Università degli Studi di Urbino Carlo Bo

Dipartimento di SCIENZE BIOMOLECOLARI

CORSO DI DOTTORATO DI RICERCA IN:

BIOMOLECULAR AND HEALTH SCIENCES

CICLO XXXVII

PRODUZIONE DI METABOLITI SECONDARI DI ORIGINE VEGETALE A PARTIRE DA COLTURE IN VITRO DI CALLI DALLA POLPA DI FRUTTA DI DIVERSE POMACEAE

(VALUTAZIONE DELL'ATTIVITÀ BIOLOGICA DEI METABOLITI SECONDARI OTTENUTI DA COLTURE IN VITRO DI CELLULE DI POLPA DI CALLO)

SSD: BIOS-07/A

Tesi redatta con il sostegno finanziario del programma
FSE-REACT-EU, PON "Ricerca e Innovazione" 2014-2020 (D.M. 1061/2021)
Azione IV.5 "Dottorati su tematiche Green"

Coordinatore: Ch.mo Prof. Ferdinando Mannello

Supervisore: Ch.mo Prof. Lucia Anna Maria Potenza

Dottoranda: Federica Gubitosa

ANNO ACCADEMICO
2023/2024

University of Urbino Carlo Bo

Department of BIOMOLECULAR SCIENCES

Ph.D. PROGRAMME IN:

BIOMOLECULAR AND HEALTH SCIENCES

CYCLE XXXVII

PRODUCTION OF PLANT-DERIVED SECONDARY METABOLITES FROM IN-VITRO CALLUS CULTURES FROM FRUIT PULP OF DIFFERENT POMACEAE

(EVALUATION OF THE BIOLOGICAL ACTIVITY OF FUNCTIONAL PLANT SECONDARY METABOLITES OBTAINED THROUGH IN VITRO CULTURE OF CALLUS PULP CELLS)

ACADEMIC DISCIPLINE: BIOS-07/A

Thesis written with financial support from the
FSE-REACT-EU programme, PON “Ricerca e Innovazione” 2014-2020 (D.M. 1061/2021)
Azione IV.5 “Dottorati su tematiche Green”

Coordinator: Prof. Ferdinando Mannello

Supervisor: Prof. Lucia Anna Maria Potenza

Ph.D. student: Federica Gubitosa

ACADEMIC YEAR
2023/2024

INDEX

ABBREVIATIONS 4

ABSTRACT 9

RESEARCH OBJECTIVE 11

INTRODUCTION 12

Natural Products: A Historical Overview 12

Plant Secondary Metabolites 14

Classification and functions of SMs in plants. 14

Plant Secondary Metabolites (SMs) Response to Environmental Factors 20

Biological Activities of Plant Secondary Metabolites 23

Uses of Plant Natural Products 29

Exploring the Applications and Techniques of Callus Culture in Plant Science 34

In vitro Culture Techniques as an Alternative System for SM production 34

Callus Culture Technique 37

Cellular and Molecular Basis of Callus Formation 38

Initiation and Maintenance of Callus Cultures 39

Factors Influencing Callus Growth and Differentiation 39

Production of Secondary Metabolites Using Callus Culture Technique 41

CHAPTER 1
CYDONIA OBLONGA MILL. PULP CALLUS INHIBITS OXIDATIVE STRESS AND INFLAMMATION IN INJURED CELLS

1.1 Introduction 44

1.2 Materials and Methods 45

1.2.1 Callus Production 45

1.2.2 Callus Extract Preparation 45

1.2.3 Chemical Characterization of SMs in Quince Callus Extract 46

1.2.4 Cell Culture 48

1.2.5 Cell Viability Assay WST8 48

1.2.6 Nitric Oxide Detection 48

1.2.7 LPS-Induced Inflammation in HaCaT Cells 49

1.2.8 Quantitative Real-Time PCR 49

1.2.9 Quantification of Intracellular ROS Production 50

1.2.10 Statistical Analysis 50

1.3 Results and Discussion 51

1.4 Conclusions 56

CHAPTER 2
LONG-LASTING PRODUCTION OF BIOACTIVE COMPOUNDS FROM MALUS X DOMESTICA BORKH VAR. MELA ROSA MARCHIGIANA PULP CALLUS CULTURE WITH ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITY

2.1 Introduction 57
2.2 Materials and Methods 59
2.2.1 Callus culture conditions and maintenance 59
2.2.2 Mela Rosa Marchigiana callus ethanolic Extract (MRME) preparation 59
2.2.3 Mela Rosa Marchigiana callus ethanolic extract analyses by GC-MS and GC-FID 60
2.2.4 DPPH scavenging activity 61
2.2.5 ABTS scavenging activity 61
2.2.6 ORAC assay 61
2.2.7 Cell cultures 62
2.2.8 Cell viability 62
2.2.9 Quantification of Intracellular ROS Production 62
2.2.10 Nitric oxide detection 63
2.2.11 Statistical analysis 63
2.3 Results and Discussion 63
2.4 Conclusions 69

CHAPTER 3
ANTI-INFLAMMATORY, ANTIOXIDANT, AND GENOPROTECTIVE EFFECTS OF CALLUS CULTURES FROM MALUS PUMILA CV. MILLER (ANNURCA CAMPANA APPLE) AND PRELIMINARY EVALUATION OF A NANOFORMULATION IN AN IN VITRO MODEL OF FERROPTOSIS

3.1 Introduction 70
3.2 Materials and Methods 72
3.2.1 Plant Material and Callus Cultures 72
3.2.2 Preparation of Extracts from Peel, Pulp, and Calli of Annurca Apple 73
3.2.3 Chemical Characterization of Extracts from Peel Pulp and Calli of Annurca Apple 73
3.2.4 Preparation of nanoformulations 76
3.2.5 Evaluation of the Physiochemical Properties of the Nanoformulations 76
3.2.6 DPPH Scavenging Activity 76
3.2.7 ABTS Scavenging Activity 77
3.2.8 ORAC Assay 77
3.2.9 Lipoxygenase Inhibition Assay 78
3.2.10 Nicking Assay 78
3.2.11 Cell Cultures 79

3.2.12 Griess Assay 79
3.2.13 Assessment of Cell Viability on HaCaT Cells 80
3.2.14 Optimization of an *In vitro* Model for Iron-Overload-Induced Oxidative Stress 80
3.2.15 Intracellular Total Iron Quantification 80
3.2.16 Intracellular Ferritin Quantification 81
3.2.17 Effects of Iron-Induced Inflammation in HaCaT Cells 81
3.2.18 Quantitative Real-Time PCR 81
3.2.19 Statistical Analysis 82
3.3. Results and Discussion 83
3.4 Conclusions 98
CONCLUSIONS 99
ACKNOWLEDGMENT 101
REFERENCES 102

ABBREVIATIONS

§: Zeta Potential

•OH: Hydroxyl Radical

2,4-D: 2,4-Dichlorophenoxyacetic Acid

3-CQA: 3-O-Caffeoylquinic Acid

4-p-CoQA: 4-P-Coumaroylquinic Acid

5-CQA: 5-Caffeoylquinic Acid

5-LO: 5-Lipoxygenase

5-p-CoQA: 5-P-Coumaroylquinic Acid

AAPH: 2,2'azobis-(2-Amidinopropane) Hydrochloride

AAPH: 2,2′-Azobis-(2-Amidinopropane) Hydrochloride

ABTS: 2,2'-Azinobis-(3-Ethyl-Benzothiazoline-6-Sulfonic Acid)

AD: Anno Domini

ADV: Adenovirus

ANOVA: Analysis Of Variance

BA: Benzyladenine

BAP: 6-Benzylaminopurine

BB4: Binding Buffer 4

BC: Before Christ

BCA: Bicinchoninic Acid

BHA: β-Hydroxy Acid

BSTFA: N, O-Bis-Trimethylsilyl-Trifluoroacetamide

CCC: Circular DNA

CHIKV: Chikungunya Virus

CMC: Critical Micellar Concentration

CNS: Central Nervous System

Co ext.: *Cydonia oblonga* callus extract

COX-2: Cyclooxygenase-2

CTRL: Control Group

DC: Dark Callus

DCF-DA: 2',7' -Dichlorofluorescein Diacetate

DEXA: Dexamethasone

DMEM: Dulbecco's Modified Eagle's Medium

DMSO: Dimethyl Sulfoxide

DNMTs: DNA Methyltransferases

DPPH: 2,2-Diphenyl-1-Picrylhydrazyl

DSA: DPPH Radical Scavenging Ability

DW: Dry Weight

EDTA: Ethylenediaminetetraacetic Acid

EGCG: Epigallocatechin-3-Gallate

EI: Electron Ionization

EMA: European Medicines Agency

ESR1: Enhancer Of Shoot Regeneration 1

EtOAc: Ethyl Acetate

EtOH: Ethanol

FBS: Fetal Bovine Serum

FDA: Food and Drug Administration

Fe_2SO_4: Ferric Sulfate

GA: Gibberellins

GC-FID: Gas Chromatography-Flame Ionization Detector

GC–MS: Gas chromatography-mass spectrometry

GERD: Gastroesophageal Reflux Disease

GPX-4: Glutathione Peroxidase-4

H1N1: Influenzavirus A subtype H1N1

H_2O_2: Hydrogen Peroxide

H_2S: hydrogen sulfide

HCl: Hydrogen Chloride

HCMV: Cytomegalovirus

HCoV-NL63: Human Coronavirus NL63

HCV: Hepatitis C Virus

HDACs: Histone deacetylases

HIV: Human Immunodeficiency Virus

HMTs: Histone Methyltransferases

HPIV: Human parainfluenza virus

HPLC-DAD-ESI-MSn: High-Performance Liquid Chromatography Coupled to Electrospray Ionization Mass Spectrometric

HRP: Horseradish Peroxidase

HRV: Rhinovirus

HSV: Herpes Simplex Virus

IAA: Indole Acetic Acid

ICAM: Intercellular Cell Adhesion Molecule

ikBα: Inhibitor kappa B-alpha

IL-1: Interleukin 1

IL-1β: Interleukin 1β

IL-6: Interleukin 6

IL-8: Interleukin 8

iNOS: Inducible Nitric Oxide Synthase

IS: Internal Standard

JNK: C-Jun N-Terminal Kinases

$K_2S_2O_8$: Potassium Persulphate

KCl: Potassium Chloride

KH_2PO_4: Potassium Dihydrogen Phosphate

$KMnO_4$: Potassium Permanganate

L DNA: Linear DNA

LC: Light Callus

LCE: Light Callus Extract

LDL: Low-Density Lipoprotein

LI: Labile Iron Pool

LPS: Lipopolysaccharide

LS: Linsmaier and Skoog medium

LTs: Leukotrienes

MEM: Minimum Essential Medium

MeOH: Methanol

MEP: Methylerythritol-4-Phosphate

MIC: Minimum Inhibitory Concentration

MRM: Mela Rosa Marchigiana

MRME: Mela Rosa Marchigiana pulp callus extract

mRNAs: messenger RNAs

MRSA: Methicillin-Resistant Staphylococcus Aureus

MS: Murashige and Skoog medium

MTT: 3-(4,5-Dimethylthiazol-2yl)-2,5-Diphenyl Tetrazolium Bromide

Na_2HPO_4: Sodium Hydrogen Phosphate

Na_2SO_4: Sodium Sulfate

NAA: Naphthalene Acetic Acid

nAChRs: Nicotinic Acetylcholine Receptors

NaOH: Sodium Hydroxide

ncRNAs: non-coding RNAs

NF-B: Blank Nanoformulation

NF-LCE: Nanoformulated Light Callus Extract

NF-TA: Nanoformulated Tormentic Acid

NF-κB: Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells

NO: Nitric Oxide

NPs: Natural Products

NrF2: Nuclear Factor Erythroid 2-Related Factor 2

OC: Open Circular Form

ON: Over Night

ORAC: Oxygen Radical Absorbance Capacity

PBS: Phosphate Buffered Saline

PCs: Phenolic Compounds

PDI: Polydispersity Index

PEG: Polyethylene Glycol

PGI: Protected Geographical Indication

PGRs: Plant Growth Regulators

PLT: PLETHORA

PMs: Primary Metabolites

PPO: Polyphenylene Oxide

PTAs: Pentacyclic Triterpenic Acids

ROS: Reactive Oxygen Species

RT: Room Temperature

SA: Scavenging Activity

SD: Standard Deviation

SEM: Standard Error of the Mean

SH: Schenk and Hildebrandt medium

SMs: Secondary Metabolites

SPF: Sun Protection Factor

TA: Tormentic Acid

TBE: Tris Borate EDTA

TBHP: Tert-butyl Hydroperoxide

TCA: Tricarboxylic Acid Cycle

TEM: Transmission Electron Microscope)

TEq: Trolox Equivalents

TLR4: Toll-Like Receptor-4

TMB: 5,5′-Tetramethyl-Benzidine

TMS: Trimethylsilyl Derivatives

TNF-α: Tumor Necrosis Factor-Alpha

TROLOX: 6-Hydroxy-2,5,7,8-Tetramethylchroman-2-Carboxylic Acid

UV: Ultraviolet Radiation

VOCs: Volatile Organic Compounds

WHO: World Health Organization

WIND: Wound Induced Dedifferentiation

WOX: WUSCHEL-Related Homeobox

WST-8: Water-Soluble Tetrazolium salt-8

ABSTRACT

This doctoral thesis focused on evaluating the biological activities of hydroalcoholic extracts derived from callus pulp cultures of three selected apples: *Cydonia oblonga Mill.*, Mela Rosa Marchigiana, and *Malus pumila* cv Miller (Annurca Campana Apple).

For each apple, the research followed the following workflow: (1) establishment of callus cultures and extract preparation, (2) qualitative-quantitative chemical characterization of SM extracts, and (3) evaluation of their biological activity.

In *Cydonia oblonga Mill.*, the hydroalcoholic extract, previously found to be rich in phenolic and triterpenic compounds, demonstrated a potent anti-inflammatory effect in *in vitro* models of LPS-stimulated RAW 264.7 macrophages and HaCaT keratinocytes. Results indicated significant reductions in NO production, and pro-inflammatory gene expression (iNOS, IL-6, IL-1β, ikBα, ICAM). Moreover, the extract reduced the radical oxygen species levels in the hydrogen peroxide (H_2O_2) and tert-butyl hydroperoxide- (TBHP) injured HaCaT keratinocytes revealing an important antioxidant activity.

For *Mela Rosa Marchigiana*, callus cultures from sub-cultures maintained high triterpenic acid content and bioactivity over the years. Comparative analysis with previous extracts confirmed consistent antioxidant, and anti-inflammatory effects, as demonstrated through cell-free (DPPH, ABTS, and ORAC) and cell-based assays (LPS-stimulated RAW 264.7 macrophages and H_2O_2 injured HaCaT keratinocytes).

In *Malus pumila cv Miller*, two callus cultures were cultivated under darkness and photoperiod conditions. The selected conditions for generating the two types of calli facilitated the production of substantial amounts of triterpenic compounds. Both calli primarily exhibit anti-inflammatory and genoprotective (tested using the lipoxygenase inhibition activity assay and LPS-stimulated RAW 264.7 macrophages, and the nicking assay respectively) effects while showing lower antioxidant activity (evaluated by (DPPH, ABTS, and ORAC assays). The callus cultivated in light conditions was chosen for encapsulation in a nanocarrier, which allowed for the assessment of its anti-inflammatory activity in a cellular model of ferroptosis on HaCaT cells. The results highlight the promise of Annurca Apple Callus extract in addressing oxidative stress and inflammation linked to excess iron. The nanocarrier system greatly improves its therapeutic effectiveness, as evidenced by gene expression analysis showing that the encapsulated extract can modulate the expression of genes associated with the ferroptosis process (COX-2, GPX-4, NrF2, IL-6, and TNF-α).

Overall, this study highlights the potential of callus culture technology for producing bioactive SMs with pharmaceutical, cosmetic, and nutraceutical applications. It also emphasizes the importance of optimizing culture conditions and leveraging advanced delivery systems to maximize the therapeutic

potential of plant-derived compounds. These findings pave the way for scalable and sustainable production strategies in bioactive compound research and industry applications.

RESEARCH OBJECTIVE

Plant secondary metabolites play crucial roles in ecosystems and human health, offering antioxidant, anti-inflammatory, and disease-preventing properties. They are valuable in pharmaceuticals, nutraceuticals, and agriculture, serving as natural pesticides and growth regulators that reduce reliance on synthetic chemicals.

Traditional extraction methods face challenges like variability in yield, environmental impact, and ethical concerns, particularly for endangered species.

Callus cultures provide a sustainable and efficient method for large-scale secondary metabolite production, bypassing the limitations of field cultivation. By creating a controlled environment, they ensure consistent production, unaffected by climate or soil conditions. This reduces pressure on wild plant populations and supports conservation. Callus cultures are scalable for industrial use, grown in bioreactors, and can be enhanced with elicitors to boost metabolite production. Adjusting factors like light, temperature, and pH optimizes yield. This approach ensures high-quality, pure metabolites with shorter production cycles, accelerating research and commercial use availability.

In this context, this thesis seeks to establish callus cultures derived from the pulp of various apple varieties, namely *Cydonia oblonga* Mill., Mela Rosa Marchigiana, and *Malus pumila* cv. Miller (Annurca Campana Apple). The study will involve the extraction of corresponding hydroalcoholic extracts, followed by testing these extracts in both cell-free and cell-based models to elucidate their potential applications in conditions wherein oxidation and inflammation are pivotal factors.

INTRODUCTION

Natural Products: A Historical Overview

Nutrition plays a pivotal role in maintaining the balance between health and disease by providing essential nutrients and bioactive compounds that support overall well-being. Plants are precious, offering vital macronutrients and micronutrients and a diverse array of secondary metabolites with health-enhancing properties. For centuries, these plant-derived compounds have been utilized for their therapeutic benefits. Modern diets rich in vegetables, fruits, grains, and legumes are especially beneficial due to their high content of carotenoids, phytochemicals, fiber, vitamins, and other essential bioactive constituents, all contributing to human health [1-3].

Plant natural products (NPs) have served as therapeutic agents for millennia. Evidence of their use dates to 2,600 BC, with Mesopotamian clay tablets describing about 1,000 plant-based remedies, including cypress and myrrh, still in use today. Similarly, the ancient Egyptian *Ebers Papyrus* (2,900 BC) lists over 700 plant-derived treatments. Traditional Chinese Medicine, documented in texts like the *Chinese Materia Medica* (1100 BC) and the *Shennong Herbal* (100 BC), and Ayurveda, originating in the first millennium BC, also feature extensive use of plant-based drugs [4-7]. In the Western world, Greek and Roman cultures laid the foundation for herbal medicine. Figures like Theophrastus, Pliny the Elder, Galen, and Dioscorides extensively documented medicinal plants between 100 BC and the second century AD. During the 5th to 12th centuries, European monasteries preserved this knowledge, while Arab scholars expanded it by integrating Chinese and Indian herbal practices. Pioneers like Avicenna advanced pharmacy and medicine, notably with the *Canon Medicinae*. By the 10th century, the merging of Arab and Greco-Roman traditions in southern Italy led to the establishment of the Salerno school, an early model for modern universities [8,9]. The invention of the printing press in the 15th century revived interest in herbal medicine, enabling the widespread dissemination of works like *The Mainz Herbal*, *The German Herbal*, and Leonhart Fuchs' *De Historia Stirpium* [6].

In 1804, Friedrich Sertürner, a German apothecary assistant, revolutionized drug discovery by isolating morphine from opium, introducing the alkaloid class of drugs. This milestone spurred extensive exploration of medicinal plants throughout the 19th century, with apothecaries like H.E. Merck extracting morphine and other alkaloids. By 1853, the focus shifted to chemical synthesis, exemplified by the production of salicylic acid [9,10]. Alexander Fleming's discovery of penicillin in 1928 marked a turning point, igniting widespread research into natural products, particularly from microbes. During and after World War II, this work laid the foundation for the modern pharmaceutical industry, with companies heavily investing in antibiotics and microbial fermentation [11,12].

By the late 20th century, natural product discovery programs became standard in pharmaceutical companies, leading to breakthroughs like the 1970s development of statins (e.g., compactin and mevinolin), revolutionizing cholesterol management and everyday medicine [13].

The success of natural product (NP) drug discovery in the 1980s and 1990s spurred a shift toward chemical synthesis to address the growing demand for new drugs. This transition enabled the development of extensive libraries of synthesized compounds using combinatorial and computational chemistry techniques. Nevertheless, NP-based drug discovery remains an active and promising research field, particularly within academia and startups, where it continues to drive innovation and exploration [14-17].

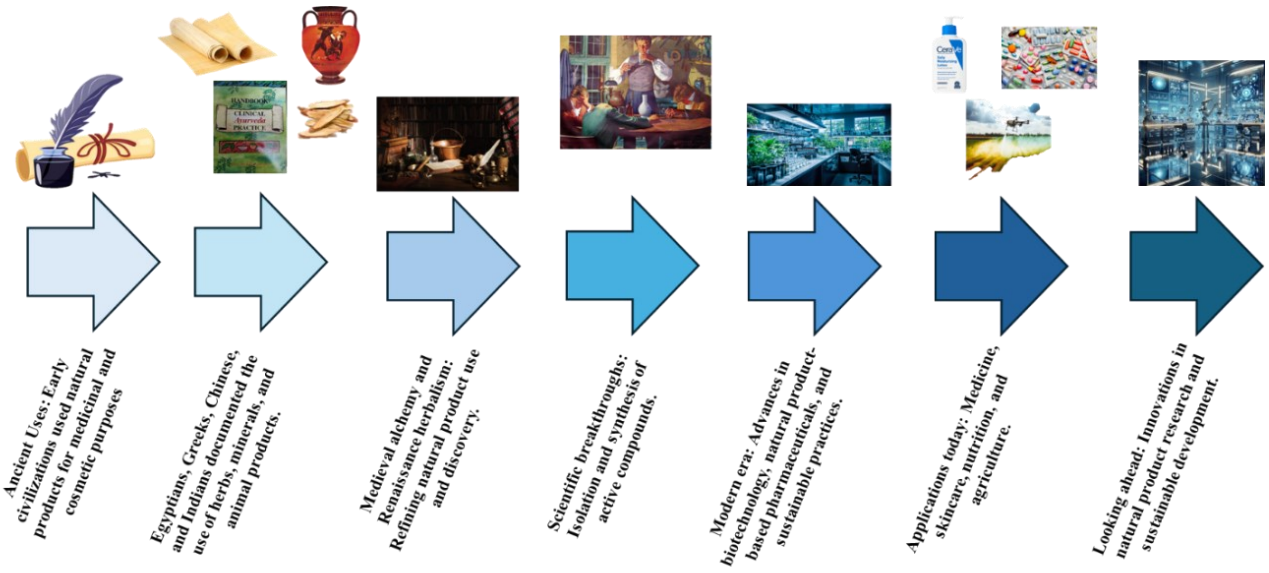


Figure 1: **The Evolution of Drug Discovery – From Ancient Herbal Remedies to Modern Synthetic Chemistry and Biotechnology.** (Imagine modified from: https://doi.org/10.1080/14786419.2017.1356838).

Plant Secondary Metabolites

Classification and functions of SMs in plants.

Plant metabolites, essential in agriculture, medicine, and pharmaceuticals, are classified into primary metabolites (PMs) and secondary metabolites (SMs). Primary metabolites, produced during the growth phase (trophophase), include carbohydrates, amino acids, fatty acids, and organic acids. These metabolites are crucial for growth, respiration, photosynthesis, hormone and protein synthesis, and energy production. They also form key components of cell structures, such as membranes, cell walls, and cytoskeletons, and play a role in genetic material (DNA and RNA). Furthermore, PMs act as signal molecules in defense responses, facilitating signal transduction and pathogen recognition [18,19].

Plant secondary metabolites (SMs), including over 12,000 alkaloids, 40,000 terpenoids, and 8,000 phenylpropanoids, are exclusively produced by plants. SMs are synthesized in response to environmental stress, with factors like temperature, chemical exposure, microbes, and insects triggering increased production. While plants generally maintain a balanced redox state, stress disrupts this balance, prompting SM biosynthesis as an adaptive mechanism [20]. SM synthesis involves complex biochemical pathways, including the tricarboxylic acid cycle (TCA), methylerythritol-4-phosphate (MEP) pathway, mevalonic and shikimic acid pathways, and the phenylpropanoid pathway, which produces compounds like lignin, flavonoids, and coumarins. These pathways yield a diverse array of SMs from limited intermediates. The production of SMs varies by cell type, developmental stage, and environmental conditions, contributing to the plant's defense, systemic resistance, and ecological roles such as protecting against herbivores, pathogens, and competing plants, and interacting with pollinators. While SMs are not essential for basic plant functions, their absence can impact long-term survival [21-23].

Certain SMs are specific to distinct plant groups and can vary among populations, individual plants, and stages of development. This variability arises from genetic differences, environmental factors like heat stress and drought, and attacks from herbivores and pathogens, reflecting the plant's adaptation to its surroundings and stressors [24-26].

SMs can be classified into three main classes based on their chemical structures and biosynthetic pathways: terpenoids, phenolic compounds, and nitrogen-containing compounds [27].

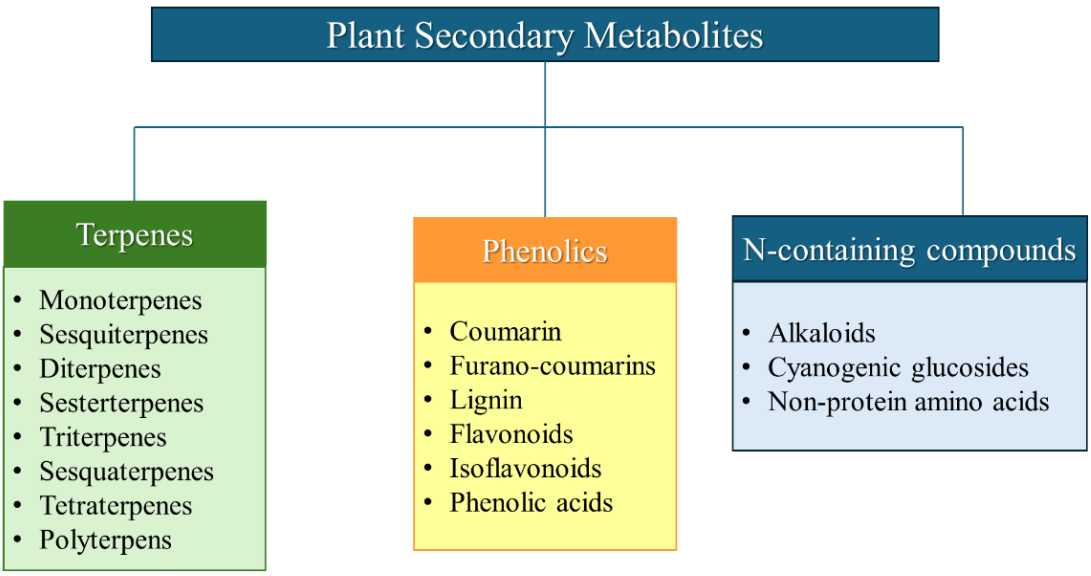


Figure 2: **Plant Secondary Metabolites classification.** (Image modified from https://doi.org/10.3390/ijpb13010003)

Terpenoids

Terpenes and terpenoids are a diverse family of natural compounds derived from 5-carbon isoprene units. Terpenes consist solely of isoprene units, while terpenoids include additional functional groups like ketones or hydroxyl rings. Classified by isoprene unit count, they follow a head-to-tail condensation pattern. Terpenoids are further categorized as aliphatic (e.g., geraniol) or cyclic (e.g., limonene) and are synthesized via the mevalonic acid pathway (cytosol) or the 2-C-methyl-D-erythritol-4-phosphate pathway (plastid) [18,20,28].

Terpenes are classified into several categories based on the number of isoprene units they contain:

- **Monoterpenes**: Composed of two isoprene units ($C_{10}H_{16}$), these compounds are found in the essential oils of plants from families such as Lamiaceae and Rutaceae. Examples include limonene (a hydrocarbon), linalool (an alcohol), and citronellal (an aldehyde).
- **Sesquiterpenes**: Consisting of three isoprene units ($C_{15}H_{24}$), these compounds are divided into acyclic (e.g., farnesol), monocyclic (e.g., bisabolol), and bicyclic (e.g., caryophyllene).
- **Diterpenes**: Composed of four isoprene units ($C_{20}H_{32}$), these molecules include compounds such as phytol, a component of chlorophyll, and taxol, known for its antitumor properties. They can be either acyclic or macrocyclic.
- **Sesterterpenes**: Contain five isoprene units and have a molecular formula of $C_{25}H_{40}$.
- **Triterpenes**: Consisting of six isoprene units ($C_{30}H_{48}$), they include compounds like squalene and tetracyclic triterpenes (e.g., dammarenes) and pentacyclic triterpenes (e.g., lupanes). They are often found in saponins (photogenic biosurfactants) and steroids, with cholesterol being a basic structure [29].
- **Sesquiterpenes**: Composed of seven isoprene units with a molecular formula of $C_{35}H_{56}$.

- **Tetraterpenes**: Contain eight isoprene units ($C_{40}H_{64}$), including carotenoids and natural rubbers from plants like those in the Euphorbiaceae family.
- **Polyterpenes**: Comprise long chains of many isoprene units.
- **Norisoprenoids**: Derived from terpenes by shortening the chain or modifying side chains.
- **Hemiterpenes**: Rare compounds, like isoprene, are found in some plants [30-32].

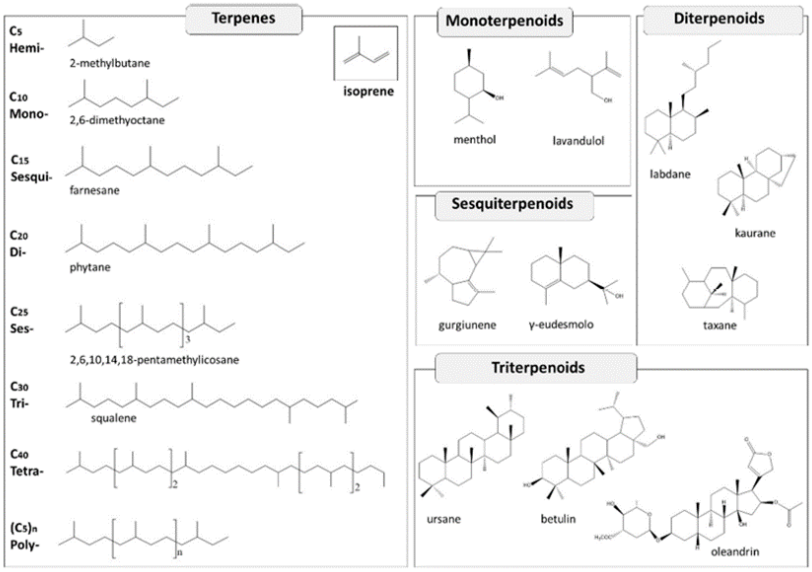


Figure 3: Examples of terpene chemical structures and their classification based on the number of isoprene units in the structure. (Image from https://doi.org/10.3390/molecules26020495).

Terpenoids play key defensive roles in plants like polypodium, peppermint, lemon, basil, sage, corn, cotton, and wild tobacco [33]. They release phytoalexins to combat pathogens and VOCs to attract herbivore predators. Monoterpenes are toxic to insects, fungi, and bacteria, while steroidal saponins like cardenolides inhibit Na+/K+ ATPase, poisoning animals [34,35]. Terpenoids also help manage stress: sesquiterpene β-caryophyllene and monoterpenes (e.g., 1,8-cineole, α-pinene) combat oxidative stress with antioxidant properties, and carotenoids provide photoprotection against photodamage and oxidative stress [36-38].

Phenolic Compounds

Phenolic compounds (PCs) are diverse plant secondary metabolites characterized by one or more phenol groups. They include 4,000–4,500 flavonoids, primarily derived from the shikimate pathway, and range from simple phenols like vanillin to complex lignins, coumarins, quinones, and tannins. PCs are classified based on their structure, including the number of aromatic rings, carbon atoms, and hydroxyl groups [18,20,28].

Phenolic compounds are broadly classified into:

- **Simple Phenols**: Single benzene ring (C6) with a hydroxyl group, e.g., resorcinol, catechol, and phloroglucinol.
- **Phenolic Acids**: Contain a carboxylic group on the benzene ring, further divided into:
    - **Benzoic Acid Derivatives (C6-C1)**: e.g., gallic acid, vanillic acid, syringic acid.
    - **Hydroxycinnamic Acid Derivatives (C6-C3)**: e.g., caffeic acid, ferulic acid, coumaric acid.
- **Flavonoids**: Two benzene rings linked by a three-carbon chain, often forming a pyran ring. Sub-classes include chalcones, flavones, flavonols, and anthocyanidins, found as aglycones or glycosides.
- **Tannins**: Polyphenolic compounds divided into:
    - **Condensed Tannins**: Flavonoid unit polymers.
    - **Hydrolyzable Tannins**: Esters of gallic or ellagic acids with a monosaccharide, usually glucose [39,40].

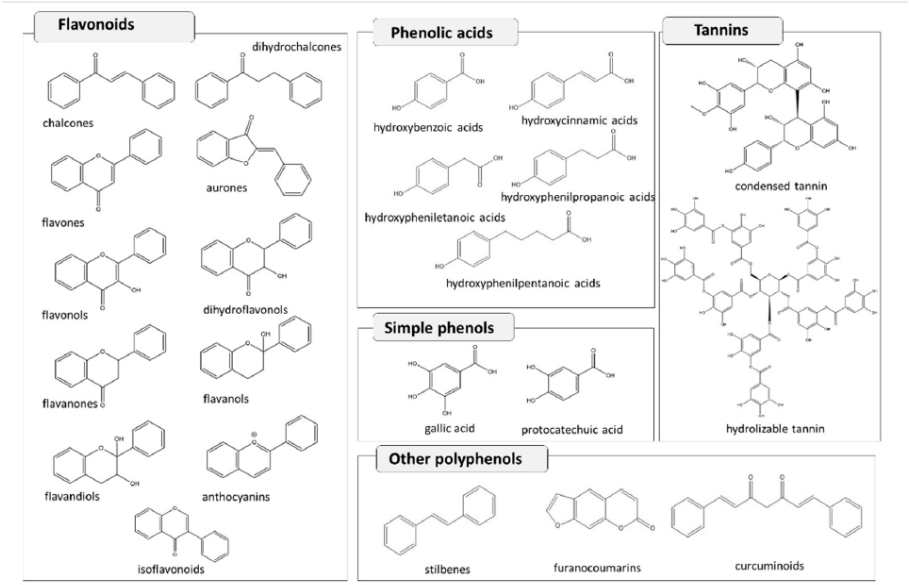


Figure 4: **Examples of polyphenols, basic nucleus, and classification.** (Image from https://doi.org/10.3390/molecules26020495).

Polyphenols are distributed across all plant organs, with phenolic acids in seeds, leaves, roots, and stems; flavonoids in aerial parts; and tannins in roots, bark, and seeds [41]. Flavonoids, the largest group of natural phenols, play key roles in nitrogen fixation, flower pigmentation, and reproduction by attracting pollinators. They deter herbivores, exhibit antioxidant properties, and help plants adapt to UV stress and extreme temperatures [42-45]. Anthocyanins absorb UV radiation and scavenge reactive oxygen species (ROS), while flavonoids like quercetin chelate transition metals to prevent oxidative damage [46,47].

Nitrogen-Containing Compounds

Nitrogen-containing compounds in plants, such as alkaloids, cyanogenic glucosides, and glucosinolates, play vital roles in plant defense. Alkaloids are the most prominent, characterized by nitrogen atoms derived from amino acid decarboxylation and distributed widely in plant orders like *Caryophyllales*, *Magnoliales*, *Rosales*, and *Gentianales*. They are typically classified based on carbon skeletons or biochemical precursors, although compounds like nicotine, derived from both nicotinamide and ornithine, complicate this system. Traditionally, alkaloids are defined as nitrogen-containing metabolites with heterocyclic rings. This group includes:

- **Proto-alkaloids**: Non-heterocyclic compounds derived directly from amino acids, e.g., hordenine, ephedrine, colchicine, and capsaicin.
- **True Alkaloids**: Heterocyclic nitrogen compounds primarily derived from amino acids such as ornithine, lysine, tyrosine, tryptophan, and phenylalanine, including tropane, pyrrolidine, and benzylisoquinoline alkaloids.
- **Pseudoalkaloids**: Not derived from amino acids but incorporate nitrogen via transamination or amination. These include purine, sesquiterpene, and steroidal alkaloids (e.g., glycoalkaloids), which are common in *Solanaceae* and *Liliaceae* families, featuring a C27 cholestane skeleton glycosylated with monosaccharides.

Unique alkaloid types include polyamine alkaloids (from putrescine, spermidine, and spermine), peptide alkaloids, and cyclopeptide alkaloids. These nitrogen-containing compounds are crucial for plant defense and exhibit diverse bioactivities, highlighting their ecological and pharmaceutical significance [48-51].

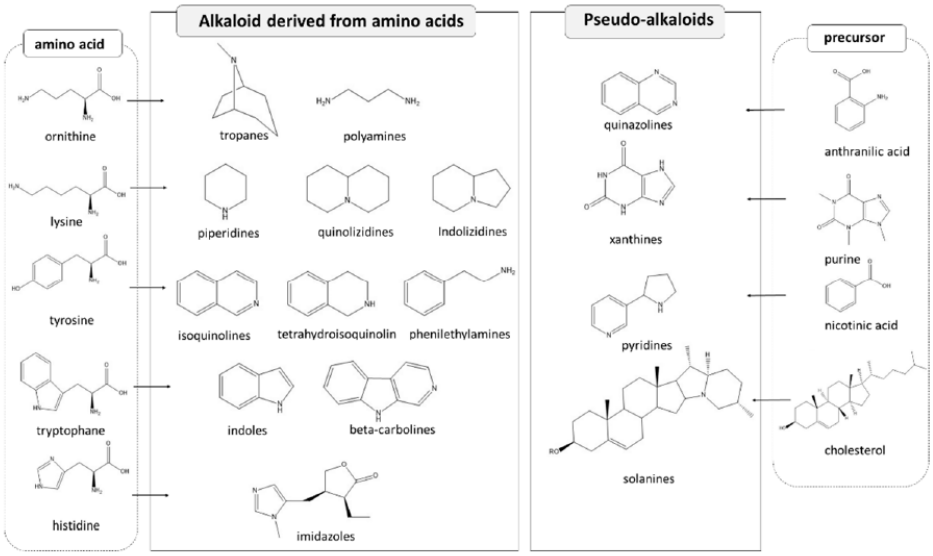


Figure 5: **Example of alkaloids (basic skeletons) and their precursors.** (Image from https://doi.org/10.3390/molecules26020495)

Alkaloids, abundant in flowering plants (*Angiospermae*), are found in various plant organs such as leaves, flowers, seeds, roots, stems, fruits, bark, and bulbs. Their distribution varies across species and depends on the plant's life cycle stage [52]. While most plant families produce one type of alkaloid, some, like *Solanaceae* and *Rutaceae*, accumulate a wide range. Alkaloid biosynthesis is genetically controlled but influenced by environmental factors like light, temperature, moisture, and soil nutrients [53-55].

Alkaloids serve multiple roles in plant metabolism, acting as nitrogen storage, growth regulators, and defense agents. Their toxicity and bitterness help protect plants from herbivores and pathogens, and some alkaloids also aid reproduction by attracting pollinators [56,57].

Other nitrogen-containing defense compounds, such as cyanogenic glycosides and glucosinolates, are less structurally diverse than alkaloids but also serve important defensive functions. Cyanogenic glycosides are found in ferns, gymnosperms, and angiosperms, while glucosinolates are common in the *Capparales* order and the *Drypetes* genus of the *Euphorbiaceae* family. These compounds are concentrated in young leaves and reproductive tissues and can increase in response to environmental stress, such as drought or high temperatures. For example, tropical *Prunus turneriana* accumulates higher cyanogenic glycoside levels in older leaves under low-light conditions, demonstrating their adaptive role in defense [58-61].

Plant Secondary Metabolites (SMs) Response to Environmental Factors

The production of phytochemicals in plants is tightly regulated by environmental factors such as light, temperature, soil, and salinity, which significantly influence growth and SMs synthesis. Environmental stresses often trigger SMs production, enhancing plant adaptation and survival. In medicinal plants, these changes can affect the type and quantity of bioactive compounds. Optimizing controlled environments, including the use of biotic and abiotic elicitors, is essential for maximizing SMs production, both *in vivo* and *in vitro*, to support drug development [62-67].

Temperature Effects on SM Production

Temperature significantly influences SMs production in plants, with effects varying by species and conditions. High temperatures often boost SMs like terpenoids, flavonoids, and alkaloids, which help mitigate oxidative stress. For instance, tomatoes increase terpene emissions under heat stress, while citrus plants may shift towards antioxidants like ascorbate instead of terpenes. Similarly, poppies and sweet basil show elevated alkaloid and flavonoid production under high temperatures. Cold stress also impacts SMs synthesis, promoting compounds like anthocyanins, phenolic acids, and glucosylated terpenoids, which aid cold tolerance. For example, tartary buckwheat upregulates anthocyanins and proanthocyanidins, *Arabidopsis thaliana* and soybeans accumulate phenolic acids and isoflavonoids, and Indian ginseng increases withanolides. Tea plants, meanwhile, produce more glucosylated terpenoids under cold conditions [20,64,68].

Drought Stress and SM Production

Drought stress, a major abiotic challenge, impacts plant morphology, physiology, and biochemistry, often enhancing SMs production. Compounds such as flavonoids, alkaloids, and terpenes increase under drought, helping plants combat oxidative damage and maintain cellular stability. For example, drought boosts flavonoid levels, including flavonols and anthocyanins, as seen in *Cistus clusii* and *Arabidopsis thaliana*, where these compounds enhance stress tolerance.

Alkaloid production also responds to drought, with increased camptothecin levels in *Camptotheca acuminata* and higher alkaloid content in *Papaver somniferum*. Similarly, drought significantly raises monoterpenes and resin acids in conifers like *Pinus sylvestris* and *P. abies* These responses highlight drought stress as a trigger for SM production, enhancing plant survival under water-limited conditions [20,64,68].

Soil Salinity and SM Production

Salt stress, a severe abiotic challenge, disrupts plant growth and metabolism by causing nutritional imbalances, osmotic stress, and oxidative damage. Its effects on SMs production vary by species and conditions. For example, high salinity doubles polyphenol levels in *Aegiceras corniculatum* but reduces phenolic and flavonoid content in broccoli. Alkaloid production is also influenced by salinity;

in *Catharanthus roseus*, NaCl increases indole alkaloids and vincristine, though prolonged exposure eventually reduces levels. Similarly, in *Rosmarinus officinalis*, salinity shifts monoterpene profiles, enhancing cineole and camphor while reducing others.

Nutrient stress further affects SMs. Potassium deficiency increases alkaloid concentrations in lupin seeds, while nitrogen fertilizers, particularly ammonium nitrate, boost alkaloid and monoterpenoid levels in plants like Douglas-fir and *Thuja plicata* during active growth. These findings highlight the complex interplay between salinity, nutrient availability, and plant SM production [20,64,68].

Soil Fertility and SM Production

Nutritional stress significantly influences plants' SMs production, with effects varying based on nutrient availability, plant species, and developmental stage. Deficiencies in nitrogen and phosphate often lead to increased flavonoid accumulation as an adaptive response. For example, in *Arabidopsis* and tomato seedlings, limited nitrogen and phosphate levels enhanced flavonols like quercetin, kaempferol, and isorhamnetin. In contrast, excessive nitrogen, as seen in *Labisia pumila*, reduced phenolic and flavonoid content.

Potassium deficiency can also boost SMs production, with alkaloid levels in lupins increasing by 205%. Additionally, nitrogenous fertilizers, particularly ammonium nitrate, stimulate alkaloid and monoterpenoid production in plants such as Douglas-fir and *Thuja plicata*. These examples highlight the complex interplay between nutrient availability and SM biosynthesis in plants [64,69].

Ultraviolet (UV) Radiation and SM production

Light plays a pivotal role in plant growth and SMs production, with its duration, intensity, and quality significantly influencing SMs biosynthesis. Longer photoperiods often boost SMs; for instance, *Ipomoea batatas* produces more flavonoids and phenolic acids under 16 hours of light, while *Xanthium* species reduce caffeoylquinic acids and flavonoid levels with shorter light durations.

UV radiation stimulates SMs production, with plants like *Brassica napus* and *Fagopyrum esculentum* increasing antioxidative flavonoids such as quercetin and luteolin under UV-B exposure. Similarly, UV enhances anthocyanins in *Daucus carota* cell cultures and glycosyl flavonoids in *Passiflora* calluses.

Light quality also affects SMs synthesis. High-intensity light triples anthocyanin levels in *Berberis microphylla*, and full sunlight increases asiaticoside in *Centella asiatica* compared to shaded conditions. However, excessive red light reduces phenolic content in *Lactuca sativa*. Shading can modulate SMs differently across plant parts; for example, 27% shading raises camptothecin in *Camptotheca acuminata* leaves but lowers it in roots. Altitude also impacts SMs, with higher solar radiation increasing rutin levels in *Hypericum perforatum* [20,64,68].

Other factors that can influence SM production

The synthesis and accumulation of SMs in plants are also influenced by environmental stresses such as soil type, composition, wounding, metal ions, circadian rhythm, and geography. For example, nutrient levels and metal stress can alter SMs profiles, while geographical differences lead to variations in SMs content. Plants' responses to combined stresses are intricate and cannot always be predicted from studies of individual stresses. Furthermore, the soil microbial community is crucial in promoting plant growth and health. Understanding the relationship between soil microbial composition and the synthesis of medicinal plant constituents is important for cultivating plants with high active ingredient content for pharmaceutical purposes [64].

Environmental factors greatly influence the production of secondary metabolites in plants. These responses help plants adapt to stresses and survive. Understanding these interactions is vital for optimizing the cultivation of medicinal plants and boosting bioactive compound production through controlled environmental conditions.

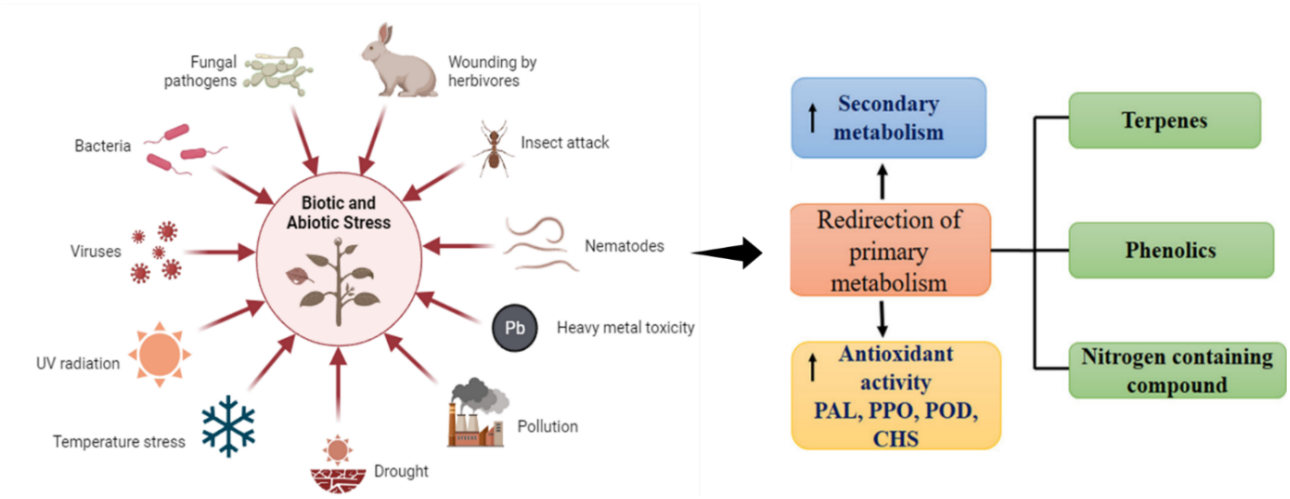


Figure 6: **Influence of exogenous elicitors on secondary metabolite production in plants.** Under natural and *in vitro* conditions, plants respond to stresses and biotic disturbances by triggering defense mechanisms through signaling pathways. These responses impact primary metabolism and bioactive compound production, with outcomes varying based on species, genotype, cultivar, developmental stage, and physiological state.

Biological Activities of Plant Secondary Metabolites

Interest in plant-derived medicines has grown significantly in recent decades due to their therapeutic potential and minimal side effects. The World Health Organization (WHO) reports that 60% of the global population relies on phytomedicines, rising to 80% in developing countries. Advances in analytical techniques like chromatography, NMR, and enzymology have enabled comprehensive studies of secondary metabolites, uncovering their biosynthesis, mechanisms of action, and therapeutic potential.

Secondary metabolites play vital roles in medicine, serving as antibacterial, antifungal, anticancer, and cholesterol-lowering agents, as well as immunosuppressants, antiparasitic agents, herbicides, and diagnostic tools. Emerging research also highlights their potential in addressing unmet medical needs, with activities such as anti-HIV, antitumor, anti-aging, and immunosuppressive effects, underscoring their expanding role in modern medicine [70-72].

Antimicrobial Activity

Infectious diseases remain a major global health issue, with antibiotic-resistant microorganisms worsening the problem and limiting treatment options. This is compounded by a lack of new antibiotic development, creating an urgent need for alternative solutions in infectious disease management. Through their secondary metabolites, plants offer a promising source of antimicrobial agents with potential therapeutic benefits due to their efficacy against pathogens and minimal side effects [73]. The development of plant-based antimicrobials poses significant challenges, primarily due to their limited activity and unfavorable pharmacokinetics. However, it is possible to enhance their efficacy through specific structural modifications [74].

Phenols are significant secondary metabolites known for their antimicrobial properties due to hydroxyl groups and aromatic rings [75]. One example is resveratrol, a polyphenol found in grapes, peanuts, blueberries, and cranberries. It acts as a plant defense against fungal infections and can inhibit various pathogens, including Gram-positive and Gram-negative bacteria. However, effective concentrations often exceed 100 mg/L. Resveratrol faces challenges in clinical use; oral and intravenous administrations lead to rapid metabolism, preventing adequate plasma levels for effective treatment. Topical application, however, can achieve sufficient bactericidal concentrations and effectively reduce acne vulgaris lesions [76-80]. Acacetin is an O-methylated flavone found in plants like *Turnera diffusa* and propolis, known for its broad antimicrobial activity. At a concentration of 64 mg/L, it enhances norfloxacin's effectiveness, halving its minimum inhibitory concentration (MIC). Acacetin significantly inhibits methicillin-resistant Staphylococcus aureus (MRSA) by targeting sortase A, and it is effective against *Enterococcus faecalis*, *Escherichia coli*, and *Pseudomonas*

aeruginosa. It also inhibits the growth of several species, including *Actinomyces naeslundii* and *Porphyromonas gingivalis*, but does not affect *Aggregatibacter actinomycetemcomitans* [81-84].

Terpenoids, including mono-, di-, and triterpenes, are well-known for their potent antibacterial properties. Their antimicrobial effects often involve disrupting metabolic pathways, such as inhibiting glutamate and aspartate metabolism [85,86]. Essential oils, which are rich in terpene-derived secondary metabolites, play a key role in plant fragrance and possess significant antibacterial activity. Among their components, carvacrol and thymol stand out for their effectiveness against pathogens such as *Mycobacterium tuberculosis* and *Mycobacterium bovis*. Carvacrol, found in essential oils from oregano, thyme, and other aromatic plants, exhibits robust antimicrobial activity due to its phenolic structure and hydrophobic nature. It is effective against a wide range of bacteria, including those responsible for foodborne illnesses, and has shown potential in combating biofilm-associated infections, particularly when combined with nanomaterials [87]. Thymol, a structural isomer of carvacrol, also displays broad-spectrum antibacterial properties. However, its practical application is limited by the high doses required for efficacy and its associated toxicity. Further research is needed to identify safe and effective concentrations to maximize its therapeutic potential [88].

Thanks to the nitrogen atom within an amine group, alkaloids can form hydrogen bonds with various biomolecules influencing their biological activities, including antibacterial ones [89]. Caffeine, a xanthine alkaloid found mainly in coffee beans, has antibacterial effects against *Pseudomonas aeruginosa* by inhibiting biofilm formation and disrupting quorum sensing. Its minimum inhibitory concentration (MIC) is 200 mg/L [90]. Berberine, an alkaloid from plants like *Berberis vulgaris* and *Coptis chinensis*, is used to treat bacterial diarrhea. It inhibits enterotoxins from *Vibrio cholerae* and *Escherichia coli* and reduces adherence in *Streptococcus pyogenes*. Berberine is also effective against *Staphylococcus aureus* and, when combined with vancomycin, enhances treatment for *Clostridium difficile* infections in mice. Additionally, it forms nanoparticles with Cinnamomi cortex (CinA) that show activity against *Methicillin-resistant Staphylococcus aureus* (MRSA) and can eliminate biofilms. These nanoparticles effectively deliver targeted antibacterial effects [91-94].

Overall, plant-derived compounds hold promise for addressing antibiotic resistance and providing alternative therapeutic options, but their practical application requires further research and development.

Antioxidant Activity

Free radicals and reactive oxygen species (ROS) are highly reactive molecules produced in biological systems. While they are vital for cellular signaling and homeostasis, excessive ROS can lead to oxidative stress, damaging lipids, proteins, and DNA. This damage is linked to diseases such as diabetes, cancer, cardiovascular disorders, and neurodegenerative diseases. Cells use their antioxidant

systems to counter ROS, and medicinal plants are particularly rich in antioxidants from phytochemicals like phenolics, terpenes, and alkaloids, which help neutralize free radicals and prevent oxidative damage [95,96].

Among various phytochemicals, phenolic compounds are particularly notable for their potent antioxidant activity, as demonstrated in both *in vivo* and *in vitro* studies. Plant families such as *Rosaceae*, *Asteraceae*, and *Lamiaceae* are especially rich in phenolic compounds. For instance, cyanidin-3-O-glucoside (an anthocyanin) and verbascoside (a phenylethanoid glycoside) have been shown to enhance cell viability and reduce reactive oxygen species (ROS) levels under hyperglycaemic conditions, suggesting their potential to mitigate the progression of diabetic retinopathy [97,98]. In addition to their antioxidant properties, phenolic compounds also exhibit significant metal-chelating activity. Extracts from plants like *Medicago sativa* and *Allium porrum* have been observed to reduce iron levels effectively in thalassemia patients, highlighting their therapeutic potential [99]. Other phenolics with both strong antioxidant and metal-chelating properties include curcumin, epigallocatechin-3-gallate (EGCG), quercetin, myricetin, kaempferol, and baicalin. These compounds have shown promise in addressing conditions associated with elevated ROS and metal ion levels, such as cancer and atherosclerosis, underscoring their importance in combating oxidative stress-related diseases [100,101].

Terpenes have garnered significant attention as a rich source of natural antioxidants, with their activities commonly evaluated through in vitro chemical assays such as DPPH, ABTS, and ORAC [102]. Andrographolide, a diterpenoid lactone isolated from *Andrographis paniculata*, has demonstrated promising potential in managing diabetes by lowering plasma glucose levels and mitigating complications such as diabetic retinopathy [103]. The antioxidant properties of essential oils from various plants, including *Satureja macrostema*, cinnamon, clove, basil, and *Origanum vulgare*, are largely attributed to the presence of terpenes such as pulegone, linalool, thymol, limonene, and carvacrol. These compounds not only neutralize free radicals but also contribute to the therapeutic applications of essential oils, underscoring their value in addressing oxidative stress-related health issues [104-106].

Although alkaloids generally have moderate to low radical scavenging ability, some studies suggest they may possess antioxidant properties. For example, indole alkaloids from *Catharanthus roseus* reduced oxidative damage in pancreatic β-TC6 cells [107]. Notably, canadine exhibited low cytotoxicity and inhibited lipid peroxidation, while other alkaloids like theobromine, linearilin, lycotoine, and betacarboline also demonstrated antioxidant activity [108-110].

Anti-inflammatory Activity

Inflammation is a complex response that protects body tissues from infections, damage, and irritants by mobilizing immune cells and stimulating blood vessels. While it serves a protective role, chronic inflammation can lead to health problems, making anti-inflammatory compounds important for symptom relief. Natural herbs like Curcuma longa (turmeric), Zingiber officinale (ginger), Rosmarinus officinalis (rosemary), and Borago officinalis (borage) have shown significant anti-inflammatory effects and hold promise for clinical use [111].

Curcumin, a phenolic compound from *Curcuma longa*, exhibits strong anti-inflammatory activity by inhibiting NF-κB expression and downregulating pro-inflammatory mediators like TNF-α, COX-2, IL-1, IL-6, and IL-8 [112]. Similarly, quercetin, abundant in fruits and vegetables such as berries, red grapes, and broccoli, reduces inflammation by suppressing TNF-α, IL-1β, and IL-6 expression and inhibiting COX-2 and iNOS. These actions alleviate insulin resistance, diabetic complications, and atherosclerosis. Moreover, quercetin contributes to cancer treatment by reducing epigenetic modifications, including DNA methylation and activity of DNMTs, HDACs, and HMTs [113,114].

Genipin, a monoterpene iridoid from *Gardenia jasminoides* fruits, has been traditionally used to treat headaches, fever, inflammation, and liver disorders. Its biodegradability, biocompatibility, and stable, non-toxic cross-linking properties make it valuable in pharmaceutical applications, particularly in anti-inflammatory drug delivery systems [115,116]. Diterpenoids such as acanthoic acid (*Acanthopanax koreanum*), ginkgolides (*Ginkgo biloba*), carnosol (*Rosmarinus officinalis*), and kahweol (*Coffea arabica*), along with triterpenoids like lupeol (*Crateva adansonii*), boswellic acid (*Boswellia serrata*), and betulin (*Betula alba*), exhibit potent anti-inflammatory effects. These compounds have shown efficacy in managing conditions such as osteoarthritis, neurodegenerative disorders, and cardiovascular diseases [117-119].

Alkaloids, widely distributed in plants, exhibit diverse anti-inflammatory activities, making them promising candidates for new drug development. Indole alkaloids such as cassiaindoline (*Cassia alata*), strictosidine (*Uncaria rhynchophylla*), and alstoyunines (*Alstonia yunnanensis*) have shown significant anti-inflammatory effects. For instance, strictosidine inhibits NO production in LPS-induced N9 cells, while alstoyunines suppress COX-2 activity [120-122]. Girinimbine, a carbazole alkaloid from *Murraya koenigii* (curry leaves), provides gastroprotection against ethanol-induced ulcers by reducing TNF-α, IL-6, iNOS, and COX-2 levels through its antioxidant and anti-inflammatory properties [123]. Berberine, an isoquinoline alkaloid from *Rhizoma coptidis*, mitigates neuroinflammation in diseases like Alzheimer's by modulating NF-κB, TLR4, TNF-α, and IL-6 [124]. Trigonelline, a pyridine alkaloid from *Leonurus japonicus*, demonstrates therapeutic potential in

cardiovascular, CNS, and diabetic disorders, with growing evidence supporting its anti-inflammatory effects [125].

Antiviral Activity

Medicinal plants have been used for centuries to combat viral infections through various preparations, including powders, decoctions, infusions, pastes, and pills. Numerous plants have demonstrated potent antiviral properties, with studies reporting a diverse range of species effective against human viruses. For example, a 2018 study by Ogbole et al. identified 27 medicinal plants from Southern Nigeria with activity against positive-stranded RNA viruses, including enteroviruses [126,127].

Plant-derived antiviral compounds have also garnered significant attention. Notable examples include aloe gel (*Aloe barbadensis*), artemisinin (*Artemisia annua*), glycyrrhizinic acid (*Glycyrrhiza glabra*), silymarin (*Silybum marianum*), and ellagic acid (*Phyllanthus urinaria*). Key bioactive classes such as alkaloids, flavonoids (e.g., quercetin), lignans (e.g., phyllanthine), polyphenols (e.g., tannins), and terpenoids (e.g., glycyrrhizinic acid) have demonstrated activity against viruses like HSV, influenza, HIV, HCV, and dengue. These findings underscore the potential of plant-based compounds in developing antiviral therapies [128-132].

L.C. Chiang et al. investigated *Plantago major* extracts and compounds against HSV-1, HSV-2, and adenoviruses (ADV-3, -8, -11). While the aqueous extract showed mild anti-herpes activity, caffeic acid exhibited potent antiviral effects with high selectivity indices. Similarly, *Sambucus formosana* ethanol extract and its phenolic compounds, especially caffeic acid, demonstrated strong activity against HCoV-NL63 by inhibiting viral replication and attachment [133,134]. Ojha et al. evaluated *Pedilanthus tithymaloides* against HSV-2, finding that the methanolic leaf extract and luteolin significantly suppressed viral replication by modulating NF-κB signaling and reducing pro-inflammatory cytokines and nitric oxide [135].

Triterpenoids have also been identified as promising antiviral agents. Glycyrrhizin, derived from licorice root (*Glycyrrhiza radix*), inhibits the replication of viruses such as HIV, influenza, and human parainfluenza virus type 2 (HPIV2). In combination with ribavirin, glycyrrhizin effectively treats H1N1 influenza by reducing inflammation and suppressing viral replication [136,137]. Guava leaves, rich in 2α-hydroxyursolic acid, exhibit potent anti-human rotavirus (HRV) activity by enhancing immune function and inhibiting viral replication. Additionally, ursolic acid has been shown to suppress rotavirus replication and interfere with the maturation of viral particles, further highlighting the antiviral potential of triterpenoids [138,139].

Isolated plant compounds, particularly alkaloids, have shown significant antiviral potential. Berberine, an isoquinoline alkaloid from *Berberis* species, is a promising candidate for antiviral drug development due to its ability to target multiple stages of viral replication. It has been shown to inhibit

the replication of human cytomegalovirus (HCMV), including drug-resistant strains, by disrupting key gene expression and protein activation in the viral replication cycle. Additionally, berberine exhibits dose-dependent antiviral activity against herpes simplex virus (HSV) by reducing viral RNA transcription, protein synthesis, and titers through the modulation of JNK and NF-κB pathways [140,141]. Tomatidine, a steroidal alkaloid from unripe green tomatoes, also displays potent antiviral effects. Studies by Troost et al. found that tomatidine inhibits chikungunya virus (CHIKV) particle production across multiple strains, acting at a post-entry stage of the replication cycle. Its antiviral activity persists up to 24 hours post-infection, effectively controlling multiple rounds of CHIKV replication, making it most effective early in the infection process [142].

Anticancer Activity

Cancer is a complex disease characterized by uncontrolled cell growth, evasion of cell death, and the ability to spread to other tissues. It results from disruptions in cellular balance due to internal factors like oxidative stress and mutations, as well as external factors such as radiation and pollution. Key features of cancer progression include uncontrolled proliferation, unlimited replication, angiogenesis, increased growth signaling, resistance to apoptosis, and metastasis. Effective treatments target these disrupted pathways to restore normal cellular function [143]. Natural products are promising candidates for cancer therapy due to their selective toxicity toward cancer cells and reduced side effects on normal cells. Nearly 49% of cancer drugs approved between 1940 and 2014 were derived from natural sources [144].

Vincristine, an alkaloid from *Catharanthus roseus* approved in 1963, is pivotal in cancer therapy, particularly for pediatric acute lymphoblastic leukemia. It inhibits tubulin polymerization, disrupting mitosis, and is especially effective in combination therapies [145]. Similarly, homoharringtonine, derived from *Cephalotaxus* species and approved in 2012 for chronic myeloid leukemia, inhibits protein synthesis and cellular signaling. Its efficacy extends to other hematological cancers, including acute myeloid leukemia, with durable responses against cancer stem and progenitor cells [146-149]. Paclitaxel, a diterpenoid from *Taxus brevifolia*, is effective against various cancers, including breast, ovarian, and lung, by binding β-tubulin and preventing microtubule disassembly essential for mitosis. This mechanism induces cancer cell death in a dose- and time-dependent manner [150]. Ingenol mebutate, derived from *Euphorbia peplus*, is FDA- and EMA-approved for actinic keratosis and exhibits potent antiproliferative effects against skin and colon cancers. It penetrates deep dermal layers, induces necrosis resistant to apoptosis, and has both cytotoxic and immunomodulatory properties [151-153].

Curcumin, a polyphenol from *Curcuma longa*, shows broad anticancer potential against cancers such as brain, pancreatic, breast, and liver cancer. It works by modulating enzymes, arresting the cell cycle,

inducing apoptosis, and inhibiting angiogenesis. Despite poor bioavailability, strategies like combining curcumin with piperine, using nanotechnology, and developing analogs are under investigation. Ongoing clinical trials, especially in combination therapies, highlight curcumin's therapeutic promise [154].

Uses of Plant Natural Products

Food Industry

The food functionalization market is rapidly growing, driven by demand for innovative bioactive ingredients and sustainable solutions. With limited natural resources, agro-industrial byproducts provide a renewable source for creating high-value functional ingredients. These byproducts enhance food products' nutritional value, health benefits, shelf life, and sensory appeal, aligning with the trend toward natural additives and clean-label products [155-157].

Preservatives play a critical role in extending shelf life and ensuring food safety, with natural alternatives gaining traction due to consumer demand [158]. Fruit peels and seeds, rich in antimicrobial properties, show promise as natural preservatives. For instance, phenolic-rich extracts from berries like blueberries and cranberries demonstrate strong antimicrobial activity, while pomegranate peel extracts inhibit pathogens like *Listeria monocytogenes* and *Escherichia coli*, extending poultry product shelf life by up to two weeks [159-162].

Antioxidants from agricultural byproducts combat oxidation, preserving flavor, color, and nutritional value while preventing toxic compound formation [163]. Studies show artichoke and eggplant peel extracts enhance food antioxidant activity and shelf life [164,165]. Natural antioxidants, such as ascorbic acid (E300), tocopherol (E306), and β-carotene (E160a), are already approved additives, offering effective alternatives to synthetic compounds like BHA in bakery, dairy, and meat products [166-168].

Natural colorants from agro-food byproducts provide cost-effective, health-beneficial alternatives to synthetic dyes, enhancing visual appeal and consumer acceptance [169]. Examples include lycopene from tomato peels and anthocyanins from fruit byproducts, which are widely approved as colorants (e.g., E160d for lycopene and E163 for anthocyanins) [170,171]. Additionally, natural additives derived from fruits and vegetables often carry health benefits due to their bioactive compounds. Incorporating banana peels into biscuits has demonstrated the potential for creating low-calorie, high-fiber products without sacrificing taste, aroma, or color. Rich in phytochemicals such as phenolic compounds, anthocyanins, carotenoids, catecholamines, sterols, and triterpenes, banana peels provide antioxidant and antimicrobial properties, making them a valuable functional ingredient [172, 173].

The food industry can address consumer demand for functional, natural, and sustainable solutions while maintaining quality and safety by utilizing agro-industrial byproducts.

Cosmetic Uses

Throughout history, humans have sought to enhance their appearance using natural and chemical products, laying the foundation for today's cosmetic industry. This sector, which includes skincare, haircare, makeup, and toiletries, has skincare alone accounting for 36% of the global market [174]. Cosmetics serve four main purposes: improving condition, modifying appearance, protecting the body, and addressing odor, with products classified into seven categories, such as personal cleansing, skin and hair care, and active cosmetics [175]. Rising awareness of risks associated with synthetic ingredients has driven demand for natural products, prompting the industry to focus on plant-derived resources [176].

Natural compounds have shown significant potential in combating skin aging by improving elasticity and reducing wrinkles. For instance, xanthones from *Garcinia mangostana* and triterpenoids from *Eriobotrya japonica* promote collagen production, while polysaccharides from red ginseng enhance hydration [177-179]. Clinical trials have demonstrated positive effects of herbal formulations, such as extracts from *Camellia sinensis* and *Polygonum cuspidatum*, and oral supplements with *Vaccinium vitis-idaea* and *Phyllanthus emblica* significantly improved skin elasticity and reduced wrinkles. Topical products like *Thymus vulgaris* creams also effectively improved facial contours and fine lines [180-182]. Skin aging is linked to reduced barrier function, leading to dryness and fragility, exacerbated by environmental factors like UV radiation. Natural products such as *Triticum aestivum* extract oil and *Fragaria ananassa* extract have been shown to enhance skin hydration and texture, while extracts from *Vaccinium vitis-idaea* and kinetin improve moisture retention and epidermal health [183-185].

Hyperpigmentation, caused by excessive melanin production, often results in conditions like melasma and age spots [186]. Tyrosinase, a key enzyme in melanin synthesis, can be inhibited by plant extracts, such as *Hibiscus sabdariffa*, which downregulates pigmentation-related genes, and *Litchi sinensis*, which inhibits melanin production in melanoma cells [187,188].

UV radiation exposure can cause skin damage, including sunburn and premature aging. While traditional sunscreens use organic and inorganic filters, natural compounds like flavonoids and polyphenols offer UV protection alongside antioxidant and anti-inflammatory benefits [188]. For example, *Hylocereus polyrhizus* extract provides SPF 35.02. It reduces oxidative stress, while grape extracts and onion peel formulations enriched with quercetin show potential as natural UV filters in sunscreens and daily moisturizers [189-192].

Natural cosmeceuticals, containing bioactive compounds, also address various dermatological conditions such as burns, wounds, scars, acne, eczema, alopecia, and even teeth whitening, demonstrating their versatility and therapeutic potential [193].

Medical/Pharmaceutical Uses

Plant-based diets play a crucial role in maintaining health and preventing chronic diseases, particularly age-related conditions. Rich in fibers, micronutrients, and bioactive compounds known as phytochemicals, such as (poly)phenols, carotenoids, sulfur compounds, alkaloids, and plant sterols, these diets improve cardiometabolic markers like blood lipids, endothelial function, inflammation, and oxidative stress, while also positively influencing gut microbiota composition. Extensive research highlights the potential of these compounds to reduce disease risks and support key physiological functions [194,195].

Plant sterols, structurally similar to cholesterol, are effective in lowering LDL cholesterol by reducing its intestinal absorption [196]. Meta-analyses confirm that consuming approximately 1.6 grams of plant sterols daily reduces LDL-C levels by 8.5%, with reductions reaching 12% at higher doses of about 3 grams per day. Foods such as spreadable fats and yogurt are efficient carriers due to the solubility of these compounds, offering a practical means of incorporating them into the diet [197-200].

Garlic, particularly its bioactive compounds like S-allylcysteine, contributes significantly to vascular health by enhancing the production of hydrogen sulfide (H_2S) and nitric oxide (NO), which promote smooth muscle relaxation and vasodilation [201]. Aged garlic extract exhibits blood pressure-lowering effects through ACE inhibition and calcium channel blocking, complementing standard antihypertensive treatments [202]. Additionally, garlic demonstrates mild lipid-lowering and anti-aggregant properties, further supporting cardiovascular protection [203,204].

Carotenoids, including lycopene, offer protective effects against cardiovascular diseases, certain cancers, and metabolic syndrome. Known for their strong antioxidant properties, carotenoids influence cellular redox balance and other physiological processes. Lycopene has been shown to mitigate components of metabolic syndrome, although its efficacy varies with dosage and study duration [205,206].

Emerging research underscores the role of plant-derived polyphenols in cancer prevention and treatment by modulating critical pathways such as Hedgehog, Notch, and Wnt/β-catenin [207-209]. These compounds, including anthocyanins and flavonoids, reduce inflammation, reverse harmful epigenetic changes, and enhance the efficacy of chemotherapy and radiotherapy [210]. Eggplants, rich in phenolic compounds like solasodine, show promise in lung cancer prevention by reducing cancer cell viability, inhibiting cell invasion, and downregulating signaling pathways like PI3K/Akt and microRNA-21 [211,212].

Plant-based foods also contribute to diabetes prevention and management. Blackcurrants, with their high anthocyanin content, outperform common diabetes medications like acarbose in reducing postprandial hyperglycemia by inhibiting glucose uptake and sugar transporters [213]. Similarly, red-

skinned onions and purple eggplants, which contain beneficial phenolic compounds, aid in diabetes management by inhibiting key enzymes involved in glucose metabolism. Cooking these vegetables enhances the bioavailability of their active compounds, improving their anti-diabetic effects [214, 215]. The growing body of evidence highlights the diverse health benefits of plant-based nutrition, showcasing its ability to support physiological functions, reduce the risk of chronic diseases, and promote overall well-being.

Agricultural Uses

For nearly 75 years, innovation in crop protection technology has been essential to meeting the growing global food demand sustainably. The need for new agrochemical classes is driven by resistance to existing products, evolving agricultural practices, integrated pest management strategies, shifting pest populations, and stricter regulations emphasizing environmental and toxicological safety [216,217]. Research often focuses on testing compounds against target organisms such as weeds, fungi, or insects, generating *in vivo* data to guide the development of effective control methods. Collaboration with pharmaceutical companies and leveraging natural products provide valuable leads in agrochemistry, as these products often target underexplored chemical spaces and exhibit desirable properties like reduced persistence and specificity to pests [218,219].

Secondary metabolites play a critical role in plant defense, producing toxic compounds, repelling pests, and activating signaling molecules to counter oxidative stress and environmental threats. These natural products offer unique chemotypes, exploring uncharted chemical spaces to uncover novel pesticides and resistance-management strategies. With low environmental persistence, high target specificity, and reduced toxicity to non-target organisms, they provide safer, effective pest control. Exploring new chemical scaffolds for agrochemicals often begins with plant-derived metabolites, particularly those integral to plant defense mechanisms [220]. For example, the alkaloid stemofoline, derived from *Stemona japonica*, acts on insect nicotinic acetylcholine receptors (nAChRs) with antifeedant and repellent effects. Although its complex structure limits synthetic accessibility, simplified derivatives, such as pyridinyl-cyanotropane compounds developed by Syngenta, have shown effective pest control while being easier to produce [221,222].

Weed management has evolved from manual methods to reliance on synthetic herbicides, which are effective but raise health and environmental concerns. This has spurred interest in natural alternatives like essential oils, which act as non-selective, contact herbicides [223]. For example, eucalyptus oil, containing citronellal and terpenes, shows rapid weed control but limited persistence due to volatilization. Innovations like microencapsulation improve its effectiveness by reducing environmental degradation [224]. Similarly, Manuka oil, rich in sesquiterpenes and leptospermone,

disrupts carotenoid biosynthesis, mimicking the action of synthetic herbicides and offering a promising natural alternative for organic agriculture [225-227].

Natural compounds, classified as biopesticides, offer reduced toxicity to non-target organisms, lower environmental persistence, and minimal risks to humans. These biopesticides include insect repellents, attractants, and various pest control agents derived from plant secondary metabolites Examples include pyrethrins, nicotine, rotenone, azadirachtin, and essential oils [228]. Azadirachtin, derived from the neem tree, disrupts insect feeding and growth by acting on microtubules, causing death while maintaining low toxicity to mammals, fish, and pollinators. Its environmentally friendly profile makes it a preferred choice in sustainable pest management [229,230].

Botanical insecticides have inspired synthetic agrochemicals. Ryanodine, an alkaloid from Ryania stems, affects calcium channels in insects, causing paralysis and death. Despite its moderate mammalian toxicity, ryanodine inspired the development of selective diamide insecticides like flubendiamide and chlorantraniliprole, which target insect ryanodine receptors with high specificity and efficacy [231-233].

While natural products in crop protection offer ecological benefits and help combat pest resistance, challenges remain, including resource availability, quality control, production costs, and environmental constraints. Innovative approaches like plant tissue culture and metabolic engineering are enhancing the scalability and effectiveness of these solutions. Although unlikely to fully replace synthetic pesticides, plant-derived products align with regulatory frameworks prioritizing sustainability and environmental conservation, cementing their role in integrated pest management [234].

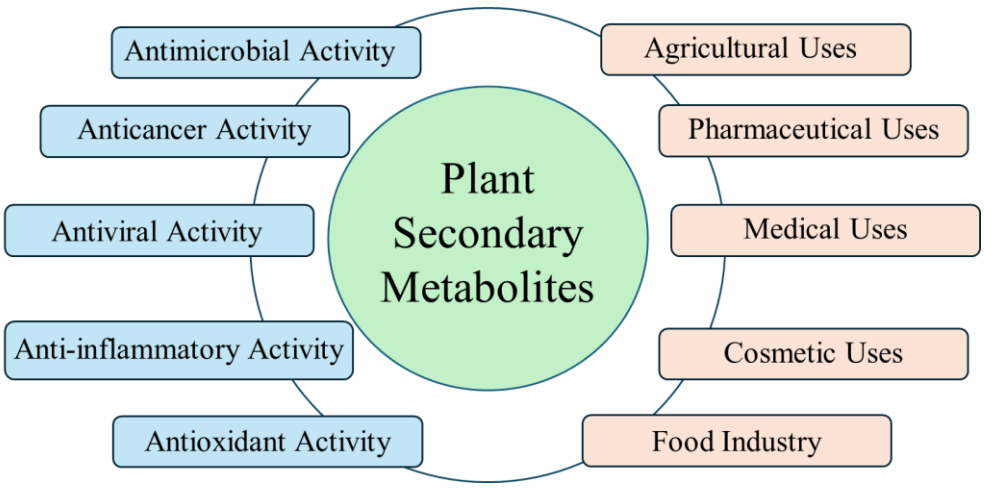


Figure 7: **Biological activities and uses of plant Secondary Metabolites**.

Exploring the Applications and Techniques of Callus Culture in Plant Science

In vitro Culture Techniques as an Alternative System for SM production

Plants have developed a highly adaptive biochemical system to manage various environmental challenges. They produce SMs in small quantities in response to factors like bacteria, viruses, fungi, insects, herbivores, and changing environmental conditions such as climate and soil. To optimize this production, plants have evolved signaling systems that can distinguish between mechanical damage and attacks by insects or herbivores and assess damage severity based on cues like insect secretions or feeding patterns. The biosynthesis of SMs is shaped by geographical location, genetics, climate, and environmental conditions. These compounds often accumulate in specific plant structures, such as vacuoles, glands, and trichomes, and may only be produced at certain stages of development. This selective accumulation helps plants adapt to their environment without disrupting normal growth and physiological processes [235,236].

As discussed in the previous chapter, these metabolites are valuable as active compounds in pharmaceuticals, food, cosmetics, and agrochemicals. However, because plants produce them in small amounts, large quantities of plant material are often needed for extraction. Historically, this reliance on traditional plant sources has led to overharvesting, endangering many plant species critical to these industries [237].

Biotechnology has recently provided valuable opportunities for producing plant-based *in vitro* systems, such as callus, cell suspension, and organ cultures, along with genetic manipulation to create desired plants and products by offering a scalable and efficient source for producing SMs.

The history of plant tissue culture traces back to 1902 when Gottlieb Haberlandt introduced the concept of plant cell totipotency. The first successful plant cell culture followed in 1922, and discoveries of plant growth regulators like indole-3-butyric acid (1926) and kinetin (1955) advanced the field. In 1962, Murashige and Skoog developed the MS medium, which became a standard for plant tissue cultures. By the 1970s, large-scale production of bioactive compounds using tissue culture was viable, and by 1987, it surpassed traditional methods for secondary metabolite production. Today, plant tissue culture is widely used for studying plant development and producing pharmaceuticals like taxol [238,239].



Figure 8: **Milestones of in vitro culture techniques development and metabolite production.** (Image modified from https://doi.org/10.1016/j.indcrop.2015.12.016)

Plant tissue and organ culture refers to the cultivation of plant cells and organs under sterile conditions, using a controlled environment with regulated light, humidity, and temperature. This system ensures consistent extract quality by maintaining uniform concentrations of desired compounds and preserving the genetic traits of high-performing clones [240,241]. It offers a sustainable alternative for producing valuable bioactive metabolites, addressing challenges like habitat loss, species extinction, and difficulties in cultivating specific plants. In vitro culture is especially beneficial for plants that are hard to grow, have long growth cycles, produce low metabolite levels, or when chemical synthesis is not feasible. Moreover, it enables the discovery of novel compounds and facilitates biotransformation processes [242].

The advantages of *in vitro* culture include:

- Independence from environmental factors
- Production can be tailored to market demand
- Consistent product quality using characterized cell lines
- Potential for novel product development via mutant cell lines
- Reduced pressure on overexploited medicinal plants
- A continuous and reliable source of natural products
- Controlled synthesis of bioactive compounds, unaffected by climate or soil
- Increased use of genetic tools to enhance secondary metabolite production
- Commercial viability for large-scale production, particularly for pharmaceuticals
- The potential for developing knowledge of biosynthetic pathways at the cellular and molecular levels
- Advances in molecular biology, such as transgenic cultures, are making plant cell cultures increasingly applicable for commercial use.

In vitro culture enables the cultivation of any plant since plant cells retain their genetic capacity for secondary metabolism and totipotency in artificial media. This method can produce both specialized and non-specialized cells with metabolic profiles like native plants. Advances in biotechnology and biochemistry have boosted yields, making in vitro culture a preferred approach for producing bioactive compounds. Beyond its research applications, *in vitro* plant propagation holds commercial potential, supporting sustainable industries focused on plant-derived compounds. It is highly effective for isolating and processing active compounds, including secondary metabolites, and has been instrumental in large-scale production for industries cultivating ornamental, vegetable, and fruit plants [236,243].

Callus Culture Technique

Plant callus forms naturally as a wound-healing tissue or as a tumor in response to pathogens or genetic disorders [244]. *In vitro*, a callus can be defined as an unorganized mass of plant cells generated from explants cultured with specific plant growth regulators (PGRs). In callus cultures, both differentiated and undifferentiated cells grow together, and further procedures are applied to induce organ differentiation. Given the variety of explants and conditions used for callus initiation and culture, plant calli exhibit considerable variability. They can differ in color (white, yellow, green, brown), structure (compact or friable), dependence on plant hormones for cell division, and regeneration potential (non-regenerating, rooty, shooty, or embryogenic) Generally, friable callus is particularly prominent and well-suited for propagation, cell suspension culture, and the production of SMs [237,244,245].

Almost any part of the plant can be used to generate callus cultures. Explants taken from plant tissues slowly grow *in vitro* into a cell mass that ranges from amorphous and colorless to pale-brown, if they are obtained under sterile conditions avoiding microbial infection, and if they are cultured on a solid gel medium supplemented with growth hormones. Regular passaging allows callus cultures to be indefinitely maintained *in vitro*. Unlike differentiated plant cells, callus cells resemble non-differentiated meristematic cells, lacking chloroplasts and having small vacuoles [238].

Callus formation from an explant involves several stages. Initially, cells divide in random planes, leading to reduced specialization and loss of organized structures. Callus can be derived from various plant parts, but successful induction depends on factors such as the physiological age of the tissue, environmental conditions before explantation, cell organization, and the size and location of the explant. Not all cells in an explant will form a callus, which should be considered based on the culture's objectives. Primary explants can include aseptically germinated seedlings or surface-sterilized roots, stems, leaves, or reproductive structures. The type and concentration of plant growth regulators, along with the culture medium, are critical for controlling callus formation. Optimal culture conditions, including gas levels within the container, are essential for callus development. For subculturing, small portions of friable callus (typically 0.2–0.5 g) are transferred to a fresh medium to sustain vigorous growth. Callus cultures serve as valuable sources for biochemical and molecular studies and can be used to produce suspension cultures, protoplasts, organogenesis, somatic embryos, and secondary metabolites, including phenolics, flavonoids, alkaloids, and other secondary metabolites from different plant species. For example, compounds such as apigenin, p-coumaric acid, and vincristine have been produced using tissue culture techniques [246]. These cultures are typically maintained in growth chambers with controlled light conditions specific to each species and genotype and at a temperature of around 25°C. Regular subculturing every 2–6 weeks, depending on the growth

rate, ensures the continual maintenance of the callus viability and prevents cell loss, which can reduce cell division and metabolite production [246, 247].

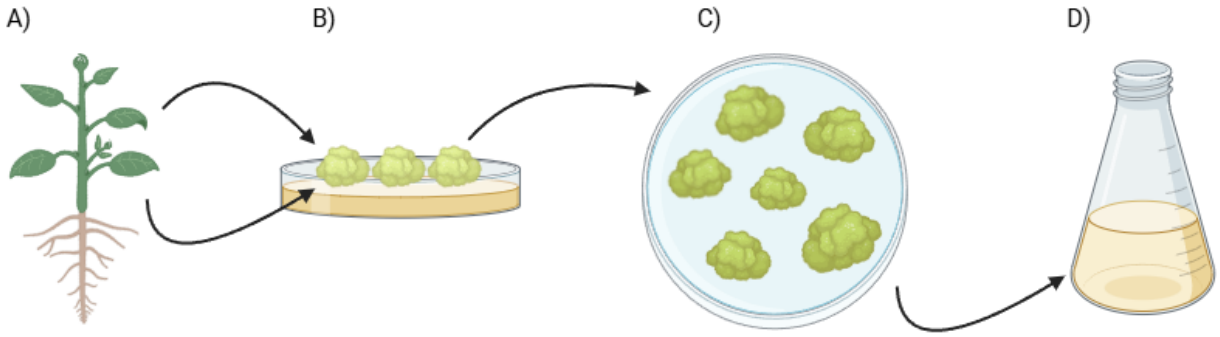


Figure 9: **Callus induction and secondary metabolite production from callus cultures. (A) Main plant and explant sources, (B) Isolation of explant, and in vitro callus induction, (C) Obtained callus, and (D) Isolation of secondary metabolites from callus.** (Image modified from https://doi.org/10.3389/fpls.2023.1132555)

Cellular and Molecular Basis of Callus Formation

Callus induction in plants is a complex and highly regulated process involving a series of cellular and molecular events (including hormonal signals, gene expression, and epigenetic modifications) that reprogram differentiated cells into a pluripotent state, allowing them to proliferate and potentially regenerate into new plant structures. This transformation is primarily initiated by wounding, which triggers hormonal changes and stress responses within the plant tissue. Key hormones, particularly auxin and cytokinin, play central roles in this process. Auxin, when present in high concentrations, activates genes such as *WOX11* and *WOX12*, which are essential for initiating the dedifferentiation of cells and promoting callus formation. These genes further stimulate the expression of downstream genes that drive cell proliferation and the formation of callus tissues. Cytokinin, on the other hand, works in tandem with auxin to regulate the balance between cell division and differentiation, crucial for successful callus formation [247-250].

The process of callus growth is initiated by the expression of wound-responsive genes like WIND1, which activates downstream targets such as ENHANCER OF SHOOT REGENERATION 1 (ESR1). This activation leads to the formation of callus tissue at wound sites. Additionally, transcription factors such as PLETHORA (PLT) proteins are crucial for maintaining the pluripotency of callus cells, ensuring they can proliferate and eventually differentiate into various plant tissues [251].

A significant aspect of callus induction is the remodeling of the cell wall, which is necessary for cells to re-enter the cell cycle and begin dividing. Enzymes like endoglucanases and pectinesterases modify

the cell wall's structure, facilitating the physical changes required for callus formation. This remodeling process is intricately regulated by non-coding RNAs (ncRNAs) that interact with messenger RNAs (mRNAs), forming regulatory networks that ensure the precise timing and coordination of gene expression [247-250].

Epigenetic modifications, such as DNA methylation and histone modification, play a critical role in callus growth by regulating gene expression patterns. These modifications can either activate or repress specific genes necessary for maintaining the callus's undifferentiated state or for triggering its eventual differentiation into new tissues. For example, the suppression of certain genes through histone modification is necessary to maintain the pluripotency of callus cells [247-250].

These mechanisms collectively ensure that callus growth is tightly regulated, allowing for effective plant regeneration and tissue culture applications.

Initiation and Maintenance of Callus Cultures

Callus formation typically begins with selecting and sterilizing explants, which are pieces of plant tissue such as leaves, stems, or roots. These explants are then placed on a solid nutrient medium that contains a specific balance of plant hormones, primarily auxins and cytokinins. For callus initiation, a balanced ratio of auxin and cytokinin is generally used to induce the formation of undifferentiated callus tissue. Wounding the explant, either physically or chemically, is often necessary to stimulate the production of wound-induced signals, which trigger the dedifferentiation of cells and the formation of callus. The presence of these wound signals, combined with the appropriate hormonal environment, leads to the reprogramming of cells within the explant to a pluripotent state, allowing them to proliferate and form a callus mass [247, 250].

Once a callus is formed, it must be maintained under specific conditions to prevent differentiation and ensure continued growth. This involves regularly subculturing the callus onto fresh nutrient medium to provide a consistent supply of nutrients and hormones. The subculture interval is typically every 3-4 weeks, depending on the growth rate of the callus. Maintaining an appropriate hormonal balance in the medium is crucial for preventing the callus from differentiating into roots, shoots, or other tissues [247,250].

Factors Influencing Callus Growth and Differentiation

Various factors, including hormonal balance, environmental conditions, genetic factors, and the type of explant used influence the growth and differentiation of callus tissues in plant cultures. Understanding these factors is crucial for optimizing callus culture techniques in plant tissue culture.

A. Hormonal Balance: The ratio of auxins (e.g., 2,4-D, IAA) to cytokinins (e.g., BAP, kinetin) is a critical factor. High auxin concentrations relative to cytokinin typically promote callus formation and cell proliferation, while a higher cytokinin-to-auxin ratio can encourage differentiation into shoots or

roots. The specific balance required can vary depending on the plant species and the desired outcome (callus proliferation vs. organogenesis) [244, 247, 250].

B. Environmental Conditions: Most callus cultures are grown in the dark or under low-light conditions to prevent differentiation. Light can trigger photomorphogenic responses that lead to the development of shoots or roots, which may be undesirable if the goal is to maintain undifferentiated callus tissue. The optimal temperature for callus growth typically ranges from 25°C to 28°C. Deviations from this range can slow down growth or lead to abnormal differentiation. Some plant species may require specific temperature conditions for optimal callus induction and maintenance [248]. High humidity levels are generally maintained to prevent desiccation of the culture medium. Adequate gas exchange is also necessary to avoid the accumulation of ethylene, which can negatively affect callus growth [238].

C. Explant Source: The type of explant used (e.g., leaf, stem, root) significantly affects callus formation. Younger, more meristematic tissues generally have a higher potential for callus induction due to their higher cell division activity. Mature tissues might require more precise hormonal treatments to initiate callus formation [247].

The physiological state of the plant from which the explant is derived, including its health, nutrient status, and growth phase, can influence the success of callus induction. Explants from actively growing plants often yield better results.

D. Genetic Factors: The genetic makeup of the plant plays a significant role in its ability to form callus and subsequently differentiate. Some genotypes are naturally more amenable to tissue culture techniques, showing higher rates of callus induction and regeneration. Others may require specific modifications to the culture medium or environmental conditions [250].

E. Culture Medium Composition: The composition of the culture medium, including the types and concentrations of macro- and micronutrients, can influence callus growth. Adequate supply of essential nutrients like nitrogen, phosphorus, and potassium is crucial for sustaining cell division and growth. Sucrose is commonly used as a carbon source in culture media. The concentration of sucrose can affect osmotic pressure and energy availability, both of which are important for callus growth and maintenance [238, 247].

F. Epigenetic Modifications: Epigenetic changes can influence the ability of cells within the callus to remain undifferentiated or to differentiate into specific tissues. These modifications can be induced by the culture conditions and the hormonal environment, and they play a role in the long-term stability of the callus culture [250].

Production of Secondary Metabolites Using Callus Culture Technique

Cultivating callus from medicinal plants provides an effective method for the sustainable and large-scale synthesis of secondary metabolites (SMs) with applications in pharmaceuticals, cosmetics, food, and related industries. This method yields bioactive phytochemicals that offer potential treatments for a diverse range of diseases. The chemical compounds synthesized from callus display a broad spectrum of chemical diversity across various classes. Extracting phytochemicals directly from calli without harming the entire plant holds significant promise for the conservation of rare and endangered plant species. Additionally, producing a plentiful supply of SMs in vitro under controlled conditions helps mitigate the influence of environmental fluctuations, seasonal variations, microbial infections, pests, and geographical limitations [251].

Optimizing and enhancing callus culture are critical steps for the accumulation and synthesis of SMs. Here some of the factors that could be optimized are reported [252].

A. Selection of cell lines and clones. The accumulation of secondary metabolites in plants is specific to the genotype. Therefore, choosing the right species and specific organ for *in vitro* callus induction is essential. It's extremely important to isolate and select cells and organ lines that have increased accumulation of metabolites [253].

B. Medium optimization. The production and synthesis of SMs in callus cultures are influenced by various chemical and physical factors. The key factors include the type of culture medium, salt strength, types and levels of carbohydrates, nitrate levels, phosphate levels, and growth regulator levels [253]. The selection of a suitable media and salt concentration is crucial. Gamborg (B5), Linsmaier and Skoog (LS), Murashige and Skoog (MS), and Schenk and Hildebrandt (SH) are widely used media for establishing callus cultures. Callus cultures commonly rely on simple sugars such as glucose, fructose, maltose, and sucrose as their primary energy sources and nutritional components. The concentration of carbohydrates significantly influences the production of metabolites. Furthermore, sugars play a crucial role as signaling molecules, impacting cell growth, development, and metabolism [254]. The ratio of ammonium to nitrate and the overall level of total nitrogen significantly affect metabolite accumulation. In general, High concentrations of ammonium ions inhibit the formation of secondary metabolites, while lower levels increase it. Phosphate concentration in the growth medium also influences the production of secondary metabolites; elevated phosphate levels foster cell growth and primary metabolism, while diminished phosphate concentrations tend to promote the biosynthesis of secondary metabolites [238, 254].

Plant growth regulators (PGRs) are indispensable for promoting growth and secondary metabolite production in callus cultures. The specific type and concentration of PGRs are pivotal factors influencing callus growth, proliferation, and metabolite accumulation. The type and concentration of

auxins or cytokinins, as well as the ratio of auxins to cytokinins, significantly impact biomass growth and product formation in callus cultures. Indole acetic acid (IAA), naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and benzyladenine (BA) are commonly used to enhance the production of secondary metabolites. Gibberellins (GA) and ethylene are also employed to induce secondary metabolite production [254, 255].

C. Optimization of the culture environment. The influence of environmental conditions such as light, temperature, medium pH, and essential gases on biomass growth and SMs accumulation has been studied. Callus culture is typically maintained within a temperature range of 17-25°C, although different plant species may have different temperature preferences for growth and metabolism. Light, as an energy source, affects both biomass growth and SMs accumulation. Various studies have examined the effects of light intensity and irradiance, including continuous light and darkness, and have found that manipulating light and dark regimes can potentially alter SMs accumulation [254]. The pH of the medium is commonly adjusted to fall between 5 and 6 before autoclaving to prevent extreme pH values. Changes in the concentration of hydrogen ions may occur due to nutrient uptake or metabolite accumulation under culture conditions. Adjusting the pH of the medium can influence membrane permeability and result in the release of secondary products into the culture medium.

D. Elicitation. An elicitor is a factor or element that enhances the biosynthetic capacity of secondary metabolites when introduced or modified in an *in vitro* culture. Elicitors can be categorized into two types: biotic and abiotic. The addition of an eliciting agent can effectively improve the production of secondary metabolites in medicinal plants through *in vitro* culture. Biotic elicitors, originating from biological sources such as microbial cell walls (e.g., chitin and polysaccharides) or plant cell walls (e.g., pectin and oligosaccharides), play a crucial role in this process by inducing defense mechanisms against pathogens. On the other hand, abiotic elicitors include non-biological factors such as heavy metals, inorganic salts, UV radiation, and high salinity, which also trigger stress responses. Phytohormones like salicylic acid and methyl jasmonate, produced by plants in response to stress or pathogen attack, are key signaling compounds that trigger secondary metabolite production and are widely used as elicitors in callus cultures. Microbes like fungi, bacteria, and yeast also activate secondary metabolic pathways, and fungal elicitors, such as Fusarium and Trichoderma species, are especially effective. Their use in callus culture has gained attention for significantly boosting the yield and quality of secondary metabolites in medicinal plants [252].

The effectiveness of elicitors depends on several factors, including concentration, exposure duration, and the developmental stage of the culture at the time of treatment. Optimizing these parameters can significantly improve the yield and quality of secondary metabolites [236, 237, 256].

Plant name	Active Ingredient	Culture medium and PGRs
Agave amanuensis	Saponins	MS + Kinetin
Artemisia annua	Artemisinin	MS + NAA + Kinetin
Plantago media	Verbascoside	B5 + IAA + Kin
Aspidosperma ramiflorum	Ramiflorin alkaloid	MS + 2-4,D + BAP + 30 g/l Sucrose
Camellia chinensis	Flavones	MS + 2,4-D + NAA
Capsicum annum	Capsiacin	MS + 2,4-D+ GA3
Centella asiatica	Asiaticoside	MS + 2,4-D + Kin
Coffea arabica	Caffeine	MS + 2,4-D + Kinetin
Coscinium fenustratum	Berberine	MS + IAA +BAP
Crataegus sinaica	Flavonoid	MS + 2,4-D + NAA + BAP
Cryptolepis buchanani	Cryptosin	B5 + 2,4-D + Kinetin
Duboisia leichhardtii	Alkaloids	LS + NAA + BA
Eriobotrya japonica	Triterpenes	LS + NAA + BA
Eucalyptus tereticornis	Sterols and phenolic compounds	MS + 2,4-D
Frangula alnus	Anthraquinones	WPM + IAA + BAP
Gentiana sp.	Glucosides	B5 + Kinetin
Nicotiana rustica	Alkaloids	LS + 2,4-D + Kinetin
Capparis spinosa	Rutin	B5 medium + 2,4-D + BAP + MeJA + SA

Table 1: **Examples of secondary metabolites produced using the callus culture technique and the corresponding culture conditions**. (Image modified from https://doi.org/10.3390/molecules27228093)

CHAPTER 1

Cydonia Oblonga Mill. Pulp Callus Inhibits Oxidative Stress and Inflammation in Injured Cells

Original article published in:

- Gubitosa, F., Fraternale, D., De Bellis, R., Gorassini, A., Benayada, L., Chiarantini, L., Albertini, M. C., & Potenza, L. (2023). Cydonia oblonga Mill. Pulp Callus Inhibits Oxidative Stress and Inflammation in Injured Cells. Antioxidants (Basel, Switzerland), 12(5), 1076. https://doi.org/10.3390/antiox12051076

1.1 Introduction

Cydonia oblonga Miller, commonly known as quince, is a small shrub in the Rosaceae family, related to apples and pears, that produces bright yellow, pome fruits. Though its raw fruit is hard, sour, and astringent, quince is widely used in jams, marmalades, jellies, dried slices, and wines due to its aromatic qualities. The fruit's high pectin content also makes it valuable in producing fruit spreads and as a thickener in the food industry [257]. Rich in essential minerals like calcium, potassium, and phosphorus, quince offers nearly double the mineral content of apples [257]. Beyond its culinary uses, quince is a potent source of bioactive compounds, including polyphenols and flavonoids, contributing to its antioxidant, antimicrobial, and anti-inflammatory properties. Despite its limited popularity as a fresh fruit due to its texture and bitterness, quince has drawn increasing attention for its potential to prevent and treat various health conditions. Its extracts have been shown to have antibacterial, antifungal, anti-diabetic, and wound-healing effects. In traditional medicine, quince has been used to treat gastrointestinal issues, respiratory ailments, and skin infections [257].

Modern studies have confirmed these benefits, showing quince's effectiveness in managing nausea and vomiting during pregnancy, as well as gastroesophageal reflux disease (GERD) [258,259]. Its seeds, leaves, and peels exhibit strong antioxidant and anti-cancer activities, with extracts demonstrating antiproliferative effects against certain cancer cells. The fruit's polyphenolic compounds have also been linked to anti-inflammatory and anti-ulcerative effects, making quince a promising natural remedy for various conditions [257,260]. Additionally, oven-dried quince has been identified as a valuable source of antioxidants, with potential applications in the pharmaceutical and food industries [257].

This study builds on the work previously conducted by our research group, where we described the production of a callus culture extract from the pulp of *Cydonia oblonga* Mill. (referred to as Co ext.), along with preparing its hydroalcoholic extract, identifying its chemical composition—including

secondary metabolites such as phenolic compounds and triterpenic acids—and its biological activity in cell-free models.

Building on these initial findings, this study focuses on evaluating the anti-inflammatory properties of Co ext. We assessed its effects in LPS-stimulated RAW 264.7 cells by analyzing nitric oxide (NO) production, and in LPS-stimulated HaCaT cells by examining the expression of key pro-inflammatory genes, including iNOS, IL-6, IL-1β, IκBα, and ICAM. In addition, we investigated the production of reactive oxygen species (ROS) in HaCaT cells that were injured using hydrogen peroxide and tert-butyl hydroperoxide.

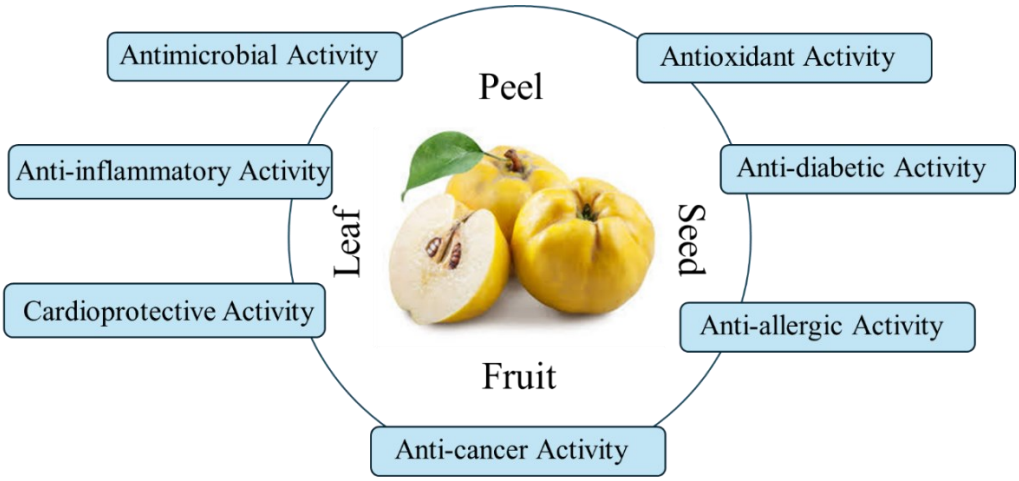


Figure 10: **Biological properties of *Cydonia oblonga* Miller.** (Image modified from https://doi.org/10.1080/10942912.2023.2205059)

1.2 Materials and Methods

1.2.1 Callus Production

C. oblonga ripe fruits were collected from an orchard located in Urbino (PU), Marche region, Italy. The apples were opened with a sterile blade under the laminar flow cabinet, after surface sterilization with ethanol 90%. Cultures were then conducted as previously described by our team [261]. Briefly, the combination of Gamborg B5 medium plus 1.77 µM BA (6-benzylaminopurine, Sigma-Aldrich) and 5.40 µM NAA (1-Naphthaleneacetic acid, Sigma-Aldrich, Milan, Italy), 30 g/L sucrose, and pH 5.8 induced the highest biomass production from pulp explants of C. oblonga fruits. Cultures were incubated in the dark at 25 ± 2 °C, and subcultures were obtained after 28 days in the same media. The callus obtained by subcultures was stored at -80 °C and lyophilized before the extraction.

1.2.2 Callus Extract Preparation

Callus extract from *C. oblonga* (Co ext.) was prepared following the previously described protocols [261,262]. Briefly, 130 mg of callus freeze-dried was homogenized in a Potter-type homogenizer. The dried material was dissolved in 15 mL of diluted ethanol solution (70%) and shaken overnight (ON).

The mixture was centrifuged at 13,000 rpm for 45 min at 4 °C, and the supernatant was recovered, filtered with 0.2 μm filters, aliquoted, dried, and weighed (dry weight—dw). Before use, each aliquot was dissolved in double distilled water at 30 mg dw/mL.

1.2.3 Chemical Characterization of SMs in Quince Callus Extract

Characterization and quantification of triterpenic acids and polyphenolic compounds in Co ext. were performed by GC–MS analyses and HPLC-DAD-ESI-MSn, respectively, as reported by De Bellis et al. [261].

Chemical characterization of quince callus and pulp extracts

Each lyophilized sample (30 mg) and 500 μL of the internal standard (IS) solution (cholesterol, 1.244 mg/mL in ethyl acetate (EtOAc)) was suspended in water (5 mL) and extracted with EtOAc (25 mL × 2). The collected organic phases were washed with H_2O (4 mL) and brine (4 mL) then dried (Na_2SO_4 anhydrous), filtered, and evaporated to dryness in vacuo and the residue was dissolved in EtOAc (4 mL) and stored at 4 °C. An aliquot (2 mL) of this solution was evaporated under argon and the residue was dissolved in pyridine (0.5 mL) and Sylon BFT (0.5 mL). Silylation of the sample was performed at 80 °C for 18 h. After this time, the reaction mixture was analyzed by GC–MS and GC-FID (all the compounds were identified by GC-MS and then quantified by GC-FID). GC–MS analyses were carried out using a Trace GC Ultra gas chromatograph coupled to an ion-trap mass spectrometer (ITMS) detector Polaris Q (Thermo Fisher Scientific, MA, USA) and equipped with a split-splitless injector. The column was a 30 m × 0.25 mm i.d., 0.1 μm film thickness, fused silica SLB-5 ms (Supelco, Sigma-Aldrich, Milan, Italy). The initial oven temperature was 240 °C programmed to 280 °C at 2 °C/ min and kept at 280 °C for 10 min, then the temperature was then raised to 310 °C at a rate of 10 °C/min and maintained at this temperature for 20 min. Samples were injected in the split (1:10) mode. The injector, transfer line, and ion source were set at 280, 280, and 200 °C, respectively. Helium was used as carrier gas at a 1 mL/min flow. The mass spectra were recorded in electron ionization (EI) mode at 70 eV electron energy with a mass range from *m*/*z* 50 to 1000 and a scan rate of 0.8 scan/sec. Identification of metabolites was carried out by comparison of the spectral data and retention times with those of standards or to the spectra from the NIST02 spectral library. The data acquisition was under the control of Xcalibur software (Thermo Fisher Scientific, MA, USA). A Fisons GC 8000 series gas chromatograph, equipped with a flame ionization detector (FID) and a split/splitless injector (Fisons Instruments, Milan, Italy) was used for the quantitation of triterpenic acids. The separation was carried out with a fused silica capillary column DB-5MS UI 30 m × 0.250 mm × 0.25 μm film thickness (Agilent, J&W, Milan, Italy). The initial oven temperature was 240 °C programmed to 280 °C at 2 °C/min and kept at 280 °C for 25 min, the temperature was then raised to 310 °C at a rate of 10 °C/min and

maintained at this temperature for 13 min. Samples were injected in the split (1:10) mode. The injector and detector were set at 280 °C. Hydrogen was used as carrier gas at a flow of 1.8 mL/min. Peak areas were integrated using a Varian Galaxie Workstation (Agilent Technologies, Milan, Italy). Quantification of triterpenic acids in the samples was performed using the internal standard method based on the relative peak area of analyte to IS (cholesterol) from the average of three replicate measurements. A calibration curve was built for each corresponding standard compound in the extracts. For this purpose, working solutions of each available standard (β-sitosterol, ursolic acid, and oleanolic acid) were prepared at a concentration ranging from 5 to 120 μg/mL with a constant concentration of the IS (60.0 μg/mL). The calibration curves obtained had a high level of linearity with a correlation coefficient (R^2) higher than 0.999 for all analytes. When standards were unavailable, the quantification of the target analyte was carried out using the calibration curve of the available standard of a similar chemical structure.

Sample preparation for HPLC-DAD-ESI-MS^n analyses of polyphenolic compounds

Each lyophilized sample (30 mg) and 30 μL of the IS solution (3- hydroxycinnamic acid, 100 μg/mL in MeOH) was dissolved in H_2O with 0.4% formic acid (2 mL) and loaded on a C18 SPE column previously conditioned with 10 mL of MeOH with 0.4% formic acid and 10 mL of H_2O with 0.4% formic acid. After loading, the column was washed with 10 mL of H_2O with 0.4% formic acid, and the phenolic fraction was eluted with 5 mL of MeOH with 0.4% of formic acid. Solvents were removed, and the residue was diluted in 1 mL of H_2O/MeOH 9:1 (v/v) with 0.4% of formic acid and transferred to an autosampler vial for the HPLC-DAD-ESI-MS^n analysis. Chromatographic analysis was performed with a Dionex Ultimate 3000 UPLC (Thermo Fisher Scientific, MA, USA) equipped with a thermostated autosampler and a column oven. The chromatographic separation was performed with a column InfinityLab Poroshell 120 EC-C18 (4.6 × 150 mm, 2.7 μm; (Agilent Technology, Milan, Italy), thermostated at 30 °C. Elution was carried out at a flow rate of 0.6 mL/min, using as mobile phase a mixture of 0.2% formic acid in methanol (A) and 0.2% formic acid in water (B) with the following gradient: 0–6 min 10% A, 20 min 35% A, 46 min 40% A, 48 min 100% A, 60 min 100% A, 62 min 10% A, 70 min 10% A. The injection volume was 20 μL. The HPLC system was coupled with a diode array detector (DAD) and an electrospray ionization mass detector (HPLC-DAD-ESI-MS^n) in parallel by splitting the mobile phase 1:1. The acquisition was carried out in full scan (*m*/*z* 50–1500) and in full scan MS^2 (*m*/*z* 50–600) selecting the precursor ion $[M–H]^-$. The quantitative analysis was carried out using an Ultimate 3000 RS Diode Array detector (Thermo Fisher Scientific, MA, USA) controlled by Chromeleon software (version 6.80). Spectral data from all peaks were accumulated in the range of 200–400 nm and chromatograms were recorded at 280 nm for (+)-catechin, (-)-epicatechin, procyanidin B2, and 3-hydroxycinnamic acid

(IS), 328 nm for 3- and 5-caffeoylquinic acid, 4-*p*- and 5-*p*-coumaroylquinic acid, 258 nm for quercetin-3-O-glucoside, respectively. Calibration curves ($R^2 > 0.999$) were prepared by diluting a stock solution of each standard [(+)-catechin, (-)-epicatechin, procyanidin B2, 5-caffeoylquinic acid, quercetin-3-O-glucoside] in H_2O/MeOH 9:1 (v/v) with 0.2% of formic acid in the range 12–3000 ng/ with a constant concentration of IS (500 ng/mL). When standards were unavailable, the quantification of the analyte was carried out using the calibration curve of available standards presenting similar chemical structures.

1.2.4 Cell Culture

HaCaT (immortalized human keratinocytes) and murine RAW 264.7 macrophages were acquired from CLS-Cell Lines Service GmbH (Eppelheim, Germany) and the European Collection of Cell Cultures (Salisbury, UK), respectively. Cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, Milan, Italy) completed with 1 mM pyruvate, 100 mM non-essential amino acids, 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 µg/mL), 1% (w/v) L glutamine. The cells were incubated under standard conditions (37 °C, 5% CO_2, 95% humidity). The growth medium was changed every 2–3 days till approximately 80% confluence was reached. All experiments were performed after 24 hours of cell adhesion.

1.2.5 Cell Viability Assay WST8

Cell viability assay was performed according to Tiboni et al. [263]. HaCaT and RAW 264.7 cells were seeded into 96-well plates (5 x 10^3 cells per well) in 100 µL complete DMEM and incubated at 37 °C for 24 h. The medium was then removed, and 100 µL of fresh medium containing Co ext. at increasing concentrations (0–4 mg/mL) was added and incubated for a further 24 h. After 24 h, 10 µL of water-soluble tetrazolium salt (WST-8) (Sigma-Aldrich, Milan, Italy) was added to each well to evaluate cell metabolic activity. Absorbance was measured at 450 nm using a plate reader (BioRad Laboratories, Hercules, CA, USA) up to a maximum of 4 h of incubation at 37 °C. Three independent experiments were performed in triplicate, and cell viability was assessed as a percentage of the control.

1.2.6 Nitric Oxide Detection

RAW 264.7 cells stimulated by lipopolysaccharide (LPS, Sigma-Aldrich, Milan, Italy) were used to evaluate anti-inflammatory properties. Determination of extracellular nitric oxide (NO) concentration was carried out by UV-visible spectrophotometer. The nitrite concentration was determined according to a linear standard curve (calcium nitrite 6.25–100 µM) [264]. RAW 264.7 cells, in the logarithmic phase, (3 x 10^4 cells/100µL) were seeded in 96-well plates and cultured for 24 h. Then, cells were co-

treated for 24 h with LPS (1 µg/mL) in the presence or absence of Co ext. 0–4 mg/mL. Dexamethasone (DEXA) 0.0039 mg/mL was used as positive control. After 24 h, aliquots of 50 µL of supernatant were taken and incubated for 10 min in the dark at room temperature with 50 µL of Griess reagent at a concentration of 40 mg/mL. Absorbance was measured at 570 nm using a plate reader (BioRad Laboratories, Hercules, CA, USA).

To exclude the possibility that the reduction in NO could be linked to a reduction in cell viability, it was assessed in the same plate using the WST-8 viability assay.

1.2.7 LPS-Induced Inflammation in HaCaT Cells

Inflammation on HaCaT cells was induced by 1 µg/mL LPS [265]. HaCaT cells, in the logarithmic phase, were seeded at a density of 6 x 10^5 cells/mL (2 mL/well) on 6-well plates and allowed to adhere for 24 h in a 37 °C incubator with 5% CO2. Then, the cells were co-treated for 24 h with LPS in the presence or absence of 1.78 mg/mL Co ext. (value derived from a previous viability study). At the end of the treatment, the medium was removed, and the cells were washed in PBS and trypsinized. The cells were stored in 350 µL of lysis buffer RLT (RNeasy mini kit Qiagen, Milan, Italy) at -80 °C until RNA extraction.

1.2.8 Quantitative Real-Time PCR

Total RNA was extracted using the RNeasy mini kit Qiagen, Milan, Italy, and then mRNA was converted to cDNA and processed by real-time PCR to evaluate the expression of iNOS, IL-6, IL-1β, ikBα, and ICAM genes related to the inflammatory process. The expression levels of the inflammatory genes were normalized with the housekeeping gene 36B4. Quantification was performed in a 20 µL volume using 2 µL of each cDNA. Specifically, 10 µL of Power up Sybr Green Master Mix, 0.6 µL of forward and reverse primers from a 10 µM preparation, and 6.8 µL of water were used. Real-time PCR was conducted using a QuantStudio 1 Real-Time PCR System thermal cycler (Thermo Fisher). The analysis was carried out with the Pfaffl method [266]. The real-time PCR thermal conditions were carried out as follows: initial denaturation at 95 °C for 10 min, and then 40 cycles at 95 °C for 15 sec and 60 °C for 1 min.

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

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

Table 2: **Employed primers for real-time polymerase chain reaction (PCR) analysis.**

1.2.9 Quantification of Intracellular ROS Production

Intracellular ROS (reactive oxygen species) was determined by FLUOstar OPTIMA fluorescence microplate reader (BMG LABTECH) using the cell-permeable fluorogenic probe 2',7' -dichlorofluorescein diacetate (DCF-DA). HaCaT cells were seeded into a 96-well plate at a density of 1 x 10^4 cells in 100 µL of growth medium for 48 h at 37 °C, then washed with PBS, and treated with different concentrations of Co ext. in DMEM without FBS and antibiotics for 2 h at 37 °C. After the treatment, the cells were washed with PBS, and then 10 µM DCF-DA was added for 30 min. The DCF-DA was removed, by washing with PBS, and ROS production was determined. A total of 0.1 mM H_2O_2 or 0.3 mM tert-butyl hydroperoxide (TBHP) was added, and after 30 min of treatment, ROS production was determined again.

1.2.10 Statistical Analysis

The statistical analyses were performed using GraphPad Prism version 6 for Windows, GraphPad Software. Values were expressed as the mean ± the standard error of the mean (SEM). The treated and control sample variables were compared using a one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. The differences between samples were considered significant with p-values < 0.05.

1.3 Results and Discussion

Epidemiological studies have linked apple consumption to a reduced risk of certain cancers, cardiovascular and respiratory diseases, and diabetes. Recent findings also suggest that apples positively affect vascular function by modulating blood pressure, plasma lipid levels, inflammation, and hyperglycemia. These health benefits are attributed to the bioactive compounds found in apples, which has led researchers to explore callus cultures derived from the fruit [267]. Oota was the first to establish apple callus cultures and found that they contained high levels of anthocyanins [268]. More recently, Verardo et al. isolated callus cultures from *Golden Delicious*, *Mela Rosa Marchigiana*, and the exotic *Acca Sellowiana* (Berg.) Burret, revealing that these cultures were richer in triterpenic acids compared to the respective peels and pulps [269,270].

In addition to apples, quince—a widely available and affordable source of beneficial phytoconstituents—has also been explored for its potential in producing nutraceuticals. In a recent study, our group successfully obtained secondary metabolites (SMs) using quince pulp callus culture [261]. In the same study, the chemical characterization of phenolic and triterpenic acid contents (listed in the tables below) of Co ext. and its biological activity in cell-free models were reported. Briefly, the total phenolic content of callus extract was 8.3 times higher than that of the pulp. The most represented phenolic compound in callus extract was 5-CQA followed by 5-p-coumaroylquinic acid (5-p-CoQA), and 3-O-caffeoylquinic acid (3-CQA). The compounds 3-CQA and procyanidin B2 were found to be more expressed in the pulp than in the callus. The main triterpenic acids present in the callus were maslinic, corosolic, annurcoic, and tormentic acids, while they were completely absent in the pulp. Other studies have been conducted on the phytochemical composition of quince focusing mainly on the polyphenolic profile of the various parts of the fruit [271-273]. No studies concerning the triterpene acids profile of quince are reported in the literature, thus our team was the first to develop quince pulp callus cultures and to characterize the triterpene acids and polyphenols contents [261].

(3)

Phenolic compounds	µg per 100.0 mg*	RSD%[a]
5-CQA	72.18 ± 0.47	0.65
5-p-CoQA	12.98 ± 0.34	2.62
(-)-Epicatechin	2.02 ± 0.05	2.48
3-CQA	1.82 ± 0.04	2.20
Procyanidin B2	0.94 ± 0.02	2.13
4-p-CoQA	0.65 ± 0.03	4.62
(+)-Catechin	0.60 ± 0.02	3.33
Quercetin-3-O-glucoside	0.18 ± 0.01	5.55
Procyanidin B type dimmer	0.11 ± 0.01	9.09
Procyanidin B type dimmer	0.10 ± 0.01	10
Total	**91.58 ± 0.91**	

(4)

Triterpenic acids	µg per 100.0 mg*	RSD%[a]
Maslinic acid	13.92 ± 0.12	0.86
Corosolic acid	8.74 ± 0.06	0.69
Annurcoic acid	6.81 ± 0.06	0.88
Tormentic acid	3.70 ± 0.08	2.16
Total	**33.17 ± 0.04**	

Tables 3 and 4: **Specific content of phenolic compounds (3) and triterpenic acids (4) in the extract obtained from 100.0 mg of lyophilized callus culture of quince.** (Tables from https://dx.doi.org/10.1016/j.jff.2022.105133). [a] Relative Standard Deviation expressed as a percentage.

This background and the known antioxidant and anti-inflammatory properties of polyphenols and triterpenic acids [274-276] prompted us to investigate the Co ext. effects in cellular models.

First, the non-cytotoxic concentration range was identified in both RAW 264.7 and HaCaT cell lines, after 24 h of Co ext. treatment by cell viability WST-8 assay. The analyses showed that none of the tested concentrations were cytotoxic, with even a significant increase in cell viability at the Co ext. concentration of 3.56 mg/mL and 1.78 mg/mL in RAW 264.7 and HaCaT cells, respectively (Figure 11).

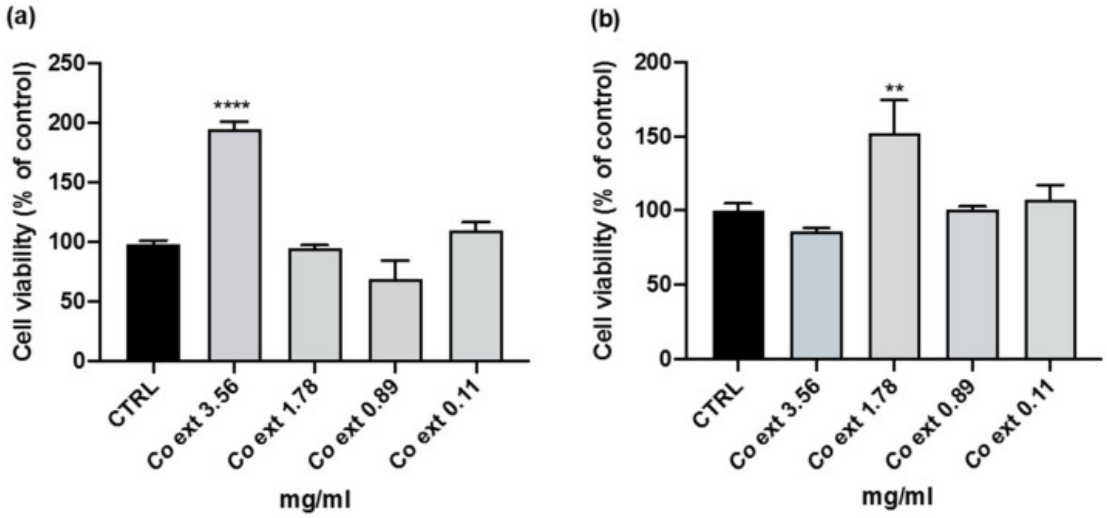


Figure 11. RAW 264.7 and HaCaT cell viability. RAW 264.7 (a) and HaCaT (b) cells were treated with different concentrations of Co ext. (0–4 mg/mL) for 24 h, and cell viability was measured by WST-8 assay. Data are the means of three independent experiments conducted in triplicate ± SEM. (** $p < 0.01$, **** $p < 0.0001$) compared to CTRL (untreated cells).

Macrophages have an important role in immune reactions, allergies, and inflammation protecting the body from foreign pathogens through phagocytosis. During this process, macrophages release a variety of inflammatory mediators, including interleukin 1 (IL-1), tumor necrosis factor-alpha (TNF-α), nitric oxide (NO), and prostaglandins [277]. LPS-activated macrophages produce nitric oxide synthase (iNOS), an enzyme that catalyzes the oxidative deamination of L-arginine to produce NO. Excess NO production by iNOS can have negative consequences, such as inflammatory diseases and septic shock [277]. NO released by macrophages stimulated with LPS is a marker associated with acute and chronic inflammation processes [278]. We selected the RAW 264.7 cells as an appropriate macrophage model to rapidly study the inflammatory cell responses with the Griess test [279].

The co-treatment of RAW 264.7 cells for 24 h with 1 µg/mL LPS and Co ext. (0–4 mg/mL) showed a significant reduction in NO release at all tested concentrations except for 0.11 mg/mL Co ext. (Figure 12a). To exclude the possibility that the NO reduction could be linked to a reduction in cell viability after LPS administration the WST-8 assay was also performed. As shown in the figure, none of the Co ext. concentrations tested led to a reduction in cell viability (Figure 12b).

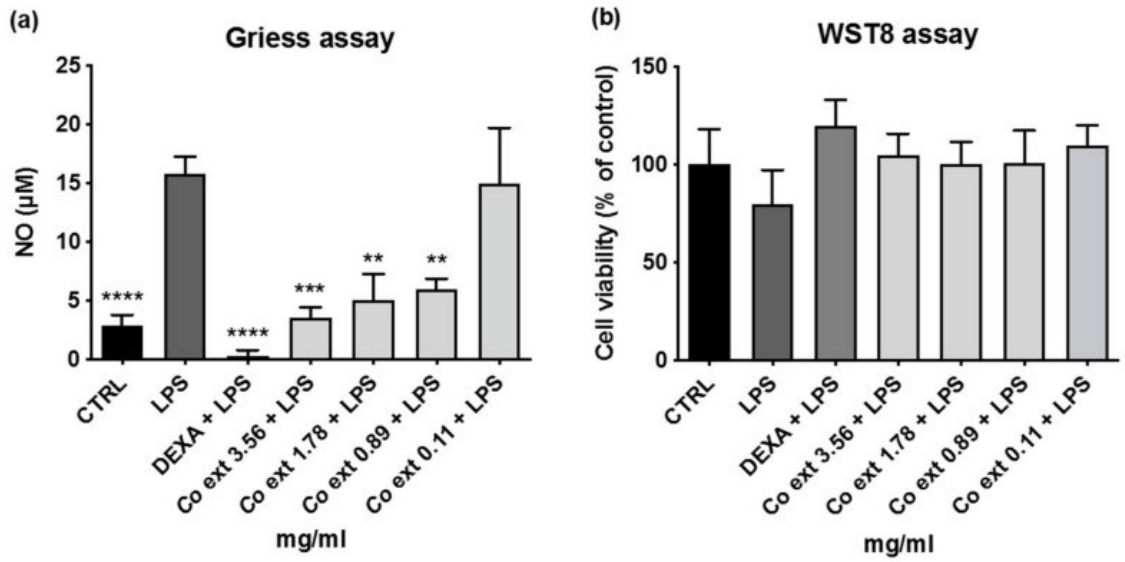


Figure 12. Extracellular NO release and cell viability evaluation after LPS, Co ext., and dexamethasone (DEXA) administration to RAW 264.7 cells. (**a**) RAW 264.7 cells were co-treated for 24 h with LPS 0.001 mg/mL in the presence or absence of Co ext. or DEXA. CTRL: negative control (untreated cells); DEXA 0.0039 mg/mL was used as an anti-inflammatory reference compound. The results are presented as nitric oxide concentration. The data show the means ± SEM of all samples compared to LPS (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Dunnett's multiple comparison test was performed. (**b**) Evaluation of cell availability (WST8 assay). The results are expressed as the percentage of control ± SEM of three independent measurements.

As a human cell model, we chose the keratinocyte cell lines (HaCaT) because they are a cellular model that allows us to study epidermal homeostasis and its pathophysiology. Keratinocytes are primarily responsible for the structural and barrier functions of the epidermis; they are also involved in activating and maintaining inflammatory and immunological responses in the skin, and they actively participate in wound repair through the secretion of growth factors, cytokines, and chemokines [280]. Given the importance of the function of the skin barrier, the effect of Co ext. was investigated in HaCaT where we evaluated how Co ext. can modulate the expression of pro-inflammatory cytokines. The anti-inflammatory effect of Co ext. on HaCaT cells was evaluated after the cotreatment for 24 h with 1 µg/mL LPS in the presence or absence of the extract by investigating the expression of iNOS, IL-6, IL-1β, ikBα, and ICAM by real-time PCR. These genes, linked to the inflammatory process, were downregulated by the co-treatment with 1.78 mg/mL Co ext. (Figure 13).

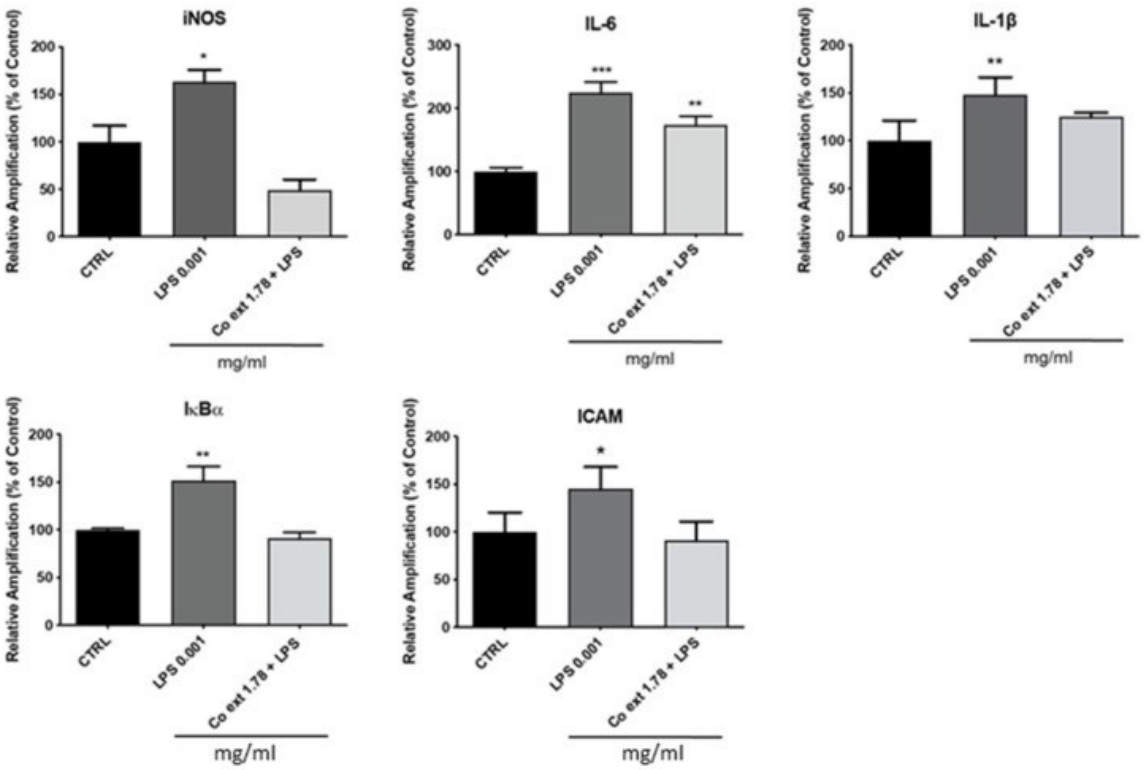


Figure 13. Anti-inflammatory activity of Co ext. on LPS-treated HaCaT cells. HaCaT cells were co-treated for 24 h with LPS 0.001 mg/mL in the presence or absence of 1.78 mg/mL Co ext. Co ext. significantly decreased the expression of all the LPS-upregulated analyzed genes. Data are the means ± SEM of three independent experiments. (* $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$). Dunnett's multiple comparison test was performed vs. CTRL.

Although a more in-depth investigation will be required, the downregulation of the selected cytokines suggests that Co ext. acts through the involvement of the NF-kB signal transduction pathway.

Preliminary studies were also carried out to evaluate Co ext. antioxidant properties. The antioxidant activity of Co ext. was investigated on HaCaT cells using the fluorescent probe DCF-DA. Using this probe, we found that the ROS production was significantly decreased by Co ext. already in the absence of an oxidative challenge (Figure 14a). The ROS production induced by 0.3 mM tert-butyl hydroperoxide (TBHP) or by 0.1 mM H_2O_2 for 30 min was significantly reduced by Co ext. (Figure 14b, c).

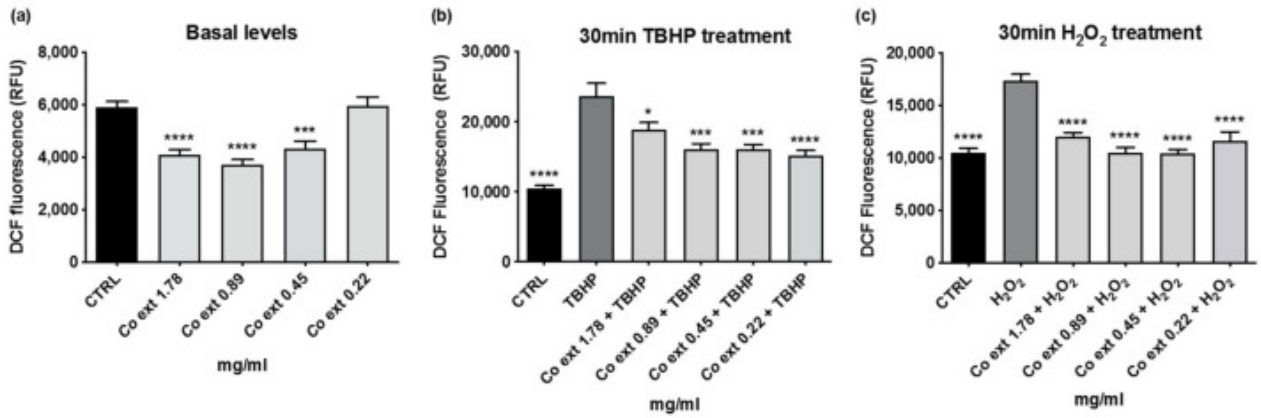


Figure 14. Antioxidant activity of Co ext. in HaCaT cells. Intracellular ROS were evaluated using the DCF-DA assay in HaCaT cells treated with different concentrations of Co ext. ROS production was detected (**a**) after ON incubation with 0 (=CTRL), 1.78; 0.89; 0.45; 0.22 mg/mL Co ext. without any oxidative stimulus and (**b**) after treatment with 0.3 mM TBHP for 30 min or (**c**) 0.1 mM H_2O_2 for 30 min. Dunnett's multiple comparison test was performed vs. CTRL (**a**), TBHP (**b**), and H_2O_2 (**c**). Data represent the mean ± SEM of at least three independent experiments. (* $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$).

These results confirmed also in a cellular model the antioxidant activity of Co ext., previously observed in cell-free models detected by DPPH, ABTS, and ORAC assays [261].

1.4 Conclusions

The results of this study further highlight and expand the beneficial effects of *C. oblonga* Mill. callus extract in cellular models, where its anti-inflammatory and antioxidant properties were demonstrated. The anti-inflammatory action was observed in both macrophage and keratinocyte cells, with a marked reduction in NO levels in immune cells, as well as significant downregulation of iNOS, pro-inflammatory IL-6, IL-1β, ikBα, and ICAM cytokine gene expression in epidermal cells. The antioxidant effect was evident through decreased ROS levels in oxidatively stressed keratinocytes. However, the limitation of our experimental approach lies in the use of cellular models, and to confirm these beneficial effects of the extract, further research in more complex systems is needed.

Despite this, these findings may contribute to the development of non-pharmacologic strategies aimed at extending the health span and preventing age-related diseases. Achieving this goal is crucial, given the socioeconomic and public health impact of an aging population.

CHAPTER 2

Long-lasting production of bioactive compounds from Malus x domestica Borkh var. Mela Rosa Marchigiana pulp callus culture with antioxidant and anti-inflammatory activity.

Original articles published in:

- Gubitosa, F., Benayada, L., Fraternale, D., De Bellis, R., Carloni, S., Potenza, L., Chiarantini, L., Gorassini, A., Verardo, G., Roselli, C., Valentini, L., Gobbi, P., Balduini, W., Ventura, N., Giannaccini, F., Kass, G. E. N., Colomba, M., & Albertini, M. C. (2024). Long-lasting bioactive compounds production from *Malus x domestica Borkh* var. Mela Rosa Marchigiana pulp callus culture with antioxidant activity: *In vitro*, *ex vivo* and *in vivo* validation. *Journal of Functional Foods, 114*, 106073. https://doi.org/10.1016/j.jff.2024.106073
- Benayada, L., Gubitosa, F., Fraternale, D., Carloni, S., Cerioni, L., Matacchione, G., Olivieri, F., Potenza, L., De Bellis, R., Chiarantini, L., Roselli, C., Valentini, L., Gobbi, P., Balduini, W., Pappagallo, N., Ventura, N., Kass, G. E. N., Colomba, M., & Albertini, M. C. (2024). In vitro anti-inflammatory activity of *Malus × domestica* var. Mela Rosa Marchigiana pulp callus extract contrasting high glucose conditions. *Journal of Functional Foods, 121*, 106420. https://doi.org/10.1016/j.jff.2024.106420

2.1 Introduction

The Mela Rosa dei Monti Sibillini is an ancient apple variety cultivated in the Sibillini Mountains of Marche, central Italy, since Roman times. This small, flat-shaped apple with a green peel and pink-reddish shades has a distinctive sweet and sour taste and a strong aroma. It is typically grown at altitudes between 400 and 900 meters [281]. After a period of neglect, local authorities have worked to revive its cultivation, particularly after the 2016 Central Italy earthquakes. Recognition by Slow Food has driven efforts to expand its growth, enhance the local economy, and promote its unique taste, aroma, and long storability, though its small size makes it less commercially competitive compared to modern varieties. Renewed interest in the Mela Rosa stems from both its cultural heritage and potential health benefits [281].

Studies have focused on its phytonutrients and biological properties, aiming to elevate its status. Research by Joice G. et al. highlighted that the apple is rich in carbohydrates, fiber, malic acid, and essential minerals like potassium, magnesium, and calcium, vital for cardiovascular and bone health. The apple is also packed with polyphenols, such as flavan-3-ols, procyanidins, dihydrochalcones, and quercetin, which provide antioxidant and anti-inflammatory benefits, supporting cardiovascular health, diabetes management, and neuroprotection. Additionally, hydroxycinnamic acids, such as chlorogenic acid, contribute to glucose regulation and weight management, while ursolic and

annurcoic acids help reduce inflammation. The apple's distinctive aroma, due to compounds like esters and terpenoids, enhances its appeal [281].

Various *in vitro* studies confirm the apple's antioxidant and anti-inflammatory properties, as well as its ability to inhibit enzymes like α-glucosidase, lipase, monoamine oxidase A, tyrosinase, and acetylcholinesterase, indicating potential use in health applications [282-284]. Animal studies further demonstrate the apple's neuroprotective and hepatoprotective effects, suggesting its value in nutraceuticals and dietary supplements. These findings underscore the Mela Rosa's promise as both a cultural treasure and a source of health-promoting compounds [285, 286].

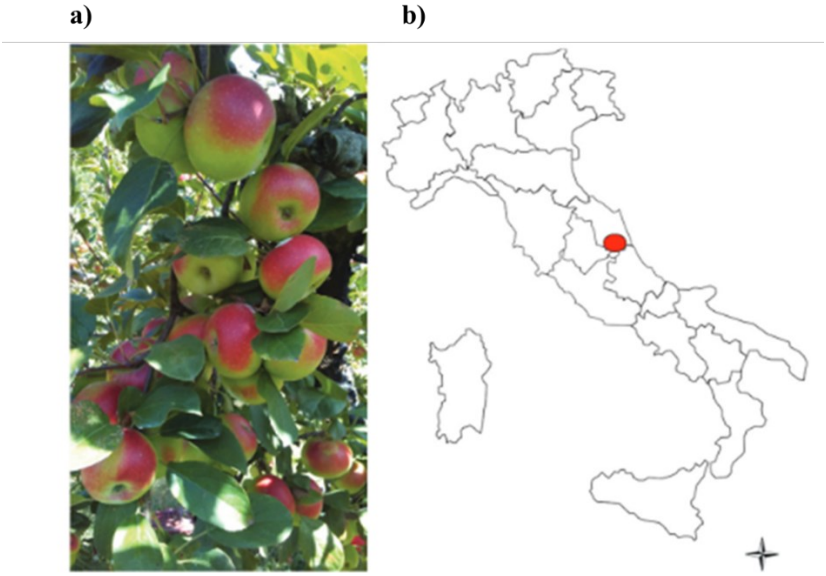


Figure 15: **Mela Rosa dei Monti Sibillini (a) and its cultivation area (b).** (Image from https://doi.org/10.1080/09637486.2019.1580684)

In 2017, our research group discovered the optimal conditions for obtaining callus culture from the pulp of Mela Rosa Marchigiana (MRM) to increase the production of important SMs that have beneficial effects on human health. Since 2017, the MRM pulp callus has been kept alive through regular sub-cultures every 28 days, and the triterpenic acid content of both MRM pulp fruit and callus methanolic extracts has been analyzed [269]. According to Table 5, the total amount of triterpenes in callus culture products was significantly higher compared to that obtained from fresh fruit pulp. Based on this result, Potenza and colleagues prepared a 70% ethanol MRM pulp callus extract (MRME) from one of the 2019 callus sub-cultures (without GC–MS/GC-FID characterization) to test its biological potential. In that study, the extract exhibited antioxidant, geno-protective, anti-inflammatory, stimulating mitochondrial biogenesis, and wound healing activities [262]. In the current study, the MRM callus ethanolic extract was prepared from one of the 2022 sub-cultures. The extraction protocol was slightly modified to reduce debris, and the triterpenic acid composition of the extract was analyzed by GC-MS and GC-FID. Experimental tests were conducted to determine if the 2022 extract still possessed antioxidant and anti-inflammatory capacity, and if so, to assess its

effectiveness. After comparing the triterpenic acid content of MRME 2022 and MRME 2019, cell-free and cell-based assays were performed to evaluate the potential of the extract against oxidative and inflammatory stress-induced damage. To compare present and previous results, we intentionally repeated some of the cell-free (i.e., DPPH, ABTS, and ORAC) assays and *in vitro* experiments previously carried out by Potenza et al. [262].

2.2 Materials and Methods

2.2.1 Callus culture conditions and maintenance

The callus used in this study and the previous one [262], comes from the same culture developed more than six years ago [269]. The MRM callus obtained in 2017 was regularly sub-cultured every 28 days and continues to be sub-cultured today. The *in vitro* culture was conducted as described by Verardo et al. [269]. Briefly, we employed the Gamborg B5 medium plus 2.0 mg/L 6-benzylaminopurine (BA, Sigma-Aldrich) and 0.2 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid, Sigma-Aldrich), plus 30 g/L sucrose, pH 5.8, as this combination turned out to give the highest biomass production from pulp explants of MRM. Callus culture was conducted in the dark at 25 ± 2 ◦C and subcultures were made after 28 days in the same medium. Every 28 days, a part of the callus obtained by each sub-culture is sub-cultured again to maintain the material, while the remaining part is harvested and stored at -20 ◦C until use. When necessary, part of the frozen material is lyophilized and employed for the extraction procedure (for details see paragraph 2.2.2). To use a less toxic extraction solvent than methanol, the extraction efficiency of 70 % ethanol following the procedure of Verardo et al. [269] has been tested. Both 100 % methanol and 70 % ethanol callus extracts were analyzed. In addition, a modified protocol of ethanol extraction has been performed, characterized by two extra sequential resuspension (in 70 % ethanol) and centrifugation steps, to minimize the debris as much as possible.

2.2.2 Mela Rosa Marchigiana callus ethanolic Extract (MRME) preparation

Freeze-dried MRM pulp callus (260 mg) was extracted in a glass potter with 15 mL of 70 % ethanol for 15 min. The homogenate was stirred overnight (ON) at 4 ◦C and then centrifuged for 45 min at 4 ◦C x 13,000g. After supernatant recovery, the pellet was extracted a second time with 15 mL of 70 % ethanol and stirred at 4 ◦C for 30 min. The supernatant was recovered by centrifugation as above, and the two supernatants were combined and dried in Savant ON. The day after, 15 mL of 70 % ethanol was added to the residue and, after stirring for 30 min at 4 ◦C, the tube was centrifuged as above and the recovered supernatant was dried to be subsequently resuspended in 15 mL of 70 % ethanol, re-centrifuged and finally the obtained supernatant aliquoted in Eppendorf (1 mL each) and dried in Savant. After weighting the pellet, each Eppendorf was stored at -20 ◦C before chemical analysis and biological tests.

2.2.3 Mela Rosa Marchigiana callus ethanolic extract analyses by GC-MS and GC-FID

The procedure for identification and quantification of triterpenic acids found in MRME used in this study was described in our previous works [261, 269, 270]. Briefly, each dried extract was spiked with internal standard (cholesterol, IS), suspended in water, and extracted twice with EtOAc. The collected organic phases were washed with H_2O, and brine, then dried (Na_2SO_4 anhydrous), filtered, and evaporated to dryness in vacuo. The residue was silylated by a mixture (1:1) of pyridine and BSTFA, and the reaction mixture was analyzed by GC–MS and GC-FID (all the compounds were identified by GC-MS and then quantified by GC-FID). GC–MS analyses were carried out using a Trace GC Ultra gas chromatograph equipped with a split-splitless injector and coupled to an ion-trap mass spectrometer detector Polaris Q (Thermo Scientific). The column was a 30 m × 0.25 mm i.d., 0.1 μm film thickness, fused silica SLB-5 ms (Supelco, Sigma-Aldrich). The initial oven temperature was 240 ◦C programmed to 280 ◦C at 2 ◦C/min and kept at 280 ◦C for 10 min the temperature was then raised to 310 ◦C at a rate of 10 ◦C/min and maintained at this temperature for 20 min. Samples were injected in the split (1:10) mode. The injector, transfer line, and ion source were set at 280, 280, and 200 ◦C, respectively. Helium was used as carrier gas at a flow of 1 mL/min. The mass spectra were recorded in electron ionization (EI) mode at 70 eV electron energy with a mass range from *m*/*z* 50 to 1000 and a scan rate of 0.8 scan/sec. Identification of metabolites was carried out by comparison of the spectral data and retention times with those of standards or to the spectra from the NIST02 spectral library or with those available in the literature. The data acquisition was under the control of Xcalibur software (Thermo Scientific). The quantitative analysis was carried out using an Fisons GC 8000 series gas chromatograph, equipped with a split/splitless injector and a flame ionization detector (Fisons Instruments, Milan, Italy). The separation was carried out with a fused silica capillary column DB-5MS UI 30 m × 0.250 mm × 0.25 μm film thickness (Agilent, J&W, Italy). The initial oven temperature was 240 ◦C programmed to 280 ◦C at 2 ◦C/min and kept at 280 ◦C for 25 min, the temperature was then raised to 310 ◦C at a rate of 10 ◦C/min and maintained at this temperature for 13 min. Samples (1 μL) were injected in the split (1:10) mode. The injector and detector were set at 280 ◦C. Hydrogen was used as carrier gas at a flow of 1.8 mL/min. Peak areas were integrated using a Varian Galaxie Workstation (Agilent Technologies, Italy). Quantification of the triterpenic acids and β-sitosterol in the samples was performed using the internal standard method based on the relative peak area of analyte to IS (cholesterol) from the average of three replicate measurements.

2.2.4 DPPH scavenging activity

The antioxidant ability of MRME was determined using the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) as described by Saltarelli et al. [287] with minor modifications. In brief, 850 µL of 100 µM DPPH• ethanol solution newly prepared was mixed with 150 µL of MRME (50 mg dw/mL). After half an hour in the dark at room temperature (RT), the absorbance was measured at a wavelength corresponding to 517 nm (UVIKON 930 UV/VIS spectrophotometer); 70 % ethanol was used as blank. Values are expressed as the MRME concentration required to scavenge 50 % of the initial DPPH radicals (IC50 mg dw/mL).

2.2.5 ABTS scavenging activity

The 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay was carried out according to Loizzo et al. [288] with some modifications. The ABTS radical is generated after mixing 7 mM ABTS solution with 140 mM potassium persulphate ($K_2S_2O_8$) and left to incubate for 12–16 h in the dark at RT. The reaction mixture was then diluted 1:90 v/v with 70 % ethanol and the absorbance was measured and should have a corresponding value of 0.70 ± 0.02 at 734 nm. 10 µL MRME of different solutions ranging from 50 to 500 µg/mL, was added to 1 mL of ABTS ethanolic solution, and incubated 4 min in the dark at RT. The absorbance decrease was measured at 734 nm (UVIKON 930 UV/VIS spectrophotometer); 70 % ethanol was used as blank. Values are expressed as the MRME concentration required to scavenge 50 % of the initial ABTS radicals (IC50 mg dw/mL).

2.2.6 ORAC assay

The ORAC (oxygen radical absorbance capacity) assay is one of the most used methods to assess the antioxidant ability of substances for research and industrial purposes. The antioxidant capacity of MRME was tested as described by De Bellis et al. [289] using a Fluostar Optima Plate reader fluorimeter (BMG Labtech, Offenburg, Germany) equipped with a temperature-controlled incubation chamber (37 ◦C) and an automatic injection pump. The following compounds were mixed as in the protocol below: 200 µL of 0.096 µM fluorescein sodium salt in 0.075 M Na-phosphate buffer (pH 7.0), 20 µL of the sample (MRME) or Trolox or 0.075 M Na-phosphate buffer (pH 7.0) as blank. The reaction was initiated with 40 µL of 0.33 M of 2,2-azobis-(2-amidinopropane) hydrochloride (AAPH). Fluorescence was read at 485 nm excitation and 520 nm emission until complete extinction. Each time, a calibration curve was made using a positive standard, the Trolox, with a concentration ranging from 50 to 500 µM in 0.075 M Na-phosphate buffer (pH 7.0). ORAC values of the tested samples are reported as µmol Trolox Equivalents (TE)/g of callus extract.

2.2.7 Cell cultures

HaCaT (immortalized human keratinocytes) cells (CLS-Cell Lines Service GmbH, Eppelheim, Germany) and murine macrophages RAW 264.7 (European Collection of Cell Cultures, Salisbury, UK) were used to investigate the biological properties of MRME. HaCaT cells were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum, 1 % L-glutamine, 1100 U/mL penicillin/streptomycin, and maintained in a CO_2 incubator at 37 ◦C and 5 % CO_2. Cell culture reagents were from Sigma-Aldrich (Milan, Italy). RAW 264.7 were grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma- Aldrich, Milan, Italy) completed with 1 mM pyruvate, 100 mM non-essential amino acids, 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 μg/mL), and 1% (w/v) L glutamine. The cells were incubated under standard conditions (37 °C, 5% CO_2, 95% humidity). The growth medium was changed every 2–3 days till approximately 80% confluence was reached. All experiments were performed after 24 hours of cell adhesion.

2.2.8 Cell viability

HaCaT cell viability after MRME treatment was analyzed by water-soluble tetrazolium (WST)-8 assay to evaluate cell metabolic activity. Briefly, cells ($5x10^3$/well) were seeded in 96-well plates and treated ON with MRME at different concentrations. After incubation the medium was removed, and cell viability was analysed. Color development was monitored at 450 nm in a multiwell plate and data were expressed as cell viability (%) vs untreated control cells.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to assess cell viability in RAW 264.7. Cells (5×10^3/well) were grown for 24 h at 37 °C and treated with different doses of MRME. Vehicle (70% EtOH)-treated cells were used as control (CTRL). Briefly, 10 μL MTT (5 mg/mL) solution was added to each well (100 μL) and incubated at 37 °C for 4 h; the obtained formazan salt was solubilized by dimethyl sulfoxide (DMSO) and measured by a microplate reader at the optical density of 570 nm. Cell viability percentage was calculated according to the equation (T/C) × 100, where T and C represent the mean optical density of the treated group and the control group, respectively.

2.2.9 Quantification of Intracellular ROS Production

The antioxidant property of MRME in HaCaT cells was analyzed using the cell-permeable fluorogenic probe 2,7-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich, Milan, Italy). Cells (1×10^4/well) were seeded in 96-well plates grown at 37 ◦C for 48 h, washed with PBS, and treated ON with different concentrations of MRME (2.24–71.5 μg/ mL). A total of 100 μM H_2O_2 was added for 30 min or 1 h at 37 ◦C. The medium was then removed, and cells were incubated with DCFH-DA (5 μM in PBS) for 30 min at 37 ◦C in the dark. ROS production was determined using the multiwell

plate reader FluoStarOptima (BMG Labtech, Ortenberg, Germany) (emission wavelength 520 nm; excitation 485 nm).

2.2.10 Nitric oxide detection

Determination of extracellular nitric oxide (NO) concentration was carried out by a UV-visible spectrophotometer. The nitrite concentration was determined according to a linear standard curve (calcium nitrite 6.25-100 μM) [264]. RAW 264.7 cells, in the logarithmic phase ($3x10^4$ cells/100 μL) were seeded in 96-well plates and cultured for 24 h. Then, cells were co-treated for 24 h with LPS (1 μg/mL) in the absence or presence of MRME. Dexamethasone (DEXA) 10 μM was used as positive control. After 24 h, aliquots of 50 μL of cell culture medium were taken and incubated for 10 minutes in the dark at room temperature with 50 μL of Griess reagent (40 mg/mL) (Bryan & Grisham,2007). Absorbance was measured at 570 nm using a plate reader (BioRad Laboratories, Hercules, USA).

2.2.11 Statistical analysis

Statistical analysis was performed using the computer program Graph Pad Prism (v4.0, GraphPad Software Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by Dunnett's or Tukey's Multiple Comparison Test was used to determine statistical differences between the experimental groups. The results are expressed as mean ± SD (or SEM). Differences were considered statistically significant when $p < 0.05$.

2.3 Results and Discussion

The ability of plant cell, tissue, and organ cultures to produce and store many valuable compounds with bioactive effects has been long recognized, but - in the last decades - it has been highly exploited considering undifferentiated callus/cell cultures as potential phytochemical factories [290]. Phytochemicals located in specialized parts of plants (for example, roots, leaves, or fruits) can be obtained in greater amounts in *in vitro* systems. Callus cultures are widely employed [291], but to our knowledge, this is the first study monitoring the same callus culture to test its SMs production ability over time.

The study was aimed to (i) test the possibility of a long-lasting bioactive compound production from *Malus* x *domestica* Borkh var. Mela Rosa Marchigiana (MRM) pulp callus culture; and (ii) investigate the callus ethanolic extract (MRME) antioxidant and anti-inflammatory activity over time. To this end, the MRM fruit pulp callus culture obtained in 2017 and kept alive by monthly sub-cultures was investigated. The results confirmed that the quantity of phytocompounds produced by the callus is much higher than that detected in the fresh fruit and that the sub-cultures of the same callus continue to produce high amounts of triterpenic acids over time (Table 5).

Compound	Pulp 2017 (100 % MeOH)	%	Callus 2017 (100 % MeOH)	%	Callus 2022 (100 % MeOH)	%	Callus 2022 (70 % EtOH)	%
β-Sitosterol	46.74 ± 1.128	96.39	120.90 ± 2.69	2.24	184.41 ± 2.93	5.59	168.63 ± 2.29	5.16
Oleanolic acid	0.10 ± 0.01	0.21	42.10 ± 1.90	0.78	120.44 ± 2.25	3.65	76.05 ± 1.16	2.33
Ursolic acid	0.38 ± 0.01	0.78	174.43 ± 3.58	3.23	334.36 ± 5.42	10.13	321.69 ± 4.10	9.84
Maslinic acid	0.43 ± 0.01	0.89	963.30 ± 17.62	17.82	361.63 ± 4.97	10.96	378.69 ± 5.19	11.58
Corosolic acid	0.41 ± 0.00	0.84	676.77 ± 3.85	12.52	337.13 ± 6.69	10.21	342.56 ± 3.71	10.48
Pomolic acid	0.16 ± 0.00	0.34	17.10 ± 1.47	0.32	79.60 ± 0.08	2.41	39.76 ± 0.60	1.22
Annurcoic acid	0.07 ± 0.00	0.14	504.57 ± 2.59	9.34	587.51 ± 8.32	17.80	595.61 ± 3.50	18.21
Tormentic acid	0.20 ± 0.01	0.41	2905.63 ± 33.51	53.76	1295.56 ± 9.07	39.25	1347.03 ± 10.04	41.19
Total	**48.49 ± 1.12**		**5404.80 ± 46.69**		**3300.63 ± 6.04**		**3270.01 ± 21.96**	

Table 5: **Triterpenic acids and β-Sitosterol content (µg/100 mg of dried pulp or callus cultures ± SD, and percentage) of MRM pulp and callus cultures after methanolic and ethanolic extraction**.

All experiments performed in this study were performed using the ethanol callus extract obtained as in Potenza et al. [262], with slight modifications. To distinguish between the two extracts, we reported them as MRME 2019 [262] and MRME 2022 (present study). Since in that paper the extract composition was not reported, the MRME 2019 characterization was also performed. As shown in Table 6, the quali-quantitative composition of both extracts was similar.

Compound	MRME 2019 (µg/mg)	%	MRME 2022 (µg/mg)	%
β-Sitosterol	2.29 ± 0.01	6.2	2.24 ± 0.02	5.2
Oleanolic acid	0.90 ± 0.01	2.4	0.96 ± 0.02	2.2
Ursolic acid	4.06 ± 0.08	11.0	4.58 ± 0.04	10.6
Maslinic acid	4.86 ± 0.06	13.2	5.25 ± 0.05	12.2
Corosolic acid	3.56 ± 0.04	9.7	5.10 ± 0.03	11.8
Pomolic acid	0.43 ± 0.02	1.2	0.47 ± 0.01	1.1
Annurcoic acid	8.40 ± 0.10	22.8	7.13 ± 0.04	16.6
Tormentic acid	12.29 ± 0.22	33.4	17.32 ± 0.08	40.2
Total	**36.78 ± 0.33**		**43.05 ± 0.02**	

Table 6: **Triterpenic acids and β-Sitosterol content (µg/mg of dried extract ± standard deviation, and percentage) found in MRME 2019 and MRME 2022.**

After analyzing the composition of MRME 2022 and comparing it with that of MRME 2019, some experiments described by Potenza and colleagues [262] were intentionally repeated, including cell-free and cell-based assays, to test the activity of the extract. DPPH and ABTS results confirmed that the antioxidant activity of the extract was maintained (Table 7). The Oxygen Radical Absorbance

Capacity (ORAC) assay was used to determine the total antioxidant capacity of MRME 2022 which resulted in a mean value of 54.22 ± 1.5 μmol Trolox Equivalent/g of MRM callus extract.

Compound	MRME 2019 IC50 (mg dw/mL) ± SD	MRME 2022 IC50 (mg dw/mL) ± SD
DPPH	8.43 ± 0.42	5.21 ± 0.04
ABTS	0.90 ± 0.06	0.75 ± 0.02

Table 7: **Antioxidant scavenging activity of MRME 2019 and MRME 2022. DPPH and ABTS are reported as mg of dried weight (dw) of callus ethanolic extract/mL with 50% scavenging ability (IC50).**

When performing the cell-based tests, the same experimental conditions as in Potenza et al. [262] with slight modifications were used. To assess the antioxidant activity, HaCaT cells were treated overnight (ON) with different MRME 2022 concentrations (0.36–71 μg/mL) to select non-cytotoxic extract concentrations to be used in the experiments. Cell viability was determined by the WST-8 assay and we found that none of the tested concentrations resulted in cytotoxic (Fig. 16a). Previous studies [262] carried out in the same cell type (HaCaT) showed that, after a 2 h pre-incubation with MRME 2019, followed by oxidative stress (100 μM H_2O_2) for 30 min and 1 h, the extract significantly attenuated the ROS production when supplied at the concentrations of 0.5 and 1 mg/ mL. To test whether lower concentrations of the extract could show antioxidant activity, a 2 h pre-incubation with different concentrations (2.24 to 71.5 μg/mL) of the extract followed by the introduction of H_2O_2 (100 μM for 30 min or 1 h) was tested. No significant results were obtained (data not shown). Then, we extended the pre-treatment time to ON using the same experimental conditions (i.e., the same concentrations as above, followed by the same stimulus). The tests showed that when treating cells ON, the extract exerted a significative antioxidant activity, revealed by a reduced ROS amount compared to the H_2O_2 experimental group, in all the concentration ranges tested, even after half an hour (Fig. 16b and 16c).

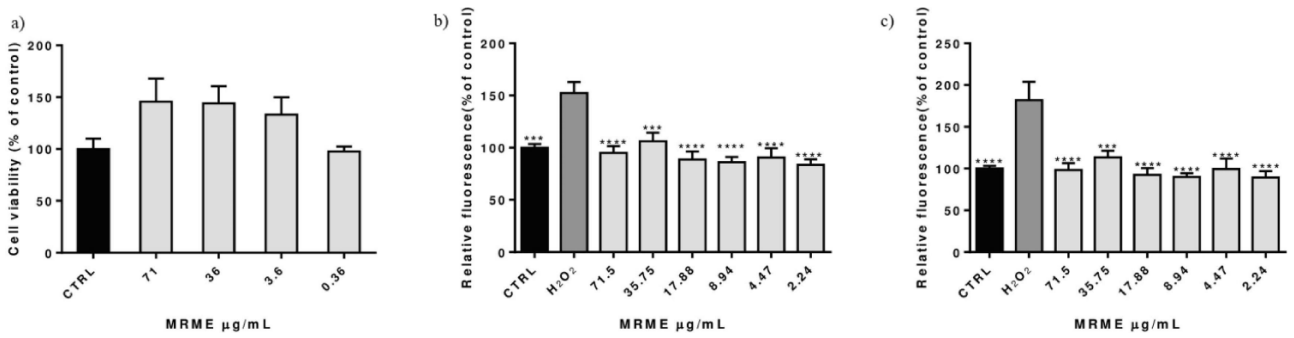


Figure 16: **Cell viability and MRME antioxidant activity.** (**a**) Cell viability after MRME 2022 treatment of HaCaT cells for 24 h evaluated by the WST-8 assay. (**b**) and (**c**) MRME 2022 effect on ROS level. Intracellular ROS were evaluated using the DCFH-DA assay in HaCaT cells treated with different concentrations of MRME. ROS production was detected after overnight incubation with 0 (=CTRL), 71.5; 35.75; 17.88; 8.94; 4.47 and 2.24 µg/mL MRME and (**b**) further introduction of 100 µM H2O2 for 30 min or (**c**) of 100 µM H_2O_2 for 1 h. Results are presented in comparison to H_2O_2. Data represent the mean ± SEM of at least three independent measurements (***p < 0.001; **** p < 0.0001; Dunnett's post hoc test).

Notably, MRME 2022 seemed to be more efficient than MRME 2019. Why so it is hard to say, but this finding could be attributed (at least in part) to a higher amount of tormentic acid (40.2 % vs 33.4 %, Table 6) observed in MRME 2022. We speculate that considering the broad spectrum of biological activities of this compound [292] and its percentage value (>30 %), it is likely to be one of the major bioactive ingredients of the extract. We also suspect that the (slightly modified) extraction method employed for MRME 2022 could have played a role in obtaining a more performing product. However, both hypotheses need to be further investigated. Besides the tormentic acid, the other triterpenic acids of the extract have already been reported as showing a strong anti-inflammatory and anti-tumoral activity and a mild antioxidant ability [293-297]. Interestingly, when testing the biological effect of the extract, we documented for the first time a significant antioxidant effect exerted by a mix of triterpenic acids. In our opinion, such a finding is likely attributable to both the synergic action of the ingredients and the high amounts produced by the callus. Please note that, as in previous studies on *P. spinosa* fruit ethanol extract [298], we deliberately did not focus on single ingredients. All experiments were intentionally carried out aiming at investigating the bioactivity of the whole extract as an antioxidant, considering the synergic effect of the compounds, rather than testing the effect (or the contribution) of each component one by one.

MRME is remarkably rich in PTAs (compared to fresh apple pulp [299], and PTAs exhibit several therapeutic characteristics, including anti-inflammatory, hypoglycaemic, and antihyperlipidemic activities [300-302]. To cite only a few interesting (certainly non-exhaustive) examples, *Potentilla chinensis*, a plant rich in tormentic acid has been demonstrated to be strongly effective against neuro-inflammation in AD mice [303]; tormentic acid was reported to ameliorate insulin resistance and to be effective for the treatment of diabetes and hyperlipidemia in high fat-fed mice [304-305]; corosolic

acid was shown to reduce glucose level in several *in vitro* and *in vivo* models [306] and to improve glucose metabolism in diabetic and prediabetic patients [307].

Potenza et al. [262] found that an MRME concentration of 0.5 mg/mL was the only concentration revealing anti-inflammatory properties without presenting cytotoxic effects in RAW 264.7 stimulated with LPS. As previously done for the antioxidant activity, also in this case we decided to see if lower concentrations of MRME could exert an anti-inflammatory activity without affecting cell viability. Moreover, we extended the treatment time to 24 hours.

Cell viability analysis showed that none of the used MRME concentrations was cytotoxic both in the absence (Fig. 17a) and presence of LPS (Fig. 17b). The evaluation of nitric oxide (NO) release showed a significant increase of NO concentration in LPS-treated RAW 264.7 cells. In contrast, co-treatment of cells with LPS (1 μg/mL) and MRME for 24 h showed a significant reduction in NO release when MRME 150 μg/mL, 75 μg/mL or 37.5 μg/mL were used (Fig. 17c).

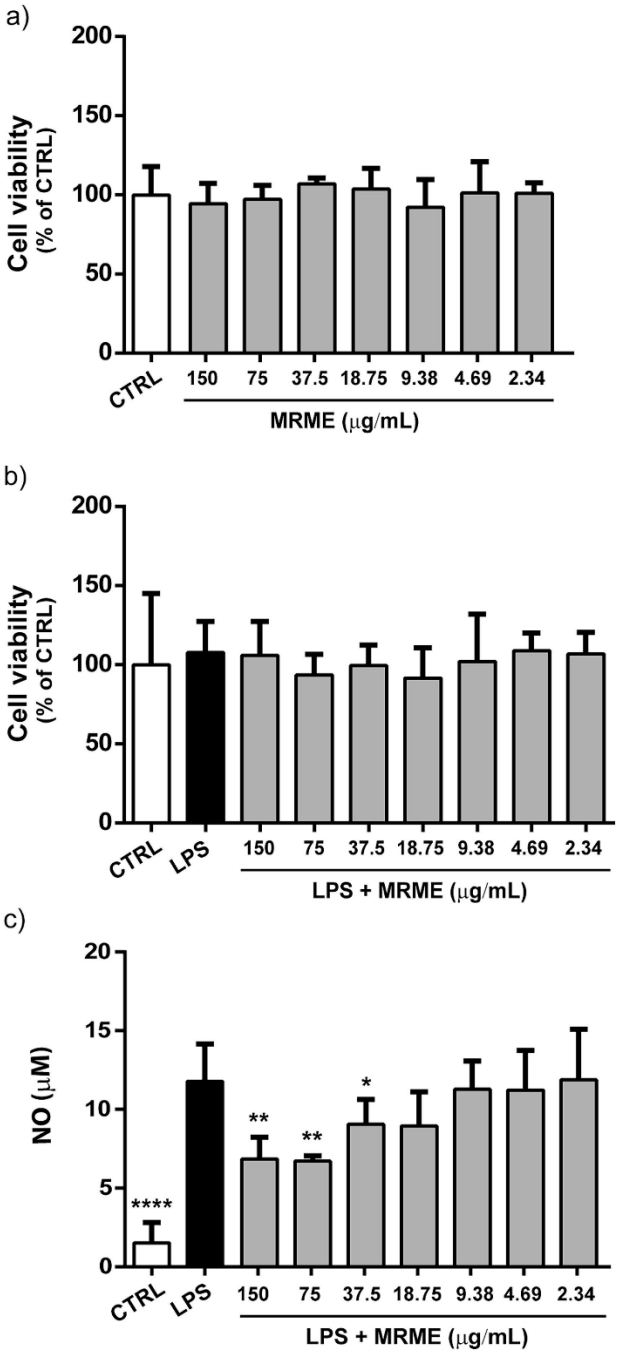


Figure 17. MRME effects on cell viability and NO production during LPS pro-inflammatory condition. (a) RAW 264.7 cell viability after treatment with different concentrations of MRME (150- 75-37.5-18.75-9.38-4.69-2.34 μg/mL) and during LPS and LPS+MRME treatments (b); (c) Nitric Oxide (NO) production (Griess Assay) in RAW 264.7 cells treated with LPS and LPS+MRME. Data are expressed as the percentage of Control (CTRL) and represent the mean ± SD of at least three independent measurements. *p <0.05; **p<0.01; ***p<0.001; ****p<0.0001 vs LPS; One-way ANOVA followed by Dunnett's multiple comparison test.

Our collaborators [299,308] have assessed the antioxidant activity of MRME in *ex vivo* and *in vivo* models. Additionally, they have examined its anti-inflammatory effects in high glucose-induced inflammation HUVEC and in LPS-stimulated U937, as well as its ability to inhibit certain metabolic enzymes. The results obtained confirm and reinforce the ability of MRME to exert antioxidant and anti-inflammatory properties in different experimental models, as well as an interesting enzyme inhibitory activity involved in metabolic syndrome.

2.4 Conclusions

MRM callus cultures generate greater quantities of triterpenic acids compared to fresh fruit pulp, and these callus sub-cultures consistently yield high levels of these acids over time. Furthermore, the bioactivity of phytochemicals remains nearly unchanged even after several years, as shown by in vitro, ex vivo, and in vivo studies [299,308]. In vivo research involving C. elegans and hippocampal slice cultures supported the notion that MRME offers protection against oxidative stress from various stimuli, suggesting it may help reduce oxidative stress-related cell damage—important for promoting healthy aging and preventing neurodegenerative disorders such as Alzheimer's and Parkinson's [299]. Moreover, MRME has been recognized as an economical source of pentacyclic triterpenic acids, which are notable for their strong anti-inflammatory characteristics. In vitro findings also highlight MRME's capability to decrease NO production in LPS-treated RAW 264.7 cells, inhibit inflammation linked to senescence, and improve endothelial and immune cell function under high-glucose conditions, indicating its potential to mitigate risks associated with diabetes-related vascular complications [308]. MRME's enzyme inhibition activities [308], alongside its anti-inflammatory benefits, make it a promising candidate for managing metabolic syndrome, overweight, and obesity—conditions commonly associated with the onset of diabetes. Based on these findings, an intriguing avenue for this research would be to assess whether the MRM pulp callus extract and/or its active components could serve as potential ingredients in a nutraceutical formulation aimed at promoting healthy aging or preventing pathologies linked to oxidative stress and inflammation.

CHAPTER 3

Anti-inflammatory, Antioxidant, and Genoprotective Effects of Callus Cultures from Malus pumila cv. Miller (Annurca Campana Apple) and Preliminary Evaluation of a Nanoformulation in an In Vitro Model of Ferroptosis

Original article published in:

- Gubitosa, F., Fraternale, D., Benayada, L., De Bellis, R., Gorassini, A., Saltarelli, R., Donati Zeppa, S., & Potenza, L. (2024). Anti-Inflammatory, Antioxidant, and Genoprotective Effects of Callus Cultures Obtained from the Pulp of *Malus pumila* cv Miller (Annurca Campana Apple). *Foods (Basel, Switzerland)*, *13*(13), 2036. https://doi.org/10.3390/foods13132036

3.1 Introduction

Annurca apples have attracted significant attention among the various apple varieties. Its historical significance traces back to the ancient Roman era, rendering it a preeminent cultivar in southern Italy. It is important to note that this variety is the most extensively cultivated in the Campania region. Given the significant economic value of the Annurca cultivar to the Campania region, the 'Istituto Sperimentale di Frutticultura' in Ciampino (Rome) established a research program in 1970 to address the cultivar's inherent limitations. This endeavor encompassed comprehensive studies of its biological characteristics, breeding techniques, and optimal storage conditions. Furthermore, a formal proposal was submitted to the European Council for the conferral of 'Protected Geographical Indication' (PGI) status under European regulation number 2081/92. This recognition seeks to facilitate the marketing of the Annurca apple cultivar at both the local and international levels [309-311]. The Annurca apple is distinguished by its unique ripening process. Following harvest, the fruit undergoes a postharvest reddening treatment using traditional structures known as 'melai'. These structures comprise a layer of straw placed on the ground to support the fruit. Subsequently, shading nets are employed to mitigate solar radiation and regulate temperature. During October, the apples are harvested and subsequently positioned on a bed of straw on the ground, which is then consistently moistened. Upon the fruits' surface turning red, they are manually rotated to ensure an even reddening. This treatment can range from 20 to 30 days depending on weather conditions and fruit characteristics. Following the reddening process, this cultivar demonstrates excellent storage properties, retaining firmness and emanating a pleasant aroma [309,311]. Several studies have shown that traditional reddening treatment may increase bioactive compounds like flavonoids and anthocyanins in Annurca fruits during storage, leading to significant antioxidant activity [312]. Important research has been conducted on the potential use of Annurca Apple in cosmeceutical

products. For example, Tenore et al. [313] explored the effects of Annurca polyphenolic extract on skin and hair, and they found that it increased hair growth, density, and keratin content in healthy subjects after 2 months.

An oleolite derived from Annurca apple, known for its high Ursolic Acid content, has been found to inhibit the activity of the elastase enzyme, which is involved in the skin aging process. In a human study, a topical formulation containing this oleolite was shown to reduce nasolabial fold and forehead wrinkles, while also significantly improving viscoelastic skin parameters [314].

Ferraro et al. [315] explored the biological effects of an Annurca Apple-based nutraceutical preparation on melanin production in cellular models of human skin and they found that it was able to induce a significant promelanogenic effect in human primary melanocytes and a melanin cytosolic accumulation consistent with tyrosinase up-regulation making this preparation attractive for the treatment of hypopigmentation disorders.

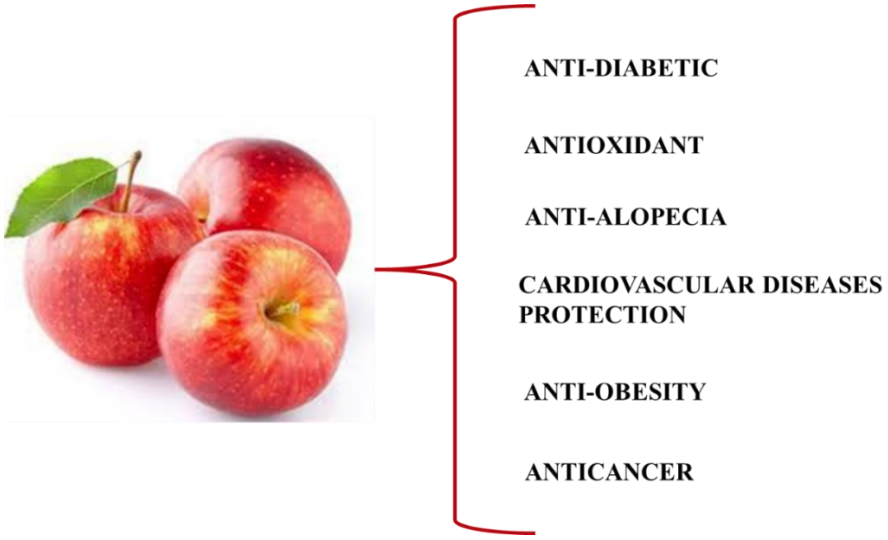


Figure 18: **Beneficial effects from Mela Annurca apple intake**. (Image modified from http://dx.doi.org/10.2174/1573401316666200217113808)

In the current study, two callus cultures were obtained from ripe Annurca apple pulp (Malus pumila cv Miller) and cultivated under different light conditions: darkness and an 18-h photoperiod. The hydro-alcoholic extracts from the calli underwent analysis using GC-MS, GC-FID, and HPLC-DAD-ESI-MSn to determine the qualitative and quantitative content of phenolic and triterpenic acids. Furthermore, we investigated their radical scavenging and antioxidant activities through DPPH, ABTS, ORAC assays, and lipoxygenase inhibition activity. Genoprotection was evaluated via nicking assay, and the anti-inflammatory effect was investigated via Griess assay on LPS-injured murine macrophages. All the analyses performed were compared with peel and pulp hydroalcoholic extracts.

In collaboration with the University of Westminster (London), the hydroalcoholic extract from Annurca apple callus grown under light conditions (LCE) was nanoformulated with Pluronic F127® and Soluplus® to enhance its stability and bioavailability. The resulting nanoformulation (NF-LCE) was characterized for its physicochemical properties. A ferroptosis model was established by exposing HaCaT cells to iron overload, and the ability of nanoformulated LCE (NF-LCE) a pure standard compound of Tormentic Acid (NF-TA) to modulate the iron-induced inflammation was evaluated through Real-Time PCR measuring the expression of genes linked to the ferroptosis process (GPX4, Nrf2, COX2, IL-6, TNF-α). The analysis was compared with the unformulated counterparts.

3.2 Materials and Methods

3.2.1 Plant Material and Callus Cultures

Mature apples were purchased from large retailers in December 2019. The fruits come from the production consortium 'Mela Annurca Campana' IGP. Ripe Annurca apple pulp was used to start callus culture induction. The culture was carried out in the laboratories of the Botanic Garden at the University of Urbino Carlo Bo. Before proceeding with the pulp explants, the fruits underwent surface sterilization by ethanol washing and burning off under a sterile laminar flow hood. The apples were subsequently sliced using a sterile scalpel and discs 5 mm in diameter and approximately 3 mm in height at 0.5 cm from the fruit skin were taken as explants using a sterilized corkscrew [269]. The explant cultures were conducted using Murashige and Skoog culture medium plus 30 g/L sucrose and supplemented with 6-benzylaminopurine (BA, Sigma-Aldrich) (8.8 µM) plus naphthaleneacetic acid (NAA, Sigma-Aldrich) (10.0 µM). The culture medium was brought to pH 5.8 before adding agar into Petri dishes in the amount of 30 mL per Petri dish. Cultures were kept in the dark at 25 ± 2 ◦C. We named this culture 'dark callus' (DC). Subcultures of callus were taken every 28 days from the same culture medium. This optimal culture condition for obtaining callus from ripe Annurca apple pulp was maintained to obtain material by subculturing every 28 days in a fresh culture medium each time. To assess any changes in the phytocomplex composition, the calli were also maintained in an 18-h photoperiod obtained with Cool White fluorescent light at 19 µmol/m^2 s, at a constant temperature of 25 ± 2 ◦C. We named this last culture 'light callus' (LC). The material collected from the sub-cultures was kept in a freezer at −20 ◦C until it was freeze-dried and then extracted. The extracts were used for chemical analysis and various biological tests. Several other fruits were selected for inclusion in the study to conduct a comparative analysis of naturally occurring metabolites in the pulp and peel of apples. Subsequently, the apple peel and pulp under consideration were promptly subjected to freeze-drying and then stored in hermetically sealed containers at a temperature of −20 ◦C until required.

3.2.2 Preparation of Extracts from Peel, Pulp, and Calli of Annurca Apple

Each 200 mg lyophilized sample underwent suspension in 11.5 mL of 70% ethanol and homogenization using a Potter-type homogenizer. The resulting homogenate was transferred into 50 mL tubes and subjected to overnight stirring at +4 ◦C. Subsequently, each tube underwent centrifugation at +4 ◦C and 13,000 rpm for 45 min. The resultant supernatant was then centrifuged at +4 ◦C and 4000 rpm for 30 min and collected in 50 mL empty tubes. The pellet was resuspended in 11.5 mL of 70% ethanol, stirred for one hour, and then centrifuged at +4 ◦C and 13,000 rpm for 45 min. The supernatant from this centrifugation was again collected and further centrifuged at +4 ◦C and 4000 rpm for 30 min. The resulting supernatants were concentrated and subjected to lyophilization using a Speed Vac Concentrator (Thermo Fisher Scientific, Waltham, MA, USA). The lyophilized samples were stored at −20 ◦C and reconstituted in 70% ethanol before use.

To prepare the nanoformulation containing the Annurca Apple Light Callus extract, the final supernatant was filtered with a 0.45µm syringe filter and concentrated using a Rotavapor R-215 (Büchi Labortechnik AG). The samples were then resuspended in 20mL of deionized H_2O and lyophilized using a FreeZone Freeze Dryer (Labconco Corporation). The lyophilized samples were stored at −20 ◦C until further use.

3.2.3 Chemical Characterization of Extracts from Peel Pulp and Calli of Annurca Apple

Each lyophilized sample (30 mg) and 30 µL of the IS solution (3-hydroxycinnamic acid, 100 µg/mL in MeOH) was dissolved in H_2O with 0.4% formic acid (2 mL) and loaded onto a C18 SPE column previously conditioned with 10 mL of MeOH with 0.4% formic acid and 10 mL of H_2O with 0.4% formic acid. After loading, the column was washed with 10 mL of H_2O with 0.4% formic acid, and the phenolic fraction was eluted with 5 mL of MeOH with 0.4% formic acid. The solvents were removed, and the residue was diluted in 1 mL of H_2O/MeOH 9:1 (v/v) with 0.4% formic acid and transferred to an autosampler bottle for HPLC-DAD-ESI-MSn analysis. Chromatographic analysis was performed with a Dionex Ultimate 3000 UPLC (Thermo Fisher Scientific, MA, USA) equipped with a thermostatic autosampler and a column oven. Chromatographic separation was performed with an InfinityLab Poroshell 120 EC-C18 column (4.6 × 150 mm, 2.7 µM (Agilent Technology, Milan, Italy)), thermostated at 30 ◦C. Elution was carried out at a flow rate of 0.6 mL/min, using a mixture of 0.2% formic acid in methanol (A) and 0.2% formic acid in water (B) as the mobile phase at the following gradient: 0‑6 min 10% A, 20 min 35% A, 46 min 40% A, 48 min 100% A, 60 min 100% A, 62 min 10% A, and 70 min 10% A. The injection volume was 20 µL. The HPLC system was coupled with a diode array detector (DAD) and an electrospray ionization mass detector (HPLC-DAD-ESI-MS^n) in parallel by dividing the mobile phase 1:1. The acquisition was performed in full

scan (m/z 50‑1500) and full scan MS^2 (m/z 50‑600) by selecting the precursor ion [M-H]− at m/z 289 for (+)-catechin and (−)-epicatechin, m/z 577 for procyanidin B2, m/z 353 for 5-O-caffeoylquinic acid (chlorogenic acid), m/z 163 for 3-hydroxycinnamic acid (IS), m/z 435‑ for phloridzin, m/z 463 for quercetin 3-O-galactoside, m/z 433 for quercetin xyloside and quercetin 3-O-arabinoside, m/z 447 for quercetin 3-O-rhamnoside, respectively. Analytes for which standards were not available, such as 3- and 4-O-caffeoylquinic acids ([M-H]− at m/z 337); procyanidin B-type dimers ([M-H]− at m/z 577), trimers ([M-H]− at m/z 865), and tetramers ([M-H]− at m/z 1153); caffeic acid hexoside ([M-H]− at m/z 341); 4- and 5-O-p-Coumaroylquinic acids ([M-H]− at m/z 337); phloretin-xyloglucoside ([M-H]- at m/z 567); quercetin pentoside ([M-H]− at m/z 433); and hexoside ([M-H]− at m/z 463), were tentatively characterized by comparison of their fragmentation patterns with those available in the literature.

Quantitative analysis of metabolites listed in Table 10 was performed using an Ultimate 3000 RS Diode Array detector (Thermo Fisher Scientific, MA, USA) controlled by Chromeleon software (version 6.80). Spectral data of all peaks were accumulated in the 200‑600 nm range and chromatograms were recorded at 280 nm for (+)-catechin, (−)- epicatechin, procyanidin B2, B-type procyanidins, phloretin derivatives, phloridzin, and 3-hydroxycinnamic acid (IS); at 328 nm for 3-, 4- and 5-O-caffeoylquinic acids, 4- and 5-O-p-coumaroylquinic acids, and caffeic acid hexoside; at 258 nm for quercetin 3-O-galactoside, quercetin-3-O-xyloside, quercetin-3-O-arabinoside, and quercetin glycosides; and at 520 nm for cyanidin derivative. Calibration curves ($R^2 > 0.999$) were prepared by diluting a stock solution of each standard [(+)-catechin, (−)-epicatechin, procyanidin B2, 5-O-caffeoylquinic acid, phloridzin, quercetin 3-O-galactoside, quercetin-3-O-xyloside, and quercetin-3-O-arabinoside] in H_2O/MeOH 9:1 (v/v) with 0.2% formic acid in the range of 12‑3000 ng/mL with a constant concentration of IS (500 ng/mL). When standards were unavailable, the analyte was quantified using the calibration curve of the available standard with a similar chemical structure. The procedure for the identification and quantification of β-sitosterol and triterpene compounds used in this study was described in detail in our previous works [261,269,270]. Briefly, the characterization of these secondary metabolites present in the Annurca apple extracts was carried out by GC‑MS analysis of the corresponding trimethylsilyl derivatives (TMS). For this purpose, a Trace GC Ultra gas chromatograph equipped with a split/splitless injector and coupled to an ion-trap mass spectrometer detector Polaris Q (Thermo Scientific) was used. The column had the following specifications: 30 m × 0.25 mm i.d., 0.1 μM film thickness, and fused silica SLB-5ms (Supelco, Sigma-Aldich). The initial oven temperature was 240 ◦C, programmed to 280 ◦C at 2 ◦C/min and kept at 280 ◦C for 10

min; the temperature was then raised to 310 ◦C at a rate of 10 ◦C/min and maintained at this temperature for 20 min. Samples were injected in the split (1:10) mode. The injector, transfer line, and ion source were set at 280, 280, and 200 ◦C, respectively. Helium was used as carrier gas at a 1 mL/min flow. The mass spectra were recorded in electron ionization (EI) mode at 70 eV electron energy with a mass range from m/z 50 to 1000 and a scan rate of 0.8 scan/s. The structural characterization of metabolites listed in Table 11 was carried out by comparison of the mass spectra of the chromatographic peaks with those of standards or to the spectra from the NIST02 spectral library, for β-sitosterol-TMS ([M]+· at m/z 488), oleanolic acid-TMS ([M]+· at m/z 600), ursolic acid-TMS ([M]+· at m/z 600), uvaol-TMS ([M]+· at m/z 586) or with those available in the literature for maslinic acid-TMS ([M]+·at m/z 472), corosolic acid-TMS ([M]+· at m/z 472), pomolic acid-TMS ([M]+· at m/z 472), annurcoic acid-TMS ([M]+· at m/z 486), and tormentic acid-TMS ([M]+ · at m/z 488). The data acquisition was under the control of Xcalibur software (version 2.0.7; Thermo Scientific). The quantitative analysis was carried out using a Fisons GC 8000 series gas chromatograph equipped with a split/splitless injector and a flame ionization detector (Fisons Instruments, Milan, Italy). The separation was carried out with a fused silica capillary column DB-5MS UI 30 m × 0.250 mm × 0.25 μM film thickness (Agilent, J&W, Leini, Italy). The initial oven temperature was 240 ◦C programmed to 280 ◦C at 2 ◦C/min and kept at 280 ◦C for 25 min, the temperature was then raised to 310 ◦C at a rate of 10 ◦C/min and maintained at this temperature for 13 min. Samples (1 μL) were injected in the split (1:10) mode. The injector and detector were set at 280 ◦C. Hydrogen was used as carrier gas at a 1.8 mL/min flow. Peak areas were integrated using a Varian Galaxie Workstation (Agilent Technologies, Leini, Italy). Quantification of the triterpenic acids and β-sitosterol in the samples was performed using the internal standard method based on the relative peak area of analyte to IS (cholesterol) from the average of three replicate measurements. A calibration curve was built for each corresponding standard compound in the extracts. For this purpose, working solutions of each available standard (β-sitosterol, ursolic acid, and oleanolic acid) were prepared at a concentration ranging from 5 to 120 μg/mL with a constant concentration of the IS (cholesterol, 60.0 μg/mL). The calibration curves obtained had a high level of linearity with a correlation coefficient (r^2) higher than 0.999 for all analytes. When standards were unavailable, the target analyte was quantified using the calibration curve of available standards of similar chemical structures.

3.2.4 Preparation of nanoformulations

All nanoformulations were prepared using a modified thin-film hydration method. 1.15mg TA (BLD Pharm, China) or hydroalcoholic extract containing the equivalent amount of TA was dissolved in 20 mL methanol with 75% Pluronic F127 (Sigma Aldrich, UK) and 25% Soluplus (BASF, UK). A rotary evaporator (Buchi Rotavapor® R-100, Flawil, Switzerland) was then used to evaporate the methanol (200 rpm and 60°C), under vacuum. Once a thin film was achieved it was then hydrated with 10 mL of Milli-Q water and mixed at 60°C, and then further sonicated using a VWR Ultrasonic cleaner bath USC300T (VWR International Limited, UK). The solution was then filtered through a sterile 0.45 μm filter to remove any unloaded active ingredients. Blank formulations (NF-B) were formulated following the same method but without any active ingredients (i.e. TA or LCE).

3.2.5 Evaluation of the Physiochemical Properties of the Nanoformulations

Particle size and zeta potential measurement by dynamic light scattering (DLS)

The size distribution and zeta potential of nanoparticles were analysed using the Zetasizer Ultra (Malvern Instruments, UK), which provided the Z_{Ave} hydrodynamic diameter, polydispersity index (PDI), and zeta potential (§). Prior to measurement, samples were processed through a 0.45 μm filter membrane. Then, 1 ml of the sample was pipetted directly into the zeta potential DTS1070 folded capillary cell (Malvern Panalytical, UK). Zeta potential was calculated from electrophoretic mobility using the Helmholtz-Smoluchowski equation by the Malvern data analysis software. Each sample was measured three times, and the mean values and standard deviations (SD) for particle size, PDI, and zeta potential were calculated [316].

Morphology of nanoparticles by transmission electron microscopy (TEM)

The morphology of the nanoparticles was analysed using TEM (Philips/FEI CM120 Bio Twin, FEI Netherlands). Samples were deposited on copper grids, and any excess droplets were removed with filter paper. After four minutes, a drop of 1% phosphotungstic acid was applied to the copper grid for negative staining. The grid was then dried at room temperature and examined using TEM.

3.2.6 DPPH Scavenging Activity

The assessment of antioxidant activity was conducted using the stable free radical DPPH· (2,2-diphenyl-1-picrylhydrazyl hydrate) following the methodology described by Saltarelli et al. [287]. In brief, 850 μL of a prepared 100 μM DPPH· ethanol solution was mixed with 150 μL of Annurca apple extracts at various concentrations (ranging from 0.46 mg/mL to 30 mg/mL) diluted in 80% EtOH. Subsequently, the reaction mixture was performed in darkness at room temperature for 30 min. A blank solution composed of 850 μL of DPPH· and 150 μL of 80% EtOH was employed as a reference. Notably, the analysis was performed on 96-well plates to facilitate multiple replicates per

sample. The determination of the extracts' DPPH radical scavenging ability (DSA) relied on the observation of reduced DPPH absorbance at 517 nm using the Bio-Rad microplate reader 'Benchmark' spectrophotometer.

DPPH Scavenging Activity (% DSA) = [A517 nm of blank − A517 nm of sample)/A517 nm of blank] × 100

Data are also reported as mg of ethanolic extract dry weight (dw) with 50% scavenging ability (EC50).

3.2.7 ABTS Scavenging Activity

To measure the antioxidant activity against ABTS· (2,2-azino-bis (3-ethylbenzothiazoline- 6 - sulphonic acid)) radical, the method described by Loizzo et al. [288] was followed with some modifications. First, the reaction mixture was prepared by combining 7 mM ABTS solution and 140 mM potassium persulphate and then incubated in the dark at room temperature for 12‑16 h to create ABTS· radical. Before use, the solution was diluted with ethanol until an absorbance of 0.70 ± 0.02 at 734 nm was achieved. Then aliquots of Annurca apple extracts at concentrations ranging from 1.87 to 30 mg/mL were added to 1 mL of the ABTS ethanolic solution. The mixture was incubated in the dark at room temperature for 4 min. Subsequently, we measured the absorbance at 734 nm using a UV Beckman spectrophotometer (Palo Alto, CA, USA) and used ethanol as a blank. The ABTS radical scavenging activity was calculated following the equation:

ABTS Scavenging Activity (% ABTS SA) = [A734 nm of blank − A734 nm of sample)/A734 nm of blank] × 100.

Data are also reported as mg of ethanolic extract dry weight (dw) with 50% scavenging ability (EC50).

3.2.8 ORAC Assay

The antioxidant capacity of Annurca apple extracts was assessed using the ORAC (oxygen radical absorbance capacity) method, following previously published procedures [289], and employed a FLUOstar Optima Plate reader fluorimeter (BMG Labtech, Offenburg, Germany). The assay comprised 200 μL of 0.096 μM fluorescein in 0.075M Na-phosphate buffer (pH 7.0), 20 μL of the sample or Trolox, or 0.075M Na-phosphate buffer (pH 7.0) as the blank. The reaction was initiated with 40 μL of 0.33M AAPH [2,2'azobis-(2-amidinopropane) hydrochloride]. Fluorescence intensity was measured at 485 nm (excitation) and 520 nm (emission) until complete extinction. A calibration curve using Trolox (ranging from 50 to 500 μM) in 0.075M Na-phosphate buffer (pH 7.0) served as the positive standard. ORAC values, expressed as mmol Trolox equivalents (TEq)/100 mg dw, were

obtained. Ten diluted callus extracts and twenty diluted pulp and peel extracts (30 mg/mL) were prepared for analysis.

3.2.9 Lipoxygenase Inhibition Assay

The assay involved the use of soybean 5-lipoxygenase following the protocol established by Saltarelli et al. [317]. Inhibition experiments were conducted to evaluate the impact on 5-lipoxygenase activity (0.18 µg/mL) using 100 µM linoleic acid as the substrate in a 50 mM sodium phosphate buffer at pH 6.8. The reaction mixture, in the absence of the enzyme, was pre-equilibrated at 20 ◦C for 20 min. Subsequently, inhibition studies were carried out in the presence of varying concentrations of Annurca apple extracts (ranging from 0.15 to 0.9 mg/mL). The measurements were recorded at 235 nm at 20 ◦C using a UV Beckman spectrophotometer. The percentage of lipoxygenase activity inhibition was calculated using the following formula:

$$\% = 1 - \{[(\Delta 235 \text{ nm of blank} - \Delta 235 \text{ nm of sample})/\Delta 235 \text{ nm of blank}] \times 100\}.$$

The determination of EC50 involved plotting a graph correlating the concentration of extracts with the percentage of inhibition of linoleic acid peroxidation.

3.2.10 Nicking Assay

The nicking assay was conducted following Sadat Asadi et al. [318] with slight adjustments. A mixture containing 125 ng/µL of pGEM-T DNA, freshly prepared H_2O_2 (5 mM), Fe_2SO_4 (0.33 mM), and EDTA (0.62 mM) was prepared. Annurca apple extracts at varying concentrations (ranging from 0.06 mg/mL to 4.5 mg/mL) were added to the mixture. The total volume was adjusted to 20 µL using phosphate-buffered saline (PBS = 8 g/L NaCl, 1.44 g/L Na_2HPO_4, 0.2 g/L KH_2PO_4, 0.2 g/L KCl, pH 7.4). A negative control containing DNA, EDTA, and PBS was also prepared. The reaction mixtures were then left to incubate for 30 min at 37 ◦C. Subsequently, the DNA samples were applied to a 1% agarose/tris borate EDTA (TBE) gel, stained using 1% Midori Green Advance DNA staining (Resnova, Genzano di Roma, Italy), and visualized under UV light using a Gel Doc 2000 (Bio-Rad, Italy). The quantification process involved densitometric analysis using Quantity One Software 4.01 (BioRad, Segrate, Italy). The retention of supercoiled DNA strands (%) was calculated using the following equation:

$$\text{Retention \%} = (\text{Intensity of supercoiled DNA of sample}/\text{Intensity of supercoiled DNA of control}) \times 100.$$

3.2.11 Cell Cultures

Murine RAW 264.7 macrophages were procured from the European Collection of Cell Cultures based in Salisbury, UK. These cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, Milan, Italy), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 mM non-essential amino acids, streptomycin (100 µg/mL), penicillin (100 U/mL), and 1% (w/v) L-glutamine. The cells were maintained in a humidified incubator with 95% humidity at 37 ◦C with 5% CO2. The growth medium was replenished every 2–3 days until approximately 80% confluence was achieved.

Immortalized human keratinocytes (HaCaT) were cultured in DMEM—GlutaMax® supplemented with 1% antibiotic/antimycotic solution, 1% L-glutamine, and 10% FBS, and maintained in a 95% humidity, 5% CO2 atmosphere at 37°C. The growth medium was replenished every 2–3 days and seeded in 96-well or 6-well plates until approximately 80% confluence was reached.

3.2.12 Griess Assay

The production of nitric oxide (NO) by RAW 264.7 cells following stimulation with bacterial lipopolysaccharide (LPS, Sigma-Aldrich, Milan, Italy) serves as an established indicator of the inflammatory process. The extracellular NO concentration was determined using a UV–visible spectrophotometer. The nitric oxide content was quantified by referencing a nitrite standard curve (calcium nitrite 6.25–100 µM) [264] that had been prepared in the fresh culture medium. RAW 264.7 cells were seeded in the logarithmic phase of 3×10^4 cells/100 µL and cultured in 96-well plates for 24 h. The experiment was then divided into four groups: control group (CTRL), lipopolysaccharide (LPS)-treated group, LPS + dexamethasone (DEXA, an anti-inflammatory compound used as a positive control), and LPS + extracts at various concentrations. The treatments were performed for 24 h at 37 ◦C. LPS was used at a concentration of 1 µg/mL and DEXA at 0.0039 mg/mL. Following overnight treatments, 50 µL aliquots of supernatants were combined with 50 µL of Griess reagent (40 mg/mL) and incubated for 10 min in the absence of light at room temperature (RT). The absorbance was measured at 570 nm employing a plate reader (BioRad Laboratories, Hercules, CA, USA). To eliminate the potential association between the decrease in NO levels and a reduction in cell viability, we performed the MTT viability assay on the same samples.

Cell viability was assessed using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assays. Following removal of the medium, cells were washed with PBS and each well was treated with a 100 µL di MTT 0.2 mg/mL solution for 1 h at 37 ◦C. Following the incubation phase, the MTT solution was extracted, and 100 µL of dimethyl sulfoxide (DMSO) was introduced to facilitate the dissolution of the resultant formazan salt. The quantification of formazan salt was carried

out by gauging the absorbance at 570 nm and employing a microplate reader. Cell viability was expressed as a percentage compared to the control and calculated using the formula:

% Cell viability = (OD test/OD control) × 100.

where OD represents optical density.

3.2.13 Assessment of Cell Viability on HaCaT Cells

An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was performed to assess potential cytotoxicity of the test samples (free TA, free LCE, NF-TA, and NF-LCE) on HaCaT cells. HaCaT cells were seeded at 30,000 cells/cm^2 in 96-well microplates and cultured in complete (DMEM)—GlutaMax® at 37◦C and 5% CO^2 until confluency. Cells were incubated with each sample at different concentrations expressed in TA concentrations, for 24, 48, and 72 h. MEM was used as the negative control. After each incubation time, a volume of 20 µL of 3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) solubilized in sterile Dulbecco's Phosphate-Buffered Saline (DPBS) was added to the wells at the concentration of 5 mg/mL [319]. After incubation at 37 ◦C for 4 h, the formazan crystals formed were dissolved in 100 µL of DMSO, with the previous medium aspirated. The plates were ultimately placed on The MaxQ 4000 benchtop orbital shaker (Thermo Fisher Scientific, Loughborough, UK) at 75 rpm for 15 min to ensure the DMSO was mixed well. The absorbance was subsequently measured at 570 nm and compared to the control cells (MEM).

3.2.14 Optimization of an *In vitro* Model for Iron-Overload-Induced Oxidative Stress

An *in vitro* iron-overload model was optimised by treating HaCat cells with varying iron concentrations (20 µM, 50 µM, 100 µM, and 200 µM) adapted from previous studies [320-323]. Cells were seeded at 10,000 cells/cm^2 and cultured in 6-well plates at 37 ◦C with 5% CO_2. Prior to iron treatment, growth media was replaced with serum-free MEM (1% L-glutamine, 1% antibiotic-antimycotic solution) and incubated for 24 hours. After 24 hours, HaCaT cells were washed with DPBS and treated with different iron concentrations for 24 hours. After the incubation period, cells were washed with DPBS and incubated with serum-free MEM for 24 hours. Finally, cells were lysed, and the lysates were collected and stored at −20◦ C. To eliminate the potential association between the increase of iron concentrations and a reduction in cell viability, an MTT-viability assay was performed at these treatment concentrations.

3.2.15 Intracellular Total Iron Quantification

Intracellular total iron content was quantified using a FerroZine™-based iron protocol according to Zariwala et al. [324] and Schiano et al. [325]. Briefly, 200 µL of 0.1 M HCl solution was added to 200 µL of cell lysates, while 200 µL of 50 mM NaOH solution was added to 200 µL of each standard of iron (from 0 to 120 µM). The blank consisted of 400 µL of 50 mM NaOH. 200 µL of freshly prepared

iron-releasing agent (equal amount of 1.4 M HCl and 4.5% (w/v) $KMnO_4$) was added to each tube. Samples were incubated in a 60°C dry bath under a fume hood for 2 hours and, at the end of incubation, cooled to room temperature for 10 minutes. Afterward, 60 µL of the iron detection reagent (6.5 mM FerroZine™, 2.5 M ammonium acetate, and 1 M ascorbic acid dissolved in water) was added into each tube, and samples were incubated for 30 minutes at room temperature. To monitor colour development, 200 µL of standard and sample tubes were placed in duplicate into a 96-well microplate. The absorbance at 550 nm was measured using a microplate reader (Fluostar Optima Plate Reader, BMG Labtech, Offenburg, Germany). The intracellular iron concentration was evaluated by normalizing the obtained results against the total protein content of each well evaluated by the BCA (Bicinchoninic Acid) assay.

3.2.16 Intracellular Ferritin Quantification

The Ferritin content of HaCaT cells was determined using a commercial ELISA kit according to the manufacturer's instructions (Eagle Biosciences, Inc.). 20 µL of calibrators, control, and samples were aliquoted in duplicates into a 96-well microplate. 200 µL of HRP-conjugate solution was then added to each well and the plate was incubated on a plate shaker (200 rpm) for 60 minutes at room temperature. Wells were then washed 5 times with 300 µL of diluted wash buffer per well. Then 150 µL of the TMB substrate was added and the plate was incubated on the plate shaker (200 rpm) for 30 minutes. At the end of the incubation time, 50 µL of stopping solution was added to each well and finally, the plate was read at 450nm within 20 minutes after the addition of the stopping solution. Ferritin concentration was standardized against the total protein concentration determined by the BCA assay.

3.2.17 Effects of Iron-Induced Inflammation in HaCaT Cells

Cells were seeded at 10,000 cells/cm^2 and cultured in 6-well plates until confluence. Cells were then iron-depleted with serum-free MEM and incubated for 24 hours at 37 °C with 5% CO_2. After 24 hours, HaCaT cells were washed with DPBS and treated for 3 hours with 3.25µM of LCE, TA, nanoformulated LCE (NF-LCE), and nanoformulated TA (NF-TA) followed by 24-hour incubation with 100 µM iron. At the end of the incubation, the medium was aspirated, cells were washed with DPBS. Cell lysates were collected with 350µL of BB4 buffer (EasyPure® RNA Kit - 50rxns - TransGen Biotech) and stored at -80°C until use.

3.2.18 Quantitative Real-Time PCR

Total RNA was extracted using the EasyPure® RNA Kit (Beijing, China). Then mRNA was converted to cDNA and processed by real-time PCR to evaluate the expression of GPX4, COX2, NrF2, IL-6, and TNF-a, genes related to the inflammatory process as well as ferroptosis. The expression levels of

the inflammatory genes were normalized with the housekeeping gene 36B4. Quantification was performed in a 20 µL volume using 2 µL of each cDNA. Specifically, 10 µL of ChamQ Universal SYBR qPCR Master Mix, 0.4 µL of forward and reverse primers from a 10 µM preparation, and 7.2 µL of water were used. Real-time PCR was conducted using a T100 Thermal Cycler (Bio-Rad Laboratories, Inc). The analysis was carried out with the Pfaffl method [266].

Real-time PCR thermal was carried out as reported in Table 8

Stage 1	Initial Denaturation	Rep: 1	95°C	30sec
Stage 2	Cycling Reaction	Reps: 40	95°C 60°C	3-10sec 10-30sec
Stage 3	Melting Curve	Rep: 1	95°C 60°C 95°C	15sec 60sec 15sec

The nucleotide sequences of the primers are summarized in Table 9:

	Sequence	**Accession Number**
GPX4	F-5'- ACAAGAACGGCTGCGTGGTGAA-3' R-5'- GCCACACACTTGTGGAGCTAGA-3'	NM_001367832.1
COX2	F-5'-CGGTGAAACTCTGGCTAGACAG-3' R-5'- GCAAACCGTAGATGCTCAGGGA-3'	NM_000963.4
NrF2	F-5'- AAACCAGTGGATCTGCCAAC-3' R-5'- ACGTAGCCGAAGAAACCTCA-3'	NM_001313902.2
IL-6	F-5'-GGTACATCCTCGACGGCATCT-3' R-5'- GTGCCTCTTTGCTGCTTTCAC-3'	NM_000600.5
TNF-a	F-5'- CTCTTCTGCCTGCTGCACTTTG-3' R-5'- ATGGGCTACAGGCTTGTCACTC-3'	NM_000594.4
36B4	F-5'-CGACCTGGAAGTCCAACTAC-3' R-5'- ATCTGCTGCATCTGCTTG-3'	NM_053275.4

Table 9: **Employed primers for real-time polymerase chain reaction (PCR) analysis.**

3.2.19 Statistical Analysis

Statistical analyses were performed using GraphPad Software (GraphPad Prism version 8 for Windows). Values were expressed either as the mean ± standard error of the mean (SEM) or standard deviation (SD). The treated and control sample variables were compared using one-way ANOVA. Dunnett's multiple comparisons test was employed to calculate the significant difference. The differences between samples were considered significant if p values were <0.05.

3.3. Results and Discussion

The literature on the properties of the Annurca apple is extensive [315, 326-328], but there is limited knowledge about callus cultures from any organ of this apple variety [329]. Based on our previous experience with various callus cultures obtained from Mela Rosa Marchigiana, Golden Delicious [269], Red Sentinel (patent n. 102020000012466), Acca Sellowiana Burret exotic fruit [270], and Cydonia oblonga Mill [261], in the present work, two callus cultures (DC and LC) derived from the pulp of Annurca apples were obtained as reported in Section 3.2.1. The experimental conditions developed in this work allowed, after two weeks, for the formation of friable, cream-colored calli above and around the explant on the cut surface, both in the culture kept in the dark and under an 18-h photoperiod. In both conditions, to allow for regular and lasting callus production, it was also necessary to add cytokinin (BA) to the culture medium, following what has been reported in previously published works [261,269,270,329]. The formation of calli in the two distinct culture conditions was documented after two months of cultivation as in Verardo et al. [269], considering the fresh and dry weight of callus obtained in each experimental condition. It is known that the development of callus culture and the production of bioactive secondary metabolites are influenced by the frequency and timing of light exposure [330-336]. however, no quantitatively significant differences were found in the weight of callus produced under the two conditions used in this work. Nor were any differences found in the texture, color, and shape of the callus obtained in the two culture conditions (Figure 19).

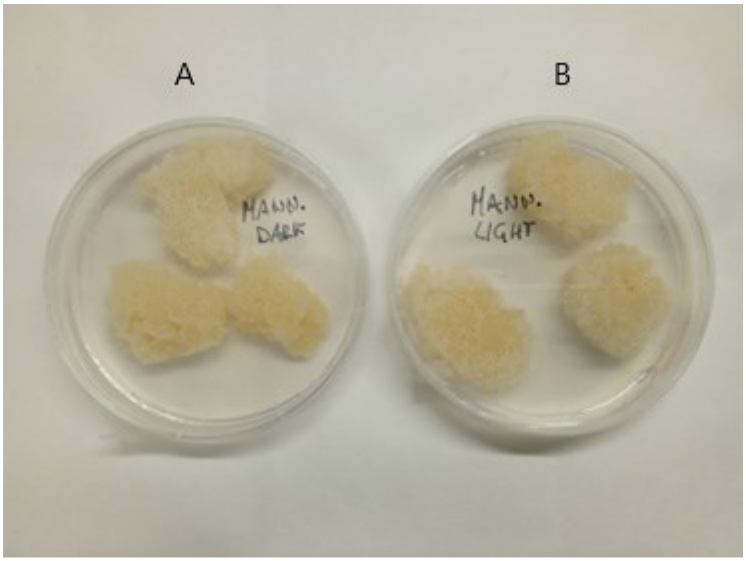


Figure 19: Annurca apple pulp-derived calli. (**A**) Callus grown in the dark ('dark callus'); (**B**) callus maintained in an 18-h photoperiod ('light callus').

Moreover, the production of secondary metabolites is comparable. The characterization and the quantification of phenolic compounds were performed by HPLC-DAD-ESI-MS^n. As reported in Table 10, the highest total phenolic content was found in the peel, followed by pulp and LC, whereas

DC showed the lowest values (111 times lower concerning peel). In the peel, the most represented phenolic compound was quercetin 3-O-galactoside (66.40 µg/100 mg dw ~16% of its total phenolic compounds), followed by chlorogenic acid (49.32 µg/100 mg dw ~12% of its total phenolic compounds) and (−) epicatechin (44.62 µg/100 mg dw ~11% of its total phenolic compounds). The pulp, on the other hand, had a considerable and higher chlorogenic acid content (61.97 µg/100 mg dw ~40% of its total phenolic compounds) than the peel, attended by (−) epicatechin (25.09 µg/100 mg dw ~16% of its total phenolic compounds) and other several compounds presented at low concentrations. Procyanidin B2 was found in both peel and pulp, with the highest concentrations in the peel but, was absent in both calli. Chlorogenic acid was the most represented compound in the LC (19.57 µg/100 mg dw ~69% of its total phenolic compounds). GC-MS analysis enabled the identification of β-sitosterol and triterpene compounds that were mainly present in the calli, less in the peel, and negligible in the pulp (Table 11). Tormentic acid (>45%) was predominantly produced in both calli in very similar concentrations, followed by corosolic (13% and 9% in LC and DC, respectively), maslinic (~12%), annurcoic (10% and 17% in LC and DC, respectively), and ursolic acid (>7%). In the peel, ursolic acid prevails with a percentage of about 67.06%, along with oleanoic (~13%) and annurcoic acids (~9%).

The inclusion of plant growth regulators (PGRs) within culture media has been identified as a significant factor influencing the production of secondary metabolites (SMs), as evidenced in the existing literature. For instance, triterpenes like betulinic acid, ursolic acid, and oleanolic acid, which are extracted from callus cultures of different sage species, can have variations based on the plant growth regulators (PGRs) used and the specific plant parts (such as stems or leaves) used to initiate the callus production. It has been demonstrated that callus obtained from leaves or stems of plants generally produces a lower concentration of secondary metabolites compared to the original plant. This difference in metabolite production could be attributed to the organization and cellular differentiation [337]. It is possible that mature apple pulp cells in culture, like in our case, may have different gene expression regulation compared to the peel and pulp of the original fruit, leading to a substantial increase in the biosynthesis and accumulation of bioactive compounds, particularly triterpene compounds, as in this instance.

In summary, both calli produced high levels of triterpene acids (mainly tormentic, corosolic, maslinic, and ursolic acids) and low levels of polyphenols, which are more abundant in peel and pulp extracts (Tables 10 and 11). Triterpenic acids in nature are primarily found on plant surfaces such as fruit peels, stem bark, leaves, and fruit waxes [338], but they are very poorly identified in the fruit pulp. No triterpene acids were found by Laezza's group [329], which showed that yeast extract added to the culture medium to produce callus from Annurca campana pulp explants induced a significant increase

in total polyphenol content. The most abundant triterpene in both callus cultures studied in this work was tormentic acid. This compound is a pentacyclic triterpene that can regulate insulin production, has a hypoglycaemic effect, and exhibits antioxidant and anti-inflammatory properties that are beneficial in healing cardiovascular disorders [339,340]. Following Babich et al. [341], alterations to the nutrient medium have the potential to bolster the production of biologically active substances by stimulating their biosynthesis. Considering the established bioeffect of this triterpene, our objective is to identify the specific medium nutrients capable of enhancing biosynthesis and yield. This initiative will facilitate the utilization of callus cultures as a reliable source of tormentic acid.

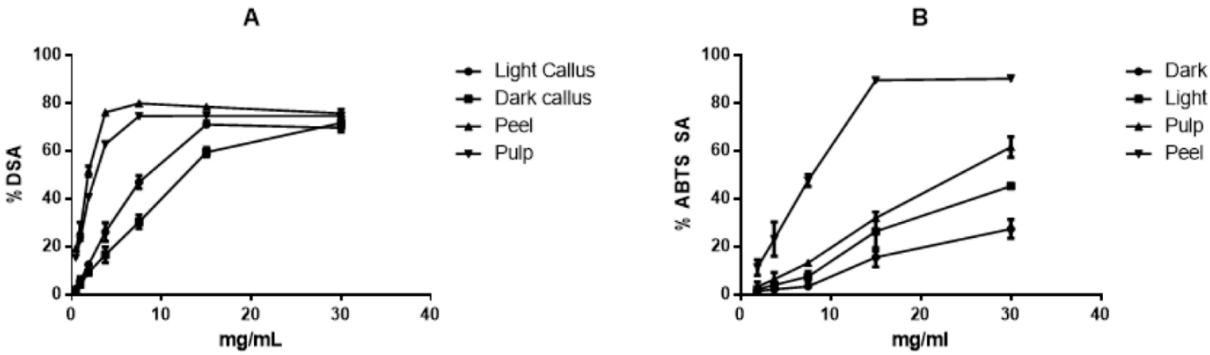
Compound	Light Callus	Dark Callus	Peel	Pulp
3-O-Caffeoylquinic acid isomer	0.05 ± 0.001	n.d.	0.18 ± 0.001	1.2 ± 0.001
Procyanidin B-type trimer isomer	n.d.	n.d.	7.38 ± 0.011	6.77 ± 0.006
Procyanidin B-type trimer isomer	n.d.	n.d.	3.87 ± 0.006	3.15 ± 0.002
Procyanidin B-type trimer isomer	n.d.	n.d.	1.67 ± 0.002	1.50 ± 0.002
Procyanidin B-type trimer isomer	n.d.	n.d.	4.27 ± 0.015	1.93 ± 0.021
4-O-Caffeoylquinic acid	0.1 ± 0.001	n.d.	0.29 ± 0.005	0.95 ± 0.008
(+) Catechin	n.d.	n.d.	8.37 ± 0.003	9.15 ± 0.002
Procyanidin B-type tetramer isomer	n.d.	n.d.	3.93 ± 0.005	1.24 ± 0.009
Procyanidin B2	n.d.	n.d.	39.6 ± 0.036	38.1 ± 0.021
5-O-Caffeoylquinic acid (chlorogenic acid)	19.57 ± 0.034	n.d.	49.12 ± 0.010	61.97 ± 0.061
Caffeoyl acid hexoside	21.6 ± 0.005	3.46 ± 0.009	0.56 ± 0.003	0.82 ± 0.001
4-O-Caffeoylquinic acid	0.43 ± 0.001	n.d.	0.43 ± 0.005	0.31 ± 0.006
Procyanidin B-type trimer isomer	n.d.	n.d.	15.32 ± 0.008	9.08 ± 0.007
4-O-p-Coumaroylquinic acid isomer	3.2 ± 0.003	n.d.	0.53 ± 0.002	0.42 ± 0.001
Cyanidin hexoside	n.d.	n.d.	n.d.	n.d.
(-) Epicatechin	0.17 ± 0.002	0.07 ± 0.001	44.62 ± 0.034	25.09 ± 0.093
5-O-p-Coumaroylquinic acid isomer	0.29 ± 0.002	n.d.	1.08 ± 0.004	0.57 ± 0.003
4-O-p-Coumaroylquinic acid isomer	n.d.	n.d.	0.54 ± 0.002	0.43 ± 0.001
5-O-p-Coumaroylquinic acid isomer	n.d.	n.d.	0.45 ± 0.007	0.38 ± 0.002
Procyanidin B-type dimer isomer	n.d.	n.d.	2.41 ± 0.002	0.94 ± 0.001
Quercetin 3-O-galactoside	1.93 ± 0.002	n.d.	n.d.	n.d.
Quercetin 3-O-xyloside	n.d.	n.d.	23.84 ± 0.010	15.6 ± 0.016
Phloretin xylosylcoside isomer	1.41 ± 0.001	n.d.	1.42 ± 0.001	1.12 ± 0.010
Quercetin 3-O-xyloside	n.d.	n.d.	6.60 ± 0.015	4.3 ± 0.008
Phloridzin	n.d.	n.d.	36.81 ± 0.001	1.71 ± 0.005
Quercetin pentoside	n.d.	n.d.	28.86 ± 0.064	0.28 ± 0.001
Quercetin 3-O-rhamnoside	0.04 ± 0.001	n.d.	13.23 ± 0.143	9.1 ± 0.009
Total	28.39 ± 0.003	3.64 ± 0.011	403.12 ± 0.493	156.16 ± 0.094

Table 10. Identification and quantification (µg/100 mg dw ± SD) of phenolic compounds in the extracts of light callus, dark callus, peel, and pulp of Annurca apple by HPLC-DAD-ESI-MS^n.

Compound	Light Callus	Dark Callus	Peel	Pulp
β-Sitosterol	148.52 ± 0.85	174.69 ± 1.47	44.61 ± 0.46	32.04 ± 0.25
Uvaol	13.45 ± 0.04	13.13 ± 0.13	n.d.	n.d.
Oleanolic acid	49.80 ± 0.45	34.92 ± 0.18	163.91 ± 0.83	3.95 ± 0.02
Ursolic acid	210.40 ± 1.29	161.48 ± 0.43	864.80 ± 0.95	21.40 ± 0.19
Maslinic acid	276.61 ± 0.88	269.74 ± 2.08	17.78 ± 0.13	n.d.
Corosolic acid	321.53 ± 0.84	201.67 ± 0.88	42.71 ± 0.22	n.d.
Pomolic acid	14.88 ± 0.01	16.57 ± 0.06	50.46 ± 0.07	n.d.
Annurcoic acid	251.89 ± 2.29	381.55 ± 2.54	128.36 ± 0.56	1.31 ± 0.01
Tormentic acid	1152.75 ± 10.35	1032.97 ± 7.05	16.25 ± 0.23	0.42 ± 0.01
Σ triterpenic acid	2277.85 ± 7.16	2098.91 ± 7.30	1283.55 ± 0.87	27.08 ± 0.22
Total	2439.82 ± 8.05	2286.73 ± 8.64	1328.15 ± 1.32	59.12 ± 0.47

Table 11. Identification and quantification (µg/100 mg dw ± SD) of β-sitosterol and triterpene compounds in the extracts of light callus, dark callus, peel, and pulp of Annurca apple, by GC-MS and GC-FID.

The different Annurca apple extracts were tested for their antioxidant activity with the use of different assays. DPPH, ABTS+, and ORAC assays were used to investigate free radical scavenging activity. As indicated in Figure 12A, all extracts exhibited a radical scavenging activity against hydroxyl radicals (DPPH scavenging activity-%DSA) in a dose-dependent manner with inhibition ranging from 70 to 76% in all four extracts at a concentration of 30 mg/mL. At lower concentrations, peel and pulp extracts were greater in DPPH scavenging activity compared to callus extracts, demonstrated by their lowest EC50 value (2 ± 0.80 and ±0.29 mg/mL in peel and pulp, respectively) (Figure 20A). The maximum ABTS scavenging activity (ABTS SA) recorded at a concentration of 30 mg/mL was 58.15% ± 3.66, 47.50% ± 0.44, 90.30% ± 0.63, 61.70% ± 4.36 for LC, DC, peel, and pulp, respectively (Figure 20B). Finally, the ORAC assay confirmed the good antioxidant activity of different Annurca apple extracts showing values ranging from 84.20 ± 4.6 to 136.6 ± 3.6 for DC and LC, and 223.3 ± 3.3 to 389.1 ± 14.0 µM Trolox equivalents/100mg for pulp and peel, respectively (Figure 20C). The higher scavenging activity demonstrated by DPPH, ABTS, and ORAC assays from in vivo-derived plant extracts (peel and pulp extracts) may be due to the extracts' higher total amount of phenolic compounds compared to callus extracts. A linear relationship between the antioxidant effect and the amount of phenolic compounds has been demonstrated [342]. In fact, the peel extract has the highest amount of polyphenols, which corresponds to the highest scavenging activity compared with the other extracts.



DPPH Assay	%DSA	EC50 mg/mL
Light Callus	70 ± 3.94	15±1.34
Dark Callus	72 ± 2. 71	17±0.51
Peel	76 ± 3.57	2±0.80
Pulp	75 ± 0.25	7±0.29

ABTS Assay	%ABTS SA	EC50 mg/mL
Light Callus	58.15 ± 3.66	24.7±1.10
Dark Callus	47.50 ± 0.44	32.1±0.95
Peel	90.30% ± 0.63	10.2±0.86
Pulp	61.70% ± 4.36	24.2±0.32

C

ORAC Assay	µmolTE/g
Light Callus	136.6 ± 3.6
Dark Callus	84.20 ± 4.6
Peel	389.1 ± 14.0
Pulp	223.3 ± 3.3

D

LOX Assay	% inhibition	EC50 mg/mL
Light Callus	62 ± 7.24	0.6±0.19
Dark Callus	69 ± 0.49	0.5±0.06
Peel	32 ± 2.18	1.6±0.18
Pulp	38 ± 1.95	1.2±0.09

Figure 20. **DPPH** (**A**), **ABTS** (**B**), **ORAC** (**C**) **tests**, and **lipoxygenase inhibition activity** (**D**). The data represent the percentage of inhibition induced by increasing concentrations of Annurca apple extracts. Each value is the mean ± SD of three independent measurements.

We also looked at how Annurca callus (DC and LC), peel, and pulp extracts inhibited lipoxygenase-catalyzed linoleic acid oxidation. We examined concentrations ranging from 0.15 to 0.9 mg/mL and found that all extracts inhibit the oxidation of linoleic acid in a dose-dependent way. At the highest concentration of 0.9 mg/mL, DC has the greatest inhibiting activity (69%), followed by LC (62%), the pulp (38%), and the peel (32%) (Figure 20D).

The presence of triterpenes in the callus extracts can explain why they inhibit the oxidation of linoleic acid more effectively than peel and pulp [343-346]. 5-lipoxygenase (5-LO) is an enzyme that converts free arachidonic acid into leukotrienes (LTs), which have been linked to a variety of medical conditions including asthma, allergic rhinitis, inflammatory bowel and dermatological pathologies, rheumatoid arthritis, cancer, osteoporosis, and cardiovascular disease. As a result, pharmaceutical therapies that disrupt the 5-LO pathway demonstrate potential therapeutic efficacy for many related illnesses [347]. Therefore, callus cultures of Annurca apple pulp, being rich in triterpene acids, can be considered an important source of substances with anti-inflammatory activity.

The DNA nicking assay was performed to assess the DNA-protective efficacy of various Annurca apple extract concentrations. On agarose gel electrophoresis, the plasmid DNA is separated into three bands. The native supercoiled circular DNA (CCC) is the quickest-moving band, the center band corresponds to linear DNA (L DNA), and the slowest-moving band is the open circular form (OC), in which DNA is broken on one strand. Compared to the plasmid DNA control, the CCC form is changed to the OC form by the hydroxyl radical (•OH) that caused DNA damage. Annurca apple extracts can reduce this damage. Nicking assays demonstrated that DC could protect DNA better than LC, but pulp has the greatest protection (Figure 21).

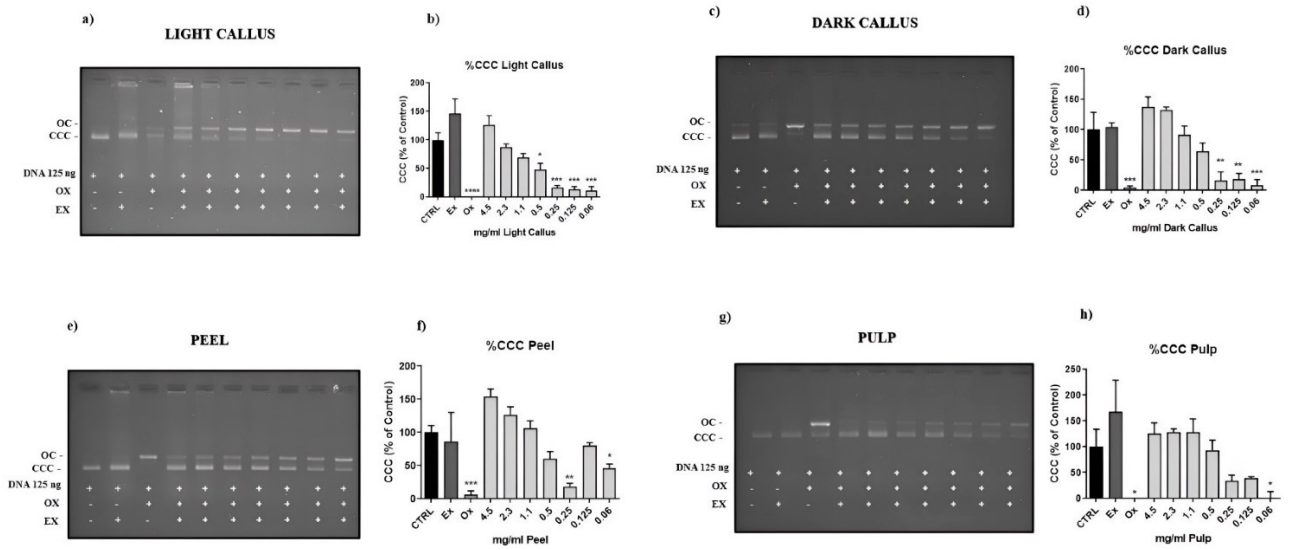


Figure 21. **Protective effect of Annurca apple extracts against strand breaks using the DNA nicking assay.** In (**a, c, e, g**), an example of agarose gel of the pGEM plasmid after incubation with the oxidant system in the presence of decreasing amounts of extracts; in (**b, d, f, h**), the quantification of gels obtained by the assay, expressed as the percentage ration between the volume of the band of the plasmid supercoiled form (CCC) after incubation with oxidant and the same band volume in the control; each value represents the mean ± SEM of three independent experiments. (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; Dunnett's post hoc test).

The results obtained from this assay are important because DNA damage is linked to several illnesses, including cancer, neurological disorders, and aging [348-350]. However, further research will be undertaken to demonstrate the genoprotective efficacy of Annurca apple extracts in cellular models.
Since the experiments described above showed that DC and LC extracts inhibited lipoxygenase-catalyzed linoleic acid oxidation more than pulp and peel extracts, suggesting a possible anti-inflammatory activity by these extracts (Figure 20D), we decided to explore the anti-inflammatory activity of Annurca apple extracts. RAW264.7 cells were chosen as a suitable macrophage model with which it is possible to quickly evaluate inflammatory cell responses with the Griess test [279,351], which evaluates NO release after treatment with bacterial LPS. The anti-inflammatory molecule DEXA was used as a positive control. The range of concentration was previously selected by MTT cell viability

assay. From the results in Figure 22a, the overnight co-treatment with 1 μg/mL of LPS and extracts at different concentrations significantly reduces NO release. In contrast to the evidence shown in the lipoxygenase inhibition assay, all the extracts showed anti-inflammatory activity with the same statistical significance value. To rule out the possibility that NO reduction resulted from a decrease in RAW264.7 cell viability following LPS treatment, a cell viability assay (MTT) was performed. Figure 22b shows that co-treated (LPS with DEXA or extract) cells are comparable to untreated ones (CTRL).

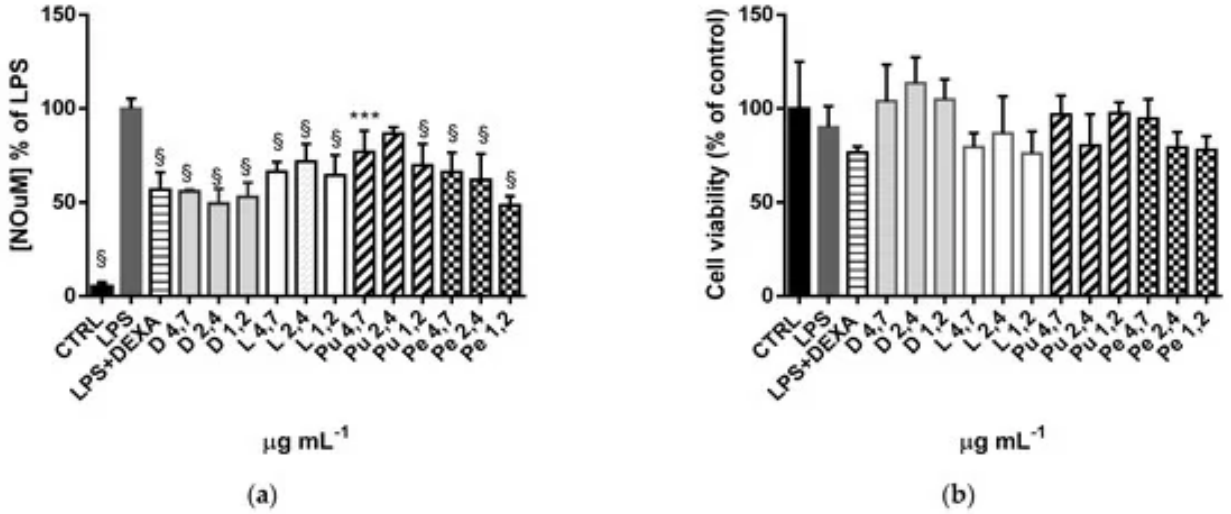


Figure 22. **Evaluation of extracellular NO release and cell viability of RAW 264.7 cells after LPS, Annurca extracts, and DEXA treatment.** (**a**) RAW 264.7 cells were co-treated overnight with LPS 1 μg mL^{-1} either in the absence or presence of Annurca extracts at different concentrations or DEXA 0.0039 mg mL^{-1}. CTRL: untreated cells. The results are expressed as NO reduction compared to LPS-treated cells (*** $p < 0.001$, § $p < 0.0001$); ANOVA followed by Dunnett's multiple comparison test was performed). (**b**) Cell viability assay on Raw 264.7 cells co-treated with LPS and Annurca extracts (MTT). The results are expressed as % of CTRL ± SD of three independent. ANOVA followed by Dunnett's multiple comparison test was performed; No statistical differences were found.

Thus, our results demonstrate that all the extracts have no cytotoxic effect on RAW 264.7 cells and possess a potent anti-inflammatory property. The lack of statistical differences between the extracts could be attributed to the fact that both triterpenes and polyphenols have anti-inflammatory properties [352-357], and they could exert a synergistic effect in all four extracts. Further experiments will be conducted to investigate if reducing the concentration of the Annurca apple extracts results in a statistically significant difference in their activity.

From these results, Annurca apple light callus extract was selected to be encapsulated in a nanocarrier, and the anti-inflammatory potential of both nanoformulated and unformulated compounds was assessed in a cellular model of ferroptosis. Tormentic Acid (formulated and unformulated, was used as a reference compound, as the main compound present in the extract [Table 11]).

Ferroptosis is a recently identified form of programmed cell death involving iron accumulation and consequent lipid peroxidation [358]. It is characterized by distinct morphological, biochemical, and genetic traits that differentiate it from other established forms of regulated cell death [359]. Ferroptosis plays a crucial role in a variety of conditions, including neurodegenerative diseases, tumors, myocardial injury, fibrosis, and potentially skin diseases [360,361]. Keratinocytes, which make up over 90% of epidermal cells, absorb around 95% of UV light that reaches the skin [362]. When skin cells are exposed to ultraviolet A (UVA) radiation (320–400 nm), there is an immediate increase in the intracellular labile iron (LI) pool, heightening cellular susceptibility to oxidative damage and potential cell death via ferroptotic mechanisms. The ROS generated by UVA exposure directly harms skin components, leading to an increased cytosolic LI pool that further exacerbates oxidative damage [363,364].

Based on existing literature [320-323], this study aimed to induce ferroptosis-related inflammation in HaCaT cells by overloading them with varying incremental iron concentrations.

To optimize an *in vitro* iron-overload model HaCaT cells were treated with different iron concentrations (20 µM, 50 µM, 100 µM, and 200 µM) for 24 hours. Ferrozine and ferritin assays were used to evaluate iron accumulation within the cells by measuring non-bound and ferritin-bound iron levels. Figure 23 illustrates a linear correlation between an increasing concentration of iron and the levels of LI but a decrease in ferritin storage at 100 µM iron concentration. This observation is consistent with findings from previous studies, indicating a decline in ferritin storage caused by increasing iron concentrations. This phenomenon occurs when the synthesis and storage capacity of ferritin is exceeded, resulting in an increase in free iron within the LI pool [365]. Additionally, during oxidative stress, iron may be released from ferritin either through reductive processes or due to the proteolytic degradation of ferritin [321,365].

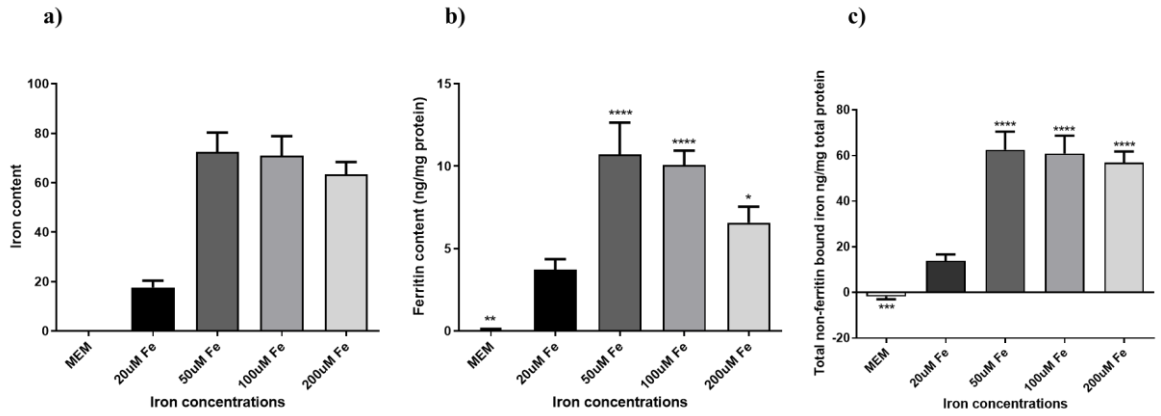


Figure 23: **Quantification of non-bound and ferritin-bound iron levels.** Intracellular iron accumulation was quantified using FerroZine™-based colorimetric assay **(a)**, and Ferritin assay **(b)**. Total non-ferritin-bound iron was obtained using the total iron concentrations obtained from the ferrozine assay subtracted from the mean ferritin concentrations from the ferritin ELISA **(c)**. The results are expressed as % of iron 20 µM ± SD (o SEM) of three independent experiments (n=3; P≤ 0.05). ANOVA followed by Dunnett's multiple comparison test was performed (**** $p < 0.0001$)

The aim was to stimulate iron-related inflammation without adversely affecting cell viability. For this reason, a cell viability assay was conducted to assess the reliability of cellular inflammatory response following the treatment with iron concentrations. As observed in Figure 24, cell viability was not significantly affected over the specified time-period.

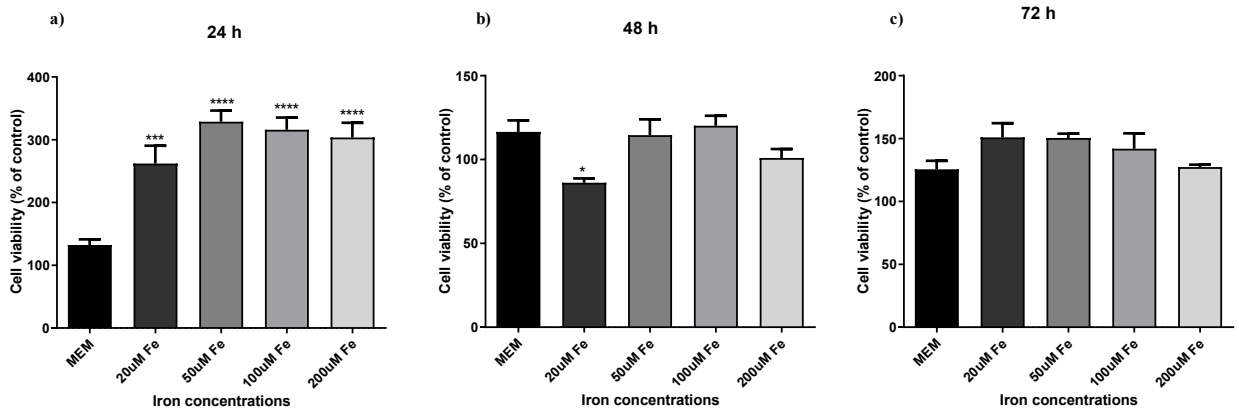


Figure 24: **Cell viability.** HaCaT cells were treated with different iron concentrations (20 µM, 50 µM, 100 µM, and 200 µM) for 24h **(a)**, 48h **(b)**, and 72h **(c)**, and cell viability was measured by MTT assay. The results are expressed as % of CTRL ± SEM of three independent (n=6; P≤ 0.05). ANOVA followed by Dunnett's multiple comparison test was performed (**** $p < 0.0001$).

From these experiments, a concentration of 100 µM was selected for further investigations, as it provided the optimal balance between inducing iron overload and preserving cell viability.

There is a growing trend toward using natural products in the realm of skin protection. A range of literature has demonstrated that plants are a rich source of natural protective molecules with anti-

aging properties that have been shown to stimulate immune responses, mitigate inflammation, bolster antioxidant defences, detoxify cells and tissues, and influence gene expression [366-368]. Triterpenes are among the most abundant natural products, with approximately 30,000 known structures. Common in medicinal plants and foods like fruits, vegetable oils, and cereals, these compounds are noted for their anticancer, anti-inflammatory, antioxidant, antiviral, and antidiabetic effects [369,370]. Pentacyclic triterpenes have garnered significant interest for their therapeutic potential and are widely marketed as dietary supplements and treatments. Triterpenic compounds are well-regarded for their effectiveness in topical formulations addressing various skin conditions, from aging to malignant melanoma [371-375]. However, their high hydrophobicity limits their effectiveness as pharmacological agents by restricting skin penetration [376]. Consequently, multiple strategies have been developed to address this limitation [375-380]. Recent advancements in pharmacology and nanotechnology have introduced nanocarriers as innovative drug delivery systems. Nanoformulation has the potential to enhance the stability of these compounds by minimizing volatility and shielding them from environmental influences like oxygen, light, moisture, and pH fluctuations [381-383]. Additionally, this strategy improves their solubility while increasing both bioactivity and bioavailability through improved cellular uptake and potential targeted and controlled release [384]. In dermatology, nanocarrier-based systems notably improve skin penetration and allow for precise cellular targeting [385,386].

In this work, we aimed to nanoformulate both the LCE and the pure standard, TA, to assess their effects using the ferroptosis model that we optimised.

Prior to the nanoencapsulation of LCE and TA, a preliminary MTT assay was conducted to assess the toxicity of LCE and to identify the optimal concentrations for nanoformulation. HaCaT cells were exposed to TA concentrations ranging from 0.203125 µM to 13 µM for 24, 48, and 72 hours. As illustrated in Figure 25, all tested concentrations, except for 13 µM, were non-cytotoxic. Interestingly, a significant increase in cell viability was observed starting at a concentration of 6.5 µM.

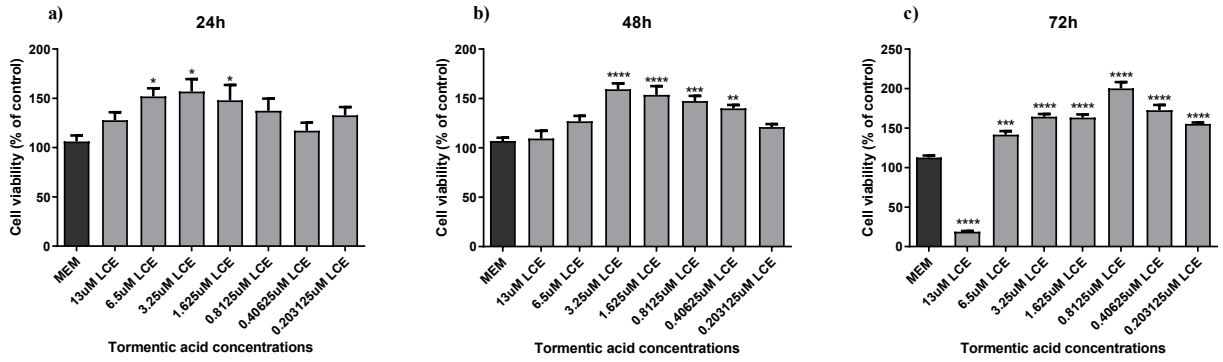


Figure 25: **HaCaT cell viability**. HaCaT cells were treated with different concentrations of LCE (13-0.203125 µM TA concentration) for 24h **(a)**, 48h **(b)**, and 72h **(c)**, and cell viability was measured by MTT assay. The results are expressed as % of CTRL ± SEM of three independent (n=6; $P \leq 0.05$). ANOVA followed by Dunnett's multiple comparison test was performed (* $p < 0.01$, **** $p < 0.0001$). Except for the 13 µM concentration, no cytotoxicity was observed at test time points.

From this MTT (Figure 25) the concentrations of 3.25 µM, 1.625 µM, and 0.8125 µM were selected to be encapsulated.

For the nanocarrier delivery systems of LCE and TA, the polymers Pluronic F127® and Soluplus® were utilised in a modified thin-film hydration process. Both Soluplus® and Pluronic F127® are notable as drug solubilizers, absorption, and permeation enhancers in formulations such as solid dispersions, micelles, and liposomes. Soluplus® is an amphiphilic non-ionic surfactant synthesized from a polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer, that rapidly forms nanocolloidal micelles in aqueous environments, lowering free energy above the critical micellar concentration (CMC). This characteristic makes it a promising carrier for nanodelivery systems, with enhanced bioavailability and improved dissolution profiles. Recent studies highlight its effectiveness in improving poorly water-soluble drugs whilst enabling sustained release [387,388]. In contrast, Pluronic F127®, composed of PEG97-PPO69-PEG97, is a versatile triblock copolymer that is commonly used as a nanocarrier material due to its safety profile, solubilizing capacity, and ability to prolong circulation time, and its pharmaceutical and medical applications were approved by FDA [389]. Additionally, Pluronic F127®-based formulations have been shown to enhance the topical bioavailability of encapsulated compounds [390-392].

The resulting nanoformulations (NF-LCE, NF-TA, NF-B) were characterized for their physiochemical properties and morphology.

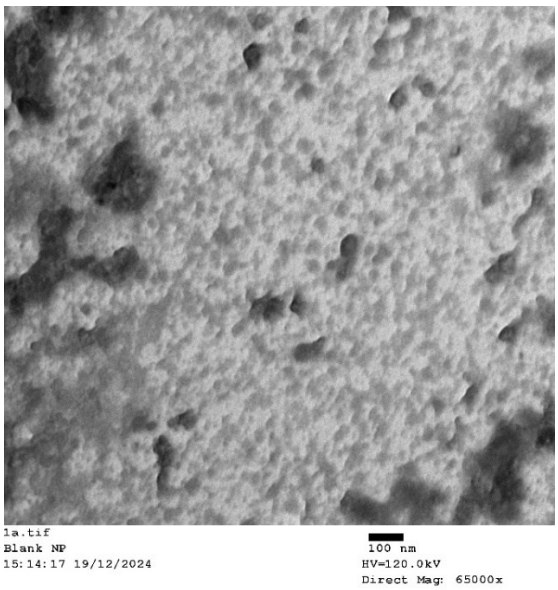
The blank nanoformulation (NF-B) exhibited a mean particle size of 75.00 ± 0.47 nm, indicating a relatively small and uniform particle size distribution. NF-TA demonstrated a slightly increased mean particle size of 81.48 ± 0.39 nm, which may result from the interaction between tormentic acid and the nanocarrier matrix or polymers, leading to the formation of marginally larger particles. In

contrast, NF-LCE showed a significantly larger mean particle size of 103.17 ± 0.87 nm. This substantial increase suggested that the bioactive compounds in apple extract contributed to the formation of larger particles, potentially due to particle aggregation or the entrapment of larger molecules inherent in the extract's complex phytochemical composition. NF-LCE exhibited the highest PDI at 0.21 ± 0.00, indicating a broader size distribution. The heterogeneity in size distribution was likely caused by the complex nature of apple extract, which might lead to less uniform particle encapsulation or interactions within the nanocarrier matrix. However, all three formulations were considered monodisperse/uniform as the PDI obtained was below 0.25 (Table 12). All three nanoformulations were regarded as neutrally charged based on the zeta potential measurements (Table 12).

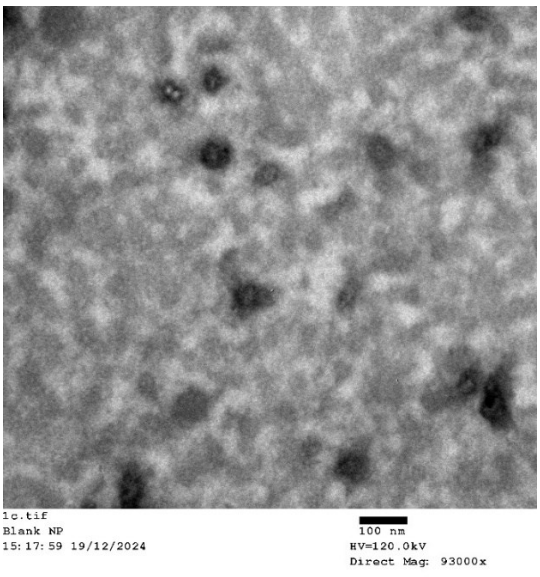
Formulation Name	Particle Size (nm)	PDI	Zeta Potential (mV)
NF-Blank	75.00 ± 0.47	0.02 ± 0.01	-5.24 ± 0.69
NF-TA	81.48 ± 0.39	0.06 ± 0.01	-4.01 ± 0.36
NF-LCE	103.17 ± 0.87	0.21 ± 0.00	-1.88 ± 0.64

Table 12: **Mean particle size, polydispersity index (PDI), and zeta potential of the formulations**. Data represent the mean ± standard deviation (SD) of three replicates.

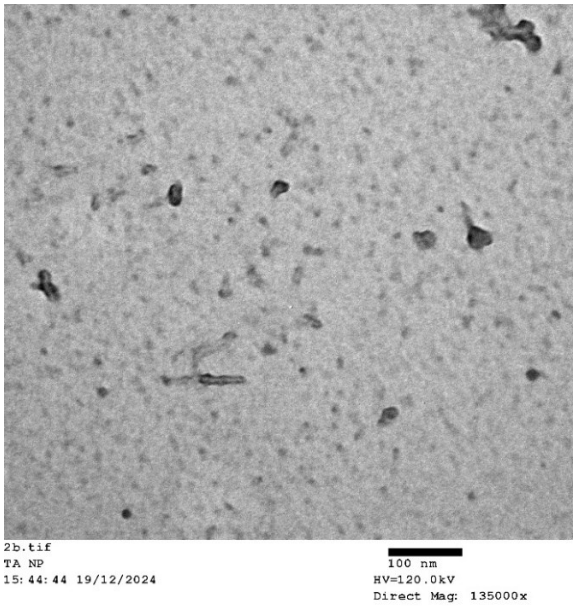
All formulations demonstrated a generally spherical morphology, with particles falling within the nanosize range in the TEM analysis. The particle sizes observed in the TEM images aligned with the mean particle sizes for each formulation conducted by DLS.



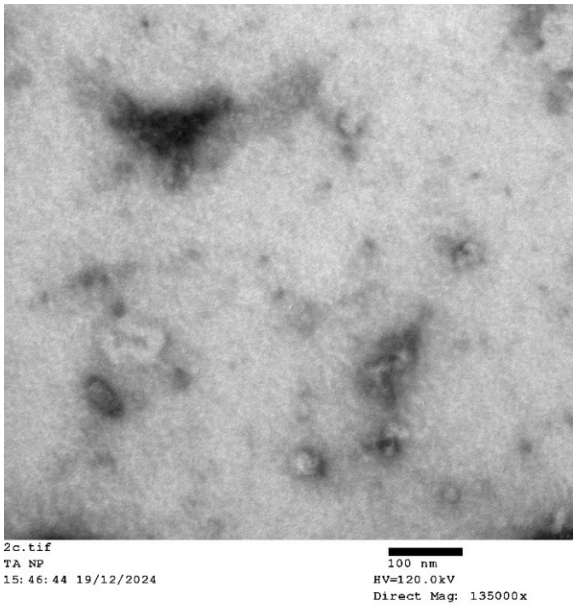
a) Blank Nanoformulation (magnification: 65k)



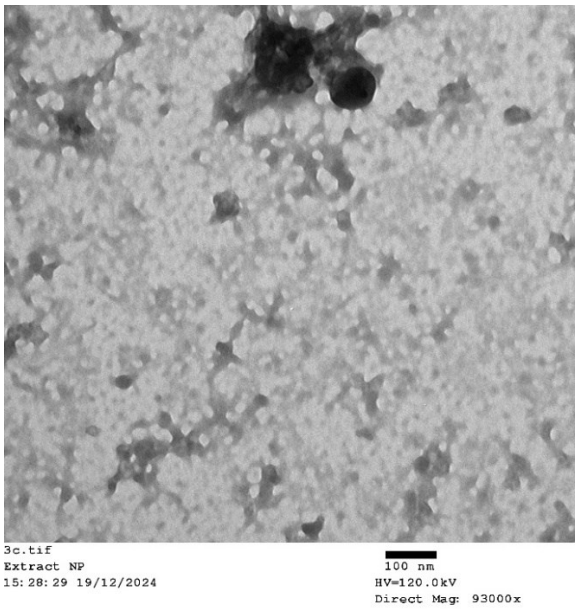
b) Blank Nanoformulation (magnification: 93k)



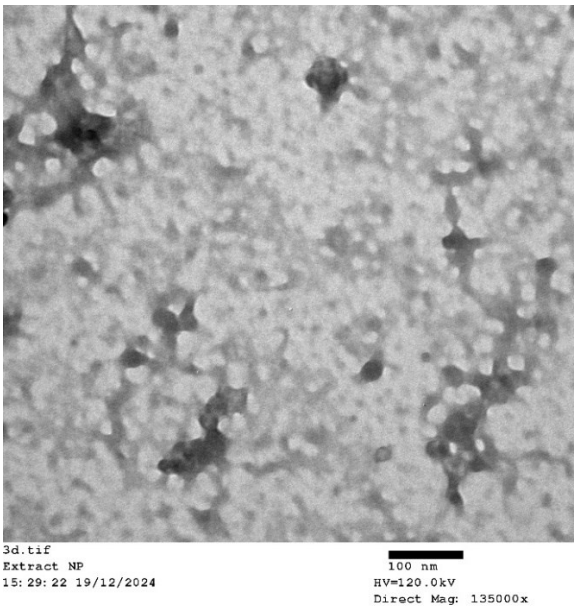
b) NF-TA (magnification: 135k)



d) NF-TA (magnification: 135k)



e) NF-LCE (magnification: 93k)



f) NF-LCE (magnification: 135k)

Figure 26: **Transmission electron micrographs of nanoparticles of the three nanoformulations** – **(a)** blank nanoformulation (magnification: 65k); **(b)** blank nanoformulation (magnification: 93k); **(c)** NF-TA (magnification: 135k); d) NF-TA (magnification: 135k); e) NF-LCE (magnification: 93k); f) NF-LCE (magnification: 135k)

Preliminary evaluations were conducted to assess the biological activity of the nanoformulated compound. An MTT cell viability assay was initially performed to determine whether the nanocarrier delivery systems impacted cell survival. In this case, as shown in Figure 27, neither the pure compound nor the nanoformulations significantly affected the viability of HaCaT cells. This indicates that both treatments did not compromise cell viability, suggesting a favorable safety profile.

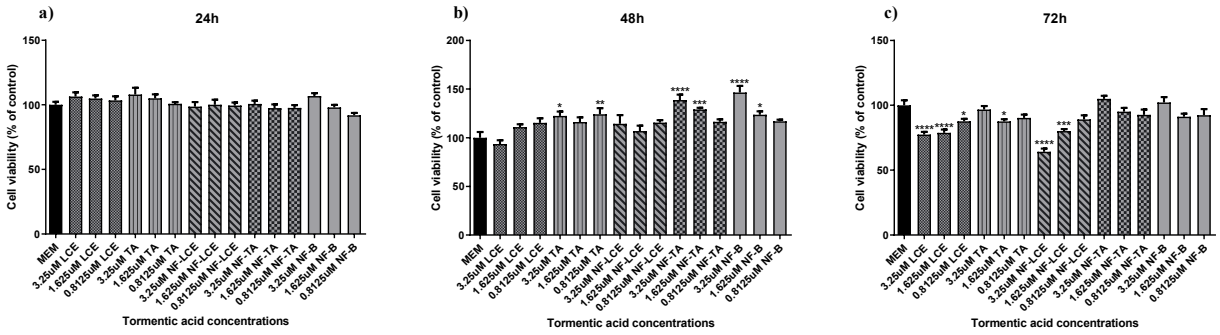


Figure 27: **HaCaT cells viability after exposure to nanoformulated compounds**. HaCaT cells were treated with different concentrations of encapsulated and non-encapsulated LCE and TA for 24h **(a)**, 48h **(b)**, and 72h **(c)**, and cell viability was measured by MTT assay. The results are expressed as % of CTRL ± SEM of three independent experiments (n=6; P≤ 0.05). ANOVA followed by Dunnett's multiple comparison test was performed (* $p < 0.01$, **** $p < 0.0001$). No cytotoxicity was observed at test time points with the selected concentrations.

This confirms that both components are biocompatible, non-toxic, and well-tolerated by the skin [393,394]. TA concentration of 3.25 µM for LCE, TA, NF-LCE, and NF-TA was selected for nanoencapsulation for further experiments. The aim was to assess whether the nanoformulated compounds (NF-LCE and NF-TA) and their unformulated counterparts (LCE and TA) could mediate iron-induced inflammation by examining gene expression. Ferroptosis exhibits unique genetic characteristics distinct from other established forms of regulated cell death. The main function of prostaglandin-endoperoxide synthase 2 (PTGS2/COX2) is metabolizing arachidonic acid into prostaglandins. This gene is upregulated during ferroptosis, making cells more susceptible to ferroptotic attributes. On the other hand, the GPX4 gene is downregulated during ferroptosis. GPX4 converts cytotoxic lipid peroxides into their corresponding alcohols in the presence of glutathione. When GPX4 activity is suppressed, lipid peroxides accumulate, leading to ferroptosis [358,359]. The transcription factor Nrf2 is vital for maintaining skin health and addressing various skin disorders. Present in all skin cell types, Nrf2 regulates oxidative stress, protecting cells from damage. Its short-term activation is crucial for preventing apoptosis caused by UVA and UVB rays. Nrf2 also controls anti-apoptotic molecules that respond to oxidative stress, particularly in the outer layers of the skin exposed to UV sunlight [395]. The data presented in Figure 28 demonstrates that iron significantly boosts the expression levels of COX2, while concurrently decreasing the levels of GPX4 and Nrf2. This suggests that iron may play a pivotal role in regulating these proteins, potentially influencing detrimental oxidative stress responses. In contrast, NF-LCE formulations exhibit a remarkable ability to counteract this effect by effectively reversing the increased COX2 expression and restoring the levels of GPX4 and Nrf2. This indicates that NF-LCE could have therapeutic potential in mitigating the impacts of iron overload on cellular processes. Elevated concentrations of intracellular iron can

lead to an increase in the expression and activity of inflammatory cytokines such as IL-6, and TNF-α [396,397]. This suggests that when iron levels within cells are high, there is a corresponding rise in the activity of these pro-inflammatory cytokines, which play a crucial role in the body's inflammatory response. In contrast, NF-LCE has been shown to significantly reduce the expression of IL-6 and TNF-α, indicating that it may have a protective effect against inflammation driven by excess iron.

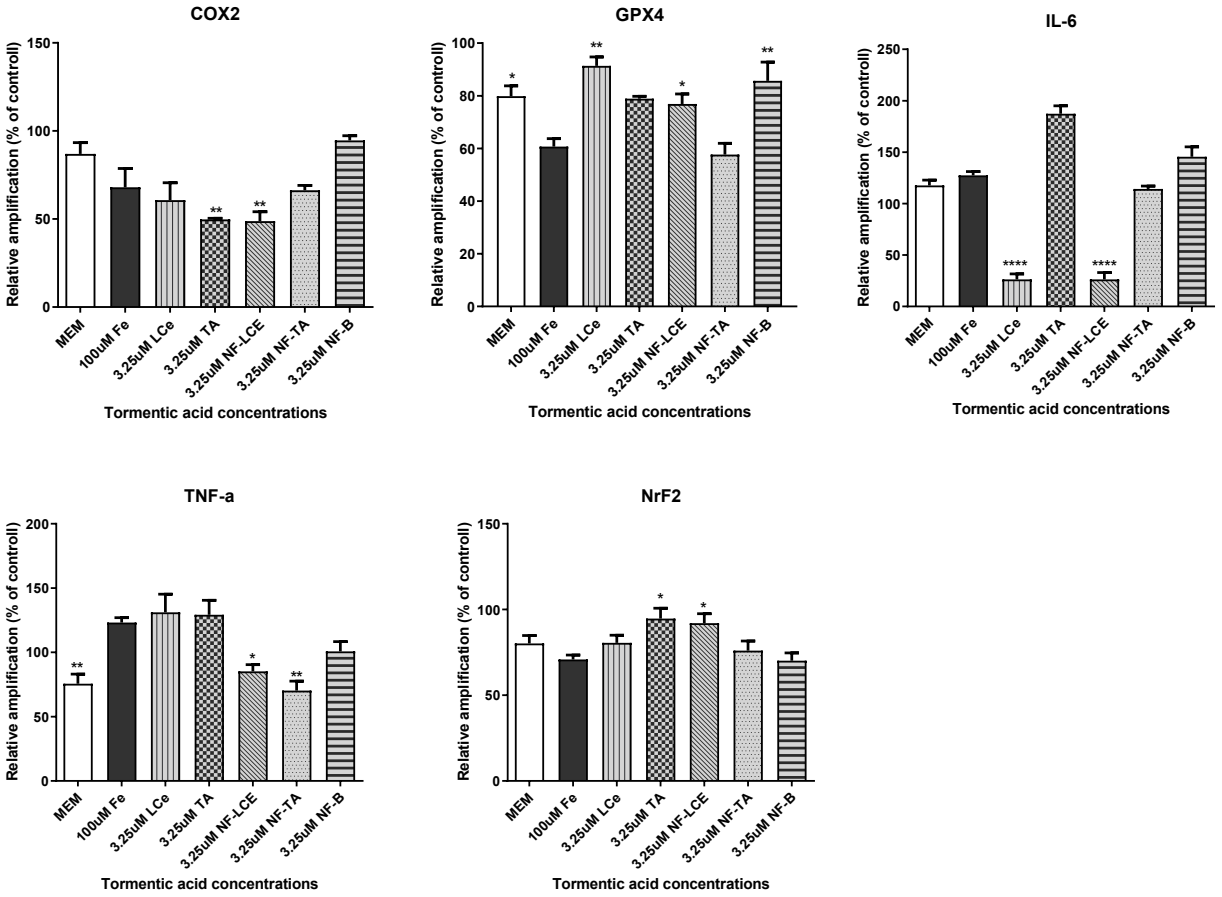


Figure 28: **Anti-inflammatory activity of formulated and non-formulated LCE and TA on iron-treated HaCaT cells.** HaCaT cells were pre-treated for 3h with formulated and non-formulated LCE and TA (3.25µM) followed by 24 h of iron treatment. NF-LCE significantly decreased the expression of anti-inflammatory genes and increased the expression of genes involved in contrasting ferroptosis-induced inflammation. Data are the means ± SEM of three independent experiments. (* $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$, ****$p<0.0001$). ANOVA followed by Dunnett's multiple comparison test was performed vs iron 100 µM.

3.4 Conclusions

The findings of this study reveal that various combinations of media and plant growth regulators, along with adjustments to other parameters such as light, can effectively promote the accumulation of specific secondary metabolites (SMs). The selected conditions for generating the two types of calli facilitated the production of substantial amounts of triterpenic compounds that had not been previously identified in apple pulp. Preliminary assessments of the biological activity of the extracts derived from the calli indicate that both calli primarily exhibit anti-inflammatory and genoprotective effects while showing lower antioxidant activity.

Nanoformulations incorporating pure LCE and standard TA were developed for the first time using Soluplus® and Pluronic F127®, demonstrating optimum physiochemical and cell viability characteristics. A cellular model was optimised and utilised to assess the efficacy of these formulations in countering the detrimental effects that occur during ferroptosis, with results revealing NF-LCE to have a significant protective effect against excess iron-mediated oxidative stress and inflammation by reducing the expression of inflammatory genes and restoring levels of antioxidant protective mechanisms. Together, our findings reinforce previous data illustrating the efficacy of LCE as a potent bioactive phytochemical agent, with nanoformulation further augmenting its therapeutic potential. This work paves the way for future studies in more complex three-dimensional models to further clarify the underlying mechanisms and optimising the formulations prior to progressing these compounds into human clinical studies to benefit skin dysfunctions as topical nutraceuticals.

CONCLUSIONS

Health promotion and chronic disease prevention are primary objectives at both national and European levels. Preventive strategies play a crucial role, and one promising avenue involves the use of medicinal herbs and phytochemicals. Over recent decades, phytochemicals have been recognized as modulators of essential cellular signaling pathways and health enhancement, making them appealing for use in healthcare as functional (bioactive) compounds. However, acquiring secondary metabolites (SMs) often requires large amounts of fresh plant material or resorting to costly chemical synthesis methods, which carry notable environmental drawbacks. Additionally, environmental elements—such as light, temperature, soil moisture, soil fertility, and salinity—can greatly influence processes related to plant growth and development, including the synthesis of SMs, resulting in varying concentrations of bioactive molecules across different plants.

In vitro propagation techniques utilizing plant cell cultures have been established to address these challenges, with the most common method involving the formation of callus cultures from established plant cells. Thus, callus derived from hairy roots, organs, or pulp-controlled *in vitro* cultures is preferred for obtaining valuable SMs and ensuring high-quality, reproducible products. This approach helps mitigate the impacts of seasonal and geographical variations while using less water and reducing waste, aligning with Green Economy principles.

Worldwide studies have focused on apples because of their rich phytochemical profile. Research indicates that consuming apples and their components can help prevent several chronic diseases, including cardiovascular and obstructive pulmonary diseases.

This study focused on three apple varieties: *Cydonia oblonga* Mill., Mela Rosa Marchigiana, and Malus pumila cv. Miller - Annurca Campana Apple. Their pulp was used to start the callus culture. The culture conditions were slightly adjusted for each variety, resulting in varying levels of secondary metabolite accumulation. Analysis of the hydroalcoholic extract revealed that *Cydonia oblonga* Mill. contains both phenolic and triterpenic compounds, whereas Mela Rosa Marchigiana and Annurca Campana Apple mainly consist of triterpenic compounds. The evaluation of the biological activities of the hydroalcoholic extracts highlighted the potential of all three extracts to alleviate inflammation and oxidative stress under different stress conditions (e.g., reduction in NO production in LPS-treated RAW 264.7, reduction of pro-inflammatory gene expression in LPS and iron-stimulated HaCaT cells, reduction of ROS production in H2O2 and TBHP-insulted HaCaT cells). In addition, the derived hydroalcoholic extracts can be successfully encapsulated and their activity improved.

In conclusion**,** **t**his study's results emphasize the positive impacts of callus-derived extracts, suggesting their potential applications in food, and bioproduct manufacturing for the pharmaceutical sector. Additionally, the research indicates that various combinations of media and plant growth

regulators, along with adjustments to factors like light, can efficiently stimulate the production of targeted secondary metabolites. Callus sub-cultures also maintain high yields of these secondary metabolites over time. Finally, employing a nanocarrier delivery system to encapsulate the extracted compounds can boost their effectiveness.

Considering these findings, a compelling direction for this research might be to assess if the apple pulp callus extract and its active compounds can be considered potential components of a nutraceutical formulation aimed at promoting healthy aging or preventing oxidative stress and inflammation-related disorders.

ACKNOWLEDGMENT

Completing this doctoral thesis has been an intense and transformative journey, filled with challenges, growth, and rewarding experiences. I am deeply grateful to everyone who has supported me along the way, both academically and personally.

First and foremost, I would like to express my sincere gratitude to my supervisor, Professor Lucia Potenza, for her invaluable guidance, patience, and encouragement throughout these years. Her expertise and insightful advice have been essential in shaping this research and in helping me grow as a scientist.

I also extend my appreciation to the members of my research group, Professors Roberta De Bellis, Maria Cristina Albertini, and Daniele Fraternale for their collaboration, insightful discussions, and unwavering support. I am grateful for the positive impact they had on my academic growth. Special thanks to Serena Benedetti for her technical assistance and for always being available to help when needed.

A special thanks go to my colleagues for their collaboration, stimulating discussions, and constant support.

I would also like to extend my heartfelt appreciation to the Centre for Nutraceuticals and its incredible research team, who welcomed me during my time abroad. My experience there was truly enriching, both professionally and personally, and I am thankful for the opportunity to have worked alongside such inspiring scientists. A special thanks to Professor Mohammed Gulrez Zariwala for his guidance, kindness, and for making my stay an unforgettable experience. Thanks to Laura, Helena, Ruchitha, and Stefanie for their support, and friendship, and for making my time in the lab both productive and enjoyable.

I am immensely grateful to my family and friends for their unconditional love and encouragement. Their support has been my source of strength, especially during the most demanding phases of this journey

A very special thank you to my boyfriend, Diego, who has been my rock throughout this entire process. His patience, understanding, and constant encouragement have meant the world to me. Thank you for being there through every late-night writing session, every moment of doubt, and every milestone—your support has made this journey infinitely more meaningful.

Finally, I gratefully acknowledge the financial support of the FSE-REACT-EU program, PON "Ricerca e Innovazione" 2014-2020 (D.M. 1061/2021), Azione IV.5 "Dottorati su tematiche Green." Their contributions have made this research possible.

This thesis is dedicated to all those who have believed in me and supported me throughout this academic journey.

REFERENCES

1. Panchawat, S., & Ameta, C. (2021). Medicinal importance of plant metabolites. In Chemistry of biologically potent natural products and synthetic compounds (pp. 1–19). Wiley. http://dx.doi.org/10.1002/9781119640929.ch1
2. Faehnrich, B., Franz, C., Nemaz, P., & Kaul, H.-P. (2021). Medicinal plants and their secondary metabolites—State of the art and trends in breeding, analytics, and use in feed supplementation, with special focus on German chamomile. Journal of Applied Botany and Food Quality, 94, 61–74. http://dx.doi.org/10.5073/JABFQ.2021.094.008
3. Arumugam, T., Sona, C. L., & Maheswari, M. U. (2021). Fruits and vegetables as superfoods: Scope and demand. Journal of Pharmaceutical Innovation, 10, 119–129.
4. Cragg, G. M., & Newman, D. J. (2005). Biodiversity: A continuing source of novel drug leads. Pure and Applied Chemistry, 77(1), 7–24. http://dx.doi.org/10.1351/pac200577010007
5. Borchardt J. K. (2002). The Beginnings of Drug Therapy: Ancient Mesopotamian Medicine. Drug news & perspectives, 15(3), 187–192. https://doi.org/10.1358/dnp.2002.15.3.840015
6. Sneader, D. (2006). Drug discovery: A history. (Sneader, Walter) Journal of Chemical Education, 83. http://dx.doi.org/10.1021/ed083p215.1
7. Cragg, G. M., & Newman, D. J. (2013). Natural products: a continuing source of novel drug leads. Biochimica et biophysica acta, 1830(6), 3670–3695. https://doi.org/10.1016/j.bbagen.2013.02.008
8. Atanasov, A. G., Waltenberger, B., Pferschy-Wenzig, E. M., Linder, T., Wawrosch, C., Uhrin, P., Temml, V., Wang, L., Schwaiger, S., Heiss, E. H., Rollinger, J. M., Schuster, D., Breuss, J. M., Bochkov, V., Mihovilovic, M. D., Kopp, B., Bauer, R., Dirsch, V. M., & Stuppner, H. (2015). Discovery and resupply of pharmacologically active plant-derived natural products: A review. Biotechnology advances, 33(8), 1582–1614. https://doi.org/10.1016/j.biotechadv.2015.08.001
9. Dias, D. A., Urban, S., & Roessner, U. (2012). A historical overview of natural products in drug discovery. Metabolites, 2(2), 303–336. https://doi.org/10.3390/metabo2020303
10. Zenk, M. H., & Juenger, M. (2007). Evolution and current status of the phytochemistry of nitrogenous compounds. Phytochemistry, 68(22-24), 2757–2772. https://doi.org/10.1016/j.phytochem.2007.07.009
11. Davies, J., & Davies, D. (2010). Origins and evolution of antibiotic resistance. Microbiology and molecular biology reviews: MMBR, 74(3), 417–433. https://doi.org/10.1128/MMBR.00016-10
12. Baker, D. D., Chu, M., Oza, U., & Rajgarhia, V. (2007). The value of natural products to future pharmaceutical discovery. Natural product reports, 24(6), 1225–1244. https://doi.org/10.1039/b602241n
13. Brown, A. G., Smale, T. C., King, T. J., Hasenkamp, R., & Thompson, R. H. (1976). Crystal and molecular structure of compactin, a new antifungal metabolite from Penicillium brevicompactum. Journal of the Chemical Society. Perkin transactions 1, (11), 1165–1170.
14. Dickson, M., & Gagnon, J. P. (2004). The cost of new drug discovery and development. Discovery medicine, 4(22), 172–179.
15. Ortholand, J. Y., & Ganesan, A. (2004). Natural products and combinatorial chemistry: back to the future. Current opinion in chemical biology, 8(3), 271–280. https://doi.org/10.1016/j.cbpa.2004.04.011
16. Sheridan C. (2012). Recasting natural product research. Nature biotechnology, 30(5), 385–387. https://doi.org/10.1038/nbt.2208

17. David, B., Wolfender, JL. & Dias, D.A. (2015). The pharmaceutical industry and natural products: historical status and new trends. Phytochemistry Reviews, 14, 299–315. https://doi.org/10.1007/s11101-014-9367-z
18. Elshafie, H. S., Camele, I., & Mohamed, A. A. (2023). A Comprehensive Review on the Biological, Agricultural and Pharmaceutical Properties of Secondary Metabolites Based-Plant Origin. International journal of molecular sciences, 24(4), 3266. https://doi.org/10.3390/ijms24043266
19. Simsek, M., & Whitney, K. (2024). Examination of Primary and Secondary Metabolites Associated with a Plant-Based Diet and Their Impact on Human Health. Foods (Basel, Switzerland), 13(7), 1020. https://doi.org/10.3390/foods13071020
20. Yeshi, K., Crayn, D., Ritmejerytė, E., & Wangchuk, P. (2022). Plant Secondary Metabolites Produced in Response to Abiotic Stresses Has Potential Application in Pharmaceutical Product Development. Molecules (Basel, Switzerland), 27(1), 313. https://doi.org/10.3390/molecules27010313
21. Godoy, F., Olivos-Hernández, K., Stange, C., & Handford, M. (2021). Abiotic Stress in Crop Species: Improving Tolerance by Applying Plant Metabolites. Plants (Basel, Switzerland), 10(2), 186. https://doi.org/10.3390/plants10020186
22. Jan, R., Asaf, S., Numan, M., Lubna, & Kim, K.-M. (2021). Plant Secondary Metabolite Biosynthesis and Transcriptional Regulation in Response to Biotic and Abiotic Stress Conditions. Agronomy, 11(5), 968. https://doi.org/10.3390/agronomy11050968
23. Patra, B., Schluttenhofer, C., Wu, Y., Pattanaik, S., & Yuan, L. (2013). Transcriptional regulation of secondary metabolite biosynthesis in plants. Biochimica et biophysica acta, 1829(11), 1236–1247. https://doi.org/10.1016/j.bbagrm.2013.09.006
24. Wink M. (2003). Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. Phytochemistry, 64(1), 3–19. https://doi.org/10.1016/s0031-9422(03)00300-5
25. van Wyk, B.-E. (2003). The value of chemosystematics clarifying relationships in the genistoid tribes of papilionoid legumes. Biochemical Systematics and Ecology, 31(8), 875-884. https://doi.org/10.1016/S0305-1978(03)00083-8
26. Ritmejerytė, E., Boughton, B. A., Bayly, M. J., & Miller, R. E. (2020). Unique and highly specific cyanogenic glycoside localization in stigmatic cells and pollen in the genus Lomatia (Proteaceae). Annals of Botany, 126(3), 387–400. https://doi.org/10.1093/aob/mcaa038
27. Twaij, B. M., & Hasan, M. N. (2022). Bioactive Secondary Metabolites from Plant Sources: Types, Synthesis, and Their Therapeutic Uses. International Journal of Plant Biology, 13(1), 4-14. https://doi.org/10.3390/ijpb13010003
28. Chiocchio, I., Mandrone, M., Tomasi, P., Marincich, L., & Poli, F. (2021). Plant Secondary Metabolites: An Opportunity for Circular Economy. Molecules, 26(2), 495. https://doi.org/10.3390/molecules26020495
29. Gonzalez, P.J., & Sörensen, P.M. (2020). Characterization of saponin foam from Saponaria officinalis for food applications. Food Hydrocolloids, 101, 105541. https://doi.org/10.1016/j.foodhyd.2019.105541
30. Arroo, R. (2007). Eberhard Breitmaier. Terpenes - Flavors, fragrances, pharmaca, pheromones. Wiley-VCH, 2006, 214 pp ISBN 3-527-31786-4. Applied Organometallic Chemistry, 21, 377-377. http://dx.doi.org/10.1002/aoc.1209
31. Zárybnický, T., Boušová, I., Ambrož, M., & Skálová, L. (2018). Hepatotoxicity of monoterpenes and sesquiterpenes. Archives of toxicology, 92(1), 1–13. https://doi.org/10.1007/s00204-017-2062-2

32. Davis, E.M., Croteau, R. (2000). Cyclization Enzymes in the Biosynthesis of Monoterpenes, Sesquiterpenes, and Diterpenes. In: Leeper, F.J., Vederas, J.C. (eds) Biosynthesis. Topics in Current Chemistry, vol 209. Springer, Berlin, Heidelberg. https://doi.org/10.1007/3-540-48146-X_2
33. Kessler, A, & Baldwin, I.T. (2001). Defensive Function of Herbivore-Induced Plant Volatile Emissions in Nature. Science. 291. 2141-4. http://dx.doi.org/10.1126/science.291.5511.2141
34. Singh, B., & Sharma, R. A. (2015). Plant terpenes: defense responses, phylogenetic analysis, regulation and clinical applications. 3 Biotech, 5(2), 129–151. https://doi.org/10.1007/s13205-014-0220-2
35. Friedman, M., Henika, P. R., & Mandrell, R. E. (2002). Bactericidal activities of plant essential oils and some of their isolated constituents against Campylobacter jejuni, Escherichia coli, Listeria monocytogenes, and Salmonella enterica. Journal of food protection, 65(10), 1545–1560. https://doi.org/10.4315/0362-028x-65.10.1545
36. Dahham, S. S., Tabana, Y. M., Iqbal, M. A., Ahamed, M. B., Ezzat, M. O., Majid, A. S., & Majid, A. M. (2015). The Anticancer, Antioxidant and Antimicrobial Properties of the Sesquiterpene β-Caryophyllene from the Essential Oil of Aquilaria crassna. Molecules (Basel, Switzerland), 20(7), 11808–11829. https://doi.org/10.3390/molecules200711808
37. Porres-Martínez, M., González-Burgos, E., Carretero, M. E., & Gómez-Serranillos, M. P. (2016). In vitro neuroprotective potential of the monoterpenes α-pinene and 1,8-cineole against H2O2-induced oxidative stress in PC12 cells. Zeitschrift fur Naturforschung. C, Journal of biosciences, 71(7-8), 191–199. https://doi.org/10.1515/znc-2014-4135
38. Bartwal, A., Mall, R., Lohani, P., Guru, S.K., & Arora, S. (2013). Role of Secondary Metabolites and Brassinosteroids in Plant Defense Against Environmental Stresses. Journal of Plant Growth Regulation, 32, 216–232 (2013). https://doi.org/10.1007/s00344-012-9272-x
39. Bravo L. (1998). Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutrition reviews, 56(11), 317–333. https://doi.org/10.1111/j.1753-4887.1998.tb01670.x
40. Panche, A. N., Diwan, A. D., & Chandra, S. R. (2016). Flavonoids: an overview. Journal of nutritional science, 5, e47. https://doi.org/10.1017/jns.2016.41
41. Robbins R. J. (2003). Phenolic acids in foods: an overview of analytical methodology. Journal of agricultural and food chemistry, 51(10), 2866–2887. https://doi.org/10.1021/jf026182t
42. Samanta, A., Das, G., & Das, S.K. (2011). Roles of flavonoids in Plants. International Journal of pharmaceutical science and technology. 6. 12-35.
43. Khoo, H. E., Azlan, A., Tang, S. T., & Lim, S. M. (2017). Anthocyanidins and anthocyanins: colored pigments as food, pharmaceutical ingredients, and the potential health benefits. Food & nutrition research, 61(1), 1361779. https://doi.org/10.1080/16546628.2017.1361779
44. Boeckler, G. A., Gershenzon, J., & Unsicker, S. B. (2011). Phenolic glycosides of the Salicaceae and their role as anti-herbivore defenses. Phytochemistry, 72(13), 1497–1509. https://doi.org/10.1016/j.phytochem.2011.01.038
45. Agati, G., Azzarello, E., Pollastri, S., & Tattini, M. (2012). Flavonoids as antioxidants in plants: location and functional significance. Plant science : an international journal of experimental plant biology, 196, 67–76. https://doi.org/10.1016/j.plantsci.2012.07.014
46. Tattini, M., Landi, M., Brunetti, C., Giordano, C., Remorini, D., Gould, K. S., & Guidi, L. (2014). Epidermal coumaroyl anthocyanins protect sweet basil against excess light stress: multiple consequences of light attenuation. Physiologia plantarum, 152(3), 585–598. https://doi.org/10.1111/ppl.12201

47. Leopoldini, M., Russo, N., Chiodo, S., & Toscano, M. (2006). Iron chelation by the powerful antioxidant flavonoid quercetin. Journal of agricultural and food chemistry, 54(17), 6343–6351. https://doi.org/10.1021/jf060986h
48. Dewick, P. M. (2002). Medicinal natural products: A biosynthetic approach. New York, NY: John Wiley & Sons.
49. Debnath, B., Singh, W. S., Das, M., Goswami, S., Singh, M. K., Maiti, D., & Manna, K. (2018). Role of plant alkaloids on human health: A review of biological activities. Materials Today Chemistry, 9, 56–72. https://doi.org/10.1016/j.mtchem.2018.05.001
50. Aniszewski, T. (2015). Alkaloids: Chemistry, biology, ecology, and applications (2nd ed.). Elsevier.
51. Kurek J. (2019). Introductory Chapter: Alkaloids - Their Importance in Nature and for Human Life. http://dx.doi.org/10.5772/intechopen.85400
52. Bednarz, H., Roloff, N., & Niehaus, K. (2019). Mass Spectrometry Imaging of the Spatial and Temporal Localization of Alkaloids in Nightshades. Journal of agricultural and food chemistry, 67(49), 13470–13477. https://doi.org/10.1021/acs.jafc.9b01155
53. Nowacki, E., & Waller, G. R. (1973). A study on the origin and protective function of alkaloids in plants. Flora, 162, 108–117. https://doi.org/10.1016/S0367-2530(17)31694-8
54. McCulley, R. L., Bush, L. P., Carlisle, A. E., Ji, H., & Nelson, J. A. (2014). Warming reduces tall fescue abundance but stimulates toxic alkaloid concentrations in transition zone pastures of the U.S. Frontiers in chemistry, 2, 88. https://doi.org/10.3389/fchem.2014.00088
55. Waller, G. R., & Nowacki, E. K. (1978). Alkaloid biology and metabolism in plants. In Alkaloids: Biochemistry, Ecology, and Medicinal Applications (pp. 85–119). Springer.
56. Mattocks A. R. (1968). Toxicity of pyrrolizidine alkaloids. Nature, 217(5130), 723–728. https://doi.org/10.1038/217723a0
57. Trigo J.R. (2011). Effects of pyrrolizidine alkaloids through different trophic levels. Phytochemistry Reviews, 10, 83–98. https://doi.org/10.1007/s11101-010-9191-z
58. Del Cueto, J., Ionescu, I. A., Pičmanová, M., Gericke, O., Motawia, M. S., Olsen, C. E., Campoy, J. A., Dicenta, F., Møller, B. L., & Sánchez-Pérez, R. (2017). Cyanogenic Glucosides and Derivatives in Almond and Sweet Cherry Flower Buds from Dormancy to Flowering. Frontiers in plant science, 8, 800. https://doi.org/10.3389/fpls.2017.00800
59. Halkier, B. A., & Gershenzon, J. (2006). Biology and biochemistry of glucosinolates. Annual review of plant biology, 57, 303–333. https://doi.org/10.1146/annurev.arplant.57.032905.105228
60. Gleadow, R.M., & Woodrow, I.E. (2000). Temporal and spatial variation in cyanogenic glycosides in Eucalyptus cladocalyx, Tree Physiology, 20(9), 591–598, https://doi.org/10.1093/treephys/20.9.591
61. Yulvianti, M., & Zidorn, C. (2021). Chemical Diversity of Plant Cyanogenic Glycosides: An Overview of Reported Natural Products. Molecules (Basel, Switzerland), 26(3), 719. https://doi.org/10.3390/molecules26030719
62. Verma, N., & Shukla, S. (2015). Impact of various factors responsible for fluctuation in plant secondary metabolites. Journal of Applied Research on Medicinal and Aromatic Plants, 2(4), 105–113. https://doi.org/10.1016/j.jarmap.2015.09.002
63. Metlen, K. L., Aschehoug, E. T., & Callaway, R. M. (2009). Plant behavioural ecology: dynamic plasticity in secondary metabolites. Plant, cell & environment, 32(6), 641–653. https://doi.org/10.1111/j.1365-3040.2008.01910.x

64. Yang, L., Wen, K. S., Ruan, X., Zhao, Y. X., Wei, F., & Wang, Q. (2018). Response of Plant Secondary Metabolites to Environmental Factors. Molecules (Basel, Switzerland), 23(4), 762. https://doi.org/10.3390/molecules23040762
65. Li, Y., Kong, D., Fu, Y., Sussman, M. R., & Wu, H. (2020). The effect of developmental and environmental factors on secondary metabolites in medicinal plants. Plant physiology and biochemistry: PPB, 148, 80–89. https://doi.org/10.1016/j.plaphy.2020.01.006
66. Zhao, J., Davis, L. C., & Verpoorte, R. (2005). Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnology advances, 23(4), 283–333. https://doi.org/10.1016/j.biotechadv.2005.01.003
67. Selmar, D., & Kleinwächter, M. (2013). Stress enhances the synthesis of secondary plant products: the impact of stress-related over-reduction on the accumulation of natural products. Plant & cell physiology, 54(6), 817–826. https://doi.org/10.1093/pcp/pct054
68. Balestrini, R., Brunetti, C., Cammareri, M., Caretto, S., Cavallaro, V., Cominelli, E., De Palma, M., Docimo, T., Giovinazzo, G., Grandillo, S., Locatelli, F., Lumini, E., Paolo, D., Patanè, C., Sparvoli, F., Tucci, M., & Zampieri, E. (2021). Strategies to Modulate Specialized Metabolism in Mediterranean Crops: From Molecular Aspects to Field. International journal of molecular sciences, 22(6), 2887. https://doi.org/10.3390/ijms22062887
69. Isah T. (2019). Stress and defense responses in plant secondary metabolites production. Biological research, 52(1), 39. https://doi.org/10.1186/s40659-019-0246-3
70. Asfaw, T. B., Esho, T. B., Bachheti, A., Bachheti, R. K., Pandey, D. P., & Husen, A. (2022). Exploring important herbs, shrubs, and trees for their traditional knowledge, chemical derivatives, and potential benefits. In Herbs, shrubs, and trees: Potential medicinal benefits, 1–26. https://doi.org/10.1201/9781003205067-1
71. Solárová, Z., Liskova, A., Samec, M., Kubatka, P., Büsselberg, D., & Solár, P. (2020). Anticancer Potential of Lichens' Secondary Metabolites. Biomolecules, 10(1), 87. https://doi.org/10.3390/biom10010087
72. Vaishnav, P., & Demain, A. L. (2011). Unexpected applications of secondary metabolites. Biotechnology advances, 29(2), 223–229. https://doi.org/10.1016/j.biotechadv.2010.11.006
73. Liu, Q., Meng, X., Li, Y., Zhao, C. N., Tang, G. Y., & Li, H. B. (2017). Antibacterial and Antifungal Activities of Spices. International journal of molecular sciences, 18(6), 1283. https://doi.org/10.3390/ijms18061283
74. Alibi, S., Crespo, D., & Navas, J. (2021). Plant-Derivatives Small Molecules with Antibacterial Activity. Antibiotics (Basel, Switzerland), 10(3), 231. https://doi.org/10.3390/antibiotics10030231
75. Brglez Mojzer, E., Knez Hrnčič, M., Škerget, M., Knez, Ž., & Bren, U. (2016). Polyphenols: Extraction Methods, Antioxidative Action, Bioavailability and Anticarcinogenic Effects. Molecules (Basel, Switzerland), 21(7), 901. https://doi.org/10.3390/molecules21070901
76. Vestergaard, M., & Ingmer, H. (2019). Antibacterial and antifungal properties of resveratrol. International journal of antimicrobial agents, 53(6), 716–723. https://doi.org/10.1016/j.ijantimicag.2019.02.015
77. Walle, T., Hsieh, F., DeLegge, M. H., Oatis, J. E., Jr, & Walle, U. K. (2004). High absorption but very low bioavailability of oral resveratrol in humans. Drug metabolism and disposition: the biological fate of chemicals, 32(12), 1377–1382. https://doi.org/10.1124/dmd.104.000885
78. Boocock, D. J., Faust, G. E., Patel, K. R., Schinas, A. M., Brown, V. A., Ducharme, M. P., Booth, T. D., Crowell, J. A., Perloff, M., Gescher, A. J., Steward, W. P., & Brenner, D. E. (2007). Phase I dose escalation pharmacokinetic study in healthy volunteers of resveratrol, a potential cancer chemopreventive agent. Cancer epidemiology, biomarkers & prevention : a

publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology, 16(6), 1246–1252. https://doi.org/10.1158/1055-9965.EPI-07-0022
79. Cottart, C. H., Nivet-Antoine, V., Laguillier-Morizot, C., & Beaudeux, J. L. (2010). Resveratrol bioavailability and toxicity in humans. Molecular nutrition & food research, 54(1), 7–16. https://doi.org/10.1002/mnfr.200900437
80. Fabbrocini, G., Staibano, S., De Rosa, G., Battimiello, V., Fardella, N., Ilardi, G., La Rotonda, M. I., Longobardi, A., Mazzella, M., Siano, M., Pastore, F., De Vita, V., Vecchione, M. L., & Ayala, F. (2011). Resveratrol-containing gel for the treatment of acne vulgaris: a single-blind, vehicle-controlled, pilot study. American journal of clinical dermatology, 12(2), 133–141. https://doi.org/10.2165/11530630-000000000-00000
81. Gomes, R., Ramirez, R., Maciel, J., Agra, M., Souza, M., Falcão-Silva, V., & Siqueira-Junior, J. (2011). Phenolic compounds from Sidastrum micranthum (A. St.-Hil.) Fryxell and evaluation of acacetin and 7,4'-DI-O-methylisoscutellarein as modulators of bacterial drug resistance. Química Nova, 34(8), 1385–1388. https://doi.org/10.1590/S0100-40422011000800016
82. Cha, J. D., Choi, S. M., & Park, J. H. (2014). Combination of acacetin with antibiotics against methicillin-resistant Staphylococcus aureus isolated from clinical specimens. Advances in Bioscience and Biotechnology, 54, 398–408. http://dx.doi.org/10.4236/abb.2014.54048
83. Komape, N. P., Aderogba, M., Bagla, V. P., Masoko, P., & Eloff, J. N. (2014). Anti-bacterial and anti-oxidant activities of leaf extracts of Combretum vendae (Combretecacea) and the isolation of an anti-bacterial compound. African journal of traditional, complementary, and alternative medicines: AJTCAM, 11(5), 73–77. https://doi.org/10.4314/ajtcam.v11i5.12
84. More, G., Lall, N., Hussein, A., & Tshikalange, T. E. (2012). Antimicrobial Constituents of Artemisia afra Jacq. ex Willd. against Periodontal Pathogens. Evidence-based complementary and alternative medicine: eCAM, 2012, 252758. https://doi.org/10.1155/2012/252758
85. Verdeguer, M., Sánchez-Moreiras, A. M., & Araniti, F. (2020). Phytotoxic Effects and Mechanism of Action of Essential Oils and Terpenoids. Plants (Basel, Switzerland), 9(11), 1571. https://doi.org/10.3390/plants9111571
86. Guimarães, A. C., Meireles, L. M., Lemos, M. F., Guimarães, M. C. C., Endringer, D. C., Fronza, M., & Scherer, R. (2019). Antibacterial Activity of Terpenes and Terpenoids Present in Essential Oils. Molecules (Basel, Switzerland), 24(13), 2471. https://doi.org/10.3390/molecules24132471
87. Marchese, A., Arciola, C. R., Coppo, E., Barbieri, R., Barreca, D., Chebaibi, S., Sobarzo-Sánchez, E., Nabavi, S. F., Nabavi, S. M., & Daglia, M. (2018). The natural plant compound carvacrol as an antimicrobial and anti-biofilm agent: mechanisms, synergies and bio-inspired anti-infective materials. Biofouling, 34(6), 630–656. https://doi.org/10.1080/08927014.2018.1480756
88. Marchese, A., Orhan, I. E., Daglia, M., Barbieri, R., Di Lorenzo, A., Nabavi, S. F., Gortzi, O., Izadi, M., & Nabavi, S. M. (2016). Antibacterial and antifungal activities of thymol: A brief review of the literature. Food chemistry, 210, 402–414. https://doi.org/10.1016/j.foodchem.2016.04.111
89. Othman, L., Sleiman, A., & Abdel-Massih, R. M. (2019). Antimicrobial Activity of Polyphenols and Alkaloids in Middle Eastern Plants. Frontiers in microbiology, 10, 911. https://doi.org/10.3389/fmicb.2019.00911
90. Chakraborty, P., Dastidar, D. G., Paul, P., Dutta, S., Basu, D., Sharma, S. R., Basu, S., Sarker, R. K., Sen, A., Sarkar, A., & Tribedi, P. (2020). Inhibition of biofilm formation of

Pseudomonas aeruginosa by caffeine: a potential approach for sustainable management of biofilm. Archives of microbiology, 202(3), 623–635. https://doi.org/10.1007/s00203-019-01775-0

91. Sack, R. B., & Froehlich, J. L. (1982). Berberine inhibits intestinal secretory response of Vibrio cholerae and Escherichia coli enterotoxins. Infection and immunity, 35(2), 471–475. https://doi.org/10.1128/iai.35.2.471-475.1982
92. Wang, D., Yu, L., Xiang, H., Fan, J., He, L., Guo, N., Feng, H., & Deng, X. (2008). Global transcriptional profiles of Staphylococcus aureus treated with berberine chloride. FEMS microbiology letters, 279(2), 217–225. https://doi.org/10.1111/j.1574-6968.2007.01031.x
93. Lv, Z., Peng, G., Liu, W., Xu, H., & Su, J. (2015). Berberine blocks the relapse of Clostridium difficile infection in C57BL/6 mice after standard vancomycin treatment. Antimicrobial agents and chemotherapy, 59(7), 3726–3735. https://doi.org/10.1128/AAC.04794-14
94. Huang, X., Wang, P., Li, T., Tian, X., Guo, W., Xu, B., Huang, G., Cai, D., Zhou, F., Zhang, H., & Lei, H. (2020). Self-Assemblies Based on Traditional Medicine Berberine and Cinnamic Acid for Adhesion-Induced Inhibition Multidrug-Resistant Staphylococcus aureus. ACS applied materials & interfaces, 12(1), 227–237. https://doi.org/10.1021/acsami.9b17722
95. Taysi, S., Tascan, A. S., Ugur, M. G., & Demir, M. (2019). Radicals, Oxidative/Nitrosative Stress and Preeclampsia. Mini reviews in medicinal chemistry, 19(3), 178–193. https://doi.org/10.2174/1389557518666181015151350
96. Krishnaiah, D., Sarbatly, R., & Nithyanandam, R. (2011). A review of the antioxidant potential of medicinal plant species. Food and Bioproducts Processing, 89, 217–233. https://doi.org/10.1016/j.fbp.2010.04.008
97. Pohl, F., & Kong Thoo Lin, P. (2018). The Potential Use of Plant Natural Products and Plant Extracts with Antioxidant Properties for the Prevention/Treatment of Neurodegenerative Diseases: In Vitro, In Vivo and Clinical Trials. Molecules (Basel, Switzerland), 23(12), 3283. https://doi.org/10.3390/molecules23123283
98. Anfuso, C. D., Giurdanella, G., Longo, A., Cosentino, A., Agafonova, A., Rusciano, D., & Lupo, G. (2022). Antioxidant Activity of Cyanidin-3-O-Glucoside and Verbascoside in an in Vitro Model of Diabetic Retinopathy. Frontiers in bioscience (Landmark edition), 27(11), 308. https://doi.org/10.31083/j.fbl2711308
99. Mirzaee, A., Abbasi, M., Sepehri, S., & Mirzaei, M. (2013). The effects of Allium porrum and Medicago sativa on iron concentration in thalassemia serums. Life Science Journal. 10. 27-31.
100. Jansen van Rensburg, Z., Abrahams, S., Bardien, S., & Kenyon, C. (2021). Toxic Feedback Loop Involving Iron, Reactive Oxygen Species, α-Synuclein and Neuromelanin in Parkinson's Disease and Intervention with Turmeric. Molecular neurobiology, 58(11), 5920–5936. https://doi.org/10.1007/s12035-021-02516-5
101. Yin, M., Liu, Y., & Chen, Y. (2021). Iron metabolism: an emerging therapeutic target underlying the anti-cancer effect of quercetin. Free radical research, 55(3), 296–303. https://doi.org/10.1080/10715762.2021.1898604
102. González-Burgos, E., & Gómez-Serranillos, M. P. (2012). Terpene compounds in nature: a review of their potential antioxidant activity. Current medicinal chemistry, 19(31), 5319–5341. https://doi.org/10.2174/092986712803833335
103. Qi, L. W., Liu, E. H., Chu, C., Peng, Y. B., Cai, H. X., & Li, P. (2010). Anti-diabetic agents from natural products--an update from 2004 to 2009. Current topics in medicinal chemistry, 10(4), 434–457. https://doi.org/10.2174/156802610790980620

104. Torres-Martínez, R., García-Rodríguez, Y. M., Ríos-Chávez, P., Saavedra-Molina, A., López-Meza, J. E., Ochoa-Zarzosa, A., & Garciglia, R. S. (2018). Antioxidant Activity of the Essential Oil and its Major Terpenes of Satureja macrostema (Moc. and Sessé ex Benth.) Briq. Pharmacognosy magazine, 13(Suppl 4), S875–S880. https://doi.org/10.4103/pm.pm_316_17
105. Sokmen, A., Gulluce, M., Akpulat, H. A., Daferera, D., Tepe, B., Polissiou, M., Sokmen, M., & Sahin, F. (2004). The in vitro antimicrobial and antioxidant activities of the essential oils and methanol extracts of endemic Thymus spathulifolius. Food Control, 15, 627–634. https://doi.org/10.1016/J.FOODCONT.2003.10.005
106. Mimica-Dukic, N., Bozin, B., Sokovic, M., & Simin, N. (2004). Antimicrobial and antioxidant activities of Melissa officinalis L. (Lamiaceae) essential oil. Journal of agricultural and food chemistry, 52(9), 2485–2489. https://doi.org/10.1021/jf030698a
107. Tiong, S. H., Looi, C. Y., Hazni, H., Arya, A., Paydar, M., Wong, W. F., Cheah, S. C., Mustafa, M. R., & Awang, K. (2013). Antidiabetic and antioxidant properties of alkaloids from Catharanthus roseus (L.) G. Don. Molecules (Basel, Switzerland), 18(8), 9770–9784. https://doi.org/10.3390/molecules18089770
108. Correché, E. R., Andújar, S. A., Kurdelas, R. R., Lechón, M. J. G., Freile, M. L., & Enriz, R. D. (2008). Antioxidant and cytotoxic activities of canadine: Biological effects and structural aspects. Bioorganic & Medicinal Chemistry, 16, 3641–3651. https://doi.org/10.1016/J.BMC.2008.02.015
109. Azam, S., Hadi, N., Khan, N. U., & Hadi, S. M. (2003). Antioxidant and prooxidant properties of caffeine, theobromine and xanthine. Medical science monitor: international medical journal of experimental and clinical research, 9(9), BR325–BR330.
110. Moura, D. J., Richter, M. F., Boeira, J. M., Pêgas Henriques, J. A., & Saffi, J. (2007). Antioxidant properties of beta-carboline alkaloids are related to their antimutagenic and antigenotoxic activities. Mutagenesis, 22(4), 293–302. https://doi.org/10.1093/mutage/gem016
111. Ghasemian, M., Owlia, S., & Owlia, M. B. (2016). Review of Anti-Inflammatory Herbal Medicines. Advances in pharmacological sciences, 2016, 9130979. https://doi.org/10.1155/2016/9130979
112. Aggarwal, B. B., & Harikumar, K. B. (2009). Potential therapeutic effects of curcumin, the anti-inflammatory agent, against neurodegenerative, cardiovascular, pulmonary, metabolic, autoimmune and neoplastic diseases. The international journal of biochemistry & cell biology, 41(1), 40–59. https://doi.org/10.1016/j.biocel.2008.06.010
113. Azeem, M., Hanif, M., Mahmood, K., Ameer, N., Chughtai, F. R. S., & Abid, U. (2023). An insight into anticancer, antioxidant, antimicrobial, antidiabetic and anti-inflammatory effects of quercetin: a review. Polymer bulletin (Berlin, Germany), 80(1), 241–262. https://doi.org/10.1007/s00289-022-04091-8
114. Devarshi, P. P., Jones, A. D., Taylor, E. M., Stefanska, B., & Henagan, T. M. (2017). Quercetin and Quercetin-Rich Red Onion Extract Alter Pgc-1α Promoter Methylation and Splice Variant Expression. PPAR research, 2017, 3235693. https://doi.org/10.1155/2017/3235693
115. Bryś, M., Urbańska, K., & Olas, B. (2022). Novel Findings regarding the Bioactivity of the Natural Blue Pigment Genipin in Human Diseases. International Journal of Molecular Sciences, 23(2), 902. https://doi.org/10.3390/ijms23020902
116. Andrade Del Olmo, J., Pérez-Álvarez, L., Sáez-Martínez, V., Benito-Cid, S., Ruiz-Rubio, L., Pérez-González, R., Vilas-Vilela, J. L., & Alonso, J. M. (2022). Wound healing and antibacterial chitosan-genipin hydrogels with controlled drug delivery for synergistic anti-

inflammatory activity. International journal of biological macromolecules, 203, 679–694. https://doi.org/10.1016/j.ijbiomac.2022.01.193

117. Salminen, A., Lehtonen, M., Suuronen, T., Kaarniranta, K., & Huuskonen, J. (2008). Terpenoids: natural inhibitors of NF-kappaB signaling with anti-inflammatory and anticancer potential. Cellular and molecular life sciences: CMLS, 65(19), 2979–2999. https://doi.org/10.1007/s00018-008-8103-5

118. Ma, T., Jia, L., Zhao, J., Lv, L., Yu, Y., Ruan, H., Song, X., Chen, H., Li, X., Zhang, J., & Gao, L. (2022). Ginkgolide C slows the progression of osteoarthritis by activating Nrf2/HO-1 and blocking the NF-κB pathway. Frontiers in pharmacology, 13, 1027553. https://doi.org/10.3389/fphar.2022.1027553

119. Habtemariam S. (2023). Anti-Inflammatory Therapeutic Mechanisms of Natural Products: Insight from Rosemary Diterpenes, Carnosic Acid and Carnosol. Biomedicines, 11(2), 545. https://doi.org/10.3390/biomedicines11020545

120. Villaseñor, I. M., & Sanchez, A. C. (2009). Cassiaindoline, a new analgesic and anti-inflammatory alkaloid from Cassia alata. Zeitschrift fur Naturforschung. C, Journal of biosciences, 64(5-6), 335–338. https://doi.org/10.1515/znc-2009-5-605

121. Ma, B., Wu, C. F., Yang, J. Y., Wang, R., Kano, Y., & Yuan, D. (2009). Three new alkaloids from the leaves of Uncaria rhynchophylla. Helvetica Chimica Acta, 92(8), 1575-1585. https://doi.org/10.1002/hlca.200900021

122. Feng, T., Li, Y., Cai, X. H., Gong, X., Liu, Y. P., Zhang, R. T., Zhang, X. Y., Tan, Q. G., & Luo, X. D. (2009). Monoterpenoid indole alkaloids from Alstonia yunnanensis. Journal of natural products, 72(10), 1836–1841. https://doi.org/10.1021/np900374s

123. Mohan, S., Hobani, Y.H., Shaheen, E., Abou-Elhamd, A.S., Abdelhaleem, A., Alhazmi, H.A., & Abdelwahab, S.I. (2020). Girinimbine from curry leaves promote gastro protection against ethanol induced peptic ulcers and improve healing via regulation of anti-inflammatory an antioxidant mechanism. Food & Function, 11, 3493-3505 http://dx.doi.org/10.1039/D0FO00053A

124. Sadraie, S., Kiasalari, Z., Razavian, M., Azimi, S., Sedighnejad, L., Afshin-Majd, S., Baluchnejadmojarad, T., & Roghani, M. (2019). Berberine ameliorates lipopolysaccharide-induced learning and memory deficit in the rat: insights into underlying molecular mechanisms. Metabolic brain disease, 34(1), 245–255. https://doi.org/10.1007/s11011-018-0349-5

125. Zhang, W., Zhang, Y., Chen, S., Zhang, H., Yuan, M., Xiao, L., Lu, Y., & Xu, H. (2021). Trigonelline, An Alkaloid From Leonurus japonicus Houtt., Suppresses Mast Cell Activation and OVA-Induced Allergic Asthma. Frontiers in pharmacology, 12, 687970. https://doi.org/10.3389/fphar.2021.687970

126. Mukhtar, M., Arshad, M., Ahmad, M., Pomerantz, R. J., Wigdahl, B., & Parveen, Z. (2008). Antiviral potentials of medicinal plants. Virus research, 131(2), 111–120. https://doi.org/10.1016/j.virusres.2007.09.008

127. Ogbole, O. O., Akinleye, T. E., Segun, P. A., Faleye, T. C., & Adeniji, A. J. (2018). In vitro antiviral activity of twenty-seven medicinal plant extracts from Southwest Nigeria against three serotypes of echoviruses. Virology journal, 15(1), 110. https://doi.org/10.1186/s12985-018-1022-7

128. Efferth, T., Romero, M. R., Wolf, D. G., Stamminger, T., Marin, J. J., & Marschall, M. (2008). The antiviral activities of artemisinin and artesunate. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America, 47(6), 804–811. https://doi.org/10.1086/591195

129. Pompei, R., Laconi, S., & Ingianni, A. (2009). Antiviral properties of glycyrrhizic acid and its semisynthetic derivatives. Mini reviews in medicinal chemistry, 9(8), 996–1001. https://doi.org/10.2174/138955709788681636
130. Polyak, S. J., Oberlies, N. H., Pécheur, E. I., Dahari, H., Ferenci, P., & Pawlotsky, J. M. (2013). Silymarin for HCV infection. Antiviral therapy, 18(2), 141–147. https://doi.org/10.3851/IMP2402
131. Kang, E. H., Kown, T. Y., Oh, G. T., Park, W. F., Park, S. I., Park, S. K., & Lee, Y. I. (2006). The flavonoid ellagic acid from a medicinal herb inhibits host immune tolerance induced by the hepatitis B virus-e antigen. Antiviral research, 72(2), 100–106. https://doi.org/10.1016/j.antiviral.2006.04.006
132. Hussain, W., Haleem, K. S., Khan, I., Tauseef, I., Qayyum, S., Ahmed, B., & Riaz, M. N. (2017). Medicinal Plants: A Repository of Antiviral Metabolites. Future Virology, 12(6), 299–308. https://doi.org/10.2217/fvl-2016-0110
133. Chiang, L. C., Chiang, W., Chang, M. Y., Ng, L. T., & Lin, C. C. (2002). Antiviral activity of Plantago major extracts and related compounds in vitro. Antiviral research, 55(1), 53–62. https://doi.org/10.1016/s0166-3542(02)00007-4
134. Weng, J. R., Lin, C. S., Lai, H. C., Lin, Y. P., Wang, C. Y., Tsai, Y. C., Wu, K. C., Huang, S. H., & Lin, C. W. (2019). Antiviral activity of Sambucus FormosanaNakai ethanol extract and related phenolic acid constituents against human coronavirus NL63. Virus research, 273, 197767. https://doi.org/10.1016/j.virusres.2019.197767
135. Ojha, D., Das, R., Sobia, P., Dwivedi, V., Ghosh, S., Samanta, A., & Chattopadhyay, D. (2015). Pedilanthus tithymaloides Inhibits HSV Infection by Modulating NF-κB Signaling. PloS one, 10(9), e0139338. https://doi.org/10.1371/journal.pone.0139338
136. Hattori, T., Ikematsu, S., Koito, A., Matsushita, S., Maeda, Y., Hada, M., Fujimaki, M., & Takatsuki, K. (1989). Preliminary evidence for inhibitory effect of glycyrrhizin on HIV replication in patients with AIDS. Antiviral research, 11(5-6), 255–261. https://doi.org/10.1016/0166-3542(89)90035-1
137. Chen, X. X., Zhou, H. X., Qi, W. B., Ning, Z. Y., Ma, Y. J., Li, Y. L., Wang, G. C., & Chen, J. X. (2015). Yao xue xue bao = Acta pharmaceutica Sinica, 50(8), 966–972.
138. Zhang, W. J., Chen, B. T., Zhu, Q. H., Chen, G. B., Wei, L. B., & Xiong, B. (2005). In vitro antirotaviral effects of quercetin and 2α-hydroxyursolic acid extracted from Psidium guajava leaves. Chin. Tradit. Herb. Drugs, 36, 76-79.
139. Tohmé, M. J., Giménez, M. C., Peralta, A., Colombo, M. I., & Delgui, L. R. (2019). Ursolic acid: A novel antiviral compound inhibiting rotavirus infection in vitro. International journal of antimicrobial agents, 54(5), 601–609. https://doi.org/10.1016/j.ijantimicag.2019.07.015
140. Luganini, A., Mercorelli, B., Messa, L., Palù, G., Gribaudo, G., & Loregian, A. (2019). The isoquinoline alkaloid berberine inhibits human cytomegalovirus replication by interfering with the viral Immediate Early-2 (IE2) protein transactivating activity. Antiviral research, 164, 52–60. https://doi.org/10.1016/j.antiviral.2019.02.006
141. Song, S., Qiu, M., Chu, Y., Chen, D., Wang, X., Su, A., & Wu, Z. (2014). Downregulation of cellular c-Jun N-terminal protein kinase and NF-κB activation by berberine may result in inhibition of herpes simplex virus replication. Antimicrobial agents and chemotherapy, 58(9), 5068–5078. https://doi.org/10.1128/AAC.02427-14
142. Troost, B., Mulder, L. M., Diosa-Toro, M., van de Pol, D., Rodenhuis-Zybert, I. A., & Smit, J. M. (2020). Tomatidine, a natural steroidal alkaloid shows antiviral activity towards

chikungunya virus in vitro. Scientific reports, 10(1), 6364. https://doi.org/10.1038/s41598-020-63397-7
143. Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. Cell, 144(5), 646–674. https://doi.org/10.1016/j.cell.2011.02.013
144. Newman, D. J., & Cragg, G. M. (2016). Natural Products as Sources of New Drugs from 1981 to 2014. Journal of natural products, 79(3), 629–661. https://doi.org/10.1021/acs.jnatprod.5b01055
145. Yoshihara, H., Yoshimoto, Y., Hosoya, Y., Hasegawa, D., Kawano, T., Sakoda, A., Okita, H., & Manabe, A. (2017). Infantile fibrosarcoma treated with postoperative vincristine and dactinomycin. Pediatrics international: official journal of the Japan Pediatric Society, 59(3), 371–374. https://doi.org/10.1111/ped.13229
146. Kantarjian, H. M., O'Brien, S., & Cortes, J. (2013). Homoharringtonine/omacetaxine mepesuccinate: the long and winding road to food and drug administration approval. Clinical lymphoma, myeloma & leukemia, 13(5), 530–533. https://doi.org/10.1016/j.clml.2013.03.017
147. Damlaj, M., Lipton, J. H., & Assouline, S. E. (2016). A safety evaluation of omacetaxine mepesuccinate for the treatment of chronic myeloid leukemia. Expert opinion on drug safety, 15(9), 1279–1286. https://doi.org/10.1080/14740338.2016.1207760
148. Allan, E. K., Holyoake, T. L., Craig, A. R., & Jørgensen, H. G. (2011). Omacetaxine may have a role in chronic myeloid leukaemia eradication through downregulation of Mcl-1 and induction of apoptosis in stem/progenitor cells. Leukemia, 25(6), 985–994. https://doi.org/10.1038/leu.2011.55
149. Lam, S. S., Ho, E. S., He, B. L., Wong, W. W., Cher, C. Y., Ng, N. K., Man, C. H., Gill, H., Cheung, A. M., Ip, H. W., So, C. C., Tamburini, J., So, C. W., Ho, D. N., Au, C. H., Chan, T. L., Ma, E. S., Liang, R., Kwong, Y. L., & Leung, A. Y. (2016). Homoharringtonine (omacetaxine mepesuccinate) as an adjunct for FLT3-ITD acute myeloid leukemia. Science translational medicine, 8(359), 359ra129. https://doi.org/10.1126/scitranslmed.aaf3735
150. Weaver B. A. (2014). How Taxol/paclitaxel kills cancer cells. Molecular biology of the cell, 25(18), 2677–2681. https://doi.org/10.1091/mbc.E14-04-0916
151. Ramsay, J. R., Suhrbier, A., Aylward, J. H., Ogbourne, S., Cozzi, S. J., Poulsen, M. G., Baumann, K. C., Welburn, P., Redlich, G. L., & Parsons, P. G. (2011). The sap from Euphorbia peplus is effective against human nonmelanoma skin cancers. The British journal of dermatology, 164(3), 633–636. https://doi.org/10.1111/j.1365-2133.2010.10184.x
152. Serova, M., Ghoul, A., Benhadji, K. A., Faivre, S., Le Tourneau, C., Cvitkovic, E., Lokiec, F., Lord, J., Ogbourne, S. M., Calvo, F., & Raymond, E. (2008). Effects of protein kinase C modulation by PEP005, a novel ingenol angelate, on mitogen-activated protein kinase and phosphatidylinositol 3-kinase signaling in cancer cells. Molecular cancer therapeutics, 7(4), 915–922. https://doi.org/10.1158/1535-7163.MCT-07-2060
153. Rosen, R. H., Gupta, A. K., & Tyring, S. K. (2012). Dual mechanism of action of ingenol mebutate gel for topical treatment of actinic keratoses: rapid lesion necrosis followed by lesion-specific immune response. Journal of the American Academy of Dermatology, 66(3), 486–493. https://doi.org/10.1016/j.jaad.2010.12.038
154. Seca, A. M. L., & Pinto, D. C. G. A. (2018). Plant Secondary Metabolites as Anticancer Agents: Successes in Clinical Trials and Therapeutic Application. International journal of molecular sciences, 19(1), 263. https://doi.org/10.3390/ijms19010263
155. Okino Delgado, C. H., & Fleuri, L. F. (2015). Orange and mango by-products: Agro-industrial waste as source of bioactive compounds and botanical versus commercial

description—A review. Food Reviews International, 32(1), 1–14. https://doi.org/10.1080/87559129.2015.1041183
156. European Commission. (2015). Closing the loop—An EU action plan for the circular economy. Brussels, Belgium: European Commission.
157. Abraha, B., Mahmud, A., Tessema, H. A., Shui, X., Fang, Y., Tsighe, N. K., & Mohammed, J. (2017). 62 Food Additives: Functions, Effects, Regulations, Approval and Safety Evaluation.
158. Kuyu, C. (2015). Review on potential use of fruit and vegetable by-products as a valuable source of natural food additives. Food Science and Quality Management, 45, 47–61.
159. Silva, S., Costa, E. M., Mendes, M., Morais, R. M., Calhau, C., & Pintado, M. M. (2016). Antimicrobial, antiadhesive and antibiofilm activity of an ethanolic, anthocyanin-rich blueberry extract purified by solid phase extraction. Journal of applied microbiology, 121(3), 693–703. https://doi.org/10.1111/jam.13215
160. Ross, K. A., Ehret, D., Godfrey, D., Fukumoto, L., & Diarra, M. (2017). Characterization of Pilot Scale Processed Canadian Organic Cranberry (Vaccinium macrocarpon) and Blueberry (Vaccinium angustifolium) Juice Pressing Residues and Phenolic-Enriched Extractives. International Journal of Fruit Science, 17(2), 202–232. https://doi.org/10.1080/15538362.2017.1285264
161. Çam, M., Içyer, N. C., & Erdoğan, F. (2014). Pomegranate peel phenolics: Microencapsulation, storage stability, and potential ingredient for functional food development. LWT - Food Science and Technology, 55(1), 117–123. https://doi.org/10.1016/j.lwt.2013.09.011
162. Kanatt, S. R., Chander, R., & Sharma, A. (2010). Antioxidant and antimicrobial activity of pomegranate peel extract improves the shelf life of chicken products. International Journal of Food Science & Technology, 45(2), 216–222. https://doi.org/10.1111/j.1365-2621.2009.02124.x
163. Jiang, J., & Xiong, Y. L. (2016). Natural antioxidants as food and feed additives to promote health benefits and quality of meat products: A review. Meat science, 120, 107–117. https://doi.org/10.1016/j.meatsci.2016.04.005
164. Larrosa, M., Llorach, R., Espín, J. C., & Tomás-Barberán, F. A. (2002). Increase of antioxidant activity of tomato juice upon functionalisation with vegetable byproduct extracts. LWT - Food Science and Technology, 35(6), 532–542. https://doi.org/10.1006/fstl.2002.0907
165. Sadilova, E., Stintzing, F. C., & Carle, R. (2006). Anthocyanins, colour and antioxidant properties of eggplant (Solanum melongena L.) and violet pepper (Capsicum annuum L.) peel extracts. Zeitschrift fur Naturforschung. C, Journal of biosciences, 61(7-8), 527–535. https://doi.org/10.1515/znc-2006-7-810
166. Caleja, C., Barros, L., Antonio, A. L., Oliveira, M. B., & Ferreira, I. C. (2017). A comparative study between natural and synthetic antioxidants: Evaluation of their performance after incorporation into biscuits. Food chemistry, 216, 342–346. https://doi.org/10.1016/j.foodchem.2016.08.075
167. Caleja, C., Barros, L., Antonio, A. L., Ciric, A., Barreira, J. C., Sokovic, M., Oliveira, M. B. P., Santos-Buelga, C., & Ferreira, I. C. (2015). Development of a functional dairy food: Exploring bioactive and preservation effects of chamomile (Matricaria recutita L.). Journal of Functional Foods, 16, 114–124. https://doi.org/10.1016/j.jff.2015.04.033
168. Baines, D., & Seal, R. (2012). Natural food additives, ingredients, and flavourings. Elsevier.

169. Sigurdson, G. T., Tang, P., & Giusti, M. M. (2017). Natural Colorants: Food Colorants from Natural Sources. Annual review of food science and technology, 8, 261–280. https://doi.org/10.1146/annurev-food-030216-025923
170. Bridle, P., & Timberlake, C.F. (1997). Anthocyanins as natural food colours—selected aspects. Food Chemistry, 58, 103-109.
171. Rizk, E. M., El-Kady, A. T., & El-Bialy, A. R. (2014). Characterization of carotenoids (Lyco-Red) extracted from tomato peels and its uses as natural colorants and antioxidants of ice cream. Annals of Agricultural Science, 59(1), 53–61. https://doi.org/10.1016/j.aoas.2014.06.008
172. Someya, S., Yoshiki, Y., & Okubo, K. (2002). Antioxidant compounds from bananas (Musa cavendish). Food Chemistry, 79(3), 351–354. https://doi.org/10.1016/S0308-8146(02)00186-3
173. Kanazawa, K., & Sakakibara, H. (2000). High content of dopamine, a strong antioxidant, in Cavendish banana. Journal of agricultural and food chemistry, 48(3), 844–848. https://doi.org/10.1021/jf9909860
174. González-Minero, F. J., & Bravo-Díaz, L. (2018). The Use of Plants in Skin-Care Products, Cosmetics and Fragrances: Past and Present. Cosmetics, 5(3), 50. https://doi.org/10.3390/cosmetics5030050
175. Shai, A., Baran, R., & Maibach, H. I. (Eds.). (2009). Cosmetics and cosmetic preparations: Basic definitions (1st ed.). Informa UK Ltd.
176. Alves, A., Sousa, E., Kijjoa, A., & Pinto, M. (2020). Marine-Derived Compounds with Potential Use as Cosmeceuticals and Nutricosmetics. Molecules (Basel, Switzerland), 25(11), 2536. https://doi.org/10.3390/molecules25112536
177. Im, A. R., Kim, Y. M., Chin, Y. W., & Chae, S. (2017). Protective effects of compounds from Garcinia mangostana L. (mangosteen) against UVB damage in HaCaT cells and hairless mice. International journal of molecular medicine, 40(6), 1941–1949. https://doi.org/10.3892/ijmm.2017.3188
178. Tan, H., Sonam, T., & Shimizu, K. (2017). The Potential of Triterpenoids from Loquat Leaves (Eriobotrya japonica) for Prevention and Treatment of Skin Disorder. International journal of molecular sciences, 18(5), 1030. https://doi.org/10.3390/ijms18051030
179. Kim, J. E., Jang, S. G., Lee, C. H., Lee, J. Y., Park, H., Kim, J. H., Lee, S., Kim, S. H., Park, E. Y., Lee, K. W., & Shin, H. S. (2019). Beneficial effects on skin health using polysaccharides from red ginseng by-product. Journal of food biochemistry, 43(8), e12961. https://doi.org/10.1111/jfbc.12961
180. Caverzan, J., Mussi, L., Sufi, B., Padovani, G., Nazato, L., Camargo, F. B., Junior, Magalhães, W. V., & Di Stasi, L. C. (2021). A new phytocosmetic preparation from Thymus vulgaris stimulates adipogenesis and controls skin aging process: In vitro studies and topical effects in a double-blind placebo-controlled clinical trial. Journal of cosmetic dermatology, 20(7), 2190–2202. https://doi.org/10.1111/jocd.13818
181. Choi, M., Oh, J. H., Shin, M. K., Lee, S. R., Lee, D. H., Jin, S. P., Cho, S., & Chung, J. H. (2015). Beneficial effects of blood group antigen synthesis-increasing natural plant extracts and monosaccharides on extracellular matrix protein production in vivo. Journal of dermatological science, 80(2), 152–155. https://doi.org/10.1016/j.jdermsci.2015.08.001
182. Uchiyama, T., Tsunenaga, M., Miyanaga, M., Ueda, O., & Ogo, M. (2019). Oral intake of lingonberry and amla fruit extract improves skin conditions in healthy female subjects: A randomized, double-blind, placebo-controlled clinical trial. Biotechnology and applied biochemistry, 66(5), 870–879. https://doi.org/10.1002/bab.1800

183. Boisnic, S., Keophiphath, M., Serandour, A. L., Branchet, M. C., Le Breton, S., Lamour, I., & Gaillard, E. (2019). Polar lipids from wheat extract oil improve skin damages induced by aging: Evidence from a randomized, placebo-controlled clinical trial in women and an ex vivo study on human skin explant. Journal of cosmetic dermatology, 18(6), 2027–2036. https://doi.org/10.1111/jocd.12967
184. Kowalska K. (2021). Lingonberry (Vaccinium vitis-idaea L.) Fruit as a Source of Bioactive Compounds with Health-Promoting Effects-A Review. International journal of molecular sciences, 22(10), 5126. https://doi.org/10.3390/ijms22105126
185. Markiewicz, A., Zasada, M., Erkiert-Polguj, A., Wieckowska-Szakiel, M., & Budzisz, E. (2019). An evaluation of the antiaging properties of strawberry hydrolysate treatment enriched with L-ascorbic acid applied with microneedle mesotherapy. Journal of cosmetic dermatology, 18(1), 129–135. https://doi.org/10.1111/jocd.12545
186. Pillaiyar, T., Manickam, M., & Jung, S. H. (2017). Recent development of signaling pathways inhibitors of melanogenesis. Cellular signalling, 40, 99–115. https://doi.org/10.1016/j.cellsig.2017.09.004
187. Li, J., Lu, Y. R., Lin, I. F., Kang, W., Chen, H. B., Lu, H. F., & Wang, H. D. (2020). Reversing UVB-induced photoaging with Hibiscus sabdariffa calyx aqueous extract. Journal of the science of food and agriculture, 100(2), 672–681. https://doi.org/10.1002/jsfa.10063
188. Lourith, N., Kanlayavattanakul, M., Chaikul, P., Chansriniyom, C., & Bunwatcharaphansakun, P. (2017). In vitro and cellular activities of the selected fruits residues for skin aging treatment. Anais da Academia Brasileira de Ciencias, 89(1 Suppl 0), 577–589. https://doi.org/10.1590/0001-3765201720160849
189. Li, L., Chong, L., Huang, T., Ma, Y., Li, Y., & Ding, H. (2023). Natural products and extracts from plants as natural UV filters for sunscreens: A review. Animal models and experimental medicine, 6(3), 183–195. https://doi.org/10.1002/ame2.12295
190. Vijayakumar, R., Abd Gani, S. S., Zaidan, U. H., Halmi, M. I. E., Karunakaran, T., & Hamdan, M. R. (2020). Exploring the Potential Use of Hylocereus polyrhizus Peels as a Source of Cosmeceutical Sunscreen Agent for Its Antioxidant and Photoprotective Properties. Evidence-based complementary and alternative medicine: eCAM, 2020, 7520736. https://doi.org/10.1155/2020/7520736
191. Soto, M. L., Falqué, E., & Domínguez, H. (2015). Relevance of Natural Phenolics from Grape and Derivative Products in the Formulation of Cosmetics. Cosmetics, 2(3), 259-276. https://doi.org/10.3390/cosmetics2030259
192. Ferreira, S. M., Gomes, S. M., & Santos, L. (2023). A Novel Approach in Skin Care: By-Product Extracts as Natural UV Filters and an Alternative to Synthetic Ones. Molecules, 28(5), 2037. https://doi.org/10.3390/molecules28052037
193. Abelan, U. S., de Oliveira, A. C., Cacoci, É. S. P., Martins, T. E. A., Giacon, V. M., Velasco, M. V. R., & Lima, C. R. R. C. (2022). Potential use of essential oils in cosmetic and dermatological hair products: A review. Journal of cosmetic dermatology, 21(4), 1407–1418. https://doi.org/10.1111/jocd.14286
194. Mozaffarian D. (2016). Dietary and Policy Priorities for Cardiovascular Disease, Diabetes, and Obesity: A Comprehensive Review. Circulation, 133(2), 187–225. https://doi.org/10.1161/CIRCULATIONAHA.115.018585
195. Doré, J., & Blottière, H. (2015). The influence of diet on the gut microbiota and its consequences for health. Current opinion in biotechnology, 32, 195–199. https://doi.org/10.1016/j.copbio.2015.01.002

196. De Smet, E., Mensink, R. P., & Plat, J. (2012). Effects of plant sterols and stanols on intestinal cholesterol metabolism: suggested mechanisms from past to present. Molecular nutrition & food research, 56(7), 1058–1072. https://doi.org/10.1002/mnfr.201100722
197. Demonty, I., Ras, R. T., van der Knaap, H. C., Duchateau, G. S., Meijer, L., Zock, P. L., Geleijnse, J. M., & Trautwein, E. A. (2009). Continuous dose-response relationship of the LDL-cholesterol-lowering effect of phytosterol intake. The Journal of nutrition, 139(2), 271–284. https://doi.org/10.3945/jn.108.095125
198. Ras, R. T., Hiemstra, H., Lin, Y., Vermeer, M. A., Duchateau, G. S., & Trautwein, E. A. (2013). Consumption of plant sterol-enriched foods and effects on plasma plant sterol concentrations--a meta-analysis of randomized controlled studies. Atherosclerosis, 230(2), 336–346. https://doi.org/10.1016/j.atherosclerosis.2013.08.012
199. Talati, R., Sobieraj, D. M., Makanji, S. S., Phung, O. J., & Coleman, C. I. (2010). The comparative efficacy of plant sterols and stanols on serum lipids: a systematic review and meta-analysis. Journal of the American Dietetic Association, 110(5), 719–726. https://doi.org/10.1016/j.jada.2010.02.011
200. Ferguson, J. J., Stojanovski, E., MacDonald-Wicks, L., & Garg, M. L. (2016). Fat type in phytosterol products influence their cholesterol-lowering potential: A systematic review and meta-analysis of RCTs. Progress in lipid research, 64, 16–29. https://doi.org/10.1016/j.plipres.2016.08.002
201. Ried, K., & Fakler, P. (2014). Potential of garlic (Allium sativum) in lowering high blood pressure: mechanisms of action and clinical relevance. Integrated blood pressure control, 7, 71–82. https://doi.org/10.2147/IBPC.S51434
202. Butt, M. S., Sultan, M. T., Butt, M. S., & Iqbal, J. (2009). Garlic: nature's protection against physiological threats. Critical reviews in food science and nutrition, 49(6), 538–551. https://doi.org/10.1080/10408390802145344
203. Rohner, A., Ried, K., Sobenin, I. A., Bucher, H. C., & Nordmann, A. J. (2015). A systematic review and metaanalysis on the effects of garlic preparations on blood pressure in individuals with hypertension. American journal of hypertension, 28(3), 414–423. https://doi.org/10.1093/ajh/hpu165
204. Steiner, M., & Lin, R. S. (1998). Changes in platelet function and susceptibility of lipoproteins to oxidation associated with administration of aged garlic extract. Journal of cardiovascular pharmacology, 31(6), 904–908. https://doi.org/10.1097/00005344-199806000-00014
205. Tapiero, H., Townsend, D. M., & Tew, K. D. (2004). The role of carotenoids in the prevention of human pathologies. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie, 58(2), 100–110. https://doi.org/10.1016/j.biopha.2003.12.006
206. Senkus, K. E., Tan, L., & Crowe-White, K. M. (2019). Lycopene and Metabolic Syndrome: A Systematic Review of the Literature. Advances in nutrition (Bethesda, Md.), 10(1), 19–29. https://doi.org/10.1093/advances/nmy069
207. Minocha, T., Das, M., Rai, V., Verma, S. S., Awasthee, N., Gupta, S. C., Haldar, C., & Yadav, S. K. (2022). Melatonin induces apoptosis and cell cycle arrest in cervical cancer cells via inhibition of NF-κB pathway. Inflammopharmacology, 30(4), 1411–1429. https://doi.org/10.1007/s10787-022-00964-6
208. Alharbi, K. S., Almalki, W. H., Albratty, M., Meraya, A. M., Najmi, A., Vyas, G., Singh, S. K., Dua, K., & Gupta, G. (2022). The therapeutic role of nutraceuticals targeting the Nrf2/HO-1 signaling pathway in liver cancer. Journal of food biochemistry, 46(10), e14357. https://doi.org/10.1111/jfbc.14357

209. Senobari, Z., Karimi, G., & Jamialahmadi, K. (2022). Ellagitannins, promising pharmacological agents for the treatment of cancer stem cells. Phytotherapy research : PTR, 36(1), 231–242. https://doi.org/10.1002/ptr.7307
210. Zhang, Z., Sang, W., Xie, L., Li, W., Li, B., Li, J., Tian, H., Yuan, Z., Zhao, Q., & Dai, Y. (2021). Polyphenol-Based Nanomedicine Evokes Immune Activation for Combination Cancer Treatment. Angewandte Chemie (International ed. in English), 60(4), 1967–1975. https://doi.org/10.1002/anie.202013406
211. Shen, K. H., Hung, J. H., Chang, C. W., Weng, Y. T., Wu, M. J., & Chen, P. S. (2017). Solasodine inhibits invasion of human lung cancer cell through downregulation of miR-21 and MMPs expression. Chemico-biological interactions, 268, 129–135. https://doi.org/10.1016/j.cbi.2017.03.005
212. Jin, M., Shi, C., Li, T., Wu, Y., Hu, C., & Huang, G. (2020). Solasonine promotes ferroptosis of hepatoma carcinoma cells via glutathione peroxidase 4-induced destruction of the glutathione redox system. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie, 129, 110282. https://doi.org/10.1016/j.biopha.2020.110282
213. Barik, S. K., Russell, W. R., Moar, K. M., Cruickshank, M., Scobbie, L., Duncan, G., & Hoggard, N. (2020). The anthocyanins in black currants regulate postprandial hyperglycaemia primarily by inhibiting α-glucosidase while other phenolics modulate salivary α-amylase, glucose uptake and sugar transporters. The Journal of nutritional biochemistry, 78, 108325. https://doi.org/10.1016/j.jnutbio.2019.108325
214. Yang, H. J., Kim, H. J., Kim, M. J., Kang, S., Kim, D. S., Daily, J. W., Jeong, D. Y., Kwon, D. Y., & Park, S. (2013). Standardized chungkookjang, short-term fermented soybeans with Bacillus lichemiformis, improves glucose homeostasis as much as traditionally made chungkookjang in diabetic rats. Journal of clinical biochemistry and nutrition, 52(1), 49–57. https://doi.org/10.3164/jcbn.12-54
215. Cattivelli, A., Conte, A., Martini, S., & Tagliazucchi, D. (2022). Cooking and In Vitro Digestion Modulate the Anti-Diabetic Properties of Red-Skinned Onion and Dark Purple Eggplant Phenolic Compounds. Foods (Basel, Switzerland), 11(5), 689. https://doi.org/10.3390/foods11050689
216. Lamberth, C., Jeanmart, S., Luksch, T., & Plant, A. (2013). Current challenges and trends in the discovery of agrochemicals. Science (New York, N.Y.), 341(6147), 742–746. https://doi.org/10.1126/science.1237227
217. Sparks, T. C., & Lorsbach, B. A. (2017). Perspectives on the agrochemical industry and agrochemical discovery. Pest management science, 73(4), 672–677. https://doi.org/10.1002/ps.4457
218. Plant, A. (2010). Crop protection chemistry: Challenges and opportunities in the 21st century. Agrow Silver Jubilee Issue, XI–XV.
219. Cantrell, C. L., Dayan, F. E., & Duke, S. O. (2012). Natural products as sources for new pesticides. Journal of natural products, 75(6), 1231–1242. https://doi.org/10.1021/np300024u
220. Sparks, T. C., Hahn, D. R., & Garizi, N. V. (2017). Natural products, their derivatives, mimics and synthetic equivalents: role in agrochemical discovery. Pest management science, 73(4), 700–715. https://doi.org/10.1002/ps.4458
221. Nauen, R., Jeschke, P., Velten, R., Beck, M. E., Ebbinghaus-Kintscher, U., Thielert, W., Wölfel, K., Haas, M., Kunz, K., & Raupach, G. (2015). Flupyradifurone: a brief profile of a new butenolide insecticide. Pest management science, 71(6), 850–862. https://doi.org/10.1002/ps.3932

222. Lind, R.J., Greenhow, D.T., Blythe, J., Goodchild, J., Hirst, E.A., Dunbar, S.J., & Earley, F.G. (2002). Cyanotropanes: novel chemistry interacting at the insect nicotinic acetylcholine receptor.
223. Dayan, F. E., Cantrell, C. L., & Duke, S. O. (2009). Natural products in crop protection. Bioorganic & medicinal chemistry, 17(12), 4022–4034. https://doi.org/10.1016/j.bmc.2009.01.046
224. Ben Abada, M., Haouel Hamdi, S., Gharib, R., Messaoud, C., Fourmentin, S., Greige-Gerges, H., & Mediouni Ben Jemâa, J. (2019). Post-harvest management control of Ectomyelois ceratoniae (Zeller) (Lepidoptera: Pyralidae): New insights through essential oil encapsulation in cyclodextrin. Pest Management Science, 75(7), 1874–1884. https://doi.org/10.1002/ps.5315
225. Mathew, C., Tesfaye, W., Rasmussen, P., Peterson, G. M., Bartholomaeus, A., Sharma, M., & Thomas, J. (2020). Mānuka Oil-A Review of Antimicrobial and Other Medicinal Properties. Pharmaceuticals (Basel, Switzerland), 13(11), 343. https://doi.org/10.3390/ph13110343
226. Douglas, M. H., van Klink, J. W., Smallfield, B. M., Perry, N. B., Anderson, R. E., Johnstone, P., & Weavers, R. T. (2004). Essential oils from New Zealand manuka: triketone and other chemotypes of Leptospermum scoparium. Phytochemistry, 65(9), 1255–1264. https://doi.org/10.1016/j.phytochem.2004.03.019
227. Dayan, F. E., & Duke, S. O. (2020). Discovery of new herbicide sites of action by quantification of plant primary metabolite and enzyme pools. Engineering, 6(5), 509–514. https://doi.org/10.1016/j.eng.2020.03.004
228. Velasques, J., Cardoso, M. H., Abrantes, G., Frihling, B. E., Franco, O. L., & Migliolo, L. (2017). The rescue of botanical insecticides: A bioinspiration for new niches and needs. Pesticide Biochemistry and Physiology, 143, 14–25. https://doi.org/10.1016/j.pestbp.2017.10.003
229. Morgan E. D. (2009). Azadirachtin, a scientific gold mine. Bioorganic & medicinal chemistry, 17(12), 4096–4105. https://doi.org/10.1016/j.bmc.2008.11.081
230. Qiao, J., Zou, X., Lai, D., Yan, Y., Wang, Q., Li, W., Deng, S., Xu, H., & Gu, H. (2014). Azadirachtin blocks the calcium channel and modulates the cholinergic miniature synaptic current in the central nervous system of Drosophila. Pest management science, 70(7), 1041–1047. https://doi.org/10.1002/ps.3644
231. Jeanguenat A. (2013). The story of a new insecticidal chemistry class: the diamides. Pest management science, 69(1), 7–14. https://doi.org/10.1002/ps.3406
232. Selby, T. P., Lahm, G. P., & Stevenson, T. M. (2017). A retrospective look at anthranilic diamide insecticides: discovery and lead optimization to chlorantraniliprole and cyantraniliprole. Pest management science, 73(4), 658–665. https://doi.org/10.1002/ps.4308
233. Sattelle, D. B., Cordova, D., & Cheek, T. R. (2008). Insect ryanodine receptors: molecular targets for novel pest control chemicals. Invertebrate neuroscience: IN, 8(3), 107–119. https://doi.org/10.1007/s10158-008-0076-4
234. Divekar, P. A., Narayana, S., Divekar, B. A., Kumar, R., Gadratagi, B. G., Ray, A., Singh, A. K., Rani, V., Singh, V., Singh, A. K., Kumar, A., Singh, R. P., Meena, R. S., & Behera, T. K. (2022). Plant Secondary Metabolites as Defense Tools against Herbivores for Sustainable Crop Protection. International journal of molecular sciences, 23(5), 2690. https://doi.org/10.3390/ijms23052690
235. Danova, K., & Pistelli, L. (2022). Plant Tissue Culture and Secondary Metabolites Production. Plants (Basel, Switzerland), 11(23), 3312. https://doi.org/10.3390/plants11233312

236. Mohaddab, M., El Goumi, Y., Gallo, M., Montesano, D., Zengin, G., Bouyahya, A., & Fakiri, M. (2022). Biotechnology and In Vitro Culture as an Alternative System for Secondary Metabolite Production. Molecules (Basel, Switzerland), 27(22), 8093. https://doi.org/10.3390/molecules27228093
237. Abdulhafiz, F., Mohammed, A., Reduan, M. F. H., Abdul Kari, Z., Wei, L. S., & Goh, K. W. (2022). Plant cell culture technologies: A promising alternative to produce high-value secondary metabolites. Arabian Journal of Chemistry, 15(11), 104161. https://doi.org/10.1016/j.arabjc.2022.104161
238. Efferth, T. (2019). Biotechnology applications of plant callus cultures. Engineering, 5(1), 50–59. https://doi.org/10.1016/j.eng.2018.11.006
239. Dias, M. I., Sousa, M. J., Alves, R. C., & Ferreira, I. C. F. R. (2016). Exploring plant tissue culture to improve the production of phenolic compounds: A review. Industrial Crops and Products, 82, 9–22. https://doi.org/10.1016/j.indcrop.2015.12.016
240. Chaturvedi, H. C., Jain, M., & Kidwai, N. R. (2007). Cloning of medicinal plants through tissue culture--a review. Indian journal of experimental biology, 45(11), 937–948.
241. Smetanska I. (2008). Production of secondary metabolites using plant cell cultures. Advances in biochemical engineering/biotechnology, 111, 187–228. https://doi.org/10.1007/10_2008_103
242. Alamgir A. N. M. (2018). Biotechnology, In Vitro Production of Natural Bioactive Compounds, Herbal Preparation, and Disease Management (Treatment and Prevention). Therapeutic Use of Medicinal Plants and their Extracts: Volume 2: Phytochemistry and Bioactive Compounds , 74, 585–664. https://doi.org/10.1007/978-3-319-92387-1_7
243. Hasnain, A., Naqvi, S. A. H., Ayesha, S. I., Khalid, F., Ellahi, M., Iqbal, S., Hassan, M. Z., Abbas, A., Adamski, R., Markowska, D., Baazeem, A., Mustafa, G., Moustafa, M., Hasan, M. E., & Abdelhamid, M. M. A. (2022). Plants in vitro propagation with its applications in food, pharmaceuticals and cosmetic industries; current scenario and future approaches. Frontiers in plant science, 13, 1009395. https://doi.org/10.3389/fpls.2022.1009395
244. Fehér A. (2023). A Common Molecular Signature Indicates the Pre-Meristematic State of Plant Calli. International journal of molecular sciences, 24(17), 13122. https://doi.org/10.3390/ijms241713122
245. Khafagi, I. (2007). Variation of callus induction and active metabolite accumulation in callus cultures of two varieties of Ricinus communis L. Biotechnology, 6, 193–201. https://doi.org/10.3923/biotech.2007.193.201
246. Ozyigit, I. I., Dogan, I., Hocaoglu-Ozyigit, A., Yalcin, B., Erdogan, A., Yalcin, I. E., Cabi, E., & Kaya, Y. (2023). Production of secondary metabolites using tissue culture-based biotechnological applications. Frontiers in plant science, 14, 1132555. https://doi.org/10.3389/fpls.2023.1132555
247. Galáz-Ávalos, R. M., Aguilar-Díaz, S., Xool-González, P. A., Huchín-May, S. M., & Loyola-Vargas, V. M. (2012). Callus, suspension culture, and hairy roots. Induction, maintenance and characterization. Methods in molecular biology (Clifton, N.J.), 877, 29–40. https://doi.org/10.1007/978-1-61779-818-4_3
248. Zhang, G., Liu, P., Zhang, G., Yao, X., Wang, X., Zhang, Y., Lin, J., Cui, Y., & Li, X. (2024). Cell wall remodeling promotes callus formation in poplar. Molecular horticulture, 4(1), 16. https://doi.org/10.1186/s43897-024-00093-4
249. Shih, S. M., & Doran, P. M. (2009). In vitro propagation of plant virus using different forms of plant tissue culture and modes of culture operation. Journal of biotechnology, 143(3), 198–206. https://doi.org/10.1016/j.jbiotec.2009.07.007

250. Lee, S., Park, Y. S., Rhee, J. H., Chu, H., Frost, J. M., & Choi, Y. (2024). Insights into plant regeneration: cellular pathways and DNA methylation dynamics. Plant cell reports, 43(5), 120. https://doi.org/10.1007/s00299-024-03216-9
251. Hussain, M. S., Fareed, S., Ansari, S., Rahman, M. A., Ahmad, I. Z., & Saeed, M. (2012). Current approaches toward production of secondary plant metabolites. Journal of pharmacy & bioallied sciences, 4(1), 10–20. https://doi.org/10.4103/0975-7406.92725
252. Rao, S. R., & Ravishankar, G. A. (2002). Plant cell cultures: Chemical factories of secondary metabolites. Biotechnology advances, 20(2), 101–153. https://doi.org/10.1016/s0734-9750(02)00007-1
253. Ochoa-Villarreal, M., Howat, S., Hong, S., Jang, M. O., Jin, Y. W., Lee, E. K., & Loake, G. J. (2016). Plant cell culture strategies for the production of natural products. BMB reports, 49(3), 149–158. https://doi.org/10.5483/bmbrep.2016.49.3.264
254. Murthy, H. N., Lee, E. J., & Paek, K. Y. (2014). Production of secondary metabolites from cell and organ cultures: Strategies and approaches for biomass improvement and metabolite accumulation. Plant Cell, Tissue and Organ Culture, 118(1), 1–16. https://doi.org/10.1007/s11240-014-0467-7
255. Ramawat, K. G. (2021). An introduction to the process of cell, tissue, and organ differentiation, and production of secondary metabolites. In K. G. Ramawat, H. M. Ekiert, & S. Goyal (Eds.), Plant cell and tissue differentiation and secondary metabolites (pp. 515–529). Reference Series in Phytochemistry. Springer, Cham. https://doi.org/10.1007/978-3-030-30185-9_35
256. Chandran, H., Meena, M., Barupal, T., & Sharma, K. (2020). Plant tissue culture as a perpetual source for production of industrially important bioactive compounds. Biotechnology reports (Amsterdam, Netherlands), 26, e00450. https://doi.org/10.1016/j.btre.2020.e00450
257. Al-Zughbi, I., & Krayem, M. (2022). Quince fruit Cydonia oblonga Mill nutritional composition, antioxidative properties, health benefits and consumers preferences towards some industrial quince products: A review. Food chemistry, 393, 133362. https://doi.org/10.1016/j.foodchem.2022.133362
258. Jafari-Dehkordi, E., Hashem-Dabaghian, F., Aliasl, F., Aliasl, J., Taghavi-Shirazi, M., Sadeghpour, O., Sohrabvand, F., Minaei, B., & Ghods, R. (2017). Comparison of quince with vitamin B6 for treatment of nausea and vomiting in pregnancy: a randomised clinical trial. Journal of obstetrics and gynaecology : the journal of the Institute of Obstetrics and Gynaecology, 37(8), 1048–1052. https://doi.org/10.1080/01443615.2017.1322046
259. Shakeri, A., Hashempur, M. H., Mojibian, M., Aliasl, F., Bioos, S., & Nejatbakhsh, F. (2018). A comparative study of ranitidine and quince (Cydonia oblonga mill) sauce on gastroesophageal reflux disease (GERD) in pregnancy: a randomised, open-label, active-controlled clinical trial. Journal of obstetrics and gynaecology : the journal of the Institute of Obstetrics and Gynaecology, 38(7), 899–905. https://doi.org/10.1080/01443615.2018.1431210
260. Essafi-Benkhadir, K., Refai, A., Riahi, I., Fattouch, S., Karoui, H., & Essafi, M. (2012). Quince (Cydonia oblonga Miller) peel polyphenols modulate LPS-induced inflammation in human THP-1-derived macrophages through NF-κB, p38MAPK and Akt inhibition. Biochemical and biophysical research communications, 418(1), 180–185. https://doi.org/10.1016/j.bbrc.2012.01.003
261. De Bellis, R., Chiarantini, L., Potenza, L., Gorassini, A., Verardo, G., De Marco, R., Benayada, L., Stocchi, V., Albertini, M. C., & Fraternale, D. (2022). High production of

secondary metabolites and biological activities of Cydonia oblonga Mill. pulp fruit callus. Journal of Functional Foods, 94, 105133. https://doi.org/10.1016/j.jff.2022.105133
262. Potenza, L., Minutelli, M., Stocchi, V., & Fraternale, D. (2020). Biological potential of an ethanolic extract from "Mela Rosa Marchigiana" pulp callus culture. Journal of Functional Foods, 75, 104269. https://doi.org/10.1016/j.jff.2020.104269
263. Tiboni, M., Elmowafy, E., El-Derany, M. O., Benedetti, S., Campana, R., Verboni, M., Potenza, L., Palma, F., Citterio, B., Sisti, M., Duranti, A., Lucarini, S., Soliman, M. E., & Casettari, L. (2022). A combination of sugar esters and chitosan to promote in vivo wound care. International journal of pharmaceutics, 616, 121508. https://doi.org/10.1016/j.ijpharm.2022.121508
264. Bryan, N. S., & Grisham, M. B. (2007). Methods to detect nitric oxide and its metabolites in biological samples. Free radical biology & medicine, 43(5), 645–657. https://doi.org/10.1016/j.freeradbiomed.2007.04.026
265. Takuathung, M. N., Potikanond, S., Sookkhee, S., Mungkornasawakul, P., Jearanaikulvanich, T., Chinda, K., Wikan, N., & Nimlamool, W. (2021). Anti-psoriatic and anti-inflammatory effects of Kaempferia parviflora in keratinocytes and macrophage cells. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie, 143, 112229. https://doi.org/10.1016/j.biopha.2021.112229
266. Pfaffl M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic acids research, 29(9), e45. https://doi.org/10.1093/nar/29.9.e45
267. Sut, S., Dall'Acqua, S., Poloniato, G., Maggi, F., & Malagoli, M. (2019). Preliminary evaluation of quince (Cydonia oblonga Mill.) fruit as extraction source of antioxidant phytoconstituents for nutraceutical and functional food applications. Journal of the science of food and agriculture, 99(3), 1046–1054. https://doi.org/10.1002/jsfa.9271
268. Oota, S. (1983). Apple flesh tissue culture and anthocyanin formation in the derived callus tissue. Journal of the Japanese Society for Horticultural Science, 52(2), 117–122.
269. Verardo, G., Gorassini, A., Ricci, D., & Fraternale, D. (2017). High Triterpenic Acids Production in Callus Cultures from Fruit Pulp of Two Apple Varieties. Phytochemical analysis: PCA, 28(1), 5–15. https://doi.org/10.1002/pca.2638
270. Verardo, G., Gorassini, A., & Fraternale, D. (2019). New triterpenic acids produced in callus culture from fruit pulp of Acca sellowiana (O. Berg) Burret. Food research international (Ottawa, Ont.), 119, 596–604. https://doi.org/10.1016/j.foodres.2018.10.037
271. Silva, B. M., Andrade, P. B., Ferreres, F., Domingues, A. L., Seabra, R. M., & Ferreira, M. A. (2002). Phenolic profile of quince fruit (Cydonia oblonga Miller) (pulp and peel). Journal of agricultural and food chemistry, 50(16), 4615–4618. https://doi.org/10.1021/jf0203139
272. Zhang, L., Rocchetti, G., Zengin, G., Ak, G., Saber, F. R., Montesano, D., & Lucini, L. (2021). The UHPLC-QTOF-MS Phenolic Profiling and Activity of Cydonia oblonga Mill. Reveals a Promising Nutraceutical Potential. Foods (Basel, Switzerland), 10(6), 1230. https://doi.org/10.3390/foods10061230
273. Wojdyło, A., Oszmiański, J., & Bielicki, P. (2013). Polyphenolic composition, antioxidant activity, and polyphenol oxidase (PPO) activity of quince (Cydonia oblonga Miller) varieties. Journal of agricultural and food chemistry, 61(11), 2762–2772. https://doi.org/10.1021/jf304969b
274. Liang, N., & Kitts, D. D. (2015). Role of Chlorogenic Acids in Controlling Oxidative and Inflammatory Stress Conditions. Nutrients, 8(1), 16. https://doi.org/10.3390/nu8010016
275. Chen, H., Yang, J., Zhang, Q., Chen, L. H., & Wang, Q. (2012). Corosolic acid ameliorates atherosclerosis in apolipoprotein E-deficient mice by regulating the nuclear factor-κB

signaling pathway and inhibiting monocyte chemoattractant protein-1 expression. Circulation journal: official journal of the Japanese Circulation Society, 76(4), 995–1003. https://doi.org/10.1253/circj.cj-11-0344

276. Baba, K., Hiramatsu, R., Suradej, B., Tanigaki, R., Koeda, S., Waku, T., & Kataoka, T. (2018). Asiatic Acid, Corosolic Acid, and Maslinic Acid Interfere with Intracellular Trafficking and N-Linked Glycosylation of Intercellular Adhesion Molecule-1. Biological & pharmaceutical bulletin, 41(12), 1757–1768. https://doi.org/10.1248/bpb.b18-00276
277. Xu, J., Zhao, Y., & Aisa, H. A. (2017). Anti-inflammatory effect of pomegranate flower in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. Pharmaceutical biology, 55(1), 2095–2101. https://doi.org/10.1080/13880209.2017.1357737
278. Dzoyem, J. P., Donfack, A. R., Tane, P., McGaw, L. J., & Eloff, J. N. (2016). Inhibition of Nitric Oxide Production in LPS-Stimulated RAW 264.7 Macrophages and 15-LOX Activity by Anthraquinones from Pentas schimperi. Planta medica, 82(14), 1246–1251. https://doi.org/10.1055/s-0042-104417
279. Taciak, B., Białasek, M., Braniewska, A., Sas, Z., Sawicka, P., Kiraga, Ł., Rygiel, T., & Król, M. (2018). Evaluation of phenotypic and functional stability of RAW 264.7 cell line through serial passages. PloS one, 13(6), e0198943. https://doi.org/10.1371/journal.pone.0198943
280. Colombo, I., Sangiovanni, E., Maggio, R., Mattozzi, C., Zava, S., Corbett, Y., Fumagalli, M., Carlino, C., Corsetto, P. A., Scaccabarozzi, D., Calvieri, S., Gismondi, A., Taramelli, D., & Dell'Agli, M. (2017). HaCaT Cells as a Reliable In Vitro Differentiation Model to Dissect the Inflammatory/Repair Response of Human Keratinocytes. Mediators of inflammation, 2017, 7435621. https://doi.org/10.1155/2017/7435621
281. Nkuimi Wandjou, J. G., Sut, S., Giuliani, C., Fico, G., Papa, F., Ferraro, S., Caprioli, G., Maggi, F., & Dall'Acqua, S. (2019). Characterization of nutrients, polyphenols and volatile components of the ancient apple cultivar 'Mela Rosa Dei Monti Sibillini' from Marche region, central Italy. International journal of food sciences and nutrition, 70(7), 796–812. https://doi.org/10.1080/09637486.2019.1580684
282. Nkuimi Wandjou, J. G., Lancioni, L., Barbalace, M. C., Hrelia, S., Papa, F., Sagratini, G., Vittori, S., Dall'Acqua, S., Caprioli, G., Beghelli, D., Angeloni, C., Lupidi, G., & Maggi, F. (2020). Comprehensive characterization of phytochemicals and biological activities of the Italian ancient apple 'Mela Rosa dei Monti Sibillini'. Food research international (Ottawa, Ont.), 137, 109422. https://doi.org/10.1016/j.foodres.2020.109422
283. Nkuimi Wandjou, J. G., Mevi, S., Sagratini, G., Vittori, S., Dall'Acqua, S., Caprioli, G., Lupidi, G., Mombelli, G., Arpini, S., Allegrini, P., Les, F., López, V., & Maggi, F. (2020). Antioxidant and Enzyme Inhibitory Properties of the Polyphenolic-Rich Extract from an Ancient Apple Variety of Central Italy (Mela Rosa dei Monti Sibillini). Plants, 9(1), 9. https://doi.org/10.3390/plants9010009
284. López, V., Les, F., Mevi, S., Nkuimi Wandjou, J. G., Cásedas, G., Caprioli, G., & Maggi, F. (2020). Phytochemicals and Enzyme Inhibitory Capacities of the Methanolic Extracts from the Italian Apple Cultivar Mela Rosa dei Monti Sibillini. Pharmaceuticals (Basel, Switzerland), 13(6), 127. https://doi.org/10.3390/ph13060127
285. Yousefi-Manesh, H., Dehpour, A. R., Nabavi, S. M., Khayatkashani, M., Asgardoon, M. H., Derakhshan, M. H., Moradi, S. A., Sheibani, M., Tavangar, S. M., Shirooie, S., Nkuimi Wandjou, J. G., Caprioli, G., Sut, S., Dall'Acqua, S., & Maggi, F. (2021). Therapeutic Effects of Hydroalcoholic Extracts from the Ancient Apple Mela Rosa dei Monti Sibillini in Transient Global Ischemia in Rats. Pharmaceuticals, 14(11), 1106. https://doi.org/10.3390/ph14111106

286. Yousefi-Manesh, H., Dehpour, A. R., Ansari-Nasab, S., Hemmati, S., Sadeghi, M. A., Shahraki, R. H., Shirooie, S., Nabavi, S. M., Nkuimi Wandjou, J. G., Sut, S., Caprioli, G., Dall'Acqua, S., & Maggi, F. (2020). Hepatoprotective Effects of Standardized Extracts from an Ancient Italian Apple Variety (Mela Rosa dei Monti Sibillini) against Carbon Tetrachloride (CCl4)-Induced Hepatotoxicity in Rats. Molecules, 25(8), 1816. https://doi.org/10.3390/molecules25081816
287. Saltarelli, R., Palma, F., Gioacchini, A. M., Calcabrini, C., Mancini, U., De Bellis, R., Stocchi, V., & Potenza, L. (2019). Phytochemical composition, antioxidant and antiproliferative activities and effects on nuclear DNA of ethanolic extract from an Italian mycelial isolate of Ganoderma lucidum. Journal of ethnopharmacology, 231, 464–473. https://doi.org/10.1016/j.jep.2018.11.041
288. Loizzo, M. R., Sicari, V., Pellicanò, T., Xiao, J., Poiana, M., & Tundis, R. (2019). Comparative analysis of chemical composition, antioxidant and anti-proliferative activities of Italian Vitis vinifera by-products for a sustainable agro-industry. Food and chemical toxicology: an international journal published for the British Industrial Biological Research Association, 127, 127–134. https://doi.org/10.1016/j.fct.2019.03.007
289. De Bellis, R., Piacentini, M. P., Meli, M. A., Mattioli, M., Menotta, M., Mari, M., Valentini, L., Palomba, L., Desideri, D., & Chiarantini, L. (2019). In vitro effects on calcium oxalate crystallization kinetics and crystal morphology of an aqueous extract from Ceterach officinarum: Analysis of a potential antilithiatic mechanism. PloS one, 14(6), e0218734. https://doi.org/10.1371/journal.pone.0218734
290. Le, V., Sukhikh, A., Larichev, T., Ivanova, S., Prosekov, A., & Dmitrieva, A. (2023). Isolation of the Main Biologically Active Substances and Phytochemical Analysis of Ginkgo biloba Callus Culture Extracts. Molecules (Basel, Switzerland), 28(4), 1560. https://doi.org/10.3390/molecules28041560
291. Zaman, G., Farooq, U., Bajwa, M. N., Jan, H., Shah, M., Ahmad, R., Andleeb, A., Drouet, S., Hano, C., & Abbasi, B. (2022). Effects of yeast extract on the production of phenylpropanoid metabolites in callus culture of purple basil (Ocimum basilicum L. var. purpurascens) and their in-vitro evaluation for antioxidant potential. Plant Cell, Tissue and Organ Culture, 150, 543–553. https://doi.org/10.1007/s11240-022-02303-7
292. Olech, M., Ziemichód, W., & Nowacka-Jechalke, N. (2021). The Occurrence and Biological Activity of Tormentic Acid—A Review. Molecules (Basel, Switzerland), 26(13), 3797. https://doi.org/10.3390/molecules26133797
293. Castellano, J. M., Ramos-Romero, S., & Perona, J. S. (2022). Oleanolic Acid: Extraction, Characterization and Biological Activity. Nutrients, 14(3), 623. https://doi.org/10.3390/nu14030623
294. He, Y., Wang, Y., Yang, K., Jiao, J., Zhan, H., Yang, Y., Lv, D., Li, W., & Ding, W. (2022). Maslinic Acid: A New Compound for the Treatment of Multiple Organ Diseases. Molecules (Basel, Switzerland), 27(24), 8732. https://doi.org/10.3390/molecules27248732
295. Mlala, S., Oyedeji, A. O., Gondwe, M., & Oyedeji, O. O. (2019). Ursolic Acid and Its Derivatives as Bioactive Agents. Molecules (Basel, Switzerland), 24(15), 2751. https://doi.org/10.3390/molecules24152751
296. Wei, H., Guo, J., Sun, X., Gou, W., Ning, H., Shang, H., Liu, Q., Hou, W., & Li, Y. (2022). Discovery of Natural Ursane-type SENP1 Inhibitors and the Platinum Resistance Reversal Activity Against Human Ovarian Cancer Cells: A Structure-Activity Relationship Study. Journal of natural products, 85(5), 1248–1255. https://doi.org/10.1021/acs.jnatprod.1c01166

297. Zhao, J., Zhou, H., An, Y., Shen, K., & Yu, L. (2021). Biological effects of corosolic acid as an anti-inflammatory, anti-metabolic syndrome and anti-neoplasic natural compound. Oncology letters, 21(2), 84. https://doi.org/10.3892/ol.2020.12345
298. Coppari, S., Colomba, M., Fraternale, D., Brinkmann, V., Romeo, M., Rocchi, M. B. L., Di Giacomo, B., Mari, M., Guidi, L., Ramakrishna, S., Ventura, N., & Albertini, M. C. (2021). Antioxidant and Anti-Inflammaging Ability of Prune (Prunus Spinosa L.) Extract Result in Improved Wound Healing Efficacy. Antioxidants (Basel, Switzerland), 10(3), 374. https://doi.org/10.3390/antiox10030374
299. Gubitosa, F., Benayada, L., Fraternale, D., De Bellis, R., Carloni, S., Potenza, L., Chiarantini, L., Gorassini, A., Verardo, G., Roselli, C., Valentini, L., Gobbi, P., Balduini, W., Ventura, N., Giannaccini, F., Kass, G. E. N., Colomba, M., & Albertini, M. C. (2024). Long-lasting bioactive compounds production from Malus x domestica Borkh var. Mela Rosa Marchigiana pulp callus culture with antioxidant activity: In vitro, ex vivo and in vivo validation. Journal of Functional Foods, 114, 106073. https://doi.org/10.1016/j.jff.2024.106073
300. Castellano, J. M., Guinda, A., Delgado, T., Rada, M., & Cayuela, J. A. (2013). Biochemical basis of the antidiabetic activity of oleanolic acid and related pentacyclic triterpenes. Diabetes, 62(6), 1791–1799. https://doi.org/10.2337/db12-1215
301. Nazaruk, J., & Borzym-Kluczyk, M. (2015). The role of triterpenes in the management of diabetes mellitus and its complications. Phytochemistry reviews : proceedings of the Phytochemical Society of Europe, 14(4), 675–690. https://doi.org/10.1007/s11101-014-9369-x
302. Oboh, M., Govender, L., Siwela, M., & Mkhwanazi, B. N. (2021). Anti-Diabetic Potential of Plant-Based Pentacyclic Triterpene Derivatives: Progress Made to Improve Efficacy and Bioavailability. Molecules (Basel, Switzerland), 26(23), 7243. https://doi.org/10.3390/molecules26237243
303. Cui, W., Sun, C., Ma, Y., Wang, S., Wang, X., & Zhang, Y. (2020). Neuroprotective effect of tormentic acid against memory impairment and neuro-inflammation in an Alzheimer's disease mouse model. Molecular medicine reports, 22(2), 739–750. https://doi.org/10.3892/mmr.2020.11154
304. Lin, C.-H., Shih, Z.-Z., Kuo, Y.-H., Huang, G.-J., Tu, P.-C., & Shih, C.-C. (2018). Antidiabetic and antihyperlipidemic effects of the flower extract of Eriobotrya japonica in streptozotocin-induced diabetic mice and the potential bioactive constituents in vitro. Journal of Functional Foods, 49, 122–136. https://doi.org/10.1016/j.jff.2018.08.005
305. Wu, J. B., Kuo, Y. H., Lin, C. H., Ho, H. Y., & Shih, C. C. (2014). Tormentic acid, a major component of suspension cells of Eriobotrya japonica, suppresses high-fat diet-induced diabetes and hyperlipidemia by glucose transporter 4 and AMP-activated protein kinase phosphorylation. Journal of agricultural and food chemistry, 62(44), 10717–10726. https://doi.org/10.1021/jf503334d
306. Xu, S., Wang, G., Peng, W., et al. (2019). Corosolic acid isolated from Eriobotrya japonica leaves reduces glucose level in human hepatocellular carcinoma cells, zebrafish, and rats. Scientific Reports, 9, 4388. https://doi.org/10.1038/s41598-019-40934-7
307. Hibi, M., Matsui, Y., Niwa, S., Oishi, S., Yanagimoto, A., Ono, T., & Yamaguchi, T. (2022). Corosolic acid improves glucose and insulin responses in middle-aged men with impaired fasting glucose: A randomized, double-blinded, placebo-controlled crossover trial. Journal of Functional Foods, 97, 105256. https://doi.org/10.1016/j.jff.2022.105256

308. Benayada, L., Gubitosa, F., Fraternale, D., Carloni, S., Cerioni, L., Matacchione, G., Olivieri, F., Potenza, L., De Bellis, R., Chiarantini, L., Roselli, C., Valentini, L., Gobbi, P., Balduini, W., Pappagallo, N., Ventura, N., Kass, G. E. N., Colomba, M., & Albertini, M. C. (2024). In vitro anti-inflammatory activity of Malus x domestica var. Mela Rosa Marchigiana pulp callus extract contrasting high glucose conditions. Journal of Functional Foods, 121, 106420. https://doi.org/10.1016/j.jff.2024.106420
309. Lo Scalzo, R., Testoni, A., & Genna, A. (2001). 'Annurca' apple fruit, a southern Italy apple cultivar: Textural properties and aroma composition. Food Chemistry, 73(3), 333–343. https://doi.org/10.1016/S0308-8146(00)00306-X
310. D'Abrosca, B., Fiorentino, A., Monaco, P., Oriano, P., & Pacifico, S. (2006). Annurcoic acid: A new antioxidant ursane triterpene from fruits of cv. Annurca apple. Food Chemistry, 98(2), 285–290. https://doi.org/10.1016/j.foodchem.2005.05.072
311. D'Abrosca, B., Scognamiglio, M., Corrado, L., Chiocchio, I., Zampella, L., Mastrobuoni, F., Rega, P., Scortichini, M., Fiorentino, A., & Petriccione, M. (2017). Evaluation of different training systems on Annurca apple fruits revealed by agronomical, qualitative and NMR-based metabolomic approaches. Food chemistry, 222, 18–27. https://doi.org/10.1016/j.foodchem.2016.11.144
312. Cefarelli, G., D'Abrosca, B., Fiorentino, A., Izzo, A., Mastellone, C., Pacifico, S., & Piscopo, V. (2006). Free-radical-scavenging and antioxidant activities of secondary metabolites from reddened cv. Annurca apple fruits. Journal of agricultural and food chemistry, 54(3), 803–809. https://doi.org/10.1021/jf052632g
313. Tenore, G. C., Caruso, D., Buonomo, G., D'Avino, M., Santamaria, R., Irace, C., Piccolo, M., Maisto, M., & Novellino, E. (2018). Annurca Apple Nutraceutical Formulation Enhances Keratin Expression in a Human Model of Skin and Promotes Hair Growth and Tropism in a Randomized Clinical Trial. Journal of medicinal food, 21(1), 90–103. https://doi.org/10.1089/jmf.2017.0016
314. Di Lorenzo, R., Maisto, M., Ricci, L., Piccolo, V., Marzocchi, A., Greco, G., Tenore, G. C., & Laneri, S. (2024). Annurca Apple Oleolite as Functional Ingredient for the Formulation of Cosmetics with Skin-Antiaging Activity. International journal of molecular sciences, 25(3), 1677. https://doi.org/10.3390/ijms25031677
315. Ferraro, M. G., Piccolo, M., Pezzella, A., Guerra, F., Maione, F., Tenore, G. C., Santamaria, R., Irace, C., & Novellino, E. (2021). Promelanogenic Effects by an Annurca Apple-Based Natural Formulation in Human Primary Melanocytes. Clinical, cosmetic and investigational dermatology, 14, 291–301. https://doi.org/10.2147/CCID.S299569
316. Chan, S. H. Y., Sheikh, K., Zariwala, M. G., & Somavarapu, S. (2023). Dry powder formulation of azithromycin for COVID-19 therapeutics. Journal of Microencapsulation, 40(4), 217–232. https://doi.org/10.1080/02652048.2023.2175924
317. Saltarelli, R., Ceccaroli, P., Iotti, M., Zambonelli, A., Buffalini, M., Casadei, L., Vallorani, L., & Stocchi, V. (2009). Biochemical characterisation and antioxidant activity of mycelium of Ganoderma lucidum from Central Italy. Food Chemistry, 116(1), 143–151. https://doi.org/10.1016/j.foodchem.2009.02.023
318. Sadat Asadi, N., Heidari, M. M., & Khatami, M. (2019). Protective effect of Berberis vulgaris on Fenton reaction-induced DNA cleavage. Avicenna journal of phytomedicine, 9(3), 213–220.
319. Mursaleen, L., Chan, S. H. Y., Noble, B., Somavarapu, S., & Zariwala, M. G. (2023). Curcumin and N-Acetylcysteine Nanocarriers Alone or Combined with Deferoxamine Target the Mitochondria and Protect against Neurotoxicity and Oxidative Stress in a Co-Culture

Model of Parkinson's Disease. Antioxidants (Basel, Switzerland), 12(1), 130. https://doi.org/10.3390/antiox12010130

320. Al-Qenaei, A., Yiakouvaki, A., Reelfs, O., Santambrogio, P., Levi, S., Hall, N. D., Tyrrell, R. M., & Pourzand, C. (2014). Role of intracellular labile iron, ferritin, and antioxidant defence in resistance of chronically adapted Jurkat T cells to hydrogen peroxide. Free radical biology & medicine, 68(100), 87–100. https://doi.org/10.1016/j.freeradbiomed.2013.12.006

321. Zhong, J. L., Yiakouvaki, A., Holley, P., Tyrrell, R. M., & Pourzand, C. (2004). Susceptibility of skin cells to UVA-induced necrotic cell death reflects the intracellular level of labile iron. The Journal of investigative dermatology, 123(4), 771–780. https://doi.org/10.1111/j.0022-202X.2004.23419.x

322. Pourzand, C., Watkin, R. D., Brown, J. E., & Tyrrell, R. M. (1999). Ultraviolet A radiation induces immediate release of iron in human primary skin fibroblasts: the role of ferritin. Proceedings of the National Academy of Sciences of the United States of America, 96(12), 6751–6756. https://doi.org/10.1073/pnas.96.12.6751

323. Breuer, W., Greenberg, E., & Cabantchik, Z. I. (1997). Newly delivered transferrin iron and oxidative cell injury. FEBS letters, 403(2), 213–219. https://doi.org/10.1016/s0014-5793(97)00056-2

324. Zariwala, M. G., Somavarapu, S., Farnaud, S., & Renshaw, D. (2013). Comparison Study of Oral Iron Preparations Using a Human Intestinal Model. Scientia Pharmaceutica, 81(4), 1123-1140. https://doi.org/10.3797/scipharm.1304-03

325. 325. Schiano, E., Novellino, E., Gámez Fernández, M. M., Tiekou Lorinczova, H., Tenore, G. C., Iannuzzo, F., Patel, V. B., Somavarapu, S., & Zariwala, M. G. (2023). Antioxidant and Antidiabetic Properties of a Thinned-Nectarine-Based Nanoformulation in a Pancreatic β-Cell Line. Antioxidants (Basel, Switzerland), 13(1), 63. https://doi.org/10.3390/antiox13010063

326. Nasso, R., Pagliara, V., D'Angelo, S., Rullo, R., Masullo, M., & Arcone, R. (2021). Annurca Apple Polyphenol Extract Affects Acetyl- Cholinesterase and Mono-Amine Oxidase In Vitro Enzyme Activity. Pharmaceuticals (Basel, Switzerland), 14(1), 62. https://doi.org/10.3390/ph14010062

327. Maisto, M., Piccolo, V., Novellino, E., Schiano, E., Iannuzzo, F., Ciampaglia, R., Summa, V., & Tenore, G. C. (2022). Optimization of Phlorizin Extraction from Annurca Apple Tree Leaves Using Response Surface Methodology. Antioxidants (Basel, Switzerland), 11(10), 1933. https://doi.org/10.3390/antiox11101933

328. Orlandella, F. M., Mirabelli, P., De Stefano, A. E., Iervolino, P. L. C., Luciano, N., D'Angelo, S., & Salvatore, G. (2022). Effects of Annurca Flesh Apple Polyphenols in Human Thyroid Cancer Cell Lines. Oxidative medicine and cellular longevity, 2022, 6268755. https://doi.org/10.1155/2022/6268755

329. Laezza, C., Imbimbo, P., D'Amelia, V., Marzocchi, A., Monti, D. M., Di Loria, A., Monti, S. M., Novellino, E., Tenore, G. C., & Rigano, M. M. (2024). Use of yeast extract to elicit a pulp-derived callus cultures from Annurca apple and potentiate its biological activity. Journal of Functional Foods, 112, 105988. https://doi.org/10.1016/j.jff.2023.105988

330. Ahmad, N., Rab, A., & Ahmad, N. (2016). Light-induced biochemical variations in secondary metabolite production and antioxidant activity in callus cultures of Stevia rebaudiana (Bert). Journal of photochemistry and photobiology. B, Biology, 154, 51–56. https://doi.org/10.1016/j.jphotobiol.2015.11.015

331. Fazal, H., Abbasi, B. H., Ahmad, N., Ali, S. S., Akbar, F., & Kanwal, F. (2016). Correlation of different spectral lights with biomass accumulation and production of antioxidant

secondary metabolites in callus cultures of medicinally important Prunella vulgaris L. Journal of photochemistry and photobiology. B, Biology, 159, 1–7. https://doi.org/10.1016/j.jphotobiol.2016.03.008

332. Kapoor, S., Raghuvanshi, R., Bhardwaj, P., Sood, H., Saxena, S., & Chaurasia, O. P. (2018). Influence of light quality on growth, secondary metabolites production and antioxidant activity in callus culture of Rhodiola imbricata Edgew. Journal of photochemistry and photobiology. B, Biology, 183, 258–265. https://doi.org/10.1016/j.jphotobiol.2018.04.018
333. Younas, M., Drouet, S., Nadeem, M., Giglioli-Guivarc'h, N., Hano, C., & Abbasi, B. H. (2018). Differential accumulation of silymarin induced by exposure of Silybum marianum L. callus cultures to several spectres of monochromatic lights. Journal of photochemistry and photobiology. B, Biology, 184, 61–70. https://doi.org/10.1016/j.jphotobiol.2018.05.018
334. Adil, M., Abbasi, B. H., & Haq, I. u. (2019). Red light controlled callus morphogenetic patterns and secondary metabolites production in Withania somnifera L. Biotechnology Reports, 24, e00380. https://doi.org/10.1016/j.btre.2019.e00380
335. Kumar, S. S., Arya, M., Mahadevappa, P., & Giridhar, P. (2020). Influence of photoperiod on growth, bioactive compounds and antioxidant activity in callus cultures of Basella rubra L. Journal of photochemistry and photobiology. B, Biology, 209, 111937. https://doi.org/10.1016/j.jphotobiol.2020.111937
336. Biswal, B., Jena, B., Giri, A. K., et al. (2022). Monochromatic light elicited biomass accumulation, antioxidant activity, and secondary metabolite production in callus culture of Operculina turpethum (L.). Plant Cell Tissue and Organ Culture, 149, 123–134. https://doi.org/10.1007/s11240-022-02274-9
337. Vergara Martínez, V. M., Estrada-Soto, S. E., Arellano-García, J. J., Rivera-Leyva, J. C., Castillo-España, P., Flores, A. F., Cardoso-Taketa, A. T., & Perea-Arango, I. (2018). Methyl Jasmonate and Salicylic Acid Enhanced the Production of Ursolic and Oleanolic Acid in Callus Cultures of Lepechinia Caulescens. Pharmacognosy magazine, 13(Suppl 4), S886–S889. https://doi.org/10.4103/pm.pm_77_17
338. Jäger, S., Trojan, H., Kopp, T., Laszczyk, M. N., & Scheffler, A. (2009). Pentacyclic triterpene distribution in various plants - rich sources for a new group of multi-potent plant extracts. Molecules (Basel, Switzerland), 14(6), 2016–2031. https://doi.org/10.3390/molecules14062016
339. Ivorra, M. D., Paya, M., & Villar, A. (1988). Hypoglycemic and insulin release effects of tormentic acid: a new hypoglycemic natural product. Planta medica, 54(4), 282–285. https://doi.org/10.1055/s-2006-962433
340. Wang, Y. L., Sun, G. Y., Zhang, Y., He, J. J., Zheng, S., & Lin, J. N. (2016). Tormentic acid inhibits H2O2-induced oxidative stress and inflammation in rat vascular smooth muscle cells via inhibition of the NF-κB signaling pathway. Molecular medicine reports, 14(4), 3559–3564. https://doi.org/10.3892/mmr.2016.5690
341. Babich, O., Sukhikh, S., Pungin, A., Astahova, L., Chupakhin, E., Belova, D., Prosekov, A., & Ivanova, S. (2021). Evaluation of the Conditions for the Cultivation of Callus Cultures of Hyssopus officinalis Regarding the Yield of Polyphenolic Compounds. Plants (Basel, Switzerland), 10(5), 915. https://doi.org/10.3390/plants10050915
342. Hariprasath, L., Jegadeesh, R., Arjun, P., & Raaman, N. (2015). In vitro propagation of Senecio candicans DC and comparative antioxidant properties of aqueous extracts of the in vivo plant and in vitro-derived callus. South African Journal of Botany, 98, 134–141. https://doi.org/10.1016/j.sajb.2015.02.011

343. Moon, T. C., Lin, C. X., Lee, J. S., Kim, D. S., Bae, K., Son, K. H., Kim, H. P., Kang, S. S., Son, J. K., & Chang, H. W. (2005). Antiinflammatory activity of astilbic acid from Astilbe chinensis. Biological & pharmaceutical bulletin, 28(1), 24–26. https://doi.org/10.1248/bpb.28.24
344. Vo, N. N. Q., Nomura, Y., Muranaka, T., & Fukushima, E. O. (2019). Structure-Activity Relationships of Pentacyclic Triterpenoids as Inhibitors of Cyclooxygenase and Lipoxygenase Enzymes. Journal of natural products, 82(12), 3311–3320. https://doi.org/10.1021/acs.jnatprod.9b00538
345. Magiera, A., Marchelak, A., Michel, P., Owczarek, A., & Olszewska, M. A. (2021). Lipophilic extracts from leaves, inflorescences and fruits of Prunus padus L. as potential sources of corosolic, ursolic and oleanolic acids with anti-inflammatory activity. Natural product research, 35(13), 2263–2268. https://doi.org/10.1080/14786419.2019.1666385
346. Pace, S., Zhang, K., Jordan, P. M., Bilancia, R., Wang, W., Börner, F., Hofstetter, R. K., Potenza, M., Kretzer, C., Gerstmeier, J., Fischer, D., Lorkowski, S., Gilbert, N. C., Newcomer, M. E., Rossi, A., Chen, X., & Werz, O. (2021). Anti-inflammatory celastrol promotes a switch from leukotriene biosynthesis to formation of specialized pro-resolving lipid mediators. Pharmacological research, 167, 105556. https://doi.org/10.1016/j.phrs.2021.105556
347. Werz O. (2007). Inhibition of 5-lipoxygenase product synthesis by natural compounds of plant origin. Planta medica, 73(13), 1331–1357. https://doi.org/10.1055/s-2007-990242
348. Evans, M. D., Dizdaroglu, M., & Cooke, M. S. (2004). Oxidative DNA damage and disease: induction, repair and significance. Mutation research, 567(1), 1–61. https://doi.org/10.1016/j.mrrev.2003.11.001
349. Markesbery, W. R., & Lovell, M. A. (2006). DNA oxidation in Alzheimer's disease. Antioxidants & redox signaling, 8(11-12), 2039–2045. https://doi.org/10.1089/ars.2006.8.2039
350. Lenart, P., & Krejci, L. (2016). DNA, the central molecule of aging. Mutation research, 786, 1–7. https://doi.org/10.1016/j.mrfmmm.2016.01.007
351. Lee, Y. S., Han, O. K., Park, C. W., Yang, C. H., Jeon, T. W., Yoo, W. K., Kim, S. H., & Kim, H. J. (2005). Pro-inflammatory cytokine gene expression and nitric oxide regulation of aqueous extracted Astragali radix in RAW 264.7 macrophage cells. Journal of ethnopharmacology, 100(3), 289–294. https://doi.org/10.1016/j.jep.2005.03.009
352. An, H. J., Kim, I. T., Park, H. J., Kim, H. M., Choi, J. H., & Lee, K. T. (2011). Tormentic acid, a triterpenoid saponin, isolated from Rosa rugosa, inhibited LPS-induced iNOS, COX-2, and TNF-α expression through inactivation of the nuclear factor-κb pathway in RAW 264.7 macrophages. International immunopharmacology, 11(4), 504–510. https://doi.org/10.1016/j.intimp.2011.01.002
353. Kim, M. H., Kim, J. N., Han, S. N., & Kim, H. K. (2015). Ursolic acid isolated from guava leaves inhibits inflammatory mediators and reactive oxygen species in LPS-stimulated macrophages. Immunopharmacology and immunotoxicology, 37(3), 228–235. https://doi.org/10.3109/08923973.2015.1021355
354. Nair, V., Bang, W. Y., Schreckinger, E., Andarwulan, N., & Cisneros-Zevallos, L. (2015). Protective Role of Ternatin Anthocyanins and Quercetin Glycosides from Butterfly Pea (Clitoria ternatea Leguminosae) Blue Flower Petals against Lipopolysaccharide (LPS)-Induced Inflammation in Macrophage Cells. Journal of agricultural and food chemistry, 63(28), 6355–6365. https://doi.org/10.1021/acs.jafc.5b00928
355. Cho, Y.-H., Kim, N.-H., Khan, I., Yu, J.M., Jung, H.G., Kim, H.H., Jang, J.Y., Kim, H.J., Kim, D.-I., Kwak, J.-H., Kang, S.C., & An, B.J. (2016). Anti-inflammatory potential of

quercetin-3-O-β-D-(“2”-galloyl)-glucopyranoside and quercetin isolated from Diospyros kaki calyx via suppression of MAP signaling molecules in LPS-induced RAW 264.7 macrophages. Journal of Food Science, 81(10), C2447–C2456. https://doi.org/10.1111/1750-3841.13497
356. Huang, H. T., Liaw, C. C., Chiou, C. T., Kuo, Y. H., & Lee, K. T. (2021). Triterpene Acids from Mesona procumbens Exert Anti-inflammatory Effects on LPS-Stimulated Murine Macrophages by Regulating the MAPK Signaling Pathway. Journal of agricultural and food chemistry, 69(22), 6271–6280. https://doi.org/10.1021/acs.jafc.1c01810
357. Guo, F., Tsao, R., Li, C., Wang, X., Zhang, H., Jiang, L., Sun, Y., & Xiong, H. (2022). Polyphenol Content of Green Pea (Pisum sativum L.) Hull under In Vitro Digestion and Effects of Digestive Products on Anti-Inflammatory Activity and Intestinal Barrier in the Caco-2/Raw264.7 Coculture Model. Journal of agricultural and food chemistry, 70(11), 3477–3488. https://doi.org/10.1021/acs.jafc.2c00102
358. Hu, W., Liang, K., Zhu, H., Zhao, C., Hu, H., & Yin, S. (2022). Ferroptosis and Its Role in Chronic Diseases. Cells, 11(13), 2040. https://doi.org/10.3390/cells11132040
359. Chen, X., Comish, P. B., Tang, D., & Kang, R. (2021). Characteristics and Biomarkers of Ferroptosis. Frontiers in cell and developmental biology, 9, 637162. https://doi.org/10.3389/fcell.2021.637162
360. Stockwell, B. R., Jiang, X., & Gu, W. (2020). Emerging Mechanisms and Disease Relevance of Ferroptosis. Trends in cell biology, 30(6), 478–490. https://doi.org/10.1016/j.tcb.2020.02.009
361. Jiang, X., Stockwell, B. R., & Conrad, M. (2021). Ferroptosis: mechanisms, biology and role in disease. Nature reviews. Molecular cell biology, 22(4), 266–282. https://doi.org/10.1038/s41580-020-00324-8
362. Mukherjee, S., Date, A., Patravale, V., Korting, H. C., Roeder, A., & Weindl, G. (2006). Retinoids in the treatment of skin aging: an overview of clinical efficacy and safety. Clinical interventions in aging, 1(4), 327–348. https://doi.org/10.2147/ciia.2006.1.4.327
363. Aroun, A., Zhong, J. L., Tyrrell, R. M., & others. (2012). Iron, oxidative stress and the example of solar ultraviolet A radiation. Photochemical & Photobiological Sciences, 11(1), 118–134. https://doi.org/10.1039/c1pp05204g
364. Vile, G. F., & Tyrrell, R. M. (1995). UVA radiation-induced oxidative damage to lipids and proteins in vitro and in human skin fibroblasts is dependent on iron and singlet oxygen. Free radical biology & medicine, 18(4), 721–730. https://doi.org/10.1016/0891-5849(94)00192-m
365. Reelfs, O., Eggleston, I. M., & Pourzand, C. (2010). Skin protection against UVA-induced iron damage by multiantioxidants and iron chelating drugs/prodrugs. Current drug metabolism, 11(3), 242–249. https://doi.org/10.2174/138920010791196265
366. Dunaway, S., Odin, R., Zhou, L., Ji, L., Zhang, Y., & Kadekaro, A. L. (2018). Natural Antioxidants: Multiple Mechanisms to Protect Skin From Solar Radiation. Frontiers in pharmacology, 9, 392. https://doi.org/10.3389/fphar.2018.00392
367. Chaiprasongsuk, A., & Panich, U. (2022). Role of Phytochemicals in Skin Photoprotection via Regulation of Nrf2. Frontiers in pharmacology, 13, 823881. https://doi.org/10.3389/fphar.2022.823881
368. Merecz-Sadowska, A., Sitarek, P., Zajdel, K., Kucharska, E., Kowalczyk, T., & Zajdel, R. (2021). The Modulatory Influence of Plant-Derived Compounds on Human Keratinocyte Function. International journal of molecular sciences, 22(22), 12488. https://doi.org/10.3390/ijms222212488

369. Mioc, M., Milan, A., Malița, D., Mioc, A., Prodea, A., Racoviceanu, R., Ghiulai, R., Cristea, A., Căruntu, F., & Șoica, C. (2022). Recent Advances Regarding the Molecular Mechanisms of Triterpenic Acids: A Review (Part I). International journal of molecular sciences, 23(14), 7740. https://doi.org/10.3390/ijms23147740
370. Mioc, M., Prodea, A., Racoviceanu, R., Mioc, A., Ghiulai, R., Milan, A., Voicu, M., Mardale, G., & Șoica, C. (2022). Recent Advances Regarding the Molecular Mechanisms of Triterpenic Acids: A Review (Part II). International journal of molecular sciences, 23(16), 8896. https://doi.org/10.3390/ijms23168896
371. Prodea, A., Mioc, A., Banciu, C., Trandafirescu, C., Milan, A., Racoviceanu, R., Ghiulai, R., Mioc, M., & Soica, C. (2022). The Role of Cyclodextrins in the Design and Development of Triterpene-Based Therapeutic Agents. International journal of molecular sciences, 23(2), 736. https://doi.org/10.3390/ijms23020736
372. Reisman, S. A., Lee, C. Y., Meyer, C. J., Proksch, J. W., & Ward, K. W. (2014). Topical application of the synthetic triterpenoid RTA 408 activates Nrf2 and induces cytoprotective genes in rat skin. Archives of dermatological research, 306(5), 447–454. https://doi.org/10.1007/s00403-013-1433-7
373. Dang, Y. Y., Liu, T., Liu, Y. D., Li, J. Y., Jing, Y., Yang, M. J., Zhang, H., Jiang, M. M., Wu, H. H., Yang, W. Z., Li, N., & Zhang, P. (2024). Anti-photoaging activity of triterpenoids isolated from Centella asiatica. Phytochemistry, 228, 114246. https://doi.org/10.1016/j.phytochem.2024.114246
374. Grudzińska, M., Stachnik, B., Galanty, A., Sołtys, A., & Podolak, I. (2023). Progress in Antimelanoma Research of Natural Triterpenoids and Their Derivatives: Mechanisms of Action, Bioavailability Enhancement and Structure Modifications. Molecules (Basel, Switzerland), 28(23), 7763. https://doi.org/10.3390/molecules28237763
375. Wang, H., Syrovets, T., Kess, D., Büchele, B., Hainzl, H., Lunov, O., Weiss, J. M., Scharffetter-Kochanek, K., & Simmet, T. (2009). Targeting NF-κB with a natural triterpenoid alleviates skin inflammation in a mouse model of psoriasis. Journal of Immunology, 183(7), 4755–4763. https://doi.org/10.4049/jimmunol.0900521
376. Günther, A., Makuch, E., Nowak, A., Duchnik, W., Kucharski, Ł., Pełech, R., & Klimowicz, A. (2021). Enhancement of the Antioxidant and Skin Permeation Properties of Betulin and Its Derivatives. Molecules (Basel, Switzerland), 26(11), 3435. https://doi.org/10.3390/molecules26113435
377. Laugel, C., Rafidison, P., Potard, G., Aguadisch, L., & Baillet, A. (2000). Modulated release of triterpenic compounds from a O/W/O multiple emulsion formulated with dimethicones: infrared spectrophotometric and differential calorimetric approaches. Journal of controlled release: official journal of the Controlled Release Society, 63(1-2), 7–17. https://doi.org/10.1016/s0168-3659(99)00169-8
378. Li, H., Peng, Q., Guo, Y., Wang, X., & Zhang, L. (2020). Preparation and in vitro and in vivo Study of Asiaticoside-Loaded Nanoemulsions and Nanoemulsions-Based Gels for Transdermal Delivery. International journal of nanomedicine, 15, 3123–3136. https://doi.org/10.2147/IJN.S241923
379. Pârvănescu Pană, R. D., Watz, C. G., Moacă, E. A., Vlaia, L., Marcovici, I., Macașoi, I. G., Borcan, F., Olariu, I., Coneac, G., Drăghici, G. A., Crăiniceanu, Z., Flondor Ionescu, D., Enache, A., & Dehelean, C. A. (2021). Oleogel Formulations for the Topical Delivery of Betulin and Lupeol in Skin Injuries-Preparation, Physicochemical Characterization, and Pharmaco-Toxicological Evaluation. Molecules (Basel, Switzerland), 26(14), 4174. https://doi.org/10.3390/molecules26144174

380. Fu, H., Wu, T. H., Ma, C. P., & Yen, F. L. (2024). Improving Water Solubility and Skin Penetration of Ursolic Acid through a Nanofiber Process to Achieve Better In Vitro Anti-Breast Cancer Activity. Pharmaceutics, 16(9), 1147. https://doi.org/10.3390/pharmaceutics16091147
381. Pateiro, M., Gómez, B., Munekata, P. E. S., Barba, F. J., Putnik, P., Kovačević, D. B., & Lorenzo, J. M. (2021). Nanoencapsulation of Promising Bioactive Compounds to Improve Their Absorption, Stability, Functionality and the Appearance of the Final Food Products. Molecules (Basel, Switzerland), 26(6), 1547. https://doi.org/10.3390/molecules26061547
382. Chowdhury, S., Kar, K., & Mazumder, R. (2024). Exploration of different strategies of nanoencapsulation of bioactive compounds and their ensuing approaches. Future Journal of Pharmaceutical Sciences, 10, 72. https://doi.org/10.1186/s43094-024-00644-y
383. Pérez-Pérez, V., Jiménez-Martínez, C., González-Escobar, J. L., & Corzo-Ríos, L. J. (2024). Exploring the impact of encapsulation on the stability and bioactivity of peptides extracted from botanical sources: trends and opportunities. Frontiers in chemistry, 12, 1423500. https://doi.org/10.3389/fchem.2024.1423500
384. Wang, S., Su, R., Nie, S., Sun, M., Zhang, J., Wu, D., & Moustaid-Moussa, N. (2014). Application of nanotechnology in improving bioavailability and bioactivity of diet-derived phytochemicals. The Journal of nutritional biochemistry, 25(4), 363–376. https://doi.org/10.1016/j.jnutbio.2013.10.002
385. Li, X., Wang, L., Fan, Y., Feng, Q., & Cui, F.-Z. (2012). Biocompatibility and toxicity of nanoparticles and nanotubes. Journal of Nanomaterials, 2012, 6. https://doi.org/10.1155/2012/548389
386. Gupta, S., Bansal, R., Gupta, S., Jindal, N., & Jindal, A. (2013). Nanocarriers and nanoparticles for skin care and dermatological treatments. Indian dermatology online journal, 4(4), 267–272. https://doi.org/10.4103/2229-5178.120635
387. Zhang, Z., Cui, C., Wei, F., & Lv, H. (2017). Improved solubility and oral bioavailability of apigenin via Soluplus/Pluronic F127 binary mixed micelles system. Drug development and industrial pharmacy, 43(8), 1276–1282. https://doi.org/10.1080/03639045.2017.1313857
388. Bonde, G. V., Ajmal, G., Yadav, S. K., Mittal, P., Singh, J., Bakde, B. V., & Mishra, B. (2020). Assessing the viability of Soluplus® self-assembled nanocolloids for sustained delivery of highly hydrophobic lapatinib (anticancer agent): Optimisation and in-vitro characterisation. Colloids and surfaces. B, Biointerfaces, 185, 110611. https://doi.org/10.1016/j.colsurfb.2019.110611
389. Agafonov, M., Volkova, T., Kumeev, R., Chibunova, E., & Terekhova, I. (2019). Impact of pluronic F127 on aqueous solubility and membrane permeability of antirheumatic compounds of different structure and polarity. Journal of Molecular Liquids, 274, 770–777. https://doi.org/10.1016/j.molliq.2018.11.060
390. Essaghraoui, A., Belfkira, A., Hamdaoui, B., Nunes, C., Lima, S. A. C., & Reis, S. (2019). Improved Dermal Delivery of Cyclosporine A Loaded in Solid Lipid Nanoparticles. Nanomaterials (Basel, Switzerland), 9(9), 1204. https://doi.org/10.3390/nano9091204
391. Niu, J., Yuan, M., Chen, C., Wang, L., Tang, Z., Fan, Y., Liu, X., Ma, Y. J., & Gan, Y. (2020). Berberine-Loaded Thiolated Pluronic F127 Polymeric Micelles for Improving Skin Permeation and Retention. International journal of nanomedicine, 15, 9987–10005. https://doi.org/10.2147/IJN.S270336
392. Kamothi, D. J., Kant, V., Jangir, B. L., Joshi, V. G., Ahuja, M., & Kumar, V. (2022). Novel preparation of bilirubin-encapsulated pluronic F-127 nanoparticles as a potential biomaterial

for wound healing. European journal of pharmacology, 919, 174809. https://doi.org/10.1016/j.ejphar.2022.174809
393. U.S. Food and Drug Administration. (n.d.). Inactive ingredient search for approved drug products. U.S. Department of Health and Human Services. Retrieved January 16, 2025, from https://www.accessdata.fda.gov/scripts/cder/iig/index.Cfm
394. Pervez, S., Nasir, F., Hidayatullah, T., Khattak, M. A., Alasmari, F., Zainab, S. R., Gohar, S., Tahir, A., & Maryam, G. E. (2023). Transdermal Delivery of Glimepiride: A Novel Approach Using Nanomicelle-Embedded Microneedles. Pharmaceutics, 15(8), 2019. https://doi.org/10.3390/pharmaceutics15082019
395. Pourzand, C., Albieri-Borges, A., & Raczek, N. N. (2022). Shedding a New Light on Skin Aging, Iron- and Redox-Homeostasis and Emerging Natural Antioxidants. Antioxidants (Basel, Switzerland), 11(3), 471. https://doi.org/10.3390/antiox11030471
396. Jian, J., Pelle, E., Yang, Q., Pernodet, N., Maes, D., & Huang, X. (2011). Iron sensitizes keratinocytes and fibroblasts to UVA-mediated matrix metalloproteinase-1 through TNF-α and ERK activation. Experimental dermatology, 20(3), 249–254. https://doi.org/10.1111/j.1600-0625.2010.01152.x
397. Liebel, F., Kaur, S., Ruvolo, E., Kollias, N., & Southall, M. D. (2012). Irradiation of skin with visible light induces reactive oxygen species and matrix-degrading enzymes. The Journal of investigative dermatology, 132(7), 1901–1907. https://doi.org/10.1038/jid.2011.476