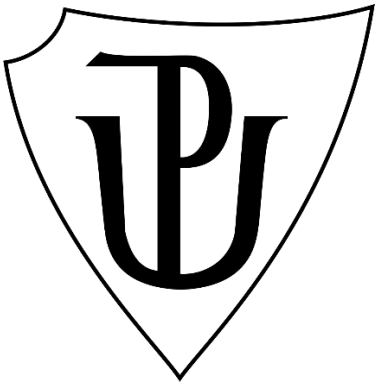
UNIVERZITA PALACKÉHO V OLOMOUCI

Přírodovědecká fakulta

Katedra biotechnologií



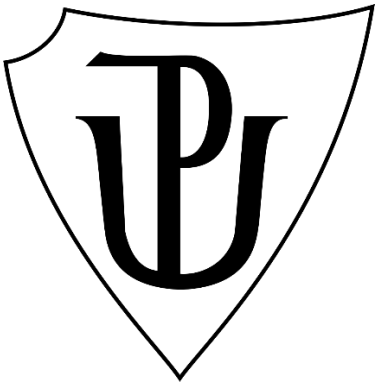
Zobrazování oxidativního stresu v lidských nádorových buňkách

BAKALÁŘSKÁ PRÁCE

Autor:	**Gaukhar Zhanysbayeva**
Studijní program:	B0512A130007 Biotechnologie a genové inženýrství
Specializace:	Biotechnologie a genové inženýrství
Forma studia:	Prezenční
Vedoucí práce:	**doc. Ankush Prasad, Ph.D.**
Rok:	2023

PALACKÝ UNIVERSITY IN OLOMOUC

Faculty of Science
Department of Biotechnology



Imaging of oxidative stress in human cancer cells

BACHELOR THESIS

Author:	**Gaukhar Zhanysbayeva**
Study program:	B0512A130007 Biotechnology and gene engineering
Branch of study:	Biotechnology and gene engineering
Form of study:	Full time
Supervisor:	**doc. Ankush Prasad, Ph.D.**
Year:	2023

Prohlašuji, že jsem bakalářskou práci vypracovala samostatně s vyznačením všech použitých pramenů a spoluautorství. Souhlasím se zveřejněním bakalářské práce podle zákona č. 111/1998 Sb., o vysokých školách, ve znění pozdějších předpisů. Byla jsem seznámena s tím, že se na moji práci vztahují práva a povinnosti vyplývající ze zákona č. 121/2000 Sb., autorský zákon, ve znění pozdějších předpisů.

V Olomouci dne ....

Podpis studenta

I declare that I have prepared the bachelor thesis independently, indicating all sources used and co-authorship. I agree with the publication of the bachelor thesis according to Act no. 111/1998 Coll., On Higher Education, as amended. I have been informed that my thesis is subject to the rights and obligations arising from Act No. 121/2000 Coll., Copyright Act, as amended.

In Olomouc ....

Student's signature

Acknowledgments:

I would like to give special thanks to doc. Ankush Prasad, Ph.D., for his valuable support, patience, and guidance throughout my studies. Many thanks to Deepak Rathi for his valuable advice and help in difficult situations. Additionally, I would like to express my gratitude to the entire Department of Biophysics and the Department of Biotechnology at the Faculty of Natural Sciences, Palacký University, Olomouc, for their valuable contributions to my work.

This work was funded by the grant no. IGA_PrF_2023_023 of Palacký University.

Bibliografická identifikace

Jméno a příjmení autora	Gaukhar Zhanysbayeva
Název práce	Zobrazování oxidativního stresu v lidských nádorových buňkách
Typ práce	Bakalářská
Pracoviště	Katedra biofyzyky
Vedoucí práce	doc. Ankush Prasad, Ph.D.
Rok obhajoby práce	2023

Abstrakt

Cílem této práce je zkoumaní oxidačního stresu u tří lidských nádorových buněčných linií (U-937, THP-1, HL-60) pomocí různých technologií. U-937 je promonocytární buněčná linie lidské myeloidní leukemie, zatímco buněčná linie THP-1 je typ lidské monocytární leukemie, která byla získána z krve jedince s akutní monocytární leukemií. Buněčná linie HL-60, byla získána od pacienta s akutní myeloidní leukémií. Diferenciace buněk byla indukována pomocí phorbol 12-myristát-13-acetátu (PMA), což vedlo k tvorbě reaktivních forem kyslíku v buňkách. Tvorba těchto reaktivních forem kyslíku byla detekována pomocí zobrazovacího zařízení s nábojovou vazbou (CCD), fotonásobiče (PMT), Western blotu a spektroskopie elektronové paramagnetické rezonance (EPR). Pomocí CCD kamery a PMT bylo v různých stavech detekováno spontánní zobrazování ultra slabých fotonů. Zatímco tvorba radikálu s centrovaným uhlíkem byla měřena v EPR spektroskopii. Kromě toho bylo studováno potenciální využití přírodních sloučenin pro jejich antioxidační účinky v různých buněčných liniích pomocí Western blottingu.

Klíčová slova

U-937, THP-1, HL-60 buněčné linie, Reaktivní formy kyslíku, volné radikály, zařízení s nábojovou vazbou (CCD), fotonásobič (PMT), Western blot, spektroskopie elektronové paramagnetické rezonance (EPR), bioaktivní sloučeniny.

Počet stran	40
Počet příloh	1
Jazyk	Anglický

Bibliographical identification

Autor's first name and surname	Gaukhar Zhanysbayeva
Title	Imaging of oxidative stress in human cancer cells
Type of thesis	Bachelor
Department	Department of Biophysics
Supervisor	doc. Ankush Prasad, Ph.D.
The year of presentation	2023

Abstract

The objectives include the investigation of oxidative stress in three human cancer cell lines (U-937, THP-1, HL-60) using different technologies. U-937 is a pro-monocytic human myeloid leukaemia cell line, while the THP-1 cell line is a type of human monocytic leukaemia that was obtained from the blood of an individual with acute monocytic leukaemia. The HL-60 cell line was obtained from a patient with acute myeloid leukaemia. Differentiation of the cells was induced by phorbol 12-myristate-13-acetate (PMA), which led to the formation of reactive oxygen species in the cells. The formation of this reactive oxygen species was detected using charge-coupled device (CCD imaging), Photomultiplier tube (PMT), Western blotting, and electron paramagnetic resonance spectroscopy (EPR). Spontaneous ultra-weak photon emission imaging was detected by CCD camera and PMT in different states. The formation of carbon-centred radicals was measured by EPR spin-trapping spectroscopy. Furthermore, the potential usage of natural compounds for their antioxidant effects in different cell lines was studied using Western blotting.

Keywords	U-937, THP-1, HL-60 cell lines, reactive oxygen species, free radicals, Charge-coupled device (CCD), Photomultiplier tube (PMT), Western blotting, Electron paramagnetic resonance spectroscopy (EPR), bioactive compounds.
Number of pages	40
Number of appendices	1
Language	English

Contents

1. Introduction 1
2. Current state of the topic 3
   2.1. Human cancer cell lines 3
   2.2. Reactive oxygen species 4
   2.3. Sources of reactive oxygen species 6
   2.4. Essential role of reactive oxygen species 7
   2.5. Oxidative stress 8
   2.6. Reactive oxygen species in disease 9
   2.7. Antioxidants and defence mechanisms 10
   2.8. The detection of ROS 11
   2.9. Bioactive compounds 13
   2.10. Chlorogenic acid 13
   2.11. Oleuropein 14
   2.12. Tomatine 16
   2.13. Tyrosol 17
3. Materials and Methods 19
   3.1. Extracts 19
   3.2. Cell lines 19
   3.3. Cell density, growth, and viability 19
   3.4. Differentiation of cell lines 20
   3.5. Treatment with bioactive compounds 20
   3.6. Cell harvesting and protein isolation. 21
   3.7. Bicinchoninic acid Assay for protein estimation 21
   3.8. Protein Immunoblotting 21
   3.9. Ultra-weak photon emission (UPE) measuring system 22
   3.10. Electron paramagnetic resonance spectroscopy (EPR) 23
4. Results and Discussion 24
   4.1. Growth curve of U-937 cells 24
   4.2. U-937 cell differentiation under phorbol-12 myristate-13-acetate (PMA) 25
   4.3. Toxicity in U-937 and THP-1 27
   4.4. Ultra-weak photon emission (UPE) imaging of U-937, THP-1 HL-60 cell cultures .. 29
   4.5. Kinetics of ultra-weak photon emission (UPE) in U-937 THP-1 HL-60 cells 31
   4.6. Spin trapping and formation of carbon-centred radicals using EPR spin trapping spectroscopy 33
   4.7. Western blotting 35
5. Conclusion 36
6. References 37

7. List of Appendices 41

Aims

- To learn the procedures for culturing human cell lines and understand the patterns of growth, viability, and mortality.
- To perform spatial and temporal imaging of oxidative stress in human cancer cells.
- To study the formation of oxidation products using electron paramagnetic resonance spectroscopy and immunoblotting.
- To understand the mechanism of biomolecule oxidation.

1. Introduction

Oxidative stress is a condition that results from an excess of free radicals and a lack of antioxidants to combat them. Increased levels of oxygen and free radicals in the body lead to oxidation of cell components, damaging their functionality. Free radicals are highly reactive molecules with an unpaired electron in their outer orbital [superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$) among others]. Proteins, lipids, and nucleic acids are altered as a result of the harmful effects of free radicals. Many diseases arise and progress as a result of exposure to these free radicals. Early detection of oxidative stress is essential to protect the body from the onset of cancer, Alzheimer's and Parkinson's disease, diabetes mellitus, inflammatory diseases, as well as psychological diseases or ageing processes (Preiser, 2012).

Bioactive compounds are organic compounds that the body cannot synthesise on its own, so it must be absorbed from food. These bioactive compounds lead to the improvement of physical and mental abilities of the human body as well as strengthen the immune system. Bioactive compounds are used for prophylactic auxiliary therapies and to support the functional activity of the body. Moreover, a great advantage of such compounds is their availability. They are found in fruits and vegetables and can easily be chemically synthesised. In this work four bioactive compounds were used: chlorogenic acid, oleuropein, tomatine, and tyrosol, as they can reduce the occurrence of oxidative stress and consequently prevent the appearance of cancer cells. Oleuropein is a compound consisting of hydroxytyrosol and an oleoside skeleton with an added carbohydrate group. This compound is found in raw olives or in olive leaves. α-Tomatine, the main steroidal glycoalkaloid and saponin in tomatoes, is predominantly present in the stems, leaves, and roots of vegetative tissues and unripe green fruits of the tomato plant (*Solanum lycopersicum* L.). Tyrosol, also known as 2-(4-hydroxyphenyl)-ethanol, is a biophenol found in various natural sources, especially in products such as olive oil and wine. Chlorogenic acid, which exists in various forms, including 3-CQA, 4-CQA, and 5-CQA, belongs to a group of secondary phenolic metabolites produced by certain plant species such as green roasted coffee, tea, chocolate, berries, apples, pears, and citrus fruits.

In this work, the preventive effects of these bioactive compounds (chlorogenic acid, oleuropein, tomatine, and tyrosol) on cell viability and oxidation of biomolecules were evaluated. Three different human cancer cell lines, U-937, THP-1, and HL-60, which are widely used in biochemical research, were used in most experiments. The U-937 cell line

is derived from human myeloid leukaemia and is characterised as monocytic. It was originally isolated from 37-year-old male histiocytic lymphoma and is commonly used to study monocyte differentiation and behaviour (Chanput et al., 2015). The HL-60 cell line used in the current study was isolated from a 36-year-old woman with acute promyelocytic leukaemia. More than 90% of these cells are promyelocytes, making them an ideal model for studying the growth and maturation of normal and cancer cells *in vitro* (Breitman et al., 1980). Another human monocytic leukaemia cell line, THP-1, has been obtained from the blood of a human with acute monocytic leukaemia. These cells have physical and differentiation properties comparable to primary monocytes and macrophages (Chanput et al., 2015). Confocal laser scanning microscopy was used to detect cell differentiation under phorbol-12-myristate-13-acetate (PMA) by visualising them *in vivo*. Overall, this work shows the physiological stress generated during the cell differentiation shown using novel *in-vivo* CCD imaging (spatial and temporal imaging) as well as the effect of bioactive compounds on reducing oxidative stress by reducing exposure to reactive oxygen species/ reactive intermediates evaluated using immunoblotting.

2. Current state of the topic

2.1. Human cancer cell lines

Monocytes are cells derived from myeloid cells and when it enters the tissues from the bloodstream, it can differentiate into macrophages or dendritic cells. Although they do not constantly multiply, they are found in the spleen, bone marrow, and blood. Chemokine receptors and pathogen recognition receptors in monocytes enable them to move from the bloodstream to tissues in response to an infection (Shi & Pamer, 2011). Specialised cells called macrophages and dendritic cells expose antigens to the immune system (Chanput et al., 2015). Phagocytosis, antigen presentation, and cytokine synthesis are the three main functions of monocytes, macrophages, and dendritic cells in the immune system (Germic et al., 2019). Monocytes and macrophages play a critical role as regulators of the innate immune response. Dendritic cells, on the other hand, play a key role in the formation of immunological memory and tolerance, initiate, and highly regulate pathogen-specific adaptive immune responses (Geissmann et al., 2010).

The human monocytic leukemia cell line THP-1 was developed in 1980 (Tsuchiya et al., 1980). It was isolated from the blood of a patient with acute monocytic leukemia. THP-1 cells share morphological and differentiation characteristics with primary monocytes and macrophages. THP-1 cells have a rounded, large cell shape and exhibit specific monocytic markers. After being exposed to phorbol-12-myristate-13-acetate, which is a differentiation inducer, almost all THP-1 cells begin to attach to culture plates and develop into macrophages (Chanput et al., 2015). U-937 is a pro-monocytic human myeloid leukemia cell line that was discovered in a 37-year-old man's histiocytic lymphoma. This cell line is simple to use and has many characteristics of monocytes. They are homogeneous, can be prepared in an almost unlimited number, and is a model cell line utilised in biomedical research. A variety of solubility stimulants cause U-937 cells to differentiate and take on the appearance and traits of macrophages. Due to their myeloid origin, U-937 cells produce many cytokines and chemokines in response to solvent stimulation (Chanput et al., 2015). The HL-60 cell line was generated in 1977 from a patient with acute myeloid leukaemia. HL-60 cells can develop into granulocyte-like cells or monocyte/macrophage-like cells depending on the reagent used. The proto-oncogene c-myc proto-oncogene has been amplified in the HL-60 cell genome, and as a result, c-myc mRNA levels are high in undifferentiated cells but quickly drop after

differentiation is induced (Birnie, 1988). These characteristics have made the HL60 cell line a desirable model for research on the development of human myeloid cells.

In our study, three different human cell lines (THP-1, U-937, and HL-60) derived from myeloid leukemia were used because they differ from each other in several parameters, and our objective was to study the physiology and/or variation in responses between cell lines. In brief, the first difference among chosen cell lines concerns the origin of the cells. THP-1 was derived from patients with acute monocytic leukemia and U-937 was derived from patients with histiocytic lymphoma; HL-60 was obtained from a patient with acute promyelocytic leukemia. Secondly, these cells have a different differentiation process. THP-1 and U-937 are pro-monocytic cell lines, whereas HL-60 consists predominantly of promyelocytes. The third difference is that all three cell lines have different morphologies. THP-1 cells are small and round, U-937 cells are larger, and more elongated, and HL-60 cells are irregularly shaped. Although all three cell lines are useful models for studying myeloid leukemia, they have distinctive characteristics that allow us to examine the effects of bioactive compounds from different angles. (Nascimento et al., 2022; Ascensao & Mickman, 1984; Harris & Ralph, 1985).

2.2. Reactive oxygen species

Reactive oxygen species (ROS) are reactive molecules derived from molecular oxygen (O_2), which is a molecule consisting of one, two, or three oxygen atoms. ROS are produced as by-products of various metabolic pathways such as mitochondrial oxidative metabolism (Ray et al., 2012). These molecules are also divided into radical ROS and non-radical ROS. The difference between radical and non-radical ROS is that radical ROS contains one or two unpaired electrons on the oxygen atom, whereas non-radical ROS have no unpaired electrons on the oxygen atom. Well-known examples of radical ROS are superoxide anion radical ($O_2^{\bullet -}$), hydroxyl radical ($HO^{\bullet}$), while well-known examples of non-radical ROS include hydrogen peroxide (H_2O_2). (Fig. 1)

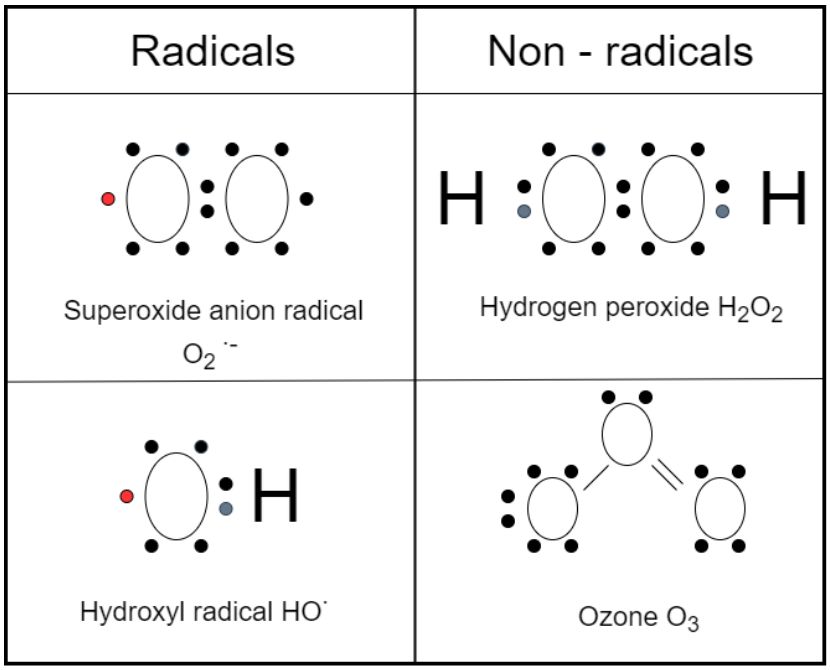


Figure 1. Examples of radical and non-radical reactive oxygen species.

Examples of ROS in detail:

- The Superoxide anion radical ($O_2^{\bullet -}$) - is produced by electron leakage from electron transport chains in mitochondria, endoplasmic reticulum, microbodies and cell walls (Held, 2010). $O_2^{\bullet -}$ is not capable of transportation across the plasma membrane and not highly toxic compared to other ROS. Moreover, its dismutation (decomposition) produces hydrogen peroxide. $O_2^{\bullet -}$ can be formed chemically, biologically, and physically. Chemical generation occurs because of the interaction of xanthine oxidase enzyme (XO) with substrate xanthine, while $O_2^{\bullet -}$ biologically can be formed because of the one-electron reduction of molecular oxygen by non-enzymatic and enzymatic reactions. Irradiation of water using y-radiation and UV radiation is the main reason for physical generation.
- Hydroxyl radical ($HO^{\bullet}$) - diatomic molecule with one unpaired electron on the oxygen atom. $HO^{\bullet}$ is a strong oxidant. It can be formed chemically and physically. Physical generation occurs because of irradiation of water using y-radiation and UV-irradiation. Chemical generation occurs due to the Fenton reaction [one-electron reduction of hydrogen peroxide by transition metals (Fe_2^{+}, Cu^{+}, and Zn^{+})] and the Haber-Weiss reaction (one-electron reduction of hydrogen peroxide by $O_2^{\bullet -}$).
- Hydrogen peroxide (H_2O_2) - non-radical signalling molecule initiates signalling pathways and cascades. H_2O_2 can act as an oxidant and reductant. Decomposition

leads to the formation of water and molecular oxygen. Can be formed by photorespiration in peroxisomes and catalysed by superoxide dismutase (SOD). (Fig. 2)

O_2 + 1e	⟶	$O_2^{\bullet-}$(superoxide)
O_2 + 2e + 2H+	⟶	H_2O_2 (hydrogen peroxide)
$H_2O_2 + O_2^{\bullet-}$	⟶	$2HO^{\bullet}$ (hydroxyl radical)
where e	=	electron

Figure 2. Reactions of radical formation.

2.3. Sources of reactive oxygen species

Reactive oxygen species are mainly produced in the plasma membrane, mitochondria, peroxisomes, and endoplasmic reticulum, where there is a higher consumption of oxygen (Fig. 3). Mitochondria are known to be the main source of ROS in the cell. As a result, they have been identified as the source of several clinical diseases. A chronic increase in ROS production causes an accumulation of ROS-associated damage in DNA, proteins, and lipids, which can lead to progressive cell dysfunction.

The largest by-product of mitochondrial oxidative phosphorylation is the superoxide anion radical ($O_2^{\bullet-}$) due to the contribution of complex I and complex III (Turrens, 2003). It is formed as a result of the leakage of electrons from the mitochondrial respiratory chain and the interaction of these electrons with O_2. Superoxide dismutase (MnSOD, SOD_2) from the mitochondrial matrix and superoxide dismutase (Cu/ZnSOD, SOD_1) from the mitochondrial intermembrane space convert the $O_2^{\bullet-}$ into H_2O_2, which can be catalysed to water (H_2O) (Suski et al., 2012).

In the case of peroxisomes, the xanthine oxidase reaction, which is found in microbodies such as peroxisomes, is the main metabolic process responsible for the generation of $O_2^{\bullet-}$. This process reduces molecular oxygen and produces $O_2^{\bullet-}$ by catalysing the oxidation of hypoxanthine to xanthine and xanthine to uric acid (Sharma et al., 2012).

In the case of plasma membrane, electron transporting oxidoreductases cause the formation of ROS. NADPH oxidase catalyses the transport of electrons from cytoplasmic NADPH to O_2, that lead to the formation of $O_2^{\bullet-}$, which then dismutates to H_2O_2 either spontaneously or through SOD (Sharma et al., 2012).

The endoplasmic reticulum (ER) is also responsible for the formation of ROS. Insufficient chaperone activity in the endoplasmic reticulum (ER) causes the accumulation of unfolded proteins, which is known as ER stress. For normal oxidative protein folding, the ER generates ROS such as H_2O_2 (Alzoghaibi, 2013).

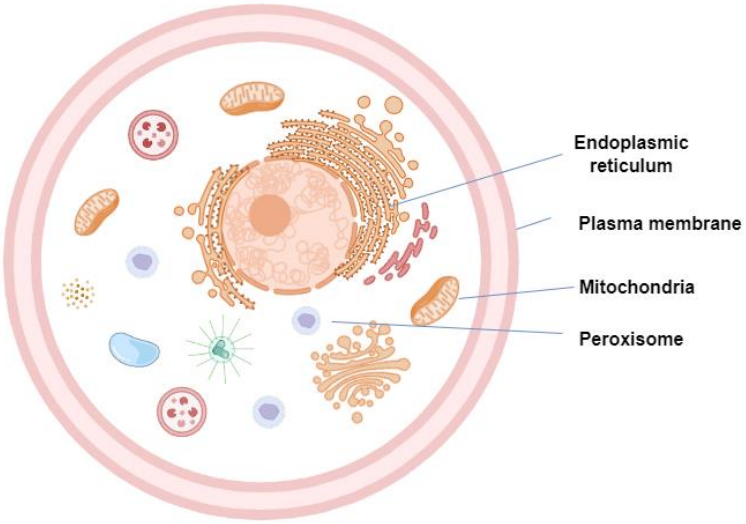


Figure 3. Localisation of reactive oxygen species in the cell.

2.4. Essential role of reactive oxygen species

ROS play an essential role in normal vascular physiology and participate in different pathways. One of the functions of ROS is the initiation of mitogen-activated protein kinase (MAPK) cascades. As a result, MPK, which was phosphorylated by MAPKK and MAPKKK, leads to the phosphorylation of target proteins (transcription factors). This cascade contributes to the specificity and amplification of transmitted signals that activate regulatory molecules in the cytoplasm and nucleus. These signals then initiate various cellular processes such as differentiation, proliferation, and development (Seger & Krebs, 1995). ROS participates in the regulation of gene expression by changing the redox state of a component of signalling pathways or transcription factors (most often oxidation of cysteine or methionine residues). ROS, as signalling molecules, regulate and maintain many physiological functions primarily through interaction with Cys residues of proteins. For example, H_2O_2 reacts with Cys thiolate anions of Cys-S- and oxidizes them to the sulfonyl form Cys-SOH, which leads to structural and functional changes in proteins. ROS take part in long distance signal transmission. The emergence of ROS production can initiate a cascade of intercellular communication, which leads to the formation of the ROS wave, which transmits a signal over long distances. Calcium is involved in this transfer, which activates NADPH oxidase after transport to another cell. ROS are

involved in the development and maintenance of the immunological defence system. When exposed to environmental pathogens, they play a role in the oxidative burst of the activation of the local inflammatory milieu. This is one of the first lines of defence that the immune system utilizes against invading pathogens (Alfadda & Sallam, 2012).

The impact of ROS can be positive and negative. The negative effects of ROS consist of the oxidation of polyunsaturated fatty acids, which causes the disruption of membranes. The emergence of different mutations since oxidative damage of DNA and enzymatic functions can be inhibited by ROS reactions. The positive effects of ROS include the expression of defence genes during stress, essential for the development and regulation of the cell cycle. It causes a hypersensitive response, which targets cell death to prevent progression of the pathogen to other neighbouring cells.

2.5. Oxidative stress

Molecular oxygen has two strongly bound electrons in separate orbitals of the valence layer that have the same spin. This property causes the oxygen base state to be triplet, and its excitation leads to a highly reactive singlet state. Under stressed conditions, the production of ROS in cancer cells increases and the level of antioxidants decreases. It leads to the oxidation of proteins, nucleic acids, and lipids under intrinsic or extrinsic factors; as a result, oxidative stress occurs. This process leads to the induction of gene mutations and changes in transcriptional processes that can cause indiscriminate damage to biological molecule, leading to loss of biomolecule's function and even the formation of cancer and other diseased conditions. On the other hand, if the production of ROS considerably decreases, reductive stress occurs and can cause pathologies from cancer to cardiomyopathy (Cristiana et al., 2018).

The interaction of ROS with biological macromolecules such as lipids, proteins, and nucleic acids causes oxidative damage (Fig. 4). According to earlier research, unsaturated bonds in membrane phospholipids are the primary targets of free radical reactions. Loss of membrane fluidity, receptor alignment, and cell lysis are the results of lipid peroxidation. (Opara, 2006).

Oxidative stress has been associated with several diseases, such as Alzheimer's and Parkinson's disease, cancer, diabetes mellitus, inflammatory diseases, as well as psychological diseases or ageing processes (Preiser, 2012).

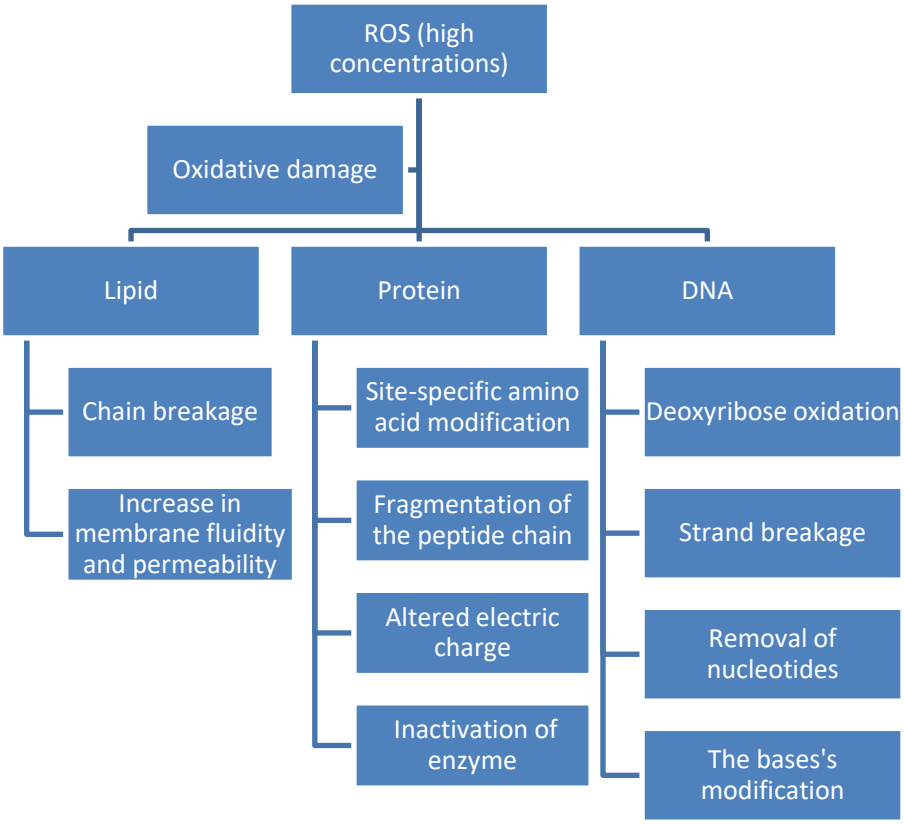


Figure 4. Oxidative damage of biological macromolecules.

2.6. Reactive oxygen species in disease

Reactive oxygen species have a variety of detrimental changes, which contribute to the development of an increasing number of diseases, as well as physiological regulating activities. Excessive amounts of ROS can cause a variety of ailments ranging from autoimmune illnesses to cardiomyopathies. There are numerous sources of ROS in the cell, including the cytosol, extracellular, and mitochondrial domains. Overproduction of ROS is harmful and can result in mitochondrial and cellular death due to apoptosome protein complex activation. Inflammatory diseases such as rheumatoid arthritis, multiple sclerosis, and atherosclerosis are often caused by excessive production of ROS, which is achieved through activation of the MAPK pathway. Several degenerative diseases are associated with oxidative damage to mitochondria, such as Parkinson's, Alzheimer's, and Huntington's disease. Individuals with mitochondrial DNA damage caused by ROS suffer from many symptoms typical of old age, such as neuromotor disorders, deafness, and dementia.

2.7. Antioxidants and defence mechanisms

An antioxidant is any substance or chemical compound whose mechanism is based on inhibition of free radical processes in the cell or destroying free oxygen radicals. Antioxidants inhibit the development of destructive processes. The level of antioxidant protection inside and outside the cell must be sufficient to counteract the toxic effects of lipid peroxides and maintain normal physiology. This balance can be impaired by excessive free radical formation or insufficient intake of nutrients containing antioxidant molecules. The maintenance of redox-antioxidant balance, which is the most important mechanism of homeostasis in living systems, is realized in the structural elements of the cell, mainly in membrane structures, such as mitochondria, chloroplast, plasma membrane (Alzoghaibi, 2013).

Antioxidants are classified into two types: enzymatic and non-enzymatic. The major enzymatic antioxidants are superoxide dismutase (SODs), glutathione reductase, and peroxidases such as catalase, ascorbate peroxidases, glutathione peroxidase. These chemical compounds have been found to play crucial roles in the antioxidant defences. SODs located in the cytosol and mitochondria, catalytically convert the $O_2^{\bullet-}$ into oxygen and H_2O_2 in the presence of metal ion cofactors such as copper (Cu), zinc (Zn), manganese (Mn). The enzyme catalase is present in the peroxisome, converts H_2O_2 to water and oxygen, while the enzyme glutathione peroxidase (GSHPx) converts the H_2O_2 into water and shows synergistic effect in the scavenging of $O_2^{\bullet-}$ (Fig. 5) (Nimse & Pal, 2015).

$$O_2^{\cdot-} \xrightarrow{(SOD)} H_2O_2 \xrightarrow{(CAT)} H_2O + \frac{1}{2} O_2$$

$$O_2^{\cdot-} \xrightarrow{(SOD)} H_2O_2 \xrightarrow{(GSHPx)} 2H_2O$$

Figure 5. Conversion of $O_2^{\bullet-}$ to H_2O_2 and its conversion to water and oxygen.

Non-enzymatic antioxidants include low-molecular-weight compounds, such as vitamins (vitamins C and E), carotenoids (xanthophylls and carotene), glutathione, coenzyme Q10 etc. Vitamin C (ascorbic acid), for instance, provides intracellular and extracellular antioxidant capacity primarily by scavenging oxygen free radicals, while vitamin E (α-Tocopherol) is concentrated in the hydrophobic interior site of the cell membranes and is the primary defence against oxidant induced membrane injury. Glutathione (GSH) is the most prevalent soluble antioxidant and is found in all cell compartments. GSH protects cells from apoptosis by interacting with pro-apoptotic and

anti-apoptotic signalling pathways. Carotenoids, mainly β-carotene, have been shown to react with peroxyl (ROO•), hydroxyl (•OH), and $O_2^{\bullet-}$. Carotenoids demonstrate antioxidant properties when oxygen partial pressure is low and are also known to impact cell apoptosis (Birben et al., 2012).

2.8. The detection of ROS

Reactive oxygen species are highly unstable molecules; detection of ROS is based on measuring the formation of products when they react with specific substances. ROS often have a short lifetime, making direct detection and identification complicated. Changes in fluorescence, colour, or luminescence of the end/oxidized products can be used to detect them (Jambunathan, 2010). The methods of ROS can be direct and indirect. Several techniques are used for the direct detection of ROS, such as electron paramagnetic resonance spectroscopy, fluorescent probes, chromatography methods, spectrophotome try methods etc. Indirect methods of ROS detection are ultra-weak photon emission and immunoblotting.

In this work, we used a confocal laser scanning microscope (CLSM), charge-coupled device (CCD) imaging, Photomultiplier tube (PMT), Western blotting, Electron paramagnetic resonance spectroscopy (EPR). The CLSM is designed to achieve the highest possible resolution by using a laser as a light source and image acquisition method. Briefly, the imaging mechanism is described as layer-by-layer imaging of a sample at the same level of depth of field, by limiting the depth of focus and using coherent point light sources. Confocal microscopy offers several advantages over conventional wide-field optical microscopy, including the ability to control the depth of field, the elimination, or reduction of background information, and the ability to collect successive optical slices from thick samples. This microscope allows imaging of both fixed and live cells and tissues.

Charge-coupled device imaging is an analogue integrated silicon chip consisting of light-sensitive photodiodes. It is based on charge-coupled device technology, which reads the electrical potential by shifting the charge from one element to another. The technical construction of a CCD is as follows: It is made up of a polysilicon material that is separated from a silicon substrate. The substrate itself changes the electrical potential near the electrodes when the voltage is applied via the polysilicon gates. Prior to exposure, a certain combination of voltages is applied to the electrodes to reset all previously generated charges and bring all elements to an equal initial state. During imaging, a

potential is created on the electrodes (during exposure, as a result of exposure to light), allowing electrons to accumulate in a particular pixel of the sensor. The photoelectric effect manifests itself, among other things, in such a way that more intense light allows more electrons to accumulate and creates a higher final charge for a given pixel.

The photomultiplier tube (PMT), consisting of a photocathode and an electron multiplier, is used to detect weak optical signals from weakly emitting sources. Photomultiplier current is amplified by a secondary electron emission. The most common types are photomultiplier tubes in which the electron flow is amplified by a system of discrete dynodes. The light incident on the photocathode emits photoelectrons, which, after being accelerated and focused by the electrodes, fall on the dynode. Several new electrons are produced as a result of the collision between the electron and the dynode and are then accelerated into another dynode. Some of the electrons is lost in the focussing and accelerating system.

Western blotting (protein blotting or immunoblotting) consists of transfer of proteins that have been separated onto a membrane (nitrocellulose or polyvinylidene difluoride (PVDF)) after electrophoresis. Usually, labelled antibodies are used to process the proteins transferred to the membrane. In this process, the primary antibody is specific for the horseradish peroxidase-attached indicated protein, and the secondary antibody is specific for the primary antibody. As several secondary antibodies can be attached to a single primary antibody in this two-step procedure, the signal is higher and the use of horseradish peroxidase as a tag allows for a highly sensitive chemiluminescent detection method. Proteins present in the cell can be detected by this western blotting method.

Electron paramagnetic resonance spectroscopy is based on the principle that paramagnetic materials placed in a constant magnetic field resonantly absorb electromagnetic radiation. It results from quantum transitions between the paramagnetic atoms' and ions' magnetic sublevels. In the super-high frequency spectrum, EPR spectra are most often observed. Using electron paramagnetic resonance, the influence of local magnetic fields on EPR spectra can be evaluated. Local magnetic fields reflect magnetic interactions in the system under study. As a result, both the structure of paramagnetic particles and their interaction with the environment can be analyzed using EPR spectroscopy.

2.9. Bioactive compounds

The bioactive compounds produced by plant metabolism have a wide range of chemical properties and perform many biochemical and physiological functions. Bioactive compounds can be produced from plants and animals. Fruits and vegetables are consumed more frequently because they contain high concentrations of nutrients that are beneficial for health, as well as their significant role in the prevention of a variety of chronic diseases. They contain an optimal balance of antioxidants including vitamins C and E, polyphenols, and carotenoids as well as complex carbohydrates, nucleotides, omega-3 fatty acids and organic acids (Kamiloglu et al., 2020). The actions of these antioxidants are varied. They can act as anti-carcinogenic, immune-supporting, antibacterial, antifungal, antiviral, anti-inflammatory, and stress-protective agents as well as regulating blood pressure. In this study, we used four bioactive compounds, namely chlorogenic acid, oleuropein, tomatine, and tyrosol, due to their antioxidant and stress protective properties (Aguilar et al., 2008).

2.10. Chlorogenic acid

The most common isomer of caffeoylquinic acid is chlorogenic acid (CGA, 3-CQA, 3-, 4- and 5-CQA). It belongs to the category of secondary phenolic metabolites (Fig. 6) produced by certain plant species such as tea, green roasted coffee, chocolate, berry fruits, apples, pears, citrus fruits and green roasted beans (Clifford, 2000; Naso et al., 2014). According to a study, coffee has the highest percentage of polyphenols among all beverages studied (Clifford, 2000). CGA is a significant and biologically active dietary polyphenol with a number of therapeutic and health benefits, including antioxidant, antibacterial, hepatoprotective, cardioprotective, anti-inflammatory, antipyretic, neuroprotective, anti-obesity, antiviral, antimicrobial, hypertension, free radical killing, and CNS stimulant activity.

The results of various scientific studies clearly show that CGA exhibits extremely beneficial anti-inflammatory and antioxidant effects (Yun et al., 2012). There is some evidence that CGA can affect lipid and glucose metabolism in inherited metabolic abnormalities (Zhang et al., 2011). In addition, CGA has demonstrated hepatoprotective effects, protecting mice from chemical or lipopolysaccharide-induced damage (Shi et al., 2013; Hemmerle et al., 1997). In another similar study, where rats with a hypercholesterolemic diet received CGA supplementation, CGA attenuated both the

decrease in plasma high-density lipoprotein concentration and the increase in total cholesterol and plasma low-density lipoprotein concentration (Wan et al., 2013). Clinical and scientific studies over the past several decades suggest that CGA administration may have antihypertensive effects (Zhao et al., 2012). CGA has been found to have antioxidant activity (Bai et al., 2022) and ability to preserve organs (spleen, thymus, and liver), which can be achieved by inhibiting blood glucose content as reported by Yuan et al., (2017), who fed CGA to mice. There is increasing scientific evidence that the metabolites of CGA, ferulic acid and caffeic acid (CA), may also have a hypotensive effect (Onakpoya et al., 2015).

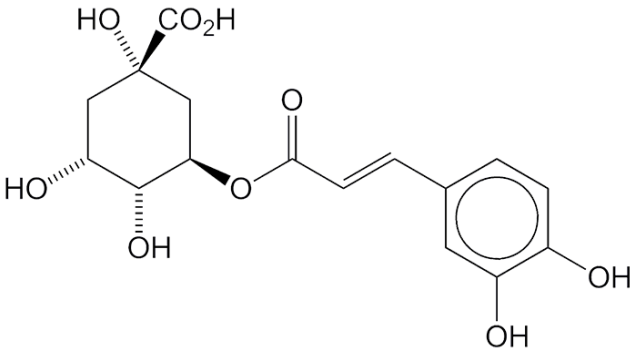


Figure 6. The structure of chlorogenic acid (source: ACS Chemistry for life; webpage: https://www.acs.org/molecule-of-the-week/archive/c/chlorogenic-acid.html).

2.11. Oleuropein

Oleuropein is an ester of hydroxytyrosol that has an oleosidic skeleton and a carbohydrate group. Oleuropein belongs to the secoiridoid class of compounds, which are common in plant families such as *Oleaceae*, *Gentianaceae*, and *Cornaleae*. *The* secoiridoid glucosides are often characterised by their oleosidic skeleton (Fig. 7). Geraniol, 8-oxogeranial, Iridotrial, 7-deoxycholic acid, 7-epiloganic acid, 7-ketologanic acid, 7-ketologanin, oleoside-11-methyl ester, and 7-1-d-glucopyranosyl-11-methyl oleoside are the substrate intermediates. Tyrosol is attached to 7-b-1-d-glucopyranosyl-11-methyl oleoside in the process of making oleuropein, which produces ligstroside and 3,4 DHPEA-EDA. While 3,4 DHPEA-EDA is transformed first into oleuropein aglycone and then into oleuropein, ligstroside is a direct pathway to oleuropein (Shamshoum et al., 2017).

Oleuropein is notably abundant in raw olive fruits and leaves. Oleuropein concentrations in young olives can reach 140 $mg \cdot g^{-1}$ on a dry matter basis (Amiot et al., 1986), while in leaves, it can range from 60 to 90 $mg \cdot g^{-1}$ (Silva et al., 2006). During the

mechanical extraction of the oil from green olives, the Oleuropein (Ole) is hydrolysed by the activity of endogenous β-glucosidase to produce oleuropein aglycone (OleA). Olive tree leaves are a traditional Mediterranean herbal medicine that have been used for centuries to treat chronic illnesses and reduce stress levels. The oleeuropein content varies depending on the cultivar, the region of production, and the state of the leaf tissue (fresh frozen, dried, or lyophilised).

In a recent study (Larussa et al., 2017), it was found that giving OleA to ulcerative colitis patients who had colon biopsies resulted in lower levels of COX-2 and IL-17, which significantly reduced colonic tissue inflammation. This data supports the use of oleuropein as an anti-inflammatory agent. In similar research, oleuropein dramatically reduced the viability of HepG2 and Huh7 cells, suppressed cell proliferation, and caused apoptosis in these hepatocellular carcinomas (HCC) cell lines. Oleuropein's ability to induce apoptosis in HepG2 liver cancer cells was linked to the stimulation of activated caspase-3, caspase-8, caspase-9, PARP, and Bax proteins and to the downregulation of antiapoptotic Bcl-2 protein (Yan et al., 2015).

Oleuropein is widely known for its high antioxidant effects, which it shares with other phenols found in olive leaves and olive oil. Oleuropein has an ortho-phenolic group in its chemical structure that can stabilize oxygen radicals through an intramolecular hydrogen bond and scavenge ROS through hydrogen donation. A significant antioxidant property is specifically conferred by an o-diOH substitution. Oleuropein due to its antioxidant and anti-inflammatory activity, as well as other features of action such as autophagy inducer and amyloid fibril growth inhibitor, and finally due to its anticancer effect, is effective in neuro- and cardiovascular disease, diabetes, chronic kidney disease, and cancer (Nediani et al., 2019).

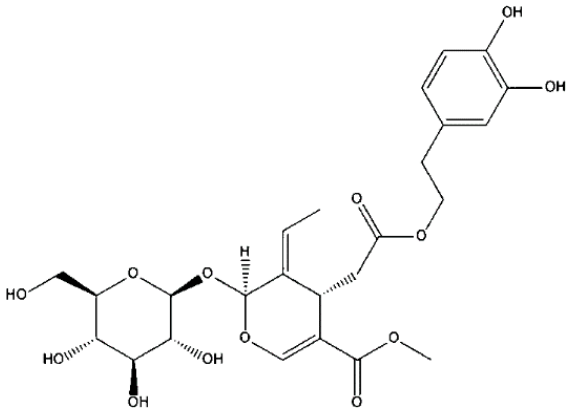


Figure 7. The structure of oleuropein (Sahin & Bilgin, 2017).

2.12. Tomatine

The tomato, which is a member of the *S. lycopersicum* L. species, contains many nutrients, including vitamins, minerals, carbohydrates, fiber, and carotenoids with demonstrated antioxidant potential. The nutritional content of mature red tomatoes is highly recognised and they also have significant anti-inflammatory, antioxidant, and anticancer properties (Faria-Silva et al., 2022). α-Tomatine has three basic structural components (Fig. 8). The first is a hydrophilic tetrasaccharide side chain (-likotetraose) made up of one D-galactose molecule, two D-glucose molecule, and one D-xylose molecule. Second, the hydrophobic part, the steroid moiety, also referred to as an aglycone, which has an oxa- and azaspirodecane system attached to a C27-cholestane skeleton (spirozolane). The polar group -NH is the third structural component (Taveira et al., 2014).

α-Tomatine, which is the main tomato steroidal glycoalkaloids (SGA) and saponin, is present mostly in the stems, leaves, and roots of vegetative tissues and unripe green fruits (*S. lycopersicum* L.) (Arena et al., 2018). The amount of α-tomatine depends on the part of the plant where the leaves and flowers have the highest concentration, and the roots have the lowest. In addition to promoting humoral and cellular immunity, α-tomatine has several biological effects, among which are antiviral, fungicide, antibiotic, anti-inflammatory, anticarcinogenic, skin anti-ageing, and others (Chao et al., 2012).

Several functions of -tomatine and its aglycon and their potential as chemo preventive or chemotherapeutic agents have recently been investigated (Bailly, 2021). α-Tomatine appears to be significantly helpful in the prevention of cancer and pathogenic gene infections, and tomatidine is generally considered as a preventive agent. Numerous studies using *in vitro* and *in vivo* models have shown that tomatine can stop the growth of many cancer cell lines, including those from the breast, colon, liver, stomach, leukemia, lung, mammary, and prostate (Chao et al., 2012).

In addition, tomatine is considered to prevent lung cancer from metastasising (Shih et al., 2009). The human lung adenocarcinoma cell line's ability to spread and infect is considerably decreased by α-tomatine (A549). This impact was associated with the deactivation of the extracellular signal-regulated kinase 1 and 2 (ERK1/2) or phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) signalling pathways, which control the activities of matrix metalloproteinase-2 (MMP-2) and matrix

metalloproteinase-9 (MMP-9) (Faria-Silva et al., 2022).

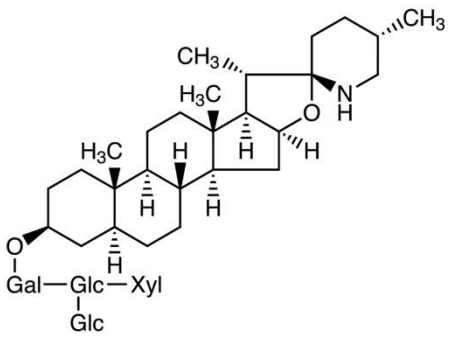


Figure 8. The structure of tomatine (from Cymit, CAS: 17406-45-0, webpage: https://cymitquimica.com/products/3B-T3636/17406-45-0/tomatine-from-tomato/).

2.13. Tyrosol

Tyrosol [2-(4-hydroxyphenyl)-ethanol], a biophenol, is found in many different natural sources, primarily in foods like wine and olive oil (Fig. 9). Tyrosol has a wide range of biological effects, including antioxidant, stress reduction, anti-inflammatory, anti-cancer, cardioprotective, neuroprotective, and many others (Plotnikov & Plotnikova, 2021).

It has been shown to be a powerful cellular antioxidant and radical scavenger (Di Benedetto et al., 2007). It can help restore the imbalance between increased oxidative stress and impaired antioxidant defence, which promotes the progression of atherosclerotic disease. Tyrosol is a relatively stable substance, which makes it much less sensitive to auto-oxidation than other polyphenols. Even in a critical state, it retains its antioxidant function. Tyrosol retained its antioxidant activity in the presence of oxidised LDL (low-density lipoprotein) when autoxidation processes had already started (Perona et al., 2006).

Several studies have shown that cell death is caused by oxidative stress, which is induced by chemical oxidants such as H_2O_2 (Cai et al., 2008). Furthermore, H_2O_2 has been shown to induce apoptotic modifications that ultimately lead to cell death in various cellular systems, including L6 cells (Xie et al., 2017). In this study, researchers used oxidative stress in L6 muscle cells to assess the antioxidant capabilities of tyrosol. This finding was further supported by Western immunoblotting results for cleaved caspase-3, which is a component of apoptotic cell death induced by H_2O_2. These data suggest that tyrosol prevents H_2O_2-induced apoptosis by inhibiting caspase-3 activation (Lee et al., 2018).

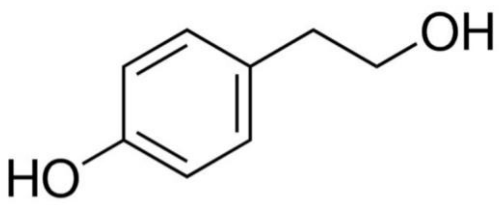


Figure 9. The structure of tyrosol (source: MERCK, PHL80166; webpage: https://www.sigmaaldrich.com/CZ/en/product/supelco/phl80166).

3. Materials and Methods

3.1. Extracts

Olive leaf (*Olea europaea* L.) were utilized to create the Oleuropein extract, while tomatine was derived from tomato leaves (*Solanum lycopersicum* L.). The methods of extraction for both tomatine and Oleuropein were previously described in published reports (Tamasi et al., 2019). Chlorogenic acid (with a purity of more than 97%) and tyrosol (with a purity of more than 98%) were commercially procured and used without any additional purification.

3.2. Cell lines

The U-937 cell line is a type of human myeloid leukaemia that has a pro-monocytic characteristic. It was obtained from the histiocytic lymphoma of a 37-year-old male and is employed in studying the behaviour and differentiation of monocytes. These cells can be prepared in large quantities and are generally uniform in nature, making them a valuable tool for research purposes (Chanput et al., 2015).

The HL-60 cell line originates from a 36-year-old woman who suffered acute promyelocytic leukemia. The majority of these cells (over 90%) are promyelocytes, and they offer a distinct model for examining how normal and cancerous cells grow and mature in a laboratory setting (Breitman et al., 1980).

The THP-1 cell line is a type of human monocytic leukemia that was obtained from the blood of an individual with acute monocytic leukemia. These cells have similar physical and differentiation characteristics to primary monocytes and macrophages (Chanput et al., 2015).

3.3. Cell density, growth, and viability

The TC20 automated cell counter from BIO-RAD Laboratories (Hercules, California) is capable of accurately counting cells using autofocus technology and a complex cell-counting algorithm that measures cell growth, viability, and density (Fig. 10). To determine the number of viable cells in a cell suspension, the trypan blue dye exclusion test (Sigma-Aldrich, GmbH, Germany) was used. Before using the Automated Cell Counter TC20 BIO-RAD, a mixture of 10 µl of cell suspension and 2.5 µl of 0.4% trypan blue dye was prepared.

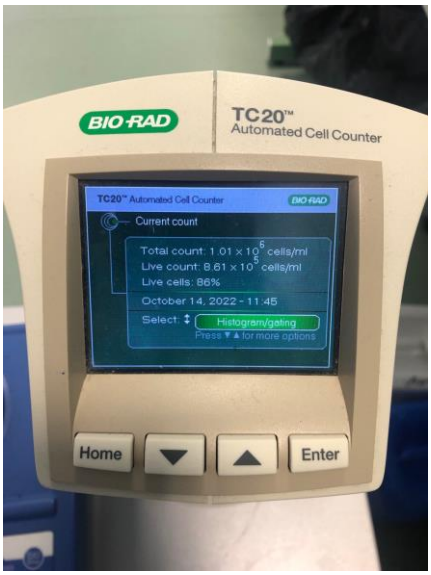


Figure 10. The TC20 automated cell counter.

3.4. Differentiation of cell lines

The U-937, THP-1 and HL-60 cell lines were grown in suspension culture containing RPMI-1640 medium. This medium includes the reducing agent glutathione, as well as high amounts of vitamins, such as biotin (vitamin B7), vitamin B12, and PABA (vitamin B10). Fetal bovine serum (FBS) and antibiotics [penicillin/streptomycin] were added to RPMI-1640 medium. The foetal bovine serum is made from whole blood, where platelets and fibrinogen are removed. All proteins that are not involved in blood coagulation, as well as all electrolytes, antibodies, antigens, hormones, and exogenous chemicals, are found in this serum. Antibiotics penicillin and streptomycin are commonly used to prevent bacterial contamination of cell cultures due to their effective combined activity against Gram-positive and Gram-negative bacteria. Suspension cultures containing cells were cultivated in an incubator at 37°C in 5% CO_2 atmosphere. For differentiation studies, cells were treated with different doses of phorbol 12-myristate 13-acetate (obtained from Sigma Aldrich (St. Louis, Missouri, United States). Based on pilot experiments and preliminary results obtained, 250 nM for U-937 cells, 75 nM for HL-60 cells, 150 nM for THP-1 cells were used to induce differentiation at 37°C in 5% CO_2 atmosphere for 72 h.

3.5. Treatment with bioactive compounds

After differentiation, the medium was removed, and a fresh medium was added. The cells were then incubated with bioactive compounds (chlorogenic acid, oleuropein, tomatine,

tyrosol) for 24 h (resting period) at 37°C in a 5% CO_2 atmosphere.

3.6. Cell harvesting and protein isolation.

Incubated cell lines were collected and centrifuged at 2000 rpm, 21°C for 10 min. After centrifugation, the pellets were resuspended in 1 mL of phosphate buffered saline (PBS) (pH 7.4) and centrifuged at 2000 rpm, RT for 10 min. The last step was repeated to remove PMA residues. Cell lysis was performed by adding 200 µl Radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris (pH 8.0), 0.5% sodium deoxycholate, 0.1% SDS and 1% NP-40) containing 1% protease and phosphatase inhibitor (v/v). Eight cycles of sonication were done (a total of 2 min., 40 sec). A UP50H ultrasonic processor was used for sonication. The cells were then centrifuged at 14,000 rpm, 4°C for 30 min. Following this step, the supernatant was collected from each cell and prepared for protein estimation.

3.7. Bicinchoninic acid Assay for protein estimation

The precise amount of protein must be determined and therefore, the Pierce BCA Protein Assay kit was utilised. Using bicinchoninic acid (BCA) in combination with the well-known conversion of Cu^{2+} to Cu^{+} by a protein in alkaline media to determine copper cations (Cu^{+}). 2400 µl of AB solution from the Pierce BCA protein estimation kit (Thermo Fisher Scientific, Paisley, UK) was mixed with samples (U-937, THP-1, HL-60 cells) and 7 standards. A 96-well plate with samples was incubated for 30 min at 37°C and the absorbance was read at 562 nm in the spectrometer.

3.8. Protein Immunoblotting

Homogenates of U-937, THP-1, and HL-60 cells separated on a 10% SDS gel were transferred to nitrocellulose membranes using the Trans-Blot Turbo transfer system (Bio-Rad, Hercules, CA, USA). Nitrocellulose membranes (Bio-Rad Laboratories) were then incubated with blocking solution buffer (5% BSA in Tris buffered saline (TBS) (pH 7.4) containing 0.01% Tween 20) overnight at 4°C. The blocked membranes were then probed with anti-malondialdehyde (anti-MDA) antibody (Abcam, Cambridge, UK) (dilution 1:5000) at room temperature for 2 h and incubated for 1 h at room temperature with HRP-conjugated anti-rabbit secondary antibody (dilution 1:10,000) (Bio-Rad, Hercules, CA, USA). Membranes were prepared for imaging using Immobilon Western

Chemiluminescent HRP Substrate (Sigma Aldrich, GmbH, Mannheim, Germany) (Fig. 11).

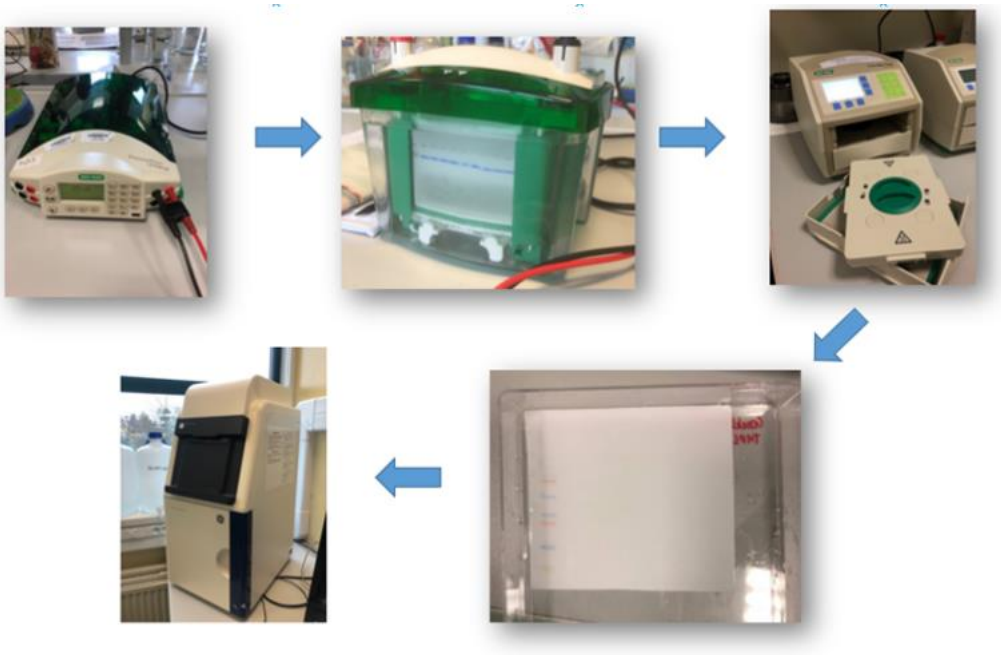


Figure 11. Brief description of the immunoblotting procedure.

3.9. Ultra-weak photon emission (UPE) measuring system

A complete dark room free of any interference is a prerequisite to avoid any interference in photon emission measurement. All ultra-weak photon imaging data for the current study were collected in an experimental dark room. Details on the adopted methodology can be accessed in published article (Prasad & Pospíšil, 2013). Two-dimensional photon emission imaging was measured in human cells utilising a sensitive CCD camera (Fig. 12). To eliminate any interference from delayed luminescence, the samples were adapted to darkness for 15 min. before processing. Briefly, the VersArray 1300B CCD camera (Princeton Instruments, Trenton, New Jersey, USA) was used with the following settings: scan rate, 100 kHz; win, 2; accumulation time, 30 min. The VersArray 1300B CCD camera has spectral sensitivity in the 350-1000 nm range with a quantum efficiency of about 90%. The CCD camera was cooled to -110°C using a liquid nitrogen cooling system. Before each measurement, the data correction was done by subtracting the background noise from the experimental data set.

For two-dimensional CCD imaging, U-937, THP-1, HL-60 cell lines were grown in suspension culture containing RPMI-1640 medium for measurement in CCD camera. 2.5 ml of each cell in a Petri dish was placed in front of the chamber to measure ultra-weak

photon emission. Cells were induced to undergo respiratory burst by exogenous addition of PMA (50μM).

Photon emission kinetics was performed using photomultiplier tube (PMT R7518P) [spectral sensitivity: 185-730 nm] (Fig. 12). PMT was cooled to -30°C using thermoelectric cooler C9143 (Hamamatsu Photonics, K.K., Iwata City, Japan). To study the kinetics of ultra-weak photon emission, photomultiplier tube (PMT) was used. U-937, THP-1, and HL-60 cell lines were grown in suspension culture containing RPMI-1640 medium and PMA was then added to the cell suspension similar to that seen in CCD imaging to mimic respiratory burst.

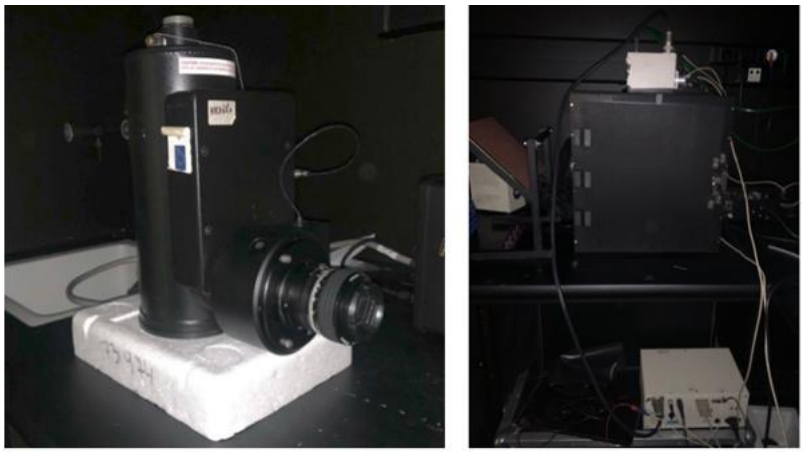


Figure 12. Charge-coupled device (CCD) imaging on the left side of the figure, Photomultiplier tube (PMT) on the right side of the figure.

3.10. Electron paramagnetic resonance spectroscopy (EPR)

To detect the formation of carbon centered radicals (R$^{\bullet}$) in cells, we used EPR spin-trapping technique using POBN (4-pyridyl-1-oxide-N-tert-butylnitrone) as the spin-trap compound. The U-937, THP-1, HL-60 cell lines were grown in suspension culture containing RPMI-1640 medium and treated with 50μM PMA to induce respiratory burst.

4. Results and Discussion

4.1. Growth curve of U-937 cells

The density of viable cells in the culture with incubation time is presented by a growth curve in which several growth phases are distinguished. The growth curve allows the growth and behaviour of the cell culture to be determined. The growth curve was monitored for 5 days to detect inaccuracies, if any, in the cell line before using the cells in the experiment (Fig. 13). The growth curve is characterized by 4 main phases. The initial phase (lag phase) - the cells grow but do not multiply. The exponential (log) growth phase is when the cells multiply intensively. Stationary phase - when the number of viable cells reaches a maximum and does not increase sharply anymore. The death phase, when metabolic wastes accumulate, nutrients are depleted, and cells die. We have observed an initial growth phase, an exponential phase, and a stationary phase.

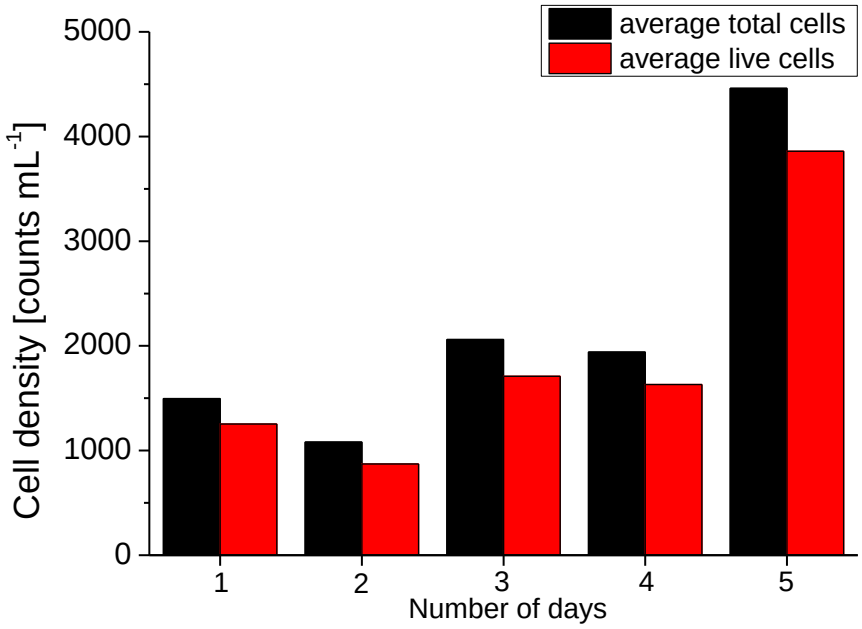


Figure 13. The growth curve of U-937 cells was monitored for 5 days using the Bio-Rad TC20 automated cell counter.

4.2. U-937 cell differentiation under phorbol-12 myristate-13-acetate (PMA)

In response to numerous stimuli, including phorbol 12-myristate 13-acetate (PMA), the human monocyte-like cell line U-937 is capable of differentiating monocytes from the promonocytic cell line into cells that resemble macrophages. Protein kinase C, a family of enzymes involved in a variety of biological processes such as cell proliferation, differentiation, apoptosis, and immunological responses, is robustly activated by PMA. PMA can lead to the phosphorylation and activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, which are enzymes that generate ROS such as $O_2^{\bullet-}$ and H_2O_2 (Smith et al., 1998). Its effects on U-937 cells depend on the concentration and duration of exposure. We measured morphological changes after 72 h of incubation with CLSM.

At low concentrations (250 nM), treatment with PMA of U-937 cells leads to increased phagocytic activity, cytokine production, and differentiation into macrophage-like cells. It can be observed that with increasing PMA concentration, the shape of the cells changes from spherical to oval with visible pseudopodia (500 nM). However, at higher concentrations (such as 1μM and 5 μM), PMA can induce apoptosis or cell death in U-937 cells (Fig. 14). Consequently, we determined that the response of U-937 cells to 250 nM PMA treatment is optimal for the cells.

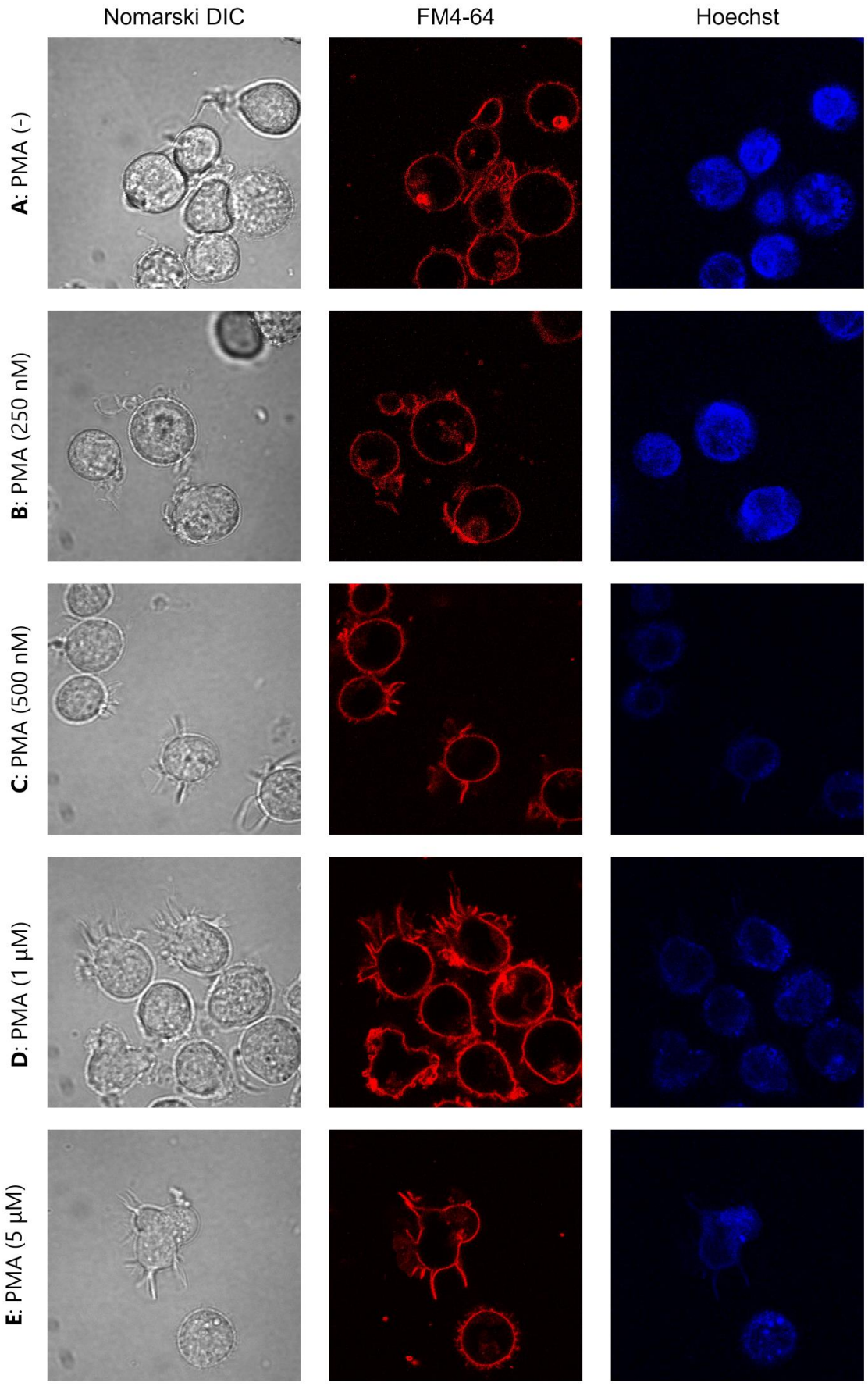


Figure 14. U-937 cells differentiation under different concentrations (250 nM; 500 nM; 1 µM; 5 µM) of PMA.

4.3. Toxicity in U-937 and THP-1

Reactive oxygen species are recognized as one of the main regulators of the immune system, neurological system, infection, and cancer development. Cell function depends on ROS homeostasis. Overproduction of ROS can cause oxidative stress and increase cell toxicity. The toxicity of U-937 and THP-1 cells was measured to determine which of the bioactive compounds (chlorogenic acid, oleuropein, tomatine, tyrosol) used are the most effective in reducing cell survival. Since different cell types were used, the goal was to find the most effective bioactive compounds for all three cell types. Cell toxicity measurements were made with an TC20 automated cell counter from BIO-RAD Laboratories, based in Hercules, California. 10 µl of cell suspension and 2.5 µl of 0.4% trypan blue dye were prepared. The device showed the total number of cells and the viable number of cells.

In the case of U-937 cells, the effects of chlorogenic acid, oleuropein, tomatine were comparable and showed a viability percentage above or equal to 80% (Fig. 15). While in the case of THP-1 cells, chlorogenic acid and oleuropein affected the cells more and viability decreased by about 75%. Tyrosol in both cases showed higher viability (Fig. 16). In general, it can be considered that all compounds are not lethal as viability around 70% or more in a cell population is considered to be in the acceptable range.

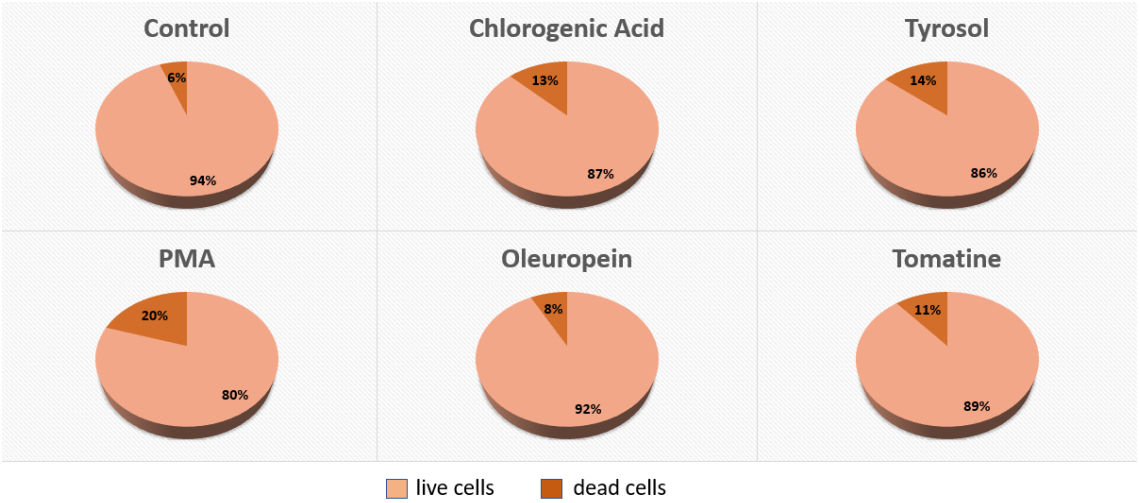


Figure 15. Toxicity of compounds on cell viability of U-937 cells.

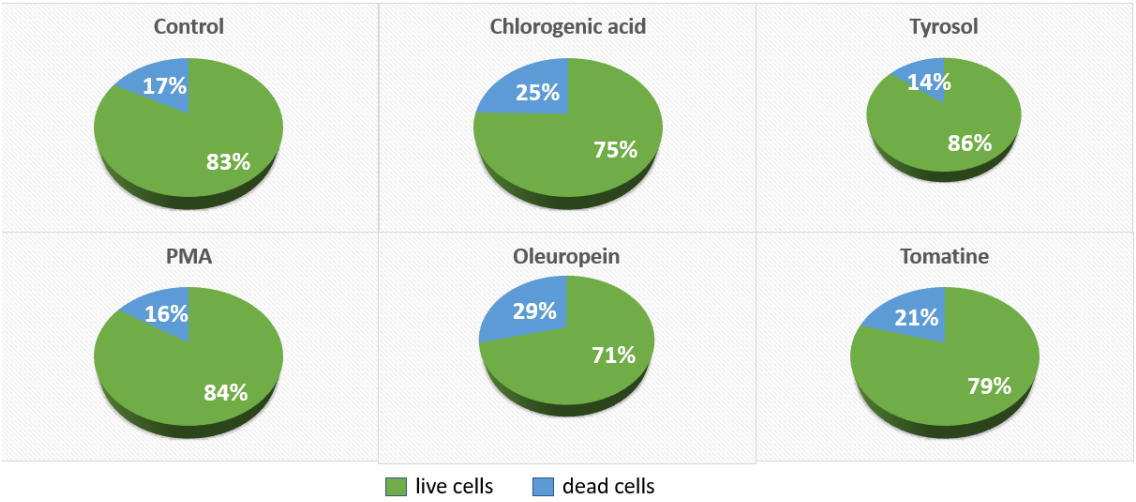


Figure 16. Toxicity of compounds on cell viability of THP-1 cells.

4.4. Ultra-weak photon emission (UPE) imaging of U-937, THP-1 HL-60 cell cultures

A CCD camera is a silicon semiconductor light-sensitive device. The collection of photons on the surface of a silicon grid with several wells or pixels serves as the basis for image generation in a compact compact device (CCD) camera. The photoelectric effect converts the photons in the well into an electrical charge, which is then sent to the image digitiser and transformed into an electrical signal representing the pixel's intensity. Using a CCD camera, we measured UPE in U-937, THP-1, and HL-60 cell cultures in different states: spontaneous emission, respiratory burst, and H_2O_2 (Fig. 17). Spontaneous emission of UPE refers to the phenomenon where living cells spontaneously emit very low levels of photons without any external stimulation. The results show that cells emit a low level of photon emissions. Respiratory burst refers to the rapid release of reactive oxygen species (ROS) by cells as a response to induction; only the U-937 cells showed sufficient amount of UPE. 500μM of H_2O_2 was exogenously used to mimic the respiratory burst and to see pronounced signals in each cell. H_2O_2 was also added to cells after removing RPMI-1640 medium to prevent their intervention.

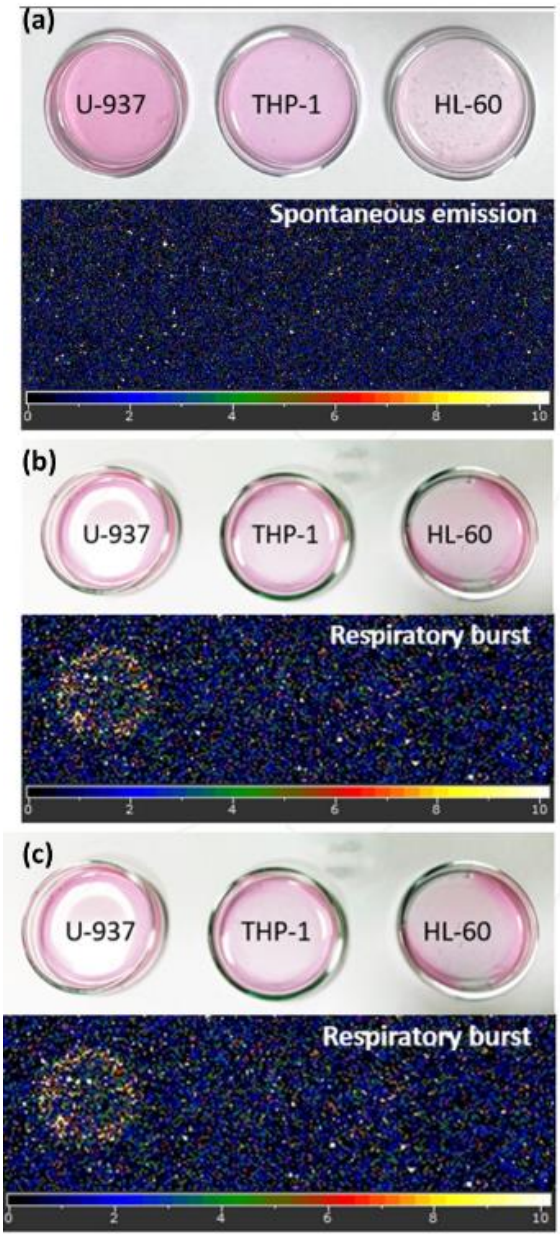


Figure 17. Ultra-weak photon emission imaging from U-937, THP-1, HL-60 cell cultures using a CCD camera. Spontaneous emission of UPE (a). Respiratory burst (b), exogenous addition of H_2O_2 (c).

4.5. Kinetics of ultra-weak photon emission (UPE) in U-937 THP-1 HL-60 cells

Spontaneous UPE was detected in U-937, THP-1 HL-60 cell cultures using a photomultiplier tube (PMT). The photomultiplier tube detects the kinetics of UPE emitted by living system. Depending on the UPE signal reflected by counts/ gate time (in our case, photon counts/s), the area below the curve or total counts can be deduced and interpreted.

In the results presented, the signal in respiratory burst (after addition of PMA), was found to be slightly higher in each cell compared to controls (Fig. 18). Mimicking the respiratory burst by adding H_2O_2 to high concentration showed a substantial enhancement in photon emission. The H_2O_2 treated cells showed a variable pattern. After adding H_2O_2 to the U-937 and HL-60 cell cultures, there was a sharp rise, after which the photon emission gradually began to decrease. However, in THP-1 cells, a typical propagation reaction is evident, where a radical and molecular oxygen interact to produce a peroxyl radical fatty acid. This unstable species generates another free fatty acid radical and lipid peroxide when it combines with a different free fatty acid.

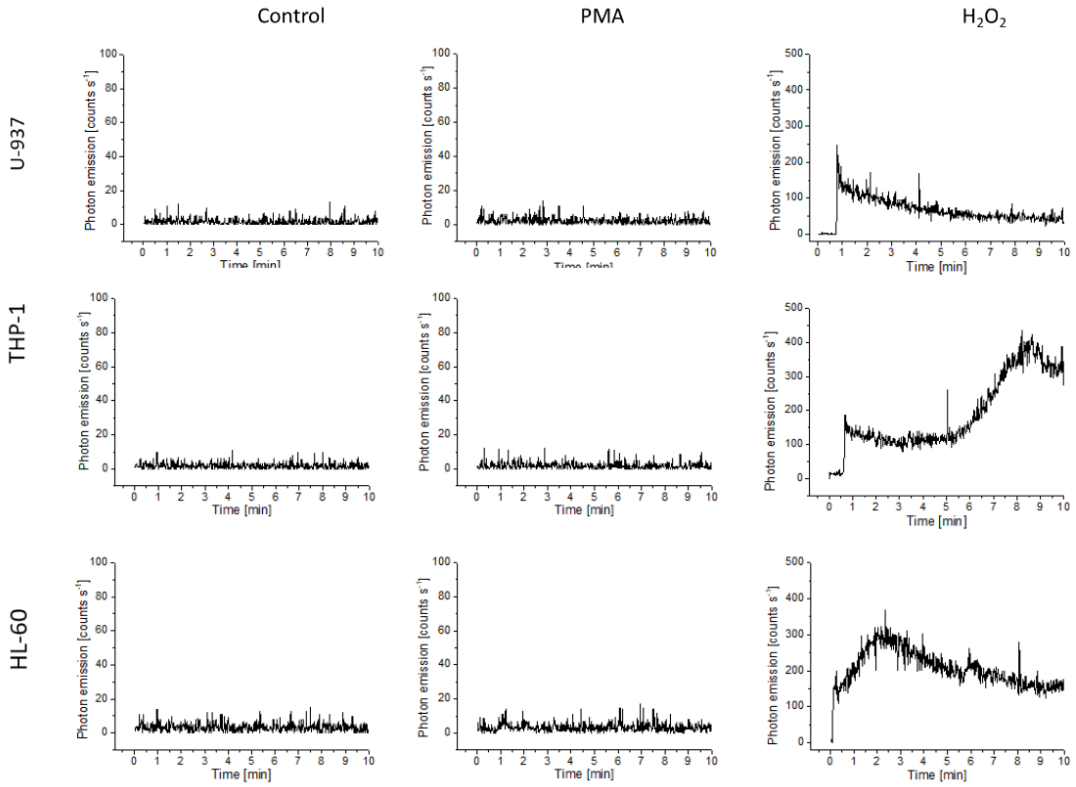


Figure 18. Ultra-weak photon emission from U-937, THP-1, and HL-60 cell cultures using PMT. *Control:* U-937, THP-1, HL-60 cell cultures. *PMA:* U-937, THP-1, HL-60 cell cultures and 50µM PMA H_2O_2: U-937, THP-1, HL-60 cell cultures in phosphate-buffered saline (PBS) with 500µM H_2O_2.

4.6. Spin trapping and formation of carbon-centred radicals using EPR spin trapping spectroscopy

The formation of carbon-centred radicals was measured by electron paramagnetic resonance (EPR) spectroscopy. EPR is an effective technique for analysing chemical species with one or more unpaired electrons of a radical. EPR spectroscopy has evolved into a direct method for identifying free radicals produced chemically or synthesised in biological systems. This technique with numerous spin trapping agents are used to identify specific radicals by forming a relatively stable spin adduct. In our experiment, the cells were differentiated within 72 h after the addition of 50μM PMA, leading to the formation of respiratory bursts in the cells [generation of hydroxyl radicals ($HO^{\bullet}$)]. Spin-trapping was performed with POBN, which react with $HO^{\bullet}$. In the results, the formation of ROS during a respiratory burst causes a pronounced enhancement in the signal (relative intensity) (Fig. 19).

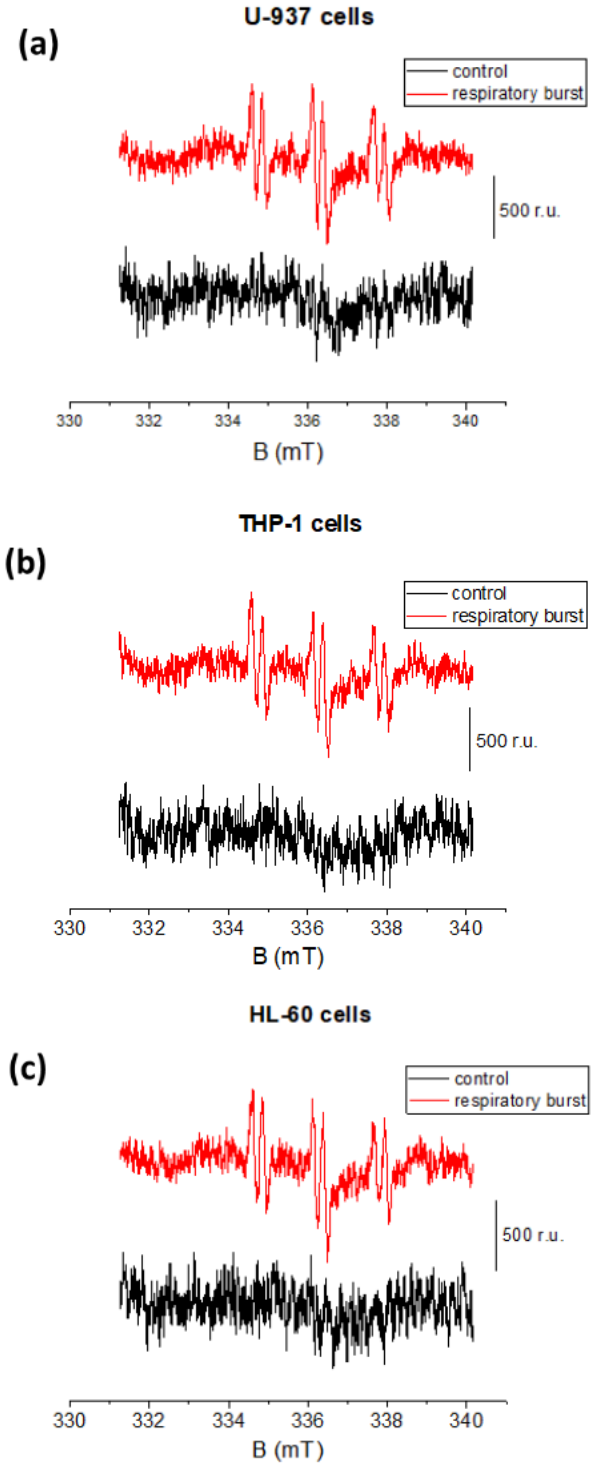


Figure 19. The formation of carbon-centered radicals in the U-937, THP-1 HL-60 cells detected using EPR spin trapping spectroscopy. (a) carbon-centered radicals in U-937 cells. (b) carbon-centered radicals in THP-1 cells. (c) carbon-centered radicals in HL-60 cells.

4.7. Western blotting

Western blotting in our experiments was used to detect malondialdehyde-oxidized proteins, which are immobilized on a nitrocellulose membrane. Cells were treated with bioactive compounds (chlorogenic acid, oleuropein, tomatine, tyrosol). The primary antibodies used in our experiments was a rabbit polyclonal anti-malondialdehyde antibody used for an incubation time of 2 h. Following washing, cells were incubated with Horseradish peroxidase (HRP) conjugated anti-rabbit polyclonal secondary antibodies. Cellular differentiation in U-937 and THP-1 cells resulted in the formation of MDA-protein adduct. The production of MDA that led to the protein modification was because of the oxidation of lipid predominantly polyunsaturated fatty acids (PUFA). In the presented result, protein modification can be seen in both cell type (U-937 and THp-1) (Fig. 20 and Fig. 21). It can also be clearly seen that the addition of bioactive compound lead to suppression of protein MDA formation in most cases.

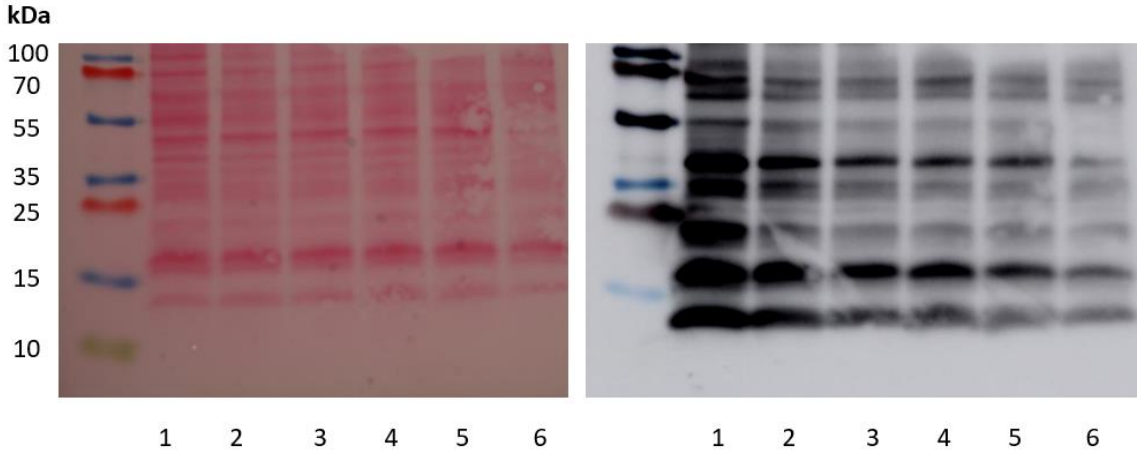


Figure 20. Identification of protein MDA adducts in U-937 cell. Lane 1: Control; Lane 2: PMA, Lane 3: Chlorogenic acid, Lane 4: Oleuropein, Lane 5: Tomatine, Lane 6: Tyrosol.

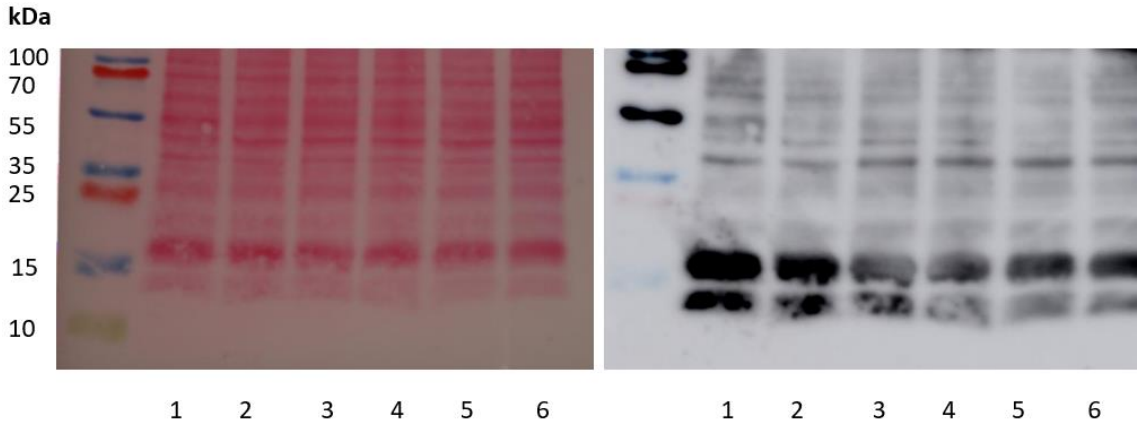


Figure 21. Identification of protein MDA adducts in THP-1 cell. Lane 1: Control; Lane 2: PMA, Lane 3: Chlorogenic acid, Lane 4: Oleuropein, Lane 5: Tomatine, Lane 6: Tyrosol.

5. Conclusion

The aim of this work was to learn handling of cell lines and culturing procedures for human cell lines such as U-937, THP-1, and HL-60 cells and to understand cell growth, viability, and mortality patterns using the TC20 automated cell counter and the trypan blue dye exclusion test. Spatial and temporal imaging of oxidative stress and ROS characterization and protein oxidation in human cancer cells was also carried out using various techniques such as CCD camera, EPR spectroscopy, PMT, Western blotting, and confocal laser microscopy. CCD camera showed ultra-weak photon emission images in U-937, THP-1, and HL-60 cell cultures in different states: spontaneous emission, respiratory burst, and the addition of H_2O_2. It was concluded that imaging of ultra-weak photon emission could be used as a non-invasive tool for the analysis of physiological conditions. The respiratory burst signal was found to be slightly higher than that of the control. Simulation of respiratory burst with H_2O_2 showed a significant increase in photon emission. Cells treated with H_2O_2 showed a different pattern in U-937 and HL-60 cell cultures, which can indicate the progression of cells towards the recovery process and/or apoptosis for instance, THP-1 cells under our experimental condition showed a typical propagation reaction. Finally, effect of several natural compounds/bioactive compounds such as (chlorogenic acid, oleuropein, tomatin, tyrosol) were used to measure the potential usage as strong antioxidants. The direct effect of the compounds was tested on protein oxidation levels under stress conditions and most of the compunds showed a suppression in protein modification.

6. References

Article citation:

ACS Chemistry for life. https://www.acs.org/molecule-of-the-week/archive/c/chlorogenic-acid.html (03.05.2023)

Aguilar, G., Sánchez, M., Martínez-Téllez, M., Olivas, G., Alvarez-Parrilla, E., De la Rosa, L. (2008). Bioactive compounds in fruits: Health benefits and of storage conditions. Stewart Postharvest Review. 4. 1-10. 10.2212/spr.2008.3.8

Alfadda, A. A., & Sallam, R. M. (2012). Reactive oxygen species in health and disease. Journal of biomedicine & biotechnology, 2012, 936486. https://doi.org/10.1155/2012/936486

Alzoghaibi M. A. (2013). Concepts of oxidative stress and antioxidant defense in Crohn's disease. World journal of gastroenterology, 19(39), 6540–6547. https://doi.org/10.3748/wjg.v19.i39.6540

Amiot, M.J., Fleuriet, A., and Macheix,, J. J., Macheix Journal of Agricultural and Food 1986 34 (5), 823-826, DOI: 10.1021/jf00071a014

Arena, M. P., Govers, C., Lotti, C., Ricciardi, L., Wichers, H. J., & Mes, J. J. (2018). The Effect of Tomatine on Gene Expression and Cell Monolayer Integrity in Caco-2. Molecules (Basel, Switzerland), 23(3), 644. https://doi.org/10.3390/molecules23030644

Ascensao, J. L., & Mickman, J. K. (1984). Differentiation of human leukemic cell lines (HL60, U937) toward macrophages is accompanied by production of colony-stimulating activity (CSA). Experimental hematology, 12(3), 177–182.

Bai, D., Liu, K., He, X., Tan, H., Liu, Y., Li, Y., Zhang, Y., Zhen, W., Zhang, C., Ma, Y. (2022). Effect of Dietary Chlorogenic Acid on Growth Performance, Antioxidant Function, and Immune Response of Broiler Breeders under Immune Stress and Stocking Density Stress. Vet. Sci. 9, 582. https://doi.org/10.3390/ vetsci9100582

Bailly, C. (2021). The steroidal alkaloids α-tomatine and tomatidine: Panorama of their mode of action and pharmacological properties. Steroids, 176, 108933. https://doi.org/10.1016/j.steroids.2021.108933

Birben, E., Sahiner, U. M., Sackesen, C., Erzurum, S., & Kalayci, O. (2012). Oxidative stress and antioxidant defense. The World Allergy Organization journal, 5(1), 9–19. https://doi.org/10.1097/WOX.0b013e3182439613

Birnie G. D. (1988). The HL60 cell line: a model system for studying human myeloid cell differentiation. The British journal of cancer. Supplement, 9, 41–45.

Breitman, T. R., Selonick, S. E., & Collins, S. J. (1980). Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. Proceedings of the National Academy of Sciences of the United States of America, 77(5), 2936–2940. https://doi.org/10.1073/pnas.77.5.2936

Cai, L., Wang, H., Li, Q., Qian, Y., and Yao, W. (2008). Salidroside inhibits H2O2 -induced apoptosis in PC12 cells by preventing cytochrome c release and inactivating of caspase cascade. Acta. Biochim. Biophys. Sin., 40, 796–802

Chanput, W., Peters, V., & Wichers, H. (2015). THP-1 and U937 Cells. The Impact of Food Bioactives on Health: in vitro and ex vivo models, 147-159.

Chao, M. W., Chen, C. H., Chang, Y. L., Teng, C. M., & Pan, S. L. (2012). α-Tomatine-mediated anti-cancer activity in vitro and in vivo through cell cycle- and caspase-independent pathways. PloS one, 7(9), e44093. https://doi.org/10.1371/journal.pone.0044093

Clifford, M. (2000). Chlorogenic acids and other cinnamates–nature, occurrence, dietary burden, absorption and metabolism. Journal of The Science of Food and Agriculture - J SCI FOOD AGR. 80. 1033-1043. 10.1002/(SICI)1097-0010(20000515)80:73.0.CO;2-T

Cristiana Filip, C. Filip, & Elena Albu, E. Albu. (2018). Reactive Oxygen Species (ROS) in Living Cells. IntechOpen

Cymit, CAS. https://cymitquimica.com/products/3B-T3636/17406-45-0/tomatine-from-tomato/ (03.05.2023)

Di Benedetto, R., Varì, R., Scazzocchio, B., Filesi, C., Santangelo, C., Giovannini, C., Matarrese, P., D'Archivio, M., & Masella, R. (2007). Tyrosol, the major extra virgin olive oil

compound, restored intracellular antioxidant defences in spite of its weak antioxidative effectiveness. Nutrition, metabolism, and cardiovascular diseases : NMCD, 17(7), 535–545. https://doi.org/10.1016/j.numecd.2006.03.005

Faria-Silva, C., de Sousa, M., Carvalheiro, M. C., Simões, P., & Simões, S. (2022). Alpha-tomatine and the two sides of the same coin: An anti-nutritional glycoalkaloid with potential in human health. Food chemistry, 391, 133261. https://doi.org/10.1016/j.foodchem.2022.133261

Geissmann, F., Manz, M. G., Jung, S., Sieweke, M. H., Merad, M., & Ley, K. (2010). Development of monocytes, macrophages, and dendritic cells. Science (New York, N.Y.), 327(5966), 656–661. https://doi.org/10.1126/science.1178331

Germic, N., Frangez, Z., Yousefi, S., & Simon, H. U. (2019). Regulation of the innate immune system by autophagy: monocytes, macrophages, dendritic cells and antigen presentation. Cell death and differentiation, 26(4), 715–727. https://doi.org/10.1038/s41418-019-0297-6

Harris, Paul & Ralph, Popoola. (1985). Human Leukemic Models of Myelomonocytic Development: A Review of the HL60 and U937 Cell Lines. Journal of leukocyte biology. 37. 407-22. 10.1002/jlb.37.4.407.

Held, P.G. (2010). An Introduction to Reactive Oxygen Species Measurement of ROS in cells

Hemmerle, H., Burger, H. J., Below, P., Schubert, G., Rippel, R., Schindler, P. W., Paulus, E., & Herling, A. W. (1997). Chlorogenic acid and synthetic chlorogenic acid derivatives: novel inhibitors of hepatic glucose-6-phosphate translocase. Journal of medicinal chemistry, 40(2), 137–145. https://doi.org/10.1021/jm9607360

Jambunathan N. (2010). Determination and detection of reactive oxygen species (ROS), lipid peroxidation, and electrolyte leakage in plants. Methods in molecular biology (Clifton, N.J.), 639, 292–298. https://doi.org/10.1007/978-1-60761-702-0_18

Kamiloglu, S., Tomas, M., Ozdal, T.,Yolci,, P., Capanoglu, E. (2020). Bioactive component analysis. 10.1016/B978-0-12-819493-5.00002-9

Larussa, T., Oliverio, M., Suraci, E., Greco, M., Placida, R., Gervasi, S., Marasco, R., Imeneo, M., Paolino, D., Tucci, L., Gulletta, E., Fresta, M., Procopio, A., & Luzza, F. (2017). Oleuropein Decreases Cyclooxygenase-2 and Interleukin-17 Expression and Attenuates Inflammatory Damage in Colonic Samples from Ulcerative Colitis Patients. Nutrients, 9(4), 391. https://doi.org/10.3390/nu9040391

Lee, K. M., Hur, J., Lee, Y., Yoon, B., Choi, S. Y. (2018). Protective effects of tyrosol against oxidative damage in L6 muscle cells, Food Science and Technology Research, Volume 24, Issue 5, Pages 943-947, Released on J-STAGE October 27, 2018, Online ISSN 1881-3984, Print ISSN 1344-6606, https://doi.org/10.3136/fstr.24.943

MERCK. https://www.sigmaaldrich.com/CZ/en/product/supelco/phl80166 (03.05.2023).

Nascimento, C. R., Rodrigues Fernandes, N. A., Gonzalez Maldonado, L. A., & Rossa Junior, C. (2022). Comparison of monocytic cell lines U937 and THP-1 as macrophage models for in vitro studies. Biochemistry and biophysics reports, 32, 101383. https://doi.org/10.1016/j.bbrep.2022.101383

Naso, L. G., Valcarcel, M., Roura-Ferrer, M., Kortazar, D., Salado, C., Lezama, L., Rojo, T., González-Baró, A. C., Williams, P. A., & Ferrer, E. G. (2014). Promising antioxidant and anticancer (human breast cancer) oxidovanadium(IV) complex of chlorogenic acid. Synthesis, characterization and spectroscopic examination on the transport mechanism with bovine serum albumin. Journal of inorganic biochemistry, 135, 86–99. https://doi.org/10.1016/j.jinorgbio.2014.02.013

Nediani, C., Ruzzolini, J., Romani, A., & Calorini, L. (2019). Oleuropein, a Bioactive Compound from Olea europaea L., as a Potential Preventive and Therapeutic Agent in Non-Communicable Diseases. Antioxidants (Basel, Switzerland), 8(12), 578. https://doi.org/10.3390/antiox8120578

Nimse, S. B. & Pal, D. (2015). Free Radicals, Natural Antioxidants, and their Reaction Mechanisms. RSC Adv.. 5. 10.1039/C4RA13315C

Onakpoya, I. J., Spencer, E. A., Thompson, M. J., & Heneghan, C. J. (2015). The effect of chlorogenic acid on blood pressure: a systematic review and meta-analysis of randomized

clinical trials. Journal of human hypertension, 29(2), 77–81. https://doi.org/10.1038/jhh.2014.46 Ray, P. D., Huang, B. W., & Tsuji, Y. (2012). Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cellular signalling, 24(5), 981–990. https://doi.org/10.1016/j.cellsig.2012.01.008

Opara E. C. (2006). Oxidative stress. Disease-a-month : DM, 52(5), 183–198. https://doi.org/10.1016/j.disamonth.2006.05.003

Perona, J. S., Cabello-Moruno, R., & Ruiz-Gutierrez, V. (2006). The role of virgin olive oil components in the modulation of endothelial function. The Journal of nutritional biochemistry, 17(7), 429–445. https://doi.org/10.1016/j.jnutbio.2005.11.007

Plotnikov, M. B., & Plotnikova, T. M. (2021). Tyrosol as a Neuroprotector: Strong Effects of a "Weak" Antioxidant. Current neuropharmacology, 19(4), 434–448 https://doi.org/10.2174/1570159X18666200507082311

Preiser, J.C. (2012). Oxidative Stress. JPEN. Journal of parenteral and enteral nutrition. 36. 147-54. 10.1177/0148607111434963

Şahin, S., & Bilgin, M. (2017). Olive tree (Olea europaea L.) leaf as a waste by-product of table olive and olive oil industry: A review. Journal of the Science of Food and Agriculture. 98. 10.1002/jsfa.8619.

Seger, R., Krebs, E. G. (1995). The MAPK signaling cascade. FASEB J. 9, 726-735

Shamshoum, H., Vlavcheski, F., & Tsiani, E. (2017). Anticancer effects of oleuropein. BioFactors (Oxford, England), 43(4), 517–528. https://doi.org/10.1002/biof.1366

Sharma, P., & Jha, A., Dubey, R., Pessarakli, M. (2012). Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. Journal of Botany. 2012. 10.1155/2012/217037

Shi, C., & Pamer, E. G. (2011). Monocyte recruitment during infection and inflammation. Nature reviews. Immunology, 11(11), 762–774. https://doi.org/10.1038/nri3070

Shi, H., Dong, L., Jiang, J., Zhao, J., Zhao, G., Dang, X., Lu, X., & Jia, M. (2013). Chlorogenic acid reduces liver inflammation and fibrosis through inhibition of toll-like receptor 4 signaling pathway. Toxicology, 303, 107–114. https://doi.org/10.1016/j.tox.2012.10.025

Shih, Y. W., Shieh, J. M., Wu, P. F., Lee, Y. C., Chen, Y. Z., & Chiang, T. A. (2009). Alpha-tomatine inactivates PI3K/Akt and ERK signaling pathways in human lung adenocarcinoma A549 cells: effect on metastasis. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association, 47(8), 1985–1995. https://doi.org/10.1016/j.fct.2009.05.011

Silva, S., Gomes, L., Leitão, F., Coelho, A., Boas, L. (2006). Phenolic Compounds and Antioxidant Activity of Olea europaea L. Fruits and Leaves. Food Science and Technology International. 12. 385-395. 10.1177/1082013206070166

Smith, M.E., van der Maesen, K., Somera, F.P. (1998). Effects of Phorbol Myristate Acetate (PMA) on Functions of Macrophages and Microglia In Vitro. Neurochem Res 23, 427–434. https://doi.org/10.1023/A:1022478005243

Suski, J. M., Lebiedzinska, M., Bonora, M., Pinton, P., Duszynski, J., & Wieckowski, M. R. (2012). Relation between mitochondrial membrane potential and ROS formation. Methods in molecular biology (Clifton, N.J.), 810, 183–205. https://doi.org/10.1007/978-1-61779-382-0_12

Tamasi, G., Baratto, M. C., Bonechi, C., Byelyakova, A., Pardini, A., Donati, A., Leone, G., Consumi, M., Lamponi, S., Magnani, A., & Rossi, C. (2019). Chemical characterization and antioxidant properties of products and by-products from Olea europaea L. Food science & nutrition, 7(9), 2907–2920. https://doi.org/10.1002/fsn3.1142

Tamasi, G., Pardini, A., Bonechi, C., Donati, A., Pessina, F., Marcolongo, P., Gamberucci, A., Leone, G., Consumi, M., Magnani, A., Rossi, C. (2019). Characterization of nutraceutical components in tomato pulp, skin and locular gel. European Food Research and Technology. 245. 10.1007/s00217-019-03235-x.

Taveira, M., Sousa, C., Valentão, P., Ferreres, F., Teixeira, J. P., & Andrade, P. B. (2014). Neuroprotective effect of steroidal alkaloids on glutamate-induced toxicity by preserving mitochondrial membrane potential and reducing oxidative stress. The Journal of steroid biochemistry and molecular biology, 140, 106–115.

https://doi.org/10.1016/j.jsbmb.2013.12.013
Turrens J. F. (2003). Mitochondrial formation of reactive oxygen species. The Journal of physiology, 552(Pt 2), 335–344. https://doi.org/10.1113/jphysiol.2003.049478
Wan, C. W., Wong, C. N., Pin, W. K., Wong, M. H., Kwok, C. Y., Chan, R. Y., Yu, P. H., & Chan, S. W. (2013). Chlorogenic acid exhibits cholesterol lowering and fatty liver attenuating properties by up-regulating the gene expression of PPAR-α in hypercholesterolemic rats induced with a high-cholesterol diet. Phytotherapy research : PTR, 27(4), 545–551. https://doi.org/10.1002/ptr.4751
Xie, F., Zhu, J., Hou, B., Wang, Y., Meng, F., Ren, Z., and Ren, S. (2017). Inhibition of NF-κB activation improves insulin resistance of L6 cells. Endocr. J., 64, 685–693
Yan, C. M., Chai, E. Q., Cai, H. Y., Miao, G. Y., & Ma, W. (2015). Oleuropein induces apoptosis via activation of caspases and suppression of phosphatidylinositol 3-kinase/protein kinase B pathway in HepG2 human hepatoma cell line. Molecular medicine reports, 11(6), 4617–4624. https://doi.org/10.3892/mmr.2015.3266
Yuan, Y., Gong, X., Zhang, L., Jiang, R., Yang, J., Wang, B., & Wan, J. (2017). Chlorogenic acid ameliorated concanavalin A-induced hepatitis by suppression of Toll-like receptor 4 signaling in mice. International immunopharmacology, 44, 97–104. https://doi.org/10.1016/j.intimp.2017.01.017
Yun, N., Kang, J. W., & Lee, S. M. (2012). Protective effects of chlorogenic acid against ischemia/reperfusion injury in rat liver: molecular evidence of its antioxidant and anti-inflammatory properties. The Journal of nutritional biochemistry, 23(10), 1249–1255. https://doi.org/10.1016/j.jnutbio.2011.06.018
Zhang, L. T., Chang, C. Q., Liu, Y., & Chen, Z. M. (2011). Effect of chlorogenic acid on disordered glucose and lipid metabolism in db/db mice and its mechanism. Zhongguo yi xue ke xue yuan xue bao. Acta Academiae Medicinae Sinicae, 33(3), 281–286. https://doi.org/10.3881/j.issn.1000-503X.2011.03.015
Zhao, Y., Wang, J., Ballevre, O., Luo, H., & Zhang, W. (2012). Antihypertensive effects and mechanisms of chlorogenic acids. Hypertension research : official journal of the Japanese Society of Hypertension, 35(4), 370–374. https://doi.org/10.1038/hr.2011.195

7. List of Appendices

List of abbreviation

BCA	bicinchoninic acid
BSA	bovine serum albumin
CA	caffeic acid
CAT	caffeic acid
CCD	charge-coupled device
CGA	chlorogenic acid
CLSM	confocal laser scanning microscopy
CNS	central nervous system
DIC	differential interference contrast
EPR	paramagnetic resonance
ER	endoplasmic reticulum
FBS	fetal Bovine Serum
GSH	glutathione
GSHPx	glutathione peroxidase
HCC	hepatocellular carcinomas cell lines
H_2O_2	hydrogen peroxide
$HO^{\bullet}$	hydroxyl radical
HRP	horseradish peroxidase
MAPK	mitogen activated protein kinase
MDA	malondialdehyde
NADPH oxidase	nicotinamide adenine dinucleotide phosphate oxidase
O_2	molecular oxygen
$O_2^{\bullet-}$	superoxide anion radical
OleA	oleuropein aglycone
PVDF	polyvinylidene difluoride
PMA	phorbol 12-myristate 13-acetate
PMT	photomultiplier tube
POBN	α-(4-Pyridyl 1-oxide)-N-tert-butylnitrone
PUFA	polyunsaturated fatty acid
RIPA	radioimmunoprecipitation assay buffer

ROS	reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute 1640
SOD	superoxide dismutase
TBS	tris buffered saline
UPE	ultra-weak photon emission
UV	ultraviolet radiation
XO	xanthine oxidase