Università degli Studi di Palermo

AREA RICERCA E TRASFERIMENTO TECNOLOGICO
SETTORE DOTTORATI E CONTRATTI PER LA RICERCA
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Settore Scientifico Disciplinare BIOS-06/A

CHEMICAL CHARACTERIZATION AND IN VITRO AND IN VIVO EVALUATION OF ANTIOXIDANT PROPERTIES OF THREE SICILIAN ENDEMIC *BRASSICA* EXTRACTS

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CICLO XXXVII
ANNO CONSEGUIMENTO TITOLO 2025

ABBREVIATIONS LIST

CAT: catalase

COX: cyclooxygenase

CYP: cytochrome P450

DMSO: dimethyl sulfoxide

DPPH: diphenyl-2-picrylhydrazyl

ER: endoplasmic reticulum

FET: fish embryo acute toxicity test

GPx: glutathione peroxidase

GR: glutathione reductase

GSH: glutathione reduced

GSSG: glutathione oxidized

H_2DCFDA: 2',7'-dichlorodihydrofluorescein diacetate

HAT: hydrogen atom transfer

Hpf: hours post fertilization

LPS: lipopolysaccharides

MDA: malondialdehyde

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

NADPH: nicotinamide adenine dinucleotide phosphate

NO: nitric oxide

NOS: nitric oxide synthases

NOX: NADPH oxidase

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

RS: Reactive Species

SET: single electron transfer

SOD: superoxide dismutase

TBARS: thiobarbituric acid reactive substances

VEGF: vascular endothelial growth factor

ABSTRACT

In recent years, scientific interest in natural compounds with antioxidant properties has significantly increased, largely due to the established link between oxidative stress and a range of chronic diseases[1]. The prolonged use of conventional medications to manage chronic conditions associated with oxidative stress can lead to drug resistance and undesirable side effects, making natural alternatives an increasingly attractive option[2]. Numerous studies have already highlighted the beneficial effects of *Brassica* species in both *in vitro* and *in vivo* models of oxidative stress[3].

Sicily, known for its rich biodiversity, is home to more than 10 endemic species of this genus. Since plants have been shown to concentrate phytochemicals in response to environmental factors like salinity, solar radiation, and other stressors[4], we chose to explore wild Sicilian *Brassica* species. We hypothesized that, due to the unique climate of Sicily, these wild *Brassica* species may show specific phytochemical profiles offering distinct and potentially potent biological effects.

The aim of this project was to evaluate the biological properties of endemic *Brassica* species through both *in vitro* and *in vivo* analyses. We selected three endemic species from different habitats: *B. macrocarpa* endemic to Favignana island, *B. rupestris* subsp. *hispida* endemic to Ficuzza wood, and *B. tardarae* endemic to Tardara gorges. methanolic extracts were prepared from the leaves, and the chemical composition was analysed by HPLC-MS/MS. The three *Brassica* species showed a similar chemical profile, containing significant amounts of glucosinolates and phenolic compounds. Notably, differences were observed in the sinapyl derivatives, which were found in higher concentrations in *B. hispida* compared to the other two species.

Subsequently, their radical scavenging activities were evaluated using the diphenyl-2-picrylhydrazyl (DPPH) assay. All three *Brassica* species exhibited scavenging activities. In consideration of these results, we continued our investigation into the potential beneficial effects of these extracts *in vitro*, using the RAW 264.7 murine macrophage cell model. Assessing toxicity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay we observed a reduction in cell viability were for *B. hispida* and *B. tardarae*, while *B. macrocarpa* proved to be safe at all concentrations tested. Moreover, using the Griess reaction we assessed in RAW 264.7 cells subjected to lipopolysaccharide (LPS)-induced oxidative stress the ability of the extracts to reduce nitric oxide (NO) production. At safe concentrations, *B. hispida* and *B. tardarae* were ineffective in reducing NO production, while *B. macrocarpa* successfully decreased NO levels. Real-time PCR analysis confirmed that *B. macrocarpa* treatment resulted in a reduction in NOS2 mRNA expression. Furthermore, in the 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) fluorescent probe assay, *B. macrocarpa*, once again, proved to be efficient in reducing the levels of ROS increased by exposure to LPS in a concentration-dependent manner.

For *in vivo* studies, in the zebrafish (*Danio rerio*) embryo the toxicity, behaviour, and antioxidant properties of *B. macrocarpa* were evaluated. Zebrafish embryos were subjected to the acute toxicity test (FET). Throughout the 96-hour treatment period, no induced malformations were observed; however, changes in spontaneous movement and heart rate were noted. Behavioural tests did not indicate significant alterations in anxiety-like, aversion, or exploratory behaviours, and all groups displayed normal swimming patterns. Exposure to sub-lethal concentrations for 26 hours did not result in any noticeable changes in the key oxidative stress markers evaluated.

By using copper sulphate ($CuSO_4$)-induced oxidative stress model, zebrafish embryos pre-treated with *B. macrocarpa* extract showed a significant reduction in ROS and NO levels compared to the untreated group. The extract also modulated the activities of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT). Additionally, lipid peroxidation and protein oxidation were reduced. However, the extract did not appear to reverse $CuSO_4$-induced DNA damage.

In conclusion, our data demonstrated that *B. macrocarpa* exhibited strong antioxidant properties, proving safe in the *in vitro* murine macrophage model, while in the FET test conducted on zebrafish embryo, showed to be safe up to 500 µg/mL. *B. hispida* and *B. tardarae* showed antioxidant effects solely in the DPPH assay and resulted to induce, in our model, cell toxicity at low concentration, hampering any further studies on their possible effects in our biological setting. Additional research is needed to further investigate their full potential.

1. INTRODUCTION

Maintaining a balance between the production of reactive oxygen species and antioxidant defences is crucial for cellular health. When this balance is disrupted, oxidative stress can play a role in the onset of various diseases. Alongside traditional pharmacological treatments, also natural extracts, present a promising approach to counteracting these detrimental processes. In this thesis we will investigate the ability of extracts derived from Sicilian *Brassica* species in the management of oxidative stress.

1.1 FREE RADICALS

Free radicals, any molecular species capable of independent existence that contains an unpaired electron in an atomic orbital, are highly reactive due to their tendency to gain or lose an electron to achieve stability and were first identified by Moses Gomberg over a century ago[5]. For many years, it was believed that these highly reactive molecules were not present in biological systems due to their extreme reactivity and short lifespan. However, in the 1950s, their presence in living organisms was confirmed, and it was quickly hypothesized that they may play a role in various pathological processes and aging[6–9]. Between the 1970s and 1990s, the prevailing view that free radicals were solely detrimental to biological systems underwent a major re-evaluation, driven by several significant discoveries. Initially, it was observed that free radicals played a crucial role in the immune response against infectious agents[10]. Later, it was discovered that vascular endothelial cells produced nitric oxide from L-arginine[11]. This discovery marked the beginning of a research path focused on free radicals, particularly their role as mediators of cellular signalling. Today, it is widely recognized that free radicals actively participate in a variety of physiological processes and can no longer be viewed solely as harmful agents. The field of research on free radicals or reactive species (RS) most common in biological systems presents numerous challenges mainly due to their low stability and high reactivity, high diversity of reactions in which they can participate, their complex spatiotemporal distribution within cellular and extracellular spaces, and their dependence on the physiological state of the organism[12].

The most common free radicals and reactive molecules in biological systems are derived from oxygen (reactive oxygen species, ROS) and nitrogen (reactive nitrogen species, RNS). ROS or RNS are produced during electron transfer reactions, where they either lose or accept electrons. These reactive species can be classified into two categories: free radicals and non-radicals[13] (Table 1).

Table 1. Common reactive oxygen and nitrogen species in biology system

ROS NAME	FORMULA	RADICAL (R) or NON RADICAL (NR)
Superoxide anion	$\cdot O_2^-$	R
Hydroxyl	·OH	R
Peroxyl	ROO·	R
Carbonate	$HCO_3\cdot$	R
Hydroperoxy	$HO_2\cdot$	R
Hydrogen peroxide	H_2O_2	NR
Hypochlorous acid	HOCl	NR
Peroxynitrite	$ONOO^-$	NR
RNS NAME	**FORMULA**	**RADICAL (R) or NON RADICAL (NR)**
Nitric oxide	NO·	R
Nitrogen dioxide	$\cdot NO_2$	R
Peroxynitrite	$ONOO^-$	NR
Dinitrogen trioxide	N_2O_3	NR
Dinitrogen tetraoxide	N_2O_4	NR

Free radicals contain an unpaired reactive electron in their outer orbit classifying them as single-electron oxidants, while non-radicals do not possess unpaired electrons and are categorized as two-electron oxidants[9,14].

Low or intermediate levels of ROS and RNS play important roles in various cellular processes, including cell proliferation, differentiation, migration, apoptosis, and necrosis[15]. Conversely, excessive accumulation of RS disrupts redox homeostasis leading to oxidative stress and subsequent damage to lipids, proteins and DNA, thus leading to cellular and tissue dysfunction and contributing to the onset of chronic diseases[1,16,17].

RS can originate from endogenous or exogenous sources as shown in Figure 1. Endogenous sources of RS include various cellular organs where oxygen consumption is high. Most intracellular ROS originate in the mitochondria, considered the redox-active compartment within the cell, accounting for nearly 90% of oxygen utilization. ATP production is closely linked to ROS formation because during oxidative phosphorylation, electrons travel from complexes I to IV of the mitochondrial electron transport chain (ETC) to form a proton gradient that drives the activity of ATP synthase. Complex I and complex III cause a partial reduction in oxygen that leads to the generation of $O_2\bullet^-$, which is released into the mitochondrial matrix[18]. Additionally, $O_2\bullet^-$ can be released into the intermembrane space via complex III. $O_2\bullet^-$ is rapidly converted to H_2O_2 by superoxide dismutase (SOD) and can then be detoxified by catalase (CAT) and glutathione peroxidase (GPx)[19].

Another crucial source of ROS is the peroxisomes, which are dynamic and metabolically active subcellular compartments[20]. They play an important role in various metabolic processes including fatty acid oxidation, ROS metabolism, purine and prostaglandin catabolism. In peroxisomes the respiratory pathway involves the transfer of electrons from various metabolites to oxygen leading to the formation of H_2O_2 but also $O_2^{\bullet-}$ OH• and NO•. This process occurs without ATP production, and the free energy released is primarily in the form of heat. The β-oxidation of fatty acids is the primary metabolic process responsible for generating H_2O_2 in peroxisomes. Several peroxisomal enzymes, including acyl CoA oxidase, D-amino acid oxidase, L-α-hydroxy oxidase, urate oxidase, xanthine oxidase, D-aspartate oxidase are involved in the production of various type of ROS[21,22].

The endoplasmic reticulum (ER) is a complex dynamic structure with essential roles, such as calcium storage, lipid metabolism, and protein synthesis. The ER maintains a relatively high turnover of H_2O_2 as its oxidative protein folding machinery continuously generates and utilizes H_2O_2[23].

ROS in the cytosol also originate from the cytochrome P450 (CYP) superfamily of enzymes, a group of heme-thiolated enzymes. This system is critical for various physiological processes, including the detoxification of xenobiotics. P450 enzymes utilize molecular oxygen, cleaving it so that one oxygen atom reacts with a substrate while the other is converted to water. However, if the transfer of oxygen to the substrate is poorly controlled, it can lead to uncoupled ROS production[24,25].

Another significant source of ROS is the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) family, a group of proteins responsible for transferring electrons across cell membranes[26,27]. These enzymes typically use oxygen as an electron acceptor, producing superoxide as a result of the reaction. NOX enzymes are specifically designed to generate ROS and include seven distinct members, each of which plays a role in various critical physiological functions. All isoforms share a common structural framework, including a catalytic core with six transmembrane helices that bind two heme groups, as well as a dehydrogenase domain that interacts with the flavin cofactor (FAD) and the substrate NADPH. However, they differ in their activation mechanisms, regulatory systems, and tissue-specific expression[28–30].

Immune cells, including macrophages, neutrophils, dendritic cells, T and B lymphocytes, and NK cells, contribute significantly to the production of ROS and RNS, participating not only in defence mechanisms against pathogens but also modulating intracellular signalling and cellular metabolism[31].

Exogenous sources of RS include factors such as radiation, cancer treatments, smoking, alcohol consumption, smoked red meats, and some metal-containing drugs (e.g., cyclosporine and gentamicin)[16,32].

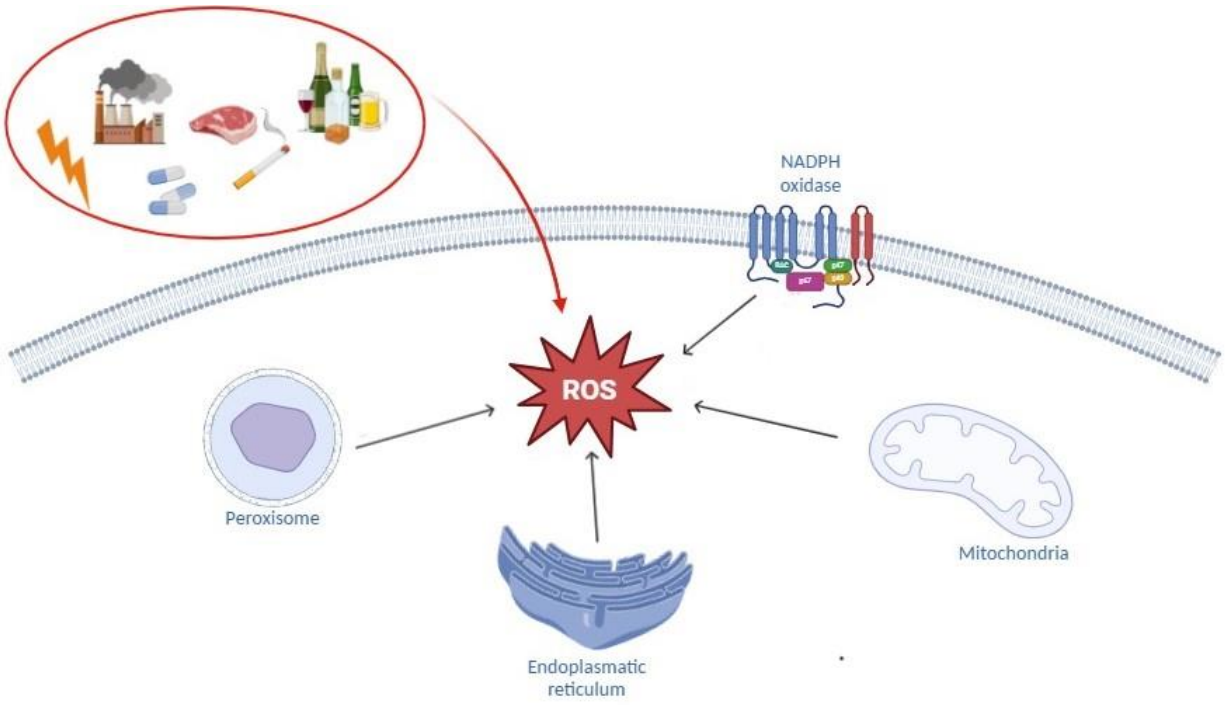

Figure 1. Sources of reactive species in cell. Created with bioRender.com (accessed December 2024). Endogenous sources (black arrows): metabolic reaction in peroxisome, endoplasmatic reticulum stress and unfolded protein, oxidative phosphorylation in mitochondira, NADPH oxidase proteins responsible for the transfer of electrons across cell membranes. Exogenous sources (red arrow) include factors such as radiation, cancer treatments, smoking, alcohol consumption, etc.

1.2 PHYSIOLOGICAL ROLE OF ROS AND RNS

ROS and RNS produced during metabolism at low to moderate levels are vital to human health. They are essential for the maturation process of cellular structures and can act as weapons for the host defence system. Indeed, phagocytes (neutrophils, macrophages, monocytes) release free radicals to destroy pathogens[33]. Activated macrophages, especially the M1 (pro-inflammatory) phenotype, release high amounts of ROS through the activation of NADPH oxidase and the production of nitric oxide by the enzyme inducible nitric oxide synthase (iNOS)[34]. This contributes to the elimination of microorganisms and damaged cells. Neutrophils, which represent one of the first lines of defence of innate immunity, are also responsible for a massive production of ROS during the "oxidative burst", a process essential for the destruction of phagocytosed pathogens[35]. Dendritic cells use ROS to facilitate the maturation and activation of T lymphocytes that together with NK (natural killer) cells, use ROS and RNS to enhance their cytotoxic function and modulate the inflammatory response[36].

In addition to their direct role in pathogen destruction, ROS and RNS play a crucial role in regulating the immune response and the inflammatory process. They influence intracellular signalling, activating key transcription factors such as NF-κB, responsible for the production of pro-

inflammatory cytokines. Furthermore, ROS production is closely linked to the activation of the mTOR pathway, which in turn determines the proliferation and differentiation of immune cells[37].

RS function is essential for redox signalling involved in various biological responses including cell proliferation, migration, differentiation, and gene expression[38,39].

Vascular NOX isoforms show differences in their activity, cellular specificity, and responses to stimuli such as agonists and growth factors, as well as in the type of RS they release upon activation. ROS, especially H_2O_2, at physiological levels function as signalling molecules to mediate angiogenesis, which is required for tissue repair and remodeling[40,41]. In endothelial cells, NOX-produced RS, localized in plasma membranes or intracellular compartments such as the cytosol and mitochondria, play a crucial role in the angiogenic response triggered by growth factors such as vascular endothelial growth factor (VEGF)[29,41]. The importance of ROS production by the immune system is clearly exemplified by patients with granulomatous disease[42]. These patients have a defective membrane-bound NADPH oxidase system which impairs their ability to produce the superoxide anion radical ($O_2^{\bullet -}$), leading to recurrent and persistent infections. On the other hand, ROS and RNS also exert beneficial effects, playing important physiological roles in the regulation of various cellular signaling pathways[39,43]. For example, nitric oxide (NO) is an intercellular messenger. It targets guanylate cyclase, binding to its heme moiety and producing cGMP as a second messenger, which ultimately leads to smooth muscle relaxation in virtually all animals. In mammals, NO biosynthesis is achieved by three distinct nitric oxide synthases (neuronal nNOS, endothelial eNOS, and inducible iNOS), which utilize L-arginine, NADPH, and O_2 as substrates. The primary role of eNOS is the regulation of blood flow, and it also contributes to the inhibition of platelet aggregation. nNOS is involved in neurotransmission and synaptic plasticity. iNOS responds to exogenous stimuli such as bacterial lipopolysaccharides and pro-inflammatory cytokines[44–46].

1.3 PHATOLOGICAL ROLE OF ROS AND RNS

In 1985, H. Sies introduced the concept of oxidative stress, defining it as "*an imbalance between oxidants and antioxidants in favour of oxidants, leading to disruption of redox signalling and control and/or molecular damage*"[47]. According to this definition, oxidative stress is primarily a quantitative issue: an excess of reactive species (RS) and a deficiency of antioxidants result in cellular damage. According to subsequent studies on redox signalling pathways, antioxidant mechanism and oxidative stress markers, Dean Jones (2006) redefines oxidative stress as "*a disruption of redox signaling and control*"[48], thus broadening the understanding of oxidative stress. This definition incorporates the functional and regulatory roles of RS in cellular physiology, moving beyond the reductionist perspective of free radical neutralization proposed by the classical definition of Sies[49,50].

An imbalance in ROS generation and scavenging by antioxidants results in pathological conditions, including cancer, neurodegenerative disorders, inflammatory disease and atherosclerosis[9,17,51–54].

Although the role of oxidative stress in many diseases is not fully understood, based on its contribution to the aetiology of various pathologies, it can be grouped into two categories:

- oxidative stress as a primary cause of disease (including radiation[55] and paraquat[56] toxicities and atherosclerosis[57]);
- oxidative stress as a secondary factor contributing to disease progression (such as hypertension and Alzheimer's disease[58]).

Oxidative stress contributes to disease through two main mechanisms:

- directly through the production of reactive species during oxidative stress, particularly •OH, $ONOO^-$ and HOCl, which oxidize macromolecules, including membrane lipids, structural proteins, enzymes and nucleic acids, leading to aberrant cellular function and death[59].
- indirectly through aberrant redox signaling. Oxidants, particularly H_2O_2 generated by cells following physiological stimulation, can act as second messengers. In oxidative stress, non-physiological production of H_2O_2 can cause abnormal redox signaling. Furthermore, increased H_2O_2 production and iron release from proteins in oxidative stress by $O_2•^-$ and $ONOO^-$ causes a marked increase in the production of lipid peroxidation products, leading to aberrant signaling[46].

Nitric oxide reacts rapidly with superoxide radicals to produce the anion peroxynitrite ($ONOO^-$), a highly reactive oxidant capable of causing significant damage to biomolecules. Peroxynitrite interferes with cellular signaling by nitrating specific amino acid residues in proteins. It also induces modifications through the nitration or oxidation of amino acids such as cysteine, tyrosine, and methionine. These chemical alterations result in changes to the structure and function of the affected proteins, potentially disrupting their normal biological activity[60,61].

Elevated levels of peroxynitrite anion are associated with tumor progression and have been implicated in the development of cardiovascular and neurological disorders, as well as cancer[62].

Lipid damage: membrane lipids, particularly the polyunsaturated fatty acid (PUFA) residues in phospholipids, are highly susceptible to oxidation by free radicals[63]. Lipid peroxidation begins when a free radical extracts a hydrogen atom from a methylene group (CH_2) of a fatty acid (LH), generating a carbon-centered lipid radical (L•). This radical can subsequently react with molecular oxygen to form a lipid peroxyl radical (LOO•)[64,65].

The process propagates as lipid peroxyl radicals (LOO•) are converted into reactive intermediates such as endoperoxides, which decompose into toxic species like malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE)[66]. These end products of lipid peroxidation cause damage to DNA,

proteins, and other biomolecules, contributing to cellular dysfunction. Malondialdehyde, in particular, reacts with proteins to form complexes known as advanced lipid peroxidation end products (ALE), which alter the functional properties of biomolecules and promote disease progression[67,68].

Under oxidative stress conditions, MDA in combination with acetaldehyde can generate immunogenic adducts (MAA) that further impair biomolecular function. Additionally, 4-HNE, another reactive carbonyl species (RCS), significantly modifies proteins by inducing cross-linking of membrane-associated proteins, such as the regulatory protein RGS4, thereby disrupting cellular signaling pathways[69].

Lipid peroxidation also leads to the loss of membrane functionality, including reduced fluidity and impaired activity of membrane-associated enzymes and receptors. These damages contribute to severe cellular dysfunction and are implicated in various pathological conditions[70].

Protein damage: Protein carbonylation, driven by ROS, is one of the most prevalent forms of non-enzymatic post-translational modification[71]. This stable modification occurs through various mechanisms, including the direct oxidation of protein-bound amino acids, oxidative cleavage of the protein backbone, and the incorporation of carbonyl groups resulting from glycoxidation and lipoxidation processes[72,73]. Carbonylation is influenced by the cellular redox environment, ROS abundance, and the proximity of proteins to ROS production sites.

Protein carbonylation is a key biomarker for aging and various diseases, as it alters both the structural integrity and functional capacity of proteins. Proteins that are heavily oxidized become inefficient substrates for ubiquitin-mediated degradation, resulting in their accumulation as covalently crosslinked protein aggregates[74]. These aggregates are highly resistant to degradation, impairing cellular functionality during aging and disease processes[75]. In contrast, mildly oxidized proteins are more susceptible to proteasomal degradation because the oxidation exposes hydrophobic amino acids, a consequence of the unfolding of targeted protein domains[76].

DNA damage: mitochondrial DNA is more susceptible to damage from ROS compared to nuclear DNA due to its close proximity to ROS production sites[77]. Among the ROS, the hydroxyl radical (OH•) is particularly reactive, attacking all DNA components and causing various forms of damage, including single-strand and double-strand breaks[78]. This radical abstracts hydrogen atoms, resulting in the formation of byproducts such as modified purine and pyrimidine bases, as well as DNA-protein cross-links.

1.4 ANTIOXIDANTS

An antioxidant is a molecule that can inhibit the oxidation of other molecules. It has been defined as "any substance that delays, prevents, or removes oxidative damage to a target molecule"[79,80]. In

another definition, an antioxidant is defined as a substance that directly scavenges ROS or acts indirectly to regulate antioxidant defences or inhibit ROS production. Thus antioxidants are molecules or enzymes that are able to delay the progression of many chronic diseases[81,82].

In recent years, there has been great interest in identifying natural and safe alternative sources of food antioxidants and in the search for natural antioxidants, especially of plant origin. Antioxidants are often added to foods to prevent radical oxidation chain reactions and act by inhibiting the initiation and propagation phase leading to the conclusion of the reaction and delay the oxidation process[83]. Antioxidants have become an indispensable group of food additives mainly because of their unique properties of extending the shelf life of food products without any adverse effect on their sensory or nutritional qualities[84,85]. Numerous scientific studies are underway to address the various health benefits of antioxidants in processes such as stress, pathogen infestation, aging, inflammation, and neurological diseases. Epidemiological studies have shown an inverse association between fruit and vegetable intake and mortality from age-related diseases and cancer, which can be attributed to their antioxidant activity. Boosting of endogenous antioxidant defences of the body or supplementation with exogenous antioxidants have been found to be a promising method of countering the undesirable effects of oxidative stress[86–88].

1.5 ENDOGENOUS ANTIOXIDANTS

Endogenous antioxidants are broadly categorized into enzymatic and non-enzymatic types, each playing a distinct role based on their specific mechanisms of action, sites of operation, and ultimate biological effects[81]. This functional diversity underscores the unique contribution of each antioxidant to maintaining cellular homeostasis.

Enzymatic antioxidants, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR), represent a highly efficient defense system[89,90]. SOD catalyzes the dismutation of superoxide radicals into hydrogen peroxide and molecular oxygen, mitigating oxidative damage[91]. Catalase subsequently decomposes hydrogen peroxide into water and oxygen, preventing its accumulation and the formation of highly reactive hydroxyl radicals[92]. GPx reduces hydrogen peroxide and lipid hydroperoxides to water and their respective alcohols, relying on reduced glutathione (GSH) as a substrate. GR, in turn, regenerates GSH from its oxidized form (GSSG), ensuring a steady supply of this critical cofactor for GPx activity[93,94].

Non-enzymatic antioxidants complement enzymatic systems by scavenging free radicals and preventing their interaction with cellular macromolecules. Examples include glutathione, which participates in redox regulation and detoxification; and small molecules like uric acid and bilirubin

that also contribute to the antioxidant network. Together, these systems form an integrated defense mechanism that protects the body against oxidative stress and its deleterious effects[82,95].

1.6 PLANTS AS A SOURCE OF ANTIOXIDANTS

Plants, including edible vegetables, fruits, spices, and herbs, have long been a source of exogenous (i.e., dietary) antioxidants as vitamins (A, C and E), phenolic compounds, carotenoids, and essential microelements (Zinc, Manganese, Selenium)[96]. It is believed that two-thirds of the world's plant species have medicinal importance, and almost all of these have excellent antioxidant potential. However, it is important to note that both antioxidant activity and content can vary significantly across different plant species, varieties, and even within specific parts of the same plant. Additionally, the efficacy of these natural products is influenced by a range of external factors[97], such as climatic and soil conditions[98,99], as well as the timing of harvest[100]. These variables can all play a crucial role in determining the antioxidant potential of the plant material.

In the realm of medicinal plants, the Amaryllidaceae family, including species such as garlic (*Allium sativum*) and onion (*Allium cepa*), makes a significant contribution to natural therapy. These plants are rich in sulphur compounds and alkaloids, such as allicin, which exhibit potent anti-inflammatory and antioxidant properties. Garlic, in particular, is known for its ability to inhibit the activity of enzymes involved in inflammation, such as cyclooxygenase (COX), and to counteract oxidative stress by scavenging free radicals[101,102].

The genus *Cephalaria*, belonging to the Caprifoliaceae family, also stands out for its flavonoids and polyphenols, which provide anti-inflammatory and antioxidant benefits. Its immunomodulatory and protective properties against oxidative stress make it a valuable resource in natural medicine[103].

The combination of these natural resources, coupled with a scientific approach to optimize their use, represents a promising strategy for effectively managing oxidative stress in a sustainable manner, while presenting fewer side effects, if any, compared to conventional drugs.

1.6.1 VITAMINS

Vitamin E is a lipid-soluble compound, with α-tocopherol being the most biologically active and widely studied form. Both tocopherols and tocotrienols serve as potent antioxidants, acting as free radical scavengers within cellular membranes and lipoproteins. Vitamin E neutralizes free radicals by directly interacting with peroxyl radicals (ROO•), which are generated during the oxidative degradation of polyunsaturated fatty acids in membrane lipids. This process involves the donation of a hydrogen atom by vitamin E, resulting in the formation of a less reactive α-tocopheroxyl radical.

The α-tocopheroxyl radical can subsequently be reduced back to active α-tocopherol by other reducing agents, including ascorbic acid (vitamin C) and glutathione[104,105].

Vitamin A is a lipid-soluble vitamin essential for several critical biological processes, including growth, cell differentiation, vision, and immune function. It exists in two primary forms: retinoids, which are derived from animal sources and include retinol, retinaldehyde, and retinoic acid, and carotenoids, like β-carotene, found in plants and converted into retinol in the body. Vitamin A has a regulatory role in the body's antioxidant defenses. Its active metabolite, all-*trans*-retinoic acid, interacts with transcription factors such as NRF2, which controls the expression of genes involved in the antioxidant response[106].

Vitamin C, or ascorbic acid, is a water-soluble vitamin renowned for its potent antioxidant properties and its critical role as a cofactor in various enzymatic processes. Acting as an electron donor, vitamin C neutralizes free radicals, thereby protecting essential biomolecules such as lipids, proteins, and DNA from oxidative damage[107]. Moreover, it has the capacity to regenerate other antioxidants, such as vitamin E, by restoring them to their active state, thus amplifying its overall protective effects.

Vitamin C actively supports immune function promoting lymphocyte proliferation, enhancing the phagocytic activity of immune cells, and modulating the production of pro-inflammatory cytokines, leading to a reduction in systemic inflammation[108].

Numerous studies have highlighted the importance of vitamin C in preventing chronic diseases associated with oxidative stress, including cardiovascular disease, type 2 diabetes, and cancer[109].

1.6.2 PHENOLS

Plants are known to produce over 200,000 chemical substances, a significant proportion of which are phenolic compounds. These substances, including flavonoids, polyphenols, and related phenolic compounds, play a vital role in plant growth and reproduction. They provide defence mechanisms against pathogens and herbivores, while also contributing to the coloration and sensory qualities of fruits and vegetables[110].

Phenolic compounds are biogenetically derived natural metabolites characterized by an aromatic ring with one or more hydroxyl groups. They are often found in nature as conjugates with mono- or polysaccharides, which enhance their stability compared to their free form. They may also be associated with esters or methyl ethers[111]. These compounds are categorized into various classes, with flavonoids and phenolic acids being the most significant in the human diet due to their beneficial effects on health[112].

A key feature of phenolic compounds is their ability to counteract oxidative stress, a property extensively studied by researchers. Their antioxidant activity is attributed to their ability to act as

hydrogen or electron donors and effectively scavenge free radicals[113]. Moreover, these compounds can chelate metals such as iron and copper, preventing the metal-catalyzed generation of free radicals[114]. The antioxidant properties of phenolic compounds are strongly influenced by their structure, particularly the number and arrangement of hydroxyl groups relative to the carboxylic group[115].

In summary, phenolic compounds not only play a critical role in plant biology but also offer significant health benefits to humans. Their complex structures, diverse classifications, and functional properties make them essential components of a balanced diet, with flavonoids and phenolic acids being among the most important contributors.

FLAVONOIDS

Flavonoids are essential for various biological functions in plants, where they play a central role in growth, development, and ripening. They also help plants adapt to environmental challenges, offering protection against biotic stressors such as viruses, fungi, bacteria, and herbivores, as well as abiotic factors like UV radiation and drought[116]. They also contribute to flower coloration, attracting pollinators, and are found in fruits like tomatoes, grapes, apples, rice, and wheat. Flavonoid levels vary depending on species, environmental factors, cultivation practices, and plant ripeness[117].

In humans, flavonoids are recognized for their health benefits. Their bioavailability and biological activity depend on their molecular structure, particularly the arrangement of hydroxyl, methoxy, and glycosidic groups. Flavonoids are classified into seven main subclasses: flavonols, flavones, isoflavones, anthocyanidins, flavanones, flavanols, and chalcones[118].

One of the most important properties of flavonoids is their ability to counteract oxidative stress. They achieve this through several mechanisms, such as scavenging ROS via hydrogen donation, chelating pro-oxidant metal ions like $Fe(II)^{2+}$ and $Cu(II)^{2+}$, and inhibiting enzymes involved in free radical production (e.g., xanthine oxidase and NADPH oxidase)[119]. They can act inhibiting enzymes such as cyclooxygenase, lipoxygenase, and inducible nitric oxide synthase (iNOS), which are responsible for inflammatory pathways[120]. Flavonoids like quercetin have been shown to suppress nitric oxide (NO) production and reduce the toxicity associated with peroxynitrite, a harmful oxidant formed from the reaction between NO and superoxide anions[121]. Moreover, they regulate key processes in cellular health, such as reducing oxidative damage to DNA, proteins, and lipids, which is crucial for preventing chronic diseases[122].

These findings suggest that flavonoids could serve as effective natural alternatives to synthetic drugs, offering strong therapeutic benefits with minimal side effects.

Given their profound health benefits, including the prevention of chronic diseases, a diet rich in flavonoid-containing foods (such as fruits, vegetables, and whole grains) or the use of flavonoid supplements is highly recommended. By incorporating these compounds into daily nutrition, individuals may enhance their defense against oxidative stress and reduce the risk of developing chronic conditions.

SINAPIC ACID AND DERIVATES

Among the hydroxycinnamic acids, the most notable compounds include caffeic acid, ferulic acid, and sinapic acid. Sinapic acid is particularly abundant in cereals and is a key phenolic compound in *Brassica* vegetables, making them one of the richest sources of sinapic acid in the plant kingdom[123]. Chemically, sinapic acid is known as 3,5-dimethoxy-4-hydroxycinnamic acid. It exists both in free form and as part of ester compounds. These esters include sugar esters (glycosides) and esters with various organic molecules. Notable sinapic acid derivatives include sinapoyl malate, predominantly found in leaves, and sinapine (sinapoyl choline), which accumulates in roots[124]. Among Brassicaceae species, sinapoyl glucose (1-O-β-D-glucopyranosyl sinapate) is the most common derivative and, together with glycosides of kaempferol and quercetin, represents one of the most critical phenolic compounds in this plant family[125].

Sinapic acid is a potent antioxidant, with studies indicating that it may be more effective than ferulic acid and, in some cases, comparable to caffeic acid. Its antioxidant activity is primarily attributed to its ability to donate hydrogen atoms, which helps neutralize free radicals. The phenoxyl radicals formed during this process are stabilized by the conjugated system present in sinapic acid, enhancing its capacity to protect cells from oxidative damage[126]. Sinapic acid derivatives, such as sinapoyl glycosides, also exhibit free radical scavenging activity, although sinapic acid itself generally outperforms its glycoside forms, except for specific methylated derivatives like methyl 2-O-sinapoyl-β-D-glucose and methyl 6-O-sinapoyl-β-D-glucose, which show slightly higher activity[126].

Additionally, it has been demonstrated that a 20 μM concentration of sinapic acid can neutralize approximately 33% of DPPH radicals[127].

1.6.3 GLUCOSINOLATES

Glucosinolates are sulfur-containing secondary metabolites typical of plants of the Brassicaceae family, such as broccoli, cabbage, cauliflower, and mustard. When plant tissues are damaged, glucosinolates are hydrolyzed by the enzyme myrosinase (thioglucosidase), generating unstable intermediates that are transformed into isothiocyanates, thiocyanates, or nitriles. These hydrolysis

products play a crucial role in the plant's defense against herbivores, parasites, and pathogenic bacteria, and also contribute to their characteristic aroma[128,129].

Chemically, glucosinolates are anions consisting of a thioidoxymate-O-sulfonate group linked to a glucose molecule and a variable side chain (R), which can be alkyl, aralkyl, or indole. Their variety derives from the modification of the side chain of the precursor amino acids before the formation of the glucosinolate nucleus, followed by further secondary modifications such as oxidation, desaturation, hydroxylation, methoxylation, sulfation and glucosylation[130].

Most of the studies focus on these derivatives, such as isothiocyanates, nitriles and thiocyanates, recognized for their bioactive properties, including the induction of cytoprotective genes, the repression of the NF-κB factor, the inhibition of histone deacetylase and cytochrome P450[131,132]. Although studies on intact glucosinolates are more limited, some research has highlighted their antioxidant potential.

Lee et al. (2014) analyzed glucosinolates in 62 broccoli varieties in *in vitro* studies, evaluating their content and antioxidant power. They observed that some varieties present significantly different levels of glucosinolates, suggesting a correlation between their chemical profile and antioxidant activity[133].

In other studies, gluconasturtiin showed a modest ability to neutralize free radicals *in vitro*, while sinigrin and glucoraphanin were investigated for their metabolism by the intestinal microbiota. It was shown that, even in the absence of myrosinase, these compounds generate bioactive metabolites capable of promoting the growth of beneficial bacteria and modulating the intestinal microbiota[134–136].

Glucoraphanin, in particular, showed a protective effect on the intestinal mucosa, reducing inflammation in murine models of colitis[137]. Sinigrin, on the other hand, has shown antioxidant and anti-inflammatory properties both *in vitro* and *in vivo*[138].

The action of these compounds can be enhanced by the presence of multiple molecules with synergistic activities. Finally, the bioactivity of glucosinolates is influenced by the composition and concentration of the compounds in the plant and by the activity of the hydrolysis enzymes. Among the many plants known for their beneficial effects on human health, particularly in counteracting oxidative stress, our focus will be on the Brassicaceae family.

1.7 ANTIOXIDANT POTENTIAL OF BRASSICACEAE

The Brassicaceae, often called Cruciferae or mustard family (Fig. 2), are a monophyletic group of about 338 genera and some 3709 species distributed all over the world except Antarctica[139]. Brassicaceae family includes numerous economically significant species, many of which have

become essential in agriculture and food industries. The wild ancestors of these plants were originally found growing along the Mediterranean coast. Over time, however, a wide range of crops and multiple domestications of these plants have taken place across Mediterranean Europe, Asia, and North America, resulting in the diverse varieties we rely on today for both culinary and industrial purposes. [140]. They are traditionally consumed in the human diet as fresh and preserved vegetables, vegetable oils and condiments.

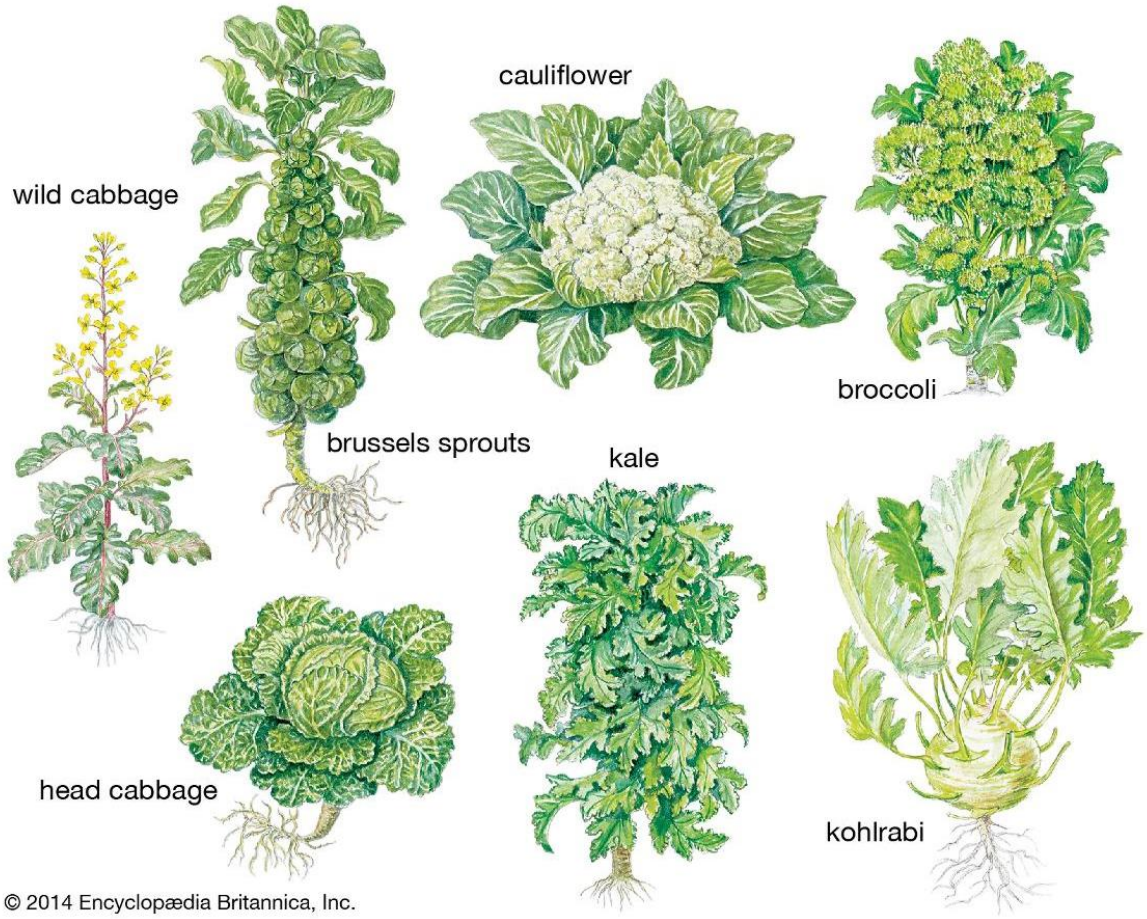

Figure 2. The different forms of cabbage include wild cabbage, brussels sprouts, cauliflower, broccoli, head cabbage, kale, and kohlrabi. Encyclopædia Britannica, Inc.

In addition to their culinary use, Brassicaceae have been extensively used in traditional medicine from ancient times. These findings allow the recognition of Brassicaceae vegetables as functional foods and numerous dietary supplements, containing a variety of extracts or compounds isolated from these vegetables, are already available on the market.

The most significant health benefits of Brassicaceae vegetables are attributed to their high content of glucosinolates. These compounds are present in all cruciferous vegetables, though their chemical structure and relative proportions can vary depending on the specific crop. Glucosinolates are considered key contributors to the health-promoting properties of these vegetables, and their levels

can be influenced by factors such as plant variety, cultivation practices, and environmental conditions[141,142].

Glucosinolates beside to be responsible for most of the Brassicaceae beneficial health effects can also exert toxic effects. In addition, cruciferous vegetables have nutritional values as they have high protein content, low fat and high value in vitamins, fibers and minerals, as well as very few calories. In addition they show high quantities of phenolic compounds triterpenes, flavonoids (mainly flavonols and anthocyanins)[143] and the hydroxycinnamic acids[144,145]. Due to the high level of vitamins, Brassicaceae crops have the potential to prevent and treat malignant and degenerative diseases and, due to the presence of folate, have the potential to reduce risk of vascular diseases, cancer and neural tube defects[144].

1.7.1 *BRASSICA* GENUS

The genus *Brassica*, the most famous, including 38 annual or perennial species[146], is divided into three sections: (1) sect. *Brassica*, (2) sect. *Brassicaria*, and (3) sect. *Melanosinapis*[147]. *Brassica* sect. *Brassica* consists of 14 species (with varius subspecies) characterized by x = 9 genome, a suffrutescent perennial habit, large size (up to two meters tall when flowering), flowers with yellow–white petals, cylindrical or subcylindrical/tetragonous fruits (siliques), globose and reticulate seeds[148,149]. The life cycle is usually between two and five to six years. The populations occur mainly around the Mediterranean basin, from Israel and Lebanon in the East to the Canary Islands and the British Isles in the West[150].

This genus includes a group of six interrelated species of worldwide economic importance, three diploid: *Brassica nigra, Brassica oleracea and Brassica rapa* and three amphidiploid: *Brassica carinata, Brassica juncea* and *Brassica napus*. The genus is categorized into oilseed, forage, condiment and vegetable crops by using their buds, inflorescences, leaves, roots, seeds and stems. *B. oleracea* and *B. rapa* include most of the vegetable *Brassica* crops and they display many choices of edible forms within each species. The greatest genetic and phenotypic variability of *B. oleracea* is found in Europe, while Asia represents the main area of diversification of vegetable *B. rapa* crops[145]. *Brassica* crops, alongside cereals, currently form the cornerstone of global food supplies. The genetic diversity inherent in *Brassica* species has not only facilitated the development of new crop varieties but has also significantly advanced the field of molecular biology and plant genetics. In the 20th century, members of the Brassicaceae family emerged as ideal model plants, gaining prominence in scientific research. Their ease of cultivation, rapid life cycle, and genetic versatility made them essential for studying fundamental biological processes, thereby contributing to ground-breaking discoveries in plant science[151].

Vegetable *Brassica* crops have the nutritional characteristics of other vegetable crops, as for example a low fat and protein content and a high value in vitamins, fiber and minerals.

Brassica crops have the highest level of vitamin C, with regard to vitamin E, also have high levels of vitamin B-6, vitamin A and β-carotene, lutein, zeaxanthin and vitamin K. Vitamin C, E and carotenoids have the potential to prevent and treat malignant and degenerative diseases, related to the reduced risk of vascular diseases, cancer and neural tube defects[152].

The most widespread and diverse group of polyphenols in *Brassica* spp. are the flavonoids (mainly flavonols and anthocyanins) and the hydroxycinnamic acids[143].

Several physical and chemical environmental factors (genetic characteristics, harvest time, hydric and osmotic stress, nutrition, photoperiod, relative humidity, seasonality, soil properties such salinity, pH, chemical composition, toxins, and the temperature), can affect the quality and quantity of phytochemicals biosynthesized by *Brassica* plants[100,153–155].

1.7.2 WILD SICILIAN *BRASSICA*

It is well known that , Sicily, home of biodiversity, is one of the centers of the diversification of wild taxa of *Brassica* sect. *Brassica*[148,150]. The 11 taxa of the section occurring in Sicily and in the small islands around are *B. incana* (in eastern Sicily), *B. macrocarpa* (endemic to the Egadi Islands), *B. rupestris* (with two subspecies: subsp. *rupestris* and subsp. *hispida* Raimondo et Mazzola), and *B. villosa* (with five subspecies: subsp. *villosa*, subsp. *bivonana* (Mazzola et Raimondo) Raimondo et Mazzola, subsp. *drepanensis* (Caruel) Raimondo et Mazzola, subsp. *tinei* (Lojac.) Raimondo et Mazzola, subsp. *brevisiliqua* (Raimondo et Mazzola) Raimondo and Geraci). More recently, new species were described on the basis of their morphological traits, namely, *B. raimondoi*, which occurs only in the cliffs of Castelmola (Messina)[156] and was recently considered a subspecies of the sympatric *B. incana*, if not synonymous, and *B. trichocarpa*, which is endemic to a single locality near Mt. Cuccio, close to Palermo[157] and the new species *Brassica tardarae* is restricted to the carbonate cliffs in the Tardara Gorges[158] (Fig. 3).

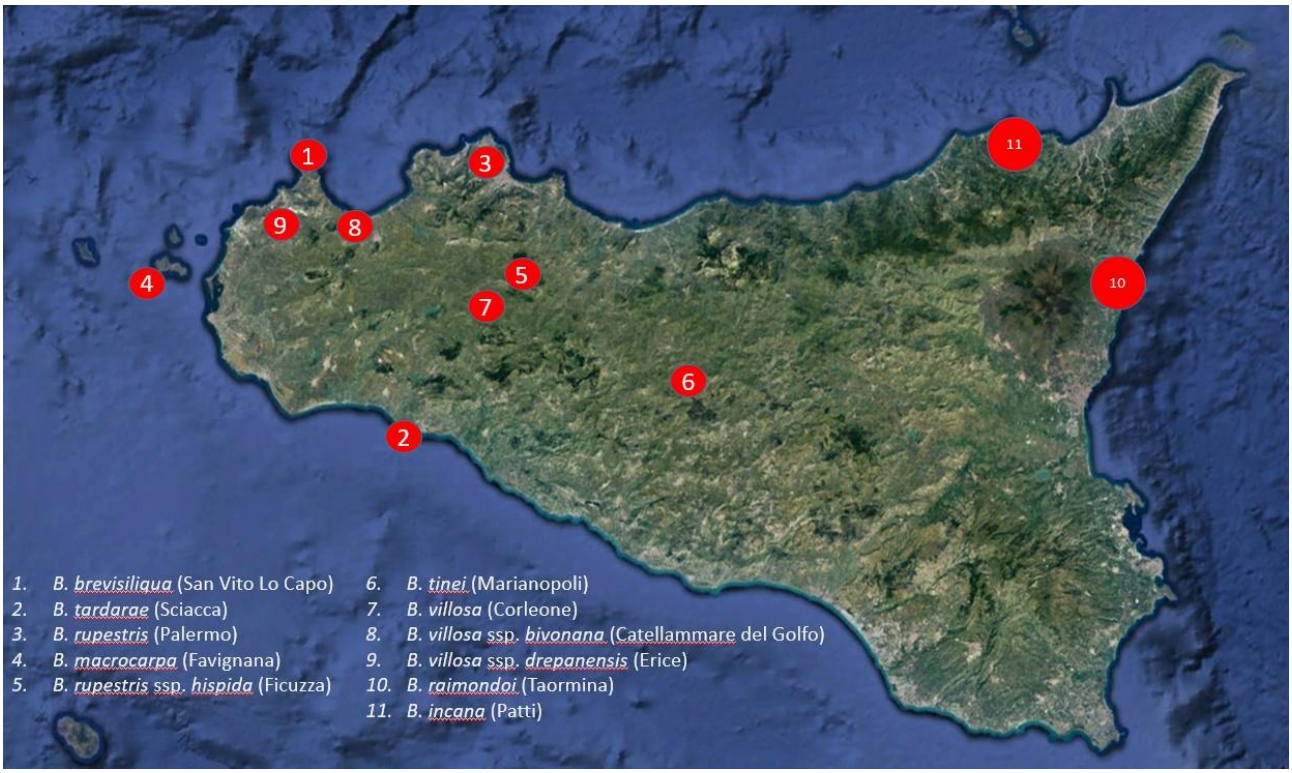

Figure 3. Growth points of the 11 endemic brassica species

B.rupestris and *B. villosa* are endemic to western Sicily, with a single disjunct population of *B. rupestris* in Calabria[159]. All populations of these taxa grow on cliffs and rocky habitats (mainly on limestone) from the sea level to 1000-1200 m above sea level (a.s.l.).

The populations are often restricted in size and distribution since most of the taxa are endemic and/or with a restricted or highly fragmented area of distribution and are often threatened by anthropic activities, grazing, fires, etc. *Brassica macrocarpa* is considered to be Critically Endangered (CR)[160], whereas *B. rupestris* s.l. and *B. villosa* s.l. are included as Near Threatened (NT) species on the IUCN Red List[161]. In previous national and regional Red Lists, according to the IUCN categories, these species were assessed as Lower Risk (LR) (*B. rupestris*. subsp. *rupestris*, *B. villosa* subsp. *bivonana*, and *B. villosa* subsp. *drepanensis*), Vulnerable (VU) (*B. villosa* subsp. *tinei* and *B. villosa* subsp. *brevisiliqua*), Endangered (EN) (*B. rupestris* subsp. *hispida*), and Critically Endangered (CR) (*B. villosa* subsp. *villosa*)[162].

These wild taxa, belonging to the same cytodeme of *Brassica oleracea*, can hybridize with cultivated forms to represent a useful genetic resource for the improvement of cultivated varieties, considering the importance of *Brassica* vegetables globally[163].

The wild taxa of sect. *Brassica* are perennial, suffruticose plants characterized by a robust growth habit, large and thickened leaves, and well-developed racemes. They feature large flowers with white to yellow petals, and their siliquae are ellipsoid to linear, with convex, woody valves that culminate

in a 0-2-seeded beak. The seeds are typically globose and have a ± reticulate surface texture. These plants are facultative chasmophytes, meaning they are not strictly confined to rocky habitats but primarily thrive on carbonate sea cliffs, as well as in gentle rocky slopes, maquis, or ruderal areas. They can be found at elevations ranging from sea level up to 1000-1200 meters. So far there are few chemical studies about wild Sicilian *Brassica*. Scialabba et al. determined the content of tocopherol (Toc), fatty acids and phytosterols only in the dry seeds of nine wild taxa of Sicilian *Brassica* and on the basis of the Toc content and the relative percentage of single isoforms they were able to discriminate between the seeds of *B.incana, B. macrocarpa, B. rupestris* group and *B. villosa* goup[164]. Furthermore, these taxa, with the highest content of T-Toc, γ-Toc and phytosterols, are potentially the most bioactive and could, therefore, be exploited in breeding programs to develop genotypes with presumed positive effects on human health[164]. At University of Catania, Branca et al., focused attention on seeds of *B. incana, B. macrocarpa, B. rupestris* and *B. villos* and *B. montana* starting to characterize the levels and profile of phytochemicals and assaying *in vitro* the differences in antioxidant capacity by the 1,1-diphenyl-2-picrylidrazil (DPPH) test[165]. Recently Picchi et al. evaluated this variability of the antioxidant traits of the same *Brassica*, concluding that the scavenging activity of the superoxide anion is significantly related to phenols, while DPPH is mainly related to the ascorbic acid content[166]. Thus although first chemical assays are starting to investigate the potential antioxidant effects, so far are scarce the biological evidence on endemic *Brassica*, encouraging the study on this topic. In Table 2 were summarised Sicilian endemic species and their biological properties.

Species	Plant extract	*in vitro* assay	cellular model	Activity	Reference
B. brevisiliqua		*Not yet analysed*	*Not yet analysed*	*Unknown*	
B. tardarae	Methanol Leaf Extract Reported for the first time in this thesis	Reported for the first time in this thesis	Reported for the first time in this thesis	Reported for the first time in this thesis	This thesis
B. rupestris subsp. rupestris	Leaf freeze-dried	DPPH, LIPOX-CROCIN DPPH, Superoxide anion radical	*Not yet analysed*	Antioxidant	165 166
B. macrocarpa	Leaf freeze-dried Methanol Leaf Extract	DPPH, LIPOX-CROCIN DPPH, Superoxide anion radical	Reported for the first time in this thesis	Antioxidant	165 166 This thesis
B. rupestris subsp. hispida	Methanol Leaf Extract Reported for the first time in this thesis	Reported for the first time in this thesis	Reported for the first time in this thesis	Reported for the first time in this thesis	This thesis
B. villosa subsp. tinei		*Not yet analysed*	*Not yet analysed*	*Unknown*	
B. villosa subsp. villosa	Leaf freeze-dried	DPPH, LIPOX-CROCIN DPPH, Superoxide anion radical	*Not yet analysed*	Antioxidant	165 166
B. villosa subsp. bivonana		*Not yet analysed*	*Not yet analysed*	*Unknown*	
B. villosa subsp. drepanensis	Ethanol Leaf Extract	ABTS, FRAP, DPPH, SOD-like Activity	LPS-stimulated RAW 264.7 Caco-2	Antioxidant ↓ ROS and NO Cytotoxic in cancer cells	235
B. incana subsp. raimondoi	Methanol Leaf Extract	DPPH, SOD-like Activity, β-Carotene Bleaching, Reducing Power, Chelating Activity Assays	H_2O_2-stimulated HepG2	Antioxidant ↓ ROS	212
B. incana	Leaf freeze-dried Methanol Leaf and Flowering Top extract	DPPH, LIPOX-CROCIN DPPH, Superoxide anion radical	MCF-7 and Caco-2	Antioxidant Cytotoxic in cancer cells ↑ LDH	165 166 215

Table 2: Sicilian endemic species and their biological properties

1.8 MODELS TO STUDY ANTIOXIDANT EFFECTS OF NATURAL COMPOUNDS

Comprehensive approaches for studying the antioxidant effects of natural compounds include various experimental models, ranging from simple biochemical assays to complex *in vivo* systems.

In this section will be described some of these key models.

1.8.1 *IN VITRO* MODEL

The most commonly employed methods for analyzing the antioxidant activity of natural compounds focus on measuring the total phenolic content, specific groups or classes of phenolic compounds, or the overall radical scavenging capacity using various radical species (such as ABTS and DPPH). Several factors can influence the accuracy of these quantifications, including the chemical nature of the analyte, the methodology used, the choice of reference standards, and the presence of interfering substances. Ideally, a standardized method for assessing the antioxidant activity of phytocomponents should meet the following criteria: (1) simplicity and reproducibility; (2) use of a biologically relevant radical source; (3) a well-defined endpoint and chemical mechanism; and (4) the ability to evaluate both hydrophilic and lipophilic antioxidants using different radical sources[82,167].

Based on the underlying mechanism, these methods can be broadly classified into two categories: hydrogen atom transfer (HAT) methods and single electron transfer (SET) methods. While both mechanisms yield similar outcomes, they differ in reaction kinetics and the likelihood of side reactions[168].

HAT-based methods

These tests measure an antioxidant's ability to neutralize free radicals by donating hydrogen atoms. HAT reactions are rapid and are independent of solvent and pH. However, the presence of reducing agents, such as metals, can lead to significant overestimations. A well-known HAT-based method is the Oxygen Radical Absorbance Capacity (ORAC) test.

Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay evaluates the antioxidant capacity of a compound by measuring its ability to inhibit the oxidation initiated by peroxyl radicals. These radicals are generated through the thermal decomposition of azo compounds, such as 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH). During the reaction, the antioxidant competes with a fluorescent probe (commonly fluorescein) for scavenging the peroxyl radicals[169]. The decrease in fluorescence intensity over time reflects the extent of radical scavenging, with higher antioxidant activity correlating to greater inhibition of fluorescence decay. This method is particularly valued for its biologically relevant radical source and suitability for both hydrophilic and lipophilic compounds.

SET-based methods

These assays evaluate an antioxidant's ability to transfer an electron to reduce oxidants, including metals, carbonyls, or radicals. The reduction of the oxidant is accompanied by a color change, which is proportional to the concentration of the antioxidant. The endpoint of the reaction is identified when the color change ceases. The SET reaction mechanism involves deprotonation and the ionization potential of the reactive functional group, making it pH-dependent. SET reactions are typically slower, and antioxidant capacity is assessed based on the percentage decrease in product rather than reaction kinetics. Prominent SET-based methods include the Trolox Equivalent Antioxidant Capacity (TEAC) test with ABTS radicals, the Ferric Ion Reducing Antioxidant Power (FRAP) assay, and the scavenging test for 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals[170].

The adoption of accurate and well-characterized methods is essential for evaluating the antioxidant potential of natural compounds *in vitro*, ensuring the comparability of results across different studies.

Ferric Ion Reducing Antioxidant Power (FRAP)

The FRAP assay is based on the ability of electron-donating antioxidants to reduce the ferric ion (Fe^{3+}) in the tripyridyltriazine complex (Fe^{3+}-TPTZ), which has a yellow coloration, to the ferrous ion (Fe^{2+}) in the blue-colored ferrous-tripyridyltriazine complex (Fe^{2+}-TPTZ). The intensity of the blue color, measurable spectrophotometrically, is proportional to the reducing power of the antioxidant. This method is rapid and simple but is limited to measuring only hydrophilic antioxidants and does not account for radical scavenging activity directly[171].

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay

The DPPH assay measures the capacity of antioxidants to neutralize the stable, purple-colored DPPH radical (DPPH•), a nitrogen-centered free radical. Upon interaction with hydrogen or electron-donating antioxidants, the radical is reduced, resulting in a color change from purple to pale yellow. The extent of discoloration is proportional to the number of electrons or hydrogen atoms transferred by the antioxidant[172]. This straightforward and widely used assay provides a qualitative and quantitative measure of antioxidant activity, although it is primarily suitable for hydrophilic compounds and may be less reliable in complex mixtures.

Trolox Equivalent Antioxidant Capacity (TEAC)

The TEAC assay evaluates the antioxidant ability of a compound to quench the blue-green colored radical cation 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{+}$•). The radical cation is generated by chemical or enzymatic oxidation of ABTS. Antioxidants reduce $ABTS^{+}$• to its colorless or less intensely coloured neutral form, with the extent of colour change proportional to the antioxidant activity. The assay is versatile, as it accommodates both hydrophilic and lipophilic antioxidants, and it uses Trolox (a water-soluble vitamin E analog) as the standard for calibration[173].

1.8.2 CELLULAR MODEL

In recent decades, it has become evident that chemical methods such as HAT and SET, while useful for initial screening, do not adequately correlate with physiological effects. This highlights the importance of developing standardized cell-based assays for a more accurate assessment of antioxidant and anti-inflammatory activities[174]. Pioneering studies by Lopez-Alarcon and Denicola, followed by Amorati and Valgimigli, emphasized that the effectiveness of an antioxidant cannot be evaluated solely based on its chemical properties[175,176]. Then various mammalian cell lines, such as RAW 264.7 macrophages, Caco-2 epithelial cells, and human fibroblasts, are widely used to study the antioxidant effects of compounds. These cells are exposed to oxidative stressors like hydrogen peroxide (H_2O_2) or lipopolysaccharides (LPS), and the ability of compounds to mitigate oxidative damage is assessed through various assays (e.g., ROS measurement, MTT assay, and antioxidant enzyme activity)[177].

Although animal models and clinical studies are more representative of physiological conditions, they are costly and time-consuming, making cell cultures a valuable intermediate approach. An effective *in vitro* model can provide preliminary insights into the bioavailability and functional activity of dietary phytochemicals. Immortalized cell lines are widely used for these studies due to their stability in proliferation, capacity to endure freezing and thawing cycles, and reproducibility in experimental conditions[178].

RAW 264.7 murine macrophage cell line has gained significant attention. Macrophages play a crucial role in the innate immune response, serving as the first line of defense against pathogens and damage signals. RAW 264.7 cells are particularly sensitive to oxidative stimuli, such as LPS, which activates Toll-like receptor 4 (TLR4) and triggers intracellular signaling cascades involving transcription factors such as NF-κB and MAPK[179]. This activation promotes the generation of ROS and upregulates oxidative stress-related mediators. Among these, nitric oxide plays a central role, serving as a biomarker of oxidative stress. NO is considered a critical biomarker and is often measured in initial screenings to evaluate the antioxidant potential of natural compounds[180].

The use of macrophages, including the RAW 264.7 model, is particularly advantageous in studies involving natural substances. Plant extracts and other phytochemicals are frequently tested using these cells due to their sensitivity to bioactive compounds. Moreover, standardization of experimental conditions is crucial for reproducibility. Facchin et al. (2022) proposed a checklist of 30 critical parameters, including cell density, LPS concentration, and incubation time (12-24 hours), to ensure reliable results[180].

The widespread use of the RAW 264.7 cell model in biomedical research not only reduces the reliance on animal models but also accelerates the screening process for new bioactive molecules while lowering experimental costs. However, variability in the temporal secretion patterns of inflammatory mediators remains a challenge, necessitating careful experimental design and interpretation of results.

1.8.3 *IN VIVO* MODEL (ZEBRAFISH EMBRYOS)

The zebrafish (*Danio rerio*), a small teleost fish, has become a globally recognized model organism in numerous scientific fields, including developmental biology, genetics, immunology, and disease research. Its versatility stems from unique characteristics, such as transparent embryos which enable direct observation of internal organs, small size, rapid development, and a high degree of genetic and physiological homology with higher vertebrates (for example zebrafish genome shares approximately 75% similarity with the human genome)[181]. These attributes make zebrafish particularly useful for drug screening and assessing the efficacy and safety of both natural and synthetic compounds. One key advantage of the zebrafish model is its ability to facilitate the analysis of new pharmacological candidates in a whole-organism context, within a short time frame and at a lower cost compared to traditional models like rodents. External fertilization, embryo transparency, and high fertility allow for detailed embryological studies, while techniques such as gene knockdown using morpholino-modified anti-sense oligonucleotides enable precise investigation of molecular mechanisms[182]. Furthermore, zebrafish are widely used in xenotransplantation studies, where human cancer cells grow, metastasize, and induce angiogenesis similarly to murine models but with enhanced efficiency and reduced cell requirements[183].

By 48 hours post-fertilization (hpf), critical organs such as the heart, intestine, and blood vessels are already formed, and by 96 hpf, the central nervous system is nearly complete, including the development of catecholaminergic neurons, glial cells, oligodendrocytes, Schwann cells, and astrocytes. This rapid anatomical organization makes zebrafish an excellent model for studying the teratogenic and toxic effects of natural and synthetic substances in early development[184].

In recent years, zebrafish have emerged as an effective model for investigating oxidative stress and inflammation-related disorders. Several studies have demonstrated that natural compounds, such as flavonoids, can modulate antioxidant defences in zebrafish exposed to various stressors, including heavy metals, UV radiation, high-fat diets, and neurotoxins[185]. These compounds also interfere with pathological processes like angiogenesis and inflammation, highlighting the zebrafish's potential as a valuable tool for evaluating novel therapeutic strategies targeting oxidative stress and inflammation.

Of particular interest is the experimental model of oxidative stress and inflammation induced by copper sulfate ($CuSO_4$). This approach mimics pathological conditions characterized by oxidative damage and inflammatory responses, providing an ideal framework to assess the protective efficacy of bioactive compounds. The effects of $CuSO_4$ exposure are highly dependent on concentration and exposure duration: high concentrations and short exposure times typically elicit acute responses, while prolonged exposure to lower concentrations results in systemic and chronic effects[186–188]. This flexibility enables the modelling of diverse pathological scenarios, ranging from transient stress to persistent inflammation.

Finally, zebrafish behavior, both at the larval and adult stages, provides valuable insights into the effects of oxidative stress and neuroactive substances. Larvae, in particular, possess a more permeable blood-brain barrier than adults, facilitating the absorption of small molecules and enabling detailed analysis of neurobehavioral changes induced by toxic or therapeutic agents[189]. Behavioral phenotypes frequently studied include spontaneous movement, responses to visual or acoustic stimuli, social behavior, and environmental preferences. Alterations in these phenotypes yield critical information on the neurotoxic or neuroprotective effects of tested compounds, aiding in the elucidation of their mechanisms of action and therapeutic potential[190,191].

A further significant advantage is that, according to European Directive 2010/63/EU on the protection of animals used for scientific purposes, experiments on zebrafish larvae up to the point of autonomous feeding do not require ministerial authorization. This exemption significantly simplifies experimental procedures, encouraging the use of the larval model for preclinical and toxicological studies while adhering to strict European ethical standards.

2. AIMS

Oxidative stress, as already mentioned, is recognized as a key factor in the pathogenesis of many chronic diseases, including cardiovascular, neurodegenerative and metabolic diseases. Despite the efficacy of many conventional drugs in treating these conditions, the prolonged use of these therapies is associated with serious side effects and resistance phenomena[51]. This has stimulated a growing scientific interest, in recent years, towards natural compounds, especially those derived from plants, as safer and more effective alternatives.

Wild plants, compared to cultivated ones, represent a promising source of bioactive phytocompounds. Environmental conditions such as salinity, intense solar radiation, are able to modify the phytochemical profile by stimulating the production of secondary metabolites including polyphenols, glucosinolates and flavonoids and concentrating them[192].

Among all the Brassicaceae family genera, the *Brassica* genus is one of the most studied due to its richness in bioactive compounds[193]. However, most research has focused on cultivated species, neglecting the potential of wild species, which may present unique phytochemical profiles and superior biological properties precisely by virtue of the ecological conditions in which they grow.

The unique climate of Sicily, characterized by high solar exposure, soils rich in mineral salts and floristic biodiversity, could contribute to confer to endemic species peculiar biological properties compared to cultivated species.

The present study aims to investigate the biological properties of Sicilian endemic species of the *Brassica* genus in experimental models of oxidative stress.

The main objective is to chemically and biologically characterize three endemic *Brassica* species from Sicily, grown in ecologically distinct environments. The species selected for this study are:

- *Brassica mocracarpa* endemic to the island of Favignana (Egadi archipelago), growing on limestone cliffs and rocky slopes at elevations ranging from 0 to 50 meters above sea level, very close to the sea where seawater can reach the plant during storms.
- *Brassica rupestris* subsp. *hispida*. growing on the slopes of Rocca Busambra, in the Ficuzza wood, at elevations ranging from 800 to 1200 m above sea level in the shade of the oaks and experiencing significant temperature fluctuations between day and night.
- *Brassica tardarae*, grows in the suggestive fluviokarst gorges of Tardara (Sciacca) among the white limestones of the rocky walls at elevations ranging from 200 to 300 m above sea level.

Leaves, the edible parts known in Sicilian folk culture for their use in cooking and medicine, from the three species were collected, and we chose the exhaustive serial extraction method, using methanol as the last solvent, to obtain extracts rich in phytocomponents.

This study aims to enhance the current understanding of the biological properties of Brassicaceae by analysing for the first time endemic Brassica species, with a particular focus on their antioxidant activities. Additionally, the investigation of wild plants could help to understand the potential advantages of utilizing non-cultivated species

HPLC-MS/MS allowed us for the first time to characterize the phytochemical profile of the species identifying and quantifying the main secondary metabolites, with particular attention to glucosinolates and polyphenols which are known to be the main active metabolites of *Brassicae*.

By *in vitro* test as DPPH assay we evaluated the antioxidant activity.

Then, the evaluation of the potential cytotoxicity of our extracts was performed in RAW 264.7 cells by MTT assay after 24 hours of treatment with increasing concentrations (0 – 1000 µg/mL).

The ability of the extracts to modulate the production of NO and ROS was tested in RAW 264.7 murine macrophages stimulated by LPS.

The extract that proved most promising in these *in vitro* studies was tested in the *in vivo* animal model, the zebrafish (*Danio rerio*) embryonic model. The *in vivo* toxicity of the extract, its impact on behavior, and its ability to modulate copper sulfate ($CuSO_4$)-induced oxidative stress were evaluated.

3. MATERIALS AND METHODS

3.1 SAMPLES: COLLECTION AND EXTRACTION

The leaves of three *Brassica* species were collected in February 2022 from distinct locations in Sicily, Italy:

- *B. macrocarpa* from Favignana Island,
- *B. rupestris* subsp. *hispida* from Ficuzza Woods (Corleone),
- *B. tardarae* from Tardara Gorges (Sciacca).

The species were carefully identified by Professor Vincenzo Ilardi, and the specimens were deposited in the STEBICEF Department, University of Palermo, Italy, with voucher number 109761, 109762 and 109763.

Sample Preparation

After collection, the leaves of each *Brassica* species were spread out and left to air-dry at room temperature for a period of 15 days. The drying process was performed in a well-ventilated area to prevent mold growth and to ensure uniform dry without significant degradation of the plant material. Once dried, the plant material was finely chopped into small pieces to increase the surface area, facilitating the extraction process.

Extraction Procedure

The finely chopped plant materials were weighed as follows:

- 76.9 g for *B. macrocarpa*,
- 39.7 g for *B. rupestris* subsp. *hispida*,
- 37 g for *B. tardarae*.

Maceration was performed using petroleum ether as the solvent. A volume of 1 L of petroleum ether was added to each sample, and the extraction was carried out for 72 hours under constant agitation. This extraction process was repeated three times to ensure complete extraction of the lipophilic compounds from the plant material.

After the petroleum ether extraction, the solvent was completely removed using a rotary evaporator (Buchi model R-210, Cornaredo, Italy), operating under reduced pressure to prevent degradation of the extracted compounds. The evaporator was set to gentle conditions to ensure complete solvent removal without compromising the integrity of the extract.

Following the petroleum ether extraction, the plant residues were subjected to a second round of extraction using methanol (MeOH) as the solvent. The same procedure was followed, using 1 L of methanol per sample, and extracting for 72 hours under constant agitation. This process was repeated three times to maximize extraction efficiency.

After the methanol extraction, the samples were filtered through Whatman No. 4 filter paper to remove any remaining plant debris, leaving behind the methanol extract.

Stock Solutions

Extract fresh stock solutions in distilled water were prepared at a concentration of 50 mg/mL to be used in subsequent *in vitro* and *in vivo* experiments.

3.2 QUALITATIVE AND QUANTITATIVE ANALYSIS OF COMPOUNDS IN THE EXTRACTS OF THREE ENDEMIC *BRASSICAE*

The chemical characterization was obtained thanks to a collaboration with Professor Stefano Dall'acqua of the University of Padova (Italy).

Glucosinolates

The dried extract of each plant (10 mg) were solubilized in 2 mL DMSO (Sigma-Aldrich, St. Louis, MO, USA) using an ultrasound bath, diluted to 10 mL with methanol/water mixture (50%, 20 mL), and the resulting solution was filtered through 0.45 µm filter membrane. A Waters Acquity UPLC equipped with triple quadrupole mass spectrometer coupled to an electrospray ionization source operating in negative ion mode was employed for the quantitative analysis of glucosinolates. A column (Waters BEH 2.1 × 100, 1.7 µm) was utilized, with a gradient of 0.1% formic acid in water (A) and acetonitrile (B), from 5% B and up to 80% B in 4 min, then 95% B in 7 min, and staying isocratic for up to 10 min. Standard solutions of glucosinolates, namely gluconapin, glucoibeverin, glucotropaeonin, glucoerucin, gluconasturcin, glucoraphanin, glucobrassicin, glucoallyssin and sinigrin, (PhytoLab GmbH & Co. KG, Vestenbergsgreuth, Germany), were utilized at concentrations of 5 µg/mL and directly infused in source with the LC flow to optimize the parameters for quantification purposes. The qualitative and quantitative data are summarized in Table 3.

Compound	[M – H]– m/z	Fragment Ion	Dwell Time	Cone	Collision Energy
sinigrin	358.0957	96.8426	0.050	36	16
gluconapin	372.0432	96.755	0.050	46	25
glucoibeverin	406.0306	69.8426	0.050	40	20
glucotropaeonin	408.0853	96.9037	0.050	38	16
glucoerucin	420.0853	96.9033	0.050	44	16
gluconasturtiin/iberin	422.0853	96.7476	0.050	46	22
glucoraphanin	436.0411	96.755	0.050	48	22
glucobrassicin	447.0865	96.7555	0.050	46	22
glucoalyssin	450.0555	96.755	0.050	48	22

Table 3. Parameters for the quantification of the glucosinolates

Phenols

The analysis was carried out using the equipment Acquity UPLC, with a triple quadrupole mass spectrometer operating in electrospray mode. A Varian MS500 ion trap operating in negative mode was employed as a secondary MS detector. A column (Agilent SB C18 3.0 × 100, 1.8 μ) was utilized to separate the compounds with a gradient of 0.1% formic acid in water (A), acetonitrile (B) and methanol (C). The gradient started at 95% A and 5% B, which was held for 0.5 min, then it reached 92% (A) and 8% (B) in 5 min. When it reached 80% (A) and 20% (B) in 15 min, started to be isocratic for up to 18 min. Then, at 25 min, it reached 50% (A), 40% (B), and 10% (C). At 35 min, it reached 20% (A), 70% (B), and 10% (C), then at 36 min, it reached 0% (A), 85% (B), and 15% (C), and it stayed isocratic for up to 38 min. Then, at 40 min, it reached 100% (B). The identification of the compounds was carried out by combining the fragmentation of the eluted compounds with the literature data on general flavonoid identification[194] and with specific literature dealing with LC-MS analysis of *Brassica* species[166]. Reference standards were utilized for a further confirmation of some of the compounds. As reference compounds, quercetin-3-O-glucoside, kaempferol-3-O-glucoside, kaempferol-7-O-glucoside, isorhamnetin-3-O-glucoside (PhytoLab GmbH & Co. KG), rutin and sinapic acid (Sigm-Aldrich, Inc., St. Louis, MO, USA) were used. The qualitative and quantitative data are summarized in Table 4.

Compound	[M – H]– m/z	Fragment Ion	Dwell Time	Cone	Collision Energy
kaempferol-3-O- glucoside	447.092	285	0.050	20	20
quercetin-3-O- glucoside	463.087	301	0.050	22	20
kaempferol-7-O- glucoside	447.092	285	0.050	20	20
isorhamnetin-3-O- glucoside	477.1072	299	0.050	35	25
rutin	609.1520	301	0.050	18	16
sinapic acid	223.063	208	0.050	10	20

Table 4. Parameters for the quantification of phenols

3.3 ANTIOXIDANT ACTIVITY: DPPH SCAVENGING ASSAY

To assess the *in vitro* antioxidant activities of the extracts from three endemic *Brassica* species, a diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich, St. Louis, MO, USA) assay was conducted. As above mentioned, the Diphenyl-2-picrylhydrazyl (DPPH) assay is a widely used method to evaluate the antioxidant activity of natural and synthetic substances. This assay is based on the ability of antioxidants to donate electrons or hydrogen atoms to neutralize the DPPH radical, a stable free radical with a deep purple color. The reduction in color intensity is directly proportional to the antioxidant capacity of the sample being tested.

The extracts of the three *Brassica* species were tested at a range of concentrations from 7.81 µg/mL to 1000 µg/mL, to establish a dose-response curve.

- For each concentration of the *Brassica* extract, 1 mL of ethanolic solution containing 0.1 mM DPPH was prepared. A specified volume of each *Brassica* extract (at concentrations of 7.81 µg/mL, 15.63 µg/mL, 31.25 µg/mL, 62.5 µg/mL, 125 µg/mL, 250 µg/mL, 500 µg/mL, and 1000 µg/mL) was added to the DPPH solution. The final volume of each reaction mixture was adjusted to 1 mL by the addition of ethanol.

- Then, samples were placed in the dark for 20 minutes. This incubation period allows the antioxidant compounds in the *Brassica* extracts to react with the DPPH free radicals, leading to a decrease in the DPPH absorbance due to the reduction of the DPPH radical.

- After the 20-minute incubation, the absorbance of the samples was measured using a UV Jasco V760 spectrophotometer.

- The samples were placed in 1.0 mL cuvettes with an optical path length of 10 mm.

- The absorbance was recorded at a wavelength of 517 nm, which corresponds to the maximum absorption of the DPPH radical. A decrease in absorbance at 517 nm indicates the reduction of DPPH by the antioxidant compounds present in the *Brassica* extracts.

The percentage of the scavenging activity was calculated according to the equation:

$$DPPH\ radical\ scavenging\ activity\ \% = \frac{[(Abs\ of\ Blank - Abs\ of\ Control) - Abs\ of\ Sample]}{(Abs\ of\ Blank - Abs\ of\ Control)} \times 100$$

3.4 *IN VITRO* CELLULAR STUDIES: CELL VIABILITY ASSAY

Toxicity of extracts from three *Brassica* species on RAW 264.7 murine macrophage cell line, was performed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Tocris, Bio-Techne, Minneapolis, MN, USA) assay. This assay measures cell viability based on the reduction of tetrazolium salts into formazan, which is proportional to the number of viable cells.

- RAW 264.7 cells (9×10^3 cells/well) were cultured in 96-well plates.
- Cells were maintained in high-glucose DMEM (Sigma-Aldrich, St. Louis, MO, USA), supplemented with: 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and 10% heat-inactivated FBS (Life Technologies, Carlsbad, CA, USA).
- Cells were incubated at 37°C with 5% CO_2 in a humidified incubator.
- Cells were cultured up to passage 11 were used in the experiments.
- After 24 hours of incubation, RAW 264.7 cells were treated with increasing concentrations of *Brassica* extracts, ranging from 7.81 to 1000 µg/mL for another 24 hours. Untreated cells were used as the control group.
- After the 24-hour treatment, 0.5 mg/mL MTT solution was added to each well and incubated for 2 hours in the dark at 37 ℃.
- Following incubation, 100 µL/well of DMSO was added to dissolve the formazan crystals formed by the reduction of MTT by metabolically active cells.
- Absorbance was measured at 570 nm using a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT, USA).
- The results were interpreted as a measure of cell viability, with a decrease in absorbance corresponding to a reduction in viable cells.

3.5 ASSAY FOR CELLULAR ANTIOXIDANT ACTIVITY: NITRIC OXIDE PRODUCTION

To determine the effective concentration and time exposure at which LPS (Escherichia coli, O55:B5, Sigma-Aldrich Inc., St. Louis, MO, USA) induces oxidative stress without affecting cell viability, RAW 264.7 cells (5×10^5 cells/well) were cultured in 6-well plates and treated with LPS at various concentrations (0.1, 0.5, and 1 µg/mL) for different time periods (0–24 hours). After treatment, cell viability was assessed, and the NO production was quantified using the Griess reagent (Sigma-Aldrich, Inc., St. Louis, MO, USA), a commonly used method to measure nitric oxide levels. The

optimal concentration and the time exposure of LPS capable to induce oxidative stress resulted to be 0.1 µg/mL for 24 hours.

Once the optimal LPS treatment conditions were identified, RAW 264.7 cells were pre-treated with safe concentrations of *Brassica* extracts (determined from preliminary toxicity studies) for 2 hours, followed by LPS (0.1 µg/mL) stimulation for 24 hours to evaluate the extract's effects on LPS-induced oxidative stress.

- At the end of treatments time, the cells were detached, rapidly homogenized and centrifuged at 10,000 g for 5 minutes.
- Next, the supernatant was incubated with Griess reagent, according to the manufacturer's instructions: 50 µL of supernatant were added to 50 µL Nitrite Assay Buffer and loaded into 96-well plate. Separately to each well were added 10 µL of Griess Reagent I, 10 µL of Griess Reagent II and 80 µL of Nitrite Assay Buffer.
- After mixed the well, the plate was incubated at room temperature for 10 minutes.
- The absorbance was read at 540 nm using a microplate reader (Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT, USA).

This absorbance measurement corresponds to the concentration of nitrite, which serves as an indirect indicator of NO production.

3.6 ASSAY FOR CELLULAR ANTIOXIDANT ACTIVITY: ROS PRODUCTION

ROS production was evaluated using the ROS Detection Assay Kit (Canvax Biotech, Cordoba, Spain) which contains the probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). The probe H_2DCFDA, upon cellular uptake, is deacetylated by intracellular esterases and subsequently oxidized by ROS to form a fluorescent product. Finally, the cell samples were analyzed using flow cytometry to quantify ROS production.

- RAW 264.7 cells (5×10^5 cells/well) were cultured in 6-well for 24 hours.

The treatment protocol for the *B. macrocarpa* extracts involved a 2-hour pre-treatment with increasing concentrations of the extracts (125-1000 µg/mL), followed by a 24-hour exposure to LPS (0.1 µg/mL). This protocol was designed to evaluate the potential modulation of ROS production by the extracts in the context of the oxidative stress response induced by LPS.

- After treatment the cells were detached and centrifuged at 1000 rpm for 5 minutes.
- The supernatant was removed and the cells were re-suspended with Working solution containing the probe H_2DCFDA and incubated at 37 °C for 30 minutes in the dark.

- After incubation time, the supernatant was removed following centrifugation (1000 rpm for 5 minutes), and the cells were washed two time with 500 µL of 1X Buffer.
- Finally, the supernatant was removed with a pipette and 1 mL of 1X buffer was added and transferred into tubes suitable for flow cytometry.

For each experimental condition, three independent flow-cytometric assays were performed, with each assay conducted in triplicate to ensure reliability. Cells were analyzed using a FACSCanto flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), in the FL1 channel (Ex/Em = 485/530 nm), and 10,000 events were recorded per sample. The data were then processed and analyzed using Floreada software, available at (https://floreada.io), which allows for the detailed analysis of flow cytometric data.

3.7 REAL-TIME PCR ANALYSIS FOR NOS2 mRNA EXPRESSION

RAW 264.7 cells (1.25×10^5 cells/well) were seeded in 24-well plates and incubated for 24 h at 37 °C with 5% CO_2. Cells were then washed with 1X PBS w/o Ca^{2+} and Mg^{2+} and treated with increasing concentrations of *B. macrocarpa* extract (125–1000 µg/mL) for 2 h. LPS (0.1 µg/mL) was subsequently added, and cells were incubated for an additional 24 h.

Total RNA was extracted using the PureLink® RNA Mini Kit (Ambion, Life Technologies, Milan, Italy) according to the manufacturer's instructions. RNA was quantified with a Nanodrop One (Thermo Fisher Scientific, Milan, Italy), and 2.5 µg of RNA was retro-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, Milan, Italy). The resulting cDNA was diluted to 100 µL with DNAse/RNAse-free water.

Real-time PCR was performed using the Applied Biosystems StepOnePlus™ Real-Time PCR System with PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Thermo Fisher Scientific, Milan, Italy). Reactions were carried out in a final volume of 20 µL containing 1–100 ng of cDNA and 200 nM of specific mouse NOS2 primers. GAPDH was used as the housekeeping gene, and expression levels of NOS2 were calculated using the $2^{-}\Delta\Delta CT$ method. PCR cycling conditions included an initial UDG activation step at 50 °C for 2 min, followed by DNA polymerase activation at 95 °C for 2 min, and 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. A melt

curve stage followed the amplification phase. Primer sequences for NOS2 and GAPDH are detailed in Table 5.

Gene	Primer Forward	Primer Reverse
GAPDH	5′-GGCCTTCCGTGTTCCTAC-3′	5′-TGTCATCATATCTGGCAGGTT-3′
NOS2	5′-CAGGAGGAGAGAGATCCGATTTA-3′	5′-GCATTAGCATGGAAGCAAAGA-3′

Table 5. Primers sequences

3.8 ZEBRAFISH MAINTENANCE AND EMBRYO COLLECTION

The *in vivo* experiments on the zebrafish embryo model were conducted at the University of Trás-os-Montes e Alto Douro (Vila Real, Portugal) during the period abroad foreseen for this doctorate.

The AB wild-type zebrafish strain was maintained at the University of Trás-os-Montes and Alto Douro (Vila Real, Portugal) in a constant temperature-light cycle (28 ◦C and 14:10 h light-dark cycle). The breeding activity was promoted by the union of zebrafish overnight with 2:1 ratio of males and female with the spawning induced by the morning light and the eggs collected after ~1 h. Then, the fertilized eggs were washed one time in Chloramine-T solution at 0.5 % w/v and washed twice with E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ and 0.33 mM $MgCl_2$, pH 7.2). The eggs with normal morphology were selected randomly and distributed in 6-well plates and maintained in E3 medium at 28.5 ◦C. All experiments were terminated at 96 hours post-fertilization (hpf), therefore, no animal test authorization was requested in accordance with European legislation (EU Directive, 2010/63/EU).

3.9 FISH EMBRYO ACUTE TOXICITY TEST (FET)

To assess the toxicity of *B. macrocarpa* extract, embryos at 2 hpf were exposed to the extract for 96 hours in 6-well culture plates, with 20 embryos per well in 5 mL of solution. The embryos were exposed to different concentrations of the extract (125, 250, 500, 1000, and 2000 µg/mL). The test solutions were replaced daily to ensure that the proper concentration of the extract was maintained throughout the exposure period. Mortality rates were recorded at 7, 24, 48, 72, and 96 hours of exposure. Following OECD guidelines[195], observations of embryo coagulation, failure of somite formation, failure of tail detachment, and lack of heartbeat were used to determine lethality. Any positive result in any of these observations means that the zebrafish embryo is dead and appears milky white to the eye while dark under the microscope. The experiment was repeated in quadruplicate and

the LC50 value was determined by probit analysis at 96 hpf. Based on the LC50 value, subsequent experiments were conducted using concentrations that were found to be safe. To evaluate the development of zebrafish embryos, 10 random embryos from each group were analyzed under an inverted microscope (Nikon SMZ-800 stereomicroscope, Japan), and the following developmental parameters were evaluated:

- spontaneous movement (at 24 hpf),
- somite formation (at 48 hpf),
- eye and otolith development (at 48 hpf),
- pigmentation (at 48 hpf),
- undetected head and tail (at 48 hpf),
- heart rate (at 48 hpf),
- hatching (at 72 hpf).
- At 96 hpf, the embryos were immobilized in a 3% methylcellulose solution, and several measurements were taken, including body length, yolk sac area, heart area, eye area, head area, and head-body angle. Digital images were captured through the inverted microscope and analyzed using Digimizer software (version 5.3.4, MedCalc Software Ltd, Acacialaan 22, 8400 Ostend, Belgium).

3.10 BEHAVIORAL ANALYSES

At the end of the 96-hour exposure period, 6 larvae from each replicate were selected for behavioral analysis using a video tracking system (TheRealFishTracker), which allowed for precise measurement of their movements in the X, Y coordinates. The behavioral assessment consisted of three main tests:

- the open field exploratory test to measure locomotor behaviour,
- an anxiety-like behaviour test using a black-and-white arena,
- an avoidance response to a bouncing ball stimulus.

The larvae were placed in 12-well agarose-coated plates, with one randomly selected embryo per well. These plates were positioned on top of a 15.6" laptop LCD screen (1366 × 768-pixel resolution), which displayed a white Microsoft PowerPoint presentation (Microsoft Corp., Washington, DC, USA). After a 5-minute acclimation period to allow the embryos to adjust to the setup, their exploratory behavior was recorded using a HD digital video camera. During the 10-minute observation period, the following behavioral parameters were analyzed:

- average speed,
- total distance travelled,

- percentage of time spent in each area,
- average distance from the center of the well,
- average absolute turn angle,
- percentage of active time.

These metrics provided insights into the general locomotor activity and exploration patterns of the embryos.

Following the exploratory behaviour analysis, the anxiety-like behaviour of the embryos was assessed using the same 12-well plate setup, with the well divided into a white and black area using the Microsoft PowerPoint display. The embryos' tendency to spend more time in either area was used as an indicator of anxiety-like behaviour, with a greater preference for the black area typically signifying increased anxiety.

Additionally, the avoidance response was evaluated by introducing a visual stimulus in the form of a bouncing red ball moving from left to right across the top half of the well. The embryos' ability to respond to this stimulus was recorded for 10 minutes, with the same experimental setup and PowerPoint presentation as in the previous tests. This test provided insight into the embryos' responsiveness to external stimuli and their ability to exhibit avoidance behavior.

3.11 OXIDATIVE STRESS MODEL AND *B. MACROCARPA* TREATMENT

20 µM of $CuSO_4$ is the dose required to induce oxidative stress in zebrafish embryos[196]. To determine the exposure time to $CuSO_4$ ($CuSO_4.5H_2O$, CAS 7758-99-8 from Merck, S.A, Lisbon, Portugal), ROS and NO levels were evaluated after 1h and 5h of exposure. A 5h exposure was chosen for the following experiment. Hatched 72 hpf healthy zebrafish embryos were selected and randomly divided into 6-well plates containing 5 mL of E3 medium (n=30/well in triplicate) in eight groups as shown in figure 4: control (E3 medium), $CuSO_4$ treated (20 µM x 5h), 125 – 250 – 500 µg/mL *B. macrocarpa* extract treated for 26 h and embryos pre-exposed (21 h) to *B. macrocarpa* extract concentrations (125 – 250 – 500 µg/mL) followed by exposure to 20 µM $CuSO_4$ for additional 5 h. At the end of the experiments biochemical analyses were carried out.

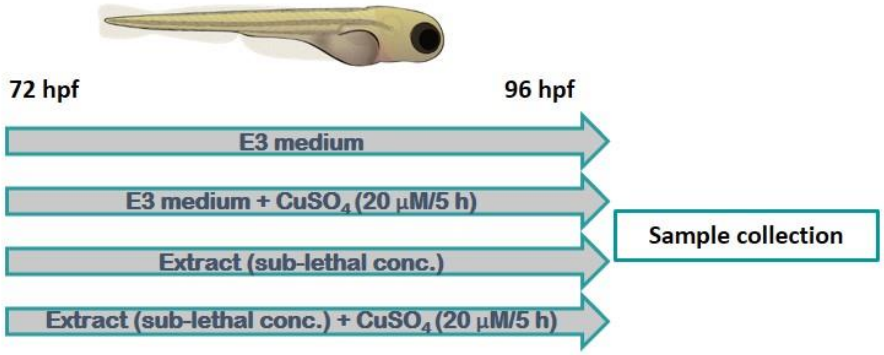

Figure 4. Scheme of the division of treatment groups

3.12 BIOCHEMICAL ANALYSES

After exposure time, treated and untreated embryos (30 embryos/group for 3 replicate) were collected in 400 µL of cold buffer (0.32 mM of sucrose, 20 mM of HEPES, 1 mM of MgCl2, and 0.5 mM of phenylmethyl sulfonylfluoride (PMSF), pH 7.4) and homogenized in a Tissuelyser II (30 Hz for 30 s - Qiagen, Hilden, Germany). The supernatant was collected at 10000g at 4 ºC for 10 min (Sigma 3K30) and total protein concentration was measured at 280 nm using a Take3 Multi-Volume plate (Take3 plate, BioTek Instruments, Winooski, VT, USA). The biomarker assessment was conducted in duplicate technical replicates using a PowerWave XS2 microplate scanning spectrophotometer or a Cary Eclipse fluorescence spectrophotometer (Varian, Palo Alto, USA) at 30°C.

Total ROS levels were determined using the dichlorofluorescein-diacetate (DCFH-DA) fluorescent probe at 485 nm excitation and 530 nm emission wavelengths, based on a DCF standard curve (0–6.25 nM). Briefly, to 20 µL of sample homogenate, 100 µL of PBS (pH 7.4) and 8.3 µL DCFH-DA (10 mg/mL in DMSO) were added. After incubation during 30 min at 37 ºC in the dark, the fluorescence was measured. The level of NO was determined using the Griess method, with some modifications. Briefly, the samples were mixed with the Griess reagent in a 1:1 ratio and incubated for 15 min at room temperature, the absorbance was read at 540 nm. Sodium nitrate was used to construct a standard curve (0–1 µM). The SOD activity was assessed using the nitrobluetetrazolium (NBT) method at 560 nm. In each well of the 96-well microplate, 10 µL of sample were mixed with 130 µL of phosphate buffer (50 mM, pH 7.4) supplemented with 1 mM EDTA, 0.5 mM hypoxanthine and 0.5 mM NBT. The reaction was started by the addition of 0.5 U/mL of xanthine-oxidase. The increase in absorbance due to dismutation of O^{2-} into H_2O_2 was recorded for 5 minutes and SOD from

bovine erythrocytes was used for construction of a standard curve (0–30 U/mL). While CAT activity was measured based on hydrogen peroxide degradation. The assay reaction consisted of 10 µL of sample with 170 µL of phosphate buffer containing 0.1 mM EDTA. The reaction was started by the addition of H_2O_2 3 mM and the decrease in absorbance was monitored at 240 nm for 5 minutes. The activity was calculated using bovine catalase as a standard (0–5 U/mL). Lipid peroxidation was assessed through the determination of thiobarbituric acid reactive substances (TBARS) at 535 nm excitation and 630 nm emission wavelengths. 10 µL of sample were added to 150 µL of TBA reagent. TBA reagent was made from thiobarbituric acid (TBA 0.5% w/v) prepared in 20% (w/v) TCA and 0.33 N HCl. To prevent artificial lipid peroxidation, 10 µL of BHT (butylated hydroxytoluene) 1mM were added. The mixture was incubated for 40 minutes at 60 °C and then 15 minutes at 4 °C; after which the fluorescence was read. The oxidative phospholipid, malondialdehyde (MDA), was estimated based on a standard curve (0–100 µM) of malondialdehyde. Protein carbonylation was determined at 450 nm based on the reaction between protein carbonyls and 2,4-dinitrophenylhydrazine (DNPH). Briefly, 20 µL of DNPH were added to 20 µL sample and incubated in the dark for 10 minutes, then 10 µL of NaOH 6M were added and incubated for another 10 minutes. DNA damage was determined by the binding of the fluorescent Hoechst 33258 to DNA at 360 nm excitation and 450 nm emission wavelengths adapting the protocol of P. Olive (1988)[197].
All data were normalized for protein content and expressed as a percentage of control.

3.13 STATISTICAL ANALYSIS

All data are presented as the mean ± standard deviation (SD). The Kolmogorov-Smirnov test was used to evaluate the normality of distribution. Statistical analysis of the data was performed using GraphPad Prism software version 6 (GraphPad Software, San Diego, CA, USA) by one- or two-way analysis of variance (ANOVA), followed by Dunnett's or Tukey's post hoc test. A $p < 0.05$ was considered to indicate a statistically significant difference.

4. RESULTS AND DISCUSSION

4.1 CHEMICAL CHARACTERIZATION

The HPLC-MS/MS analysis allowed us to identify seven glucosinolates and different phenolic compounds in the three *Brassica* species.

Glucosinolate identity was confirmed by injection of reference standards, and seven derivatives were identified as shown in Table 6.

In the chemical analysis of the glucosinolates present in *B. macrocarpa*, sinigrin was found to be the predominant compound, constituting a substantial 92.8 mg/g. Gluconapin followed as the second most abundant glucosinolate at 17.26 mg/g, with the remaining five glucosinolates present in significantly smaller quantities.

Similarly, in *B. hispida*, sinigrin (93.27 mg/g) was the sole significant glucosinolate, while gluconapin was present in trace amounts (0.18 mg/g). The other glucosinolates were detected only in minimal quantities. In *B. tardarae*, sinigrin was also the main compound, though it was present in lower quantities compared to the other two species (62.99 mg/g). Notably, this species contained a significant amount of glucobeverin (30.66 mg/g), a metabolite that was found in only trace amounts in the other two species.

Compound	M-H m/z	*B. macrocarpa* mg/g	*B. hispida* mg/g	*B. tardarae* mg/g
Sinigrin	358.0957	92.80	93.27	62.99
Gluconapin	372.0432	17.26	0.18	0.10
Glucoibeverin	406.0306	0.06	0.03	30.66
Glucotropaeonin	408.0853	0.17	0.10	1.66
Glucoerucin	420.0853	0.06	0.00	0.01
Gluconasturtiin/iberin	422.0853	0.05	0.00	0.08
Glucoraphanin	436.0411	0.03	0.00	0.16
Total		**110.43**	**93.58**	**95.66**

Table 6. Amount of glucosinolates (mg/g dried weight) identified in endemic Sicilian Brassica extracts by HPLC-MS/MS analysis.

The presence of high amounts of sinigrin in our wild *Brassica* species was not surprising, considering that other wild *Brassica* species, *B. villosa* subsp. *drepanensis* and *B. atlantica*, as well as the cultivated red cabbage, contain high amounts of total glucosinolates and in particular sinigrin[198,199]. Moreover, a comparative research on the glucosinolate profile revealed that young leaves of *B. macrocarpa* possess a peculiar content of sinigrin compared to the cauliflower cultivars[200]. Sinigrin

has been found to exert diverse biological activities such as anticancer, anti-inflammatory, antibacterial, antifungal and antioxidant agents[201–208].

The differences in glucosinolate content observed in our three species may be dependent on the conditions of the growing area. Branca et al. (2013) reported the phytochemical analyses of freeze-dried leaf powder of various Sicilian wild *Brassicae* after transplantation in the experimental farm of the University of Catania and found a qualitative profile similar to our samples but quantitatively different, supporting that the growing area could influence the phytocomponents[165]. Not only the growing area but also the climatic conditions influence the total glucosinolate content as demonstrated by ciska et al. (2000) by analyzing the glucosinolates of *Brassica oleracea* species growing in the same place but in different years with dissimilar climatic conditions (rainfall levels, temperatures)[209].

Flavonoids are present in high concentrations in the epidermis of leaves and fruits and have important and varied roles as secondary metabolites, being involved in processes like UV protection, pigmentation, stimulation of nitrogen-fixing nodules and disease resistance[210,211]

In our samples, quercetin and kaempferol derivatives were identified as glycosides presenting different sugar moieties in all three *Brassica* species and in similar amount (Table 7), in agreement with the polyphenolic fingerprints of other Sicilian wild species[212,213]. while *B. hispida* stands out for its high content of isorhamnetin derivatives. Among the three species, *B. macrocarpa* contains the highest amount of quercetin derivatives, with a concentration of 116.52 mg/g.

Quercetin, kaempferol and isorhamnetin have been found most commonly as O-glycosides[166,212–215]. Conjugation occurs most frequently at position 3 of the C-ring, but substitutions can also occur at positions 5, 7, 4´, 3´ and 5´[216]. The variety of sugar conjugates is high, but in *Brassica* vegetables they appeared mainly conjugated to glucose[125].

The beneficial effects of flavonoids are already widely demonstrated in literature[217]. The bioavailability and biological activity of flavonoids can vary depending on whether they are in free form (aglycones) or conjugated with sugars. It seems that O-glycosylation reduces the bioactivity of these molecules, as observed in several *in vitro* and in cellular model studies[218,219].

In vivo data on the influence of glycosylation on human health benefits are still limited. Xie et al (2022) demonstrated that *in vivo* aglycones possessed higher activity but unstable structures than the corresponding glycosidic flavonoids. Glycosylation would improve their stability enabling to maintain antioxidant activities after digestion, in rats[220].

Compound	M-H m/z	*B. macrocarpa* mg/g	*B. hispida* mg/g	*B. tardarae* mg/g
Quercetin-3-O-hexoside-7-O-hexoside	625.13	15.30	23.76	13.51
Quercetin-3-O-hexoside-7-O-hexoside	625.13	15.65	12.58	12.94
Quercetin-3-O-hexoside-7-O-hexoside	625.13	18.46	23.19	21.56
Quercetin-3-O-dihexoside-7-O-hexoside	787.13	30.65	12.85	29.56
Quercetin-3-O-dihexoside-7-O-hexoside	787.13	5.52	8.97	5.41
Quercetin-3-O-trihexoside-7-O-hexoside	949.23	19.05	4.93	1.77
Quercetin-3-O-trihexoside-7-O-hexoside	949.23	7.30	3.80	7.46
Kaempferol-3-O-glucoside	609.143	0.36	1.02	4.05
Kaempferol-3-O-dihexoside-7-O-hexoside	771.2	29.22	16.53	27.55
Kaempferol-3-O-dihexoside-7-O-hexoside	771.2	8.39	7.41	8.51
Kaempferol-3-O-trihexoside-7-O-hexoside	933.25	18.12	12.94	17.68
Kaempferol-3-O-trihexoside-7-O-hexoside	933.25	8.11	12.86	4.96
Isorhamnetin-dihexoside	639.15	16.57	22.27	16.09
Rutin	609.14	12.83	13.12	12.35
Quercetin-3-O-glucoside	463.09	4.59	7.24	3.71
Kaempferol-7-O-Glucoside	447.09	3.83	12.25	3.82
Isorhamnetin-3-O-glucoside	477.11	3.34	5.86	3.25
Total		**217.29**	**201.58**	**194.18**

Table 7. Amount of flavonoids (mg/g dried weight) identified in endemic Sicilian Brassica extracts by HPLC-MS/MS analysis.

Regarding sinapyl derivatives, as shown in Table 8, *B. hispida* contains a notably higher amount (325.67 mg/g) compared to the other two species, *B. macrocarpa* (79.13 mg/g) and *B. tardare* (135.22 mg/g). Notably, in *B. hispida*, the isomers of disinapoyl-gentiobioside and disinapoyl-feruloyldiflucoside are present in significantly higher concentrations than in the other two species.

The beneficial effect of Sinapic acid and its derivatives, in conditions of oxidative stress, is already known in the literature, indicating a potential use in food processing and pharmaceutical industry[221–224].

Compound	M-H m/z	*B. macrocarpa* mg/g	*B. hispida* mg/g	*B. tardarae* mg/g
Sinapic acid	223.06	2.34	2.48	1.92
Sinapic acid hexoside	385.11	2.97	33.20	7.44
Sinapic acid hexoside	386.11	40.82	37.58	46.77
Sinapic acid hexoside	387.11	6.82	5.71	6.53
Disinapoyl-gentiobioside	753.2	2.02	101.99	20.42
Disinapoyl-gentiobioside	754.2	1.75	27.86	17.82
Sinapoyl-feruloyldiglucoside	723.21	7.02	9.74	16.28
Trisinapoyl-diglucoside	959.28	13.28	26.50	16.28
Disinapoyl-feruloyldiflucoside	929.27	2.11	80.61	1.76
Total		**79.13**	**325.67**	**135.22**

Table 8. Amount of sinapyl derivatives (mg/g dried weight) identified in endemic Sicilian Brassica extracts by HPLC-MS/MS analysis.

4.2 *IN VITRO* ANTIOXIDANT ASSAY: DPPH

Taking into consideration the composition of *Brassicae* extracts, which highlights the presence of potential bioactive compounds with well-recognized antioxidant activity, and previous studies which have demonstrated that *Brassica* plants exhibit antioxidant potential in several *in vitro* assays[143,225–228], we initially evaluated the potential *in vitro* antioxidant effect of our extracts by performing DPPH colorimetric assay. The addition of extracts at increasing concentrations (7.81 – 1000 µg/mL) to the DPPH solution caused a decolorization from purple to yellow indicating a reduction of the DPPH free radical by antioxidant compounds, which can be detected by spectrophotometric determination. The percentage of inhibition, as shown in the figure 5, becomes significant starting from a concentration of 125 µg/mL for all three *Brassica* specimens analyzed. Furthermore, *B. macrocarpa* showed a more pronounced scavenging capacity, reaching 83% inhibition at the highest concentration tested in comparison to *B. hispida* (69%) and *B. tardarae* (68%).

The observed radical scavenging activity can be attributed to the presence of some compounds, such as glucosinolates and flavonoids present in large quantities in our extracts. In fact, the correlation between the scavenger activity and the content of these compounds has been evaluated in numerous studies[229–231].

The data obtained are extremely encouraging, highlighting a greater scavenging activity of our wild *Brassicae* compared to *Brassicae* cultivars as reported in the literature[232–234]. Furthermore, recent studies on leaf extracts of Sicilian endemic *Brassicae* species have detected scavenger activities similar to ours, as in the case of *B. drepanensis*[235], or lower, as observed for *B. fruticulosa*, which at

the concentration of 1000 µg/mL reaches an activity of 40%[214]. Similar values to ours were also recorded for *B. incana*[215] and *B. raimondoi*[212].

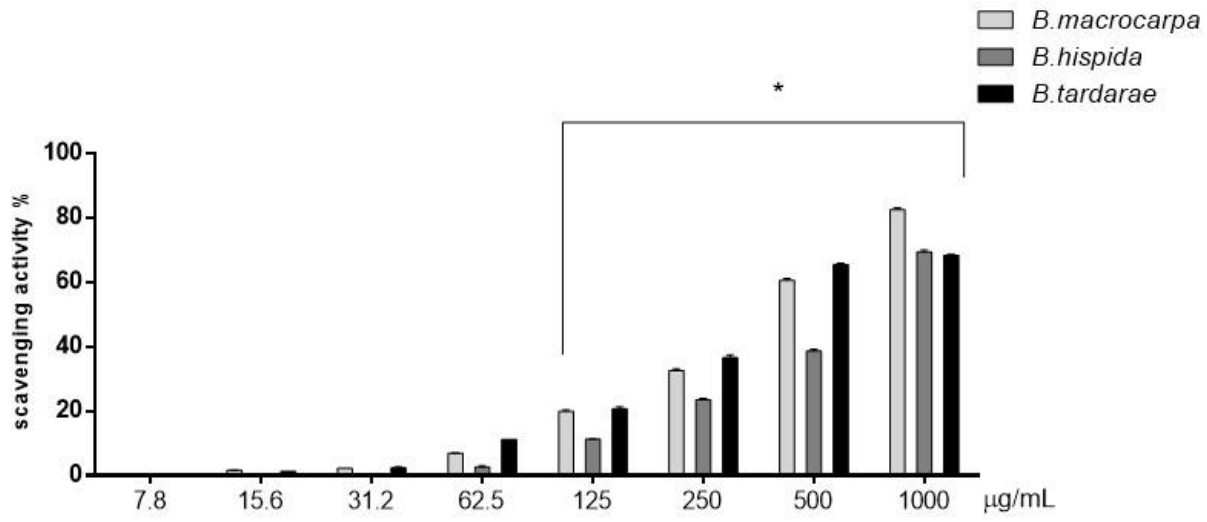

Figure 5. B. macrocarpa, B. hispida and B. tardarae extract antioxidant activity determined by DPPH scavenging capacity. Data are expressed as mean ± SD (n= 3) and reported as a percentage of DPPH alone. * means significantly different according to one-way ANOVA (p < 0.05.)

4.3 ANTIOXIDANT EFFECTS OF *BRASSICA* EXTRACTS IN MURINE RAW 264.7 MACROPHAGE CELLULAR MODEL

4.3.1 CELL VIABILITY

At first, to evaluate any potential cytotoxicity of our extracts cell viability was assessed in RAW 264.7 cells by MTT assay. Cells were treated for 24 hours with increasing concentrations (0 – 1000 µg/mL) of *Brassica* extracts. The results, expressed as a percentage of the control (untreated cells) (Fig. 6), revealed that *B. macrocarpa* extract did not affect cell viability at any of the concentrations tested. In contrast, *B. hispida* and *B. tardarae* extracts exhibited cytotoxicity starting from concentrations 62.5 µg/mL and 500 µg/mL, respectively. Although the ANOVA test did not show a statistically significant difference for the 250 µg/mL concentration of *B. tardarae* (with cell viability remaining at 82%), this concentration was excluded from subsequent studies. This decision was based on the observation of morphological changes in the macrophages, including the presence of few protrusions, which suggested the onset of oxidative stress (Fig. 7).

The observed cytotoxicity could be attributed to the presence of high amounts of sinapyl derivatives. There are few studies in the literature evaluating the safety of sinapic acid on cellular models and even fewer for its derivatives[221,236], and these studies revealed toxicity only at very high concentrations. In our samples, *B. hispida* contains a total amount of synapyl derivatives four times higher, and *B. tardarae* twice as high, compared to *B. macrocarpa*. This may suggest a correlation between the reduction in cell viability and the increased content of sinapyl derivatives.

However, due to the extract's complex composition, it is difficult to determine whether the detected compound or others are responsible for the observed effects. For these reasons, it would be prudent to perform fractionations of this methanol extract in future studies to identify the components responsible for the observed effects.

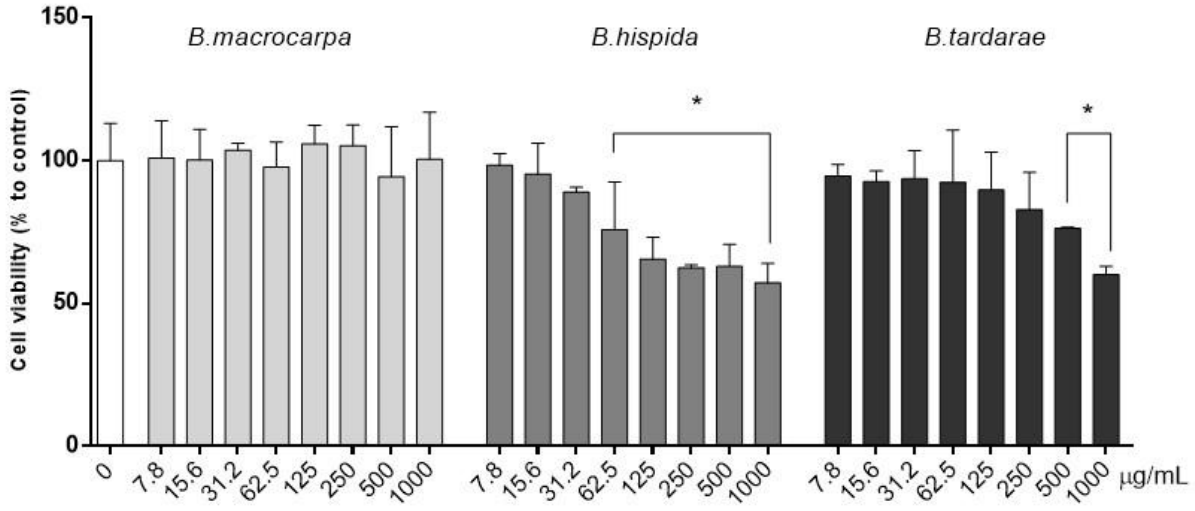

Figure 6. Cell viability, assessed by MTT assay, of RAW 264.7 cells treated for 24 h with B. macrocarpa, B. hispida and B. tardarae extracts. Values are the mean ± SD (n=3) and reported as a percentage of untreated cell. * means significantly different according to one-way ANOVA ($p < 0.05$).

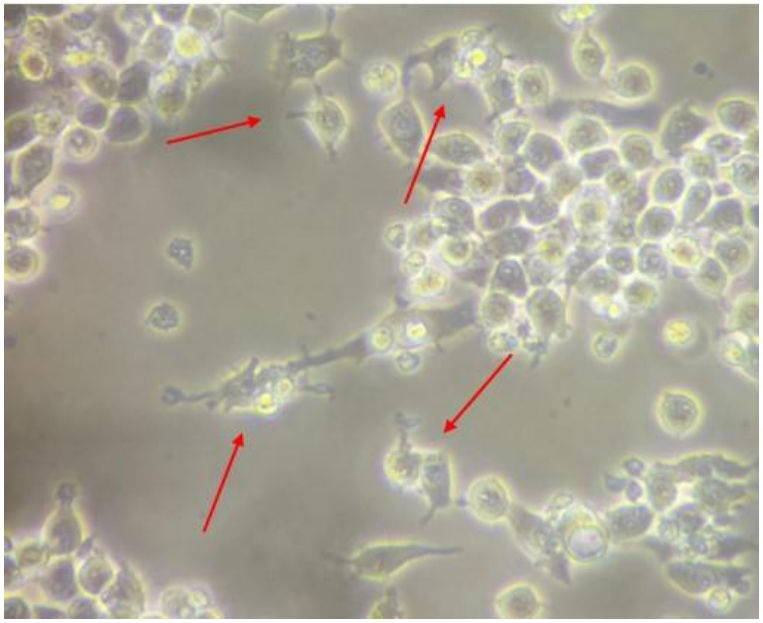

Figure 7. Raw 264.7 cells treated with B. tardarae extract at concentration of 250 µg/mL for 24 hours. cells indicated by red arrows show typical stress morphology.

4.3.2 NO PRODUCTION

Since *Brassica* extracts have shown potent *in vitro* radical scavenging activity, we aimed to investigate whether they could have an effect on modulating NO production as nitrogen radicals.

To evaluate potential antioxidant property of *Brassica* extracts lipopolysaccharide (LPS)- stimulated RAW cells was used as model. LPS-stimulated RAW 264.7 cells are widely used to study oxidative stress and inflammatory responses. When RAW 264.7 cells are treated with LPS, they produce reactive oxygen species (ROS) and nitric oxide (NO)[180]. The oxidative stress induced by LPS can lead to cellular damage, including lipid peroxidation, protein oxidation, and DNA damage. This cellular model is widely used to test the efficacy of potential therapeutic agents, such as antioxidants and anti-inflammatory compounds, in reducing oxidative stress and inflammation[237].

Preliminary experiments were conducted to determine the optimal concentration and exposure time of LPS that would not affect cell viability but would still induce oxidative stress. The results, obtained through MTT and Griess assays, indicated that a concentration of 0.1 µg/mL of LPS (Fig. 8A) applied for 24 hours (Fig. 8B) was effective in increasing NO levels, by about 430%, without compromising cell viability. Additionally, the experiments revealed that macrophages exposed to 0.1 µg/mL LPS for 24 hours exhibited the typical morphological changes associated with activation. Specifically, the cells transitioned from their normal small, round shape to larger cells with protrusions and vacuoles,

further confirming the induction of oxidative stress and the cellular response to LPS exposure (Fig 9.) as already observed by Choy (2007) or Nguyen (2020)[238,239].

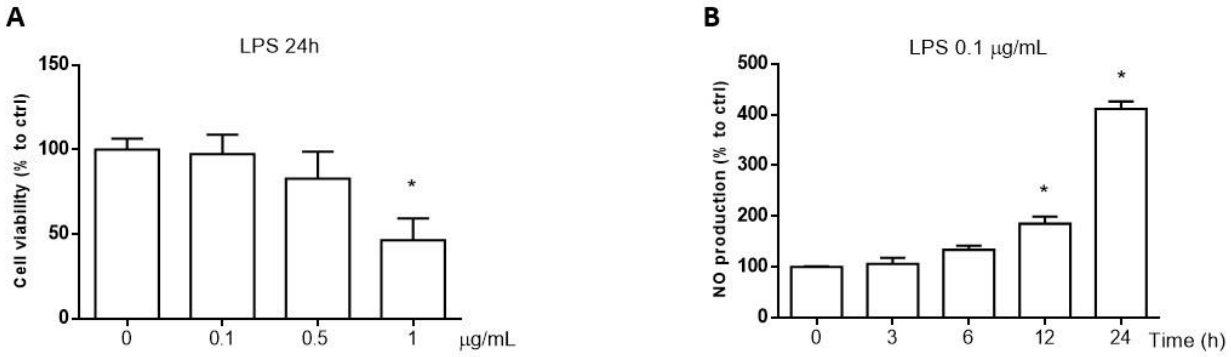

Figure 8. Concentration/Time effects of exposing RAW 264.7 cells to LPS. (A) Effects of LPS (0.1-0.5-1 µg/mL) exposure on cell viability for 24h. (B) Time-response curve of NO production by cells exposed to LPS (0.1 µg/mL). Values are the mean ± SD of three independent experiments expressed in percentage of the untreated cells. * means significantly different according to one-way ANOVA ($p < 0.05$).

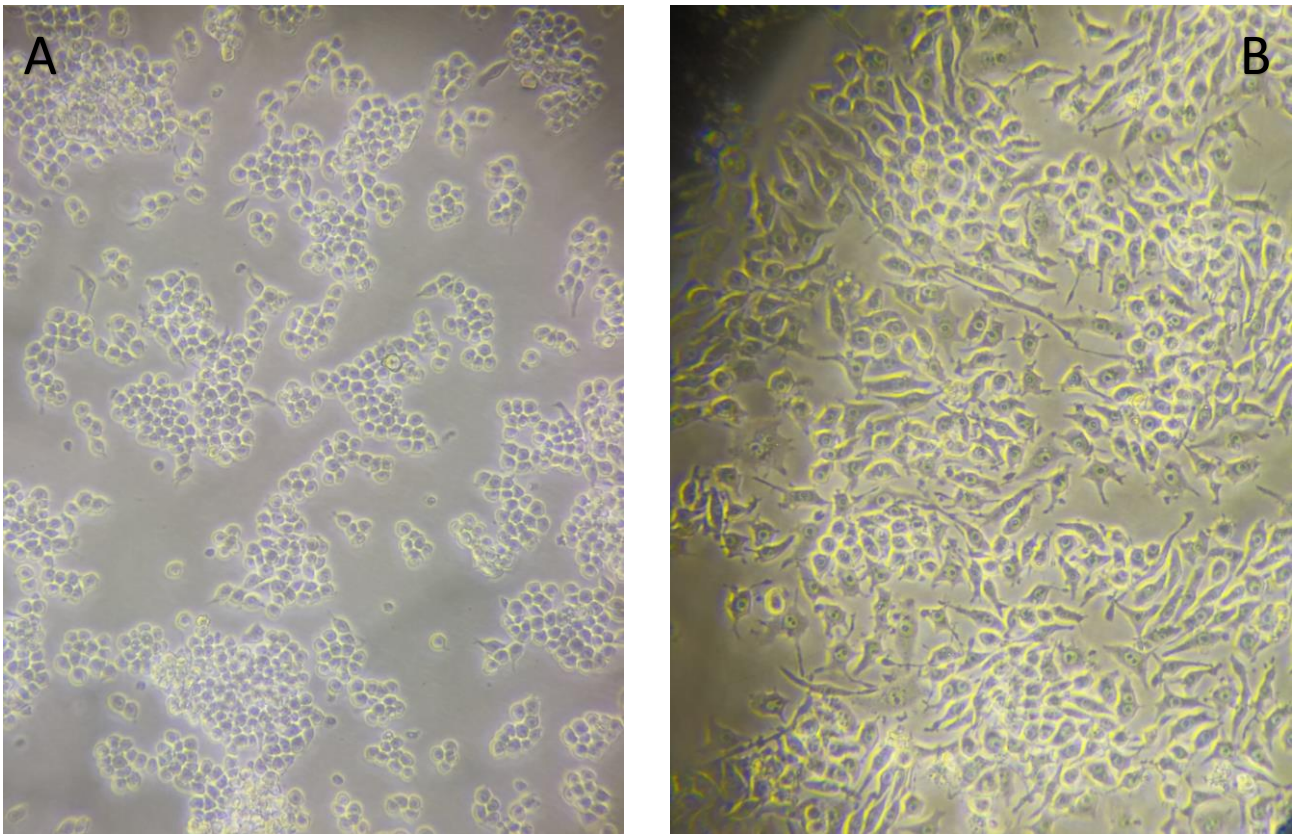

Figure 9. Microscope photo (40x) of RAW 264.7 cells: (A) Control condition (DMEM medium only): the cells appear small and round. (B) Stress condition (LPS 0.1 µg/mL for 24 h): the cells exhibit numerous protrusions and contain intracellular vacuoles.

To investigate the potential antioxidant effects of *Brassica* treatments, nitrite content in the culture media was measured using the Griess reagent. In this experiment, RAW 264.7 cells were first pre-treated with the extract for 2 hours, followed by a 24-hour exposure to LPS to induce oxidative stress. The concentrations tested were selected based on previous viability assessments (7.8-1000 for *B. macrocarpa*, 7.8-31.2 for *B. hispida* and 7.8-125 for *B. tardarae*). The nitrite levels were then quantified to assess the potential antioxidant activity of the *Brassica* extracts in mitigating the oxidative effects induced by LPS exposure. As shown in the figure 10, only *B. macrocarpa* (Fig. 10A) reduced LPS-induced NO levels in a concentration-dependent manner starting from 125 µg/mL. For the last concentration tested, a reduction in NO levels by about 58% was observe compared to the group treated with LPS alone. In contrast, no significant change was observed in the nitrite levels for *B. hispida* (Fig. 10B) and *B. tardarae* (Fig. 10C).

The inhibition of NO production could be attributed to a synergistic effect of the phytocomponents. Conforti et al. (2011), analyzing 21 extracts from wild Mediterranean plant species, observed a correlation between radical scavenging activity and phenol content, whilst no relationship with amount of polyphenolics and the inhibition of NO production in RAW 264.7 cells stimulated by LPS, was revealed[240]. Indeed, high phenolic and sulforaphane content was suggested to be responsible for the antioxidant effects and the inhibition of NO release from LPS-stimulated RAW 264.7 cells induced by a methanolic extract of cultivated *B. oleracea var. italic*[241]. However, the observed effects were less potent obtained compared to our *B. macrocarpa* extract. In contrast, Choi et al. (2023), studying natural extracts from "parental" *B. rapa* and *B. rapa* with high glucosinolate content, found that the latter exhibited a stronger antioxidant activity, which was attributed to the glucosinolates[229]. The lack of activity observed for *B. hispida* and *B. tardarae* could be likely due to the concentration of the extract used. In fact, due to the toxicity we cannot test higher concentrations. Notably, *B. macrocarpa* exhibit significant effects at concentrations higher then 125 µg/mL. A similar effect was reported by Malfa et al. (2022) for the Sicilian endemic *B. drepanensis*[235]. Lee et al. (2017) further demonstrated the effect of sinigrin, the glucosinolate present in the highest quantity in our samples, on RAW 264.7 cells stimulated with LPS. They observed a concentration-dependent reduction in NO levels, with their maximum tested concentration of 100 µg/mL of sinigrin corresponding to the estimated amount present in our extract at 1000 µg/mL[208]. This once again confirms that glucosinolates, together with phenols, play a key role in the modulation of oxidative stress.

Based on the findings from these preliminary analyses, *B. hispida* and *B. tardarae* were excluded from further investigation. As a result, the focus of subsequent studies shifted to *B. macrocarpa*, which demonstrated potential antioxidant activity.

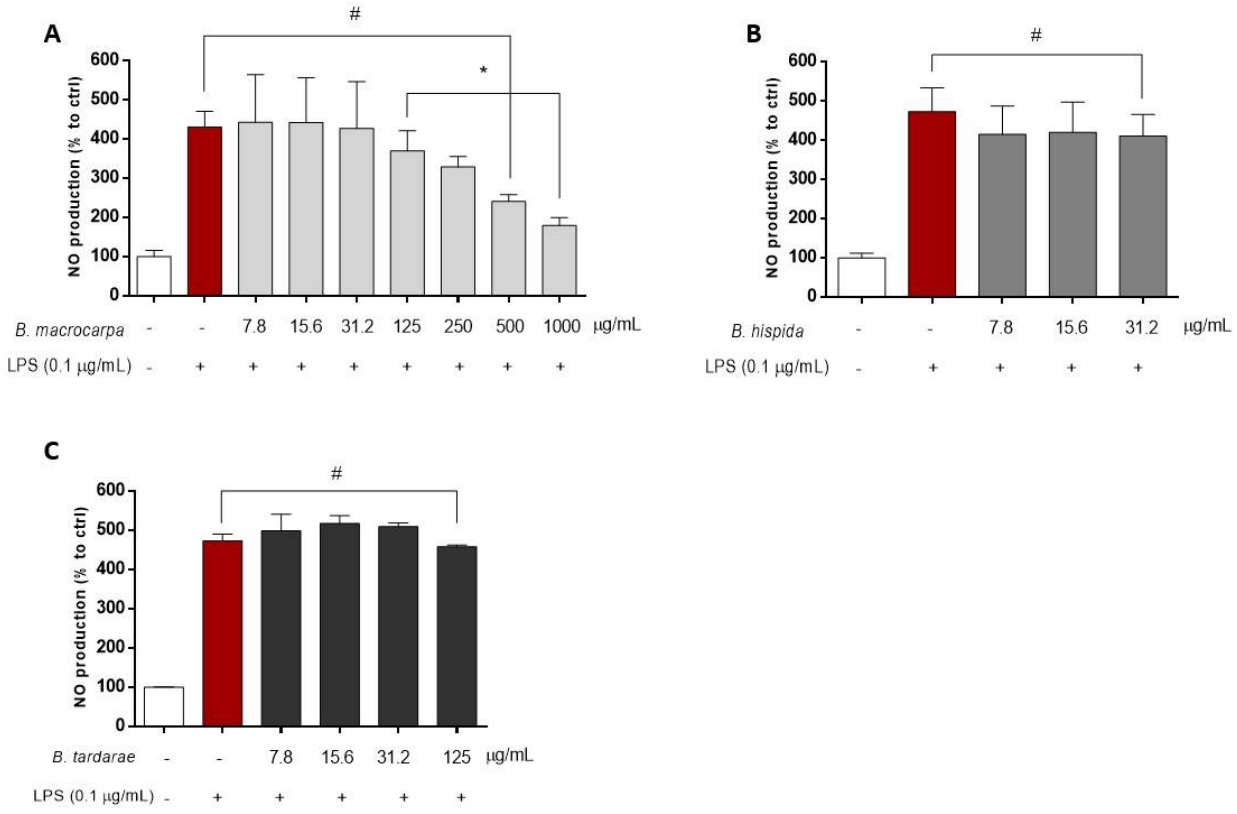

Figure 10. Effects of (A) B. macrocarpa (7.81-1000 µg/mL), (B) B. hispida (7.81- 31.25 µg/mL) and (C) B. tardarae (7.81-125 µg/mL) extracts on NO production in LPS-stimulated RAW 264.7 cells. Values are the mean ± SD (n=3), expressed as percentage of the control (untreated cells). Statistical significance calculated by one-way ANOVA: *p < 0.05 vs. LPS only. # p < 0.05 vs. control (untreated cells).

4.3.3 MODULATION OF mRNA EXPRESSION BY *B. MACROCARPA* EXTRACT

Given the effects of *B. macrocarpa* in modulating LPS-induced NO levels in RAW 264.7 cells, the mRNA expression of nitric oxide synthase 2 (NOS2), a key enzyme involved in NO production, was evaluated. LPS induced in RAW 264.7 cells a marked increase in NOS2 mRNA expression (RQ = 226). As shown in the figure 11, 2-hour pre-treatment with concentrations of *B. macrocarpa* that reduced NO levels (ranging from 125 to 1000 µg/mL), followed by 24 hours of exposure to LPS, significantly modulated the expression of NOS2. Similar to the observed reduction in nitric oxide production, a concentration-dependent decrease in NOS2 expression was also recorded. At the highest concentration tested (1000 µg/mL), *B. macrocarpa* induced a reduction in NOS2 mRNA expression by 78% compared to the LPS alone. These findings suggest that *B. macrocarpa* may regulate NOS2 expression as part of its mechanism for controlling NO production in response to inflammatory stimuli. NOS2 downregulation could be attributed to the presence of glucosinolates and

in particular to sinigrin, since, it has already been reported that both are able to reduce nitric oxide levels precisely by acting on the level of NOS2 gene expression[208,229,241]. However, further studies are needed to elucidate the exact mechanisms involved.

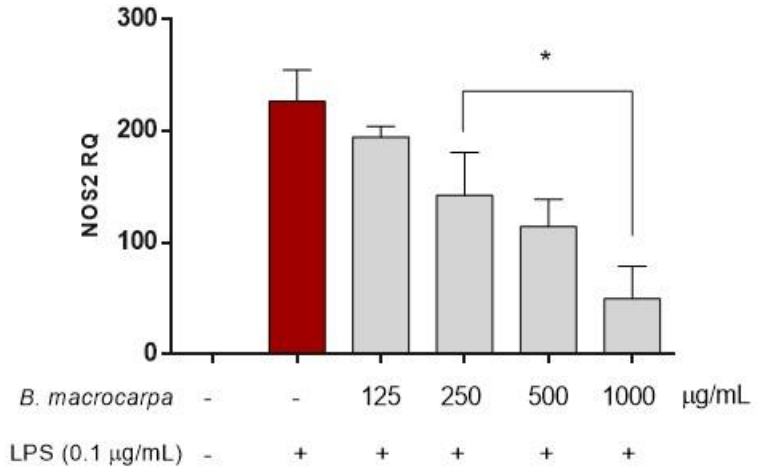

Figure 11. Effects of increasing concentrations (125-1000 µg/mL) of B. macrocarpa extract on NOS2 mRNA expression level in RAW 264.7 cells stimulated with LPS (0.1 µg/mL) and assessed by qRT-PCR. Data are the mean ± SD (n=3). * means significantly different according to one-way ANOVA (p < 0.05).

4.3.4 MODULATION OF ROS PRODUCTION BY *B. MACROCARPA* EXTRACT

The ability of *B. macrocarpa* extract to reduce reactive oxygen species (ROS) levels in RAW 264.7 macrophages cells exposed to LPS was also investigated. Like nitric oxide, ROS also play physiological roles in signalling. However, excessive production of ROS leads, as above reported, to oxidative stress status. Several studies have shown that secondary metabolites of plants are able to reduce ROS levels, especially flavonoids and glucosinolates[120,242,243].

To evaluate the possible modulatory effect on ROS production of our extract, macrophages were pre-treated with various concentrations of *B. macrocarpa* extract (ranging from 125 to 1000 µg/mL) for 2 hours, followed by a 24-hour exposure to LPS at a concentration of 0.1 µg/mL. A concentration-dependent reduction in ROS levels was observed, with significant effects starting at 250 µg/mL of the extract. At the highest concentration tested (1000 µg/mL), the ROS level was reduced by 70% (Fig 12.). These results confirmed that *B. macrocarpa* extract exhibits strong antioxidant activity, effectively reducing oxidative stress in RAW 264.7 macrophages.

A similarly effect in reducing ROS levels was showed for *B. Raimondoi* by Malfa et al. (2023) in the HepG2 cellular model stressed by H_2O_2, with a 50% reduction already observed at a concentration of 200 µg/mL[212]. *Brassica drepanensis*, also endemic to Sicily, exhibited an effect similar to our *B.*

macrocarpa in reducing ROS levels in LPS-treated- RAW 264.7 starting from 100 µg/mL in a dose-dependent manner[235].

Our finding was consistent with *in vitro* DPPH assay results, supporting the potential of *B. macrocarpa* as a natural antioxidant that could help to modulate oxidative stress in pathological conditions.

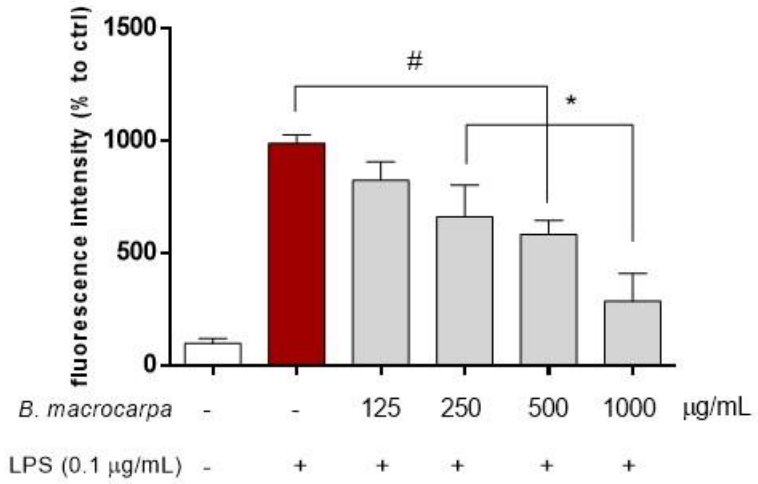

Figure 12. Effects of B. macrocarpa extract (125 - 1000 µg/mL) on ROS production in LPS-stimulated RAW 264.7 cells. ROS production was measured by the H_2DCF-DA assay and expressed as the mean of the emitted fluorescence intensity in percentage of the control group (untreated cells). Values are the mean ± SD (n=3). Statistical significance calculated by one-way ANOVA: *p < 0.05 vs. LPS only. # p < 0.05 vs. control (untreated cells).

4.4 ANTIOXIDANT EFFECTS OF *B. MACROCARPA* EXTRACT IN ZEBRAFISH EMBRYO *IN VIVO* MODEL

4.4.1 *IN VIVO* FISH EMBRYO TOXICITY TEST (FET)

Given the interesting results obtained in cellular model we moved for the last part of the study on *in vivo* model using the zebrafish embryo model to evaluate at first the safety of the extract and then to investigate also *in vivo* its antioxidant potential *in vivo*.

Zebrafish embryos were treated with increasing concentration of extract (125-2000 µg/mL) starting from 2 hpf up to 96 hpf, following OECD guidelines (Test No 236, paragraph 20: "the highest concentration tested should preferably result in 100% lethality, while the lowest concentration tested should preferably give no observable effect")[195]. The survival rate was calculated by optical monitoring, performed every 24 h. The figure 13 shows the percentage of mortality, expressed as percentage of dead embryos respect to the initial number of embryos.

Embryos exposed to *B. macrocarpa* extract at concentrations ranging from 125 µg/mL to 500 µg/mL showed no mortality throughout the treatment period. However, larvae treated with 1000 µg/mL and 2000 µg/mL concentrations exhibited mortality starting at 48 hours (96h-LC_{50} 762.1 ± 20.5 µg/mL). Meanwhile, according to the OECD (2013) LC_{50} values that ranges from 10 to 100 mg/L are harmful, 1 to 10 mg/L are toxic, and any value less than 1 mg/L are highly toxic[244].

Since the LC_{50} value of the extract was higher than the OECD values, it could be concluded, at this stage, that this methanolic extract is non-toxic and safe for consumption, at least for concentrations lower than its LC_{50} value. However, it is important to note that mortality rate alone is not the definitive criterion for assessing the safety of a plant extract. The impact of the extract on the overall development of the organism must also be taken into account[245].

These data establish a baseline for the selection of non-lethal concentrations suitable for subsequent experiments on the biological effects of *B. macrocarpa*.

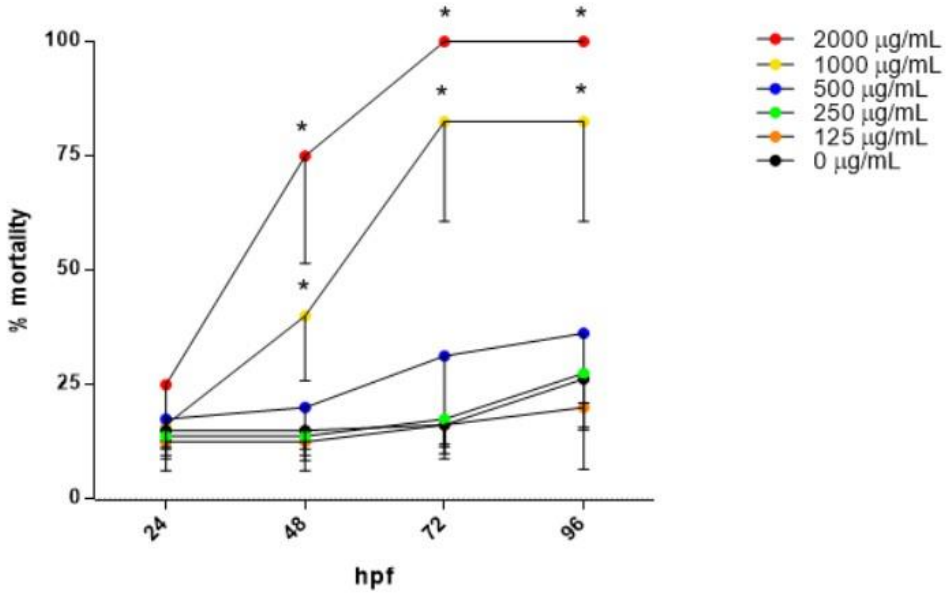

Figure 13. Mortality rate of zebrafish embryo exposed to increasing concentrations of B. macrocarpa extract (125-2000 µg/mL) for 96 h. Data are presented as mean ± SD (20 embryos/group and performed in quadruplicate). Two-way ANOVA followed by Dunnett's multiple comparisons test was performed. *p<0.05 vs. control (untreated embryo).

During the 96 h of exposure to increasing concentrations of the extract, in addition to calculate embryo mortality, the fish embryo toxicity (FET) test was conducted. The FET test on zebrafish allowed the assessment of potential teratogenic effects of the extract on embryonic development.

Analysing the different endpoints stated by OECD we observed that at 24 hpf, the formation of somites and the detachment of the head and tail were normal in all treated groups compared to the control (100% in all embryo analysed: 10 randomly embryos for each group in quadruplicate). However, the number of spontaneous movements was significantly increased at the two highest concentrations when compared to the control group ($p < 0.05$) (Fig. 14A).

At 48 hpf, the heartbeat rate was significantly reduced in embryos exposed to the sub-lethal concentrations of 250 μg/mL (162 ± 11 heartbeats/60") and 500 μg/mL (157 ± 5 heartbeats/60") compared to the control group (186 ± 19 heartbeats/60"; $p < 0.05$, each) (Fig. 14B). This reduction in heart rate, may lead to a decrease in the efficiency of blood circulation within the embryos. As a result, it may impede the supply of essential nutrients and oxygen, both crucial for normal embryonic development. This restriction in energy supply may, in turn, negatively affect the overall growth and development of the embryos[246]. However, we did not observe any effect on the hatching rate or the embryonic development. In fact, at 72 hpf, the hatching rate was also assessed, showing no significant difference between treated and untreated embryos, as demonstrated in Figure 14C.

Morphological aspects at sub-lethal concentrations were evaluated at 96 hpf using a microscope equipped with a camera, and the images were subsequently analyzed using Digimizer software. The parameters analysed are reported in Table 9. At 96 hpf, the evaluation of morphological aspects in zebrafish exposed to sub-lethal concentrations revealed a development similar to that of the control group, with just a few minor malformations observed. The overall morphology, including the backbone angle, body shape (size, eye, head, yolk, pericardial area), and general edema parameters, appeared normal. No significant alterations were noted in these developmental features, indicating that the sub-lethal concentrations did not induce major morphological abnormalities, suggesting the potential safety of these concentrations.

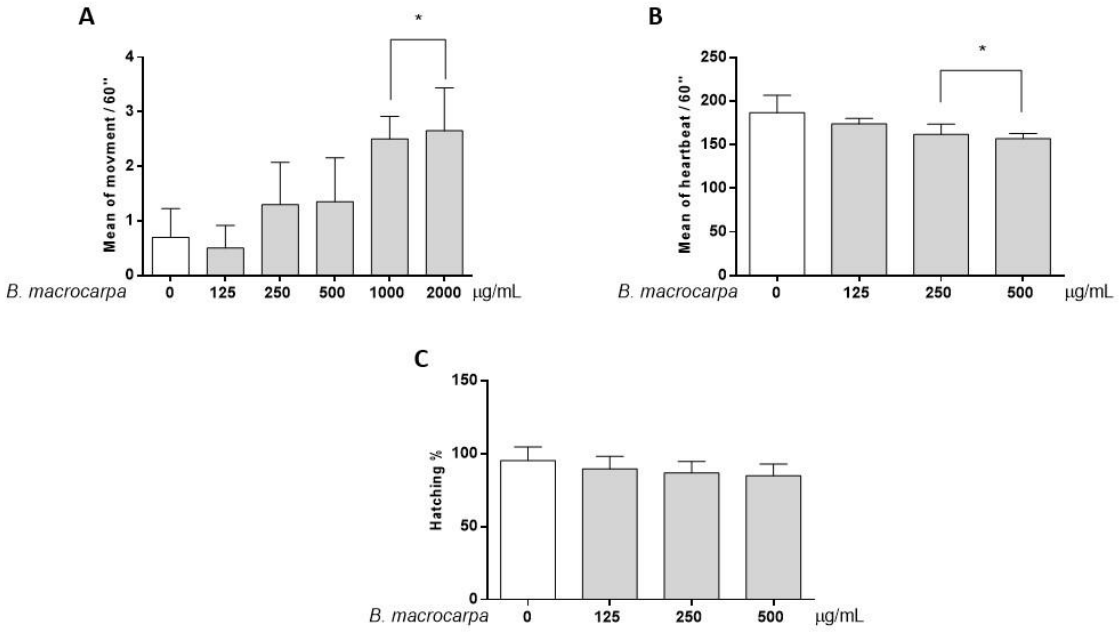

Figure 14. Effects of the increase concentrations of B. macrocarpa extract exposures on early stage zebrafish embryos development. (A) spontaneous movements measured in 60"at 24 hpf, (B) heart rate measured in 60" at 48 hpf and (C) hatching rate measured at 72 hpf. Data are the mean ± SD of 10 embryos/group in 4 replicate. * means significantly different according to one-way ANOVA ($p < 0.05$).

Development parameters	0 µg/mL	125 µg/mL	250 µg/mL	500 µg/mL
Malformation %	10 ± 8.16	15 ± 12.91	10 ± 11.55	10 ± 11.55
Edema %	10.28 ± 8.18	10,90 ± 8.25	15.27 ± 12.11	14.75 ± 5.28
Size (mm)	3.76 ± 0.12	3,78 ± 0.11	3.86 ± 0.11	3.743 ± 0.12
Angle °	157.70 ± 1.41	161,80 ± 2.34	160.30 ± 2.58	156.2 ± 2.22
Head (mm^2)	0.28 ± 0.01	0,27 ± 0.03	0.27 ± 0.02	0.252 ± 0.01
Eye (mm^2)	0.10 ± 0.01	0,09 ± 0.01	0.10 ± 0.01	0.089 ± 0.01
Pericardium (mm^2)	0.11 ± 0.02	0,11 ± 0.01	0.10 ± 0.01	0.091 ± 0.01
Yolk (mm^2)	0.43 ± 0.03	0,45 ± 0.02	0.45 ± 0.02	0.447 ± 0.01

Table 9. Developmental effects of 96 h B. macrocarpa exposure on zebrafish larvae analized by Digimizer software. Data are the mean ± SD of 10 embryos/group in 4 replicate. *$p < 0.05$ vs. control (untreated embryos) according to one-way ANOVA.

4.4.2 LARVAE BEHAVIOR ANALISES

Zebrafish larvae are widely used to evaluate the effects of various substances, including natural extracts, on the nervous system and motor behavior. In recent years, their use in behavioral research, particularly in neuroscience and neuropharmacology, has increased significantly. Zebrafish larvae show behavioral similarities to other vertebrates, such as anxiety and stress, making them ideal for evaluating how certain substances affect behavior[190,247,248].

In our study, at 96 hpf, the behavior of larvae exposed to sub-lethal concentrations of *B. macrocarpa* extract from 2 hpf was evaluated.

A total of six larvae per group, with four replicates, were selected for the tests. The behavioral assessments included locomotor activity, aversive stimulus response, and anxiolytic-like behavior. In the locomotor behavior test (open field test), the larvae were placed in a controlled environment to observe their natural swimming patterns and movement. This test helps to evaluate general motor activity and exploratory behavior. The larvae were also subjected to an aversive stimulus response test, where a red ball moved across the upper part of the well. The larvae's response to the moving stimulus was measured by recording the time spent in the area without the aversive stimulus, giving an indication of their avoidance behavior. Additionally, to evaluate the potential anxiolytic effect of the extract, a light/dark preference test was performed. The well was divided into two distinct areas: one exposed to light and the other kept dark. The larvae behavior was assessed by measuring the time spent in each zone, with a preference for the light-exposed area potentially indicating an anxiolytic effect, while a preference for the dark area could suggest anxiety-like behavior. The results of these tests are summarized in Table 10.

In the open field test, no statistically significant difference was observed in the mean distance travelled, speed, immobility, absolute turning angle, or distance from the center ($p > 0.05$). Similarly, in the light/dark test, as well in the aversion test, no difference was detected between treated larvae and controls ($p > 0.05$).

These findings, together with the results obtained in the FET test, demonstrate that sub-lethal concentrations of *B. macrocarpa* extract (125–500 µg/mL) are devoid of toxicity, including any potential neurotoxic effects.

Behavioral parameters	0 µg/mL	125 µg/mL	250 µg/mL	500 µg/mL
Distance (cm)	27.28 ± 18.90	28.51 ± 13.78	28.32 ± 21.85	17.52 ± 17.50
Speed (mm/s)	1.14 ± 0.71	1.13 ± 0.83	0.74 ± 0.30	0.71 ± 0.33
Immobility time (%)	46.56 ± 11.22	45.06 ± 9.83	40.51 ± 16.09	52.00 ± 17.78
Centre distance (mm)	7.84 ± 0.41	7.50 ± 0.35	6.64 ± 1.33	7.32 ± 0.55
Absolute turn angle (°)	4.23 ± 1.41	4.69 ± 0.79	3.93 ± 0.76	4.42 ± 1.25
Time in the lit side (%)	55.43 ± 9.56	66.24 ± 10.48	63.17 ± 11.07	50.13 ± 16.41
Time in the non-stimulus area (%)	66.77 ± 12.08	72.35 ± 17.64	53.40 ± 21.89	50.65 ± 11.28

Table 10. Larval behavioural analysis after 96 h of B. macrocarpa exposure at sub-lethal concentration. Data are the mean of 6 embryos/group in 4 replicate ± SD. Video recordings of the larvae subjected to behavioral tests were analyzed with TheRealFishTracker software. * means significantly different according to one-way ANOVA: $p < 0.05$ vs. control (untreated embryos).

4.4.3 OXIDATIVE STRESS PARAMETERS AND *B. MACROCARPA* TREATMENT

At first, we evaluated whether the *B. macrocarpa* extract itself could induce oxidative stress.

30 larvae per group, in triplicate, were exposed to sub-lethal concentrations of the extract (ranging from 125 to 500 µg/mL) at 72 hpf. The larvae were treated for a period of 26 hours. Following the treatment period, both treated and untreated larvae were collected, homogenized, and subjected to biochemical assays to evaluate major oxidative stress parameters. The results were normalized to protein content and expressed as percentages to control, as shown in Figure 15.

The 26-hour treatment with *B. macrocarpa* extract did not affect the levels of ROS and NO (Fig. 15A and 15B), the activities of the antioxidant enzymes SOD and CAT (Fig. 15C and 15D), resulting comparable to those of the control ($p > 0.05$). Furthermore, no damage was observed to lipids (Fig. 15E), proteins (Fig. 15F), or DNA (Fig. 15G) at any concentrations tested ($p > 0.05$).

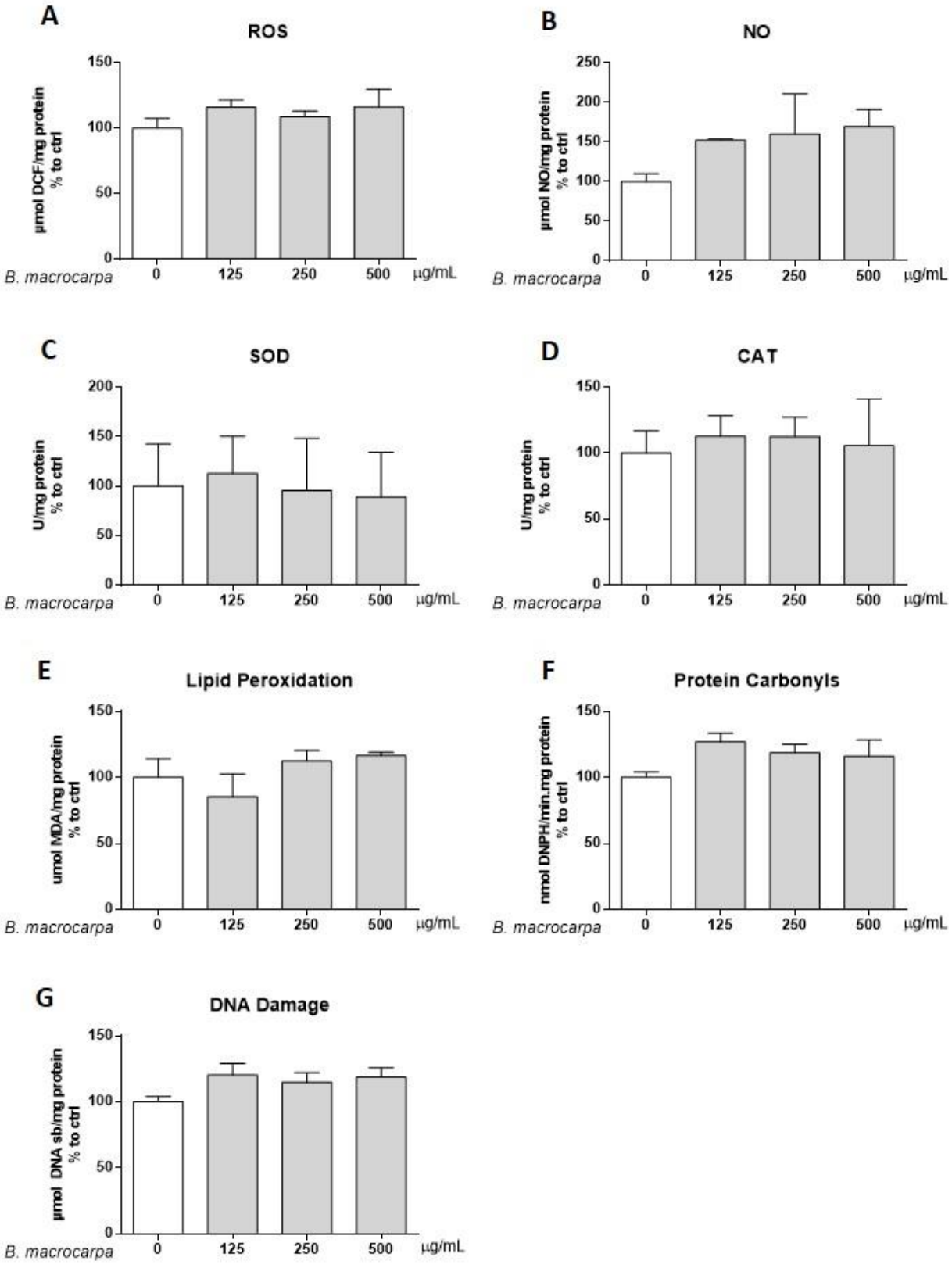

Figure 15. Effect of 26 h of treatment with sub-lethal concentrations of B. macrocarpa extract (125 – 500 µg/mL) on healthy zebrafish larvae at 72 hpf. (A) Effects on ROS production. (B) Effects on nitric oxide production. (C) Effects on SOD enzymatic activity. (D) Effects on CAT enzymatic activity. (E) Effects on lipid peroxidation levels. (F) Effects on protein oxidation levels. (G) Effects on DNA damage levels (single-strand DNA). Data are the mean of 3 different experiments conducted in technical duplicate ± SD expressed as a percentage of the control group. * means significantly different according to one-way ANOVA: $p < 0.05$ vs. control (untreated embryos).

4.4.4 $CuSO_4$-INDUCED OXIDATIVE STRESS IN ZEBRAFISH LARVAE AND *B. MACROCARPA* TREATMENT

Zebrafish larvae exposure to $CuSO_4$ is an ideal model for inducing oxidative stress in zebrafish[249]. $CuSO_4$ induced reactive oxygen species (ROS) production, which in turn leaded to oxidative damage to cellular macromolecules, disrupting normal embryonic development and causing various morphological and physiological defects. Preliminary studies were conducted to determine the optimal exposure time to $CuSO_4$ able to induce a status of oxidative stress in zebrafish larvae without resulting in larvae death. Larvae were exposed to a concentration of 20 µM $CuSO_4$, for 1 h or 5 h and levels of oxidative mediators were assessed, in particular the levels of ROS and NO, which are the main markers of stress, were measured.

The results, expressed as a percentage to control (larvae exposed only to E3 medium = time 0h), indicate that a significant increase in ROS levels by 61% and NO levels by 165% compared to the control was observed after 5 h of exposure to 20 µM $CuSO_4$ (Fig. 16).

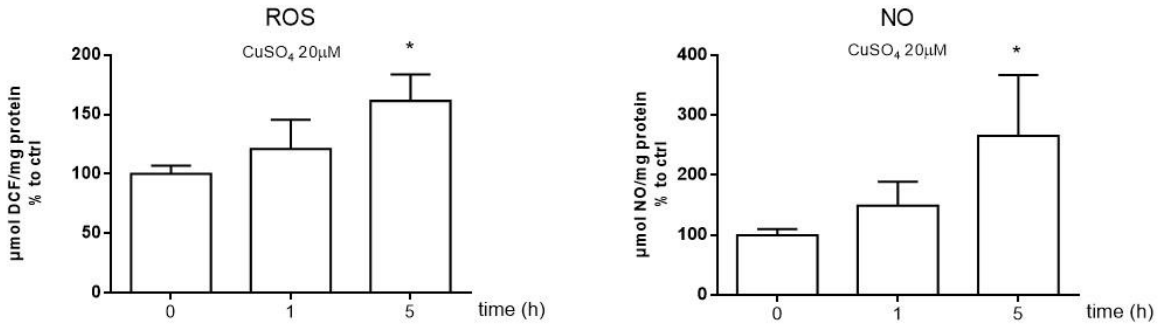

Figure 16. Effect of 20 µM $CuSO_4$ exposure zebrafish larvae at 72 hpf on ROS and NO production at different time points. Data are the mean ± SD (n=3) expressed in percentage to control group. One-way ANOVA test was performed. *p < 0.05 vs. control (untreated larvae).

To investigate the potential effects of *B. macrocarpa* extract on the oxidative stress conditions, a well-defined experimental protocol was established, consisting in 21-hour pre-treatment of zebrafish embryos with the extract at sub-lethal concentrations (125, 250, and 500 µg/mL). These concentrations were carefully selected as they *per se* did not significantly alter the oxidative stress parameters. Following the pre-treatment, the embryos were exposed to 20 µM $CuSO_4$ for an additional 5 hours to induce oxidative stress.

The figure 17 shows the results of the analysis of biochemical parameters to evaluate whether the extract could have antioxidant effects on $CuSO_4$-induced oxidative stress. As expected, ROS levels

(Fig. 17A) significantly increased in larvae exposed to $CuSO_4$ compared to the control. Treatment with the extract reduced ROS levels in a concentration-dependent manner, reaching significance at the concentration of 500 μg/mL, which reduced the ROS level by 26% compared to the increase observed in the group treated with $CuSO_4$.

NO levels (Fig. 17B) also increased after 5 hours of exposure to $CuSO_4$. The extract modulated NO production in a concentration-dependent manner, also reaching statistical significance at the concentration of 500 μg/mL, which reduced NO levels by 60% compared to the $CuSO_4$ group.

5 hours of exposure to $CuSO_4$, induced also a significant increase in SOD and CAT activities (Fig. 17C and 17D), compared to the control. Treatment with the extract significantly reduced the activity of both enzymes, bringing them back to control levels.

Moreover, exposure to $CuSO_4$ caused an increase in MDA levels, the final product of lipid peroxidation (Fig. 17E). Treatment with *B. macrocarpa* was effective in preventing lipid peroxidation. At 250 μg/mL and 500 μg/mL of the extract the MDA levels showed a decrease of 39% and 46%, respectively, compared to the increase observed in the group treated with $CuSO_4$.

$CuSO_4$ induced protein oxidation revealed by an increase in protein carbonyl level of about 67%. *B. macrocarpa* extract at the concentration of 500 μg/mL decrease the level of protein carbonylation of 31% compared to the increase observed in the group treated with $CuSO_4$.

On the contrary, the extract was not able to reduce DNA damage caused by exposure to $CuSO_4$. Although the levels of damage were decreased (15 % respect to $CuSO_4$-treated group for the concentration of 500 μg/mL), the reduction did not reach statistical significance ($p > 0.05$).

$CuSO_4$ was effective in inducing ROS and NO production indicating oxidative stress. To avoid oxidative stress and maintain the cellular redox state in balance, cells use endogenous antioxidant enzymes. SOD and CAT enzymes are considered the main line of defense against ROS production under stress conditions[250]. By activating these enzymes, cells respond to acute challenges[251]. ROS formed by copper sulphate exposure are highly reactive and can be converted into H_2O_2 by SOD and then into oxygen and water by CAT. Interestingly, $CuSO_4$ increasing the activity of SOD and CAT indicated the accumulation of hydrogen peroxide and other oxygen radicals. Moreover, increased MDA levels, a byproduct of cellular lipid peroxidation, further suggested the induction of oxyradicals in copper-treated embryos, as also demonstrated by Cai et al. (2012) in cobalt-induced embryotoxicity[252].

B. macrocarpa extract was able to significantly counteract oxidative stress in zebrafish larvae exposed to $CuSO_4$, decreasing the enhancement of ROS and NO levels, as well lipid peroxidation and protein carbonylation.

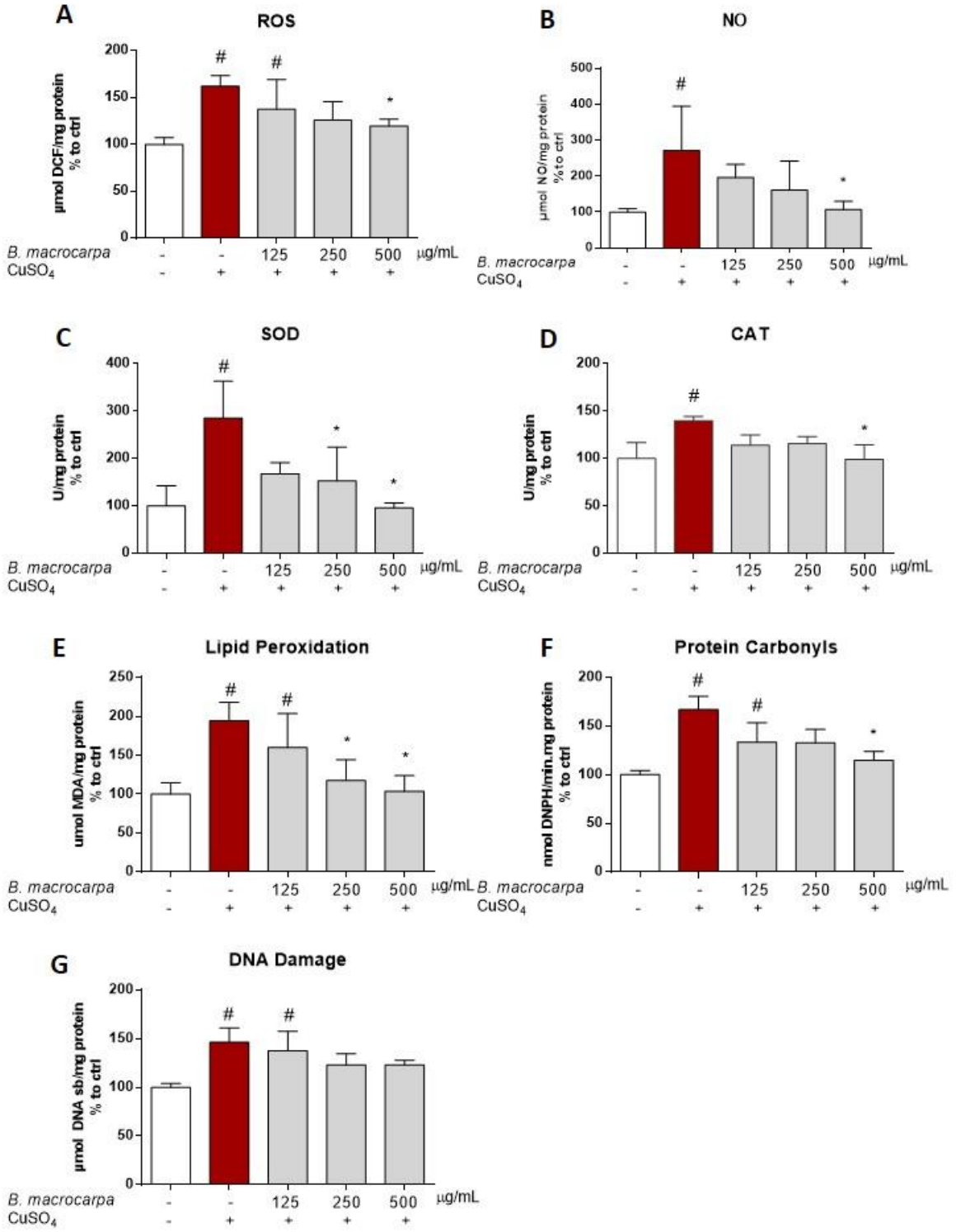

Figure 17. Effect of 21 h of pre-treatment with increasing concentrations of B. macrocarpa extract (125 – 250 - 500 µg/mL) followed by 5 h of $CuSO_4$ exposure zebrafish larvae at 72 hpf. (A) Effects on ROS production. (B) Effects on NO production. (C) Effects on SOD enzymatic activity. (D) Effects on CAT enzymatic activity. (E) Effects on lipid peroxidation levels (MDA levels). (F) Effects on protein oxidation levels (protein carbonyls levels). (G) Effects on DNA damage levels (single-strand DNA levels). Data are the mean of 3 different experiments conducted in technical duplicate ± SD expressed as a percentage of the control group. Statistical significance calculated by one-way ANOVA: *p < 0.05 vs. $CuSO_4$ only. # p < 0.05 vs. control (untreated embryos).

5. CONCLUSIONS

This project aimed for the first time, to explore the chemical composition and biological properties of three endemic Sicilian *Brassica* species using both *in vitro* and *in vivo* models. The 3 selected species, each growing in distinct habitats, provided a unique opportunity to analyze and compare their phytochemical profiles and bioactivity.

By HPLC-MS/MS analysis, it was found that the three *Brassica* species share a similar phytochemical profile, although with some differences in compound concentrations. Glucosinolates and flavonoids were present in comparable amounts across the samples, with sinigrin being the predominant glucosinolate and quercetin and kaempferol as the main flavonoids, primarily in their glycosylated forms. However, a notable variation was observed in the sinapyl derivatives, which were found in higher concentrations in the *B. hispida* extract. This could be attributed to its unique growing conditions: *B. hispida* thrives in wooded areas where there are pronounced temperature fluctuations between day and night, and it grows at elevations above 1200 meters.

In vitro scavenging activity assays demonstrated that all three *Brassica* extracts exhibited antioxidant potential, with activity starting from a concentration of 125 µg/mL.

In a cellular model using murine macrophages (RAW 264.7), *B. macrocarpa* was found to be safe at all tested concentrations. In contrast, *B. hispida* was safe up to 31.2 µg/mL, while *B. tardarae* was safe up to 250 µg/mL.

RAW 264.7 cells when exposed to oxidative stress conditions induced by LPS-stimulation, only *B. macrocarpa* showed a strong antioxidant activity reducing NO and ROS levels, alongside a downregulation of NOS2 mRNA expression.

In zebrafish embryo *in vivo*, FET test showed that *B. macrocarpa* is safe up to 500 µg/mL. Under oxidative stress conditions induced by $CuSO_4$, a 21-hour pre-treatment with *B. macrocarpa* at a concentration of 500 µg/mL, significantly reduced key oxidative stress markers, such as ROS and NO levels, protein carbonylation, lipid oxidation, and the activity of antioxidant enzymes SOD and CAT.

These findings lay a strong foundation for future research focused on unraveling the mechanisms behind the antioxidant activity of these natural extracts and investigating their potential as complementary treatments for managing oxidative stress-related conditions.

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244. Norazhar, A. I., Ibrahim, W. N. W., Saleh Hodin, N. A., Mohd Faudzi, S. M. & Shaari, K. Zebrafish Embryotoxicity and Teratogenic Effects of Christia vespertilionis Leaf Extract. *Pertanika J. Trop. Agric. Sci.* **45**, (2022).
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249. Wang, Y. *et al.* Protective effect of surfactin on copper sulfate-induced inflammation, oxidative stress, and hepatic injury in zebrafish. *Microbiol. Immunol.* **65**, 410–421 (2021).
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252. Cai, G. *et al.* The effects of cobalt on the development, oxidative stress, and apoptosis in zebrafish embryos. *Biol. Trace Elem. Res.* **150**, 200–207 (2012).

COURSES, CONFERENCES AND PUBLICATIONS

Courses with final evaluation

1. October 2022 Course "Chimica dei pigmenti e delle sostanze naturali", Laurea Magistrale in Conservazione e Restauro dei Beni Culturali. Reference Teacher prof. Maurizio Bruno (48 hours)
2. October 2022 Course "Genomica Funzionale", Laurea Magistrale in Biologia Molecolare e della Salute. Reference Teacher prof. Salvatore Feo (48 hours)
3. March 2023 Course "Writing a Scientific Proposal" Reference Teacher prof. Scilabra Simone (8 hours)
4. October 2023 Course "Antibacterial, Antitumor Activity and Drug-resistance Acquisition: Cellullare Targets and Molecular Mechanisms" Reference Teacher prof. Patrizia Cancemi and prof. Rosa Alduina (12 hours)
5. May 2024 Course "Epigenitic Mechanisms of Gene Regulation" Reference Teacher prof. Vincenzo Cavalieri (6 hours)

Conferences/workshop attendance

1. 15th Annual Meeting of Young Researchers in Physiology (SIF)13-15 June 2022
2. First STeBICeF Young Researcher Workshop, Palermo, 12 Genuary 2023
3. Nutraceuticals and Functional Foods, IRIB-CNR Palermo, Palermo, 3 february 2023
4. 95° congresso della Società Italiana di Biologia Sperimentale (SIBS), Trieste 12-15 april 2023
5. Second Workshop of Technologies and Science for Human Health, Palermo 23-25 october 2023
6. Third Workshop of Technologies and Science for Human Health, Palermo 28-30 october 2024

Oral Presentation at Conferences/workshop

1. A. Cicio, M.G. Zizzo, C. Luparello, M. Bruno, V. Ilardi, R. Serio
   ANTI-INFLAMMATORY AND ANTI-OXIDANT ACTIVITY OF ENDEMIC SICILIAN SPECIES OF GENUS *BRASSICA*: *IN VITRO* AND *IN VIVO* STUDIES
   15th Annual Meeting of Young Researchers in Physiology (SIF)13-15 June 2022.

2. Cicio A., Sut S., Dall'Acqua S., Bruno M., Luparello C., Zizzo M.G., Serio R.

CHEMICAL CHARACTERIZATION AND CYTOTOXIC AND ANTIOXIDANT ACTIVITY EVALUATION OF THE ETHANOL EXTRACT FROM THE BULBS OF PANCRATIUM MARITIMUM COLLECTED IN SICILY

95° congresso della Società Italiana di Biologia Sperimentale (SIBS), Trieste 12-15 april 2023

3. Cicio A., Terracina F., Bruno M., Ilardi V., Luparello C., Serio R., Zizzo M.G.
   CHEMICAL COMPOSITION AND ANTIOXIDANT ACTIVITY OF METHANOLIC EXTRACT OF *BRASSICA MACROCARPA*
   Second Workshop of Technologies and Science for Human Health, Palermo 23-25 october 2023

4. Cicio A., Félix L., Monteiro S.M., Serio R., Zizzo M.G
   TOXICTY, ANTIOXIDANT AND ANTI-INFLAMMATORY EFFECTS OF *BRASSICA MACROCARPA* GUSS LEAF EXTRACT IN COPPER SULFATE-INDUCED INFLAMMATION IN ZEBRAFISH (*DANIO RERIO*) EMBRYO
   Third Workshop of Technologies and Science for Human Health, Palermo 28-30 october 2024

Presented Posters at Conferences/workshop

1. M.G. Zizzo, A. Cicio, R. Serio
   EVALUATION OF *IN VITRO* ACTIVITIES OF GUANINE BASED PURINES IN RAT UTERUS.
   Europhysiology 2022 Copenaghen 15-19 settembre 2022

2. V. Maresca, L. Capasso, P. Cianciullo, P. Bontempo, A. Nebbioso, A. Cicio, N. Badalamenti,
   M. Bruno, A. Basile
   CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITIES OF *PRANGOS FERULACEA* ESSENTIAL OILS
   XL Convegno Nazionale della Divisione di Chimica Organica, CDCO Palermo 11-15 September 2022

3. Cicio A., Zizzo M.G., Bruno M., Ilardi V., Serio R.
   EFFECTS OF THE ESSENTIAL OIL FROM A SICILIAN ACCESSION OF *PRANGOS FERULACEA* IN RAT INTESTINAL SMOOTH MUSCLE
   First STeBICeF Young Researcher Workshop, Palermo, 12 Genuary 2023

4. Terracina F., Cicio A., Baiamonte C., Puleo G., Scirè S., Pizzolanti G., Zizzo M.G., Licciardi M.
   PHYCOCYANIN GASTRO-RESISTANT MICROPARTICLES PRODUCED BY SPRAY-DRYING FOR THE TREATMENT OF INFLAMMATORY BOWEL DISEASES
   CRS Italy Local Chapter Workshop, Palermo, 5-7 October 2023

5. Zizzo M.G., Cicio A., Bruno M., Serio R.
   TOCOLYTIC ACTION AND UNDERLYING MECHANISMS OF *PRANGOS FERULACEA* (L.) ESSENTIAL OIL ON RAT UTERUS
   73rd SIF National Congress, Pisa 6-8 September 2023

6. Cicio A., Félix L., Monteiro S.M., Serio R., Zizzo M.G.
   BIOLOGICAL PROPERTIES OF *BRASSICA MACROCARPA* GUSS LEAF EXTRACT IN ZEBRAFISH EMBRYO INFLAMMATION MODEL
   2° meeting – Zebrafish per One Health, Roma 24-25 October 2024

Papers (published, submitted)

1. A. Napolitano, M. Di Napoli, G. Castagliuolo, N. Badalamenti, A. Cicio, M. Bruno, S. Piacente, V. Maresca, P. Cianciullo, L. Capasso, P. Bontempo, M. Varcamonti, A. Basile, A. Zanfardino
   THE CHEMICAL COMPOSITION OF THE AERIAL PARTS OF *STACHYS SPREITZENHOFERI* (LAMIACEAE) GROWING IN KYTHIRA ISLAND (GREECE), AND THEIR ANTIOXIDANT, ANTIMICROBIAL, AND ANTIPROLIFERATIVE PROPERTIES
   Phytochemistry. 2022 Nov; 203:113373. doi: 10.1016/j.phytochem.2022.113373.

2. M. G. Zizzo, A. Cicio, S. Raimondo, R. Alessandro, R. Serio

AGE-RELATED DIFFERENCES OF γ-AMINOBUTYRIC ACID (GABA)ERGIC TRANSMISSION IN HUMAN COLONIC SMOOTH MUSCLE
Neurogastroenterology and Motilitythis 2022 Mar;34(3):e14248. doi: 10.1111/nmo.14248

3. G. D'Agostino, A. Cicio, A. Vaglica, V. Ilardi, M. BrunoTHE CHEMICAL COMPOSITION OF THE AERIAL PARTS ESSENTIAL OIL *OF AMMI CRINITUM* GUSS. (APIACEAE) ENDEMIC OF SICILY (ITALY)
Natural Product Research 2022 Sep 1;1-5. doi: 10.1080/14786419.2022.2117175

4. A. Cicio, N. Badalamenti, M. Bruno
THE ETHNOBOTANY, PHYTOCHEMISTRY, AND BIOLOGICAL PROPERTIES OF GENUS *PHAGNALON* (ASTERACEAE): A REVIEW
Natural Product Research, 2022 Aug 17:1-15. doi: 10.1080/14786419.2022.2112039

5. M. G. Zizzo, A. Cicio, F. Corrao, L. Lentini, R. Serio
AGING MODIFIES RECEPTOR EXPRESSION BUT NOT MUSCULAR CONTRACTILE RESPONSE TO ANGIOTENSIN II IN RAT JEJUNUM
Journal of Physiology and Biochemistry, 2022 Nov;78(4):753-762. doi: 10.1007/s13105-022-00818

6. Cicio, A., Serio, R., Zizzo, M.G.
ANTI-INFLAMMATORY POTENTIAL OF *BRASSICA*CEAE-DERIVED PHYTOCHEMICALS: *IN VITRO* AND *IN VIVO* EVIDENCE FOR A PUTATIVE ROLE IN THE PREVENTION AND TREATMENT OF IBD
Nutrients, 2023, 15(1), 31 doi: 10.3390/nu15010031

7. Zizzo, M.G., Cicio, A., Serio, R
INHIBITION OF UTERINE CONTRACTILITY BY GUANINE-BASED PURINES IN NON-PREGNANT RATS
Naunyn-Schmiedeberg's Archives of Pharmacology, 2023, 396(5), pp. 963–972 doi: 10.1007/s00210-022-02366-5

8. Cicio, A., Sut, S., Dall'Acqua, S., Bruno, M., Luparello, C., Serio, R., Zizzo, M.G.

CHEMICAL CHARACTERIZATION AND CYTOTOXIC AND ANTIOXIDANT ACTIVITY EVALUATION OF THE ETHANOL EXTRACT FROM THE BULBS OF *PANCRATIUM MARITIMUN* COLLECTED IN SICILY
Molecules, 2023, 28(10), 3986 doi: 10.3390/molecules28103986

9. Zizzo, M.G., Cicio, A., Bruno, M., Serio, R.
ESSENTIAL OIL OF SICILIAN *PRANGOS FERULACEA* (L.) LINDL. AND ITS MAJOR COMPONENT, B−OCIMEN, AFFECT CONTRACTILITY IN RAT SMALL AND LARGE INTESTINE
Journal of Ethnopharmacology, 2023, 313, 116531 doi: 10.1016/j.jep.2023.116531

10. Zizzo, M.G., Cicio, A., Bruno, M., Serio, R.
INHIBITORY EFFECT AND UNDERLYING MECHANISM OF ESSENTIAL OIL OF *PRANGOS FERULACEA* LINDL (L.) ON SPONTANEOUS AND INDUCED UTERINE CONTRACTIONS IN NON-PREGNANT RATS
Biomedicine and Pharmacotherapy, 2023, 167, 115570 doi: 10.1016/j.biopha.2023.115570

11. Tinnirello, V., Zizzo, M.G., Conigliaro, A., Tabone, M., Rabienezhad Ganji, N., Cicio, A., Bressa, C., Larrosa, M., Rappa, F., Vergilio G., Gasparro, R., Gallo, A., Serio, R. Alessandro, R., Raimondo, S.
INDUSTRIAL-PRODUCED LEMON NANOVESICLES AMELIORATE EXPERIMENTAL COLITIS-ASSOCIATED DAMAGES IN RATS VIA THE ACTIVATION OF ANTI-INFLAMMATORY AND ANTIOXIDANT RESPONSES AND MICROBIOTA MODIFICATION
Biomedicine and Pharmacotherapy, 2024, 174, 116514 doi: 10.1016/j.biopha.2024.116514

12. Cicio, A., Aloi, N., Sut, S., Longo, V., Terracina, F., Dall'Acqua, S., Zizzo, M.G., Bruno, M., Ilardi, V., Colombo, P., Luparello, C., Serio, R.
CHEMICAL CHARACTERIZATION, FREE RADICAL SCAVENGING, AND CELLULAR ANTIOXIDANT PROPERTIES OF THE EGADI ISLAND ENDEMIC *BRASSICA MACROCARPA* GUSS LEAF EXTRACT
Biomolecules 2024, 14, 636. doi.org/10.3390/ biom14060636

13. Naselli, F., Volpes, S., Cardinale, P.S., Micheli, S., Cicio, A., Scoglio, S., Chiarelli, R., Zizzo, M.G.,
    Picone, P., Caradonna, F., Nuzzo, D.
    FIRST EVIDENCE OF EPIGENETIC MODULATION OF HUMAN GENE METHYLATION BY MICROALGA *APHANIZOMENON FLOS-AQUAE* (AFA) IN INFLAMMATION-RELATED PATHWAYS IN INTESTINAL CELLS
    Clinical Epigenetics (paper in revision)

Other activities:

1. Training online Stabulari ATeN Center (November 2021)
2. Course FAD (IZSLER): Sperimentazione animale – corso base: dal concetto delle 3RS alla normativa vigente (December 2021)
3. Training ATeN Center: confocal imaging from basic to advanced (February 2022)
4. Course FAD (IZSLER): Elementi base per l'approccio dei ricercatori all'utilizzo degli animali ai fini scientifici (October 2022)
5. Course FAD (IZSLER): Legislazione nazionale ed etica livello 1, moduli 1 e 2, DM 5 agosto 2021 (November 2022)
6. Course FAD (IZSLER): Zebrafish come organismo modello: approcci sperimentali *in vitro* e *in vivo* nella ricerca scientifica (Jun 2024)