate, shikimate, or phenylpropanoid pathway (Harborne, 1980). They play a fundamental role in the ecology and physiology of plants, protecting them against different stresses, both abiotic and biotic, such as adverse environmental and pathogenic attacks.

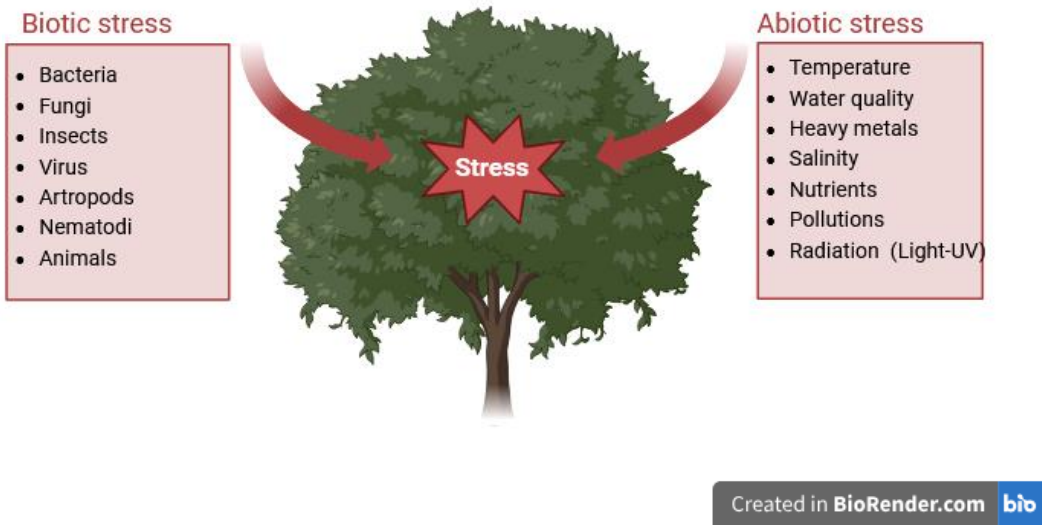


Figure 2. Environmental biotic or abiotic elements that may induce stress in plants.

Moreover, the production of these metabolites is species-specific and dependent on different factors, including the period of growth, environmental or climatic factors, and the adaptability of the plant.

Since each plant species produces a typical mix of phytochemicals, these can sometimes also be used as taxonomic characters in plant classification (Bhattacharya, 2019; Umoh, 2020).

Since ancient times, plants have been used to treat a lot of diseases for their curative properties as medicinal plants before the development of contemporary therapies. *Aloe vera* plant, for example, has been used for a long time to address various skin issues, including burns, wounds, and inflammatory conditions, even when its specific biomolecules were not known. Moreover, beyond its dermatological applications, it exhibits therapeutic potential in cancer prevention, antioxidant activity, diabetes management, and lipid regulation. Many of these properties are related to the numerous bioactive compounds, including vitamins, enzymes, essential minerals, sugars, fatty acids, and other components such as salicylic acid, lignin, and saponins which are present in the plant (Sánchez et al., 2020). Also, chamomile has always been employed in herbal medicine, such as an anti-oxidant, anti-inflammatory, analgesic, antimicrobial, anticancer, and anti-allergic agent, although the chemical composition was unknown in the past times (Sah et al., 2022).

Nowadays the awareness that most of the beneficial properties of plants are attributable to their composition of secondary metabolites has significantly increased scientific interest in these molecules and the study of their potential health benefits.

2.1 Classification

Generally, phytochemicals can be classified into different classes based on their chemical structures: alkaloids, terpenes, and phenolic compounds. Phenolic compounds are the class that received major attention for their important health-promoting properties. Their structure contains one or more aromatic rings with two or more hydroxyl groups. According to the chemical structure, phenolic compounds are divided into two main different classes, flavonoids and non-flavonoids, which together constitute about 8,000 different chemicals (Zagoskina et al., 2023).

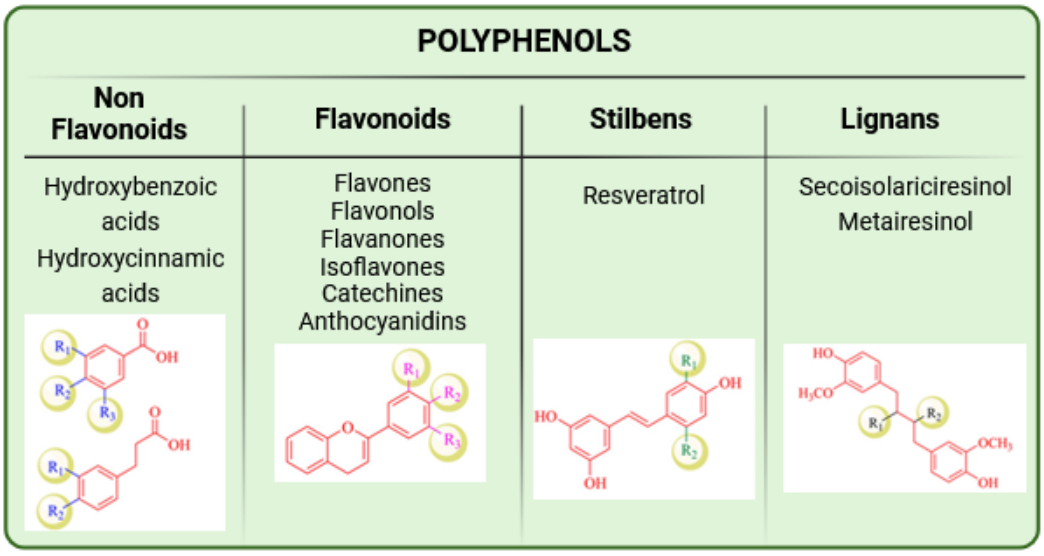


Figure 3. Different classes of polyphenols and their schematic structures (modified from Amawi, H. et al. Nutrients 9, 911, 2017).

Flavonoids represent a significant subclass of phenolic compounds characterized by low molecular weight and widespread presence in plant organisms. The fundamental structure of flavonoids consists of a diphenyl propane backbone (C6-C3-C6), featuring two aromatic rings linked by a three-carbon chain. They can be divided into six main groups, each distinguished by a heterocyclic C-ring: flavones (such as apigenin and luteolin) and flavonols (such as quercetin and myricetin) also grouped as anthoxanthins, flavanones (such

as naringenin and hesperidin), catechins (such as epicatechin and gallocatechin), anthocyanidins (such as cyanidin and pelargonidin), and isoflavones (such as genistein and daidzein). Furthermore, flavonoids can exist as either aglycones or glycosides, depending on the presence of glycosyl groups. They are predominantly found in fruits and vegetables; for instance, catechins are particularly abundant in the tea plant (a member of the Camellia family), cacao (Theobroma cacao), and wine (Dwyer and Peterson, 2013; Fang et al., 2007; Katz et al., 2011).

The class of non-flavonoid compounds includes two major groups, hydroxybenzoic acids and hydroxycinnamic acids, also called phenolic acid. The principal components of the first group are gallic, protocatechuic, vanillic and syringic acids, which are also the most abundant in nature. Instead, among the hydroxycinnamic acids groups, there are caffeic, ferulic, synapinic, and coumaric acids, that are present principally in the specific trans isomeric form. Typically, they are conjugated with glucose molecules or exist as esters.

2.2 Role of Secondary Metabolites

As it has been reported, both flavonoid and non-flavonoid compounds, show multiple functions, including anti-inflammatory, antibacterial, cardioprotective, neuroprotective, anticancer, and antioxidant properties. In many cases, the mechanism of action of these compounds has also been revealed. It has largely emerged that polyphenols activate different signaling pathways that vary in relationship to the cell type (normal or cancerous) or to their concentration.

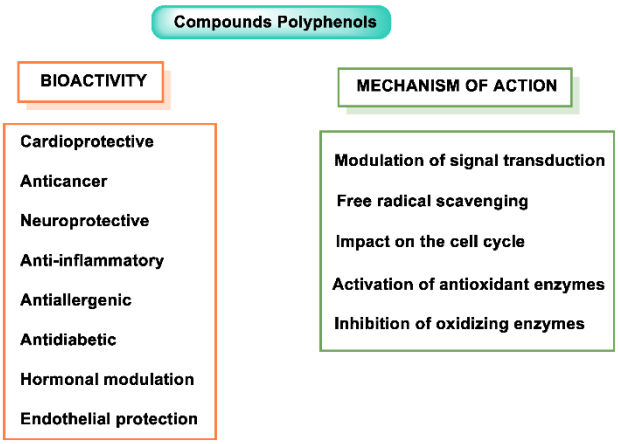


Figure 4. Bioactivity and mechanism of polyphenols action (from Andrés C. et al., Processes 11, 2771, 2023).

❖ *Anti-inflammatory role*

The immunomodulatory effects of polyphenols have been demonstrated in various studies, revealing their ability to influence immune cell populations, regulate cytokine production, and alter the expression of pro-inflammatory genes. Among non-flavonoid components, curcumin, extracted from turmeric plants and mustard, was the most studied for its potent antioxidant and anti-inflammatory properties (Krishnaiah et al., 2011). Curcumin is also involved in the downregulation of Nf-κB (Nuclear factor κB) by targeting PPARγ (peroxisome proliferator-activated receptor gamma) in cigarette smoke-induced inflammation both in *in vivo* and *in vitro* (Li et al., 2019).

The use of polyphenols has been linked to a direct influence on the quantity and differentiation of immune cells. In male C3H/HeN mice, oral intake of polyphenols induced an increase in T helper 1 cells, natural killer cells, macrophages, and dendritic cells within Peyer's patches and the spleen, so to have an immunomodulation for prevent diseases (Karasawa et al., 2011).

Numerous dietary polyphenols, like curcumin, chlorogenic acid, epigallocatechin-3-O-gallate, quercetin, and resveratrol, have demonstrated a potential to mitigate arthritis, meant as a pathological condition characterized by joint inflammation. This disease arises from the dysregulation of various pro-inflammatory mediators, as well as the overexpression of adhesion molecules, matrix metalloproteinases, and the proliferation of synovial fibroblasts. Therefore, compounds capable of downregulating these factors are considered promising for arthritis prevention (Khanna et al., 2007).

❖ *Polyphenols in cardiovascular diseases*

Numerous studies regarding polyphenols have been conducted to investigate their impact on cardiovascular diseases (CVD), the main cause of death in the world. Environmental factors such as smoking, high saturated fat diets, and physical inactivity are well-documented risk factors for CVD. Epidemiological studies consistently suggest that a regular diet rich in polyphenol-containing foods (fruits, vegetables, cocoa, tea, and wine) may offer protective benefits for cardiovascular health Mink et al., 2007). In particular, many flavonoid subclasses of dietary polyphenols (anthocyanidins, flavan-3-ols, flavones, flavonols, and proanthocyanidins) are associated with a decreased incidence of CVDs (McCullough et al., 2012).

Polyphenols from wine enhance the production of endothelial nitric oxide (NO), which in turn promotes the relaxation of blood vessels in conditions such as stroke or hypertension (Serino and Salazar, 2018). Resveratrol, a natural polyphenol found in wine, has demonstrated protective effects against various forms of cardiovascular diseases, also influencing endothelium-independent vasodilation through mechanisms involving different membrane channels (Zekrumah et al., 2023).

Positive effects of polyphenol uptake may be related to the increase in the antioxidant defenses, the ability to low blood pressure, improve endothelial function, and inhibit platelet aggregation as well as inflammatory responses (Rodriguez-Mateos et al., 2024).

❖ *Polyphenols in Neurodegenerative Diseases*

Neurodegenerative disorders such as Parkinson's and Alzheimer's appear to be triggered by a combination of factors, including neuroinflammation, excitotoxicity caused by glutamate, oxidative stress, iron accumulation, and/or a decrease in natural antioxidants. Concerning dietary interventions for these diseases, numerous studies have documented the bioavailability of polyphenols in systemic circulation, suggesting that polyphenols can cross the blood-brain barrier. Consequently, these compounds are potential candidates for direct neuroprotective and neuromodulatory effects.

Moderate wine consumption may reduce the incidence of certain neurological conditions. Furthermore, a consistent intake of flavonoid-rich foods has been associated with a 50% reduction in the risk of dementia (Holland et al., 2020).

Flavonoids may protect the brain through various mechanisms, such as safeguarding vulnerable neurons, enhancing existing neuronal functions, or promoting neuronal regeneration. (Islam et al., 2025). For instance, polyphenols have been shown to protect neurons from oxidative stress and Aβ-induced neuronal damage (Hole and Williams, 2021), as well as aging-associated disorders (Deepika et al., 2024).

❖ *Antibacterial role of polyphenols*

Polyphenols possess antibacterial properties against a wide range of bacteria, including both Gram-positive and Gram-negative strains, as well as fungi. These compounds, whether used alone or in combination with specific antibiotics, have been shown to enhance antimicrobial efficacy against various microbial types. Consequently, polyphenols may help to reduce drug dosages and mitigate the side effects associated with antibiotics. Preclinical studies have

provided compelling evidence that polyphenolic compounds, both independently and when combined with existing antibiotics, effectively combat the development of antibiotic resistance. However, the understanding of the underlying mechanisms and structure-activity relationships remains unclear (Sharma et al., 2024). Various studies indicate that the antibacterial activity of polyphenols is associated with their ability to inhibit bacterial biofilm formation through multiple mechanisms, including the inhibition of bacterial motility, surface adhesion, and the expression of virulence factors linked to pathogenic behavior. Additionally, polyphenols interact with the cell membranes of Gram-positive and Gram-negative bacteria, disrupting the phospholipid bilayers (Bae et al., 2022).

Among the numerous properties of polyphenols, their remarkable capacity to function as potent antioxidants is undoubtedly well-known. This capability plays a crucial role in safeguarding the body against damage induced by oxidative stress. Furthermore, there is a strong correlation between the role of polyphenols in mitigating oxidative stress and their potential antitumor effects.

3. Exploring the antioxidant and anticancer power of polyphenols and their role in oxidative stress

3.1 Reactive Oxygen Species and the State of Oxidative Stress: Understanding the Connection

Oxidative stress is a condition of impairment of the balance between the production of free radicals that, when accumulated, leads to the oxidation of macromolecules, and the presence of antioxidant defense systems that are important to counteract the reactive species formed.

The main reactive species include those derived from oxygen (ROS) and nitrogen (RNS), which can be in free or not-free radical form. Reactive oxygen species consist of three main species produced by the Fenton reaction, in which partial reduction of oxygen occurs. They are $O_2^{\bullet -}$ (superoxide radical), $HO^{\bullet}$ (hydroxyl radical), and H_2O_2 (hydrogen peroxide). Among these, the first two are free radicals with an unpaired electron, whereas the last is in a non-radical form.

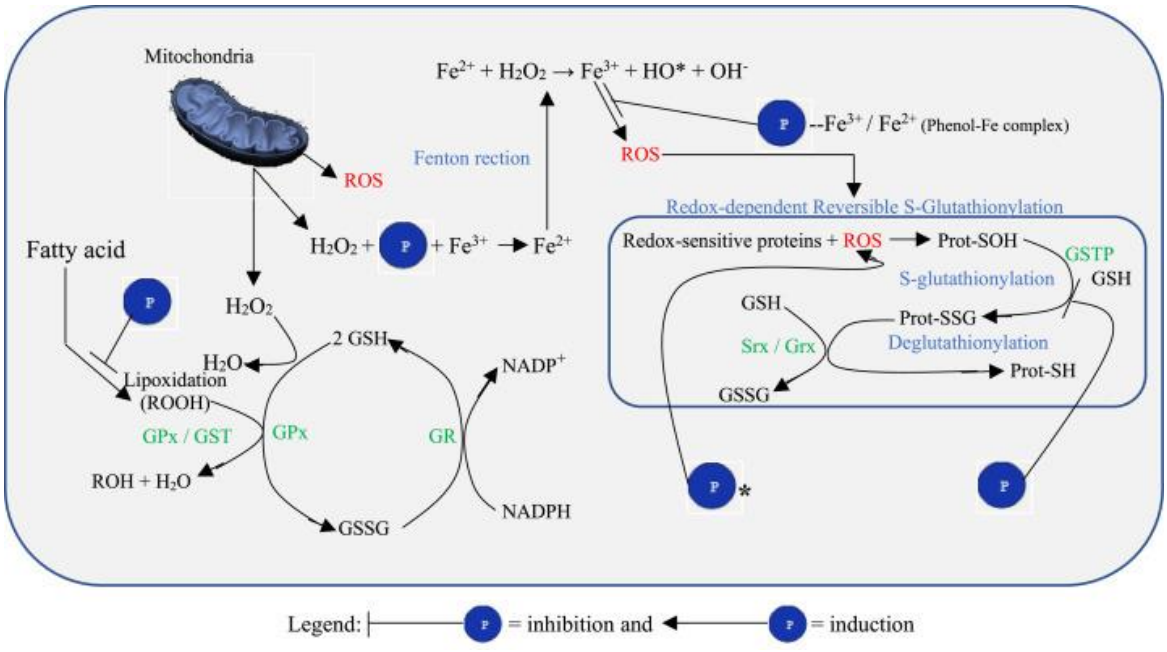


Figure 5. Overview of mechanisms of ROS production (from Dzah, C. et al. Adv. Redox Res. 11, 100099, 2024).

Each reactive species has a different reactivity, which is related to its potential to damage the biomolecules.

Reactive species can have exogenous or endogenous causes. The first is related to the exposition to UV or gamma radiation, ultrasounds, drugs, air pollutants, foods, xenobiotics, and toxins. The endogenous ROS are generated during metabolism or by the cell itself (like white blood cells) through ROS-producing enzymes. Other sources of endogenous ROS are represented by the physiological metabolism of mitochondria, through electron chain transport, and by the action of membrane-bound NADPH oxidase.

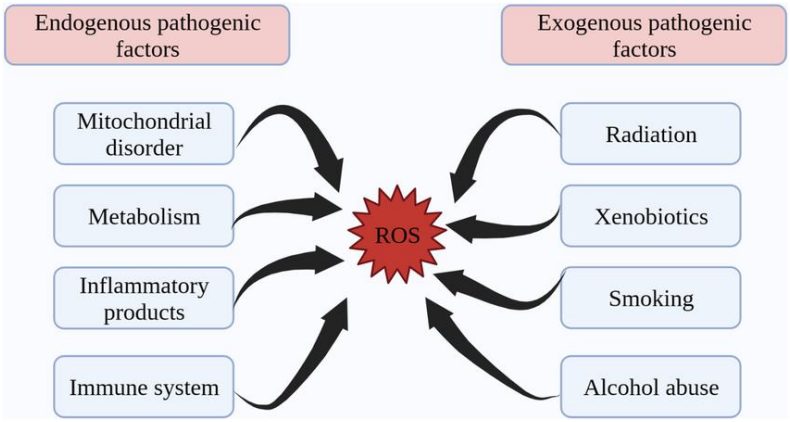


Figure 6. The main endogenous and exogenous pathogenic inducers of ROS (from Zhang, Z. et al Front. Endocrinol. 14, 1112363, 2023).

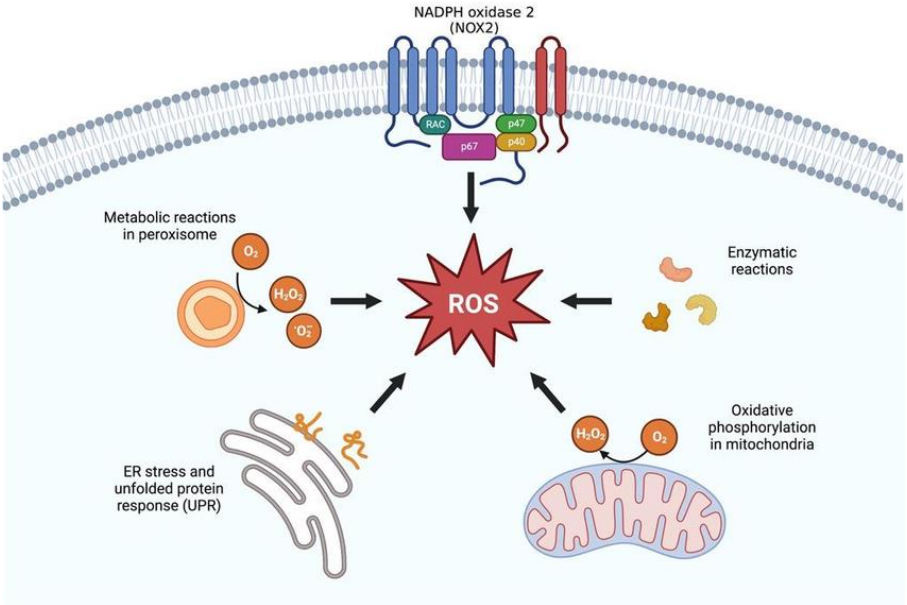


Figure 7. Different endogenous mechanisms of ROS production (from Donate-Correa et al. Antioxidants 12, 239, 2023)

Although for many years ROS were considered inducers of a harmful condition, named oxidative stress, recently, it has been largely demonstrated that moderately high ROS levels are involved in different physiological processes, behaving as modulators of intracellular signaling pathways. In particular, hydrogen peroxide, the less toxic oxygen reactive species, is responsible for a post-translational modification of protein cysteine residue, indicated as sulfenylation (Mu et al., 2024).

The constant exposition to ROS led the cells to develop defense systems to prevent and counteract the production of reactive species. The defense mechanisms fall into two categories, antioxidant enzymes, and small low molecular weight molecules.

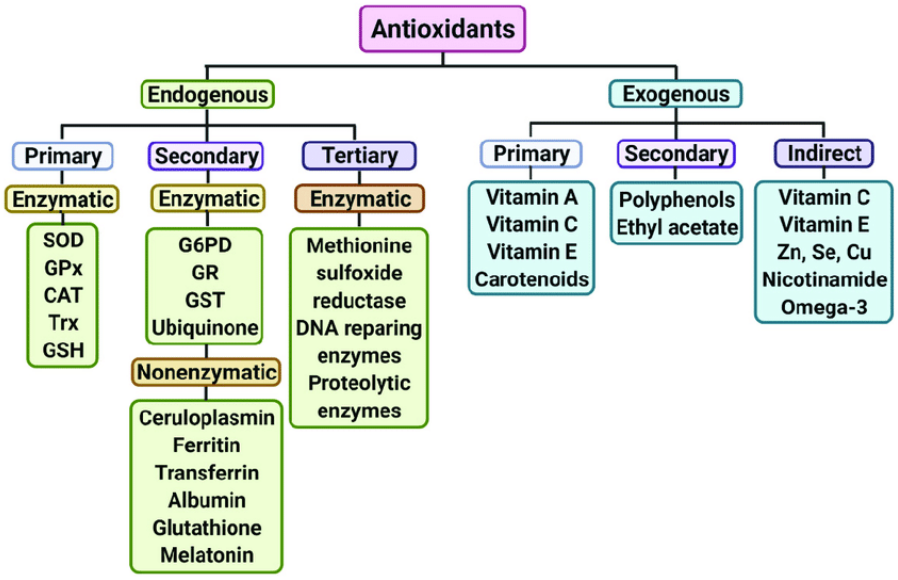


Figure 8. Schematic classification of endogenous and exogenous antioxidant systems in cells (from Riveros et al. Antioxidants 11, 540, 2022).

The enzymatic defenses refer to several proteins, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). SOD activity is able to convert superoxide radicals into hydrogen peroxide both in the cytoplasm and the mitochondrial matrix. Three SOD isoforms exist with different cellular localization, SOD-1 in the cytosol, SOD-2 in mitochondria, and SOD-3 in extracellular compartments. CAT is an enzyme appointed to hydrogen peroxide detoxification into water and oxygen. Moreover, hydrogen peroxide can also be a substrate of GPx which reduces H_2O_2 in water through the oxidation of two molecules of glutathione (Jena et al., 2023).

Heme oxygenase-1 (HO-1) is another molecule of the antioxidant defense system. This enzyme is involved in heme degradation, producing carbon monoxide (CO), biliverdin, and ferrous ion (Fe^{2+}). Biliverdin is quickly converted into bilirubin by bilirubin reductase, which acts as a neutralizer of reactive oxygen species. Moreover, the activation of HO-1 leads to an increase of ferritin expression which sequestering Fe^{2+} mitigates its pro-oxidative property through the Fenton reaction (Chiang et al., 2021).

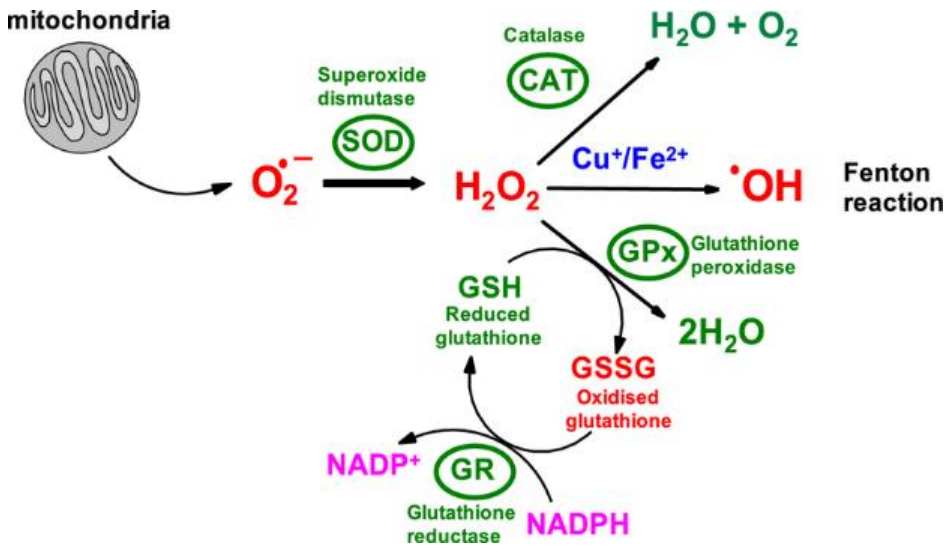


Figure 9. Schematic diagram of enzymatic antioxidant reactions (from Jomova et al. Arch. Toxic. 98, 1323, 2024).

The group of small antioxidant factors consists of molecules that can directly interact with reactive radicals, effectively neutralizing them. In the process, they may transform into new, less reactive free radicals which have a longer lifespan, and pose less of a threat than the radicals they have reduced. For instance, ascorbic acid, an oxidant scavenger, oxidizes to ascorbyl acid, which can be reduced back to its active form by glutathione (Foyer and Kunert, 2024).

The endogenous defense systems are under the control of key regulators. Among these, the antioxidant transcriptional factor Nrf2 (nuclear factor erythroid 2–related factor 2) plays a central role in the control of the expression and modulation of different proteins, such as SOD, catalase, glutathione peroxidase, heme oxygenase-1. Nrf2 is one of the Cap'n'Collar (CNC) subfamily of basic leucine zipper (bZIP) transcription factors. It contains seven NRF2-ECH homology (Neh) domains, each with specific roles in regulating its

transcriptional activity. Among these, the Neh2 domain is responsible for the interaction with the specific negative regulator, Keap1 (Kelch domain of Kelch-like-ECH-associated protein 1), that leads to Nrf2 ubiquitination and consequently proteasome-dependent degradation, while the Neh6 domain mediates the Keap1-independent degradation (Taguchi and Yamamoto, 2017).

The fine regulation of Nrf2 depends on the constitutive degradation by Keap-1, so the basal expression of its target genes is preserved to support essential housekeeping functions. However, under stress conditions, Keap1 functions as a sensor due to the presence of three cysteine residues. This mechanism prevents the degradation of Nrf2, leading to its accumulation in the cell and subsequent translocation into the nucleus, where it activates an antioxidant transcriptional program (Baird and Yamamoto, 2020). When oxidative damage is massive, it can lead to cellular senescence or cell death.
Additionally, other cellular factors, such as NF-κB, can play a role as a redox-sensitive system. Under standard physiological conditions, NF-κB is kept in a non-active state through the interaction with the inhibitory protein IκB. However, under stress conditions, IκB becomes phosphorylated, leading to its detachment from NF-κB. This enables NF-κB to translocate into the nucleus, where it drives the expression of pro-inflammatory cytokines and other related molecules. The activation of this pathway is linked to elevated levels of the pro-inflammatory enzyme COX-2, interleukin 1β, increased release of TNF-α, and initiation of apoptotic processes, evidenced by caspase-3 cleavage. These effects can be effectively inhibited by the addition of antioxidants like vitamins C and E or sulfasalazine, a pharmacological blocker of NF-κB activation.

However, when the balance between reactive species production and antioxidant cellular systems is destructed, the massive damage, that occurs by the excessive concentration of reactive species, culminates in the development of a condition recognized as oxidative stress. This abnormal condition can lead to the accumulation of genetic mutations, protein dysfunction, and lipid peroxidation, potentially triggering various diseases such as cancer, neurodegenerative disorders, and cardiovascular diseases. (Luo et al., 2020).

3.1.1 Oxidative Stress in Cancerous and Normal Cells

Cancer cells exhibit an oxidative environment characterized by a more elevated free radical production compared to normal cells. The unbalanced condition depends on different factors, mitochondrial dysfunction or oncogenic mutations, and can lead to the activation of cell

proliferation, survival, and cancer progression. Elevated ROS levels in cancer cells can induce the inactivation of PTEN and consequently the activation of PI3K/AKT signaling which results in cell proliferation (Arfin et al., 2021). The survival factor AKT regulates cell proliferation through the inactivation of pro-apoptotic proteins FoxO and Bax. AKT-dependent FoxO phosphorylation results in the inactivation of negative regulatory molecules of the cell cycle, such as p27 (Zhang et al., 2011). Moreover, it was found that PTEN can be negatively regulated by the increase in H_2O_2 levels in different types of cancer (Zhang et al., 2017).

In cancer cells, high ROS levels are maintained by an increased expression of antioxidant systems. For example, in certain types of cancer, Nrf2 is often upregulated, leading to increased expression of antioxidant proteins that regulate ROS levels and preserve cellular balance. Consequentially, the upregulation can create a condition where cancer cells maintain high ROS levels to proliferate and survive, avoiding excessive oxidative damage that could trigger cell death (Xue et al., 2020). However, very high ROS levels in cancer cells may lead to cell death pathways. The activation of the intrinsic apoptosis pathway can occur when mitochondrial ROS levels reach a so high level which mediates the release of cytochrome *c* and the activation of executioner caspases, resulting in apoptotic cell death. Also, a relationship between extrinsic apoptotic pathway and high ROS levels exists, *via* transmembrane death receptor activation. (Redza-Dutordoir and Averill-Bates, 2016).

Overall, what has been reported so far indicates that from the therapeutic point of view, targeting oxidative stress could be a challenge. On one side, strategies that increase ROS levels, such as pro-oxidant molecules, aim to overcome the oxidative threshold leading to cell death; conversely, antioxidant therapies can reduce ROS damage and make less aggressive cancer progression and metastasis. However, the heterogeneity of cancers and their redox state adaptation require a sophisticated approach to effectively exploit oxidative stress for cancer treatment.

In normal cells, as reported above, free radicals are naturally produced during the cellular physiological functions, especially during the electron respiratory chain into the mitochondria. They play an essential role in different cellular pathways, such as proliferation, differentiation, immune response, signaling transduction, and adaptive gene expression (Jalmi and Sinha, 2015; Martinvalet and Walch, 2022; Reczek and Chandel, 2015). An important role of reactive oxygen species is the oxidative burst, the first defense

of the immune system against different pathogens (Chen and Junger, 2012). However, when ROS production exceeds the antioxidant system capacity, an irreversible damage of biological macromolecules such as DNA, proteins, and lipids occurs. In normal cells, oxidative damage can cause an increase in the rate of mutations of mitochondrial DNA with the consequent ageing in various human tissues (Chen et al., 2025).

The increase in cellular ROS can also lead the cells to proliferative resting and the induction of a senescent phenotype. Oxidative stress and cellular senescence are related to the development of different diseases, such as neurodegenerative disorders. Indeed, oxidative stress has been shown to play a critical role in the pathophysiology of dementia. Moreover, a relationship has been established between certain oxidative stress biomarkers and cognitive function.

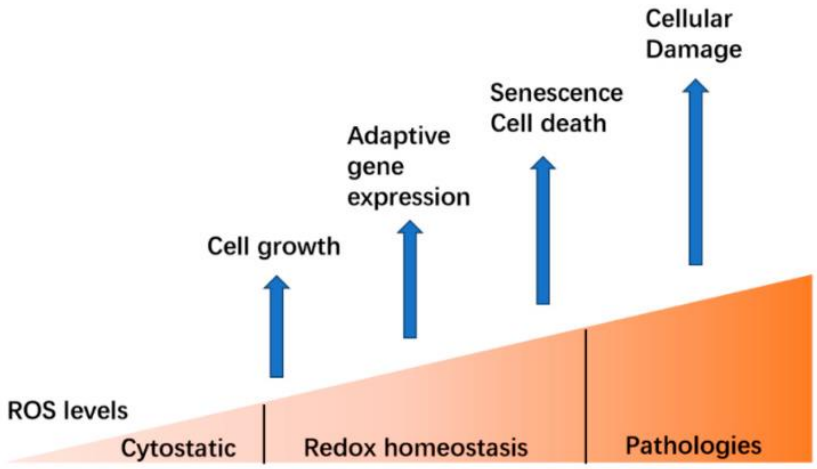


Figure 10. ROS levels dictate hormetic cellular signalling and induce biological outcomes (from Hong, Y. et al. Antioxidants 13, 312; 2024).

3.1.2 Cell Death Pathways Triggered by Oxidative Stress

Oxidative stress and autophagy

Scientific evidence highlights that the accumulation of ROS can play a crucial role in the activation of the autophagic pathway.

Autophagy is an important physiological cellular response that usually works at moderately low levels in cells, but in cellular stress conditions, as in many cases of nutrient starvation, it can be strongly triggered (Levine and Kroemer, 2008). Through autophagy, cells can degrade intracellular organelles, such as mitochondria and ribosomes, thereby producing catabolites to be used again for biosynthesis and energy production, as well as the organelles,

if damaged, can be eliminated to maintain cellular functions. During the process, specialized vesicles, autophagosomes, encapsulate cellular organelles or molecules and subsequently fuse with lysosomes, where the degradation takes place and low molecular weight metabolites are produced to help cells survive in nutrients starvation and stressed environment, in both normal and cancer cells. Moreover, the autophagic process has also been known to play a role in cell differentiation (Riffelmacher et al., 2018).

Three types of autophagy exist, macroautophagy, chaperone-mediated autophagy, and microautophagy. All three types, even if have different ways of transporting the material to be degraded, share the elimination of the cargo by lysosomal vesicles (Wu et al., 2020).

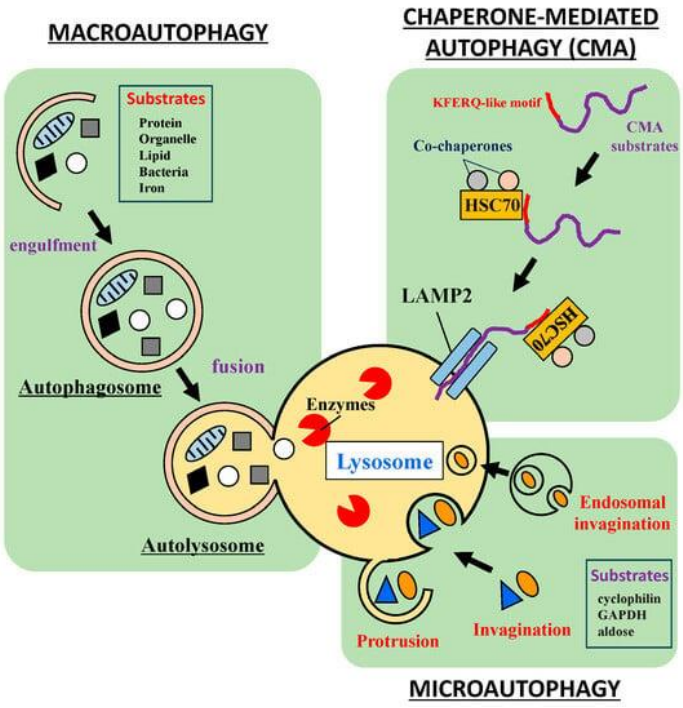


Figure 11. Schematic representation of the different autophagic pathways (from Watanabe et al. Int. J. Mol. Sci. 24, 13804, 2023).

Microautophagy is a non-specific lysosomal degradation mechanism, that results in the direct invagination of cytoplasmic material or whole organelles at the lysosomal membrane boundary. The chaperone-mediated autophagy is a selective type of autophagy, in which the cargos consist of a specific cytosolic group of proteins. Selectivity occurs through the recognition of a specific targeting motif by cytosolic chaperone proteins. This interaction directs the substrate toward lysosomal degradation (Fred Dice, 1990). Different studies

highlight that in most cells, this type of autophagy plays a crucial role in maintaining protein quality control by removing oxidized and damaged proteins during stress.

In most cases, cellular homeostasis is principally managed by macroautophagy. The macroautophagic pathway includes both regulatory and effector proteins, which are involved in different steps of the process. The first is the formation of the phagophore membrane induced by the activation of the ULK1 multiprotein complex and PI3K (phosphatidylinositol-3 kinase) complex, which is an important regulatory factor of the autophagic process for the formation of phosphatidylinositol-3-phosphate (Dunlop et al., 2011). The next step is nucleation, in which ATG proteins are implicated, followed by the elongation and expansion of the phagophore membrane, that surrounds the damaged components (Muhammad Riaz et al., 2024).

This event is mediated by the association of Atg12-Atg5 factors and the lipidation with phosphatidylethanolamine of the transmembrane protein LC3I in LC3II that remains until the autophagosome is fused with lysosome. The final steps consist of the fusion of the autophagosomes with lysosomes and the activation of lysosomal hydrolases which destroy their contents.

An important protein that represents a connection point between autophagic and apoptotic pathways is Beclin-1, a member of the BH3-domain subfamily that is involved in the early stage of autophagy. BH3 proteins linked to stress sensors can dislocate Beclin-1 from its complex, thereby initiating autophagy. Additionally, they can release pro-apoptotic factors such as Bax and Bak, which also contribute to the process of apoptosis.

In addition to Beclin-1 which is involved in the early stages during the maturation of autophagosome and in cell death by the interaction with PI3K complex and Bcl-2, other proteins can be considered markers of autophagic pathway, such as p62. This protein is involved in the recognition of ubiquitinated misfolded proteins and organelles, which are brought into the autophagosome through the interaction between p62 and LC3II (Hizomi Arani et al., 2022; Kang et al., 2011; Moscat and Diaz-Meco, 2009).
It was largely demonstrated that autophagy could be triggered by the increase in ROS levels. Notably, during oxidative stress a lot of cellular components are subjected to damage and autophagy occurs to eliminate oxidized proteins or other cellular components. Sometimes, the degradation of the components can save the cells, but in other cases, this elimination is

not sufficient, and it culminates in the activation of apoptotic cell death. Therefore, a strict and complicated relationship between ROS and autophagy and vice versa exists.

Different studies underline the crosstalk between the increased levels of antioxidant enzymes in stress conditions and the enhancement of autophagy (Gao, 2019; Pajares et al., 2018). Nrf2 factor which activates the transcription of different antioxidant enzymes, can also regulate p62, inducing positive feedback that sustains Nrf2 itself. In other words, p62, phosphorylated by mTORC1, binds Keap1 in the Nrf2 binding site, allowing the release of Nrf2 which translocates into the nucleus, while the p62-Keap1 complex is recruited to the autophagosome through LC3 and degraded by the autophagolysosome (Ichimura et al., 2013; Jain et al., 2010).

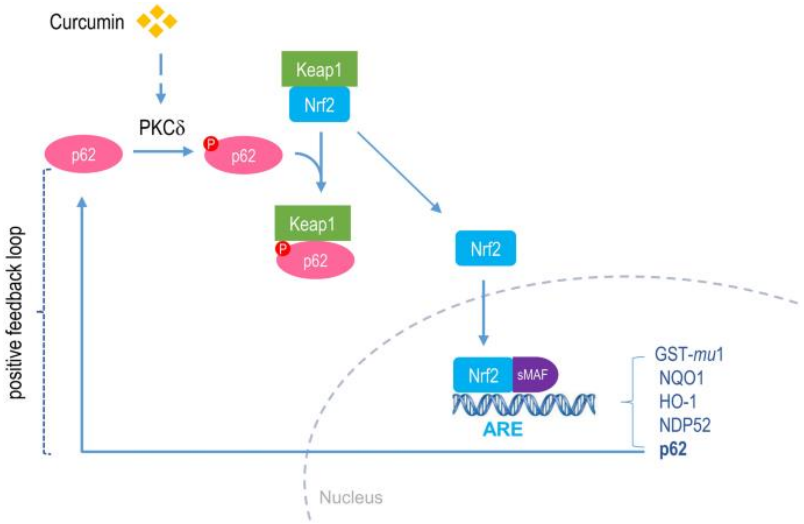


Figure 12. p62 - Nrf2 positive feedback loop (from Park et al. Sci. Rep. 11, 8430, 2021).

In this context, autophagy can help the cells to restore a healthy condition, but if the oxidative stress remains at high levels, the apoptotic pathway can be triggered.

Three different types of apoptosis (intrinsic, extrinsic, and ER stress-mediated) exist. Briefly, the extrinsic pathway is triggered by different death ligands that bind to the TNF (Tumor Necrosis Factor) receptor superfamily; the intrinsic pathway, also referred to as the mitochondrial pathway, involves the release into the cytoplasm of apoptogenic factors such as cytochrome *c* in response to cellular stress. The pathway induced by ER stress is activated when a massive accumulation of misfolded proteins occurs within the reticulum (Iurlaro and Muñoz-Pinedo, 2016). This pathway engages three key components of the unfolded protein

response (UPR) which collectively work to restore ER physiological condition. However, if the damage is maintained, apoptosis is initiated (Sano and Reed, 2013).

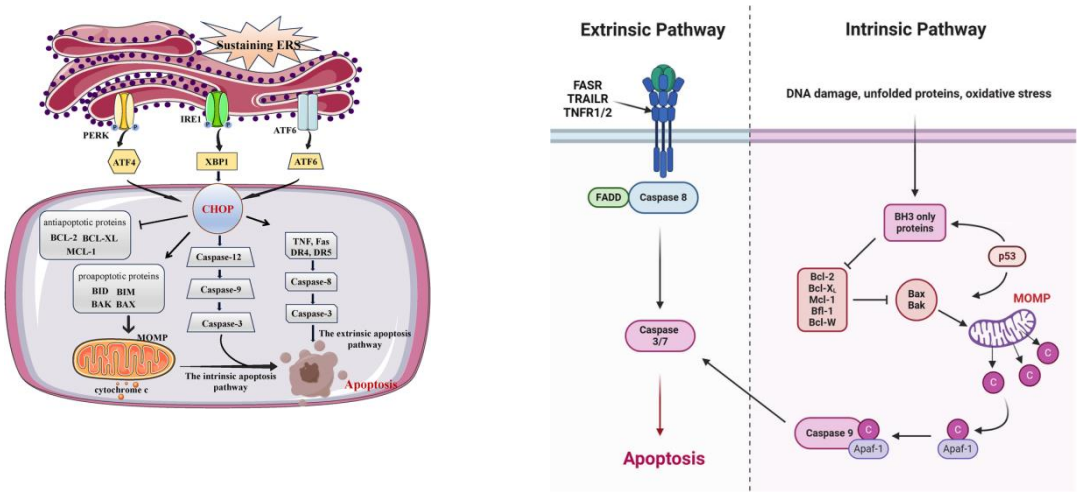


Figure 13. The three different types of apoptosis, ER-stress mediated, extrinsic, and intrinsic pathways, are shown (from Cheng et al. Front. Pharmacol. 15, 2024; Nwosi Cell death Dis 15, 2024).

When oxidative stress dramatically damages different cellular components, mitochondrial membrane alteration can occur, with the consequent induction of an intrinsic apoptotic pathway. Numerous studies report that counteracting oxidative stress by treatment with natural antioxidant molecules, such as polyphenols, or by using *N*-acetyl-cysteine, a powerful antioxidant molecule, can restore the cell physiological conditions and block the apoptotic pathway, thus saving the cell fate (Liu et al., 2024; Shi et al., 2024).

3.2 Antioxidant role of polyphenols

Polyphenols thanks to their chemical structure have a great antioxidant potential and can act in the prevention of various diseases. The discovery of the sequence “oxidative stress /antioxidant potential of secondary metabolites /good health” exponentially increased the research on these molecules in the last 20 years. A great number of papers that report the preventive effects of the different molecules of the polyphenol family against oxidative stress are present.

Among the phenolic acids, gallic acid represents the most abundant and widespread in the vegetal kingdom. Previous studies have established that gallic acid treatment has anticancer effects related to its ability to affect oxidative stress and regulate the oxidative conditions of the cells (Velderrain-Rodríguez et al., 2018; You and Park, 2010). However, different studies

revealed that both antioxidant and pro-oxidant effects of gallic acid can be responsible for anticancer properties. Apoptosis occurs in hepatocarcinoma cells when treated with a compound derived from gallic acid which, in the first hours of treatment, considerably increases ROS level and excessive mitochondrial stress (Huang et al., 2021). Likewise, another study revealed that gallic acid treatment significantly increased the level of H_2O_2 and O_2 in prostate cancer cells with the consequent activation of the apoptotic pathway (Russell et al., 2012). The antioxidant effects of gallic acid are relevant not only in pathological conditions but also in healthy cells.A study conducted by Yang et al. revealed that in human keratinocytes and fibroblasts, in normal and hyperglycaemic conditions, gallic acid accelerated cell migration and activated the proteins involved in wound repair, suggesting that gallic acid could be an effective therapeutic agent for treating wounds associated with metabolic complications (Yang et al., 2016).

3.2.1 Dual Roles of Polyphenols: Antioxidant Guardians and Pro-Oxidant Triggers

As mentioned, polyphenols exhibit antioxidant effects mainly due to their chemical structure, which enables them to act in three ways: as scavenger antioxidant species, inducers of antioxidant defense of the cells, and lastly by direct interaction with transition metal ions. The scavenger activity is carried out by either donating lone-pair electrons to form complexes with both transition metal ions and other pro-oxidant molecules or by the transfer of hydrogen atoms to free radicals.

The antioxidant role is also attributed to the inhibition of pro-inflammatory cytokines induced by ROS. Phenolic molecules are able to form complexes with transition metal ions in order to prevent the Fenton reaction reducing ROS production. Moreover, the formation of mono- or multi-complexes between polyphenols and metal cations is influenced by the pH of the cellular environment.

On the other hand, the pro-oxidant role of polyphenols can be explained by their ability to form phenoxyl radicals, that can generate, in the presence of oxygen, $O2^{-*}$, H_2O_2, quinones, and semiquinones. The generation of a pro-oxidant environment indirectly induces the activation of antioxidant systems of the cells that counteract this imbalance of ROS levels. Supporting this, a study conducted on erythrocytes demonstrated that anthocyanins and flavonoids protect against oxidative stress by activating antioxidant defenses through a pro-oxidant mechanism (Tedesco et al., 2021). Polyphenols contained in green tea can induce oxidative stress in a dose-dependent manner by the inhibition of AKT pro-survival signaling

pathway and lead prostate cancer cells to death. Moreover, the direct connection between AKT inhibition and cell death induced by the oxidative stress from green tea polyphenols was demonstrated (Posadino et al., 2017).

The pro-oxidant activity of polyphenols is also significantly affected by cellular pH. In acidic environments (pH <4), where H^+ ions dominate, reduced transition metals like Fe^{2+} are more soluble and stable, promoting pro-oxidant reactions, such as the Fenton reaction. Under these conditions, polyphenols exhibit limited deprotonation and low Fe-chelating capability, leaving free Fe ions to generate oxidants. Conversely, at a pH range of 4–9, polyphenol deprotonation increases, facilitating the formation of stable polyphenol-Fe complexes and reducing the availability of Fe ions for Fenton reactions (Pan et al., 2022). The number of hydroxyl groups in polyphenols enhances their capacity to bind high-charge-density metal ions (like Fe^{3+}, Fe^{2+}, Cu^{2+}, Zn^{2+}) (Hider et al., 2001). However, if deprotonation occurs without metal complexation, Fenton reactions can occur, sustaining ROS production.

The antioxidant or pro-oxidant effects of polyphenols are also dependent on their concentration. The relationship between concentration and redox properties is not always linear, indeed higher concentrations of polyphenols do not always lead to stronger antioxidant or pro-oxidant effects. For example, Trolox, an analog of vitamin E, showed antioxidant effects at low concentrations in HeLa cell cultures but exhibited pro-oxidant activity at higher concentrations (Giordano et al., 2020). On the contrary, extracts from *Aspalathus linearis* exhibited pro-oxidant effects at lower concentrations (0.1 mg/mL) and antioxidant effects at higher concentrations (1.5 mg/mL) (Joubert et al., 2005). Polyphenols like ferulic or caffeic acid show antioxidant behavior at low concentrations and pro-oxidant behavior at higher concentrations (Maurya and Devasagayam, 2010). Therefore, while polyphenols primarily exhibit antioxidant effects, several studies have reported that they can also function as pro-oxidant molecules depending on the treatment time and dosage.

Taken together, the different studies support the concept that a high concentration of polyphenols rather than acting as an antioxidant can lead to pro-oxidant-induced damage, and in contrast, low concentrations have an antioxidant effect. Given these variations, the antioxidant or pro-oxidant properties of polyphenols should be evaluated on a case-by-case basis, considering concentration, redox activity of all reactants, and pH. It is also crucial to account for differences in polyphenol concentrations between physiological conditions and in vitro experiments (Barreiro-Sisto et al., 2024).

Closely associated with the pro- or antioxidant properties of polyphenols is their ability to counteract cancer cell proliferation. Flavonoids could have chemo-preventive effects by modulating pro-survival or death pathways, such as autophagy or apoptosis, inducing cell cycle arrest, or stimulating the antioxidant cell defense. The anti-tumor properties of polyphenols have been extensively studied, making it difficult to provide a specific example for each bioactive molecule.

In several cancer models, including prostate, cervical, lung, breast, and colon cancers, quercetin, the most represented flavonoid, inhibits cell proliferation by inducing apoptosis and/or causing cell cycle arrest, such as G2/M or G1 arrest (Hasan et al., 2022; Tubtimsri et al., 2025). Additionally, quercetin actives the intrinsic apoptotic pathway in metastatic ovarian cancer cells by modulating Bcl-2 family proteins. Although quercetin is known as a potent antioxidant, capable of chelating metals, and scavenging oxygen free radicals, in different cancer cells quercetin behaves as a pro-oxidant molecule that induces DNA damage through reactive oxygen species generation (Hassanpour and Doroudi, 2023).

Among the phenolic acids with anti-tumor effects commonly found in plants, there are protocatechuic, syringic, and caffeic acids. Protocatechuic acid (3,4-dihydroxybenzoic acid) is present in melissa, cinnamon, rosemary, cauliflower, berries, lentils, and plum. It was found that in a model of liver cancer, it induced cytotoxicity through the stimulation of c-Jun N-terminal kinase (JNK) and p38 of MAPK family members, which are closely linked to cell death signals triggered by membrane receptors and extracellular stress (Yip et al., 2006). Moreover, in other cellular models, such as ovarian cancer cells, protocatechuic acid has been shown to induce cytotoxicity and reduce colony formation. This effect is achieved through cell cycle arrest in the G2/M phase and activation of the apoptotic pathway. Additionally, the treatment increased the levels of the autophagy-related protein LC3II (Xie et al., 2018).

Also syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid), which is abundant in olives, pumpkin, dates, grapes, spices, and honey, induces apoptosis in glioma cells. Once again, this effect is mediated through G2/M phase cell cycle arrest, modulation of Bax, Bcl-2, caspase-3, and p53 expression, as well as a reduction in mitochondrial membrane potential (Li et al., 2023; Srinivasulu et al., 2018).

The anticancer efficacy of caffeic acid, contained in olives, coffee beans, tea, wine, and fruits, is well known both in in vitro and in vivo models. In human melanoma cells, it

decreases cell viability and induces apoptosis through the increase in the level of gene expression of caspases. Also in this case, the effect of this phenolic acid is related to the enhancement of ROS levels and impairment of mitochondrial function (Pelinson et al., 2019).

4. The importance of the extraction method for the recovery of bioactive molecules

Having demonstrated the numerous health benefits of polyphenols, it becomes necessary to investigate the best extraction method for optimal recovery, being their bioavailability quite low. In agreement with a green point of view, the isolation and extraction methods should be eco-friendly, inexpensive, and safe for the operator. It may also be necessary to use a mathematical or statistical model to identify the best extraction conditions.

Generally, the choice of extraction method is influenced by the chemical nature of the substances, the size, and the possible presence of interfering molecules. Therefore, the extraction time, temperature, ratio solvent/matrix, as well as the choice of the solvent, which could be crucial for the successful extraction, must be considered. Moreover, sometimes it can be added a step before the extraction to remove all superfluous components, lipids, terpenes, and chlorophylls. Polyphenols can be extracted from fresh, dry, or frozen matrix, but it is critical a pre-treatment to grind and homogenize the matrix. Furthermore, the phenolic content is greatly influenced by the drying method; in fact, freeze-drying retains more polyphenols in the matrix compared to air-drying (Abascal et al., 2005).

The extraction methods can be divided into conventional and unconventional processes. The first one refers to solid-liquid extraction, liquid-liquid extraction, and maceration. Unconventional methods, including supercritical fluid extraction and subcritical water extraction, have been introduced to enhance yield and, in some cases, to make more green the extraction technique (Alara et al., 2021). However, polyphenol content optimization from the matrix remains a common feature in all types of extraction.

Conventional methods, which often involve combining a solvent such as alcohols (methanol, ethanol, diethyl ether, or ethyl acetate) with water at varying ratios, are preferred, despite several drawbacks. However, their use needs to be adjusted for the maximum recovery of polyphenol components. It is also necessary to consider the appropriate temperature, time, and pH for the extraction procedure. In this regard, the range temperature for the conventional extraction never exceeded 70 °C because a group of polyphenols, like anthocyanin, is thermolabile. Also, the extraction time is another critical point, since polyphenols are easy to degradation and sensitive to oxidation (Brglez Mojzer et al., 2016). Some studies show that ethanol and methanol are the best solvents for the extraction of polyphenols from the vegetal matrix, but it needs to consider cost, toxicity, and efficiency

extraction (El Mannoubi, 2023; Gil-Martín et al., 2022; Jiménez-Moreno et al., 2019). Ethanol is widely chosen because of its less toxic nature, low inflammability, and safety solvent. Moreover, ethanol represents the best choice for biological studies (Almohasin et al., 2023; Nguyen et al., 2020).

Alternatively, the unconventional methods are considered a new perspective of green extraction aimed at increasing the yield of extracted polyphenols. Recently, the unconventional methods that have received more attention are supercritical fluids and subcritical water extraction techniques. The new green technologies are environmentally friendly and do not use unsafety solvents, but they need to reach the perfect combination of pressure and temperature to optimize the extraction of the compounds to be studied.

Supercritical fluids extraction is a method that requires CO_2 in a supercritical state as solvent. This fluid is used as a green solvent for its non-toxicity and flammability, low cost and high available solvent, and high purity. The only crucial point is represented by its low polarity. The supercritical state of CO_2 is held employing low pressure and temperature reaching the critical point at 7.3 bar and 31.6 °C, in which there is no distinction between liquid or gas phase, so it has both gas and liquid properties. Often it is necessary to modify the polarity of the solvent by adding a co-solvent, such as ethanol and/or water, to enhance the extraction of more polar molecules (Da Silva et al., 2016).

A similar unconventional method is the subcritical water extraction technique, which involves the use of water in its subcritical state as a solvent. In this process, water is maintained in a liquid form under high pressure and within a temperature range of 100 to 374 °C (Soto Ayala and Luque De Castro, 2001). Water in this state is made more similar to organic solvents, like ethanol or methanol, since at higher temperatures the hydrogen bonds are less powerful due to the lower dielectric constant (Teo et al., 2010).

Recent studies have demonstrated that the supercritical fluid method can extract phenolic compounds from pomace generated in the industrial processing of orange juice, and grape seed from cacao fruit (Da Porto and Natolino, 2017; Espinosa-Pardo et al., 2017; Silva et al., 2024). However, since the yield of extraction can be different based on the variation of the extraction parameters, the addition of a co-solvent is necessary to enhance the polyphenols content and the antioxidant activity of the extract, as demonstrated by Quispe-Fuentes et al. in mandarin peel extract (Quispe-Fuentes et al., 2022). Several authors described that by

using a temperature of 60 °C and a pressure near 300 bar, the highest yield of phenolic compounds is reached as reported in peach fruit extraction (Espinosa-Pardo et al., 2014).

Also the subcritical water extraction was demonstrated to be an effective method for extracting polyphenolic compounds from a wide range of natural matrices. The technique allows for the extraction of compounds with high biological activities, while avoiding the employment of toxic solvents. The characterization of the extract obtained with subcritical water of chamomile flowers revealed the presence of catechin, gallic acid, ferulic acid glucoside, quercetin-3-glucoside, and other compounds responsible for its pharmacological activities (Cvetanović et al., 2015).

Moreover, HPLC analysis of extracts obtained at different temperatures and pressures revealed that the polarity of subcritical water was highly selective for extracting phenolic acids. Flavonoids such as rutin, luteolin, and apigenin were detected in lower concentrations, as reported in tea extract (Aćimović et al., 2021).

From what has been described, it is clear that there are many aspects to explore regarding the study of the biochemical potential of the molecules contained in agricultural waste, and within this context, my PhD project fits.

AIMS OF THE THESIS

My research project was supported by a grant from the green-based PON R&I 2014/2020, financed by the European Union.

In line with the objectives of the European program, the primary goals of my PhD project were twofold. First, to focus on the reuse of agri-food by-products from the Sicilian wine-making industry, which is closely connected to the concept of the circular economy. Second, to extract phenolic components from these by-products using alternative, environmentally friendly methods. Additionally, I aimed to explore the antioxidant and anticancer potential of these phenolic compounds.

Sicily is one of the most important Italian regions in which there is a lot of winery industries. For this reason, large quantities of waste are produced during wine production. If all these by-products could be used for advantageous purposes, it would achieve a dual objective: less environmental impact due to disposal and also improvement of human health through the extraction of bioactive compounds.

The winery by-products are represented by grape stems, grape pomace, and wastewater. Grape pomace, which contains skins, seeds, and other solid particles, is the most important residue and represents 20-25% of the total weight of grapes processed. Grapes can be different in maturation, cultivar, pedoclimatic conditions, and period of harvesting, consequently, the polyphenolic composition will be necessarily different, and the phenolic combination will be unique.

Although the literature includes some studies on polyphenols in wine and winemaking by-products, few specifically focus on Sicilian winemaking by-products (Amico et al., 2008; Di Stefano et al., 2022). Thus, this study is the first, to our knowledge, that evaluates the bioactive compounds of the Pinot Gris grape farmed in the western area of Sicily.

In this project, polyphenols were extracted from grape pomace employing different methods: conventional solid-liquid extraction and unconventional supercritical fluid and subcritical water extraction.

The main objectives were:

- ✓ the identification of the optimal extraction method and conditions for the maximum recovery of polyphenols and the characterization of extracts obtained by the different extraction methods;

- ✓ the analysis of the effects of the extracts on two cancer cell lines (colon and breast cancer cells) and on non-tumor cells and the study of biochemical pathways involved.

MATERIALS AND METHODS

White grape pomace recovery and extraction procedures

Grape pomace was supplied by Testa-Ferrarella's agri-food industry, (Trapani, Sicily) from *Vitis vinifer*a cultivar "Pinot gris", 2023 and 2024 harvest. The crude matrix (including grape skins, seeds and stems) was sun-dried by the industry and then stored at -80 °C until needed.

Before extraction, the crude material was ground into a fine powder. To optimize the extraction method, initial tests were conducted to identify the optimal chemical-physical conditions. Lipid components of the matrix were removed by using an organic solvent (*n*-hexane) in a 1:5 (*w/v*) ratio. The dried, defatted material was then subjected to extraction using a 57:43 (*v/v*) ethanol: water mixture as the solvent, with a solid-to-liquid ratio of 1:5 (*w/v*), for 60 mins at 58 °C to isolate polyphenolic compounds from the grape pomace. These parameters are derived from the Box Behnken Design (BBD), a statistical method which permits to optimisation of the recovery of total polyphenols and flavonoids (Abd-El-Aziz et al., 2022). This method was based on 3^3 factorial designs (Design Expert V.8). Two series of variables are considered: the independent variables: % solvent (0, 50 and 100% EtOH), temperature (25, 42.5 and 60 °C), pH (3, 6 and 9), and time (10, 35 and 60 min))., and the dependent variables (total content of polyphenols and flavonoids). The rate w/v of powder and solvent (1:5) was maintained constant.

Afterwards, the extract was centrifuged at 2,500 RCF for 15 mins, followed by 13,000 RCF for 20 mins at 4 °C. The resulting hydroalcoholic extract (HE) was filtered through a 0.22 µm filter and stored at -20 °C until further use. The dry weight of HE, determined after lyophilization, was 62.5 mg/g of grape pomace.

The supercritical fluid extraction was conducted by MATER Soc. Cons. a.r.l., the partner agency of the project. The supercritical fluid extraction system was equipped with a 1L extraction vessel, three separators in series (S1, S2, S3), a tank where CO_2 is stored, and a tank for its recycling. The grounded matrix, about 0.9 kg, was added to the extraction vessel, and the parameters were set. Different temperatures and pressures were employed for the extraction, 55, 60, and 70 °C and 150-280 bar, respectively. The flow rate of CO_2 was 25 kg/h and the extraction time 6 h, corresponding to 150 kg of CO_2.

The subcritical water extraction was conducted with a bench-top high-pressure system. The primary components of the system include a 200 mL stainless steel extractor vessel, a 1 L stainless steel water reservoir, a Chamber Oven XU032 (France Etuves) to control the temperature of both the reservoir and the extractor, a separator, and a high-pressure pump.

For each extraction experiment, 180 g of white grape pomace matrix was loaded into the extractor vessel. The operating pressure was kept at 100 bar to ensure that water stayed in the liquid phase at all the tested temperatures (120, 140, and 160 °C). The following parameters were held constant during all extractions: water flow rate (20 g/min) and total extraction duration (15 mins).

The aqueous extract obtained was collected in three sequential fractions using separate containers: 0–5 min, 5–10 min, and 10–15 min, which correspond to the fractions SWE $^{.1}$, SWE $^{.2}$, SWE $^{.3}$, respectively. Immediately after collection, the extracts were centrifuged at 3,880 RCF for 15 mins, and after at 15,500 RCF for 20 mins at 4 °C. Lastly, the extracts were filtered through a 0.22 µm filter and stored at -20 °C until further use. The dry weights of 1 mL of each extract were calculated after lyophilization and are reported in table.

Table 1. Dry Weight (DW) of the different fractions of SWE obtained using different temperatures (120, 140, and 160 °C) and recovered after diverse times of extraction (SWE $^{.1}$, SWE $^{.2}$, SWE $^{.3}$).

Extracts	Dry Weight (DW)
SWE $^{120.1}$	20 mg/mL
SWE $^{120.2}$	28.8 mg/mL
SWE $^{120.3}$	16 mg/mL
SWE $^{140.1}$	30 mg/mL
SWE $^{140.2}$	19 mg/mL
SWE $^{140.3}$	15.5 mg/mL
SWE $^{160.1}$	34.1 mg/mL
SWE $^{160.2}$	27.9 mg/mL
SWE $^{160.3}$	21.3 mg/mL

Total Polyphenolic Content Determination

The total polyphenol concentration was measured using the Folin-Ciocalteu colorimetric assay, with gallic acid used to produce the standard curve. Specifically, 100 µL of Folin-Ciocalteu reagent was added to 100 µL of the extract (diluted 1:100 in distilled water). After mixing in 800 µL of 5% sodium carbonate (*w/v*), the samples were incubated at 40 °C for 20 mins. They were then rapidly cooled on ice, and absorbance was measured spectrophotometrically at 765 nm (OPSYS MR, Dynex Technologies, Chantilly, VA, USA). The total polyphenol concentration was calculated as µg of gallic acid equivalent (GAE)

using an equation derived from the gallic acid calibration curve. The concentration of the extracts used in the experiments was based on their polyphenol content and expressed as µg GAE/mL.

Total Flavonoid Content Determination

The total flavonoid content was determined according to Attard. et al. (Attard et al., 2022) with some modifications (Shraim et al., 2021). For this assay, the extract was mixed with a solution of alumium chloride ($AlCl_3$) 0.3% (*w/v*) for 1 min. 200 µL of 1 mM NaOH was added and after 10 mins, the absorbance was measured spectrophotometrically at 410 nm (OPSYS MR, Dynex Technologies, Chantilly, VA, USA). The total flavonoid content was expressed in µg quercetin equivalent/mL since quercetin was used for the calibration curve.

Determination of Antioxidant Activity by DPPH (2,2-Diphenyl-1-picrylhydrazyl)

DPPH radical scavenger activity of grape pomace extracts was determined according to Attard et al. (Attard et al., 2022). A stock solution of DPPH 60 µM was prepared daily in methanol, and it was maintained at 4 °C in the dark. 50 µL of the extract was added to one well, and it was further reduced to the lowest concentration through a series of two-fold dilutions in a 96-well microtiter plate. 150 µL of DPPH solution was added to each well, and the reaction was conducted in the dark for 30 mins at room temperature. Two negative controls were prepared; the DPPH alone and the extract without DPPH. The absorbance was measured at 540 nm using a microplate reader (OPSYS MR, Dynex Technologies, Chantilly, VA, USA).

The DPPH free radical scavenging activity, expressed as IC_{50} (50% inhibitory concentration) was calculated by the following equation:

$$\%\ inhibition = \frac{[(Abs_{Blank} - Abs_{control}) - Abs_{sample}]}{Abs_{Blank} - Abs_{control}} X100$$

Qualitative and semi-quantitative analysis of the extract

A UPLC-Q Exactive Orbitrap-HRMS system (Thermo Fisher Scientific™, Bremen, Germany), which includes a Dionex Ultimate 3000 liquid chromatograph coupled with a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer and fitted with a heated

electrospray ionization (HESI) source, was employed to analyze the grape pomace extract sample. The chromatographic separation was performed using a Luna C18(2) column (150 x 2.0 mm, 5 µm) with a pre-column, and the mobile phases were composed of 0.1% formic acid (*v/v*) in water (Phase A) and 0.1% formic acid in methanol (Phase B). A gradient method with a flow rate of 200 µL/min was used as follows: start with 5% B, hold for 2 mins; increase to 95% B over 18 mins, hold for 2 mins; decrease to 5% B over 18 mins, and hold for another 2 mins, making a total run time of 40 mins. The injection volume was 1 µL.

Both full mass scan and targeted SIM (t-SIM) methods were applied. The Orbitrap was operated under the following conditions: alternate switching between negative (-) and positive (+) ESI full scan mode and t-SIM; sheath gas flow rate at 30 AU; discharge voltage at 3.0 kV; capillary temperature at 300 °C; resolution at 35,000 FWHM; AGC target set to $5*10^6$; maximum injection time at 200 ms; and a scan range of 80–1000 m/z. Calibration curves were constructed using five different concentrations for p-hydroxybenzoic acid, protocatechuic acid, p-coumaric acid, gallic acid, kaempferol, and quercetin. In cases where reference compounds were not available, structurally similar substances were used for calibration.

Cell Cultures and Conditions

Human colon cancer (HCT116), breast cancer (MDA MB-231), and human bronchial epithelial (HBE) cell lines, provided by the Interlab Cell Line Collection (ICLC, National Institute of Cancer Research, Genoa, Italy), were used to assess the biological effects of the extracts. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 2 mM glutamine, 10% heat-inactivated fetal bovine serum (FBS) and, 100 U/mL penicillin and 100 µg/mL streptomycin. 1% non-essential amino acids was added for the breast cancer cells. The cells were maintained in an incubator at 37 °C in a humidified atmosphere in the presence of 5% CO_2.

For the experiments, cells were seeded in the appropriate culture plates at 70–80% confluence for western blotting and MTT assays, or 40–50% for cell cycle and fluorescence analyses. For the experiments, the cells were seeded in appropriate culture plates at a confluence of 70-80% for Western Blotting and MTT tests, or at 40-50% for cell cycle and fluorescence analysis. The two percentages differ because, for cell cycle and fluorescence analysis, it is necessary that the cells are not too dense and do not form aggregates, which could interfere with the experimental outcomes. Following overnight incubation, cells were

treated for the designated times. 5 mM *N*-acetyl cysteine (NAC) and 100 nM bafilomycin A1 (BafA1) were used as pre-treatments for 1 hour. Sodium arsenite ($NaAsO_2$) was used at the non-toxic concentration (0.5 mM) for 1 h. Control cells, which were not exposed to the treatments, were incubated with the vehicle alone and analyzed concurrently.

Cell Viability Assays

Cell viability was assessed following treatment with varying concentrations of the extracts over specified times. The colorimetric MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Merk, Milan, Italy) was used to evaluate cellular metabolic activity as an indicator of cell viability and proliferation (Lauricella et al., 2003). The cells were seeded in 96-well plates in the presence of 200 µL of culture medium, and test agents were added after overnight incubation. After the treatments, 20 µL of MTT solution (5.5 mg/mL) was added. The resulting formazan crystals were dissolved in a lysis buffer solution (20% sodium dodecyl sulfate, 40% *N*, *N*-dimethylformamide, pH 4.7), and the absorbance was measured at 570 nm by using a microplate reader (OPSYS MR, Dynex Technologies, Chantilly, VA, USA). The absorbance value is directly proportional to the number of viable cells, and the viability of the treated cells was expressed as a percentage of the optical density (OD) values relative to the untreated cells.

Propidium iodide (PI) staining was also employed to evaluate cell viability. Cells were seeded in 24-well plates with 1 mL of culture medium for this analysis. After adhesion and treatments, cells were washed in PBS (Phosphate Buffered Saline pH 7.4) and incubated with PI (1 µg/mL) for approximately 10 mins in the dark at room temperature. Images were captured at 200X magnification using an Optika microscope (OPTIKA S.r.l., Ponteranica (BG), Italy) equipped with a computer imaging system (OPTIKA PROVIEW, version x64, 4.11.20805.20220506).

Cell Cycle Analysis

To assess cell cycle distribution, cells were collected through trypsinization (0.025% trypsin-EDTA; Life Technologies Ltd, Monza, Italy) and resuspended in a hypotonic solution containing 25 µg/mL Propidium Iodide, 0.1% sodium citrate, 0.2% Igepal, and 10 µg/mL RNase A. The distribution of cells across different cell cycle phases was analyzed using a Cytoflex Flow Cytometer (Beckman Coulter Life Sciences) with Cytoflex software. Cell debris and clumps were excluded by appropriate gating. The percentage of cells in the

subG0-G1 phase was used as an indicator of DNA fragmentation. At least 30,000 events per sample were analyzed, with the data stored in list mode files. The results shown in the figures are representative of three independent experiments.

Western Blotting Analysis and Antibodies

Western blotting analysis was conducted to examine the expression levels of proteins involved in the activated pathways. After treatment, cells were detached and lysed with RIPA buffer (1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors, pH 7.4) at 4 °C to prevent protein degradation. Cellular lysates were sonicated (following the instructions: 3 cycles of 10 seconds, intervals of 5 seconds) at a frequency of 20-30 kHz (Soniprep 150, Cellai Srl, Milano, Italia). Protein concentration was determined using the Bradford Protein Assay (Bio-Rad Laboratories S.r.l., Milan, Italy), and 30 µg of each protein sample was dissolved in sample buffer (50 mM Tris-HCl, 100 mM β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol, pH 6.8) and loaded onto a polyacrylamide for gel electrophoresis under denaturing conditions. A protein molecular weight marker was also included as an internal standard during electrophoresis. Following separation, proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories Srl, Segrate, Italy) by electroblotting. The membranes were then incubated with specific primary antibodies against the target proteins. Anti-HO-1 (GTX101147) was sourced from Gene Tex (GeneTex, Prodotti Gianni, MI, Italy); anti-SOD-2 (sc-133), Nrf2 (Sc-722), γH2AX (Sc-517348), anti-AKT 1/2/3 (Sc-8312) and anti-21 (Sc-6246) from Santa Cruz Biotechnologies (Santa Cruz, CA, USA); anti-62 (P0068), anti-Actin (A4700) and anti-Tubulin (T9026) from Merck Millipore (Merck-Sigma Aldrich, MI, Italy); and anti-Caspase-3 (96625), anti-PARP1 (9542), and anti-LC3 (2775S) from Cell Signaling Technology (Danvers, MA, USA); anti-phospho-AKT (Ser473) (AF887) from R&D SYSTEMS (Minneapolis, MN, USA). The antibody signals were detected using HRP-conjugated secondary antibodies (Promega, Fisher Scientific, MI, Italy) and enhanced chemiluminescence (ECL^{TM}) reagents (Amersham, Fisher Scientific, Milan, Italy) visualized with a ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA, USA). In some experiments, membranes were stripped with an appropriate stripping buffer to remove antibody complexes and re-probed with different antibodies to detect additional target proteins. Western blot quantification was normalized by actin or tubulin signal and relative to untreated cells (value 1).

Reactive Oxygen Species Detection

Reactive oxygen species (ROS) levels were evaluated by fluorescence microscopy and flow cytometry using the oxidation-sensitive dye 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Molecular Probe, Life Technologies, Eugene, OR, USA). In this regard, 20 mM of a stock solution of H_2DCFDA was reconstituted in DMSO and stored at -20 °C until use.

Cells were seeded in 24-well plates at a density of 2×10^4 cells/cm² for microscopy analysis. After treatment, cells were washed with PBS and incubated with 2 μM H2DCFDA dye for 15 minutes in the dark at 37 °C and 5% CO_2. Simultaneously, cells were stained with 2.5 μg/mL Hoechst 33342 to visualize nuclei under the same conditions. Following the incubation, excess dye was removed, and cells were washed with PBS. Fluorescence was analyzed using a fluorescence microscope: the signal from 2',7'-dichlorofluorescein (DCF), indicative of intracellular oxidation, was visualized with a FITC filter (488 nm excitation wavelength, 530 nm emission wavelength), while the Hoechst 33342 signal was observed using a DAPI filter (372 nm excitation wavelength, 456 nm emission wavelength). The images were captured at 200X magnification using an Optika IM3FL4 fluorescence microscope with a computer imaging system (OPTIKA PROVIEW, version x64, 4.11.20805.20220506). Relative ROS quantification was performed by using ImageJ software.

For flow cytometric analysis of ROS, cells were seeded on 6 well-plates, and after treatment cells were washed, trypsinized, centrifuged at 100 RCF, and stained with 0.5 μM H_2DCFDA for 10 mins in pre-warmed phenol red-free medium. After incubation, cells were immediately analyzed using a Cytoflex Flow Cytometer (Beckman Coulter Life Sciences) with Cytoflex software.

Evaluation of Autophagosomes

Monodansylcadaverine (MDC) staining was used to identify the autophagosomes, the acidic vesicular compartments involved in the autophagic pathway. Cells were stained with 0.05 mM MDC in PBS at 37 °C for 15 mins. After incubation, cells were washed three times with PBS and immediately examined under a fluorescence microscope using a DAPI filter with an excitation wavelength of 372 nm and an emission wavelength of 456 nm. Images were acquired at 200X magnification using an Optika IM3FL4 fluorescence microscope

connected to a computer imaging system (OPTIKA PROVIEW, version x64, 4.11.20805.20220506).

Statistical Analysis

Data are presented as mean ± S.D., and statistical analyses were performed using the Student's t-test and one-way analysis of variance (ANOVA). Comparisons were made between the control (untreated) group and all treated samples. Statistical significance was determined using one-way ANOVA, followed by Bonferroni's post-hoc test. P-values of less than 0.05 were considered statistically significant.

RESULTS

PART 1. CHARACTERIZATION AND STUDY OF THE EFFECTS OF HYDROALCOHOLIC EXTRACTS FROM SICILIAN WHITE GRAPE POMACE

In the first phase of the project, when the extraction technique with the most innovative methods had not yet been developed, research focused on the extraction of the polyphenolic components from the matrix obtained from local farms using a traditional extraction method consisting of a mixture of ethanol/water. The use of ethanol as a hydroalcoholic solvent is still considered a suitable and environmentally friendly method (Rodríguez De Luna et al., 2020) for the extraction of phenolic compounds from different matrices.

1.1 Chemical composition and characterization of Hydroalcoholic Extract (HE) from Sicilian white grape pomace

Sicilian white grape pomace refers to the solid residue produced by local winemaking industries. The extraction method used was based on solid-liquid extraction principles. However, since the extraction parameters are crucial for maximizing the yield of the components, an optimization study was carried out in collaboration with the MCAST Institute (Malta College of Arts, Science and Technology, Paola, Malta).

The analysis showed that the optimal parameters for extraction from white grape pomace were: 57% ethanol/water solution, 58 °C temperature, extraction time of 60 mins, and neutral pH. Colorimetric assays revealed that hydroalcoholic extract (HE) contains 30 ± 5 mg GAE/g DW (dry weight) of total polyphenols (TPC) and 23.6 ± 4 mg QE/g DW of total flavonoids (TFC). The antioxidant activity of HE was investigated using the DPPH scavenging assay and revealed that the concentration capable of reducing 50% of free radicals (IC_{50}) was about 138 ± 7.4 μg DW/mL. Table 2 shows the results obtained from TPC, TFC, and DPPH assays.

Table 2. Mean values ± standard deviation (SD) of total phenolic and flavonoid content, and antioxidant capacity of hydroalcoholic extract (HE).

	HE
Total Phenolic Content (TPC)	30 ±5 mg GAE/g DW
Total Flavonoid content (TFC)	23.6 ±4 mg QE/g DW
IC_{50} DPPH analysis	138 ±7.4 μg DW/mL

To better characterize the individual components of the extract, high-resolution mass spectrometry was performed in collaboration with the ATEN Center, University of Palermo. The analysis identified 17 out of 23 compounds in the extract, as shown in Table 3. The predominant component of the phenolic acid group was gallic acid (20.96 mg/100 g DW), while catechin, epicatechin, and quercetin as the main compounds of the anthoxanthins group (91.96, 52.74, and 20.04 mg/100 g DW, respectively).

Table 3. Compounds identified in the grape pomace HE. For each component the relative retention time (R.T.), exact mass (*m/z*), and concentration expressed as mg of molecule per 100 g of dry weight (DW) was reported. NF, not found. (from Affranchi et al., Biomolecules 3;14(9):1111,2024).

Phenolic Acids			
Analytes	**R.T.**	**mg/100 g DW**	**m/z**
Gallic acid	6.6	20.96	169.01425
Protocatechuic acid	9.0	4.472	153.01824
Caffeic acid	11.4	NF	179.03389
Caftaric acid	9.7	3.22	311.03976
Syringic acid	11.5	NF	197.04445
p-coumaric acid	12.8	0.528	163.03897
Ferulic acid	13.0	NF	193.05063
Fertaric acid	11.2	1.728	325.05541
p- hydroxybenzoic acid	10.8	1.232	137.02332
Anthoxanthins (flavones and flavonols)			
Analytes	**R.T.**	**mg/100 g DW**	**m/z**
Procyanidin B1	8.9	11.488	577.13405
Catechin	9.8	91.96	289.07066
Epicatechin gallate	11.7	5.408	441.08162
Procyanidin B2	9.4	5.792	577.13405
Epicatechin	11.0	52.736	289.07066
Quercetin 3-O-glucoside	13.6	5.312	463.0871
Quercetin 3-O-glucuronide	14.0	9.08	477.06637
Quercetin 3-O-galactoside	12.9	NF	463.0871
Quercetin 3-O-rhamnoside	13.3	NF	447.09219
Kaempferol 3-O-glucoside	14.4	0.896	447.09219
Quercetin	15.7	20.04	301.03428
Kaempferol	16.8	3.608	285.03936
Stilbenes			
Analytes	**R.T.**	**mg/100 g DW**	**m/z**
trans-Resveratrol	14.9	NF	227.07027
trans-Polydatin	12.3	0.584	389.12309

1.2 Grape pomace HE shows an antiproliferative effect in colon and breast cancer cell lines

Grape pomace HE was tested for its ability to modify the cell viability of two cancer cell lines, colon (HCT116) and breast (MDA MB-231) cancer cells. Firstly, different HE concentrations were used to assess its cytotoxic ability by using an MTT assay. Results reported in Figure 1a suggest that cell viability was reduced in a time- and dose-dependent manner when HE was used at concentrations greater than 25 μg GAE/mL. Furthermore, HCT116 cells showed a greater response to the treatment, with a viability reduction of

approximately 70% after 48 hours and 90% after 72 hours of treatment with 75 µg GAE/mL. Differently, in MDA MB-231 cells the reduction of cell viability never exceeded 40% even after 72 h of treatment with the highest concentration (150 µg GAE/mL). The results of the MTT assay were confirmed by the propidium iodide (PI) staining. As shown in Figure 1b, after 72 h of treatment with 75 µg GAE/mL HE, almost all HCT116 cells were positive for propidium iodide staining, confirming the induction of cell death. In contrast, MDA MB-231 cells did not exhibit significant PI positivity, indicating a lower sensitivity of breast cancer cells to the HE treatment.

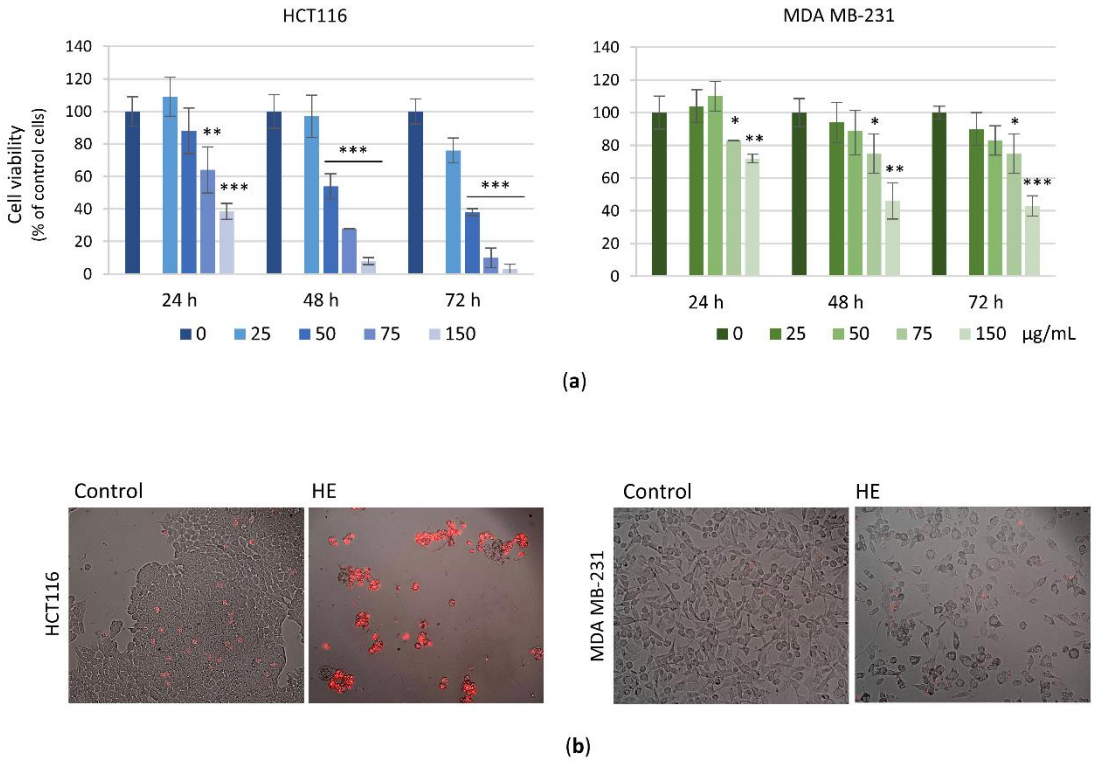
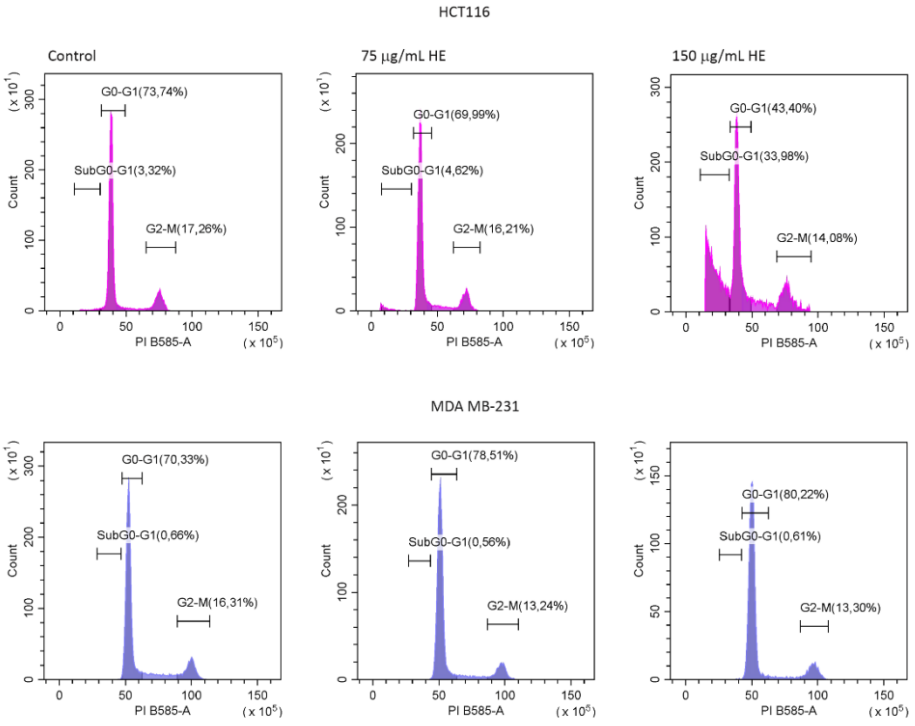


Figure 1. Time- and dose-dependent effects of HE on HCT116 and MDA MB-231 cells. (a) Cell viability was assessed using the MTT assay after 24, 48, and 72 h of HE treatment, with HE concentrations ranging from 25 to 150 µg GAE/mL. The results, which are representative of three independent experiments, are expressed as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. untreated control. (b) Representative merged images of bright field and propidium iodide-stained cells after 72 h of treatment with 75 µg GAE/mL HE. Cells were visualized under a fluorescent microscope at 200× magnification, and the images were captured using OptiKa Proview software. (from Affranchi et al., Biomolecules 3;14(9):1111,2024).

To further confirm these results, the analysis of cell cycle distribution was performed using the highest HE concentrations (75 and 150 µg GAE/mL) for 24 and 48 h of treatment. The analysis presented in Figure 2 shows an increase in the percentage of HCT116 cells confined in the subG0-G1 phase of the cell cycle, indicating DNA fragmentation, from 3.32% in the

control to 33.98% in cells treated with the highest concentration of HE for 48 h. A similar result was obtained after 24 h of treatment (Figure 2b). In contrast, in MDA MB-231 cells, no significant increase was observed in the percentage of cells in the subG0-G1 phase after HE treatment. However, a moderate rise in the percentage of cells in the G1 phase was detected, suggesting that in MDA MB-231 cells HE treatment is responsible for a block of cell proliferation (Figure 2).



(a)

	HCT116	SubG0-G1	G0-G1	G2-M	MDA-MB231	SubG0-G1	G0-G1	G2-M
24 h	Control	3.64 ± 2.3	47.45 ± 4.3	30.65 ± 5.3	Control	0.68 ± 0.03	61.1 ± 11	21.5 ± 7.3
	75 µg/mL HE	9.69 ± 0.9	53.39 ± 11.1	29.88 ± 8.9	75 µg/mL HE	0.77 ± 0.29	70.15 ± 11	18 ± 6.8
	150 µg/mL HE	23.74 ± 2.2	42.84 ± 1.7	28.74 ± 0.7	150 µg/mL HE	0.62 ± 0.02	74.33 ± 8.1	17.98 ± 6.6
48 h	Control	2.77 ± 1.2	67.9 ± 7	21.87 ± 5.5	Control	0.83 ± 0.2	64.8 ± 7.7	19.47 ± 4.4
	75 µg/mL HE	5.29 ± 1.8	65.6 ± 2.1	20.3 ± 0.3	75 µg/mL HE	0.7 ± 0.2	72.7 ± 8.1	17.1 ± 5.4
	150 µg/mL HE	35.46 ± 11	41.87 ± 7.7	13.68 ± 2.6	150 µg/mL HE	0.6 ± 0.02	71.4 ± 11.4	19.3 ± 8.3

(b)

Figure 2. Effects of HE on the cell cycle distribution of HCT116 and MDA MB-231 cells. (a) Flow cytometric analysis of cell cycle distribution after 48 h of treatment with 75 and 150 µg GAE/mL HE. (b) Summary table showing the percentage of cells in the different phases of the cell cycle after 24 and 48 h of treatment with 75 and 150 µg/mL GAE HE. Data are presented as mean ± SD (from Affranchi et al., Biomolecules 3;14(9):1111,2024).

1.3 Study of the biochemical pathways responsible for the antiproliferative effect triggered by HE

To investigate the biochemical mechanisms underlying the different behavior of HCT116 and MDA MB-231 cells following HE treatment, molecular pathways associated with apoptosis, survival, and cell cycle arrest were examined.
Initially, Western blotting analysis was focused on the evaluation of two proteins involved in the canonical apoptotic pathway, caspase-3 and poly-ADP-ribose polymerase-1 (PARP1). Caspase-3 is the main executive protease of the apoptotic pathway. In the cells, it is expressed in a pro-enzymatic form that is activated through a cleavage by upstream caspases following an apoptotic insult (Ponder and Boise, 2019). PARP1 plays an important role in the DNA repair mechanism. It is subjected to cleavage, producing different fragments involved in various cell death pathways. The formation of the 89 kDa fragment induced by caspase-3 is largely considered a general apoptotic marker, and it is indicative of the presence of the active form of caspase-3 (Chaitanya et al., 2010; Mashimo et al., 2021).

As shown in Figure 3, after 48 h of treatment, a remarkable reduction of the level of the pro-caspase-3 and the appearance of the 89 kDa fragment of PARP1, indicative of the activation of caspase-3, were observed only in HCT116 cells. These data suggested the involvement of the apoptotic pathway in HCT116 cell death (Figure 3 left panel).

The data regarding the expression of these proteins in MDA MB-231 cells confirmed the previous findings. In MDA MB-231 cells, the activation of the canonical cell death pathway did not appear to be involved. Indeed, the level of pro-caspase-3 was not influenced by HE treatment, and the canonical PARP1 fragment was not detected (Figure 3 right panel). However, it is important to underline the appearance of only a non-canonical PARP1 fragment (66 kDa) probably caused by MMP-2 (Matrix MetalloProteinases-2) cleavage (Kwan et al., 2004).

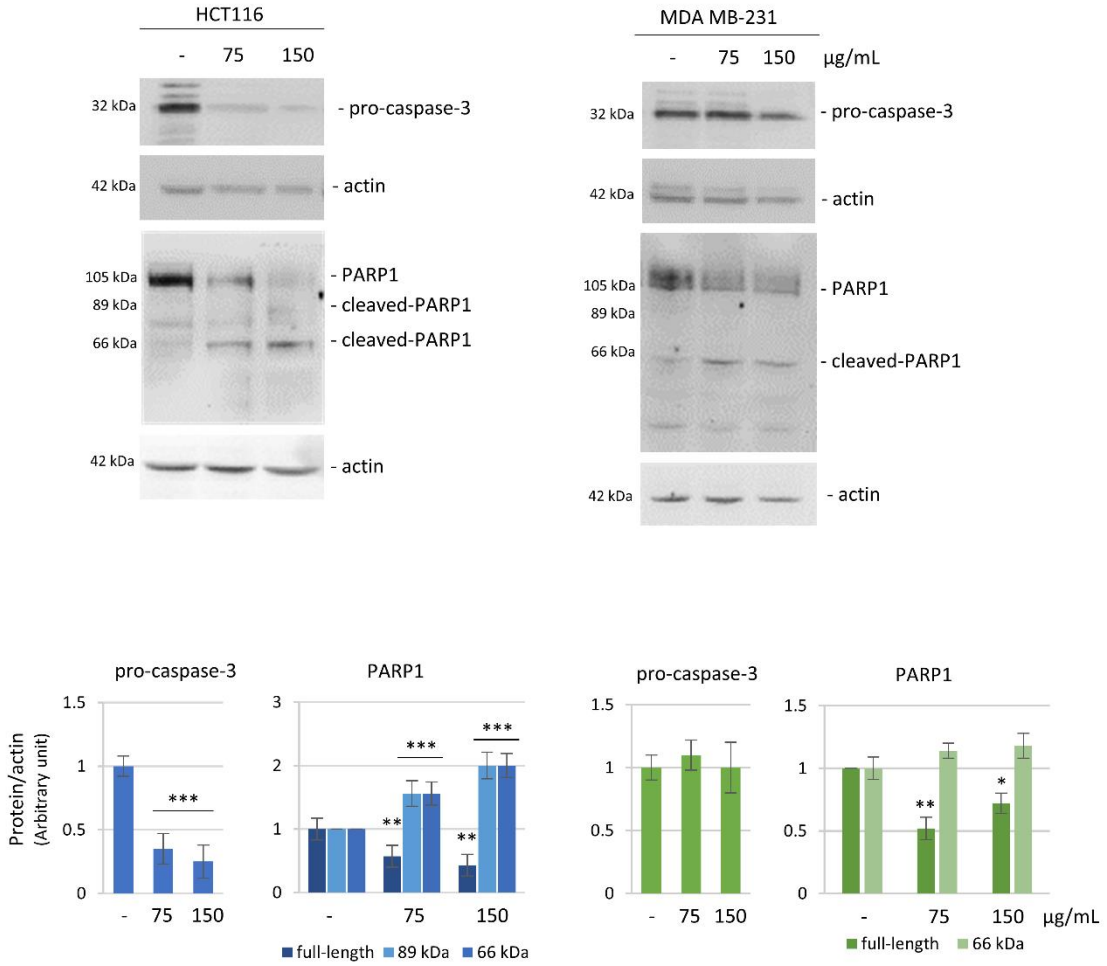


Figure 3. HE effect on the expression of apoptotic markers. Western blot analysis of pro-caspase-3 and PARP1 after 48 h of treatment with 75 and 150 µg GAE/mL HE in HCT116 cells (left panel) and MDA MB-231 cells (right panel). Actin blot was used as a loading control to normalize protein levels. Densitometric analysis, performed using Quantity One software (version 4.6.6), is shown in the histograms below. The results are representative of three independent experiments, and values are expressed as mean ± SD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. untreated control (from Affranchi et al., Biomolecules 3;14(9):1111,2024).

The different responses of the two different cancer cell lines to HE treatment suggested a likely different expression level of some pro-survival proteins, which are often involved in response to cell death insults.

For this purpose, the level of γH2AX, that is the phosphorylated form of histone H2AX at the residue Ser139, was evaluated as a marker of DNA double strand breaks, which is implicated in the early phase of apoptosis (Lauricella et al., 2019). After 48 h of HE treatment, an increase of γH2AX occurred only in HCT116 cells, while in MDA MB-231

the level of this protein was maintained at the basal condition also after the highest dose of HE employed (Figure 4).

Then, the level of AKT factor was evaluated. This serine/threonine protein kinase is involved in an important survival pathway, and its phosphorylation in Ser473 makes it fully active (Yung et al., 2011). Western blotting analysis showed that HE treatment markedly reduced the levels of total and phosphorylated form of AKT only in HCT116 cells; this differs in MDA MB-231 cells where there was an increase in pAKT level (Figure 4).

Lastly, the level of p21/WAF1 was also analyzed. This factor promotes cell cycle arrest by inhibiting the cyclin-dependent kinases (CDK). However recent literature has underlined the conflicting role of p21/WAF1 in cancer cells being involved either in tumorigenesis or in tumor suppression. This different behavior seems to be also related to a pro- or anti-apoptotic role (Lai et al., 2020). Our results showed that the p21 level was notably increased in HCT116, whilst in MDA MB-231 cells only a slight increase was observed (Figure 4).

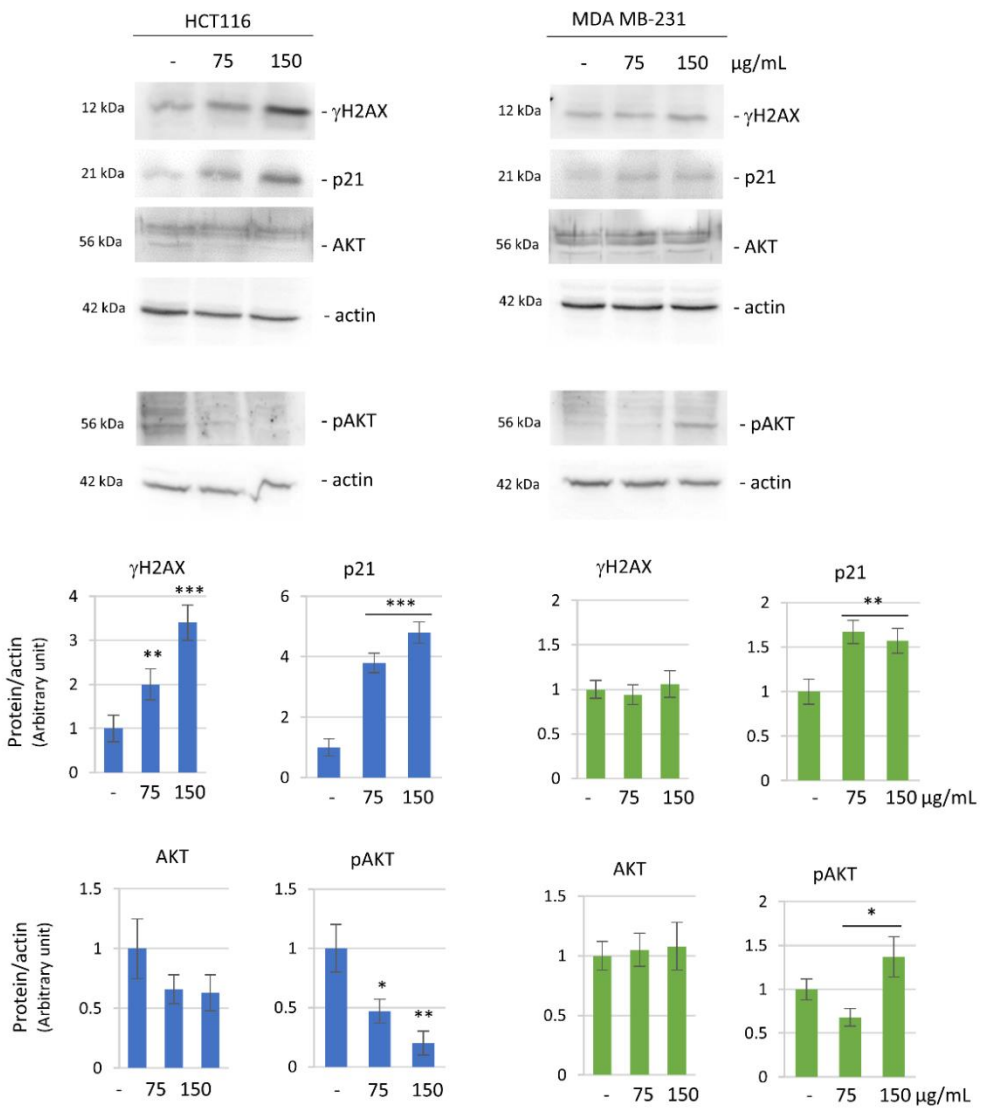


Figure 4. HE effect on the expression of some pro-survival markers. Western blot analysis of γH2AX, p21, AKT, and phosphorylated AKT (pAKT) under basal conditions and after 48 h of treatment with 75 and 150 µg GAE/mL HE in HCT116 cells (left panel) and MDA MB-231 cells (right panel) are shown. Actin blot was used as a loading control to normalize protein levels. Densitometric analysis, performed using Quantity One software, is shown in the histograms below. The results are representative of two independent experiments with similar outcomes, and values are expressed as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. untreated control (from Affranchi et al., Biomolecules 3;14(9):1111,2024).

1.4 HE treatment increases Reactive Species of Oxygen (ROS)

Evaluating reactive oxygen species (ROS) levels is essential for understanding their dual role in tumor cells. Their moderate or high levels can sustain both tumor progression and different cell death pathways, such as apoptosis, depending on the context (Iqbal et al., 2024).

To investigate the effects of HE treatment on ROS levels in the cellular models used, the intracellular ROS production over time, with different concentrations of the extract alone or in combination with a powerful antioxidant agent, *N*-acetyl-cysteine (NAC) (Zafarullah et al., 2003) were assessed.

In Figure 5a, the increase in green fluorescence was attributable to the conversion of H_2DCFDA, a non-fluorescent molecule, to fluorescent dye DCFDA, an event which is a consequence of ROS production. In both HCT116 and MDA MB-231 cells, the effect was detectable, nevertheless, it was more evident in HCT116 cells. Moreover, the increase in green fluorescence was prevented by NAC treatment in both cell lines.

Additionally, the continuous monitoring of the experiment over time revealed that in HCT116 cells, the increase in ROS levels occurred early during HE treatment, returning to levels comparable to untreated cells after 24 h. In contrast, a different pattern was observed in MDA MB-231 cells. In these cells, the increase in ROS level, indicated by the high fluorescence, persisted up to 24 h (Figure 5b). Furthermore, in HCT116 cells, the increase in ROS levels induced by HE was almost completely prevented by the presence of NAC. In contrast, a weaker NAC protective effect was observed in MDA MB-231 cells, particularly at 24 h, when the highest ROS levels were detected (Figure 5b).

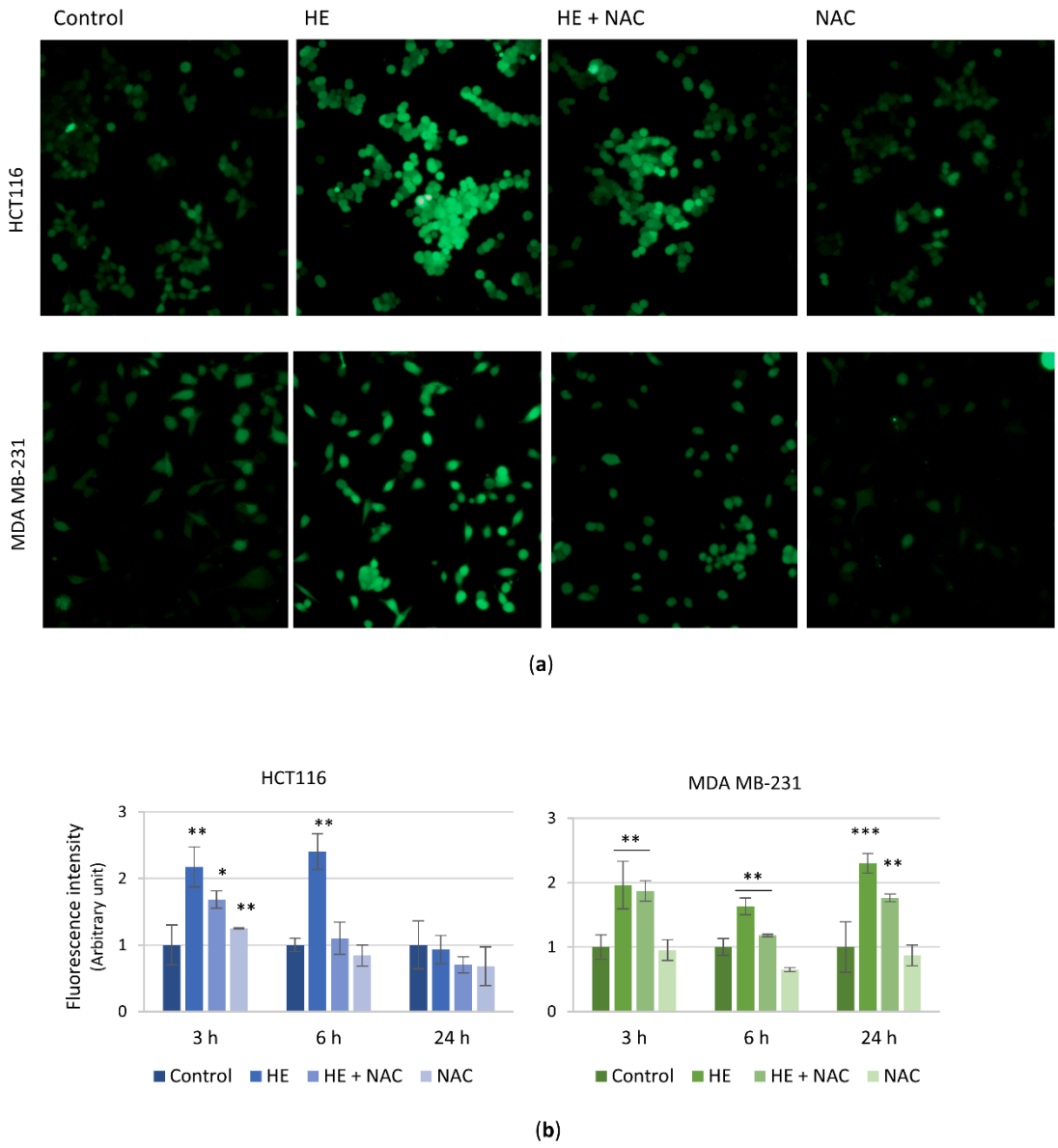


Figure 5. HE effects on reactive oxygen species (ROS) production. HCT116 and MDA MB-231 cells were treated with 150 µg/mL GAE of HE for 3, 6, and 24 h alone or in combination with 5 mM NAC. Magnification 200× (a) Fluorescence images are representative of the effects observed after 6 h of treatment. (b) Time-dependent analysis of fluorescence intensity was carried out through ImageJ software (version IJ 1.46r). Results are representative of three independent experiments and fluorescence intensity values are expressed as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. untreated control (from Affranchi et al., Biomolecules 3;14(9):1111,2024).

The reduction of ROS level observed starting 24 h of HE treatment could be a consequence of the activation of antioxidant systems in response to the stress that, as anticipated in the Introduction, can defend the cell promptly. To assess this, the levels of several antioxidant markers were tested both in HCT116 and MDA MB-231 cells. Figure 6 demonstrates that after 24 h treatment, a significant increase in the level of heme oxygenase–1 (HO-1), the first enzyme that counteracts the reactive species (Salerno et al., 2024), occurred in HCT116 cells. The raised level of HO-1 was possibly related to the increase of Nrf2, a well-known transcription factor promoting its expression (Zhang et al., 2021). At the same time, superoxide dismutase (SOD), another antioxidant protein that neutralizes the superoxide by-product (Palma et al., 2020), highly increased its expression by about six-fold compared to control cells after 24 h of HE exposure. These effects were significantly (considering HO-1 and Nrf2) or moderately (considering SOD-2) reduced when HE treatment was preceded by exposure to the antioxidant NAC. Similar effects, but much less evident, were observed in MDA MB-231 cells, where only a moderate rise of the antioxidant markers occurred, and a lower protective effect of NAC was observed (Figure 6). So, the persistence of high levels of ROS in these cells even at 24 h is consistent with these results.

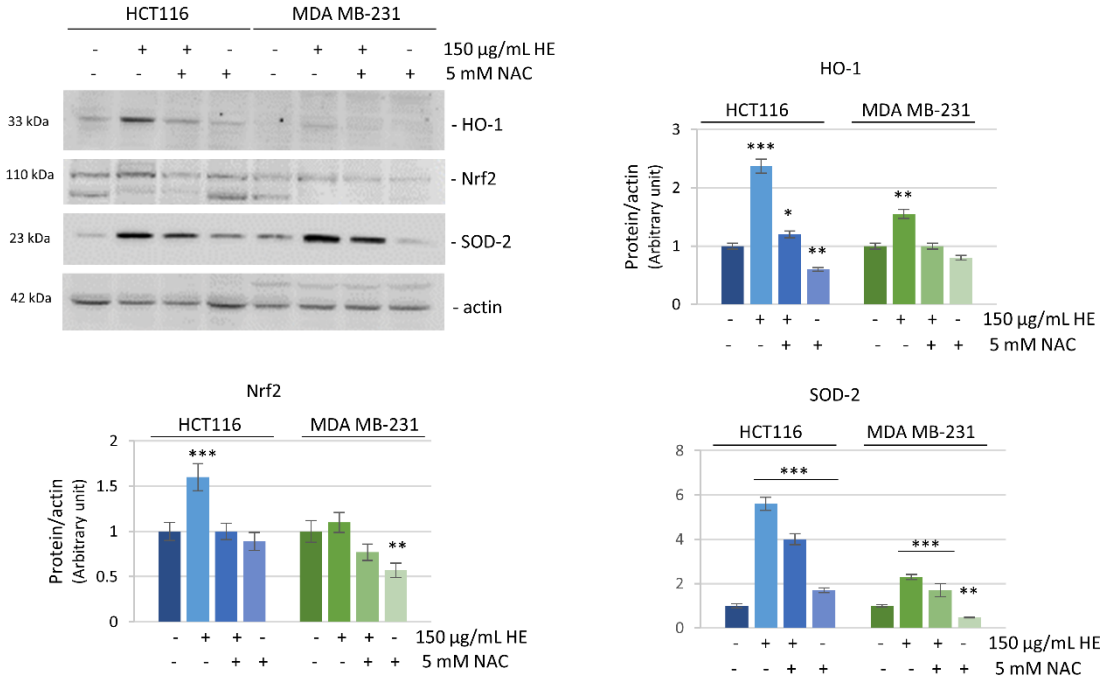


Figure 6. Evaluation of antioxidant enzyme expression. Western blot analysis of antioxidant proteins (HO-1, Nrf2, and SOD-2) after 24 h of treatment with 150 µg GAE/mL HE in HCT116 and MDA MB-231 cells. Protein levels were normalized to actin. Densitometric analysis, performed using Quantity One software, is shown in the histograms. The results are representative of three

independent experiments, with values expressed as mean ± SD. *p<0.05, **p<0.01, ***p<0.001 vs. untreated control (from Affranchi et al., Biomolecules 3;14(9):1111,2024).

1.5 A possible pro-survival role of autophagy in HE-induced mechanism is related to ROS increase

The strong increase in ROS level in the first hours of treatment suggested that another mechanism was involved in helping cell recovery. In several cellular systems, it was demonstrated that ROS increase is associated with the activation of an autophagic response (Devenport and Shah, 2019; Lauzier et al., 2019; Yun et al., 2020). Furthermore, previous studies have shown that HCT116 cells exhibit a greater sensitivity to the activation of the autophagic pathway (Celesia et al., 2022; Cernigliaro et al., 2019; Notaro et al., 2023).

Since autophagy is a process responsible for the degradation of cytoplasmic components in acidic vacuoles, the fluorescent dye monodansylcadaverine (MDC), which labels acidic vacuoles, was used in order to analyze the potential induction of autophagy in our models.

The images in Figure 7a show that HE treatment is associated with an increase in fluorescence related to the presence of acidic compartments in both cell lines. Cells remained positive to the probe up to 6 h of treatment, although HCT116 cells exhibited more responsiveness compared to MDA MB-231 cells. The presence of autophagic vacuoles gradually decreased in a time-dependent manner, until the fluorescence was comparable to that of untreated cells after about 48 h of treatment (Figure 7b).

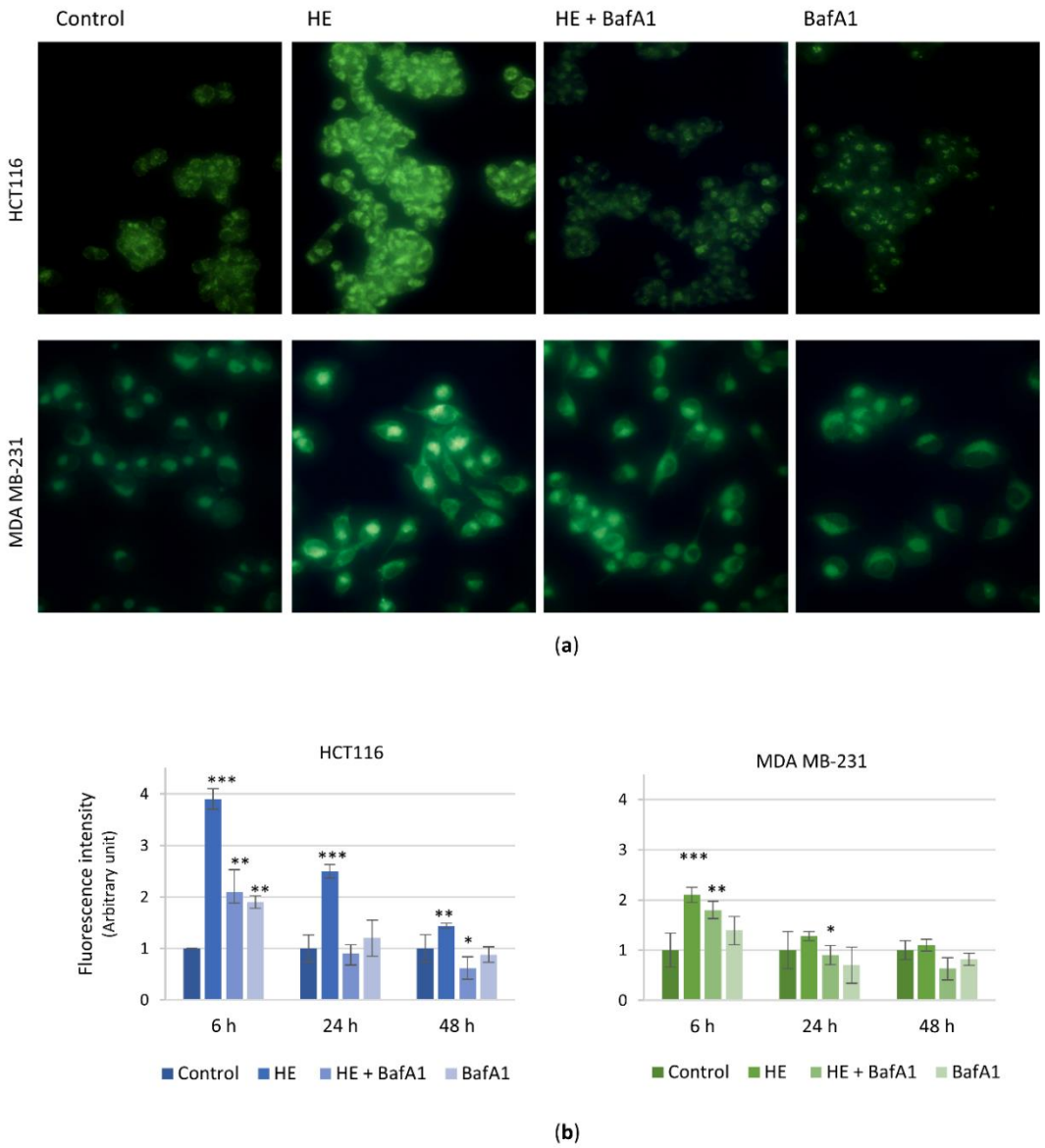


Figure 7. Autophagosome staining following HE treatment in HCT116 and MDA MB-231 cells. Cells were exposed to 150 µg GAE/mL HE alone or combined with 100 nM bafilomycinA1. After 6, 24, and 48 h, the cells were incubated with 50 µM MDC for 10 minutes and examined under a fluorescence microscope. Magnification 200x. (a) Representative images were taken after 6 h of treatment. (b) The histograms present a time-dependent analysis of fluorescence intensity, performed using ImageJ software. Data are representative of two independent experiments with similar outcomes, and fluorescence intensity values are shown as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to untreated control (from Affranchi et al., Biomolecules 3;14(9):1111,2024).

To further confirm the activation of the autophagic pathway, the levels of two specific autophagic markers, LC3 and p62, were evaluated by using western blotting analysis.

LC3 (microtubule-associated protein 1 light chain 3) is a cytosolic protein (LC3I) recruited to the membrane of the autophagosomes where it is cleaved and lipidated (LC3II); p62/SQSTM1 is an autophagosome cargo protein that recruits other proteins for selective

autophagy. It functions as an adaptor factor between LC3 and ubiquitinated substrates (Klionsky et al., 2016). To monitor the follow-up of the autophagic markers, a time- and dose-dependent experiment was performed. As shown in Figure 8, p62 and LC3II levels were high at 24 h of HE treatment in both cell lines; subsequently, in HCT116 cells the levels of the proteins decreased reaching those observed in untreated cells after 48 h of HE treatment, whereas in MDA MB-231 cells, the expression of the two markers remained elevated overall time.

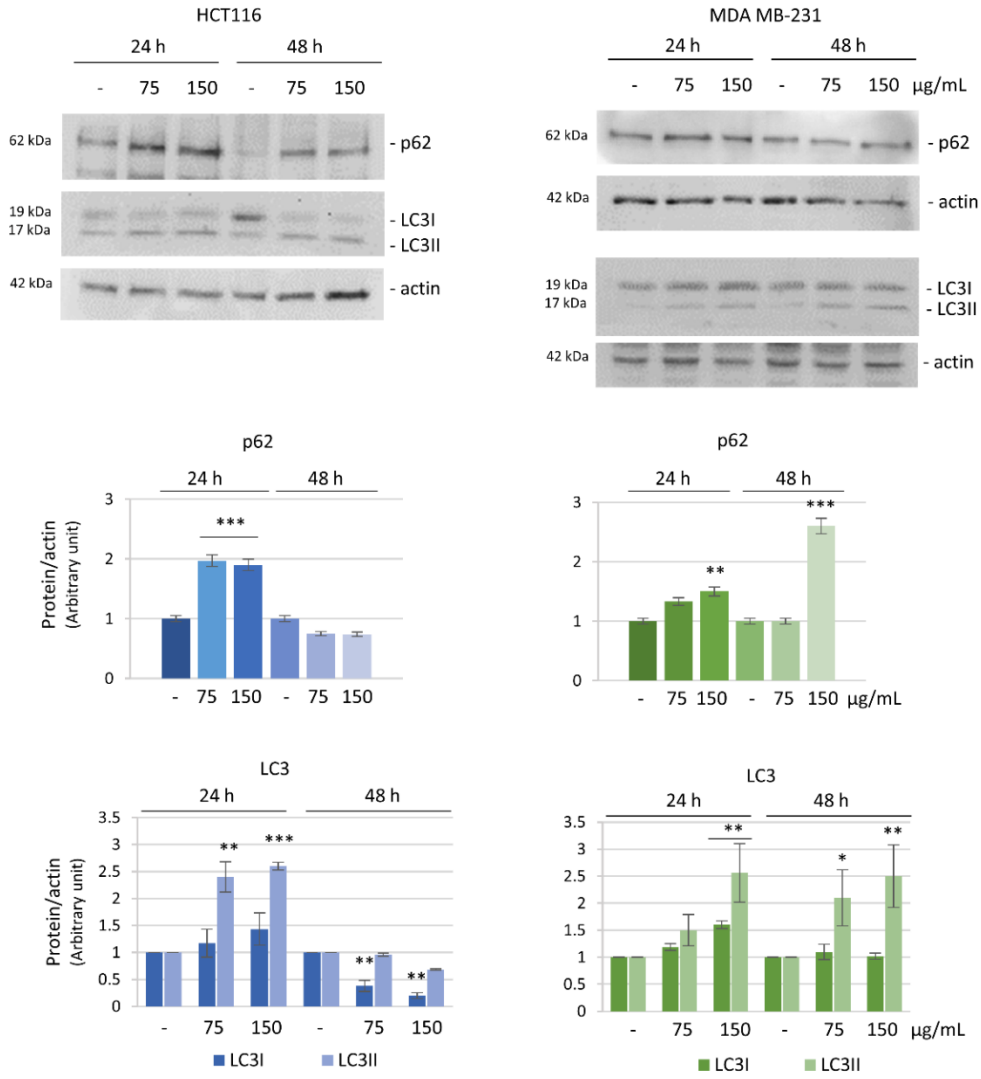


Figure 8. Assessment of autophagic pathway markers. Western blotting analysis of the autophagic markers p62 and LC3 following treatment with 75 or 150 µg GAE/mL HE for 24 or 48 h in HCT116 and MDA MB-231 cells is shown. Protein levels were normalized to actin. Densitometric analysis was performed using Quantity One software, and the results are presented in the histograms. Data represent three independent experiments, and values are expressed as mean ± SD. * $p < 0.05$, ** $p <$

0.01, *** p < 0.001 compared to untreated control (from Affranchi et al., Biomolecules 3;14(9):1111,2024).

The effects of HE treatment relative to the formation of autophagic vacuoles were counteracted when a pre-treatment with bafilomycin A1 (BafA1), a potent inhibitor of autophagic flux (Mauvezin and Neufeld, 2015), was applied (Figure 7). Concerning the level of p62 and LC3, high expression levels were observed in the cells treated with the combination of HE and BafA1 or in the presence of BafA1 alone (Figure 10), confirming that bafilomycin A1 disrupts the autophagic flux.

Pre-treatment with bafilomycin A1 did not affect cell viability in HCT116 cells when it was used combined with HE, while, interestingly, it was responsible for the decrease of the percentage of viable cells when used in combination with HE on MDA MB-231 cells. This evidence suggests a pro-survival role of autophagy, which helps to save breast cancer cells from the effects of HE treatment (Figure 9a).

Further, to evaluate if the cytotoxicity of the combination HE/Baf1 in MDA MB-231 cells was due to the activation of the apoptotic cell death pathway, the level of pro-caspase-3 was evaluated. Figure 9b demonstrates that, despite the occurrence of a cytotoxic event when autophagy was inhibited in HE-treated cells, caspase-3 and thus, the canonical apoptotic pathway, are not involved.

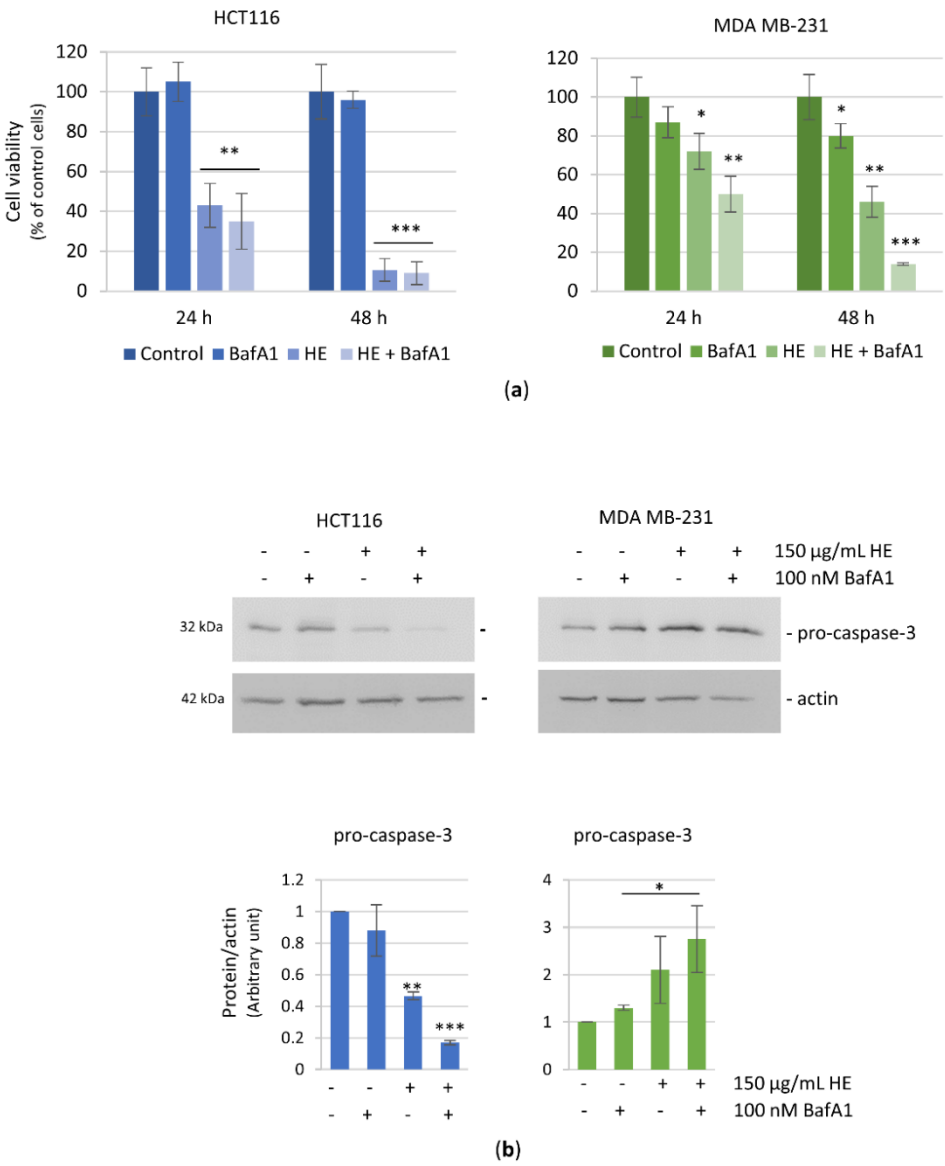


Figure 9. The impact of the autophagy inhibitor bafilomycin A1 (BafA1) on cell viability and apoptotic markers. (a) Cell viability was assessed using the MTT assay after 24 and 48 h of treatment with 150 µg GAE/mL HE alone or in combination with 100 nM bafilomycin A1. The results represent three independent experiments and are shown as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to untreated control. (b) Western blot analysis of pro-caspase-3 expression after 24 h of treatment with 150 µg/mL GAE of HE alone or with 100 nM bafilomycin A1 in HCT116 and MDA MB-231 cells. Protein levels were normalized to actin. Densitometric analysis was performed using Quantity One software, and the results are presented in the histograms. Data represent two independent experiments with similar outcomes and are shown as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to untreated control (from Affranchi et al., Biomolecules 3;14(9):1111,2024).

Lastly, to assess a relationship between ROS increase and the activation of autophagy, the effects of the antioxidant NAC on the levels of p62 and LC3II were evaluated. Western blotting reported in Figure 10 showed the reduction of the expression of the two proteins in the cells treated with the combination of NAC and HE compared to cells treated with HE alone.

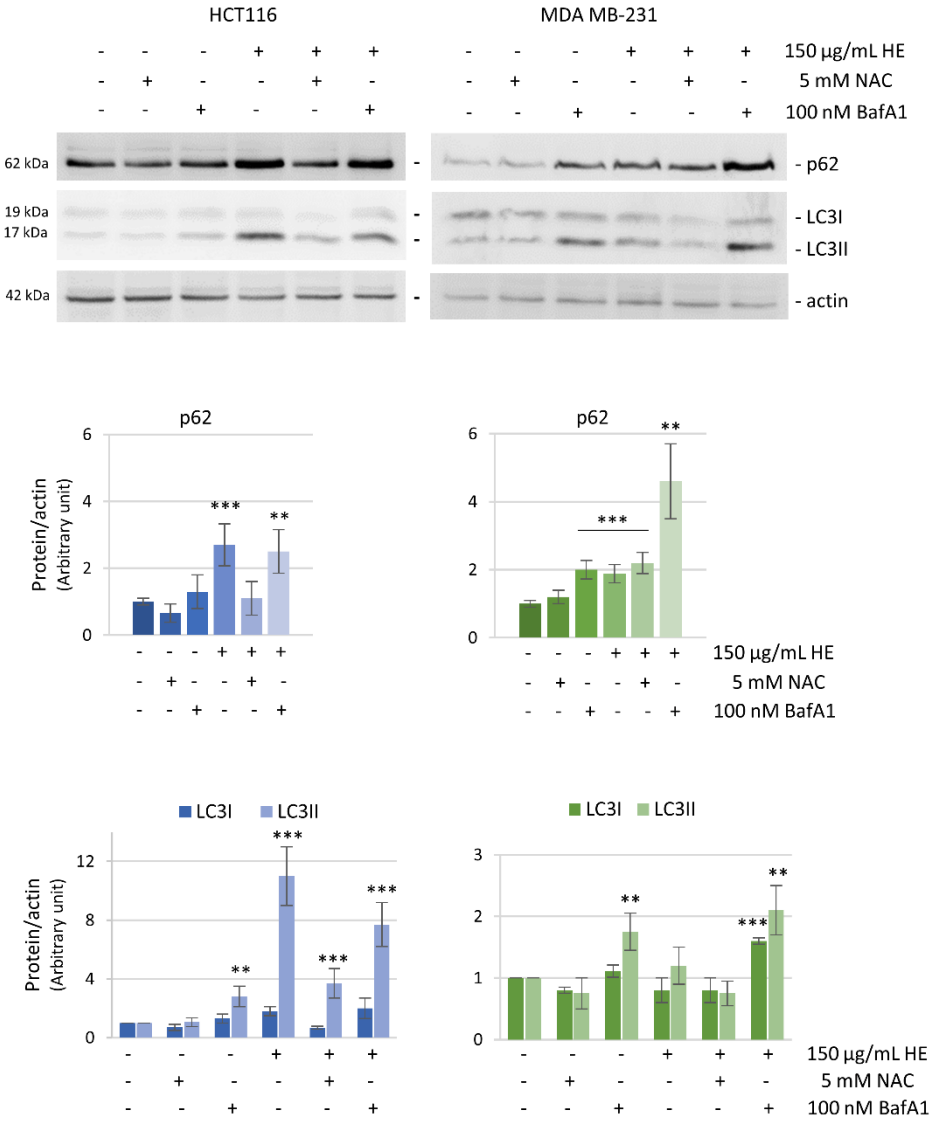


Figure 10. Relationship between ROS and autophagy in HCT116 and MDA MB-231 cells. Western blot analysis of p62 and LC3 after 24 h of treatment with 5 mM NAC, 100 nM bafilomycin A1 (BafA1), either alone or in combination with 150 µg GAE/mL HE. Actin was used as a loading control to normalize protein levels. Densitometric analysis, performed using Quantity One software, is presented in the histograms below. Data are representative of two independent experiments with consistent results and are expressed as mean ± SD. ** $p < 0.01$, *** $p < 0.001$ compared to untreated control (from Affranchi et al., Biomolecules 3;14(9):1111,2024).

PART 2. BEYOND TRADITIONAL EXTRACTION: THE GREEN SCIENCE OF SUPERCRITICAL AND SUBCRITICAL TECHNIQUES

In the first phase of the project, a traditional method was employed to extract grape pomace components. However, in accordance with the requirements of the financing agency, I worked with the project partner agency MATER to implement supercritical fluid and subcritical water extraction techniques, aiming to utilize a more environmentally friendly approach.

Firstly, Sicilian white grape pomace was subjected to Supercritical Fluid Extraction (SFE). As reported in the literature, SFE, employing CO_2 in a supercritical state as a solvent, could be a good method to extract polyphenols, also in the presence of ethanol as a co-solvent. Despite the different parameters (various combinations of temperature and pressure) used in this project for the extraction, the results obtained by the characterization were unsatisfying since no polyphenols were detected in the extracts (data not shown).

Therefore, in collaboration with MATER, subcritical water extraction, a relatively new technique for extracting less-polar compounds, was used. The technique takes advantage of water in a subcritical state to extract organic components acting on the polarity variation.

White grape pomace powder was subjected to subcritical water extraction with an instrument from the MATER industry. The extraction allowed different fractions of Subcritical Water Extract (SWE) to be collected, as described in the Materials and Methods.
The characterization shown in Table 4 revealed that phenolic acids were abundant in all the extracts analyzed. Notably, gallic acid and protocatechuic acid were the most abundant, especially in the extract obtained at the highest extraction temperature (SWE 160 °C). Regarding flavonoid components, the highest concentrations were found in the extract obtained at the temperature of 140 °C (SWE 140 °C).

Table 4. Compounds identified in the grape pomace extracts were obtained through the subcritical water extraction method (SWE). The extraction temperature and the recovery fraction are reported at the top. For each component, the concentration was expressed as mg /100 g of dry weight (DW). The retention time (R.T.) and exact mass (m/z) of each compound are the same as those indicated in Table 3. NF, not found.

Phenolic Acid (mg/100 g Dry Weight)									
	SWE 120°C			SWE 140°C			SWE 160°C		
Analytes	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Gallic Acid	16.125	27.86	27.325	41.893	39.211	48.193	61.965	56.423	52.972
Protocatechuic acid	3.7	7.194	6.369	10.1	10.842	14	17.642	15.71	13.981
Caffeic Acid	0.145	0.968	0.425	1.173	1.1684	2.3935	0.4809	0.5233	0.6572
Caftaric Acid	2.875	3.097	2.2875	14.136	10.336	8.9477	NF	NF	NF
Syringic Acid	NF	NF	NF	NF	NF	NF	5.6393	4.491	3.5258
p-Coumaric Acid	0.065	0.3889	0.1875	0.51	0.5	0.5419	NF	NF	NF
Ferulic Acid	NF	NF	NF	2.9466	2.4789	2.9355	NF	NF	NF
Fertaric Acid	1.055	0.4757	0.45	1.8466	NFR	NF	NF	NF	NF
p-hydroxybenzoic Acid	0.235	0.4931	0.4313	1.243	1.426	1.561	0.5865	0.5304	0.4977
Anthoxanthins (flavonoids and flavonols) (mg/100 g Dry Weight)									
Procyanidin B1	3.9	6.691	5.925	17.326	19.663	25.793	NF	NF	NF
Catechin	13	19.92	20.525	45.783	67.131	84.445	0.3988	0.362	0.352
Epicatechin gallate	0.0425	2.2118	0.525	4.9	4.3842	7.3225	0.0792	NF	NF
Procyanidin B2	2.35	4.25	3.5125	4.49	5.321	6.4709	NF	NF	NF
Epicatechin	7.865	13.056	12.913	31.373	42.256	49.45	0.2317	0.2043	0.174
Quercetin *3-O*-glucoside	0.41	0.836	0.587	3.68	3.69	4.167	0.076	NF	NF
Quercetin *3-O*-glucuronide	0.585	0.705	0.5875	6.4866	6.936	7.258	NF	NF	NF
Quercetin *3-O*-galactoside	NF	NF	NF	0.1033	0.1	0.14	NF	NF	NF
Quercetin *3-O*-rhamnoside	NF	NF	NF	NF	NF	NF	NF	NF	NF
Kaempferol *3-O*-glucoside	NF	NF	NF	0.78	0.6579	0.7742	NF	NF	NF
Quercetin	NF	NF	NF	11.423	11.781	18.193	0.9765	0.7706	0.366
Kaempferol	NF	NF	NF	0.4433	0.4262	0.7677	0.0264	0.0215	NF

2.1 Assessment of the potential cytotoxic effects of SWE extracts on cancer and normal cells

The analysis was initially performed to assess the impact of the different SWEs on cell viability of the human bronchial epithelial (HBE) cell line, used as an *in vitro* model of normal non-tumor cells and in HCT116 and MDA MB-231 cancer cells.

The aim was to find the SWE concentrations that would be non-toxic to normal cells and evaluate the cytoprotective effects.

To this purpose, the cells were treated for 48 h with the three fractions of SWE obtained from the three extraction temperatures (120, 140 and 160 °C), using two different concentrations, 75 and 100 µg GAE /mL, referring to the polyphenol content evaluated with Folin-Ciocalteau method. Among all the fractions, the first one obtained at the highest temperature of 160 °C was selected ($SWE^{160.1}$) since it reduced the viability of tumor cells while having a minimal impact on HBE cells. The data presented in Figure 11, obtained by MTT assay, indicated a reduction in cell viability of approximately 65% for HCT116 cells and 40% for MDA MB-231 cells, at the highest concentration tested in comparison to a 25% decrease in the viability of HBE cells.

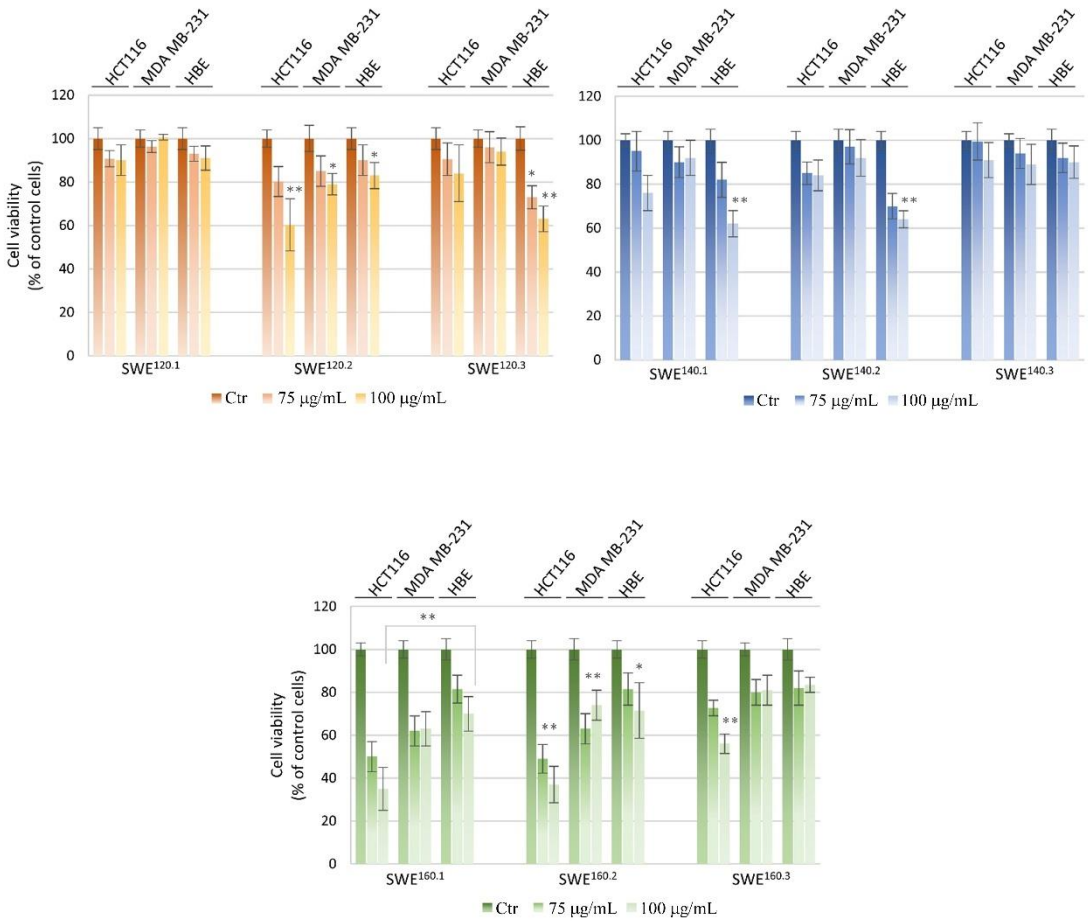


Figure 11. Dose-dependent effects of the different fractions SWE^{120}, SWE^{140}, and SWE^{160} on HCT116, MDA MB-231, and HBE cells. Cell viability was assessed by MTT assay after 48 h of SWE treatment. SWE was used at concentrations of 75 and 100 µg GAE/mL. Results are representative of three independent experiments and expressed as mean ± SD. * $p < 0.05$, ** $p < 0.01$ vs. untreated control.

The morphological analysis supported the cell viability findings. HCT116 cells, selected from the two cancer cell lines, and normal HBE cell lines were observed under a microscope after treatment with $SWE^{160.1}$ for 48 h. HE-treated HCT116 cells were clearly fewer in number and exhibited distinct morphological changes compared to control cells, while treated HBE cells showed no noticeable differences with respect to the untreated cells (Figure 12).

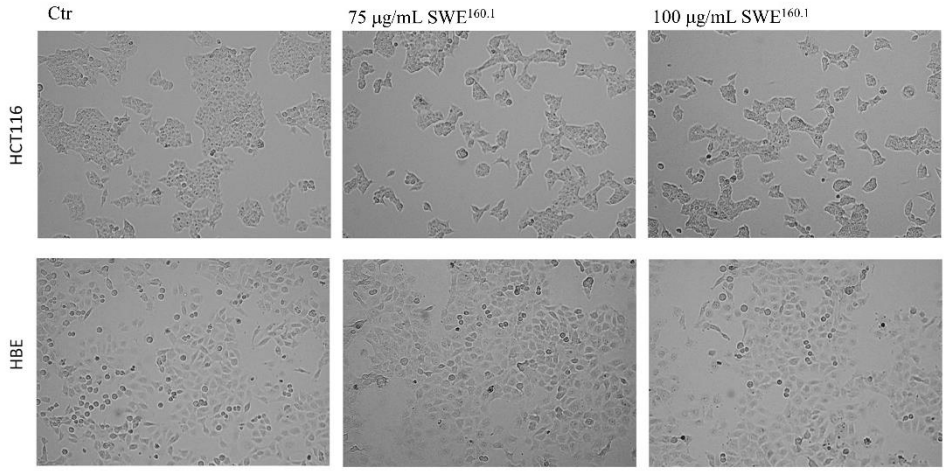


Figure 12. SWE induced the appearance of apoptotic features exclusively in tumor cells. Representative images of tumor cells (HCT116) and normal cells (HBE) after treatment with 75 and 100 µg GAE/mL of $SWE^{160.1}$ for 48 h are shown.

Since the cytotoxic effects of grape pomace extracts had already been highlighted in tumor cells, although obtained with a different extraction method (hydroalcoholic solution), the second part of my research project used epithelial bronchial cells to better characterize the antioxidant power of the SWE. Using HBE cells provided the opportunity to explore the antioxidant potential of grape pomace on non-tumor cells.

2.2 The study of the antioxidant power of SWE in HBE cells

To assess the potential antioxidant capacity of $SWE^{160.1}$ on HBE cells, the variation of ROS content was first analyzed using the H_2DCFDA probe. Since ROS in normal cells play a role in cellular aging and can contribute to the development of various diseases, investigating the

antioxidant properties of different molecules is essential for preventing diseases associated with oxidative stress.

The analysis of ROS level after treatment with the extract selected ($SWE^{160.1}$) was performed using flow cytometry. As depicted in the graph (Figure 13), after two h of treatment with 100 μg/mL of the subcritical water extract, a 78% reduction in fluorescence was observed compared to control cells.

Moreover, to better define if SWE can counteract an occurring oxidative stress condition, ROS were induced by using sodium arsenite ($NaAsO_2$). It is well known that exposure to arsenic induces a variety of oxidative stress biomarkers, including DNA damage, lipid peroxidation, decreased antioxidant defense levels, and changes in gene expression (Ruiz-Ramos et al., 2009).
Interestingly, SWE was also able to counteract $NaAsO_2$-induced stress. Indeed, the percentage of fluorescent cells decreased from 23.06% to 5.75%.

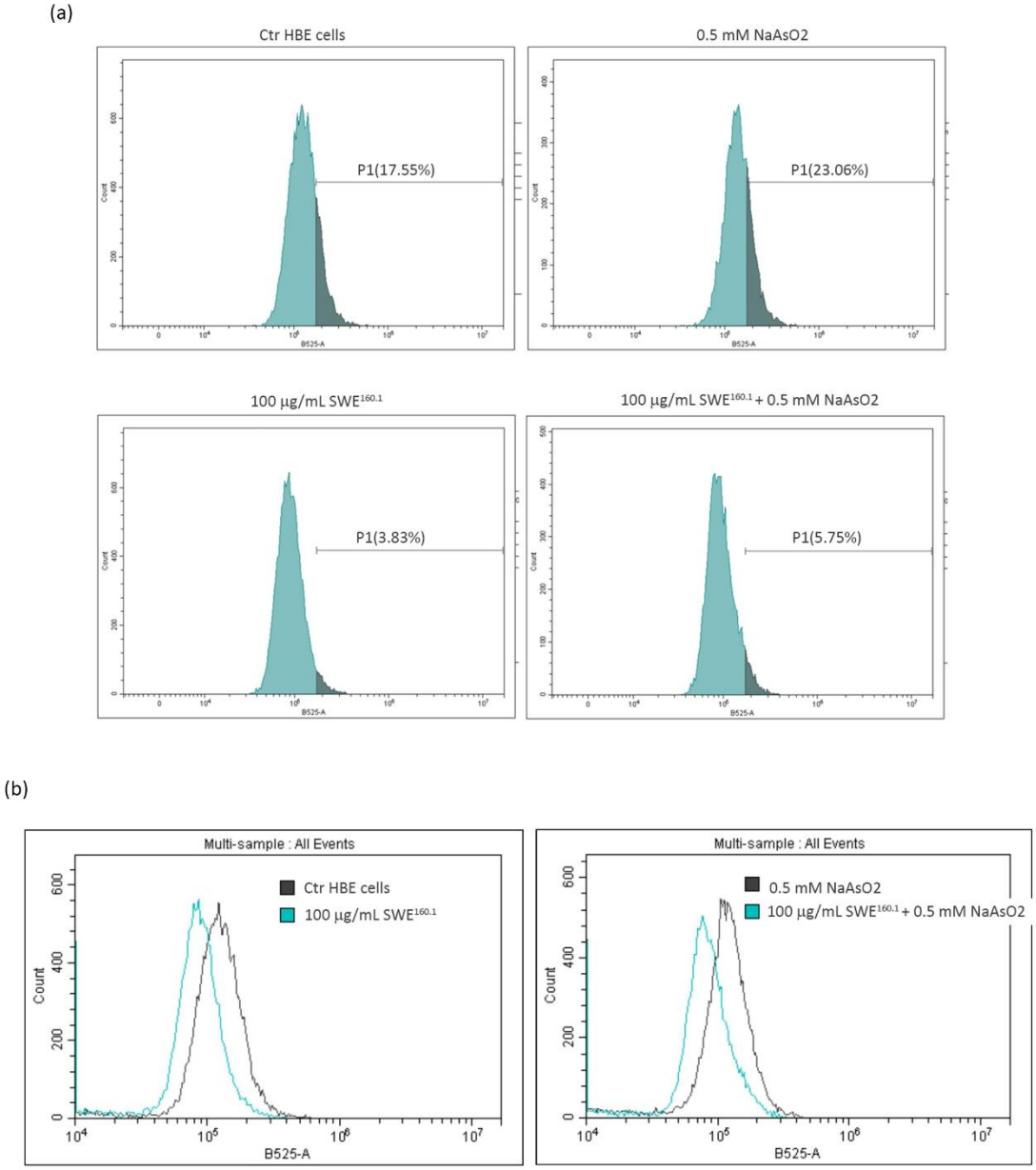


Figure 13. SWE$^{160.1}$ acts as an antioxidant in HBE cells. Flow cytometric analysis to identify the different levels of ROS. (a) HBE cells were treated with 100 µg GAE/mL of SWE$^{160.1}$ for 2 h and with 0.5 mM $NaAsO_2$ for 1 h, alone or in combination. After treatment, cells were trypsinized and incubated with a 0.5 µM H_2DCFDA probe. Peaks are representative of three independent experiments. (b) Merge plot of the fluorescence intensity.

We also used fluorescence microscopy to assess ROS expression in $NaAsO_2$-treated cells and observe any potential changes when sodium arsenite treatment was preceded by two h of SWE incubation. Images reported in Figure 14 underline, once again, the antioxidant power of SWE in normal cells. Furthermore, preliminary data (not shown) suggest that SWE pre-treatment may protect cells from the morphological changes induced by sodium arsenite.

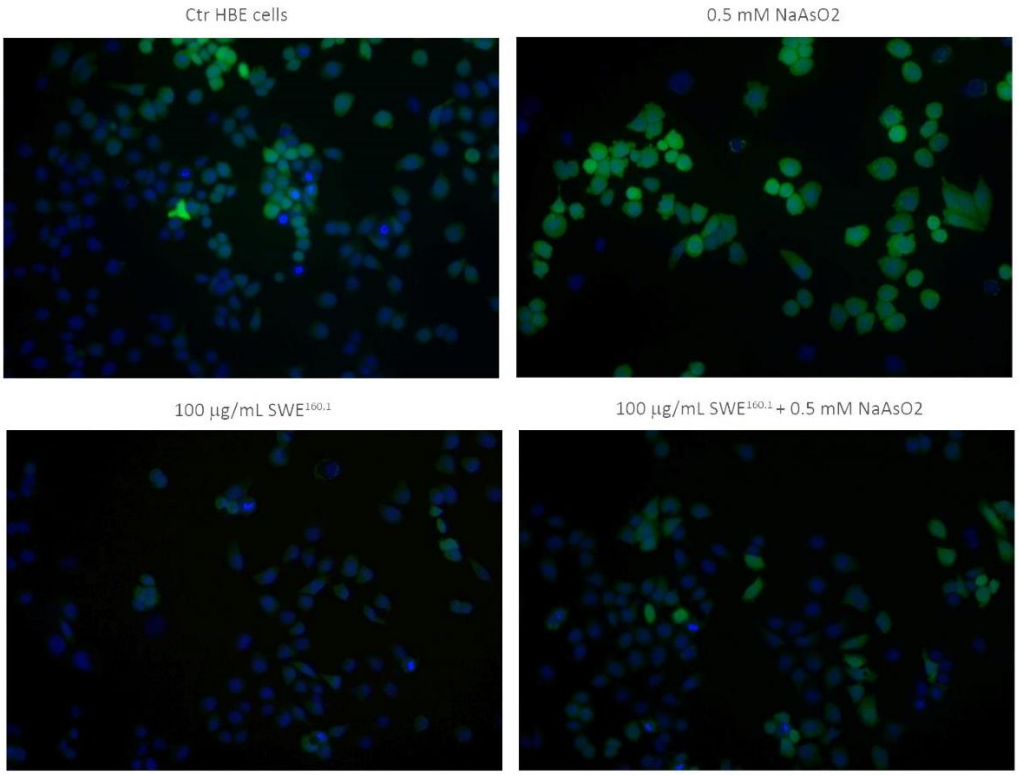


Figure 14. Fluorescent images of the antioxidant power of SWE. HBE cells were treated with 100 µg GAE/mL of $SWE^{160.1}$ for 2 h and with 0.5 mM $NaAsO_2$ for 1 h, alone or in combination. Magnification 200×. Fluorescence images are representative of ROS observed after incubation of the treated and untreated cells with 2 µM H_2DCFDA (green signal) and 2.5 µg/mL Hoechst 33342 (blue signal) to stain nuclei.

The observed reduction in ROS levels can be attributed to both the scavenging effect of the extract components and the upregulation of antioxidant enzymes involved in maintaining redox balance. To investigate this, the levels of the key antioxidant enzymes, HO-1 and SOD-2, were evaluated (Figure 15). After 24 h of treatment with 100 µg GAE/mL $SWE^{160.1}$, a threefold increase in both HO-1 and SOD-2 levels was noted.

This finding suggests that the early reduction in ROS levels was due to the scavenging activity of the extract, while in the late stages, it was also linked to the upregulation of antioxidant enzyme expression.

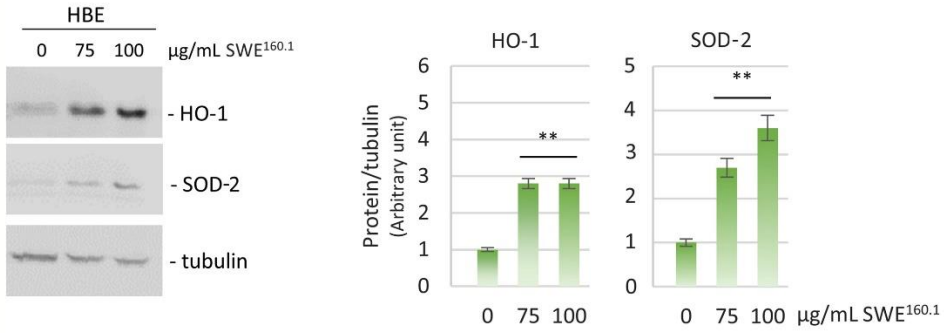


Figure 15. Evaluation of antioxidant enzyme expression after SWE treatment. Western blotting analysis of antioxidant enzymes (HO-1 and SOD-2) after 24 h of treatment with 100 µg GAE/mL SWE in HBE cells. Protein levels were normalized to tubulin. Densitometric analysis, performed using Quantity One software, is shown in the histograms. The results are representative of three independent experiments, with values expressed as mean ± SD. ** $p < 0.01$ vs. untreated control.

DISCUSSION

The multifaceted biological properties of grape pomace, including its antioxidant, antimicrobial, prebiotic, and anti-proliferative activities, have been extensively documented in scientific literature, underscoring its significant impact on human health and its different potential applications (Davinelli and Scapagnini, 2022; Dias et al., 2021). These findings reinforce the importance of grape pomace as a by-product with wide-ranging implications for health and industry since it still contains molecules, such as secondary metabolites, with high biological impact. These molecules, which are essentially produced by the plants to protect themselves from stress conditions, also have numerous properties on human health. However, the quality and quantity of secondary metabolites still present in grape pomace are strongly influenced by different environmental factors, such as soil conditions, sun exposure, and pedoclimatic conditions. Therefore, territoriality is an important factor that needs to be considered (Radulescu et al., 2024).

This study specifically aimed to enhance the valorization of Sicilian wine-making industry by-products by highlighting their antitumor and antioxidant properties. By employing different green methods of extraction and optimizing different parameters, three types of extracts were obtained: hydroalcoholic extract (HE), supercritical fluid (SFE), and subcritical water extract (SWE). When compared to other studies conducted by using white grape pomace from Marche (Italy) (Ferri et al., 2017) or Switzerland (Ghidossi et al., 2020) acid gallic content of HE and SWE$^{160.1}$ extracts was surprisingly the most abundant, 20.96 mg/100 g DW of HE with respect to ethanoic grape pomace extract from Marche and 61.965 mg/100 g DW of SWE with respect to 20.46 mg/100 g DW of a subcritical water extract obtained at 150 °C by using grape pomace from Switzerland. The data confirm how climatic conditions can influence the composition of secondary metabolites in plants.

In the first part of this PhD project, a traditional method of extraction which employs ethanol as a solvent was used. Although this is a conventional approach, ethanol is still regarded as a green solvent. Employing optimized extraction parameters obtained by using a Box Behnken Design method in collaboration with the MCAST (Malta) during my period abroad, HE proved to be particularly rich in anthoxanthins such as catechin, epicatechin, and quercetin, and it also contained phenolic acids, among which gallic acid was the most abundant.

The investigation primarily focused on the biochemical mechanisms sustaining the anti-proliferative and pro-apoptotic effects of the grape pomace HE in two cancer cell lines: HCT116 colon cancer cells and MDA MB-231 triple-negative breast cancer cells. These cell

lines were selected because they are representative of two highly prevalent and aggressive tumor types. Despite their different origins, these models allowed for an in-depth analysis of the molecular pathways involved in apoptosis and autophagy, both of which are crucial processes in the antitumoral response. The results suggest new insights into the differential cellular responses induced by HE, contributing to a deeper understanding of its therapeutic potential.

Previous studies from my research group demonstrated that HCT116 cells are an effective model for evaluating the effects of natural compounds, as evidenced by their response to a crude extract from mango fruit peel cultivated in Sicily (Lauricella et al., 2017). The current study revealed that HCT116 cells exhibit a pronounced sensitivity to HE, undergoing apoptosis through a change in the balance from pro-survival to pro-apoptotic factors. This constitutes the first report on the effects of grape pomace on this aggressive colon cancer model. Previously, Perez et al. have examined crude or partially purified grape pomace extracts from Spanish sources, demonstrating limited activity in less aggressive colon cancer models like CaCo-2 and HT-29 cells (Pérez-Ortiz et al., 2019). This comparative analysis underscores the effectiveness of the optimized HE preparation detailed in this study, emphasizing its advancements over this previous study.

In addition to the involvement of apoptotic cell death demonstrated by the activation of caspases and the presence of cells confined in the subG0-G1 phase of the cell cycle, in colon cancer cells, HE treatment also led to the reduction of the pro-survival factor phospho-AKT and a significant increase in γH2AX, a marker of DNA damage and a critical regulatory point to promote repair or steer cells towards apoptosis in cases of catastrophic genomic damage (Prabhu et al., 2024). Therefore, our findings suggest that in HCT116 cells, HE can manipulate the dynamic equilibrium between survival and death factors in relation to the apoptotic pathway.

Conversely, in the case of MDA MB-231 breast cancer cells, whilst HE similarly reduced cell viability, no signs of apoptotic cell death, such as morphological changes or cell cycle alterations, were observed. Consistent with this, pro-caspase-3 reduction was absent, and PARP1 degradation exhibited a unique fragment (66 kDa), which could be related to the cleavage by the MMP-2, as reported (Chaitanya et al., 2010). Moreover, the active phosphorylated form of AKT increased, suggesting the activation of a pro-survival pathway. Notably, HE extract exerted opposing effects on AKT phosphorylation in the two cell lines, highlighting a resistance mechanism in breast cancer cells compared to colon cancer cells.

Previous evidence demonstrated that polyphenols could exhibit either pro- or anti-oxidant roles, and the effect could be related to the induction of different processes in tumor systems, such as autophagy, which is intricately linked to the tumor progression.

The evaluation of ROS showed that HE treatment elevated ROS levels in both cell lines within the initial six hours. In HCT116 cells, however, ROS levels returned to normal within 24 h, a change linked to the activation of antioxidant responses, including the upregulation of the transcription factor Nrf2 and the antioxidant enzymes SOD-2 and HO-1. Conversely, in MDA MB-231 cells, ROS levels remained persistently elevated, coupled with a weaker antioxidant response.

Based on these findings, the study also explored the role of autophagy, a process that can either support tumor cell survival or contribute to cell death. Both cell lines exhibited increased levels of autophagic markers (p62 and LC3II) and the formation of acidic vacuoles upon HE treatment. However, the dynamics of autophagy differed significantly. In HCT116 cells, autophagic marker levels peaked at 24 h and declined by 48 h, suggesting the conclusion of the autophagic process. This reduction in autophagy seems to correlate with the strong activation of p21. Indeed, some studies show that p21 inhibition enhances autophagy in HCT116 cells (Maheshwari et al., 2022).

In contrast, autophagy in MDA MB-231 cells remained sustained at 48 h, indicating an ongoing process that likely supports cell survival. This hypothesis was corroborated by the addition of bafilomycin A1, an autophagy inhibitor, which significantly enhanced the cytotoxic effects of HE in MDA MB-231 cells but had no substantial impact on HCT116 cells. Interestingly, even under autophagy inhibition, no evidence of apoptosis was detected in MDA MB-231 cells, suggesting the activation of a different cell death pathway.

The data reported do not allow us to clarify the reasons for the different behavior of the two cancer cell lines. A hypothesis could be related to a different ROS threshold. While HCT116 cells rapidly succumbed to oxidative stress and initiated apoptosis, MDA MB-231 cells maintained elevated ROS levels, potentially supporting cell survival. This hypothesis aligns with findings indicating higher basal ROS levels in MDA MB-231 cells compared to non-malignant breast cells (Sarmiento-Salinas et al., 2019). Notably, pre-treatment with the antioxidant NAC effectively neutralized HE-induced oxidative stress in HCT116 cells but had a lesser impact on MDA MB-231 cells.

Another significant difference between HCT116 and MDA MB-231 cells is the state of the p53 protein, a well-known tumor suppressor and pro-apoptotic factors. Differently from HCT116 cells, MDA MB-231 cells present a mutant p53, which in these cells confers a gain-of-function (GOF) activity. (Olszewski et al., 2019). In the future, it will be important to investigate the role of the two forms of p53 and their relationship with the effects of HE.

A recent study underscores that the extraction technique and the method employed to evaluate the antioxidant activity are decisive and make difficult the comparison among different matrices (Bocsan et al., 2022). Thus, having explored the role of the hydroalcoholic extract of Sicilian grape pomace in the second part of my PhD project, the study was focused on the effects of an extract obtained using a more eco-friendly method of extraction by the same starting matrix.

Initially, supercritical fluid extraction was employed as a green methodology. This approach, which utilizes carbon dioxide (CO_2) in its supercritical state, is considered a non-toxic, cost-effective solvent and allows for the production of high-quality extracts free from solvent residues.

Although supercritical fluid extraction is overall used for the extraction of non-polar molecules, recent studies indicate that this method is also suitable for extracting phenolic compounds. The selectivity of extraction is strictly related to pressure and/or temperature parameters (Casas et al., 2010; Manna et al., 2015; Tyśkiewicz et al., 2018). However, despite the bibliographic evidence, even after multiple variations of extraction parameters, the chemical characterization failed to identify bioactive molecules, such as polyphenols, comparable to those present in the HE extract. A possible explanation for the poor extraction performance could depend on the observation that when exposed to high temperature and pressure during extraction, the molecules may bind with other matrix components (Khoddami et al., 2013) or even undergo hydrolysis. The yield of polar molecules could potentially be increased by adding very high concentrations of co-solvent. However, this increase tends to reduce the selectivity of CO_2 and hinders its efficient diffusion through the matrix. Additionally, from an economic perspective, adding a co-solvent is not appealing, as it would require more energy along with longer post-processing steps for concentration, isolation, and purification.

Therefore, always in collaboration with the MATER partner agency, the extraction method was shifted to an alternative yet equally eco-friendly technique: subcritical water extraction.

This method takes advantage of the extraction capabilities of water in its subcritical state, where water is maintained at a temperature and pressure below its critical point.

The obtained extracts were analyzed for their polyphenol and flavonoid content. The content of polyphenols in the extract obtained at the highest temperature was significantly higher compared to hydroalcoholic extract, especially for the phenolic acids, like gallic acid and protocatechuic acid. However, the resulting flavonoid content was very low, likely due to the thermal degradation of certain heat-sensitive compounds (Cheng et al., 2021; Ho and Chun, 2019).

Among the extracts obtained with subcritical water extraction, $SWE^{160.1}$ was selected to study the antioxidant power in non-tumor human bronchial epithelial (HBE) cells because it was able to negatively modulate the viability only in tumor cells without causing cytotoxic effects on HBE cells. The extract exhibited a strong antioxidant activity. This property was probably associated with the scavenging activity of the extract, which not only reduced basal ROS levels but also counteracted the increase in ROS levels due to acute stress. Moreover, the extract also modulated the expression of antioxidant enzymes HO-1 and SOD-2, thus demonstrating a potent antioxidant power in a normal cell model, initially scavenging free radicals and subsequently stimulating the cell antioxidant defense response.

Overall, the first phase of the study demonstrates that the antitumor potential of grape pomace HE is mediated through a pro-oxidant mechanism, which induces oxidative stress and disrupts the balance between redox homeostasis and autophagy. The effects of HE are highly context-dependent, triggering apoptosis in colon cancer cells and promoting a cytostatic response in breast cancer cells through a pro-survival autophagy pathway.

On the other hand, the different effect of the SWE in HBE cells could be related to either the different composition of the extract due to the method of extraction or a synergic effect of the phenolic components of the extract.

In conclusion, the findings support the concept of valorizing agricultural waste products within a circular economy framework, offering novel insights into the development of sustainable and effective methods for the recovery of bioactive molecules that exhibit antitumor and antioxidant effects in cancer and normal models, respectively. From a molecular perspective, the data support the need to study biochemical pathways in different experimental models to highlight potential contrasting roles of the molecules.

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I hope that one day my daughter will look at me proudly for this.

To my daughter, Beatrice.

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