University of Sfax

Faculty of Sciences Sfax

Unit of Biotechnology and Pathologies,
Higher Institute of Biotechnology Sfax

Tunisian Republic

Ministry of Higher Supérieur And
Scientific Research

University of Messina-Italy

Dep. of Chemical Biological
Pharmaceutical And Environmental
Sciences

Thesis

For the degree of

DOCTOR in Biology

in cotutela with the PhD course in

Applied Biology and Experimental Medicine

by

BEN AMOR Ichrak

Comparative study of the antiviral effect of cultivated and wild olive leaf extracts: The herpes Simplex (HSV) and the Epstein- Barr virus (EBV) as study models

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Acknowledgments

I thank my **ALLAH** for giving me the will, courage, and patience to accomplish this work.

I would like to thank

My supervisor, Pr. Hammadi ATTIA

at the National Engineering School of Sfax
Head of Laboratory of Analysis Valorization and Food Safety (LAVASA lab), National Engineering School of Sfax at the University of Sfax.
I thank you for entrusting me with this work. You welcomed me into your team and directed me with kindness. Please find in this work the expression of my high consideration.

My supervisor, Pr. Maria Teresa SCIORTINO

at Dep. of Chemical Biological Pharmaceutical and Environmental Sciences a Messina
Head of Molecular Virology Lab at the University of Messina.
I take this opportunity to express my deep gratitude to you. May you find here the expression of my deep respect and profound gratitude. I am grateful for my time working with you

My co-supervisor, Dr. Bochra GARGOURI

Assistant Professor at the Faculty of Sciences of Sfax
Member of LAVASA laboratory
I am deeply touched by your morality, solicitude, human qualities, availability, and support and assistance you constantly provide me.
Please accept the expression of my sincere gratitude, respect, and deep dedication.

This work is dedicated to the memory of ***Pr. Saloua LASSOUED*** We will never forget you, and you are always in our hearts.

Pr Radhouane GDOURA

Professor at the Faculty of Sciences of Sfax
I want to express my sincere thanks for the honor you have given me by accepting me to chair the jury of this thesis despite your busy schedule.
May you find here the expression of my deep respect and profound gratitude.

Pr Faïza FAKHFEKH

Professor at the Faculty of Sciences of Sfax
I thank you for accepting to be the reviewer of my doctoral thesis. May you find here the expression of my most sincere thanks.
Please find in this work the expression of my high consideration.

Pr Giuseppina MANDALARI

at Dep. of Chemical Biological Pharmaceutical and Environmental Sciences a Messina
Thank you for agreeing to evaluate my research, give me constructive feedback, and take the time to review my manuscript. Thank you for your precious scientific skills for the achievement of this work.

Pr Maria MAISANO

At Dep. of Chemical Biological Pharmaceutical and Environmental Sciences a Messina

Thank you very much for the honor you have given me by agreeing to examine the thesis despite your busy schedule.
Please accept my respect and high consideration.

Pr Monia ENNOURI

At Olive Institute Sfax

Thank you for the honor you have bestowed upon me by agreeing to judge my work.
May you find here the expression of my deep respect and profound gratitude.

Pr Clara URZI

At Dep. of Chemical Biological Pharmaceutical and Environmental Sciences a Messina

Thank you very much for the honor you have given me by agreeing to examine the thesis despite your busy schedule.
Please accept my respect and high consideration.

I would like to thank

Pr Domenico TROMBETTA, Dr Antonella SMERIGLIO and Dr Manuela D'ARRIGO

At Dep. of Chemical Biological Pharmaceutical and Environmental Sciences a Messina
You have done me a great honor to have allowed me to benefit from your skills.
Your warm welcome and your availability have deeply touched me. May this work express my respect and my deep gratitude.

Maria Musarra-PIZZO and Rosamaria PENNISI

At Dep. of Chemical Biological Pharmaceutical and Environmental Sciences a Messina
Your dynamic spirit, your competence, and your generosity deserve our admiration.
Please accept the expression of my sincere gratitude, respect, and deep dedication.

I would also like to thank **Pr Mongi SAOUDI** at Animal Ecophysiology Laboratory at Sciences Faculty of Sfax for the welcome he gave me for the realization of specific experiments. May this work express my respect

I would also like to thank all the laboratory members of Analysis Valorization and Food Safety (LAVASA lab), especially **Pr Souhail Besbes**, **Pr Mohamed Ali Ayadi, Dr. Haifa Sebii**, and all Biology Department members of the Faculty of Sciences Sfax (**Dr. Imen KALLEL, Dr. Ahmed BAYOUDH**, **Dr. Riadh BEN MANSOUR)**

Ichrak Ben Amor

Dedication

I dedicate this milestone in my life to

My dearest parents
Ammar & Aziza
I am thankful for having you in my life
I can't find any words that can reflect all my love and gratitude for you both
You have guided my first steps towards the path of knowledge and learning.
You have encouraged my ambitions with your love and support and the advice you have given me in my training and education to realize one of your dreams
Thank you for making me what I am
My deepest gratitude will never equal your efforts.
May God give you a long life full of joy, happiness, and health.

The memory of my dear grandmothers
I hope this work will testify to my deep gratitude for eternal love.
May God, the Almighty, offer you paradise as your eternal resting place.

My dear brother Atef and my dear sister Achwek

With all the love I have for you
May this work express my great attachment and gratitude for every sign of affection to the joyful moments shared together.
May ALLAH keep us always united. I wish you happiness, health, joy, and much success.

My dear fiance Abdallah

Your love, continued support, and encouragement have always been a great help
Thank you for always being at my side
May ALLAH always keep us united and happy for life
May ALLAH preserves us for the worst and the best.

My dear uncles, aunts, their wives and husbands, and my dear cousins (BenAmor and Abdessamed Family)
Please find in this work the expression of my most profound respect and my most sincere affection.

My dearest friends Ahlem CHIRA and Asma ELAGUEL
For their help and support, their kindness, sympathy, and encouragement.
Thank you for being a sincere friend.
I express to you all my gratitude and my most sincere affection.
May ALLAH protect you and grant you an easy life and a bright future.

My second family
Please find in this work the expression of my most profound respect and my most sincere affection.

My dears Sondoss Cheffi, Mohamed Hassairi, Mounira Maalej, Emna Ayadi and Abderazzek Msahed

Words are not enough to express the love and affection I have for you
I dedicate this work to you with all my wishes of happiness, health, and success

All my dear friends " **Youssra, Ines, Manel, Nawel, Maryem**" with whom I have this great pleasure to share moments of
continuous happiness

All my teachers and all the people who have contributed in some way to my training and the training and to the realization of this work in the best conditions.

All those I love, to all those who love me.

Ichrak Ben Amor

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List of abbreviations

ATP: Adenosine triphosphate

BHT: Butylhydroxytoluene

CAT: Catalases

CC_{50}: 50 % cytotoxic concentration

CD: Conjugated diene

DMSO: Dimethyl Sulfoxide

DNA: Deoxyribonucleic acid

DTPA: diethylenetriaminepentaacetic acid

DPPH: (2,2-Diphenyl-2-picrylhydrazyl

EBV: Epstein-Barr virus

EC_{50}: 50 % effective concentration

EBNA1: Epstein–Barr nuclear antigen 1

EDTA: Ethylene diamine tetraacetic acid

FCS: Fetal calf serum

FBS: Fetal bovine serum

FRAP: Ferric reducing antioxidant power

GPx: Glutathione peroxidases

H_2O_2: Hydrogen peroxide

HIV: Human immunodeficiency virus

HPLC: High-performance liquid chromatography

HSV: herpes simplex virus

O_2-˙: Superoxide anion

OESA: *Olea europea L. var. sativa*

OESY: *Olea europea var. sylvestris*

OD: Optic density

LB: B lymphocytes

LT: T lymphocytes

LMP: Epstein–Barr virus (EBV) latent membrane protein

LDL: low-density lipoprotein.

MA: Membrane antigens

MBC: Minimum bactericidal concentration

MIC: Minimum inhibitory concentration

MDA: Malondialdehyde

MFC: Minimum fungicidal concentration

MS: Mass spectrometry

MTT: 3 (4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide.

NO: Nitric oxide

NPC: Nasopharyngeal cancer

PBMC: Peripheral blood mononuclear cells

PBS: Phosphate-buffered saline

RNA: Ribonucleic acid

ROS: Reactive oxygen species

SOD: Superoxide dismutases

TCA: Trichloroacetic acid solution (15 %).

TBA: Thiobarbituric acid solution (0.8 %).

TBARs: Thiobarbituric acid-reactive species

TPA: 12-O-Tetradecanoyl-phorbol-13-acetate.

TSH: Thyroid-stimulating hormone

T3: Triiodothyronine

VLDL: Very Low Density Lipoprotein

VCA: Viral capsid antigens

ZEBRA: BamHI **Z** **E**pstein-**B**arr virus **r**eplication **a**ctivator

Introduction

Herbal medicine is the oldest form of healthcare known to humanity. Herbs have been used by all cultures throughout history (Astin et al. 1998; Yawar, 2001). It was an integral part of the development of modern civilization. The plants provided food, clothing, shelter, and since the dawn of civilization, man used plants for their medicinal and edible value (Hertog et al. 1993; Zhang et al. 2001). Today, scientists isolate active compounds from medicinal plants using this method. Despite the significant advances observed in modern medicine in recent decades, plants still make an essential contribution to health care. The use of plants as a source of medicinal value is a relatively old concept because of their advantages, safety, efficacy, and availability worldwide (Astin et al. 1998). Indeed, a large Tunisian plants species are well known for their ethnopharmacological uses and therapeutic practices in traditional medicine and gastronomy, making them good candidates for industrial use (Balasundram et al. 2006). Therefore, natural products are promising candidates for broad application. For example, *Olea europaea* L. is a woody oil tree of European Mediterranean islands and South-East Tunisia, widely used in the extra virgin olive oil-associated diet (Astin et al. 1998).

Olive leaves are among the most common traditional herbal teas used amongst Mediterranean people to cure certain conditions (Pereira et al. 2007). For this reason, interest in the potential health benefits of olive leaves has increased amongst scientists across various fields. The olive tree *(Olea europaea* L.) is one of the most important fruit trees in Mediterranean countries. They cover 8 million ha, accounting for almost 98% of the world crop (Pereira et al. 2007). In Tunisia, olive agriculture is one of the most important agricultural activities (Bouaziz et al. 2005). Various studies have reported the antioxidant, hypoglycemic, antihypertensive, antimicrobial, and anti-atherosclerotic effects of olive leaves (Lockyer et al. 2016; Liu et al. 2017; Borjan et al. 2020). This property is linked to the fact that the leaves are rich in polyphenols luteolin- and apigenin-7-O-glycosides to be the predominant flavonoids in olive leaves, followed by rutin (BenAmor et al. 2021).

Polyphenols modulate oxidative stress in cancer cells through modulation of signal transduction and the expression of specific genes related to cell proliferation and cell death (Rahman et al. 2006). Furthermore, as a piece of evidence, polyphenol compounds trigger apoptotic programmed cell death pathways in human gastric carcinoma cells via manipulation of ROS content of the cancerous cells (Rahman et al. 2006).

ROS can react with biological molecules, such as DNA, proteins, or lipids, generating mutations and damaging membranes, leading to cell and tissue injuries (Ksouri et al. 2009). Plants are known for resisting and destroying these toxic ROS since they are equipped with a powerful antioxidant system that includes enzymatic and non-enzymatic components (Ksouri

et al. 2009). These antioxidant compounds can delay the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions (Tepe et al. 2005). Furthermore, recent studies have shown that several olive species are an essential source of biologically active molecules (Van der Watt and Pretorius 2001; Weber et al. 2007; Duros et al. 2008). In addition to their role as antioxidants, these compounds exhibit a broad spectrum of biological activities such as antimicrobial, anti-inflammatory, anti-carcinogenic, anti-allergic, anti-thrombotic, cardioprotective, and vasodilatory effects (Verzelloni et al. 2007; Balasundram et al. 2006).

Olea europaea leaves extracts have been screened and exhibit a positive effect on health: reduces the incidences of cancer and heart and blood vessel diseases, influences the gut microbial balance, exerts anti-oxidative, anti-inflammatory, and antimicrobial activity against bacteria, fungi, and viruses (Somova et. 2003; Skerget et al. 2005).

In this context of this thesis will be structured three distinct parts:

- **Phytochemical Characterization of *Olea Europaea* Leaf Extracts by LC-DAD-ESI-MS analysis and their antioxidant power in a chemical and biological system using HeLa cell and PMBC.**
- **Anti-HSV-1 and antimicrobial of *Olea Europaea* leaf extracts.**
- ***Olea Europaea Sativa* exhibits antioxidant and antiviral activities against Epstein Barr Virus.**

Background

I. Medicinal plants

1. Definition

Medicinal plants have been used to relieve and cure human diseases since ancient times. Their therapeutic properties are due to hundreds, even thousands, of natural bioactive compounds. Therefore, it is a plant that can be cultivated and exploited for its medicinal properties for commercial purposes, especially since, in many cases, it is a cheap product compared to the price of many pharmaceutical drugs (Benariba et al. 2013).

Herbal medicines are considered therapeutic potential against many diseases, but neither their active components nor their mechanisms of action are fully understood (Ivanova et al. 2005).

In the last decade, herbal medicine has been widely used to treat several human and animal diseases. In addition, essential oils or natural products derived from traditional plants have been experimented with for their antioxidant, antibacterial, anti-parasitic, antifungal, antiviral, and cytotoxic activities, as well as for food preservation and safety (Sze et al. 2010; Solorzano and Novales 2011; Anthony et al. 2005; Reichling et al. 2009; Tajkarimi et al. 2010).

2. *Olea Europaea*

2.1. History

Olea europaea was cultivated mainly in the Mediterranean basin for at least 3500 years. It covers about 8 million hectares, representing about 98 % of the world's harvest (Eddouks et al. 2007).

In ancient Greece and Rome, it was an emblem of fertility and a symbol of peace and glory. The scientific name of the tree "Olea" comes from a word that meant "oil" to the ancient Greeks (Miliauskas et al. 2004).

The Mediterranean basin remains a privileged area compared to the rest of the world to cultivate the olive tree thanks to its adequate climate in terms of temperature and hydrometry (Loumou et Giourga, 2003).

2.2.Botanic description

The olive tree *Olea europaea* belongs to the *Oleaceae* family, including about 30 genus and 600 species. Its genus, Olea, is composed of 33 species. There are two subspecies, the cultivated olive tree or common olive tree (*Olea europaea sativa*) and the wild olive tree or oleaster (*Olea europaea sylvestris*) (Dupont et Guignard 2007; Spichiger et al. 2000).

The botanical classification of the olive tree, according to (Ghedira 2008), is the following:

- Branch: Magnoliophyta

-Subphylum: Magnoliophytina

-Class: Magnoliopsida

-Subclass: Dialypetals

-Order: Lamiales

-Family: Oleaceae

-Gender: Olea

-Species: *Olea Europaea L*

2.3. Morphological characteristics

The olive tree is a perennial tree with evergreen, hard, grey-green leaves and having an elongated shape (Bruneton 2009). The blade is lanceolate and ends in a mucro. The edges of the limbus role upon themselves. The upper surface of the leaf is smooth and shiny (Wright et al. 2007). The flowers are deposited in clusters on a long stalk (the olive tree produces two kinds of flowers, a perfect one which contains both sexes, male and female, and a staminate). They bloom in small white clusters, and each bunch will give a single fruit. Its ovoid fruit (drupe), it has a fusiform nucleus (Aillaud et al. 2013). Its tough wood is rot-proof and is used in cabinet making, blooms in May – June (Bruneton 2009). The trunk is grey-green and smooth until its tenth year, turning a dark grey colour (Sánchez-Romero 2021). The root system adapts to the soil structure; it remains at a 500 to 700 cm depth and is mainly located under the trunk (Loussert and Brousse, 1978).

Figure 1: Olive Tree

2.4.Geographical distribution

The olive tree (*Olea europaea*) is one of the oldest agricultural tree crops in the Mediterranean basin with great cultural and economic importance. Several works have focused

on assessing the distribution and variability between cultivated and wild olives (Lavee 2013). For botanists, the range of the olive tree is synonymous with the "Mediterranean region." The olive tree (*Olea europaea L.*) has been cultivated for a very long time around the Mediterranean and especially in: Spain, Italy, Greece, Turkey, France, Tunisia, Algeria, and Croatia (Lee et al. 2009). However, the plantations in California, Australia, and South Africa influence this geographical distribution by climatic and pedological factors (Gaussorgues 2009; Carrión et al. 2010; Lumaret et al. 2004).

Tunisian olive heritage is estimated at more than 65 million trees, covering an area of 1,680,000 hectares. Tunisia occupies the fourth position in the world regarding the number of trees and the second in the area (Karray 2009). The Tunisian varietal heritage consists of a wide variety of cultivars spread from North to South. Nevertheless, the olive groves are essentially made up of two main types: Chemlali, which occupies 60 %, and Chetoui of 35 % of the olive-growing area of the country (Haloui et al. 2010).

Figure 2: Sites and Geographical Distribution Of Varieties And Types Of Olive Trees In Tunisia (Trigui et al. 2002).

2.5.Difference between wild olive and cultivated olive

Morphological, biological, and genetic differences exist between the two subspecies of *Olea europaea*. *Olea europaea sativa* (the cultivated olive tree) is a tree that can reach 20 meters in height with a curved trunk and cylindrical branches whose cracked bark presents lanceolate leaves and fruits of variable shape depending on the variety considered and with very high oil content. *Olea europaea sylvestris* oleaster is a small shrub with small round or slightly elongated dark green leaves and spherical fruits containing very little oil. It is slower growing than the cultivated olive tree (Botineau 2010).

Moreover, these two subspecies are characterized by variable reproduction. It is self-fertilization for the cultivated variety and cross-fertilization for the wild olive trees (Aillaud et al. 2013).

(1) **(2)**

Figure 3: (1) *Olea Europaea Sativa* / (2) *Olea Europaea Sylvestris*

2.6.*Olea europaea* products

In addition to olive oil as the main product, the olive industry produces other leaves and olives. Indeed, the olive tree is characterized by fruit, an essential component. Olive oil has mainly beneficial effects on human health due to its high unsaturated fatty acids, vitamin E, and polyphenols (Kiai et Hafidi 2014). Olive leaves are widely used in different fields (therapeutic, pharmaceutical, cosmetic, and food industries) with several forms, whether in extract, powder, or herbal tea (El et Karakaya 2009).

2.7.Bioactive compounds of *Olea europaea* and their benefits

Since ancient times, *Olea europaea* has been widely used in traditional medicine for many diseases. It is considered an aromatic and medicinal plant and a reservoir of natural compounds with beneficial effects (Bisignano et al. 2010).

According to (Makowska-Wąs et al. 2017), bioactive molecules derived from olive products and leaf extracts are represented as follows:

-Flavonoids

-Derivatives of hydroxycinnamic acid, with the predominance of verbascoside

-Substituted phenols (hydroxytyrosol, tyrosol, and vanillic acids)

-Secoiridoids such as oleuropein

In addition, the monounsaturated fatty acids available in olive leaves, such as oleic acid, decrease plasma lipids including LDL and VLDL and prevent cardiovascular disease (Huang et Sumpio 2008).

Olive tree polyphenols have an enormous capacity to scavenge free radicals and show synergistic behavior when combined, occurring naturally in olive leaves and, therefore, in their extracts (Visioli et al. 2002). Among these polyphenols, hydroxytyrosol and tyrosol contribute to bitter taste, astringency, and resistance to oxidation (Visioli et al. 2002).

Although every element of the olive tree contains secosteroid, oleuropein, the leaves are its richest source (Makowska-Wąs et al. 2017). It is the essential biophenol with antioxidant, hypotensive, hypoglycemic, cholesterol-lowering, antiseptic, anti-inflammatory, antimicrobial, and anti-tumor activities (Visioli et al. 2002; Hamdi et Castellon 2005; Pereira et al. 2007; Ghedira 2008).

3. Olive leaves

3.1. Description

The leaves of the olive tree are of elongated oval form, persistent opposition. They are carried by a short stalk, a dark green shiny on the upper surface, and a light silvery green with a prominent midrib on the underside (Bruneton 2009). They are odorless and bitter. The foliage is evergreen, but this does not mean that its leaves are immortal. They live on average three years, then yellow and fall, mainly in summer (Argenson et al., 1999).

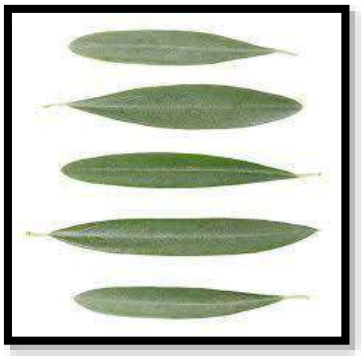


Figure 4: Olive tree leaf (Argenson et al. 1999).

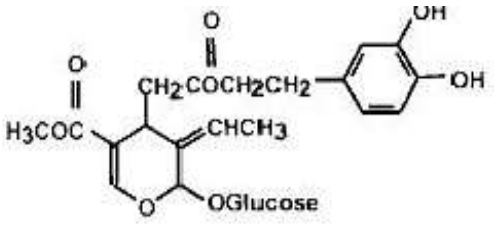
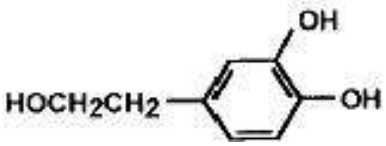
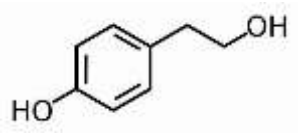
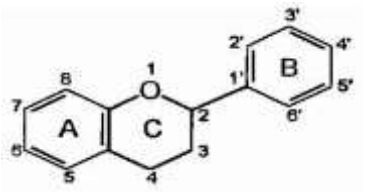
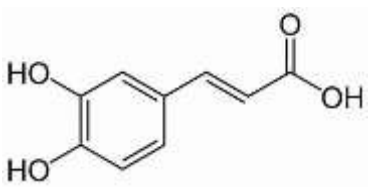
3.2. Bioactive compounds of leaves

The richness in bioactive compounds characterizes the olive leaf: total polyphenols (25.3 mg/g), flavonoids (58 mg/g), and oleuropein (9.27-13.43 %) (Lee et al. 2009).

Indeed, Mahani et al. quantified some identified compounds in an ethanolic extract of olive leaves using high-performance liquid chromatography (HPLC) (Mahani et al. 2010). The results showed that oleuropein (356 mg/g), hydroxytyrosol (4.89 mg/g), tyrosol (3.73 mg/g), and caffeic acid (49.41 mg/g) were the main compounds.

Oleuropein belongs to the secosteroids, abundant in *Oleaceae* and several other plants. Iridoids and Secoiridoids are usually derived from oleosides, a type of glucoside (Haris 2010; Ansari et al. 2011).

Table 1: Chemical structures of the most abundant bioactive compounds in the extract of olive leaves (Pereira et al. 2007).

Compounds	Chemical structure
Oleuropein	
Hydroxytyrosol	
Tyrosol	
Flavonoids	
Caffeic Acid	

3.3. Biological activities and pharmacological properties

Olive leaves have been widely used in traditional remedies in European and Mediterranean countries and are rich in triterpenes, flavonoids, secoiridoids, including oleuropeoside and phenolic acids (Lee et al. 2009).

For centuries man has been cultivating the olive tree and has discovered numerous healing and preventive powers against certain diseases.

Historically, the *Olea Europea* leaves are used, in the form of tea, in traditional herbal medicine to treat certain diseases such as malaria and hypertension (Ghedira 2008). In addition, they have been widely used in the form of an extract or a powder or tea to remedy fever and other diseases such as malaria (Lee et al. 2009). In addition, decoction and mouthwash are used to treat oral aphthosis, gingivitis, and glossitis (inflammatory and infectious conditions of the tongue). In addition, they are used to facilitate urinary and digestive elimination functions (Bruneton 2009). Several reports have shown that olive leaf extract can lower blood pressure in animals and increase blood flow in the coronary arteries, relieve arrhythmias and prevent intestinal muscle spasms (Benavente-García et al. 2000). All parts of the tree are used for healing: fruit, leaf, flower, bark, and olive oil (El et Karakaya 2009).

Multiple studies have shown that the leaves are diuretic and recommended in moderate hypertension. Furthermore, the extract is an adjuvant in mild forms of diabetes (during pregnancy or in obesity) (Ghedira 2008). The study carried out by Jemai et al. shows that it decreases the blood TSH level with an increase of T3, probably due to a stimulation of the enzyme that converts T4 into T3 (Jemai et al. 2008). In addition, leaves possess antimicrobial properties against some microorganisms such as bacteria, fungi, and mycoplasma (Pereira et al. 2007; Ghanbari et al. 2012). They also have the highest capacity to scavenge free radicals compared to different parts of the olive tree (Savournin et al. 2001).

In vitro studies have shown that oleuropein, the bioactive component of the leaves, acts as an anti-tumor compound, with efficacy against the viability of breast cancer cells in women (Hamdi et Castellon 2005; Menendez et al. 2007).

Furthermore, the extracts prepared from olive leaves have been reported an anti-HIV activity (Huang et al. 2014).

Researchers have also demonstrated the anti-inflammatory properties of oleuropein in animals, the primary polyphenol of olive products via its. The consumption of oleuropein at a dose of 0.015 % in the diet protects the skeleton from the aging process mimicked by castration coupled with an inflammatory process. These polyphenols with specific properties open

fascinating perspectives for preventing osteoporosis, both postmenopausal (Gardès Albert et al. 2003).

II. Free radical

Our cells and tissues can be subjected to a wide variety of physical (trauma, irradiation, hyper or hypothermic), chemical (acidosis, toxins), and metabolic (exposure to xenobiotics, deprivation of a hormonal or growth factor) aggressions. Most of these aggressions lead to a common expression called oxidative stress, due to the exaggeration of a physiological phenomenon, usually very controlled the production of free radicals (Walker et al. 1982).

1. Definition

Free radicals can be defined as chemical species (atoms or molecules) containing one or more single electrons in their outer orbits (Valko et al. 2007). This makes free radicals very unstable and generally gives a considerable degree of reactivity to the free radical towards other molecules to recover electrons by oxidizing them (Goudable et Favier 1997; Valko et al. 2007; Borg et al. 2010). These radicals are derived from oxygen by one-electron reductions such as the superoxide anion $O_2^{\cdot -}$, hydroxyl radical OH-, peroxyl radical ROO-, alkoxyl radical RO-. Other oxygen-derived species are called active oxygen species, such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), ozone (O_3), nitro-peroxide, or peroxynitrite acid (ONOOH) are not free radicals but are also reactive and can be radical precursors (Favier 2006). Oxygen-derived radicals represent the largest radical species generated in living systems (Valko et al. 2007). The set of free radicals and their precursors is often referred to as reactive oxygen species (ROS) (Favier 2006; Migdal et Serres 2011).

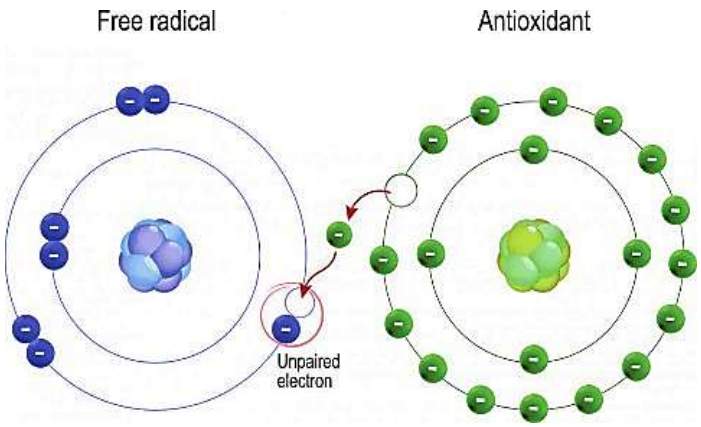


Figure 5: Free radical

Table 2: Name and chemical symbol of the different free radicals and non-radical species

	Name	Chemical symbol
Radical species	Superoxide anion	$O_2^{\bullet -}$
	Hydroxyl radical	$OH^{\bullet}$
	Peroxyl radical	$ROO^{\bullet}$
	alkoxyl radical	$RO^{\bullet}$
	Nitrogen monoxide	$NO^{\bullet}$
Non-radical species	Singlet oxygen	$^1O_2$
	Ozone	O_3
	Hydrogen peroxide	H_2O_2
	Nitroperoxide	ONOOH

2. Sources of free radicals

2.1.Endogenous sources

ROS can come from different cellular compartments such as the plasma membrane, the endoplasmic reticulum (ER), the peroxisomes, the cytoplasm, and mainly the respiratory chain of mitochondria. In addition, other enzymatic activities also provide ROS, for example, cytochromes P450 and especially NADPH oxidases during inflammation (Barouki 2006; Cillard et Cillard 2006).

a. The mitochondrial respiratory chain

The mitochondria are responsible for a significant part of energy production in the form of ATP. During the functioning of the mitochondrial respiratory chain, there is a continuous production of superoxide anions. This production remains low under normal conditions but can increase when respiration becomes intense or inflammation (Kovacic et al. 2005; Stowe et Camara 2009).

b. NADPH oxidase

NADPH oxidase is a superoxide anion ($O_2^{\bullet -}$) producing enzyme. The latter is formed by molecular oxygen reduction mediated by NAD(P)H oxidases and xanthine oxidase or by redox reactive compounds such as the semi-ubiquinone compound of the mitochondrial electron transport chain (Babior 1999).

2.2.Exogenous sources

ROS can also be produced by exogenous agents such as ultraviolet (UV) radiation, ionizing radiation (X or γ), heavy metals (mercury, arsenic), electric fields, cigarette smoke residues, and alcohol ingestion (Favier 2006).

3. The physiological roles of free radicals

ROS can be both beneficial and harmful. They can play an essential role in many physiological processes. However, they can be factors in many cardiovascular and immunological pathologies. At moderate concentrations, nitric oxide (NO), superoxide anion, and ROS play important roles as mediators and regulators of signaling processes. On the other hand, ROS are harmful to living organisms and damage all major cellular constituents (Valko et al. 2007).

3.1.Cellular signal transduction

A suggested model for activating signal transduction events during oxidative stress: H_2O_2 is sensed by a cellular receptor. Its detection leads to the activation of a mitogen-activated protein kinase (MAPK) cascade and a group of transcription factors controlling different factors cellular pathways. The presence of H_2O_2 is linked to changes in Ca^{2+} and calmodulin levels. The activation or induction of a Ca^{2+} -calmodulin kinase can also activate or suppress the activity of transcription factors. The regulation of gene expression by the different transcription factors leads to the induction of various defense pathways, such as scavenging of (ROS) and heat shock proteins (HSP) and photosynthesis (Favier 2006; Groeger et al. 2009).

3.2.Programmed cell death (apoptosis)

Apoptosis is a particular form of programmed cell death, which plays an indispensable role in the development and homeostasis of multicellular organisms. High concentrations of ROS induce apoptotic cell death in various cell types, suggesting that ROS contributes to cell death when generated in the apoptotic process (Dröge 2002).

3.3.Anti-radical defense systems

To counter the harmful effects of reactive oxygen species, the body uses an arsenal of antioxidants. Indeed, an antioxidant is defined as any substance capable of competing with other oxidizable substrates at a relatively low concentration, thus delaying or preventing the oxidation of these substrates (Berger 2006). Thus, the defense systems are divided into enzymatic antioxidants and non-enzymatic antioxidants.

a. Enzymatic systems

Enzymatic antioxidants are considered the first line of defense of our body against reactive oxygen species. The line of defense allows them to maintain their concentrations at a basal level. Indeed, they have an excellent affinity for ROS, with which they react very quickly to neutralize them. This system consists of enzymes such as superoxide dismutases (SOD), catalases (CAT), and glutathione peroxidases (GPx).

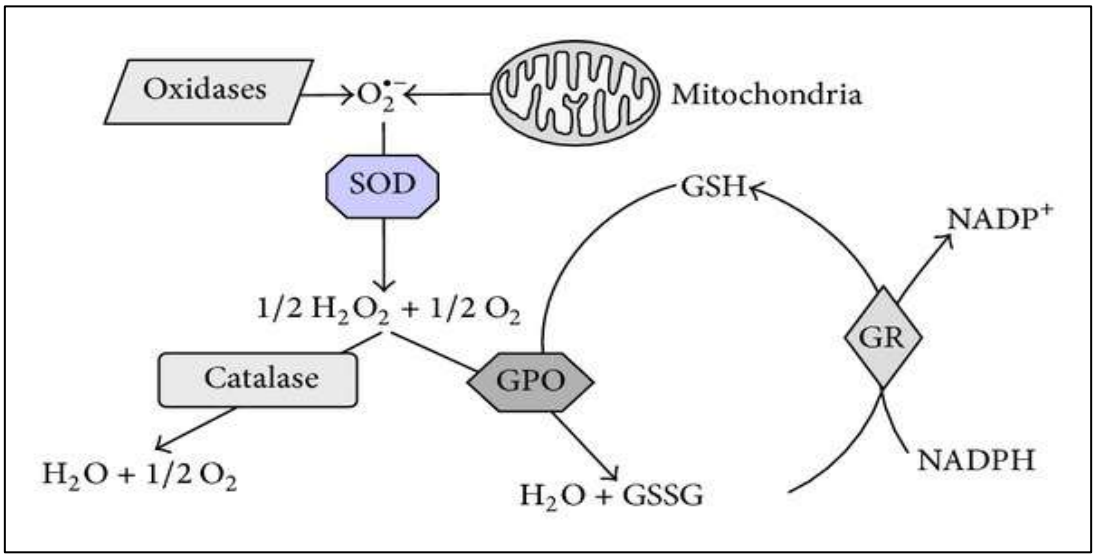


Figure 6: Schematic illustration of enzymatic defense systems

- **Superoxide dismutases**

Superoxide dismutases are the first and most important defense against ROS (Zelko et al. 2002). SOD is an intracellular metalloenzyme present in most organisms.

It catalyzes the dismutation of superoxide anion into hydrogen peroxide and molecular oxygen (Zelko et al. 2002).

$$2O_2^{\bullet-} + 2\ H^+ \xrightarrow{SOD} O_2 + H_2O_2$$

- **Catalase (CAT)**

Catalase is an intracellular enzyme in many tissues, located mainly in peroxisomes (Valko et al. 2007). It acts in synergy with SOD since its role is to accelerate the dismutation of hydrogen peroxide into water and molecular oxygen (Sorg 2004).

$$H_2O_2 \xrightarrow{CAT} O_2 + 2\ H_2O$$

- **Glutathione peroxidase (GPx)**

It is an enzyme located in the cytosol and the mitochondrial matrix. It has a significant role in the degradation of organic peroxides (ROOH), lipid peroxides, and hydrogen peroxide (H_2O_2) (Valko et al. 2007). These reactive derivatives are reduced by the oxidation of reducing substrates like glutathione (GSH) (Sorg 2004).

b. Non-enzymatic systems

Unlike antioxidant enzymes, most of these components are not synthesized by the body and must be supplied by the diet. These exogenous substances, mainly vitamin E (tocopherols), vitamin C (ascorbic acid), and glutathione, act as free radical scavengers.

- **Glutathione**

It is a tripeptide whose intracellular concentration is important. The thiol function confers glutathione an antioxidant role, i.e., a reducer (electron or H atom donor). In addition, it exerts numerous oxidized species, particularly hydrogen peroxide hydroxyl radicals (Gardès-Albert et al. 2003).

$$\mathbf{GSH + \cdot OH \rightarrow GS\cdot + H_2O}$$

$$\mathbf{GSH + R\cdot \rightarrow GS\cdot + RH}$$

The reduced form of glutathione (GSH) is the principal regulator of intracellular redox and is abundant in cells. In addition, glutathione acts as a direct scavenger of free radicals, a Co-substrate for the enzymatic activity of glutathione peroxidase. SOD is a cofactor for several other enzymes, and forms conjugates in endo and xenobiotic reactions (Gérard-Monnier et Chaudiere 1996).

- **Vitamin E**

Vitamin E is the fat-soluble antioxidant with the highest molar concentration in cells (Ohrvall et al. 1996). It plays a protective role by preventing the propagation of lipid peroxidation in the cell membrane induced by oxidative stress. It also neutralizes free radicals and acts in two different ways: either directly by scavenging ROS or by regulating antioxidant enzymes such as GPx, CAT, and SOD (Vertuani et al. 2004).

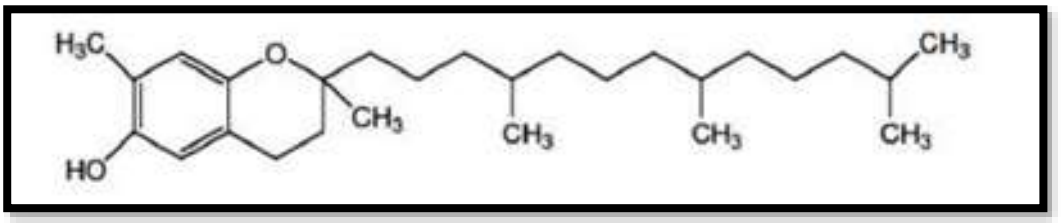


Figure 7: Structure of vitamin E (Flora et al. 2008)

- **Vitamin C (ascorbic acid)**

This vitamin is a water-soluble antioxidant that is not synthesized by our body (Evans et al. 2002). Therefore, its concentration depends mainly on a diet. Its role is essential in the intra- and extracellular compartments. It prevents LDL oxidation produced by various reactive oxygen species generating systems and protects different biological substrates like DNA, proteins, and fatty acids (Evans et al. 2002).

After the oxidation of dehydroascorbic acid, it takes an intermediate radical form (ascorbyl radical) after the loss and neutralizes singlet oxygen (Singh et al. 2005).

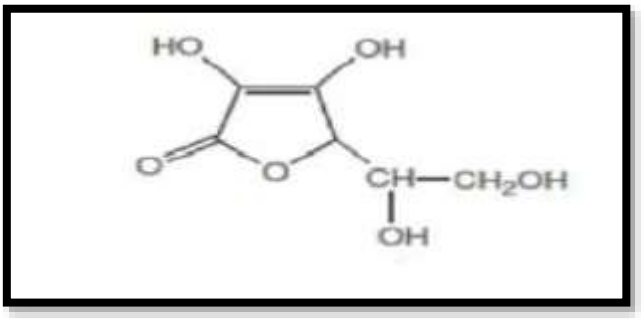


Figure 8: Structure of ascorbic acid (Evans et al. 2002).

4. Oxidative stress

4.1.Definition

The imbalance between the production of free radicals and reactive metabolites, called oxidants or reactive oxygen species, and their elimination by protective mechanisms, named oxidative stress (Reuter et al. 2010). Oxidative stress is an abnormal circumstance that sometimes crosses our cells or tissues when subjected to an imbalance between pro-oxidants and antioxidants, producing tissue damage through oxidative modifications of cellular biomolecules (Favier, 2003).

4.2.Origin of stress

Under normal circumstances, the antioxidant/pro-oxidant balance is in equilibrium. However, the endogenous overproduction of pro-oxidant agents of inflammatory origin, a nutritional deficit in antioxidants or even environmental exposure to pro-oxidant factors (tobacco, alcohol, UV and gamma rays, herbicides, and toxic metals), intense physical activity, or poorly managed all factors that can cause oxidative stress (Nève 2002).

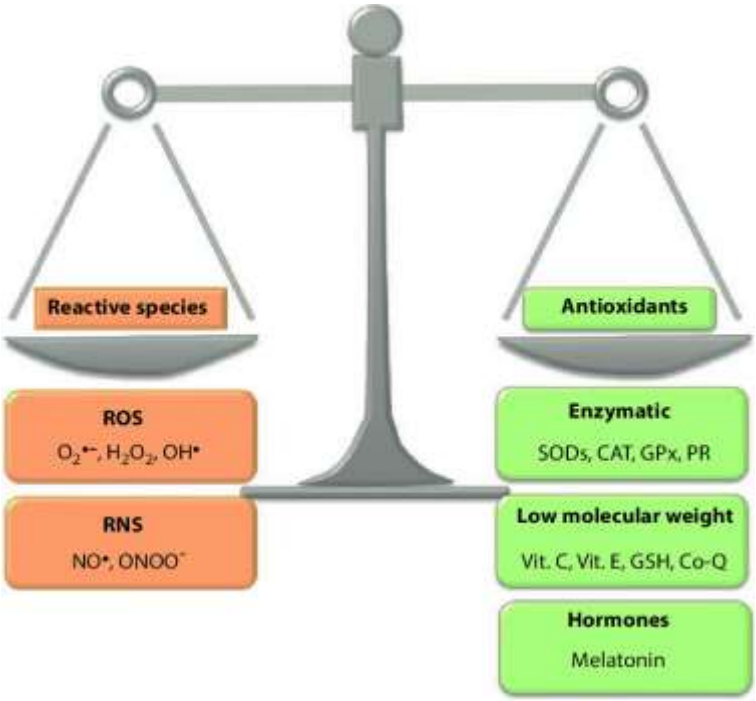


Figure 9: Pro-oxidant/antioxidant balance in biological systems (Ramiro-Cortijo et al. 2017).

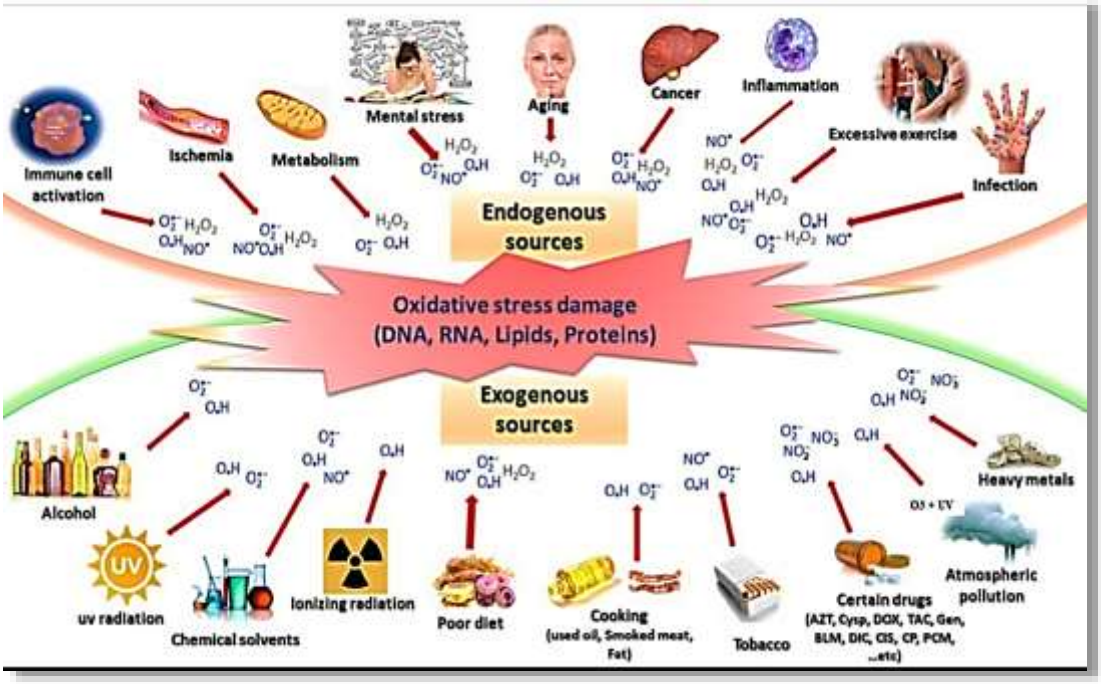


Figure 10: Origin of oxidative stress.

4.3.Biological markers of oxidative stress

ROS accumulation results in cellular and tissue damage that is often irreversible (Sorg 2004). The biological targets susceptible to radical attacks are lipids, proteins, and nucleic acids (Valko et al. 2007).

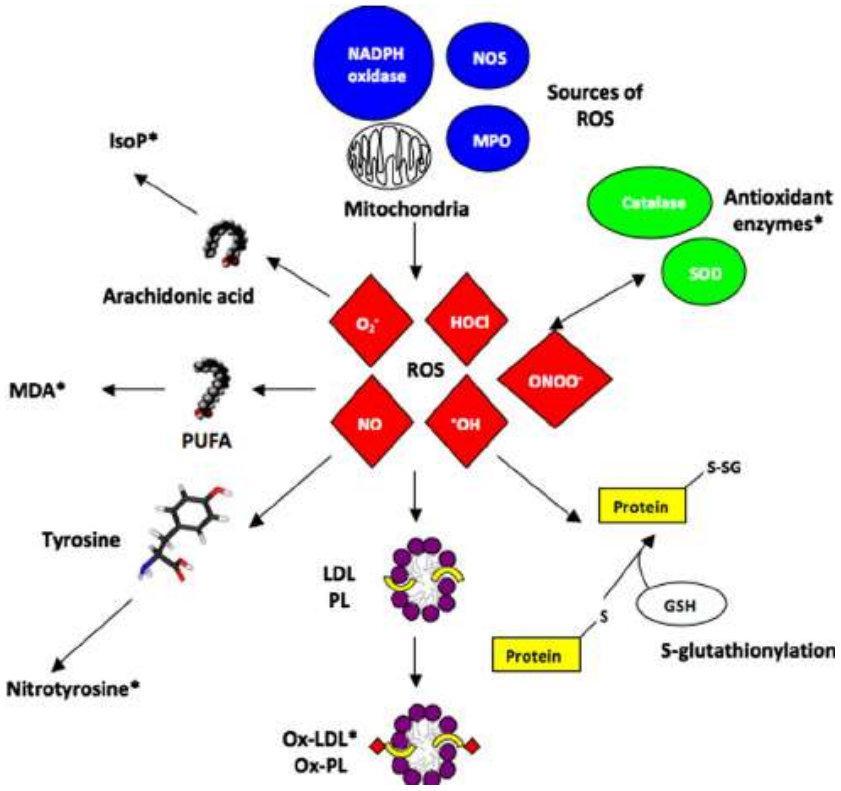


Figure 11: Formation pathways of selected biomarkers of oxidative stress (Ho et al. 2013).

a. Lipid peroxidation

Lipids, and mainly their polyunsaturated fatty acids, are the privileged target of the attack by the hydroxyl radical capable of tearing off hydrogen on the carbons located between two double bonds to form a conjugated diene oxidized in peroxyl radical. This lipid peroxidation reaction forms a chain reaction because the peroxyl radical formed is transformed into peroxide in contact with another fatty acid, including a new conjugated diene radical (Cadet et al. 2002; Favier, 2003). This reaction occurs in three stages: Initiation, propagation, and termination (Favier, 2003).

According to Lefèvre et al., lipid peroxidation products such as isoprostane, malondialdehyde (MDA), or 4-hydroxynonenal are the most commonly used markers to assess an oxidative stress situation (Lefèvre et al. 1998).

b. Alteration of DNA

ROS is the most important endogenous source of DNA damage. They can induce numerous covalent modifications such as lesions at nucleotide bases (purines and pyrimidines), single or double-strand breaks in the oligonucleotide chain, or bridging with protein residues. These

permanent changes in genetic material represent the first step involved in mutagenesis, carcinogenesis, and aging (Valko et al. 2007).

c. The oxidation of proteins

Like lipids, proteins can also target radical or oxidative reactions by undergoing modifications under the action of radical and non-radical ROS. The most sensitive proteins to attack are mainly those with a sulfhydryl (SH) group (Favier, 2003). The oxidative modifications of these proteins cause the introduction of a carbonyl group on the side chain of amino acids.

Important damages can be induced: significant structural modifications such as chain fragmentation formation of intra or intermolecular cross-linkages, which affect their functions and activities (Martínez-Cayuela 1995; Valko et al. 2007). Proteins modified in this way become more sensitive to the action of proteases and then directed to proteolytic degradation at the level of the proteasome (Jung et al. 2007).

4.4. Pathologies related to oxidative stress

The interaction of ROS with different biological molecules leads to the alteration of the organism's homeostasis. This is why oxidative stress is involved in many pathologies as a triggering or associated factor. Moreover, most pathologies related to oxidative stress appear with age because aging decreases antioxidant defenses and increases mitochondrial production of ROS with a reduction of the efficiency of repair and degradation systems of oxidative constituents (Favier, 2003; Sohal et al. 2002). Many epidemiological and clinical studies have demonstrated the involvement of oxidative stress in the development of a large number of different human pathologies ranging from atherosclerosis to cancer and inflammatory, cardiovascular, neurodegenerative diseases, and diabetes (Sánchez 2017).

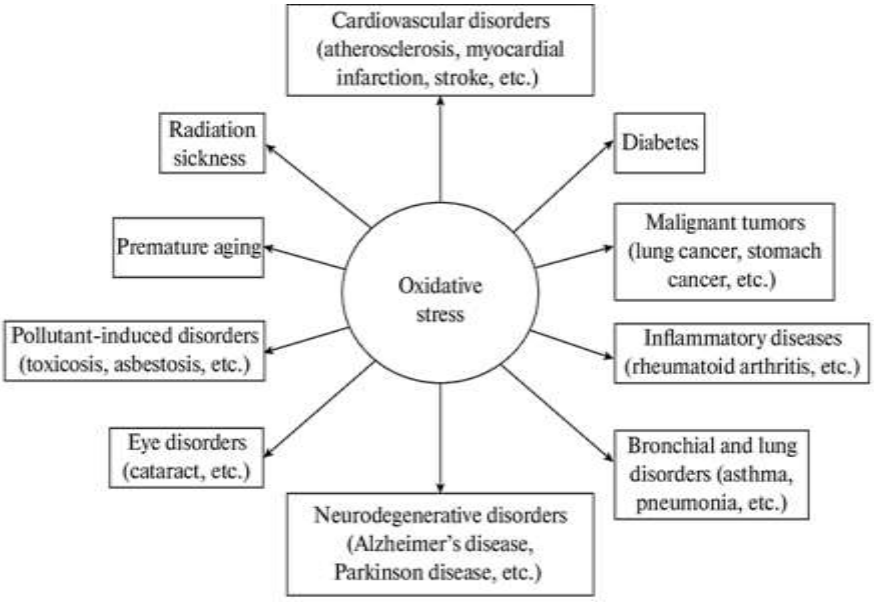


Figure 12: Pathologies caused by oxidative stress (Sharapov et al. 2018).

III. Herpes Simplex Virus

1. Definition

1.1.Classification

Genital herpes simplex virus (HSV) infection is widespread worldwide, with epidemiological surveys demonstrating rising infection rates in most countries (Fleming et al. 1997; Bonnar 2020). Herpes simplex virus (HSV) is a common human pathogen, causing infections of orofacial mucosal surfaces (HSV-1) and genital mucosal surfaces (HSV-2) (Taylor et al. 2002). HSV is the most common cause of genital ulcer disease in industrialized nations, and infections may be due to HSV types 1 or 2. Herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2) are members of the *Herpesviridae* family that cause a variety of clinically significant manifestations in adults and neonates (Rathbun et Szpara 2021).

Herpes simplex virus 1 (HSV-1) infection is common worldwide, with 45 % to 98 % of the world population and 40 % to 63 % of the people in the United States reportedly HSV-1 seropositive.

HSV-1 and HSV-2 are members of the *Herpesviridae* family of DNA viruses, grouped with varicella-zoster virus in the *alphaherpesvirus* subfamily. The alphaherpesviruses are characterized by short reproductive cycles, host cell destruction during active replication, and the ability to establish lifelong latency in sensory neural ganglia (Rathbun et Szpara 2021).

1.2.Transmission

HSV is transmitted primarily through oral mucosal contact and causes orofacial or labial infection due to viral particles in wounds, saliva, labial, or perioral surfaces (Taylor et al. 2002). HSV-1 can also be transmitted to the genital area during oral sex, causing genital herpes. In rare cases, a mother can transmit HSV-1 genital herpes to her newborn during delivery, resulting in neonatal herpes (James et al. 2014).

1.3.Viral structure and genomic

a. Viral structure

HSV is a large (150- 200 nm diameter) enveloped virus with a distinct virion structure characteristic of the herpesviruses (Taylor et al. 2002). Like *Herpesviridae* members, the HSV-1 virion comprises four main structures: envelope, tegument, capsid, and the viral genome (Pandey et al. 2020).

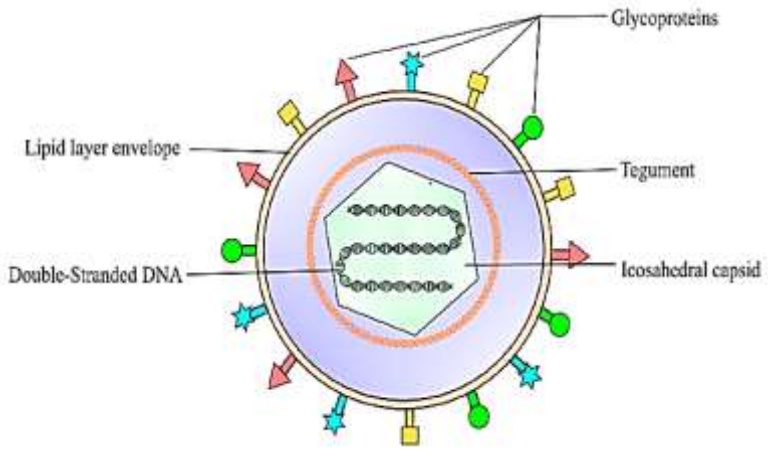


Figure 13: Herpes simplex virus structure (Pandey et al. 2020).

b. Genome Organization

Herpes simplex virus-1 (HSV-1) is a neurotropic double-stranded DNA virus belonging to the *Alphaherpesvirinae* family, a sub-family of *Herpesviridae* (Rovnak et al. 2015; Koujah et al. 2019). The HSV genome is organized into two components, a unique long (UL) region (106.5 kb), a unique short (US) region (13.5 kb), two copies of a long inverted repeat (RL) (8.75 kb each), and two copies of a short inverted repeat (RS) (6.25 kb each) (Taylor et al. 2002; Watson et al. 2012). Guanine and Cytosine (G+C) base composition varies from 32 to 75% depending on the viral species under consideration (Watson et al. 2012; Jiao et al. 2019).

Around 84 different proteins encoded by the herpes simplex virus 1 (HSV-1), at least 4 proteins, all located in the tegument of the virion, interact with mRNAs (Sciortino et al. 2007). Of these,

the proteins encoded by the U_S11, U_L47, and U_L49 open reading frames (ORFs) bind RNAs, whereas the fourth, encoded by the U_L41 ORF, items as an RNase (Smiley 2004).

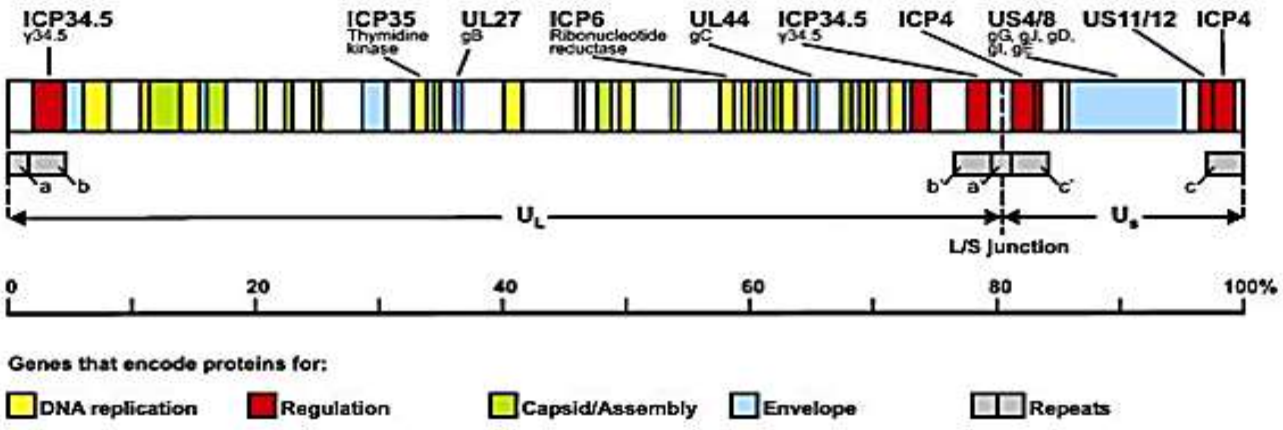


Figure 14: HSV-1 genomes (Argnani et al. 2005)

HSV-1 genome is divided into two unique segments called long (U L) and short (U S). Short regions of repeated sequence (a/b/c and a'/b'/ c') occur at the genome ends and between the L and S segments. As DNA is replicated, the L and S segments invert at a high rate, creating four genome isomers. The four occur at equal frequencies in most wild-type HSV-1 populations. The genome encodes 74 proteins. Most genes encoding proteins are located in the L or S regions, and they are named according to their location within L or S. A schematic representation of the positions of the genes encoding these proteins in the HSV-1 genome is depicted above. Genes that are modified or deleted to achieve tumor-specific targeting and replication are also indicated.

2. Tropism

Herpes simplex viruses (HSV) go into their host via mucosal epithelia, skin, or cornea. In contrast, humans are the only natural host. Primary infection in the epidermis is followed by penetration of the nervous system and establishing latent infection in neurons. Upon reactivation, HSV is transported back to the epithelium to reinfect epithelial cells (Schelhaas et al. 2003).

The first step consists of the attachment of the virus particles. It is mediated by the HSV envelope glycoprotein C (gC) and/or gB with cell surface heparan sulfate proteoglycans (Herold et al. 1994). HSV-1, like a multitude of viruses, employs glycosaminoglycan (GAG) as initial attachment receptors during infection (Musarra-Pizzo et al. 2021). The initial contact facilitates subsequent binding to a coreceptor, which is required to enter the cell. The viral envelope protein gD serves as a virus ligand for all HSV coreceptors identified. gD coreceptor binding is followed by fusion of the viral envelope and the plasma membrane, which is activated in concert with further viral envelope glycoproteins, such as gB, gH and gL (Spear 1993).

3. Viral cycle

HSV displays both lytic and latent modes of interaction with its natural human host. Primary infection of epithelial cells produces the lytic response virus replication followed by cell death. Progeny virus particles then infect adjacent sensory neurons, establishing a lifelong latent interaction. The latent viral genome is maintained in an extrachromosomal state in which only a restricted portion of the genome is transcribed. Latent genomes occasionally reactivate into the lytic cycle, producing a limited amount of progeny virus that gives rise to secondary infections of the epithelial sites enervated by the latently infected neurons. According to (Everett 1989; Lussignol et Esclatine 2017), the stages of HSV infection are:

(1) Receptor binding and membrane fusion

(2) Release of the viral nucleocapsid and tegument into the cell cytoplasm and transport of the nucleocapsid to the nuclear pore

(3) Release of viral DNA into the nucleus

(4) Transcription and translation of the viral immediate-early (IE) and early (E) genes

(5) Viral DNA synthesis

(6) Transcription and translation of the viral late (L) genes

(7) capsid assembly and DNA packaging

(8) Release of progeny virions

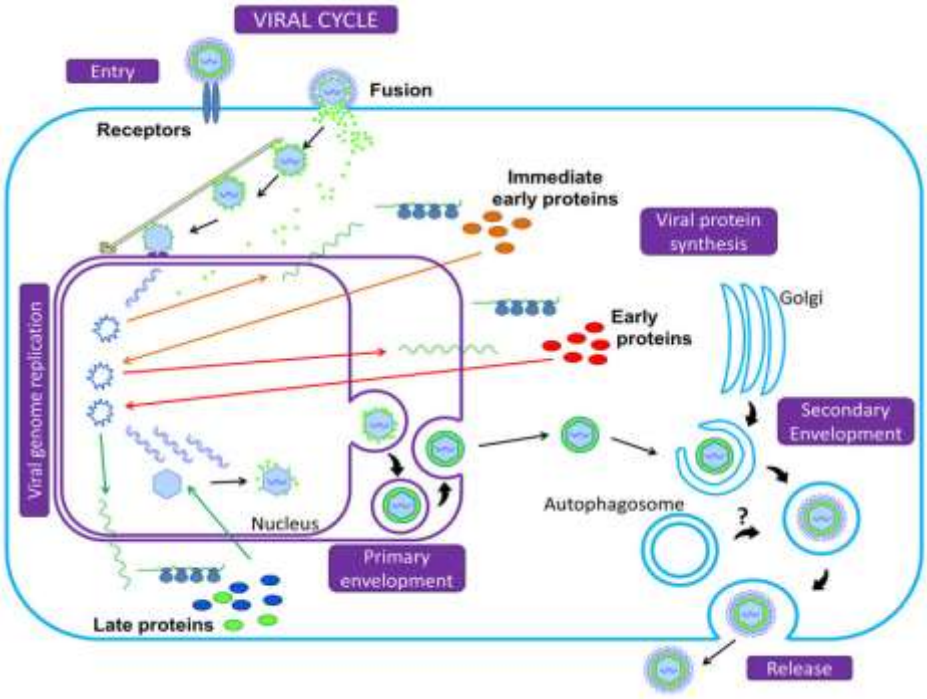


Figure 15: Herpesvirus replication cycle (Lussignol et Esclatine 2017).

4. HSV genes

Five viral immediate-early (IE) genes are expressed first, and four of these (ICP0, ICP4, ICP22, and ICP27) encode regulatory proteins that stimulate expression of the viral early (E) and late (L) genes. The E genes are activated next, giving rise to proteins required to replicate the viral genome. Viral DNA replication then ensues, augmenting IE-dependent expression of the L genes that encode the structural components of the virion.

These virion regulators are located in the viral tegument and, as such, are injected into the newly infected cell immediately upon fusion of the viral envelope with the host cell plasma membrane. Therefore, these proteins are strategically poised to influence the earliest events in the viral replication cycle. In the best-known case, the abundant tegument protein VP16 activates transcription of the viral IE genes, thereby contributing to the initial launch of the lytic program of gene expression (Herr 1998). In addition, the tegument contains HSV, the virion host shutoff protein encoded by HSV gene UL41. HSV is an mRNA-specific RNase that triggers rapid shutoff of host cell protein synthesis, disruption of preexisting polyribosomes, and degradation of host mRNAs in the absence of de novo viral gene expression (Smiley 2004). The cycle of productive HSV replication in a cell (Taylor et al. 2002). The stages of HSV infection are:

(1) Bind the receptor and membrane fusion

(2) Issue of the viral nucleocapsid and tegument into the cell cytoplasm and transport of the nucleocapsid to the nuclear pore

(3) Release of viral DNA into the nucleus

(4) Transcription and translation of the viral immediate-early (IE) and early (E) genes

(5) Synthesis of viral DNA

(6) Transcription and translation of the viral late (L) genes

(7) Capsid assembly and DNA packaging

(8) egress of progeny virions.

4.1. Immediate early genes

Table 3: Immediate early gene

Protein name	Functions
ICP0	-Transactivator of classes of HSV genes (Quinlant et Knipe 1985; Stow et Stow 1986). -Binds to cellular proteins involved in protein degradation (Everett 1989).
ICP4	-Transcription of early and late genes (Dixon et Schaffer 1980). -Regulates its expression by binding to consensus sites within the ICP4 promoter (Kristie et Roizman 1984).
ICP27	-Essential for early and late genes transcription (Fontaine-Rodriguez et Knipe 2008; Strain et Rice 2011). -Redistributes snRNPs and inhibits splicing (Tang et al. 2016). -Shuttles between nucleus and cytoplasm synthesis of some E proteins (Malik et al. 2012).
ICP22	-Modification of the host RNA polymerase II (Stelz et al. 2002) -Optimal expression of ICP0 and late genes (Jovasevic et Roizman 2010 ; Kolb et al. 2011).

4.2. Essential HSV DNA replication proteins

Table 4: HSV DNA proteins

Protein Name	Gene	Activity
Origin-binding protein	**UL 9**	• Binds HSV origins (oriL and oriS). • DNA helicase activity (Boehmer 1998).
Single-stranded DNA-binding protein (ICP8)	**UL29**	• Binds ssDNA •Stimulates DNA polymerase and helicase-primase activities (Heilbronn 2003).
Helicase-primase complex	**UL5/UL52** **UL8**	• DNA helicase and primase activities (Chen et al. 2011). • Stimulates UL5/UL52 complex (Crute et al. 1988).
DNA-polymerase holoenzyme	**UL30** **UL42**	• DNA polymerase and exonuclease activities (Sawtell et Thompson 2016). • Promotes processive DNA synthesis (Gibbs et al. 1988).

5. Disease caused by HSV

HSV has been implicated in several diseases and pathologies:

- Mucocutaneous infection (most common), including genital herpes
- Ocular infection (including herpes keratitis)
- Central nervous system (CNS) infection
- Neonatal herpes

HSV rarely causes fulminant hepatitis in the absence of cutaneous lesions.

In patients with HIV infection, herpetic infections can be particularly severe. Progressive and persistent esophagitis, colitis, perianal ulcers, pneumonia, encephalitis, and meningitis.

HSV outbreaks may be followed by erythema multiforme, possibly caused by an immune reaction to the virus.

IV. The Epstein-Barr Virus

1. Definition

1.1. Classification

The Epstein-Barr virus is a human herpesvirus of the family *Herpesviridae* (Human Herpesvirus type 4 HHV4), subfamily *Gammaherpesvirinae*, genus Lymphocryptovirus. Lymphocryptoviruses are endemic in the whole world. Primates are their exclusive hosts (Seigneurin 1999).

EBV is the first human virus associated with human tumors of lymphocytic or epithelial origin. This virus can infect primate B cells *in vivo* and *in vitro* (Sculley et al. 1990). EBV is a highly prevalent oncogenic virus carried by over 90 % of the adult human population worldwide as a largely non-pathogenic infection and is the only human-specific lymphocryptovirus (Inman et al. 2001; Oussaief et al. 2009). It exists in two types: 1 and 2 (Sculley et al. 1990). Although they differ by only a few genes, these two viral types have different biological properties and a particular geographical distribution (Crawford 2001).

1.2.Transmission

Human infection with EBV usually occurs through contact with either oral oropharyngeal secretions containing the infectious virus. EBV replicates in the cells of the oropharynx, and almost all HIV-positive individuals actively shed the virus in saliva. Transmission in transplant recipients is also possible via EBV-infected B cells present in the organ donor's blood (Cohen 2000; Aromseree et al. 2017).

1.3. Viral structure and genomic

a. Viral structure

The viral particle has a more or less spherical shape with cubic symmetry and a size of 150-200 nm (Carbone et al. 2008). The different parts of this particle are:

- A protein core with a toroidal shape is associated with a double-stranded DNA molecule.
- A nucleocapsid with icosahedral symmetry, consisting of 162 capsomeres (Seigneurin 1999).
- A tegument between the nucleocapsid and the envelope.
- A viral envelope with external glycoprotein spicules (Thorley-Lawson et Edson 1979).

The capsid itself is surrounded by a tegument and an envelope consisting of a double lipid layer, into which viral glycoproteins are inserted in the form of spicules (Szakonyi et al. 2006; Matsuura et al. 2010; Germini et al. 2020).

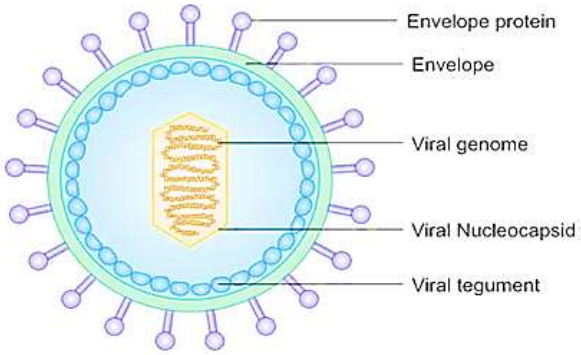


Figure 16: Structure of Epstein-Barr virus (Odumade et al. 2011).

b. Genomic structure

The EBV genome was completely sequenced in 1984 (Baer et al. 1984). It is a double-stranded DNA molecule of 172 kilobases (Kb) and encodes 100 to 150 proteins. The guanine and cytosine nitrogen bases make up 59 % of the viral DNA (Dambaugh et Kieff 1982). It is divided into several unique domains, separated by internal repeats (IR) (IR1-IR4); the 3000 bp IR1 sequence divides the genome into a unique long domain (UL) and a unique short domain (US). Each end has 538 bp terminal repeats (TRs), which allow circularization of the genome under certain circumstances during DNA replication. The DNA molecule is always in linear form (Cohen 2000).

A difference in genomic structure distinguishes EBV from viruses of the same family. However, it has significant similarities with cellular genes. Indeed, a GGGGCAGGA repeat sequence of the BKRF-1 gene, which codes for the viral antigen EBNA-1, has been detected in cellular

DNA (Helleret al. 1985). Similarly, BZLF-1, the lytic viral cycle transactivator gene, shows structural and functional homology with cellular DNA transcription activator genes (Jun/fos) (Packham et al. 1990). In addition, the BHRF-1 gene has sequence homology with a cell-derived oncogene gene BCL-2 (Pearson et al. 1987).

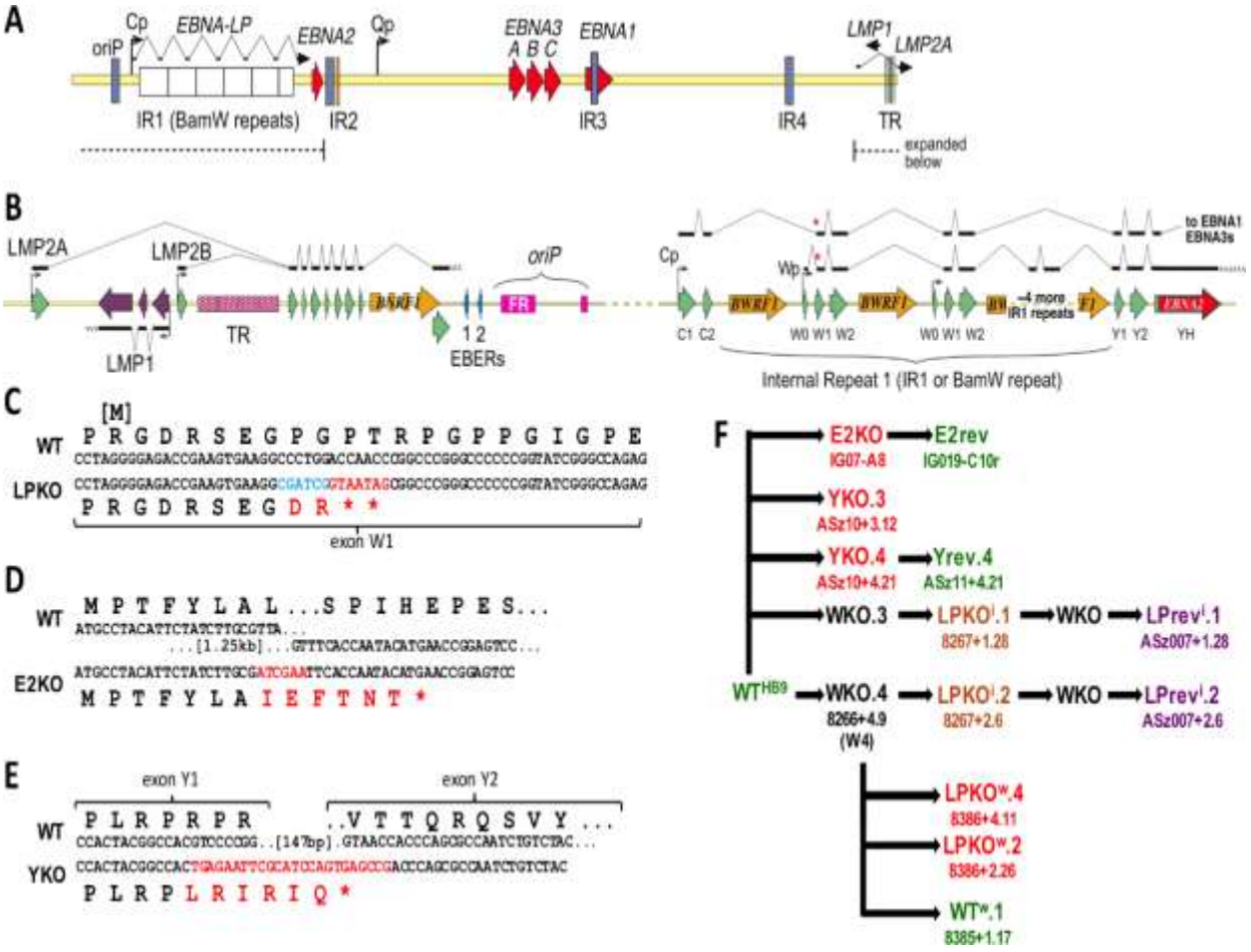


Figure 17: Schematic representation of the EBV genome (Szymula et al. 2018).

A. Schematic representation of the EBV genome showing the latency genes and internal repeat 1 (IR1) across which the repetitive domain of EBNA-LP is transcribed. The region from the LMP genes to EBNA2 (dotted line) crosses the termini of the linear genome and is shown in **B.** Promoters Cp and Wp are indicated by bent arrows; exons are green (right facing) or purple (left facing) arrows, with intact ORFs indicated in other colors. Names of EBNA exons are shown below the relevant arrow. The splicing of latency genes is indicated above (plus-strand transcripts) or below (minus strand transcripts) the exons. Two examples of the alternatively spliced EBNA transcripts indicate variable numbers of W exon pairs in EBNA-LP, variable use of Cp and Wp promoters, and differential splicing producing either EBNA2 or other downstream EBNAs. Variations in the W1 splice acceptor from C2 or W0 (red asterisks) define whether a transcript encodes EBNA2 or EBNA-LP. C-E Mutations introduced into recombinant viruses. Parental B95-8 sequence is shown above the introduced mutation used to generate: **C.** EBNA-LP knockout; **D.** EBNA2 knockout; **E.** EBNA-LP Y exon knockout. **F.** Flow chart showing a set of recombineering steps used to generate the recombinant EBVs constructed for this study. Note that viruses whose IR1 is mutated are made via an intermediate (WKO) in which IR1 has been entirely deleted. Colored names indicate recombinant BACs used to generate the viruses used in experiments– Green names are wild-type in sequence and phenotype; Red names are mutants; LPrevi is shown in purple, as it contains a point change compared to wild-type that was intended to be phenotypically neutral.

2. Cellular Tropism

The target cells of the Epstein-Barr virus are B lymphocytes (LB). However, other cells may also harbor it, such as epithelial cells and T lymphocytes (LT) (Kasahara et Yachie 2002). Susceptible techniques such as in situ hybridization, immunohistochemistry, and PCR performed on the salivary gland and tonsil cells from patients with infectious mononucleosis (IM) have shown that EBV has strict lymphotropic during primary infection and that virus persistence occurs mainly in memory B cells (Sixbey et al. 1984; Allday et Crawford 1988).
During primary infection, EBV transits from the oropharynx to the B cells crosses the epithelium by transcytosis, impressing the crypts of the oropharyngeal epithelium to infect the B cells in immediate contact with it (Faulkner et al. 2000). Once infected, the B cells will increase by expressing all latency genes. Against these infected B cells, an immune response will then develop, essentially mediated by cytotoxic T cells (CTL) (Faulkner et al. 2000; Kasahara et Yachie 2002).

3. Viral Cycle

EBV can infect its target cells in latent and lytic modes. The latent cycle does not result in virus production but allows viral persistence in B lymphocytes with reduced viral antigen expression. The lytic or productive cycle corresponds to the cascade expression of different viral proteins, enabling new virions (Niller et al. 2008).

3.1.Latent viral cycle

Latency corresponds to a specific phase of the virus-host interaction, during which the viral genome persists without producing infectious particles.

a. Different types of latency

The analysis of viral genes expressed in latently infected cells has led to the definition of 4 types of latency:

- **Latency 0:** Is demonstrated in memory B cells (Thorley-Lawson et Edson 1979; Young et Rickinson 2004). It is characterized by the expression of mRNAs of the latency antigens EBNA-1, LMP2-A, and two other non-polyadenylated transcripts (Epstein-Barr Encoded small RNAs) (EBER1 and 2 RNA) (Thorley et Edson 1979).
- **Latency I:** Associated with African Burkitt's lymphoma (Rowe et al. 1987; Gregory et al. 1990). It is characterized by the expression of the viral nuclear antigen EBNA-1 and a complex group of alternatively spliced transcripts (BARTs) and two other non-polyadenylated transcripts, EBER1 and 2 (Thorley et Edson 1979).

- **Latency II**: is associated with undifferentiated nasopharyngeal cancer and Hodgkin's lymphoma. It is characterized by the expression of the viral antigens EBNA-1, LMP-1, and LMP-2 and the mRNAs EBERs and BARTs (Fåhraeus et al. 1988; Brooks et al. 1992; Busson et al. 1992).
- **Latency III:** Is observed in B cells from patients with infectious mononucleosis (IM), B lymphoma, and B lymphoblastoid cell lines established *in vitro* (Thorley et Edson 1979; Young et Rickinson 2004). It is characterized by the expression of 6 nuclear antigens (EBNA-1, 2, 3A, 3B, 3C and LP), 3 membrane antigens (LMP-1, LMP-2A, LMP-2B) as well as the mRNAs EBERs and BARTs (Young et Rickinson 2004).

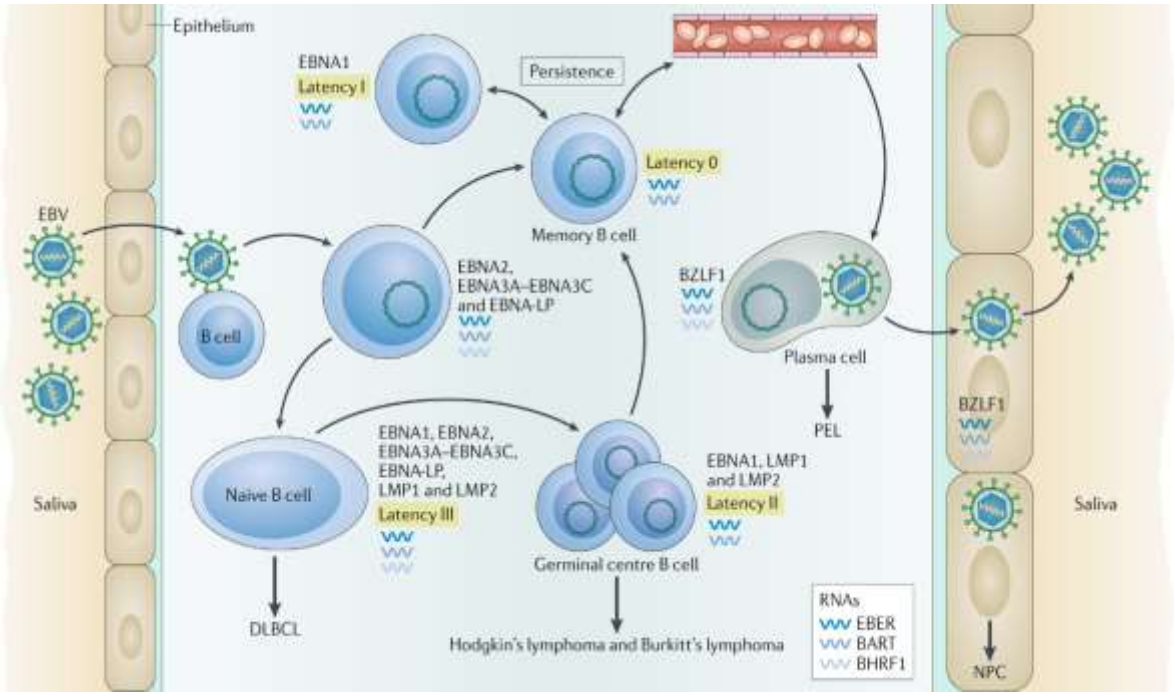


Figure 18: Models of latent Epstein–Barr virus infection to reach viral persistence (Münz 2019).

b. Latent viral cycle proteins

Infection of LBs with EBV establishes a latent cycle marked by the expression of a limited number of viral genes. The viral proteins of the EBV latent cycle include 6 nuclear proteins (EBNAs) and 3 membrane proteins (LMPs). In addition to the proteins, two genes are also transcribed in large quantities but not translated into proteins: EBER-1 and EBER-2. Analysis of mutated or deleted genes has shown that some proteins are essential for cell transformation (or immortalization), particularly EBNA-2, EBNA-3A, EBNA-3C, and LMP-1 (Bornkamm et Hammerschmidt 2001; Cohen 2003).

The EBNA-2, EBNA-3, and EBER genes show polymorphism that allows isolates into 2 viral types, EBV-1 and EBV-2. The viral proteins associated with latency and their currently known functions are listed in Table 5.

Table 5: Nomenclature of EBV latent cycle proteins and their main functions.

Viral proteins	Location	Main functions
EBNA-1	Nuclear	-Maintenance of the viral genome in episomal form.
EBNA-2	Nuclear	-Immortalization of LBs activates the cellular genes CD21 and CD2 (Farrell et al. 1989). -Modulates the cellular oncogene and the promoters of the LMP-1, LMP-2A, LMP-2B genes (Yue et al. 2005). -Acts on the Cp promoter of the nuclear antigens EBNA-1, 2, 3A, 3B, 3C, and LP (Kis et al. 2006).
EBNA-3A	Nuclear	-The repressor of EBNA-2 action binds to transcription factor RBP-Jkappa. -Participates in LB immortalization (Zhao et al. 2011)
EBNA-3B (or EBNA-4)	Nuclear	-Binds to RBP-Jkappa (Zhao et al. 2011).
EBNA-3C (or EBNA-6)	Nuclear	-Activates transcription of CD23 cell genes in non-endemic Burkitt lines and LMP-1 in the Raji line (Chen et al. 2014)
EBNA-LP (or EBNA-5)	Nuclear	- Cofactor of EBNA-2. It associates with the pRB and p53 proteins (Inman et al. 2001).
LMP-1	Membrane	-Major oncogene and transactivator (Yoshida et al. 2017).
LMP-2A	Membrane	-Prevents viral reactivation and maintains the latency phase (Engels et al. 2001)
LMP-2B	Membrane	-Modulates the activity of LMP-2A (Rovedo et Longnecker 2007).

3.2.Lytic viral cycle

There is a chronological expression of markers of the lytic cycle (Seigneurin 1999). The expression of immediate early antigens does not require protein synthesis. The expression of early antigens is independent of DNA synthesis. Finally, late antigens are expressed only after DNA synthesis.

a. The immediate early antigens

Induction of the lytic cycle in protein synthesis inhibitors allowed the identification of two transcripts corresponding to immediate early genes, BZLF-1 (1 Kb) and BRLF-1 (0.8 Kb). Their maximum transcriptional level is reached two hours after induction of the lytic cycle. Both play a significant role in activating the early and late viral antigen cascade (Bristol et al. 2010).

The ZEBRA or Zta (BamHI Z Epstein-Barr virus Replication Activator) protein is critical in viral reactivation. This protein belongs to the b-Zip (basic leucine zipper) family. It is the product of the BZLF-1 gene, which is a viral gene homologous to the cellular transcription factor c-fos, which binds to AP-1-type consensus motifs (activator proteins), also known as Z-specific response elements (ZREs) (Petosa et al. 2006; Flower et al. 2011). ZEBRA is the first viral protein expressed, which marks the entry into the lytic phase. This immediate early antigen activates its target genes by binding to the BZLF-1 response element (ZRE), which is present in many EBV early gene promoters and at the promoter of BZLF-1 itself BRLF-1 (Farrell et al. 1989; Packham et al. 1990). Thus, it serves as a transcriptional transactivator of its promoter and the promoters of immediate early genes, direct action of ZEBRA on viral replication via binding to the origin of lytic viral replication, has been demonstrated. In addition, ZEBRA represses the Cp and Wp latency genes (Atanasiu et al. 2006; Yu et al. 2007).

The ZEBRA protein acts on the anti-EBV immune response by lowering the expression of major histocompatibility complex molecules MHC-1 and MHC-2 (Zuo et al. 2009). As a result, it interferes with cell signaling systems, affects cell cycle progression, and induces apoptosis. At the same time, its expression can be affected by specific cellular components and chemical agents (Wang et Cao 2003; Imajoh et al. 2012).

The BRLF-1 gene product, Rta, activates several early viral genes (Cox et al.1990; Morrison et al. 2001). Like ZEBRA, Rta binds to specific DNA sequences (Murata 2014). However, this ability is more complex than ZEBRA, suggesting that its function is limited to activating viral gene expression (Murata 2014).

b. Early antigens

Two early antigens are defined according to their cellular distribution in the host cell, diffuse early antigen (EA-D) localized in the nucleus and cytoplasm and restricted early antigen (EA-R) localized in the cytoplasm. Most early antigens have been characterized as viral replication proteins (DNA polymerase, ribonucleotide reductase, and thymidine kinase) (Pearson et al. 1987; Verma et al. 2009; Sugimoto et al. 2019).

c. Late antigens

They are expressed after viral replication. Their function is not fully defined. The vast majority of late viral markers are structural proteins or factors involved in modifying the host cell to facilitate virus packaging and exit (Young et Rickinson 2004).

Table 6: Lytic proteins: their locations and functions

Viral proteins	Location	Functions
ZEBRA (or BZLF1, Z, Zta or EB1) and Rta or BRLF1	Nucleus	-Transcriptional regulators initiate and promote lytic infection (Deng et al. 2001). - Replication of viral DNA and induction of expression of early lytic cycle genes (Gruffat et al. 2002)
VCA	Nucleus and cytoplasm	-Formation of the virus particle (Neuhierl et al. 2002).
gp350/200	Cell membranes and virion envelope	-Binds with the complement receptor CD21 to allow virus attachment to the surface of lymphocytes (Sorem et al. 2009).
gp42-gp25-gp85	Cell membranes and virion envelope	-Form a ternary glycoprotein complex that interacts with HLA class II molecules and allows endocytosis of EBV virion (Wen et al. 2007).
gp110	Endoplasmic reticulum membrane	Production of virions (Neuhierl et al. 2002).-

4. EBV life cycle

4.1.Primary infection: Entry of the virus into the host cell

EBV binds to the cell membrane by interacting with the viral envelope glycoprotein gp350/220 and the molecule CD21, a receptor for the C3d and C3g fractions of complement. CD21 is present on certain T cell lines and epithelial cells. The viral envelope glycoprotein gp85 is responsible for fusing the cell membrane and the virus envelope (Depil et al. 2004). EBV entry then requires the formation of a complex between the four glycoproteins gp85 (gH), gp25 (gL), gp110 (gB) and gp42. This complex allows gp42 to interact with an HLA class II molecule and cause endocytosis of the virus (Depil et al. 2004).

4.2.Latent viral cycle

Latency corresponds to a specific phase of the virus-host interaction, during which the viral genome persists without the production of infectious particles (Young et Rickinson 2004; Thierry et al. 2012; Münz 2019).

4.3.Lytic viral cycle

The EBV genome is amplified 100 to 1,000 times in the lytic phase. Then, replication occurs entirely within the cell nucleus (Thierry et al. 2012).

Entry into the lytic phase is determined by the expression of the transactivator protein Zebra (BamH1 Z replication activator) (Münz 2019). The regulation of the expression of this protein seems to be the key to the transition from latency to viral production. It activates the transcription of its gene, induces the expression of early genes, the replication of viral DNA from the two origins of lytic replication (DR and DL), and the expression of late genes VCA (viral capsid antigens) and MA (membrane antigens): gp350/220, gp140, gp110 and gp85 (Klein et al. 2007).

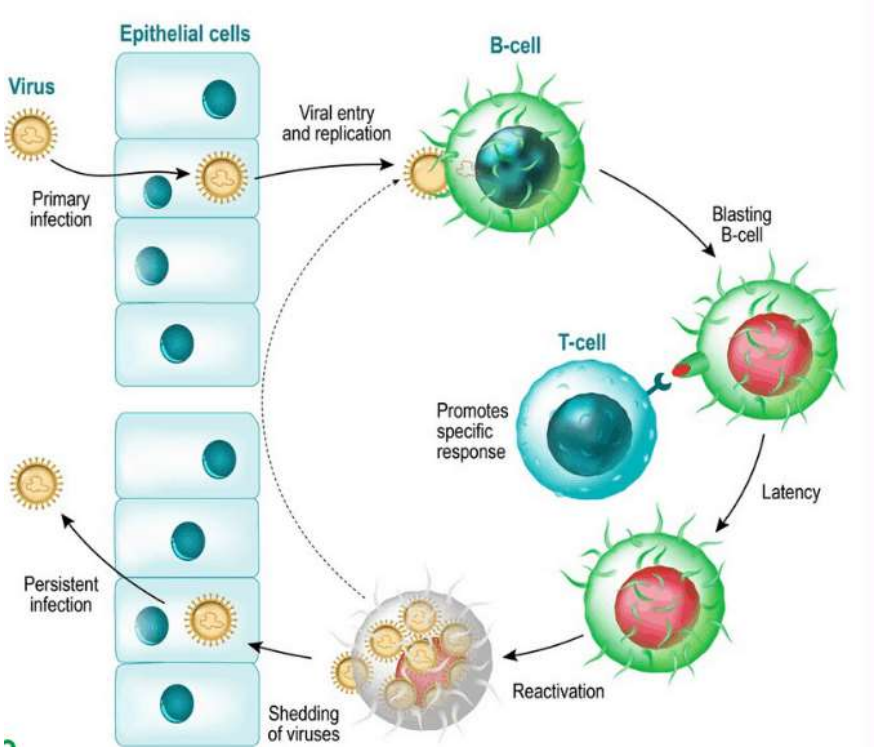


Figure 19: The replication cycle of the Epstein-Barr virus (EBV).

5. EBV and oxidative stress

According to Lassoued et al., EBV is involved in establishing an oxidative stress state *in vitro* from the early stages of viral infection (Lassoued et al. 2008).

EBNA-1 is the only viral protein expressed in all EBV-bearing malignancies. The expression of this protein induces chromosomal aberrations DNA double-strand breaks. These signs of genomic instability are associated with the production of reactive oxygen species (Gruhne et al. 2009).

Gargouri et al. showed the presence of lipid peroxidation and disruption of antioxidant enzyme activity following induction of the EBV lytic cycle (Gargouri et al. 2009). More recent studies by Gargouri et al. have shown that EBV lytic cycle induction is associated with ROS production and increased expression of CAT and SOD genes (Gargouri et al. 2011).

6. Diseases Caused by EBV

EBV has been implicated in several diseases, including infectious mononucleosis, Burkitt's lymphoma, Hodgkin's lymphoma, stomach cancer, nasopharyngeal carcinoma, multiple sclerosis, and lymphomatoid granulomatosis (Tagliavini et al. 2013; Germini et al. 2020; Zanelli et al. 2021).

The progression of asymptomatic or primary infections to:

- Any one of various Epstein-Barr virus-associated lymphoproliferative diseases. Such as chronic active EBV infection, EBV+ hemophagocytic lymphohistiocytosis, Burkitt's lymphoma, and Epstein Barr virus-positive diffuse large B-cell lymphoma.
- Non-lymphoid cancers such as Epstein-Barr virus-associated gastric cancer, soft tissue sarcomas, leiomyosarcoma, and nasopharyngeal cancers.
- Epstein-Barr virus-associated non-lymphoproliferative diseases such as some cases of the immune disorders of multiple sclerosis and systemic lupus erythematosus and the childhood disorders of Alice in Wonderland Syndrome and acute cerebellar ataxia.

Materials And Methods

A. Materials

1. Sample Origin and preparation

The samples of the olive leaves used were collected during October 2018 from Sfax, southeast of Tunisia. The choice was directed towards the most important olive variety Chemlali, *Olea europaea L. var. sativa* (OESA), and *Olea europaea var. sylvestris* (OESY).

2. Cell Lines

2.1. HeLa cell lines

HeLa was the first human cell line anchored in culture and has since become the most widely used line for studying human molecular cell biology. For example, one of the first uses of HeLa cells was to develop the poliovirus vaccine (Gey et al. 1952; Scherer et al. 1953).
It is a transformed line expressing HPV18 (human papillomavirus). This adherent line is obtained from tumor cells from a 31-year-old female cervical cancer patient named Henrietta Lacks, who later died in 1951 (Gey et al. 1952; Skloot 2011).

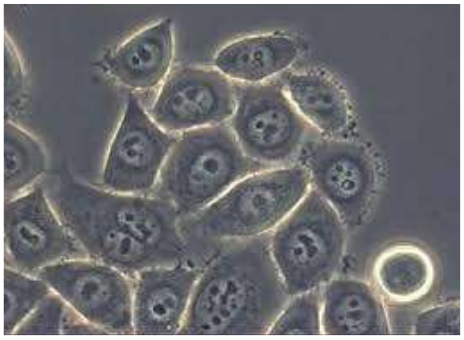


Figure 20: Microscopic observation of HeLa cell lines

2.2. Raji cell lines

An EBV-positive B lymphoblastoid line is obtained from an African Burkitt lymphoma and is characterized by an abortive viral cycle. This lymphomatous line is non-EBV-producing. The viral genome of this line is characterized by two deletions, one in the gene encoding the EBNA-3C latency protein the other in the BALF2 ORF encoding the 135 Kd DNA polymerase protein. These two deletions probably explain the underexpression of the latency genes in this cell line during the induction of the lytic viral cycle resulting in abortive infection (Hatfull et al. 1988). A fraction of less than 0.1 % of Raji cells spontaneously enters the lytic viral cycle (Rickinson et al. 1987).

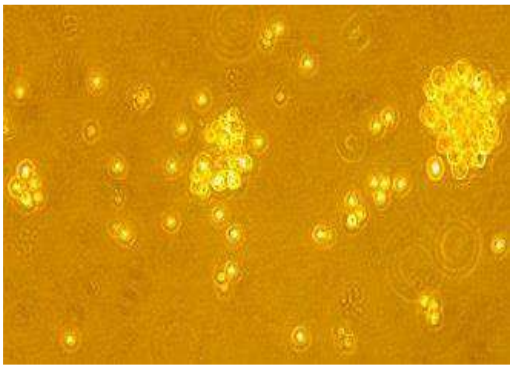


Figure 21: Microscopic observation of Raji cell lines

2.3. Vero cell lines

Vero cells were derived from the kidney of an African green monkey (*Cercopithecus aethiops*) in 1960. Vero cells are one of the most common mammalian continuous cell lines used in research (Ammermanet al. 2008). It was the first aneuploidy attachment-dependent cell to produce human biological products and simultaneously establish cell lines and cell banks of different generations.

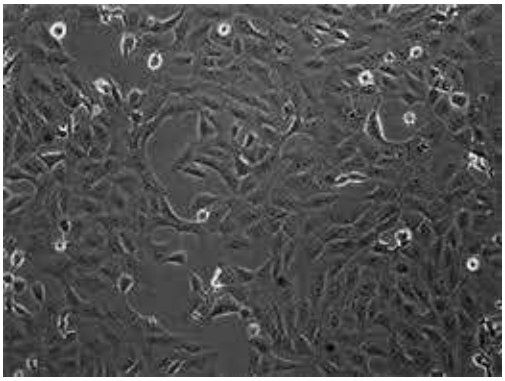


Figure 22: Microscopic observation of Vero cell lines

3. Culture Medium

3.1. RPMI 1640 (Rosewell Park Memorial Institute)

Culture medium (Gibco) is used for the culture of HeLa and Raji lines. The medium is supplemented with 2 g/L sodium bicarbonate ($NaHCO_3$). The pH is adjusted to 7.2 with 1N HCl, and the medium is filtered through a 0.22 µm filter, then supplemented with 10 % fetal calf serum (Gibco), 1 ‰ gentamicin, and 2 mM L-glutamine.

3.2.Dulbecco's Modified Eagle Medium (DMEM)

DMEM (Gibco) is a widely used basal medium for supporting the growth of many different mammalian cells. Cells successfully cultured in DMEM include primary fibroblasts, neurons, glial cells, HUVECs, and smooth muscle cells, as well as cell lines such as 293, Cos-7, and PC-12.

Vero cell lines were cultured in Eagle's minimum essential medium (EMEM, Lonza, Belgium), with 6 % fetal bovine serum (FBS, Euroclone) and a mixture of penicillin (100 U/mL) and streptomycin (100 µg/mL) (Lonza, Belgium).

4. Solutions and Chemicals

PBS (10 X): 8 % NaCl; 0.2 % KCl; 3 % Na_2HPO_4; 0.2 % KH_2PO_4.

MTT [0.5 mg/mL]: 3 (4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide.

TPA [8Nm]: 12-O-Tetradecanoyl-phorbol-13-acetate.

Luminol [10-2 M]

TCA: trichloroacetic acid solution (15 %).

TBA: thiobarbituric acid solution (0.8 %).

Formic acid and methanol were LC-MS grade and purchased from Merck (Darmstadt, Germany). Reference standards oleuropein, rutin, luteolin-7-O-glucoside, and apigenin-7- O-rutinoside were purchased from Extrasynthese (Genay, France), whereas luteolin-7 Orutinoside was purchased from Merck (Darmstadt, Germany). Other chemicals were of analytic grade.

5. Antibodies

- Anti-GFP (sc-9996) and Anti-GAPDH (sc-32233) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States). In addition, goat anti-mouse immunoglobulin G (IgG) antibody-HRP conjugate was purchased from Merk, Millipore.
- Primary antibody (serum from patients with nasopharyngeal cancer NPC).
- Secondary anti-human IgG antibody.

6. Microbial Strains and Culture Conditions

A range of strains obtained from the University of Messina's in-house culture collection (Messina, Italy) was used for the susceptibility studies: *Staphylococcus aureus* ATCC 6538, methicillin-resistant S. aureus ATCC 43300 (MRSA), 12 clinical isolates of S. aureus obtained from the pharynges (strains 26, 526, 531, 550, 808, 814), from duodenal ulcers (strains 8, 14), from hip prostheses (strains 3, 6, 32, 84), *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 9027, and *Candida albicans* ATCC 10231. All bacterial strains were grown in Mueller–Hinton Broth (MHB, Oxoid, CM0405) at 37 °C (18–20 h), whereas *C. albicans* was cultured in RPMI 1640 at 30 °C (24 h).

B. Methods

I. Sample preparation

OESA and OESY olive leaves were air-dried in the dark for three weeks, after which a mechanical grinder powdered them. The extraction was carried out using a mixture of water/ethanol (50:50, v/v) by simple maceration for 24 h with gentle stirring. Finally, the freeze-dried extracts were stored at 4 °C until further use.

II. Phytochemical Screening

1. Total Phenols

a. Principle

The total phenol content of *Olea europaea* leaf extracts was determined by kim et al. using the Folin-Ciocalteu reagent (Kim et al. 2009).

The method is based on the transfer of electrons in an alkaline medium from phenolic compounds to form a blue chromophore constituted by a phosphotungstic/ phosphomolybdenum complex where the maximum absorption depends on the concentration phenolic compounds.

b. Procedure

The total phenol content was determined according to Smeriglio et al. Briefly, 50 µL of OESA, OESY (0.5–4.0 mg/mL), and gallic acid as the reference standard (75.0–600 g/mL) were added to the Folin-Ciocalteu reagent (1:10 v/v) and brought to 1 mL with deionized water (Antonella Smeriglio et al. 2016). After 3 min, 10 % sodium carbonate (500 mL) was added, and the sample was left in the dark at room temperature (RT) for 1 h and mixed every 10 min. The absorbance was recorded at 785 nm by a UV–Vis spectrophotometer (Shimadzu UV-1601, Kyoto, Japan). The results were expressed as g of gallic acid equivalents (GAE)/100 g of dry extract (DE).

2. Flavonoids

a. Principle

Quantification of flavonoids was performed using Zhishen et al. method with aluminum trichloride and soda (Zhishen et al. 1999). Aluminum trichloride forms a yellow complex with flavonoids, and soda forms a pink complex that absorbs in the visible range at 510 nm.

b. Procedure

The flavonoid content was evaluated according to Smeriglio et al. Briefly, 200 µL of OESA, OESY (0.375–3.0 mg/mL), and rutin as the reference standard (0.125–1.0 mg/mL) were added

to 2 mg/mL of AlCl3 (1:1, v/v) and brought to 1.6 mL with 50 mg/mL sodium acetate (Smeriglio et al. 2018). After 2.5 h, the absorbance was recorded at 440 nm by a UV–Vis spectrophotometer (Shimadzu UV-1601, Kyoto, Japan). The results were expressed as g of quercetin equivalents (QE)/100 g DE.

- # LC-DAD-ESI-MS Analysis

1. Principle

Identification of phenolic compounds was made through liquid chromatography (HPLC) coupled to mass spectrometry (MS) using the electrospray ionization interface (ESI) (Carrillo-López et Yahia 2013).

2. Procedure

The phytochemical analysis of OESA and OESY was carried out using an Agilent high-performance liquid chromatography system (HPLC 1100 series) equipped with a UV–Vis photodiode array (PDA-G1315) detector and an ion trap mass spectrometer detector (IT-6320). An electrospray ionization (ESI) source was used in full scan mode, monitoring the precursor ions between m/z 50 and m/z 1000 in negative polarity by using the following parameters: capillary voltage, 3.5 kV; drying gas temperature, 350 °C; nitrogen flow, L/min; and nitrogen pressure, 50 psi. Data processing was carried out by Agilent 6300 Series Ion Trap LC/MS system software (version 6.2). The chromatographic separation was achieved by a Luna Omega PS C18 column (150 mm × 2.1 mm, 5 µm; Phenomenex, CA, USA) using solvent A (0.1% formic acid) and solvent B (methanol) as the mobile phase.

The elution program was the following: 0–2 min, 5 % B; 2–10 min, 25 % B; 10–20 min, 40 % B; 20–30 min, 50 % B; 30–40 min, 100 % B; 40–45 min, 5 % B; and 45–60 min, 5 % B. The flow rate was 0.3 mL/min, whereas the column temperature and the injection volume were 25 °C and 5 µL, respectively. UV–Vis spectra were recorded in the range of 190–700 nm, and chromatograms were acquired at 254, 280, 340, 370, and 520 nm. The acquisition wavelength chosen to show and compare the phytochemical profile of both extracts, at which all of the identified peaks were visible, was 254 nm. Peaks were identified by comparing the retention time, mass, and UV–Vis spectra with literature data and, when available, with reference standards (oleuropein, rutin, luteolin-7-O-glucoside, luteolin-7 Orutinoside,apigenin-7-O-rutinoside).

IV. Antioxidant capacity assays

1. Phosphomolybdenum assay

a. Principle

This technique is based on the reduction of molybdenum Mo (VI) present as molybdate ions MoO_4^{2-} to molybdenum Mo (V) $MoO^{2}+$ in the presence of the extract to form a green phosphate/Mo (V) complex at acid pH (Prieto et al. 1999).

b. Procedure

Extracts samples were mixed with 1 mL of the phosphomolybdenum reagent (600 mM sulfuric acid, 4 mM ammoniummolybdate, 28 mM sodium phosphate (Prieto et al. 1999). Then, incubation of mixture at 95 °C for 90 min and cooled to room temperature. The absorbance was measured at 695 nm. A standard curve was constructed using ascorbic acid to estimate the percentage of molybdenum reduced by the tested extract. EC50 (mg/mL) corresponds to the effective concentration at which the total antioxidant activity (TAA) at 50 % was obtained by interpolation from linear regression analysis.

2. 2,2-Diphenyl− 1-picrylhydrazyl (DPPH) free radical scavenging activity assay

a. Principle

DPPH- (2,2-Diphenyl-2-picrylhydrazyl) is a stable purplish free radical that absorbs at 517 nm (Cao et al. 2009). In the presence of anti-free radical compounds, the DPPH- radical is reduced and changes its color to yellow (Figure 23). This method is based on measuring the ability of antioxidants to trap the 2,2-diphenyl-1-picrylhydrazil (DPPH-) radical. The latter is reduced to the hydrazine form (non-radical) by accepting a hydrogen atom. The effect of extracts on DPPH is measured by the procedure described by (Osawa et Namiki 1981).

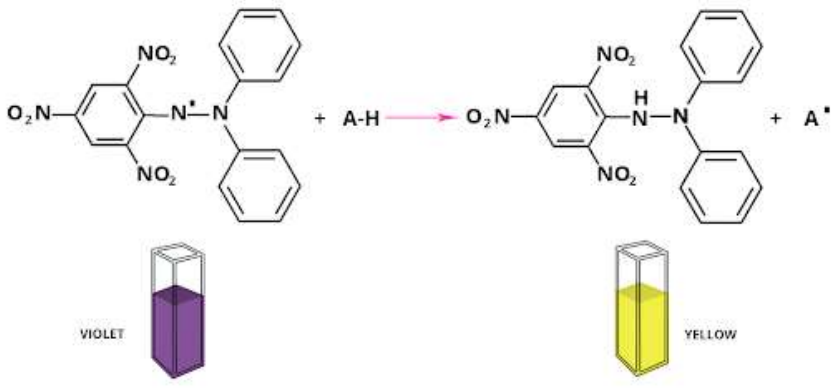


Figure 23: Principle of DPPH (Teixeira et al. 2013)

b. Procedure

The antioxidant activity was determined by monitoring the scavenging of the free radical DPPH in the presence of the OESA and OESY. The working solution was obtained by mixing the stock solution (200 µL in 1 mL Me OH) with 500 µl of DPPH (0.2 mM) purchased from Merck (Darmstadt, Germany). The final volume was brought to 1 mL with (distilled water), shaken vigorously, and allowed to reach a steady-state at room temperature for one h. Then, the mixture was poured into an optical glass cuvette and immediately placed in a spectrophotometer. The absorbance was taken at 517 nm using Backman Spectrophotometer (Bichrom Libra S32 (Beckman, Fullerton, CA) (Osawa and Namiki, 1981). Butylhydroxytoluene (BHT) (SIGMA) was used as a standard antioxidant. The DPPH radical scavenging activity was calculated according to the following equation:

$$\mathbf{I\ \%} = \frac{\mathbf{A0-A1}}{\mathbf{A0}} \times \mathbf{100}$$

Where A0 was the absorbance of the total DPPH (blank, without extract) and A1 the absorbance of the sample.

3. Ferric reducing antioxidant power (FRAP) assay

a. Principle

The iron reducing activity of our extracts is determined according to the method described by Oyaizu based on the reduction of Fe^{3+} present in the $K_3Fe(CN)_6$ complex to Fe^{2+} (Oyaizu 1986).

b. Procedure

The reducing power of OESA and OESY was determined according to the method of Oyaizu (Oyaizu 1986). According to this method, aliquots of various concentrations of the standard and extracts (0.06 to 1 mg /mL) were mixed with 1 mL of (pH 6.6) 200 mM phosphate buffer and 1 mL of (1%) potassium ferricyanide [$K_3Fe\ (CN)_6$]. The mixture was incubated at 50 °C for 20 min. Then, 1 mL of (10 %) trichloroacetic acid (TCA) was added to the mixture, centrifuged at 3000 rpm for 10 min. The upper layer of solution (1.5 mL) was mixed with 1.5 mL of deionized water and 0.1 mL (0.1%) ferric chloride solution ($FeCl_3$). The absorbance was measured at 700 nm in a UV spectrometer. A blank was prepared without adding extract. Ascorbic acid was used as standard at various concentrations (0 to 1 mg/mL).

4. Scavenging Activity of Nitric Oxide (NO)

a. Principle

The method used for evaluating the anti-antioxidant activity is based on nitrite reduction. Therefore, the test used to assess the anti-oxidant potential of molecules determines their capacity to inhibit NO production in the reaction medium (Marcocci et al. 1994).

b. Procedure

NO scavenging activity of extracts was determined as previously described (Kavoosi et al. 2012). Briefly, 0.1 mL of extracts (0–0.3 mg/mL in DMSO) was incubated with 0.5 mL of sodium nitrite (0.01 mg/mL in 100 mM sodium citrate pH 5) at 37 °C for 2 h. After incubation, 0.5 mL of Griess reagent was added, and the absorbance (A) was read at 540 nm using a spectrophotometer (Pharmacia, Uppsala, Sweden). The equation obtained the percentage of RNS scavenging:

$$\mathbf{NO \cdot \ scavenging\ effect\ (\%) = (A_0 - A_1)/A_0 \times 100}$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

5. Determination of the antioxidant activity by a chemical reaction (Chemiluminescence)

a. Principle

Another more sensitive chemical method is chemiluminescence to verify the antioxidant activity of OESA and OESY. This technique is a chemical reaction that emits energy in light in a probe that reacts with the reactive forms of oxygen.

The luminol (3-aminophthalhydrazide), used in our case, will be oxidized to give 3-aminophthalate, which is in an excited energy level and will return to its ground state, emitting light that the luminometer will detect at 425 nm (Gargouri et al. 2011).

b. Procedure

The following compounds were added in each well of a chemiluminescence plate: OESA and OESY at different concentrations, 50 µL luminol, 20 µL $FeSO_4$, and 20 µL H_2O_2. Indeed, the two chemical agents, H_2O_2 and $FeSO_4$, were used to induce the Fenton reaction. This is because $FeSO_4$ produces different types of ROS (superoxide anion, hydrogen peroxide, and the hydroxyl radical) through the ferrous ion (Fe^{2+}) reaction with oxygen. On the other hand, H_2O_2 is a non-toxic ROS. Still, in the presence of a small dose of transition metal anion, hydrogen peroxide can interact with the superoxide anion to produce, according to the Fenton reaction, the hydroxyl radical, which is very active.

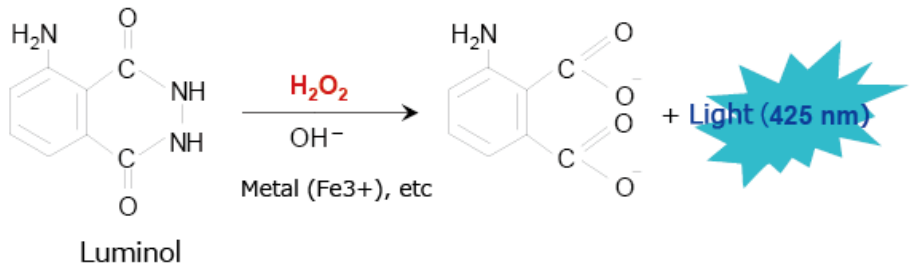


Figure 24: Chemiluminescence Reaction Caused by Luminol.

V. *In Vitro* Cell Culture

1. Maintenance of cells in culture

All cell lines are cultured in 25 cm^2 culture flasks (Iwaki). Cell transplantation is performed every 3 to 5 days, depending on the type of cell line and the initial cell concentration.

Cells that have reached saturation concentration are centrifuged for 10 min at 1000 rpm, then suspended in 1 mL of RPMI medium with 10 % FCS. After counting in the presence of trypan blue, the cells are recultured at a concentration of 2.10^5 cells/mL.

2. Freezing and defrosting of cells

a. Freezing

At the end of the exponential growth phase, the cells are centrifuged for 10 min at 1000 rpm. The cell pellet is suspended in 1 mL of FCS with 10 % dimethylsulfoxide (DMSO), then transferred to cryotubes. The cells are frozen progressively by placing them for 24 hours at -80° C, then in liquid nitrogen.

b. Defrosting

Cells are thawed rapidly in a water bath at 37° C. To remove all traces of DMSO, cells are washed in RPMI medium and centrifuged for 10 min at 1000 rpm. After counting in the presence of Trypan blue, the cells are cultured in 10 % FCS complete medium.

3. Trypan blue exclusion test (cell count)

The Trypan Blue Exclusion Test (Sigma, Germany) is based on the evaluation of cell membrane integrity. It is a vital dye, generally excluded from the living cell, which stains dead cells blue. It is a specific technique for counting cells and assessing cell death. It counts the number of cells present in a given volume of cell suspension under an optical microscope. The counting is done on a Malassez cell.

In an eppendorf tube, 20 µL of cell suspension is diluted with 20 µL of Trypan blue. After homogenization, a small volume is put into the Malassez cell for counting.

The following formula gives the concentration in a number of cells per mL:

N= n x 10 x 1000 x dilution factor

With n: number of cells per square.

4. Cytotoxicity test (MTT test) in HeLa cell, in PBMC and Raji cell line

a. Principle

MTT (3 (4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide) (Sigma, Allemagne) yellow initially, is the substrate of a mitochondrial enzyme succinate dehydrogenase. This enzyme can catalyze certain covalent bonds of MTT, which converts it to formazan salt (purple salt) (Figure 25), which is insoluble in aqueous media. This reaction can be followed quantitatively by spectrophotometry. The OD value at 570 nm reflects the activity of mitochondrial cytochromes. This activity can be considered as an index of cell proliferation (Mosmann 1983)

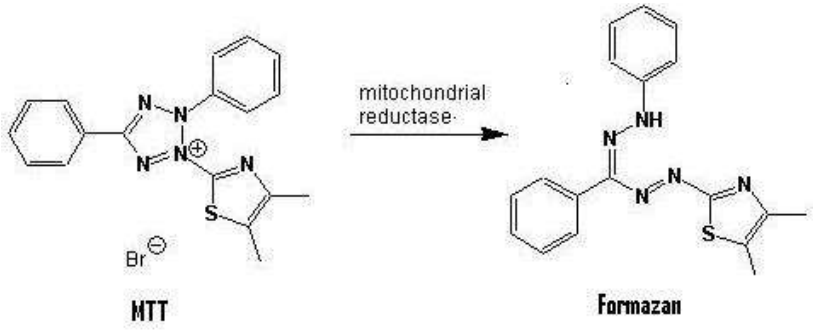


Figure 25: Principle of MTT reduction.

b. Procedure

HeLa cells and PBMC (2×10^5 cells/mL) were grown on microtiter plates (200 µL of cell suspension/well) in 96 well microplates with serial dilutions of extract. Cells were treated with various concentrations of OESA and OESY (0.23 to 30 mg/mL) for 48 h and then submitted to the MTT.

Raji cells (6×10^4) were growth in 96 wells microtiter plates at 37 °C in a 5 % CO_2 incubator for 24 h and after they are exposed to serial dilutions of OESA (0.16, 0.31, 0.625, 1.25, 2.5, 5, 10 mg/mL) for 72h. Then we follow the cytotoxic protocol as described at the beginning.

Then, 20 µl of MTT solution (5 mg/mL in PBS) (Sigma) was added to each well, and the plates were additionally incubated for 4 h at 37 °C in a CO_2 incubator. After incubation, 180 µL of

growth medium was removed from each well and replaced with DMSO/methanol (50:50) to dissolve the formazan crystals. Finally, the place was shaken on the microtiter plate shaker at room temperature to facilitate the complete solubilization of formazan crystals. The absorbance was measured at 570 nm with a microplate reader (Elx 800 microplate reader), and the % of cytotoxicity was calculated.

$$\% \, \boldsymbol{Cytotoxicity} = \mathbf{100} - \left(\frac{\boldsymbol{DO\ treated}}{\boldsymbol{DO\ non\ treated}}\right) \times \mathbf{100}$$

VI. Induction of oxidative stress

Cells were adjusted to 3×10^6 cells / mL in 25 cm^2 flasks and incubated at 37 °C. Oxidative stress was induced, after 72 h, by adding H_2O_2 to the cells at a final concentration of 0.2 mM, for 1 h. The oxidation was performed in phosphate-buffered saline (PBS).

Adherent cells were detached using trypsin/EDTA solution and centrifuged at 3000 rpm for 10 min.

Oxidative stress was induced using 0.2 mM H_2O_2 for 1 h. The activities of CAT, SOD, and GPx were assessed in cell lysates.

To assay the capacity of plant extracts to protect HeLa cells from ROS-mediated oxidative injury, cells were preincubated simultaneously for 72 h in the presence of different concentrations of ethanol extracts and the oxidative stress-inducing agent (0.2 mM of H_2O_2).

1. Protein determination by the Bradford method

Protein concentration is determined by the Bradford method, using bovine serum albumin (BSA) as the standard (Bradford 1976). First, proteins are diluted in 800 µL of sterile water, and then 200 µL of Bradford's reagent is added. The OD is read at 595 nm.

2. Evaluation of the antioxidant activity *in vitro*

a. Malondialdehyde (MDA) determination

For evaluation of MDA production rate, thiobarbituric acid-reactive species (TBARs) assay was used. Adherent cells were detached using trypsin/EDTA solution and centrifuged at 3000 rpm for 10 min. The pellet was resuspended in 500 µL of deionized water and lysed by five cycles of sonication during 20 s (Sonic,vibracell). One milliliter of TBA solution (15 % trichloroacetic acid, 0.8 % thiobarbituric acid, 0.25 N HCl) was added. The mixture was heated at 95 °C for 15 min to form an MDA-TBA adduct. Optical density (OD) was measured by a

spectrophotometer (Biochrom, Libra S32) at 532 nm. Values were reported to a calibration curve of 1,1,3,3-tetra ethoxy propane (1.1.3.3 TEP).

b. Determination of catalase activity

Catalase activity was measured as described by Aebi (Aebi 1984). This method is based on the principle that the absorbance at 240 nm decreases because of the dismutation of H_2O_2. Under standard conditions, the amount of H_2O_2 converted into H_2O and O_2 in 1 min under normal conditions is accepted as the enzyme reaction velocity. The number of catalase units was determined as follows:

$$\mathbf{U/mL = [(3.45 \times slope)/0.05] \times (1000/50\ \mu L)}$$

c. Assay of superoxide dismutase activity

SOD activity was determined by spectrophotometry (420 nm) using the pyrogallol assay as described previously and modified as follows: the rate of autoxidation of pyrogallol in Tris-cacodylic diethylenetriaminepentaacetic acid (DTPA) buffer (pH 8-8.2) was determined (A1) (Jiang et Chen 1992). The autoxidation of pyrogallol was evaluated under the same conditions after adding 25 µL of cells lysate (A2). The percentage inhibition of pyrogallol oxidation was determined using the formula:

$$\mathbf{\% \ Inhibition = [(A1\text{-}A2)/\ A1] \times 100}$$

d. Assay of glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was measured according to Flohe and Gunzler (Flohé et Günzler 1984). The supernatant obtained after centrifuging 5 % cells at 1500 g for 10 min followed by 10000 g for 30 min at 4 °C was used for GPx assay. One mL of the reaction mixture was prepared, which contained 0.3 mL of phosphate buffer (0.1 M, pH 7.4), 0.2 mL of GSH (2 mM), 0.1 mL of sodium azide (10 mM), 0.1 mL of H_2O_2 (1 mM) and 0.3 mL of cells supernatants. After incubation at 37° C for 15 min, the reaction was terminated by adding 0.5 mL 5 % TCA. Tubes were centrifuged at 1500 ×g for 5 min, and the supernatant was collected. 0.2 mL of phosphate buffer (0.1 M pH 7.4) and 0.7 mL of DTNB (0.4 mg/mL) was added to 0.1 mL of reaction supernatant. After mixing, absorbance was recorded at 420 nm.

VII. Purification of peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells have a round nucleus such as a lymphocyte, monocyte, or macrophage (Pourahmad et Salimi 2015). In humans, the frequency of these populations varies from individual to individual, but in general, lymphocytes range from 70-90 %, monocytes from 10-20 %, while dendritic cells are rare, showing only 1-2 % (Kleiveland 2015).

These blood cells are a critical link in the immune system to fight infection and adapt to intruders (Pourahmad and Salimi 2015).

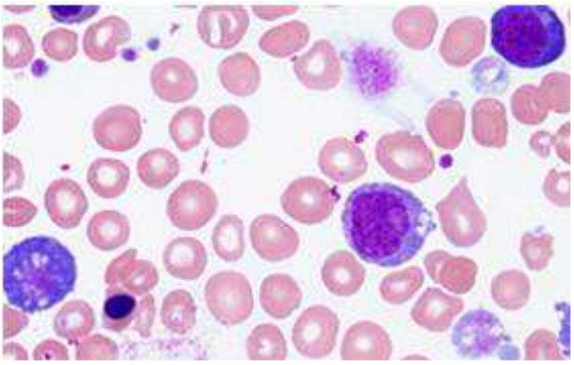


Figure 26: Microscopic observation of peripheral blood mononuclear cells in humans.

1. Principle

The Ficoll gradient technique performs PBMC isolation. The Ficoll solution has a density of 1.077. Blood elements have different densities: d (PBMC) < 1.077 while d (red blood cells and granulocytes) > 1.077. At the plasma-Ficoll interface, the PBMC are positioned and form an opalescent ring.

2. Procedure

Peripheral venous blood is collected from a healthy individual into a tube containing ethylene diamine tetraacetic acid (EDTA). The blood is then diluted with sterile PBS and carefully poured over the Ficoll, along the tube wall, which must be kept inclined to avoid mixing. After centrifugation for 25 min at 350 g with gentle deceleration, a white ring of PBMC is obtained superimposed between a Ficoll below and the plasma on top. Next, the PBMC suspension is collected in a new conical tube, avoiding Ficoll.

Cells are washed twice with HBSS (Hanks' saline solution) with 10 min centrifugation at 250 g. The final cell pellet is suspended in a complete RPMI 1640 medium for cell culture handling.

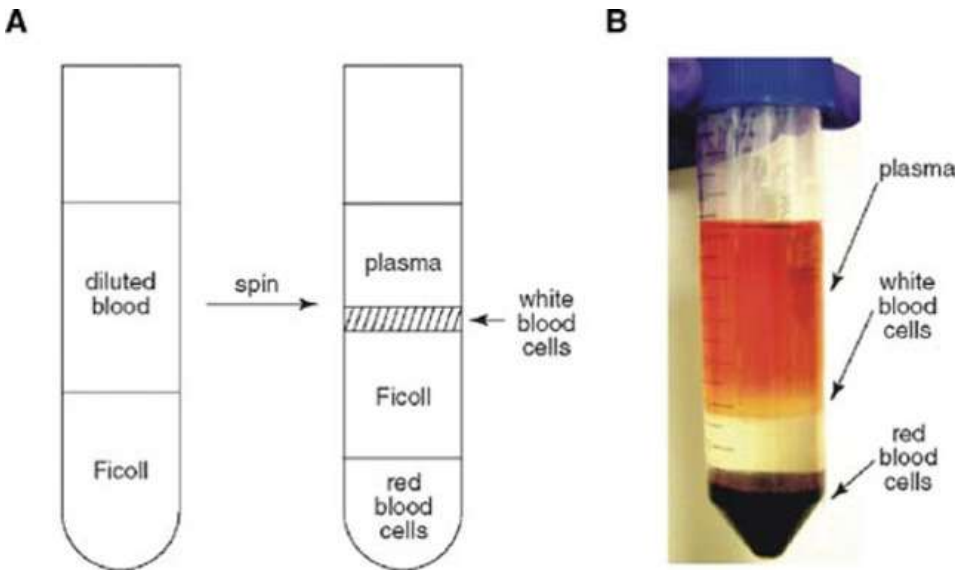


Figure 27: (A) Scheme shows blood separation by Ficoll gradient. (B) The actual blood separation after Ficoll gradient centrifuge (Lan et al. 2007).

VIII. Antiviral Assay using HSV Virus

1. Cell Lines and Viruses

VERO cell lines (American Type Culture Collection) were propagated in minimal essential medium (EMEM) and supplemented with 6% fetal bovine serum (FBS) (Lonza, Belgium) at 37 °C under 5 % CO_2. The prototype HSV-1 (F) strain was kindly provided by Dr. Bernard Roizman (University of Chicago, Chicago, IL, USA). The HSV-1 viral stocks were obtained from cell-free supernatant of Vero cells infected with HSV-1. In addition, the recombinant virus HSV-1-VP26GFP expressing a GFP-tagged VP26 protein was propagated in Vero cells as previously described (Siracusano et al. 2016).

2. Cell Proliferation Assay

As previously described, the cell viability assay was performed (Musarra-Pizzo et al. 2020). Briefly, Vero cells were grown in 96-well plates and treated with different concentrations of OESA and OESY extracts (0.05 mg/mL, 0.1 mg/mL, 0.2 mg/mL, 0.4 mg/mL, 0.8 mg/mL and 1 mg/mL) for 72 h. According to the manufacturer's instructions, the cell viability was determined with a cytotoxicity bioassay kit (Lonza Group Ltd., Basel, Switzerland). The GloMax® Multi Microplate Luminometer (Promega Corporation, 2800 Woods Hollow Road, Madison, WI, USA) combined with the ViaLight™ plus cell proliferation and cytotoxicity bioassay kit was used to detect the emitted light intensity related to ATP

degradation. As previously reported, the measured luminescence value was converted into the cell proliferation index (%) (Bisignano et al. 2017).

3. Plaque Reduction Assay

The antiviral activity was evaluated by plaque reduction assay. With gentle shaking, the Vero cells were seeded on 24-well plates and infected with the virus inoculum for 1 h at 37 °C. The virus was diluted to yield 60 plaques/100 µL. A time-of-addition approach was used: (i) the virus inoculum was added on Vero cells and, after the incubation time, the monolayers were covered with a medium containing 0.8% methylcellulose in the presence of OESA and OESY extracts; and (ii) the virus inoculum was added on Vero cells pre-treated with OESA and OESY extracts and, after infection, the monolayers were covered with a medium containing 0.8 % methylcellulose. The concentrations used for the antiviral assay were as follows: 0.1 mg/mL, 0.2 mg/mL, 0.4 mg/mL, 0.8 mg/mL, and 1 mg/mL. Acyclovir was the control at various concentrations (1, 10, and 20 µM). After three days, the cells were fixed, stained with crystal violet, and visualized with an inverted microscope (Leica DMIL, Nuloch, Germany) for plaque detection. After the incubation time, the inoculum was removed, and the monolayers were overlaid with Dulbecco's Modified Eagle's Medium containing 0.8 % methylcellulose in the presence of the extracts. The plates were incubated at 37 °C with 5 % CO_2 for 72 h, and the plaques were visualized by staining the cells with crystal violet.

4. The Binding Assay

Vero cells (4×10^5 cells/well) were cultured in 12-well plates and uninfected (mock) or infected with HSV-1 VP26GFP virus at a multiplicity of infection (MOI) of 0.5 PFU/cell. The infection was carried out by pre-treating the cells and the viral suspension with 0.1 mg/mL of OESA and 0.2 mg/mL of OESY, respectively, for 1 h. After incubation, the virus was adsorbed on pre-treated cells for 1 h at 4 °C. The infection was carried out at 4 °C to allow only the binding of the virus, but not the entry into the cells during the infection step. The virus inoculum was then removed, and the monolayers were incubated with a medium containing the extracts. Medium alone or containing DMSO were used as the controls.

5. Western Blot Analysis and Antibodies

Cellular proteins were extracted from Vero cells using SDS sample buffer 1× (62.5 mM of Tris; Dithiothreitol (DTT) 1 M; 10 % glycerol; 2 % SDS; 0.01 % Bromophenol Blue), and immunoblot analysis was performed using an equal quantity of proteins.

The proteins were revolved on SDS 10 % polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (BioRad Life Science Research, Hercules, CA, USA). The membranes were probed overnight at 4 °C with specific antibodies to detect GFP-VP26 protein. In addition, specific proteins were detected with a secondary anti-mouse antibody linked to horseradish peroxidase (HRP). Finally, GAPDH was used as a loading control.

The chemiluminescence was detected using Western HRP substrate (Merk, Millipore, Burlington, MA, USA). Immunoblot band intensity was quantified by densitometry analysis using the TINA software (version 2.10, Raytest, Straubenhardt, Germany). Anti-GFP (sc-9996) and Anti-GAPDH (sc-32233) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States). Goat anti-mouse immunoglobulin G (IgG) antibody-HRP conjugate was purchased from Merk, Millipore.

6. Viral DNA Extraction and Real-Time PCR Analysis

According to the manufacturer's instructions, cellular DNA was extracted from TRIzol-lysed cells (Life Technologies, Carlsbad, CA, USA). Briefly, the DNA solution was precipitated from the interphase and organic phase with 100 % ethanol. Then, the DNA pellet was washed twice with 0.1 M of sodium citrate in 10 % ethanol and dissolved in 8 mM NaOH. Real-time PCR analysis was performed by using a specific TaqMan HSV-1 probe in a Cepheid Smart Cycler II System (Cepheid Europe, Maurens-Scopont, France); 1 µg of DNA template was mixed with 1 µM of deoxyribonucleotide triphosphate (dNTP) mix, 0.5 µM of forward and reverse primers, 1 µM of TaqMan probe, 1 × NH_4 reaction buffer, 2 mM of $MgCl_2$, and 5 U/µL of DNA polymerase BIOTAQ (BIO-21040 Bioline) in a total volume of 25 µL.

The oligonucleotide primers used were: HSV-1 Fw 50-catcaccgacccggagagggac; HSV-1 Rev 50gggccaggcgcttgttggtgta, HSV-1 TaqMan probe 50-6FAM-ccgccgaactgagcagacacccgcgc- TAMRA, (6FAMis 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine). The amplification was performed as follows: (i) 10 min at 95 °C, (ii) 30 s at 95 °C for 40 cycles, (iii) 30 s at 55 °C, (iv) 30 s at 72 °C, and (v) 5 min at 72 °C. A negative sample was used as the amplification control for each run. The quantitation of HSV-1 DNA was generated using GAPDH as a housekeeping gene with the comparative Ct method. DNA extracted from Vero cells infected and treated with Acyclovir (20 µM) was used as a positive control.

IX. Antimicrobial Assay

The minimum inhibitory concentration (MIC), the minimum bactericidal concentration (MBC), and the minimum fungicidal concentration (MFC) of OESA and OESY were

determined by the broth microdilution method, according to CLSI (CLSI. 2002). The tested concentrations ranged from 2000 to 1.9 µg/mL of either OESA or OESY dissolved in DMSO. The final concentration of DMSO did not exceed 1 % in each sample. The MIC was defined as the lowest concentration that completely inhibited bacterial growth after 20 h. The MFC was defined as the lowest concentration that completely inhibited fungal growth after 48 h. The MBCs were determined by seeding 20 µL from clear MIC wells onto Mueller–Hinton agar (MHA, Oxoid) plates. The MBC was defined as the lowest extract concentration that killed 99.9 % of the final inocula after 24 h incubation.

X. EBV early antigen (EA) induction in Raji cells of OESA

For the induction of the lytic cycle, Raji cells (3×10^6 /well) were stimulated with 8 nM [TPA] (12-O-Tetradecanoyl-phorbol-13-acetate) (Sigma) for 30 min. Then, the cells were collected and centrifuged for 10 min at 1000 rpm (Gargouri et al. 2009). Next, the pellet is washed once with PBS (1 X) and then centrifuged for 10 min at 1000 rpm. Finally, the pellet was resuspended in 1 ml of deionized water and lysed by 10 cycles of sonication for 20 s at 37 % (Sonisc, vibracell).

1. Malondialdehyde (MDA) determination

The thiobarbituric acid reactive species assay performed MDA determination. Raji cell lysate (3×10^6 / well) previously TPA treated, were added to 700 µL reagent TBA / TCA (thiobarbituric acid / trichloroacetic acid) (15 % trichloroacetic acid, 0.8 % TBA, 0.25 N HCl). The mixture was heated at 95 °C for 15 min to form an MDA-TBA adduct. This assay measures the MDA bound to proteins. Protein concentration was calculated using the Protein Assay Kit from Bio-Rad (France), and bovine serum albumin served as the standard (Briante et al. 2002). Optical density was measured with a spectrophotometer (Biochrom, Libra S32) at 532 nm. Values were compared to a calibration curve of 1,1,3,3-tetra ethoxy propane (Gargouri et al. 2009).

2. Conjugated Dienes (CD) determination

Conjugated diene (CD) assay evaluated the lipid oxidation and was determined as described before with some modification (Kurien et Scofield 2003). Briefly, 25 µL of Raji cells lysate (3×10^6 / well) were extracted with 3 mL of chloroform/methanol (v/v) solution and centrifuged at 3000 rpm for 15 min. Then, 2 mL of the organic phase was transferred into another clear tube dried at 45 °C and dissolved in methanol. The absorbance was measured at 233 nm.

3. Indirect Immunofluorescence assay (IFA)

Cells were adjusted to 3×10^6 cells / mL and incubated at 37 °C for 48 h. Then, they were treated with TPA [8 nM] and OESA for 30 min, set for an additional 48 h, and centrifuged for 5 min at 1000 rpm. The pellet was incubated with the primary antibody (collected by serum of a patient with nasopharyngeal cancer (NPC), diluted 1/500 in PBS (1X), for 20 min at 4 °C. After, the pellet was rinsed briefly in phosphate-buffered saline twice and incubated with diluted fluorescently labeled antihuman IgG secondary antibody solution for 20 min at 4 °C in the dark. Finally, the secondary antibody was decanted, and the cells were washed three times with PBS for 5 min each in the dark. The sample was suspended in PBS slides to be analyzed on the fluorescence microscope (ZEISS, Axiostar plus, Germany) (magnification, 40X). The negative control consisted of untreated HeLa cells, and the positive controls consisted of Raji cells treated TPA. The assay was used to detect the inhibitory activity of EBV lytic cycle induction mediated by OESA.

XI. Statistical Analysis

The statistical analysis was performed with GraphPad Prism 8 software (Graph-Pad Software, San Diego, CA, USA) using one-way variance analysis (ANOVA).

The significance of the p-value was indicated with asterisks (*, **, ***, ****), which show the significance of the p-value less than 0.05, 0.01, 0.001, 0.0001, respectively. The half-maximal cytotoxic concentration (CC50) and the half-maximal effective concentration (EC50), and Half-maximal inhibitory concentration (IC50) values were calculated by using non-linear regression analysis.

Results And Discussion

Chapter 1: Phytochemical Characterization of *Olea europaea* Leaf Extracts and their antioxidant power in a chemical and biological system using HeLa cell and PBMC

Olea europaea leaves have an important place in the Mediterranean diet effects of olive leaves, such as antioxidant, hypoglycemic, antihypertensive, antimicrobial, and antiatherosclerotic, have been reported in various studies (Wang et al. 2008). This property can be linked to the fact that the leaves are rich in polyphenols luteolin- and apigenin-7-O-glycosides to be the predominant flavonoids in olive leaves, followed by rutin (Ben-Amor et al. 2021). Furthermore, polyphenols modulate oxidative stress in cancer cells through modulation of signal transduction and the expression of specific genes related to cell proliferation and cell death (Rahman et al. 2006). As evidence, polyphenol compounds trigger apoptotic programmed cell death pathways in human gastric carcinoma cells via manipulation of ROS content of the cancerous cells (Rahman et al. 2006).

ROS can react with biological molecules, such as DNA, proteins, or lipids, generating mutations and damaging membranes, leading to cell and tissue injuries (Ksouri et al. 2009). However, plants are known for resisting and destroying these toxic ROS since they are equipped with a powerful antioxidant system that includes enzymatic and non-enzymatic components (Ksouri et al. 2009). These antioxidant compounds can delay the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions (Tepe 2005). Furthermore, recent studies have shown that several olive species are an essential source of biologically active molecules (van der Watt et Pretorius 2001; Weber et al. 2007; Meot-Duros et al. 2008). In addition to their role as an antioxidant, these compounds exhibit a broad spectrum of biological activities such as antimicrobial, anti-inflammatory, anti-carcinogenic, anti-allergic, anti-thrombotic, cardioprotective, and vasodilatory effects (Verzelloni et al. 2007; Balasundram et al. 2006)

The human body produces reactive oxygen species (ROS), such as superoxide anion radical, hydroxyl radical, and hydrogen peroxide, by many enzymatic systems through oxygen consumption. These ROS can be beneficial as signal transducers and growth regulators (Finkel 2001). However, large amounts of these ROS can be produced during oxidative stress and may

be dangerous because of their ability to attack numerous molecules, including proteins and lipids (Yoshikawa et Naito 2000). It has been reported that ROS largely contributes to cellular aging mutagenesis and coronary heart disease through several ways, including membrane destabilization (Yoshikawa and Naito 2000; Favier 2003; Mcguire et al. 1982; Sohal et al. 2002). DNA breakage and oxidizing low-density lipoproteins (LDL) (Bergeron et al. 2010). The cell can reduce the impact of ROS either by an endogenous system implicating enzymes or by an exogenous system using antioxidants, vitamin C, and a-tocopherol (Yoshikawa and Naito, 2000; Favier 2003). Among antioxidant enzymes, catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) play key roles (Gargouri et al. 2009).

In this part, we prepare and compare two olive leaf extracts from *O. europaea* var. *sativa* (OESA) and *O. europaea* var. *sylvestris* (OESY) from Sfax (south-east of Tunisia), to characterize the contents, as well as the antioxidant activity, was then chemically determined by in vitro assay (DPPH, FRAP, Phosphomolybdenum assay and Scavenging Activity of Nitric Oxide (NO)) and biologically using the HeLa cell line and PMBC to investigate the lipid peroxidation (MDA marker) profile for both and to determine CAT, SOD and GPx activities in HeLa cell line. The cytotoxic effect of this extract was determined by MTT assay, so all the used concentrations did not show any cytotoxic effect for the cell culture.

I. Results

1. Phytochemical Analysis

Preliminary phytochemical screening showed that the hydroalcoholic leaf extract of *O. europaea var. sativa* (OESA) showed a higher content of total phenols (5.04 ± 0.29 g GAE/100 g DE vs. 4.40 ± 0.38 g GAE/100 g DE) and flavonoids (35.25 ± 3.19 g QE/100 g DE vs. 31.10 ± 1.81 g QE/100 g DE) for the leaf hydroalcoholic extract of *O. europaea var. sylvestris* (OESY). In addition, comparative RP-LC-DAD-ESI-MS analysis of OESA and OESY extracts (Figure 28) revealed the presence of many polyphenols characteristic of olive leaves.

Seventeen and eighteen compounds were identified in the OESA and OESY leaf extracts. Table 7 shows the phytochemical profile of both extracts by listing the compounds according to their elution order. The numbers refer to the peaks shown in Figure 28. Secoiridoids represented the most abundant class of compounds identified in OESA (55.38 %), followed by flavones (21.90 %), terpenoids (11.48 %), and phenolic acids (4.95 %). Minor compounds identified were other flavonoids (2.73 %), ellagitannins (1.97 %), phytosterols (0.84 %), and stilbenoids (0.76 %). On the contrary, in OESY, the content of secoiridoids (24.97 %) was almost comparable to the content of phenylethanoids (23.07 %), followed by flavones (19.02

%), ellagitannins (11.27 %), hydrolyzable tannin (8.69 %), and phenolic acids (5.87 %). Minor compounds identified were other flavonoids (4.64 %), terpenoids (1.91 %), and phytosterols (0.56 %). Bold numbers in Table 7 refer to the most abundant compounds identified in both leaf hydroalcoholic extracts. Oleoside/secologanoside (32.68 %) was the most abundant compound identified in the OESA, followed by oleuropein (18.03 %), luteolin-7-O-rutinoside (13.86 %), oleanolic acid (11.48 %), and luteolin-7-O-glucoside (6.14 %). On the contrary, decaffeoylverbascoside (23.07 %) was the most abundant compound identified in OESY, followed by oleuropein hexoside (11.07 %), gallagic acid (10.38 %), luteolin-7-O-rutinoside (9.84 %), and valoneic acid dilactone (8.69 %).

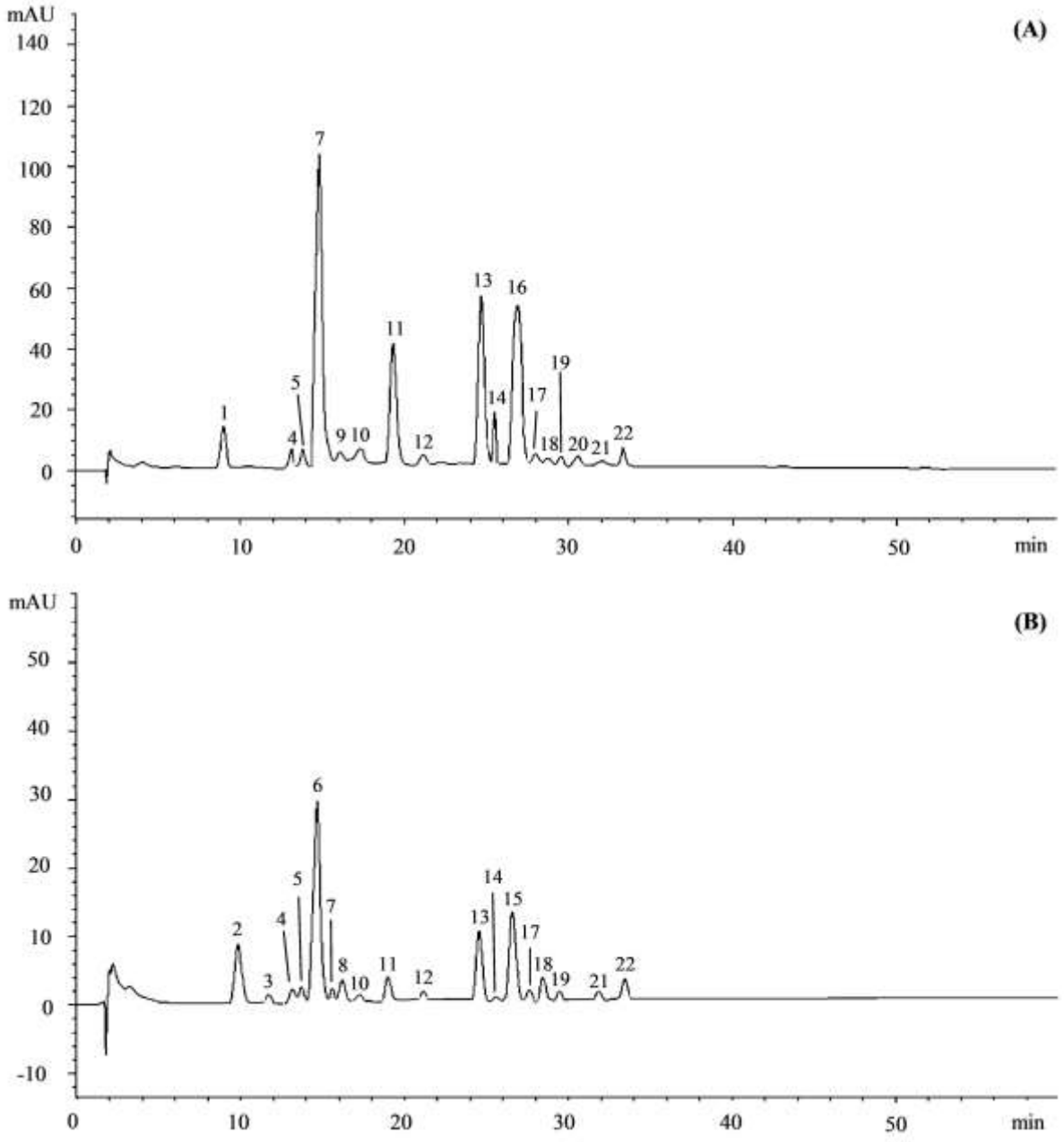


Figure 28: Representative LC-DAD chromatograms of *O. europaea L. var. sativa* (OESA, panel A) and *O. europaea var. sylvestris* (OESY, panel B) leaf hydroalcoholic extracts acquired at 254 nm. Peak numbers refer to the compounds listed in Table 7.

Table 7: Phytochemical profile of the leaf hydroalcoholic extract of *O. europaea L. var. sativa* (OESA) and *O. europaea var. sylvestris* (OESY) by LC-DAD-ESI-MS analysis.

Peak n.	Compound	RT[1]	λ_{max}	$[M-H]^-$	MW[2]	Area %[3]	
						OESA	OESY
1	Dihydroxybenzoic acid hexoside pentoside	9.083	296	447	448	4.95	-
2	Valoneic acid dilactone	9.9	256;365	469	470	-	8.69
3	Dicaffeoylquinic acid	11.7	218;327	515	516	-	5.87
4	Gallagic Acid	13.309	234;296	603	604	0.97	10.38
5	Diellagilactone	13.831	252;377	601	602	1.00	0.89
6	Decaffeoylverbascoside	14.886	236;280	461	462	-	23.07
7	Oleoside/Secologanoside	14.955	244	389	390	32.68	0.94
8	Epicatechin 3-p-hydroxybenzoate	16.199	282;318	409	410	-	2.55
9	Elenoic acid hexoside	16.230	238	403	404	0.73	-
10	Hydroxyoleuropein isomer I	17.431	232;282	555	556	1.06	2.94
11	Oleanolic acid	19.461	232	454	455	11.48	1.91
12	Hydroxyoleuropein isomer II	21.390	232;282	555	556	0.58	3.75
13	Luteolin-7-O-rutinoside	24.821	258;344	593	594	13.86	9.84
14	Rutin	25.560	256;358	609	610	1.99	0.80
15	Oleuropein hexoside	26.855	232;282	701	702	-	11.07
16	Oleuropein	26.950	232;286	539	540	18.03	-
17	Apigenin-7-O-rutinoside	27.421	252;336	577	578	1.90	2.26
18	Luteolin-7-O-glucoside	28.326	268;342	447	448	6.14	6.92
19	Hydroxyphloretin 2′-O-xylosylglucoside	29.455	250;340	583	584	0.74	1.29
20	Vitisin A	30.440	388;510	560	561	0.76	-
21	β-Sitosteryl ferulate	32.140	240;294;318	590	591	0.84	0.56
22	Lucidumoside C	33.500	240;284	583	584	2.30	6.27

2. Antioxidant assays

a. Phosphomolybdenum assay

Quantitative analysis of antioxidant activity by the phosphomolybdenum method reveals that both wild and cultivated *Olea europaea* leaf extracts have a high ability to reduce ammonium phosphomolybdate. OESA extract has a reducing activity in the order of 0.86 mg EAA /g extract and OESY equal to 3.46 mg EAA /g extract.

b. DPPH radical scavenging activity assay

The DPPH assay is based on reducing the stable radical, DPPH, forming a non-radical form in the presence of hydrogen donating antioxidant. The antioxidant activity is shown by the reduction of purple-colored DPPH to the yellow-colored derivatives. Aliquots of various concentrations of OESA and OESY extracts (0, 0.125, 0.25, 0.5, 1 mg /mL) and standard BHT were mixed with DPPH (0.2 mM) and incubated for 1 h in the dark. After 1 h of reaction, the

absorbance of the mixtures was read at 517 nm. The scavenging effect of OESA and OESY was calculated as reported in Materials and Methods, and the results were expressed as a percentage of radical scavenging activity. The results show that OESA has a 1 mg/mL concentration and a significantly higher antioxidant activity (84 %) than OESY (77 %). However, OESY showed the lowest half-maximal inhibitory concentration (IC_{50}) and consequently the most increased anti-radical activity (0.136 mg/mL vs 0.120 mg/mL of OESA).

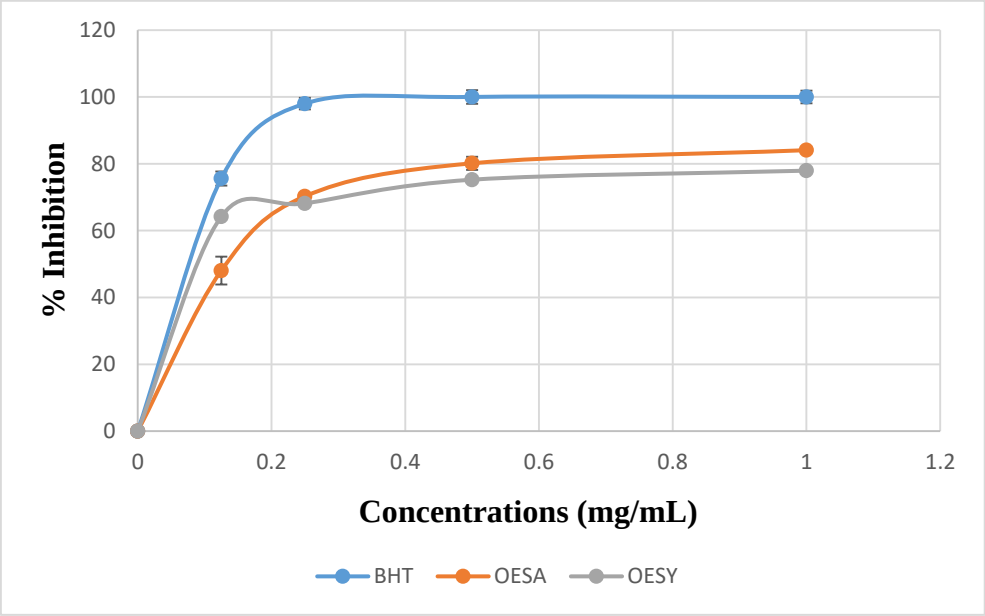


Figure 29: Percentage of radical scavenging capacity for OESA and OESY

Aliquots of various concentrations of OESA and OESY extracts (0, 0.125, 0.25, 0.5, 1 mg /mL) and standard BHT were mixed with DPPH and incubated in the dark. The antioxidant activity of OESA and OESY was measured by spectrophotometer at 517 nm. Results were expressed as mean inhibition percentage (%) ± standard deviations (n=3). BHT was used as a reference standard.

c. Ferric reducing antioxidant power

The antioxidant activity of OESA and OESY was further confirmed by the FRAP assay that measures the ability of the sample to reduce the Fe^{3+} to Fe^{2+}. According to the procedure, various concentrations of OESA and OESY extracts (0 to 1 mg /mL) and acid ascorbic were mixed with FRAP reagents. Given that FRAP assay is a redox-linked colorimetric reaction, the reduction of ferric ion (Fe^{3+}) to ferrous form (Fe^{2+}) by antioxidants produces an intense blue light revealed as a change in absorption at 593 nm.

Like the antioxidant activity of ascorbic acid, the reducing power of OESA and OESY increased with increasing concentration to at 1 mg/mL equal to 90 % and 86 %, respectively (Figure 30). The IC50 of OESA and OESY for FRAP assays are presented in table 8; for OESA and OESY,

the IC50 was (0.209 mg/mL and 0.392 mg/mL, respectively) compared with ascorbic acid value (0.025 mg/mL).

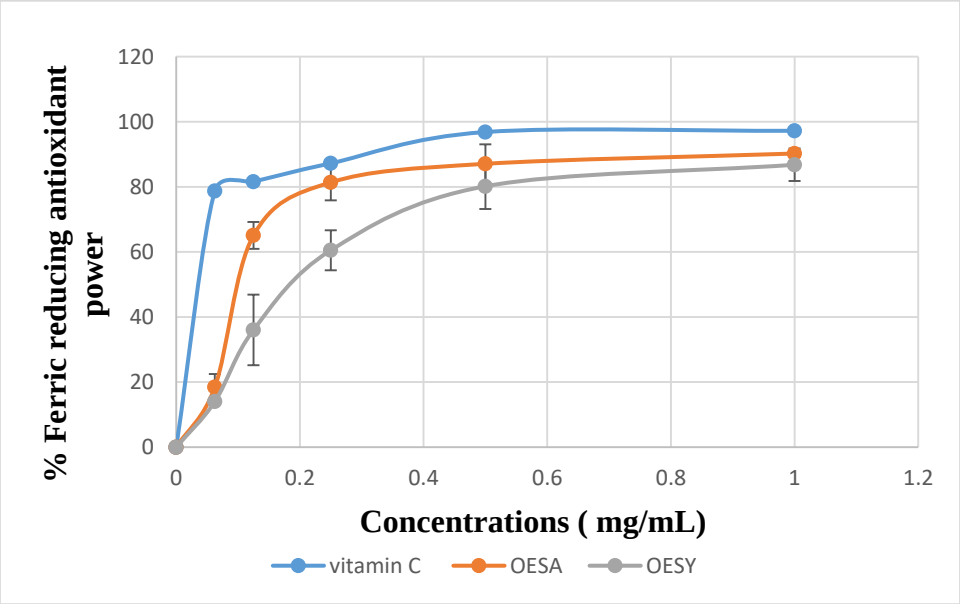


Figure 30: Ferric reducing antioxidant power (FRAP) assay of OESA and OESY

Various concentrations of OESA and OESY extracts (0 to 1 mg/mL) and acid ascorbic were mixed with FRAP reagents. The reduction of ferric ion (Fe^{3+}) to ferrous form (Fe^{2+}) by OESA and OESY. Results were expressed as mean inhibition percentage (%) ± standard deviations (n=3). Ascorbic acid was used as standard at various concentrations (0 to 1 mg/mL).

d. Evaluation of antioxidant activity by the nitrite (NO) test

The nitric oxide radical scavenging activity of OESA and OESY showed higher activity. We can note that the capacity to scavenge NO free radicals depends on increased extracts concentration. The results show that the percentage of inhibition increases with the increase of the concentration for OESA and OESY and the standard used (Vit C). At the concentration 1 mg/mL the NO radical scavenging activity is 93 % and 90 % for OESA and OESY, respectively. To evaluate the antioxidant power, the inhibitory concentration (IC_{50}) of the extracts was used. The results obtained showed that the extracts of cultivated and wild *Olea europaea* leave present the same values of IC_{50}, which is about 0,18 mg/mL (Table 8).

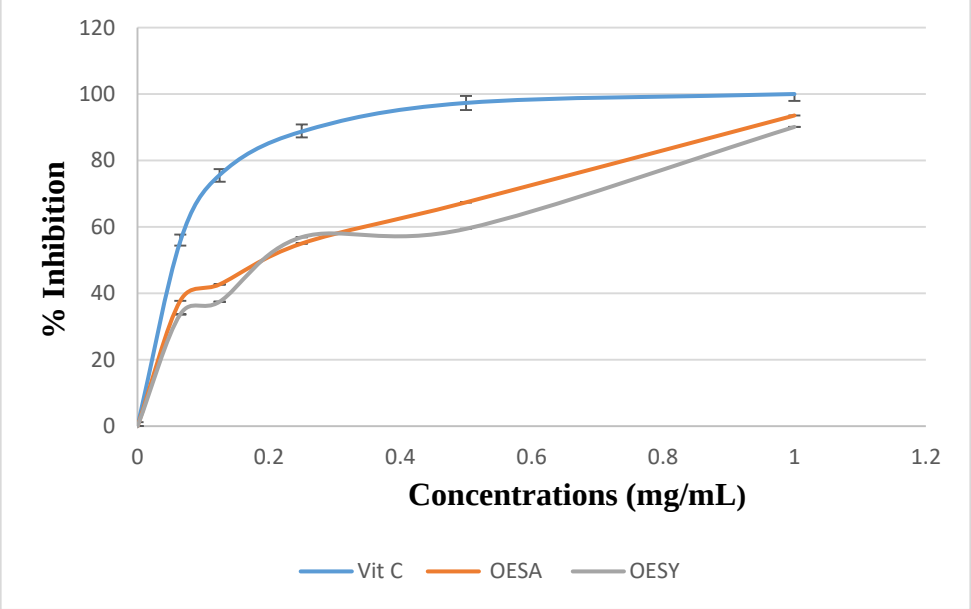


Figure 31: NO scavenging effect of olive leaves extracts

Various concentrations of OESA and OESY extracts (0 to 1 mg/mL) and Vit C were mixed with Nitric reagents. The results were expressed as mean inhibition percentage (%) ± standard deviations (n=3). Vit C was used as a reference standard.

Table 8: The IC50 of OESA and OESY compared with the standard antioxidants, BHT and Ascorbic acid

ASSAY	IC_{50} OESA	IC_{50} OESY	IC_{50} BHT	IC_{50} Ascorbic acid
DPPH	0.136 mg/mL	0.120 mg/mL	0.09 mg/mL	-
FRAP	0.209 mg/mL	0.392 mg/mL	-	0.025 mg/mL
NO	0.18 mg/mL	0.18 mg/mL	-	0.05mg/mL

e. Chemiluminescence assay

The chemiluminescence technique was used to determine the potency of our olive leaf extracts to neutralize reactive oxygen species and the product of the Fenton reaction to the OH radical. Our results showed that our extracts had significant antioxidant activity ($p < 0.05$) (Figure 32).

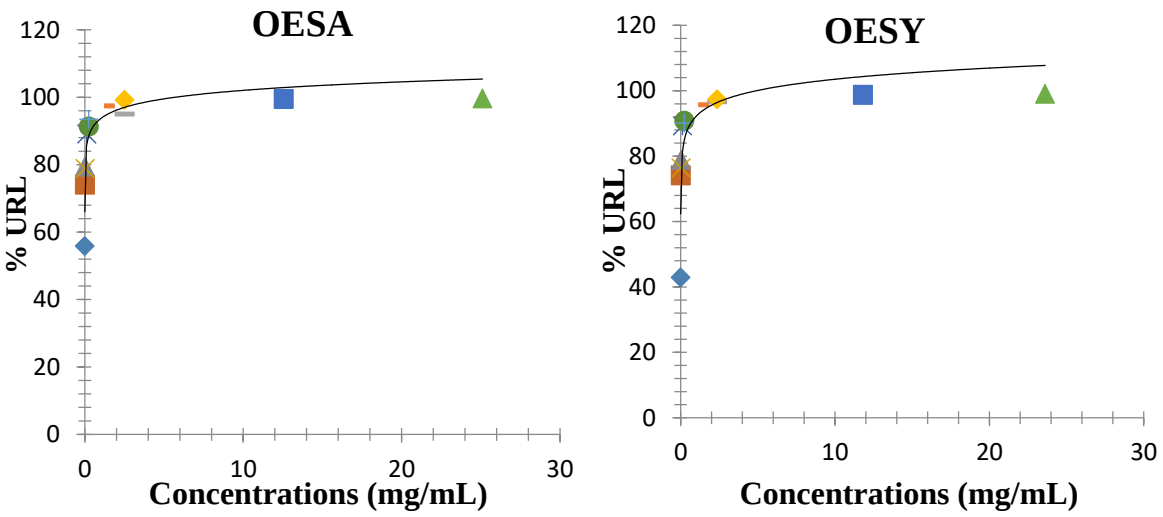


Figure 32: Anti-radical activity of *O europaea L. var. sativa* (OESA) and *O. europaea var. sylvestris* (OESY) leaf extracts. Results were expressed as a percentage (% URL).

3. Cytotoxicity effect of OESA and OESY on HeLa cell lines

To investigate the cytotoxic effect of OESA and OESY on the HeLa human cell lines, cells were treated with various concentrations of OESA and OESY (0.23 to 30 mg/mL) for 48 h and then submitted to the MTT test (Figure 33). Data showed that OESA and OESY displayed an inhibition effect on human cells grown in a dose-dependent manner. Therefore, the IC_{50} of OESA and OESY was evaluated to 15.09 mg/mL and 14.53 mg/mL, respectively. Hence doses under this concentration were used for biological antioxidant activity investigation. Therefore, two doses of the extract were chosen: 0.47 and 0.23 mg/mL, which induce less than 20 % cytotoxicity.

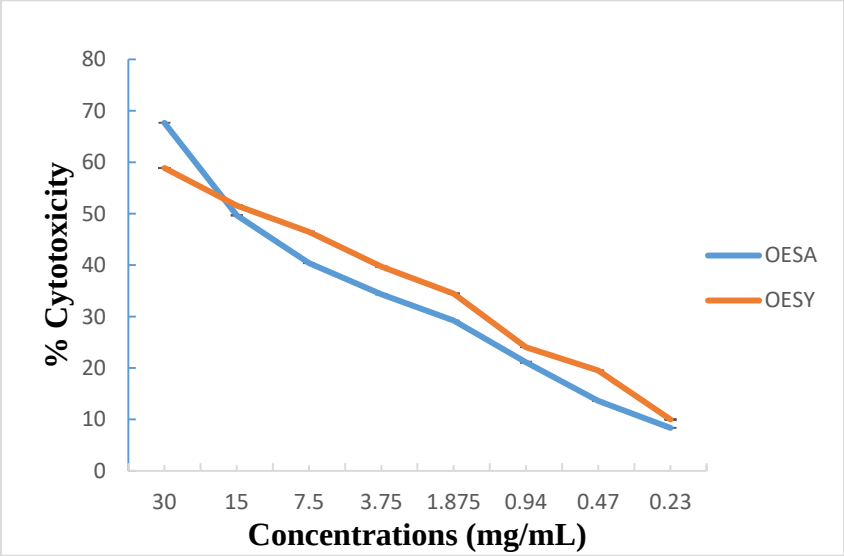


Figure 33: Cytotoxic effect of OESA and OESY on HeLa cell line

The inhibitory effect of different doses on cell growth was determined by MTT assay. Cells were treated with OESA and OESY at different concentrations (0.23; 0.47; 0.94; 1.875; 3.75; 7.5; 15; 30 mg/mL). The percent growth reduction was calculated from the extinction difference between treated cell culture and the control. Results are the mean ± SD of triplicate experiments.

4. Biological antioxidant activity in human cell culture Lipid peroxidation

The investigation of the biological antioxidant activity of OESA and OESY was carried out in the HeLa human cell line. Cells were cultured with or without the addition of OESA and OESY for 72 hours. Oxidative stress was induced by adding 0.2 mM of H_2O_2 for 1 h. In addition, malondialdehyde, is lipid peroxidation marker, was evaluated. The oxidative treatment resulted in an increase in TBARs concentration compared with control cells. As shown in Figure 34, significant protection against ROS-inducing damage was obtained with both used concentrations in a dose-dependent manner. In fact, a significant decrease in TBARs levels was obtained with the concentration of 0.47 mg/mL, and 0.23 mg/mL compared to H_2O_2 treated cells ($p < 0.001$).

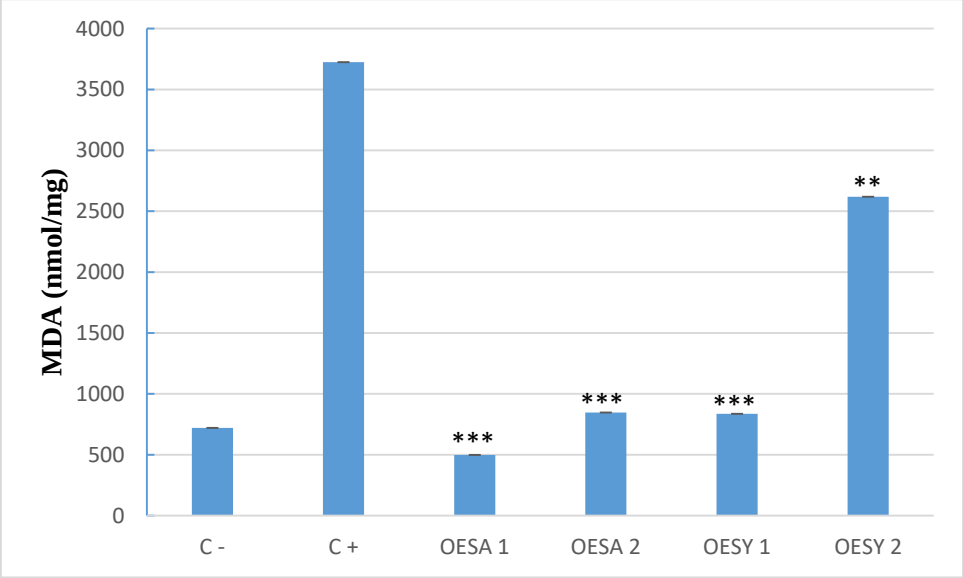


Figure 34: MDA level in OESA and OESY supplemented HeLa cell lines.

Cells were cultured simultaneously in 25 cm^2 flasks with 0.47 and 0.23 mg/mL of OESA or OESY and H_2O_2 (0.2 mM) for 30 min.
Untreated cells (C-). Cells treated with H_2O_2 (C+). OESA 1: cells treated with OESA at 0.47 mg/mL OESA 2: cells treated with OESA at 0.23 mg/mL OESY 1: cells treated with OESY at 0.47 mg/mL OESY 2: cells treated with OESY at 0.23 mg/mL.

5. Antioxidant enzyme activities

Bioactivity of OESA and OESY on catalase, SOD, and GPx antioxidant enzymes was measured in HeLa cells. As shown in Table 9, induction of oxidative stress with H_2O_2 led to an increase in the catalase, SOD, and GPx activities which can be explained by an adaptation of the enzymatic antioxidant system of the cells to the ROS production/ addition. Interestingly, treatment of cells with given concentrations of OESA and OESY induced a significant decrease in the catalase, SOD, and GPx activities ($0.01<p<0.001$).

Table 9: Effect of OESA and OESY cells pretreatment on CAT, SOD, and GPx Activities

Treatment and parameters	C-	C+	OESA 1	OESA 2	OESY 1	OESY 2
SOD Units/mg protein	41.78±1.22	32.35 ± 2.94	41.45 ±1.76 ***	38.20 ±1.47 ***	39.32 ±1.41	29.90±5.97
CAT µmol H_2O_2/mg protein	34.26± 0.80	19.03± 1.46	32.84±1.20 ***	29.52 ± 2.57 ***	30.99± 2.6 ***	14.88± 2.9 ***
GPx µmol GSH/min/mg protein	0.93±0.001	0.19±0.009	0.30±0.01 ***	0.20±0.02 ***	0.35±0.006 ***	0.24±0.008 ***

***** p<0.001**

6. Cytotoxicity effect of OESA and OESY on PBMC

A number of 2 × 10^5 cells were cultured in a 96-well plate for 24 h in the presence and absence of the different concentrations of the extract. The percentage of cytotoxicity was calculated after comparison with the controls. Our results showed a cytotoxic effect of OESA and OESY on PBMC depending on the concentrations used. The concentrations of the extracts retained for the rest of the work are those which present less than 50 % of cytotoxicity (figure 35). Two concentrations were chosen for the antioxidant power study of each extract: 0.11 µg/mL and 0.014 µg/mL.

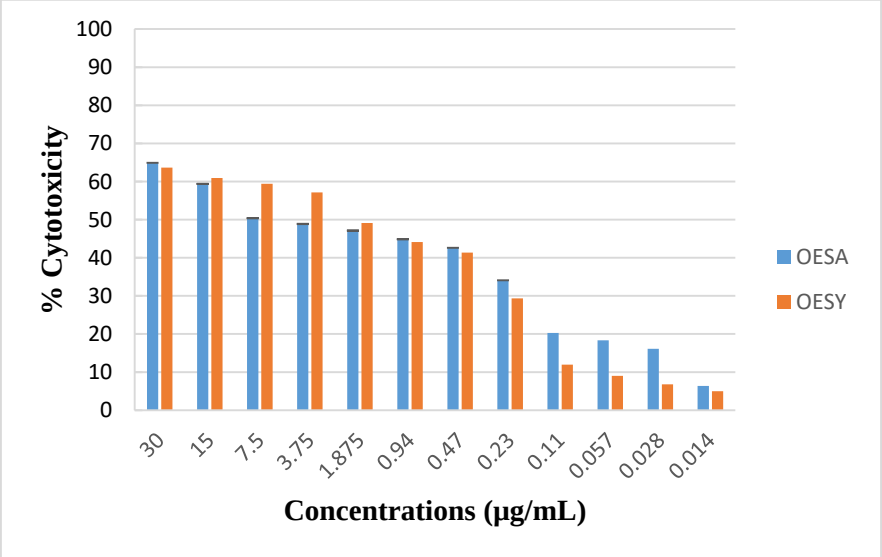


Figure 35: Study of the cytotoxicity of OESA and OESY in PBMC

7. Evaluation of malondialdehyde production in PBMC

PMBC is treated with H_2O_2, which induces a radical attack at the membrane level and generates lipid peroxidation. This is evaluated by the determination of MDA by the TBARS method.

Our results showed a highly significant increase in MDA levels following H_2O_2 only (C+) treatment ($p < 0.001$). Conversely, the treatment of cells simultaneously with olive leaf extracts and H_2O_2 induced a significant decrease in MDA levels compared to those treated with H_2O_2 alone ($p < 0.001$). Indeed, this decrease is dose-dependent since the 0.014 µg/mL concentration of OESA and OESY generates the best reduction in MDA levels.

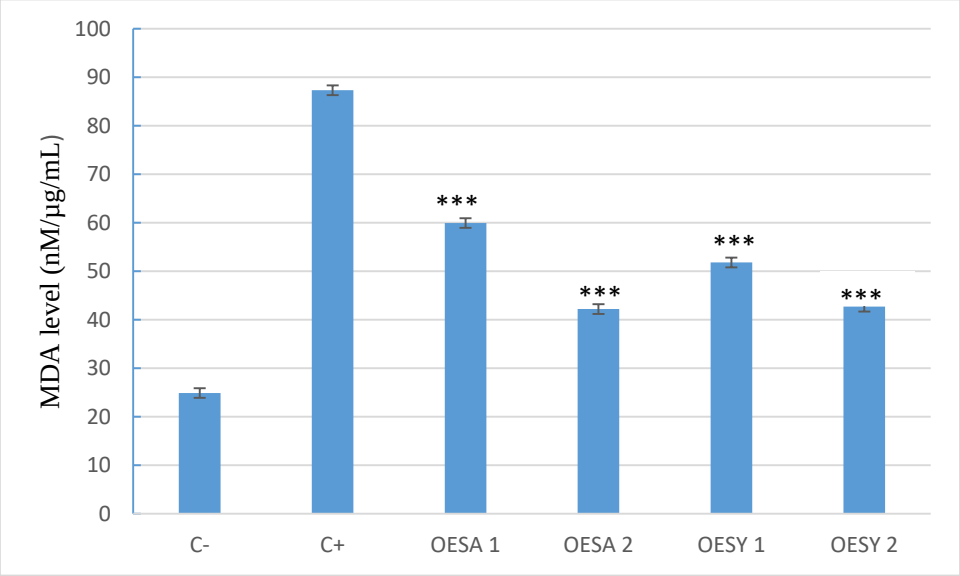


Figure 36: Determination of lipid peroxidation marker MDA on unstimulated, stimulated, and treated PBMC with different concentrations OESA and OESY

II. Discussion

Plant extracts and pure compounds have been widely tested for their antioxidant activity. Olive leaves have perfect biological activities, such as antihypertensive, anti-atherogenic, anti-inflammatory, hypoglycemic, and hypocholesterolemic effects (Briante et al. 2002; Romero et al. 2016). In the present study, the RP-LC-DAD-ESI-MS analysis revealed the presence of many polyphenols in OESA and OESY extracts. The results indicated luteolin- and apigenin-7-O-glycosides as the predominant flavonoids in olive leaves, followed by rutin.

These data are from previous observations in olive leaf extracts obtained from five cultivars grown in southern Spain and Portuguese olive cultivars (Benavente-García et al. 2000; Meirinhos et al. 2005). Moreover, Pereira and coworkers identified rutin, apigenin-7-O-glucoside, and luteolin-7-O-glucoside as the most abundant flavonoids in an aqueous extract of

olive leaves from northeast Portugal (Pereira et al. 2007). These results were recently corroborated by Makowska-Wa¸s and coworkers, who identified luteolin-7-O-glucoside, rutin, and apigenin-7-O-glucoside as the predominant flavonoids in wild olive leaves harvested in South Portugal (Makowska-Wąs et al. 2017). Regarding secoiridoids, oleuropein generally represents the most characteristic oleoside derivative in olive leaves. However, its content and bioconversion occur in different districts of the olive tree according to other factors, such as plant maturation, cultivar type, and harvest time (Termentzi et al. 2015). Indeed, as observed in Table 7, although only OESA showed the presence of oleuropein (18.03 %), OESY contained oleuropein hexoside (11.07 %), as well as the highest content of hydroxyl-oleuropein isomer I and II (2.94 % and 3.75 % vs. 1.06 % and 0.58 % in OESA). On the contrary, OESY showed the highest tannins content (19.96 % vs. 1.97 % in OESA), and it was a rich source of de-caffeoyl verbascoside (23.07 %), absent in OESA. This phenylethanoid derivative of verbascoside, a typical hydroxycinnamic derivative of olive fruit, is generally only found in small amounts in olive leaves (Makowska-Wa¸s et al. 2017). However, this metabolite in olive leaves may significantly vary depending on pedo-climatic conditions (Termentzi et al. 2015). Furthermore, a reverse relationship exists between the oleuropein and verbascoside content, which could explain the absence of oleuropein in OESY (Termentzi et al. 2015). However, beyond the different varieties, it is well-known that biotic and abiotic stressors play a pivotal role in influencing the polyphenolic content of olive leaves and the relative quantitative distribution of the main metabolites (Talhaoui et al. 2015).

In this study, we assessed the antioxidant activity of *Olea europaea*. Our ammonium phosphomolybdate potency was 0.8 ± 0.2 mg and 3.4 ± 0.5 mg of OESA and OESY, equivalent to 1 mg of vitamin C (ascorbic acid). Our results agree with Marino et al.'s studies, proving that *Olea europaea* leaves could reduce molybdenum by reducing activity equal to 0.90 mg EAA /g extract (Marino et al. 2014). Thus, another study conducted by Lfitat et al. on the extract of wild *Olea europaea* leaves showed a reducing power of about 15.02 mg EAA /g extract (Lfitat et al. 2021).

In the present study, the antioxidant activity OESA and OESY was evaluated. Our results indicate that OESA and OESY showed antioxidant activity. Furthermore, the DPPH radical scavenging assay showed a more potent antioxidant activity (IC=0.136 mg/mL and 0.120 mg/mL, respectively, for OESA and OESY). Thus, our results agree with other studies, which demonstrated potent antioxidant activity in the DPPH radical-scavenging assay (Briante et al. 2002; Romero et al. 2016; Bouaziz et al. 2004; Kontogianni et Gerothanassis 2012; Čabarkapa

et al. 2017). Indeed, our results agree with the work done by Hayes et al., who reported that extracts from wild and cultivated olive leaves could scavenge the DPPH radical with IC_{50s} in the range of 0.17 mg/mL 0.15 mg/mL, respectively (Hayes et al. 2011). Moreover, our results are better than those found by Addab et al. since they showed that the extract of the leaves of wild Algerian olive tree presents an IC_{50} equal to 84.74 mg/mL and the studies made by Bouallagui et al. on the extract of cultivated olive tree Kef determined an IC_{50} value of about 23.7 mg/mL (Addab et al. 2020; Bouallagui et al. 2019).

Our results indicated that OESA and OESY exhibit an intense (90 % and 86 at 1 mg/mL, respectively) activity for FRAP assay. These results agree with other studies, which confirm the higher antioxidant effect of olive leaf extracts (Yang et Ouyang 2012; Yancheva et al. 2016). Presented results follow the analysis of Lafka, who demonstrated the highest antioxidant potential of olive leaf Agrielia variety extracts in Greece (Lafka et al. 2013). Comparison of our olive variety (Chemlali) shows that it has a very high effect compared to other types from other regions. For example, Yancheva et al. showed that the extract of olive leaves from the Bulgaria region has an antioxidant power with IC_{50} of about 51.3 mg/mL; the Kalamon variety is characterized by an IC_{50} equal to 85.1 mg/mL (Yancheva et al. 2016). Thus, the results of Malheiro et al. show that the extracts of leaves from the Trás-Os-Montes region (North-East of Portugal) have inhibitory concentrations of the order of 420 mg/mL (Malheiro et al. 2011).
Our results are better than those found by Addab et al. They showed that the extracts of wild and cultivated olive leaves have inhibitory concentrations in the range of 58.28 mg/mL and 59.91 mg/mL, respectively (Addab et al. 2020).

The reducing power of *Olea europaea* species is probably due to hydroxyl groups in the phenolic compounds that can serve as electron donors. Therefore, antioxidants are considered as reducing and inactivating oxidants. Some previous studies have also shown that the reducing power of a compound can serve as a significant indicator of its potential antioxidant activity (Ayala-Zavala et al. 2012). The presence of reducing agents (as antioxidants) causes the conversion of the Fe^{3+} ferricyanide complex to the Fe^{2+} iron form. Although iron is essential for oxygen transport for respiration and enzyme activity, it is a reactive metal that catalyzes oxidative damage in living tissue and cells (Bourgou et al. 2008).

To evaluate the antioxidant power (NO scavenging activity), our results showed that both extracts of *Olea europaea* leaves show low antioxidant activity compared to vitamin C. Indeed, our results agree with the work performed by Adinortey et al., and they highlighted an anti-radical power of the leaves of cultivated *Olea europaea* with an IC50 equal to 0.163 mg/mL

(Adinortey et al. 2018). On the other hand, Lins et al. revealed a higher reducing power of *Olea europaea* Chemlali leaves extract with a low IC50 (0.048 µg/mL) (Lins et al. 2018). Indeed, our results agree with the work performed by Adinortey et al., who revealed an antiradical power of cultivated *Olea europaea* leaves with an IC50 equal to 0.163 mg/mL (Adinortey et al. 2018). On the other hand, other work showed a higher reducing power of *Olea europaea* Chemlali leaf extract with a low IC50 value; 0.048 µg/mL (Lins et al. 2018).

These extracts seem to be good candidates for their antioxidant activities, view their ability to scavenge free radicals, ion chelator, and that the differences in activity could be due to differences in their composition such as in polyphenols flavonoids, and functional groups (Zeng et al. 2014).

The high antioxidant activity of olive leaf extracts could be attributed to their high total content of bioactive molecules such as polyphenols and flavonoids. It has been reported that polyphenols are one of the most effective antioxidant constituents of the plant. Furthermore, a positive correlation between phenolic composition and antioxidant activity has been proven (Velioglu et al. 1998; Arnous et al. 2002). At the same time, flavonoids are likely to react with the most reactive oxygen species (Fuhrman et al. 1995). Therefore, the antioxidant action of these phytonutrients is not only exerted by the inhibition and deactivation of free radicals; it is also manifested by the neutralization of oxidative enzymes and the chelation of trace metal ions responsible for the production of ROS (Halliwell 2009; Cotelle 2001).

This difference in the antioxidant capacity of our study and other works could be due to the difference of the region, the adaptation of the plant can explain the period of cultivation of the olive tree or it to the climate and the conditions where it is found (Miliauskas et al. 2004).

The cytotoxic effect of olive leaf extracts were studied, and IC_{50} of OESA and OESY was evaluated to 15.09 mg/mL and 14.53 mg/mL, respectively. Our results agree with Zeriou et al., who showed that the phenolic extract olive leaves from Tlemcen in Algeria exhibit cytotoxic effects on human colorectal cancer cell lines HCT116 and HCT8 in a dose-dependent manner (Zeziou et al. 2017). Indeed, treatment with the phenolic extract of oleaster leaves at 20 µg/mL for 24 h reduced the number of colon cancer cells by about 50 %, without significantly affecting the viability of normal colon cells CCD 841. Other studies on MKN45 tumor cells showed that the extract of phenolic compounds from *Olea europaea* olives decreased cell growth and viability in a dose and time-dependent manner (Amiri-nowdijeh et al. 2019). Other work performed on *Olea europaea* olive oil demonstrated cytotoxic effects on two different cancer cell lines T-47D and MCF7 (Widyaningrum et al. 2020).

On the other hand, recent work performed showed that methanolic and aqueous extracts of the leaves of *Olea europaea*, from the cultivar Souri de Gaza, do not show a cytotoxic effect on healthy human PBMCs; 100% maintenance of cell viability despite increasing the doses from 75 to 1200 µg/mL (Isleem et al. 2020).

To investigate the antioxidant activity of our extract on the cells model, we choose two concentrations that induce less than 20 % of toxicity according to the results shown in Figure 33. The concentrations used are 0.47 and 0.23 mg/mL in all the experiences. HeLa cells are subjected to oxidation by H_2O_2 solution to assess lipid peroxidation and antioxidant enzyme activity. The oxidative treatment with 0.2 mM of increased MDA levels due to enhancing the lipid peroxidation reaction. The pretreatment of cells by the OESA and OESY resulted in the reduction of the production rate of the TBARs level, as shown in Figures 34 and 36.

Moreover, the addition of H_2O_2 in the culture medium increased the catalase and superoxide dismutase activities and glutathione peroxidase. Indeed, the cultivated leaf extract treatment causes a higher increase than that with the wild leaf extract. The increase in CAT activity may be due to the reactivity by active principles present in the extracts, which may cause an increase in CAT activity by capturing free radicals. Treatment with olive leaf extracts induced an increase in SOD and GPx enzymatic activity compared to the positive control with a difference depending on the concentration of the extract. Our results agree with work carried out by Bahi et al., which showed that the extract of *Olea europaea* exhibits a significant increase in the antioxidant enzyme activity (Bahi et al. 2015). The antioxidant activity could be explained by the high content of *Olea europaea* on polyphenol, flavonoid, and anthocyanins (Benamor et al. 2021).

This study revealed that the hydroethanolic extract of *Olea europaea* had significant antioxidant activity as determined by chemical and biological assays. Therefore, *Olea europaea* could be used as a potential source of natural antioxidants and bioactive molecules.

Chapter 2: Anti-HSV-1 and antimicrobial of *Olea europaea* leaf extracts

O europaea contains a mixture of polyphenolic compounds, among them oleuropein, oleuropein aglycone, eleanolic acid, and hydroxytyrosol, which are readily absorbed and bio available. The biological activities of *O europaea* are mainly derived from these compounds (Visioli et al. 2002). Fredrickson demonstrated that *O europaea* has potent antiviral activities against the herpes virus, hepatitis virus, rotavirus, bovine rhinovirus, canine parvovirus, and feline leukemia virus (Fredrickson 2000). Indeed, the antiviral effect of olive extracts from Iran has been demonstrated against herpes simplex virus type 1 (HSV-1) in Vero cells (Motamedifar et al. 2007).

Herpes simplex virus represents a persistent human pathogen that resides in infected hosts for their lifetime (Arvin et al. 2007). Indeed, following primary infection, the HSV-1 can undergo a lytic infection in epithelial cells and a latent infection in sensory neurons (Arvin et al. 2007). Since HSV infections are often subclinical, the infection is widely becoming one of the world's most prevalent sexually transmitted infections (STIs) (C. Bisignano et al. 2017). The infection can be severe in immunocompromised hosts and may involve the central nervous system, which, if left untreated, may be associated with 70 % of mortalities (Tyler 2004). Acyclovir is widely used to treat primary and recurrent HSV-1 infections (Sarisky et al. 2002). Based on this evidence, this part of the work aims to study antiviral properties. The antiviral mechanism exerted by OESA and OESY extracts was assessed against HSV-1.
Furthermore, some of these compounds have demonstrated antimicrobial activity by inhibiting the growth of a wide variety of bacteria and fungi (Hirschman 1972; G. Bisignano et al. 2010).

The antibacterial effect of olive leaves has been correlated with the presence of olive phenolic compounds, such as tyrosol (TyEDA) and hydroxytyrosol (HyEDA) (Medina et al. 2013). *O. europaea* contains a mixture of polyphenolic compounds, including oleuropein, oleuropein aglycone, oleanolic acid, and hydroxytyrosol, which are readily available absorbed and bioavailable.

Based on this evidence, this part of the work aims to study antiviral and antimicrobial properties. First, the antiviral mechanism exerted by OESA and OESY extracts was assessed

against HSV-1. In addition, the antimicrobial potential of OESA and OESY was evaluated against *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans*.

I. Results

1. Cytotoxicity of olive leaves extracts on cells cultures

To examine the cytotoxicity effect of olive leaves extracts on Vero cells, we incubated the cells in different concentrations of OESA and OESY extracts for 72 h. Samples were then collected, and the quantification of the emitted light intensity related to ATP degradation was measured. Based on these results, the CC_{50} values were 0.2 mg/mL and 0.82 mg/mL for OESA and OESY, respectively (Figure 37).

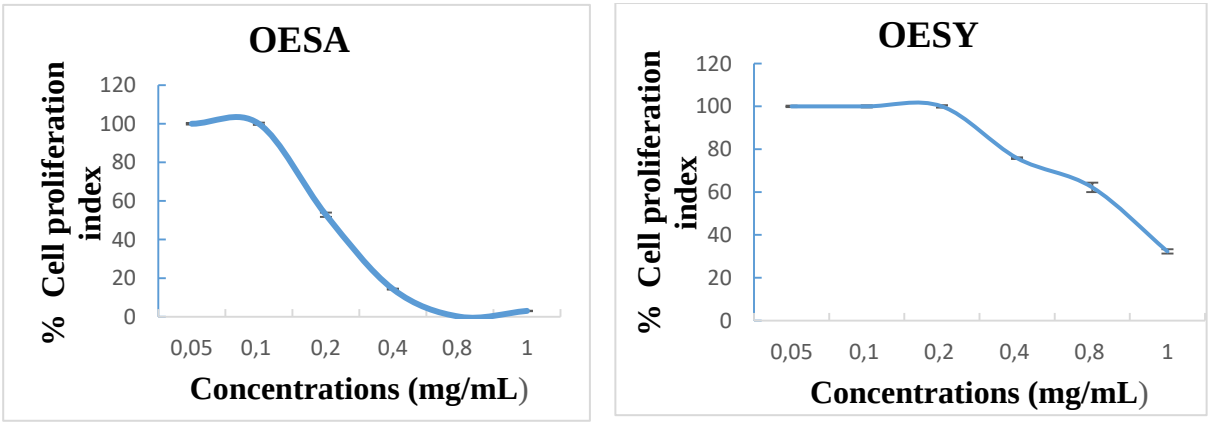


Figure 37: Viability assay in Vero cells treated with olive leaf extracts OESA and OESY.

Vero cells were treated with different concentrations of olive leaf extracts (0.05 mg/mL, 0.1 mg/mL, 0.2 mg/mL, 0.4 mg/mL, 0.8 mg/mL, 1 mg/mL). 72 h post-treatment, the cells were collected, and the luminescence value was converted into the cell proliferation index (%) as described in the Materials and Methods. The assay was performed as the means of triplicates ± SD.

2. Antiviral Activity of OESA and OESY

A plaque reduction assay determined the antiviral effect of OESA and OESY against herpes simplex virus type 1 (HSV-1). In addition, a time-of-addition assay was used. For pre-infection treatment assay, Vero cells were treated with the extracts for 1 h and then infected with HSV-1 and incubated for 1 h at 37 °C. The inoculum was removed after the incubation time, and the monolayers were overlaid with a medium containing 0.8 % methylcellulose. For post-infection treatment assay, Vero cells were infected with HSV-1 and incubated for 1 h at 37 °C. After the incubation time, the inoculum was removed. The monolayers were overlaid with a medium containing 0.8 % methylcellulose in the presence of the ethanolic extract of leaf of OESA and OESY. The plates were incubated at 37 °C, and 5 % CO_2 for 72 h, and the plaques were

visualized by staining cells with crystal violet. Acyclovir was used as a control. The results showed a dose-dependent antiviral activity for both OESA and OESY (Figure 38). However, as indicated in Table 10, the selectivity index (SI) was higher for OESY (SI between 4.1 and 7.4) compared to OESA (SI between 1.3 and 1.6), and the pre-infection treatment condition was found to be more effective compared to the post-infection treatment for both type of extracts (Table 10).

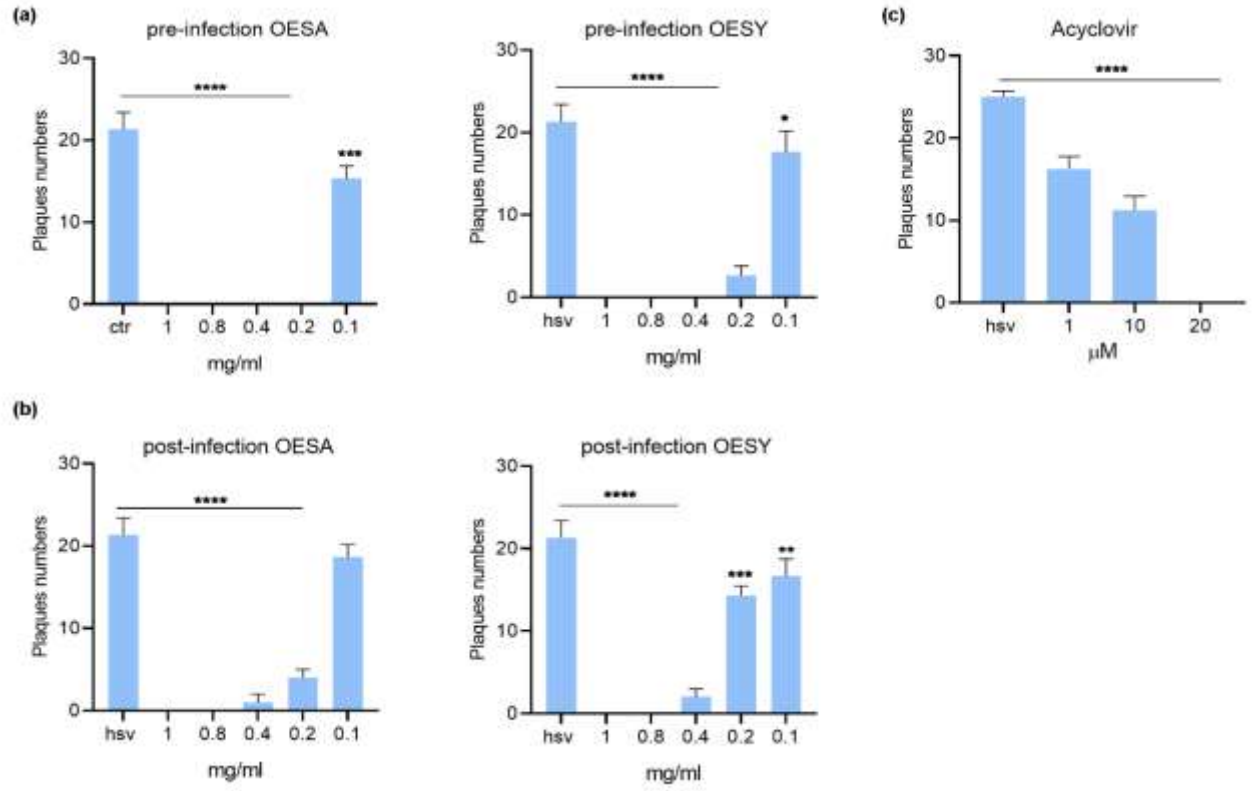


Figure 38: Plaque reduction assay from time-of-addition assay

(a) pre-infection condition and (b) post-infection condition treatments, and (c) Acyclovir control. Results are the mean ±SD of triplicate experiments, and asterisks indicate significant *p*-value (* $p<0.05$, ** $p<0.01$, and *** $p<0.001$).

Table 10: Selectivity index (SI), cytotoxicity (CC50), and antiviral activity (EC50) of OESA and OESY leaves.

Extracts	CC_{50} (mg/ml)	EC_{50} (mg/ml)	SI
OESA			
pre-infection	0.2	0.12	1.6
post-infection	0.2	0.15	1.3
OESY			
pre-infection	0.82	0.11	7.4
post-infection	0.82	0.2	4.1

CC_{50}: 50 % cytotoxic concentration; EC_{50}: 50 % effective concentration; SI ratio EC_{50}/CC_{50}

3. OESA and OESY prevent the binding of HSV-1 on Vero cells

Results obtained from the plaque reduction assay indicated that the SI was higher when the cells were pre-treated with the extracts before infection. To confirm the hypothesis that both extracts could prevent the binding of the virus, a binding inhibition assay was performed as indicated in Materials and Methods. The binding inhibition assay was carried out by using a recombinant HSV-1-VP26GFP virus. The infection was carried out at 4 °C to allow the binding of the virus to the cell receptors, but not the entry. After 24 h post-infection, the expression of the VP26GFP protein was detected by western blot analysis, and quantitative real-time PCR was carried out to quantify the viral DNA. As shown in Figure 39, the pretreatment of cells and viruses with both OESA (0.1 mg/mL) and OESY (0.2 mg/mL) reduced the accumulation of VP26 viral protein and viral DNA compared to the DMSO control. Moreover, OESY showed a more significant antiviral activity than OESA, confirming the previous results from plaque assay.

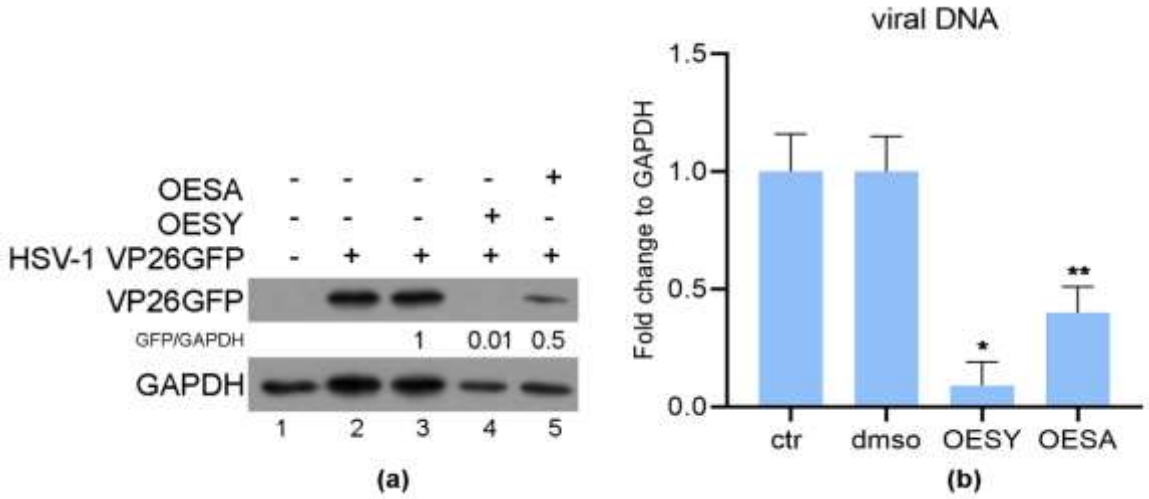


Figure 39: OESA and OESY prevent the binding of HSV-1

Vero cells infected or not with HSV-1 VP26GFPas described in Materials and Methods. Samples were processed 24h p.i. for western blot analysis (a) and real-time PCR (b). Data are expressed as a mean (± SD) of at least three experiments, and asterisks (* and **) indicate the significance of p-values less than 0.05 and 0.01, respectively.

4. Antimicrobial potential

The MIC values of OESA and OESY against the tested strains are shown in Table 11. Both extracts were active against the Gram-positive strains included in the study (MIC values between 7.81 and 15.62 μg/mL and between 15.62 and 31.25 μg/mL for OESY and OESA, respectively). In contrast, no activity was found against the Gram-negative bacteria and the yeast. As expected, the MIC values were lower for *S. aureus* ATCC 6538, demonstrating the

higher sensitivity of this strain compared to the MRSA strain. Thus, the activity was always bacteriostatic rather than bactericidal. The comparison amongst the two extracts showed a great antimicrobial potential exerted by OESY rather than OESA, reflecting the different phytochemical compositions (Table 11). This trend does not relate to the total phenol content, which was higher in OESA. However, the phytochemical profile of the two extracts may be responsible for the different antimicrobial effects: whereas secoiridoids were the most abundant class of compounds in OESA (55.38 %), followed by flavones (21.90 %) and terpenoids (11.48 %), the content of secoiridoids (24.97 %) was almost comparable to phenylethanoids (23.07 %) in OESY, followed by flavones (19.02 %) and ellagitannins (11.27 %).

Table 11: MICs of OESY and OESA (expressed as µg/mL) against Gram-positive bacteria, Gram-negative bacteria, and the yeast *C. albicans*.

Strain	OESY	OESA
***E. coli* ATCC 10536**	NA	NA
***S. aureus* ATCC 6538**	7.81 – 15.62	15.62 – 31.25
***S. aureus* ATCC 43300**	500-1000	1000
***P. aeruginosa* ATCC 9027**	NA	NA
***C. albicans* ATCC 10231**	NA	NA

MICs, minimal inhibitory concentrations; OESY, extract of *O. europaea* var. *sylvestris*; OESA, extract of *O. europaea* var. *sativa*. NA, not active.

II. Discussion

LC-DAD-ESI-MS analysis of OESA and OESY extracts identifies different compounds with antiviral and antimicrobial effects justified in this part. The hydroalcoholic extracts from olive leaves extract had significant antiviral activity against HSV-1. It has also been reported that olive leaf extracts exhibit antiviral activities against human immunodeficiency virus type 1 (HIV-1) (Lee-Huang et al. 2003; Bao et al. 2007; Lee-Huang et al. 2007). Furthermore, *Olea europaea* exhibits antiviral activity against viral hemorrhagic septicemia rhabdovirus (VHSV) (Lee-Huang et al. 2007). *Olea europaea* contains a mixture of polyphenolic compounds: oleuropein Oleuropein also possesses a well-documented antiviral activity (Motamedifar et al. 2007). Its efficacy against hemorrhagic septicemia rhabdovirus (VHSV), hepatitis B virus (HBV), and human immunodeficiency virus (HIV) was demonstrated (Ma et al. 2001). The

beneficial effect of oleuropein against VHSV is exerted through a virucidal effect, reducing virus infectivity and avoiding cell-to-cell fusion of uninfected cells (Lee-Huang et al. 2007).

Alternatively, the viral particle integrity could be damaged, as previously observed for flavanones effect on HSV-1 (Micol et al. 2005). Data presented here indicate that both OESA and OESY exert a dose-dependent broad-spectrum antiviral activity against HSV-1. However, the selectivity index (SI) was higher for OESY (SI between 4.1 and 7.4) compared to OESA (SI between 1.3 and 1.6). Moreover, as indicated from the time-of-addition assay and the binding inhibition assay, a significant antiviral effect was obtained by pretreating the cells before adding the virus rater then adding the compounds after the infection. Overall, our findings demonstrate that OESA and OESY interfere with the virus attachment to cell receptors and thus reduce HSV-1 entry and replication on Vero cells.

LC-DAD-ESI-MS analysis identifies compounds that have an antimicrobial effect. The hydroalcoholic extracts from *O. europaea* leaves were effective against *S. aureus* strains. As often reported with plant-derived extracts, the present study confirmed that Gram-positive strains were more susceptible than Gram-negative bacteria (Talhaoui et al. 2015; Filocamo et al. 2015). Amongst the Gram-positive human pathogens, *S. aureus* and methicillin-resistant *S. aureus* (MRSA) play a crucial role, being responsible for many infections, including skin, respiratory, and bone joint infection, as well as endocarditis, bacteremia, and toxic shock syndrome (Muscarà et al. 2021). Due to the increased spread of multi-drug resistance, more effort has been focused on novel antimicrobial agents against *S. aureus* and MRSA. According to many reports, phenolic compounds isolated from olive leaves have substantial antimicrobial activity (El et Karakaya 2009; Karygianni et al. 2014). The effectiveness of certain phenolic compounds presents in olive leaves, including caffeic acid, oleuropein, rutin, and verbascoside against *S. aureus* has been reported (Pereira et al. 2007; Brahmi et al. 2012). Therefore, we hypothesize these compounds and potential synergistic effects within the extracts were responsible for the activity reported here against *S. aureus*. The high relative proportion of decaffeoylverbascoside, a caffeoyl phenylethanoid glycoside in OESY, may be related to the more substantial antimicrobial effect against *S. aureus* than OESA.

Our results agree with Djenane et al., who showed a vigorous antibacterial activity of olive leaf extracts of the variety chemlali (cultivated variety) against Gram- and Gram + bacteria (Djenane et al. 2012). Bisignano et al. showed antibacterial activity against Gram- and Gram + bacteria, extract of wild olive leaves (Bisignano et al. 2019). Other studies confirmed that leaf extract from northeast Portugal in culture medium showed to induce or repress the antimicrobial

activity (Malhadas et al. 2017). Olive leaf aqueous extracts were screened for their antimicrobial activity against *B cereus, B subtilis, S aureus* (Gram +), *E coli, P aeruginosa, K pneumoniae* (Gram -) bacteria, and *C albicans* and *C neoformans* (fungi) (Tepe et al. 2004; Pereira et al. 2007; Korukluoglu et al. 2008). In opposition, other studies demonstrated that Gram + bacteria are more sensitive to plant extracts than Gram- bacteria (Bisignano et al. 2019). Olive leaf antimicrobial activity against *Staphylococcus aureus* and *Bacillus cereus, Escherichia coli,* and *Salmonella enteritidis* was studied (Somova et al. 2003; Borjan et al. 2020). Furthermore, another study has shown that olive leaves extract showed a potent antibacterial effect against *P aeruginosa, K pneumoniae* and *S aureus* (Markin et al. 2003).

Chapter 3: *Olea europaea Sativa* exhibits antioxidant and antiviral activities against Epstein Barr Virus

EBV is a human γ- herpes virus which commonly infects people living in developing countries, generally in early adulthood, provoking infectious mononucleosis. Similar to other herpesviruses, EBV establishes lifelong latency following primary infection.

An increasing number of diseases, such as rheumatoid arthritis, were also associated with the EBV lytic cycle (Sarban et al. 2005; Toussirot et Roudier 2007). Furthermore, Gargouri et al. demonstrated that EBV is implicated in the genesis of oxidative damages (Gargouri et al. 2009; 2011). Moreover, the production of oxygen radicals during EBV infection increased (Lassoued et al. 2008).

Based on this, this study aimed to investigate the antioxidant and antiviral activities of OESA. Besides, given that the EBV infection is correlated to the onset of oxidative damage and the released oxygen radicals participate in EBV pathogenesis, the lipid peroxidation and the conjugated diene products (DC) resulting by free radical oxidation of polyunsaturated lipids were studied on human Burkitt's lymphoma-derived cell line (Raji) by malonaldehyde (MDA) and DC determination, respectively. The Raji cells harbor multiple copies of EBV genomes and can be activated to express EBV early antigens and induce viral cycle by treatment with tumor promotors (Gargouri et al. 2011). The phorbol ester 12-O-Tetradecanoyl-phorbol-13-acetate (TPA) was employed to generate the lytic cycle in latently infected cells carrying EBV and verify by indirect immunofluorescence the inhibition of TPA-induced EBV early antigens by OESA.

I. Results

1. MTT cell proliferation assay

The cytotoxic effect of OESA on Raji cell lines by the MTT assay. Then, (6×10^4 /well) were cultured in 96-well plates for 48 h, with dilutions of OESA (0.16 mg/mL, 0.31 mg/mL, 0.625 mg/mL, 1.25 mg/mL, 2.5 mg/mL, 5 mg/mL, 10 mg/mL) for 72 h. The percentage of cytotoxicity was calculated as reported in Materials and Methods. Starting from the concentration of 10 mg/mL to 2.5 mg/mL, OESA induced strong cytotoxicity. A moderate

effect was detected following 0.625 mg/mL of OESA; conversely, lower concentrations did not show cytotoxicity. Besides, based on these results, the CC50 values, indicative of 50 % cytotoxic concentration, were 0,8773 mg/mL. Therefore, the 0.31 mg/mL concentration of OESA was selected for further experiments (Figure 40).

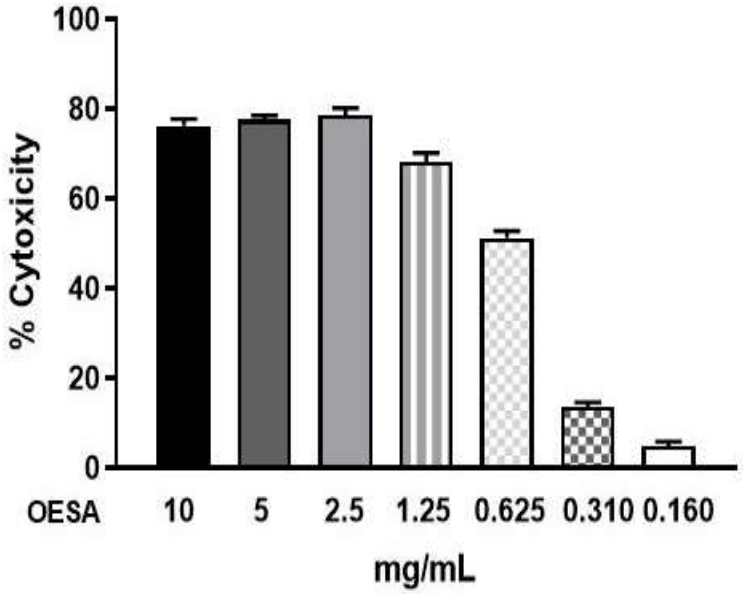


Figure 40: Cytotoxicity effect of OESA in Raji cells.

Raji cells (6×10^4 /well) were exposed to serial dilutions of OESA (0.16, 0.31, 0.625, 1.25 mg/mL, 2.5, 5, 10) for 72h and further incubated with MTT solution in dark for 4h. The absorbance was measured at 570 nm, and the % of cytotoxicity was calculated.

2. Evaluation of lipid peroxidation: MDA and DC determination

Lipid peroxidation is a reaction to oxidative degradation of polyunsaturated fatty acids mediated by oxygen-derived free radicals. Several studies reported that EBV lytic cycle induction generates the oxidative damages involved in the EBV's pathogenicity. A final product of the polyunsaturated fatty acids peroxidation in the cells during oxidative stress is MDA. After induction of the EBV lytic cycle, the levels of MDA were measured on Raji cells treated with TPA and OESA (0.3 mg / mL). The Raji cells were exposed to the minimal and sufficient concentration of TPA (8 nM) able to induce the EBV lytic cycle. The MDA levels were observed after 48h, which matches with the peak of the lytic cycle. Our data show a significant rise in MDA adduct level in Raji cells after EBV lytic cycle induction compared to the basal level of MDA. Conversely, the level of lipid peroxidation declined significantly in OESA treated cells (p <0.05) (Figure 41).

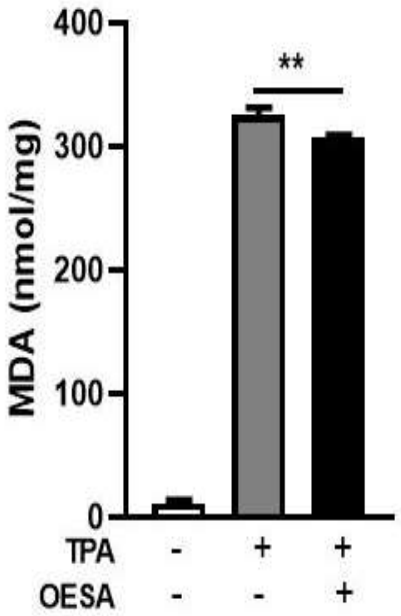


Figure 41: MDA assay: effect of OESA on MDA production in Raji cells after 48h induction of viral cycle.

Raji cells were exposed or not to TPA (8nm) and OESA (0.31 mg/mL) simultaneously at a non-cytotoxic concentration of 0.3 mg / mL. The level of MDA produced is evaluated by the determination of thiobarbituric acid reactive substances. The results are expressed in nmol/mg of protein (*: $p < 0.05$).

To further confirm the role of OESA as a scavenger of lipid peroxidation, CD levels were measured after induction of the lytic cycle. CD is produced during the initial stages of lipid oxidation and by breaking down the polyunsaturated fatty acids. Therefore, Raji cells are untreated or treated with TPA alone or combined with OESA (0.3 mg / mL). Our data show a significant reduction in CD-level in Raji cells after EBV lytic cycle induction ($p < 0.05$) (Figure 42).

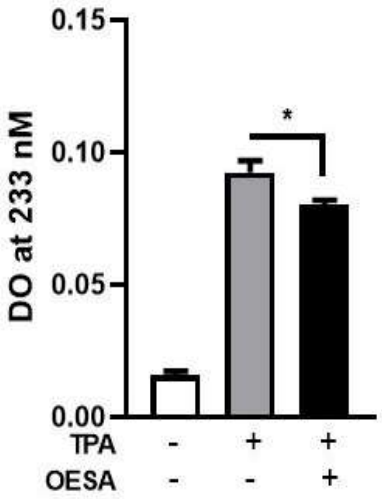


Figure 42: Conjugated diene levels determination on Raji cells treated with OESA after 48h induction of viral cycle.

Raji cells were exposed or not to TPA [8nm] and OESA (0.31 mg/mL) simultaneously at a non-cytotoxic concentration of 0.3 mg / mL. The levels of DC produced were evaluated by measuring the OD at 233 nm (*: p <0.05).

3. Detection of inhibitory activity on the EBV activation mediated by OESA

The detection of inhibitory activity of OESA on the EBV lytic cycle induction, Raji cells were stimulated with TPA, exposed to OESA (0.31 mg/mL), and processed to IFA as reported in Material and Methods. Briefly, cell extracts were subjected to incubation with primary antibody, collected by serum of a patient with nasopharyngeal cancer to detect EA-EBV antigens released after TPA induction. A fluorescence dye-conjugated secondary antibody revealed the binding of primary antibodies to viral antigens. In addition, the fluorescence signals were indicative of reaction.

The EBV activation inhibitory activity detection was measured by counting fluorescence cells and graphically reported as % positive fluorescence cells. HeLa cells ($3x10^6$) were cultured parallel and used as a negative control; conversely. Raji cells treated with TPA were employed as a positive control. A protective effect of OESA against EBV lytic cycle induction in Raji cells lines was observed. A statistically significant decrease in the percentage of fluorescence was observed after simultaneous treatment with TPA and OESA (****P <0,0001) (Figure 43).

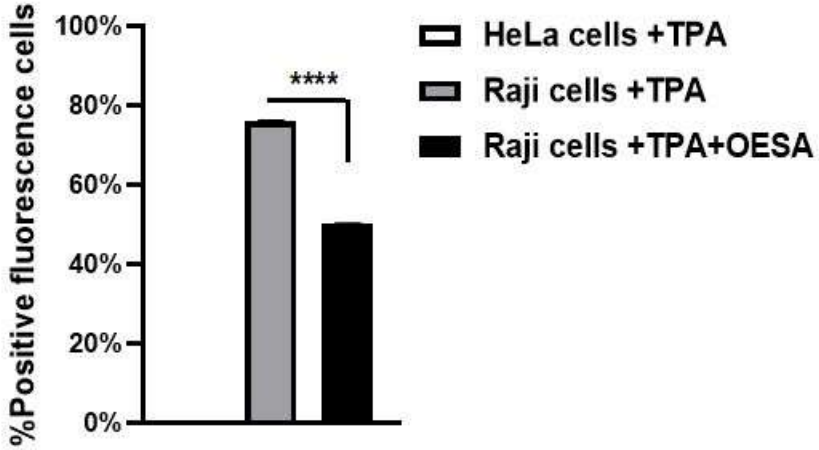


Figure 43: Antiviral effect of OESA in the Raji cells line.

Raji cells were stimulated with TPA, exposed to OESA (0.31 mg/mL), and incubated with primary antibody, collected by serum of a patient with nasopharyngeal cancer. The labeled secondary antibody is sequentially added to bind to the primary antibody. The values represent the percentage of fluorescence cells. **** Indicate significant differences compared to the OESA-untreated sample. (****P <0.0001).

II. Discussion

Many studies have supported evidence about the pharmacological properties of olive leaves on human health. The consumption of olive extracts, commercially widespread in the Mediterranean regions, was strictly associated with a reduced risk of developing diseases due to the capability to exert an antioxidant, hypoglycemic, antihypertensive, antimicrobial, and anti-atherosclerotic activity (Briante et al. 2002; Romero et al. 2016). Some studies associate the protective effect to the nature of these compounds constituted by a mixture of phytochemical compounds. The most abundant compound in olive leaves is oleuropein, which has been patented in treating viral infections to formulate antiviral compositions (Fredrickson et al. 2000). Recently there was an increasing interest in discovering natural antioxidants taken with the diet to compensate for the imbalance between production and accumulation of reactive oxygen species in the human body. This process can negatively damage several cellular structures and trigger human diseases. The onset of oxidative stress allows for an excess of hydroxyl radical, which can cause a radical chain reaction known as lipid peroxidation. This phenomenon damages cell membranes and lipoproteins and is cytotoxic and mutagenic.

The free radicals increased production appears to be a feature of EBV infection. The oxidative modifications of lipids, proteins, and DNA were reported on Raji cells after EBV lytic cycle induction (Lassoued et al. 2008). In addition, studies reported that EBV infection triggers

the oxidative stress related to MDA increase within cells, activating transcription factors, such as STAT3 and NF-Kb (Kwok Fung Lo et al. 2006). Therefore, once we assessed the antioxidant efficacy of OESA, it was necessary to verify the complete assessment of lipid oxidation resulting from EBV infection. The monitoring of lipid peroxidation was done by measuring the secondary lipid peroxidation products generated by decomposition of long-chain polyunsaturated fatty acids, MDA, and DC (Ayala et al. 2014).

To evaluate the effective concentration of OESA useful to affect the oxidative process induced by EBV induction, the toxicity assay was performed on Raji cells. Lower concentrations of OESA did not showed cytotoxicity (CC50= 0,8773 mg/mL) (Figure 40). Therefore, the 0.31 mg/mL concentration of OESA was selected to monitor lipid peroxidation after EBV induction. As reported by Gargouri et al., we used the non-stressing dose (8 nM) of TPA to induce the EBV lytic cycle, and we detected a low basal level of MDA and DC on Raji cells (Gargouri et al. 2009). The results showed that the TPA-treated cells exhibited maximum MDA production, indicating that these samples undergo lipid peroxidation due to induction of the lytic cycle (Figure 40). Otherwise, we observed that the concomitant treatment with TPA and OESA extracts causes a significant decrease in MDA and DC levels on Raji cells (Figure 41 and Figure 42). Thus, it was evident that OESA exhibits high antioxidant activity. Our results coincide with the data reported by Dekanski et al., who described a decrease in MDA levels on gastric mucosa cells treated with OESA extracts and revealed that it exhibits antiviral activity against EBV (Dekanski et al. 2009). This phenomenon was confirmed by reducing antigens-antibody immunocomplex reported by IFA assay (Figure 43). The results showed that Raji cells simultaneously exposed to TPA and OESA exhibited a percentage of positive fluorescence cells lower than TPA treated cells (****P <0,0001). This suggests that OESA treatment protects against EBV lytic cycle induction. These results reflect previous studies which reported the antiviral activity of olive leaf extract preparations towards ILTV virus, VHSV virus, HIV-1, and HSV-1 (Lee-Huang et al. 2003; Micol et al. 2005; Motamedifar et al. 2007).

The EBV maintains a lifelong infection, persistent and intermittent, which could be potentially associated with the formation of oxidative stress and trigger inflammatory processes and several diseases (Dalpke et al. 2003; Sarban et al. 2005; Toussirot et Roudier 2007). This work suggests using OESA in the preventive treatment and deactivation of EBV.

Conclusion And Prospect

Conclusion and Prospects

For thousands of years, humanity has traditionally used various plants found in its environment to treat all kinds of diseases. These plants represent an important natural source of secondary metabolites with a wide range of biological activity. Recently, scientific studies show essential added values of olive leaves, mainly their beneficial properties for human health.
Indeed, attention has been focused on the leaves of *Olea europaea* as a source of antioxidants, which can be used to protect against oxidative stress that is the direct cause of various pathological conditions such as cancer.

The first part of my work is based on two extracts obtained by simple maceration of the leaves of cultivated and wild *Olea europaea* of the Chemlali Sfax variety. The quantitative analysis of the prepared extracts showed that the extract of cultivated olive leaves is richer in total phenols and flavonoids than that of wild leaves. Then, the evaluation of the antioxidant effect of the extracts was first measured in a chemical system, namely the DPPH, FRAP, NO, and TAC test. We have highlighted that both extracts present a significant antioxidant activity. Then, we proposed studying the extracts' antioxidant effect in a biological system (cell culture). This effect was studied by determining MDA products of lipid peroxidation in the Hela line and PBMC. Simultaneous treatment of cells with H_2O_2 and extracts of wild and cultivated *Olea europaea* leaves resulted in protection against lipid peroxidation. This protection is reflected in a highly significant decrease in MDA level in the HeLa cell line and PBMC ($p < 0.001$).
The activity of antioxidant enzymes was studied by evaluating the levels of SOD and catalase in the Hela line treated simultaneously with H_2O_2 and non-cytotoxic concentrations of these extracts. Indeed, treatment with olive leaf extracts induced a highly significant increase in SOD and catalase enzymatic activity ($p < 0.001$).

In the second part of my work, we accrue the current study revealed that polyphenols from *O. europaea* leaf extract exhibit an antiviral effect against HSV-1 and antimicrobial activity against *S. aureus*, which was more robust for the methicillin-sensitive strains. Furthermore, our findings demonstrated that *O. europaea* leaf extracts significantly neutralized the virus in Vero cells after one hour of viral adsorption. Thus, it is possible to hypothesize that one potential mechanism for this inhibitory effect of *O. europaea* leaf extracts on the HSV-1 lytic cycle is the blocking of virion entry into the cells.

In the third part of the research, the analysis of olive leaf extract revealed an anti-oxidant activity and protection against lipid peroxidation. In addition, the leaves of *Oleo europaea* cultivated from Sfax showed an anti-viral effect against EBV lytic cycle induction. Oxidative stress during viral replication can play a crucial role in developing and progressing diseases.

Thus, we can conclude that *Oleo europaea* leaves might be used as a natural supplement to fight oxidative stress and as an adjuvant therapeutic remedy against viral infections. Furthermore, the richness of *Olea europaea* in chemical substances can represent a new potential source of bioactive molecules in therapy and a start of novel pharmacological treatment.

It is desirable to complete and deepen this work by:

- To test olive leaf extracts' antioxidant and anti-tumor effect on other cell lines and an animal model.
- To study the effectiveness of these extracts in pathological cases, using them in long-term protection as treatment or as an adjuvant.
- To consider histological sections of skin fragments marked with hematoxylin-eosin by letting them incubate in different non-cytotoxic concentrations of extracts of the leaves of *Olea europaea* to evaluate the protective effect of the extracts.

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Abstract

Due to the richness of bioactive compounds, Olea europaea leaf extracts exhibit health effects. The present research evaluated the antioxidant antibacterial, and antiviral effects of leaf extracts obtained from *Olea europaea L. var. sativa* (OESA) and *Olea europaea var. sylvestris* (OESY) from Tunisia. LC-DAD-ESI-MS analysis identified different compounds that contributed to the observed biological properties. This work evaluated antioxidant activities *in vitro* using Phosphomolybdenum assay, the DPPH free radical scavenging assay (DPPH), the Ferric reducing antioxidant power (FRAP) assay, and Scavenging Activity of Nitric Oxide as well as by pretreatment of HeLa cells and PBMC exposed to H_2O_2. Our findings *Olea europaea* exhibited antioxidant activity in both chemical and biological tests. Especially, pretreatment of HeLa cells with different extract concentrations conferred protection against lipid peroxidation and modulated activities of two antioxidant enzymes, catalase, SOD, and Glutathione peroxidase. In biological system, *Olea europaea* exhibited a 50 % cytotoxic concentration evaluated as 15.09 mg/ml and 14.53 mg/ml. Incubation of HeLa cell line with no cytotoxic concentrations resulted in special protection from oxidative stress induced by H_2O_2, evidenced by a decrease of MDA levels and an increase of antioxidant enzymes activities compared to cells treated with H_2O_2 alone.

In the second part, we study the anti-bacterial and anti-HSV-1 effects. Both OESA and OESY were active against Gram-positive bacteria (MIC values between 7.81 and 15.61 µg/mL and between 15.61 and 31.25 µg/mL against Staphylococcus aureus ATCC 6538 for OESY and OESA, respectively). The antiviral activity against herpes simplex type 1 (HSV-1) was assessed on Vero cells. The cell viability results indicated that *Olea europaea* leaf extracts were not toxic to cultured Vero cells. The half-maximal cytotoxic concentration (CC50) values for OESA and OESY were 0.2 mg/mL and 0.82 mg/mL, respectively. Furthermore, both a plaque reduction assay and viral entry assay demonstrated antiviral activity.

In the third part, we focused on antioxidant activity and antiviral activity against Epstein Barr Virus. During EBV infection, the free radicals increased, triggering lipid oxidation. Therefore, monitoring the secondary lipid peroxidation products was done by measuring malonaldehyde (MDA) and conjugated dienes (DC). As an inductor of the lytic cycle, the simultaneous treatment of Raji cells with OESA and TPA generated a significant decrease in MDA levels and DC ($p < 0.05$). Besides, Raji cells simultaneously exposed to TPA and OESA exhibited a percentage of EBV-positive fluorescence cells lower than TPA treated cells (****P <0.0001).

We conclude that *Olea europaea* leaves antioxidant effects and suggests a potential source of natural antioxidant molecules with possible applications in industry and medicine. In addition, *Olea europaea* leaf extracts demonstrated a bacteriostatic effect and remarkable antiviral activity. Also, OESA treatment has a protective effect against EBV lytic cycle induction.

Keywords: *Olea europaea*, LC-DAD-ESI-MS analysis antioxidant activity; antimicrobial effect; antiviral activity; herpes simplex virus 1, Epstein Barr Virus

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