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Related-oxidative stress effect and biological activities of biomolecules from Algerian medicinal plants on the model of sera from Crohn's disease patients

Members of the jury :

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Option : **Biochimie et Pharmacotoxicologie**

Présentée et soutenue par :

M^elle^ RAMLI IMAN

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2021/2022

بسم الله الرحمن الرحيم

قال الله تعالى

"وَقُلِ ٱعْمَلُواْ فَسَيَرَى ٱللَّهُ عَمَلَكُمْ وَرَسُولُهُۥ وَٱلْمُؤْمِنُونَ"

صدق الله العظيم

Dedication

To the Greatest ALLAH …. Alhamoulillah

To my parents SAKINA AROUSSI and HOCINE RAMLI, the light to my path, This work could never be accomplished without you.

To myself, Iman…., You have been a hard worker, patient, descent and humble, I thank you…., I cherish you…..

To my sisters Siham, Dalal, Chahrazed, Khawla, Fayrouz and my Brothers Ibrahim and ALI.

To the souls of my beloved Grandmothers "Ma Yamina" and "Ma Nowa"

To the little nephews: Hocine, Mohamed, Ahmed, Adam, Youcef and the little Yasmin Amna.

To my friends Wassila and Yacine and their beloved children.

To Ilhem Selatnia, Lynda GALI, Hanane Dgeghim, and Amina.

To Salvatore R,

To sabrina
To Redouane

To all of my colleagues, Valentina, Massimo, Antonella, Bianca, Claudio, Maria Antonietta, JiJi, Paula, Alessandro, Marcello, Daniele, Giang Phan and Sepideh.

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Abbreviations & Symbols used

3-MST	3-mercaptopyruvate sulfurtransferase	**8-OHDG**	8-hydroxy-2-deoxyguanosine
$AlCl_3$	aluminum chloride	**$Al(NO_3)_3$**	aluminum chloride
ABTS	(2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid	**AP-1**	Activator protein-1
CH_3COOK	potassium acetate	**BHA**	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene	**BSA**	Bovine albumin serum
CAT	Catalase	**CBS**	Cystathionine-β-synthase
CD	Crohn's disease	**CRP**	C-reactive protein
CD4	Clusters of differentiation		
Cl^-	Chlroine	**COPD**	Chronic obstructive pulmonary disease
CHL	Chloroform	**COX-2**	Cyclooxygenase 2
CQA	Cafoeylquinic acids	**CSE**	Cystathionine-γ-lyase
DC	Dendritic cell	**CSF**	Colony stimulating factors
EC-SOD	Extracellular SOD	**DNA**	Deoxyribonucleic acid
DSS	Dextran sulfide sodium	**DPPH**	(2,2-diphenyl-1-picryl-hydrazyl-hydrate
EtOH	Ethanol	**EtOAc**	ethyl acetate
ETC	electron transport chain	**GM-CSF**	Granulocyte-Macrophage Colony-Stimulating factor
G	Gravity	**GAE**	Gallic acid equivalent
GI	Gastrointextinal tract	**GPx**	Glutathione peroxidase
FBS	Newborn-calf serum	**FCS**	Foetal calf serum

GSH	Glutathione	**GSSG**	Glutathione disulfide
H_2DCFDA	Dichlorodihydrofluorescein diacetate	**HPLC-DAD-ESI-MS**	high performance liquid chromatography equipped with photodiode array detection-mass
H_2O_2	Hydrogen peroxide	**H_2S**	hydrogen sulfide
HIV	Human immunodeficiency viruses-1	**HIF-1**	Hypoxia-Inducible Factor-1
HMGB1	High-mobility group box 1	**HCl**	hydrogen chloride
HBSS	Hanks' Balanced Salt Solution		
HUVEC	Human umbilical vein endothelian cells	**IL**	Interleukin
IBD	Inflammatory bowel dieseases	**IFN-γ**	Interferon-gamma
iNOS	Inducible nitric oxide synthase	**HOBr**	Hypobromous
HOCl	Hypochlorous acid	**JAK**	Janus kinase
STAT	Transducer and activator of transcription	**L-Arg**	L-arginine
Keap1	Kelch-like ECH-associated protein 1	**L-Cys**	L-cysteine
LDL	Low-density lipoproteins		
MAPK	Mitogen-activated protein kinase	**MHC**	Major histocompatibility complex
MDA	Malon dialdehyde	**MPO**	Myeloperoxidase
MMA	Mitochondrial metabolic activity		
L-Met	L-methionine	**MMP3**	Matrix Metallopeptidase 3
mtDNA	Mitochondrial DNA	**m/z**	Molecular masse range

MTT	3-(4,5-dimethythiazol2-yl)-2,5-diphenyl tetrazolium bromide	**NADPH**	Nicotinamide Adenine Dinucleotide Phosphate
NF-κB	Nuclear factor-kappa B	**Nrf2**	Nuclear factor erythroid 2 related factor
NH_4Ac	Amonium acetate	**NLRP3**	NOD-like receptor, pyrin domain containing 3
NO	Nitric oxide	**NIR**	Nitrite reductase
NLR	NOD-like receptor	**n-BuOH**	n-Butanol
NOS	Nitric oxide synthase	**NOX**	NAPDH oxidase
O_2^-	Superoxide anion	**$^1O_2$**	Singlet molecular oxygen
O_2	Diatomic oxygen gas	**O_3**	Ozone
•OH	Hydroxyl radicals	**OMM**	Oxidative mithochondrial metabolism
Ox-LDL-C	Oxidised low density lipoprotein cholesterol	**$ONOO^-$**	Peroxynitrite
P53	Tumor protein 53	**OS**	Oxidative stress
PAMPs	Pathogen-associated molecular patterns	**PRDXs**	Peroxiredoxins
PRR	Pattern Recognition Receptors	**PGE2**	Prostaglandine E
		QE	Qercetin equivalent
RNA	Ribonucleic acid	**RES**	Reticuloendothelial systems
NLRP3	NOD-like receptor protein 3	**RO·**	Alkoxyl radical
ROO·	Peroxyl radical	**ROOH**	Organic hydroperoxides
RSH	Thiols	**ROS**	Reactive oxygen species
RNS	Reactive nitrogen species (RNS	**RSI**	Reactive Species Interactome’

RSS	Reactive sulfur species	**R-SS-R**	Disulfide
RS	Reactive species	**SD**	Standard error
SMC	Smooth muscle cell		
SOD1	Superoxide dismutase 1	**SOD3**	Superoxide dismutase 3
Th	T helper	**TLR**	Toll like receptors
TNFR	TNF receptors	**TTRX**	Thioredoxin
TNF-α	Tumor necrosis factor α	**TFF3**	Trefoil factor 3
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid	**t_R**	Time of retention
HT-29	colonic epithelial cells	**UV**	Ulcerative colitis
WHO	World health organization	**v/v**	Volume/Volume

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Introduction

Introduction

Immunity constitutes the natural defense system of our organisms against damaging stimuli (Jantan *et al*., 2015). Based on the function, immune system can be classified into innate immune system (non-specific) and adaptive immune system (Vesely *et al*., 2011). Inflammation is a defense mechanism of the immune system against tissue damage caused by a variety of stimuli. It is known that oxidative stress; an imbalance in the generation and elimination of cellular reactive oxygen species (ROS) (Sies and Jones, 2020); and inflammation are interrelated processes (Marrassini *et al*., 2018) , and were found to be tightly linked to many disorders, such as autoimmune diseases, stroke, diabetes mellitus, and cancer (Tohma *et al*., 2016) . Interactions in the immune system are a systemic defense mechanism, but when overly deployed can be harmful to the host physiology and may contribute to the development of several non-immune and immune chronic inflammatory disorders (Jantan *et al.,* 2015).

Crohn's disease (CD) is an inflammatory bowel disease (IBD) characterized by chronic progressive inflammation with a multifactorial etiology affecting the gastrointestinal tract (GI), eventually leading to an uncontrolled immune response with impaired gut mucosal homeostasis (Cosnes *et al.,* 2011). CD possesses complex and non-specific symptomatology translated into a long-lasting subclinical disorder activity requiring complicated medication and difficult monitoring (Cosnes *et al.,* 2011). It is generally accepted that oxidative stress, plays a critical role in CD onset and progression. Nonetheless, the redox-associated mechanisms underpinning IBD pathogenesis are not yet understood. While the specific causative agents remain ambiguous, a growing body of literature evidence suggests that oxidative stress represents a common thread among the multiple mechanisms resulting from the interplay between environmental factors, impaired immune system, microbiota dysbiosis, and host genetics in CD pathogenesis (Alemany-Cosme *et al.,* 2021; Alzoghaibi, 2013; Basílio *et al.,* 2021; Luceri *et al.,* 2019).

Parallelly, cellular and molecular damage can be caused by the continuous release of ROS and their related products into the microenvironment of the inflamed mucosal lesions, thus maintaining the chronic excessive immune response (Bourgonje *et al.,* 2019). This is typically associated with infiltrating effector lymphocytes and increased production of proinflammatory cytokines into the intestinal mucosa, ultimately creating a loop in the continuous production of

reactive species (Bourgonje *et al.,* 2019). At a molecular scale, ROS overproduction leads to cellular proteins' oxidation, thereby affecting gene regulation, DNA damage, ion transport, intermediary metabolism, and mitochondrial function accompanied by an endogenous antioxidant system reduced activity (Bourgonje *et al.,* 2020; Luceri *et al.,* 2019). Human and animal studies also implicated oxidative stress-associated mitochondrial dysfunction in IBD pathogenesis as early steps mechanisms of redox signalling and redox regulation depend essentially on mitochondrial dynamics (Ardizzone *et al.*, 2008; Pietta, 2000; Wang *et al*., 2021; Xu *et al*., 2017).

However, the role of the mitochondrial metabolic imbalance in CD-associated inflammation is not yet well defined. At the tissue level, the inflamed intestinal mucosa redox state impairment propagates into the intestinal tract's deep layers and mirrors through the systemic circulation (Bourgonje *et al*., 2020). Indeed, several studies reported high levels of oxidative stress in IBD patients' serum/plasma (Ardizzone *et al.,* 2008; Bourgonje *et al.,* 2020, 2019; Luceri *et al.,* 2019) and revealed expanded intestinal microvasculature typical of CD patients. Since intestinal microvessels are lined by vascular endothelial cells, the last could be an essential component of the intestinal circulation implicated in IBD development (Wang *et al.,* 2021).

When the endogenous antioxidant systems cannot efficiently balance the cellular impaired redox state, supplementation of exogenous antioxidants may be necessary (Pietta, 2000). Agents that are able to activate or induce the immune system mediators or components are recognized as 'immunostimulants'. These agents are capable of enhancing the immune system against autoimmunity, cancer, allergy, and infection. Furthermore, it is recognized that lipid peroxidation is associated with inflammation and oxidative stress, both of which have been acknowledged to play an important role in the initiation and progression of chronic diseases (Zhong *et al*., 2019). Moreover, protein denaturation is another well-documented aspect of inflammation, and the role of ROS to stimulate this process is established (Marrassini *et al.,* 2018).

The wise use of natural antioxidants may prevent many diseases which could be a promising lead if taken together with conventional drugs (Tohma *et al.,* 2016). There is an increasing interest to use herbal therapies as multi-component agents to modulate the complexity of the immune system in the prevention of related disorders instead of treating them. Interestingly, a broad range of immunomodulatory and/or anti-inflammatory effects have been contributed to

natural components due to their related human immune system bioactivities. Herbal therapies known for their efficiency in reducing inflammation have been used in traditional medicines since the ancient times. These herbal derived molecules have been shown to possess immunomodulating properties (Choi *et al*., 2016; Jantan *et al*., 2015).

Among these bioactive compounds, flavonoids; secondary metabolites found essentially in plants, vegetables and fruits; are also known for their potent antioxidant ability showing a considerable capacity to attenuate tissue damage or fibrosis related to macromolecules degradation (Choi *et al*., 2016). Several studies *in vitro* and in animal models have demonstrated their potential to inhibit the onset and development of inflammatory disorders According to WHO, near 80 % of individuals around the world use herbal medicines as standard therapeutic modalities along with conventional drugs and in Africa, the number encompasses the 85 % (Choi *et al.,* 2016). However, the fact that general population considers herbal medicines safe, it is not always shown in scientific studies, since critical issues of safety and toxicity related to certain herbal medicines consumption and in many instances demonstrated serious complications and death (Choi *et al.,* 2016).

A. maritima L. (Asteraceae) is a perennial herbaceous plant, broadly distributed over the Mediterranean basin and Africa. This plant has antispasmodic merits and is able to relief bronchial impairments and urinary tract issues (Dirar *et al.,* 2014; Abdelgaleil, 2011). The phytochemical composition of *A. maritima* includes a variety of secondary metabolites, such as pseudoguaianolides, sesquiterpene lactones, coumarins, triterpenes, and sterols. Sesquiterpene lactones have been shown to prove diverse bioactivities, including anti-inflammatory, antimicrobial, and antitumor antispasmodic properties (Mohamed *et al*., 2020).

Bituminaria bituminosa (L.) C. H. Stirton is a perennial wild legume plant, widely distributed throughout the Mediterranean basin, traditionally used in the treatment of spasms, fever, and epilepsy (Azzouzi *et al.,* 2014). It is also considered as a source of pharmaceutically active compounds with relevant bioactivities such as furanocoumarins (psoralen, angelicin), pterocarpans (erybraedin C, bitucarpin A), and flavonoids (daidzin, isoorientin) (Azzouzi *et al*., 2014; Maurich *et al*., 2004; Nelson *et al.,* 2020; Walker *et al.,* 2006). Its phytoconstituents have shown antimicrobial effect against different strains like *Staphylococcus aureus*, *Klebsiella pneumonia* and *Escherichia col*i, besides anti-HIV activity, and were able to prevent and treat solid organ transplantation rejection, besides other anti-proliferative, estrogenic, hepatic-

protective, anti-allergy, anti-inflammatory, apoptotic, and anti-tumor properties (Maurich *et al*., 2004; Pazos-Navarro *et al.,* 2011; Zhou *et al*., 2000).

In this study we are aiming to investigate the beneficial activities of various extracts from *B. bituminosa* and *A. maritima,* sampled from the east of Algeria:

-Investigate the phenolic profile composition through a phenolic and flavonoids content and a HPLC-DAD-ESI-MS fingerprint of the studied plants.

-Evaluate the acute toxicity of our plants *in vivo* and in cell culture on primary Human umbilical vein endothelial cells (HUVECs).

-Test the *in vitro* antioxidant ability of the plants' extracts using chemical and intracellular methods.

-Evaluate the cytoprotective effect of *A. maritima and B. bituminosa* phenolic extracts against the redox state impairment elicited by H_2O_2 and sera from CD patients on primary Human umbilical vein endothelial cells (HUVECs).

-Assess the immunostimulatory effect of these plants *in vivo.*

-Investigate the anti-inflammatory capability of our extracts using the protein denaturation method.

-Suggest a probable structure-Activity relationship between the phenolic content and the biological activities of our tested extracts.

Chapter I

Oxidative stress and inflammation

Highlights

-The term oxidative stress has been evoked in different associations related to research coming from different fields of interest.
-(OS) pervades the chemical, biological, biochemical, and clinical-medical literature
-(OS) plays an important role in many disease states.
-Chronic inflammation is a pathological condition characterized by continued active inflammation response and tissue destruction
-Inflammation and oxidative stress are closely related and tightly linked pathophysiological processes.

I. 1. The concept of Oxidative Stress

Oxidative stress is a global concept in biomedicine, that denotes an imbalance between oxidants and antioxidants in favor of the oxidants in given matrices (Sies, 2020), overcoming the reducing capacity of the human antioxidant defense system, which has the role of the elimination of ROS production excess, to avoid the oxidative action of this highly reactive species on cellular components (nucleic acids, lipids, proteins or carbohydrates) and thereby their resulting adverse effects. In general, high Oxidative stress can result from a an over production of ROS and/or a poor antioxidant defense system, which depends partially on exogenous molecules that can act as antioxidants, like vitamin C, vitamin E, carotenoids and polyphenols (Bouayed and Bohn, 2012).

However, recent findings have begun to demonstrate a minimal or moderate concentrations of ROS in human tissues are necessary for optimum cellular functioning, by the reason of their critical role in many physiological functions, such as the stimulation of cell signaling, gene expression, immune responses regulation and the fostering of antioxidant mechanisms as stress responsive-mediators (Valko *et al.*, 2007a; Bouayed and Bohn, 2010; Kung *et al.*, 2021). Thus the double-edged effects of ROS are well recognized (Bouayed and Bohn, 2012).

Within this basic concept, an open metabolic system with a steady state redox balance is preserved, and any deviation in the different set points maintaining its basal redox tone is considered a stress. Other implicit definitions related to Oxidative stress we may mention (Figure 1)

(i) Reductive stress: a deviation in the opposite side of the redox homeostasis.

(ii) Oxidative eustress: that includes physiological deviations and is an essential part of redox control and physiological redox signaling.

(iii) Oxidative distress: supraphysiological deviations (Sies, 2020).

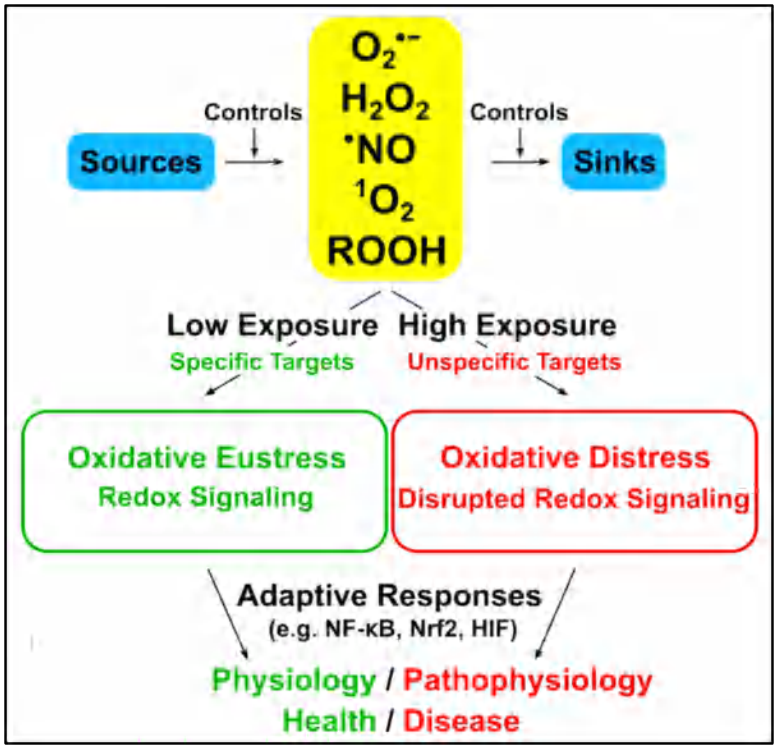

Figure 1. Different concepts of (OS) and their relationship with redox signaling (Sies, 2020).

I. 1. 1. Pro-oxidant Stress

This term has been used for the first time by Levander in his 1979 studies related to metal toxicity, in describing the effects of lead, but also by Ceruttistating this term in his analysis of the role of oxidants in the promotion of tumors (Sies and Jones, 2007).

I. 1. 2. Reactive Species

Reactive species are highly reactive compounds that have a crucial role in human metabolism (Halliwell and Gutteridge, 2015). They can be generated from oxygen (O_2), from the reduction or oxidation of nitrogenous compounds, and from the redox conversion of sulfur-containing compounds. These reactions result in the formation of ROS, RNS, and RSS, respectively. They are involved in various cell signaling processes and are able to modify cysteine thiols (redox switches), causing structural and functional modulation of proteins, particularly, enzymes (e.g., protein kinases and phosphatases), transcription factors, ion/solute transporters, and structural proteins (Cortese-Krott *et al.*, 2019). Since, the physiological functions of reactive species and

their chemical interactions are various a new term, 'Reactive Species Interactome' (RSI), has recently been introduced (Cortese-Krott *et al.*, 2017).

I. 1. 3. Reactive Species Interactome (RSI)

The RSI present an overall concept, of a frame work that aims to characterize:

(i) The chemical interactions between different reactive species,

(ii) The pathways integrated in their generation via the cellular intermediary metabolism,

(iii) The responsible elements of redox regulation transduction involved in the adapted downstream modulation of intracellular targets regarding the metabolic variation needs,

(iv) Their role in sensing the eventual changes in the extracellular environment.

Among the RSI compounds (Figure 1), Superoxide onions (O_2-) derived species (i.e., ROS) are the best-established. In aerobic redox metabolism, O_2i s an electron acceptor leading to the formation of superoxide anions (O_2-), hydrogen peroxide (H_2O_2),and hydroxyl radicals (•OH), collectively so called ROS, although they have very distinguishable chemical reactivities and different biological effects (Cortese-Krott *et al.*, 2019). Parallelly, NO-derived species which are named reactive nitrogen species (RNS) (Saijo *et al.*, 2010).

RNS are able to modulate protein tyrosine residues, alter the mitochondrial energy production, and disrupt the structure of lipids and DNA strands (Campbell and Colgan, 2019). A promising role in the RSI has been attributed to hydrogen sulfide (H_2S) deriving from reactive sulfur species (RSS), while Thiols (R-SH) have a critical role in redox machinery, (Figure 2) (Ono *et al.*, 2014; Cortese-Krott *et al.*, 2017).

I. 1. 4. Redox Switches as Biological Targets of Reactive Species

Redox switches consist target proteins with functional cysteine groups (i.e., protein thiols) and their biological function is impaired through chemical and reversible modifications induced by reaction with reactive species. They constitute the central building blocks of redox switches and are highly redox-sensitive to (reversible) thiol modifications in response to different reactive species. Redox switches can undergo a range of oxidative alterations depending on the

reactive species type that induces the modification. These frequently result in particular impairments in the structural or functional state of the protein.. Metabolic enzymes, protein kinases and phosphatases, ion channels, transporters, structural proteins, and transcription factors are all examples of redox switches. (Bourgonje *et al.*, 2020).

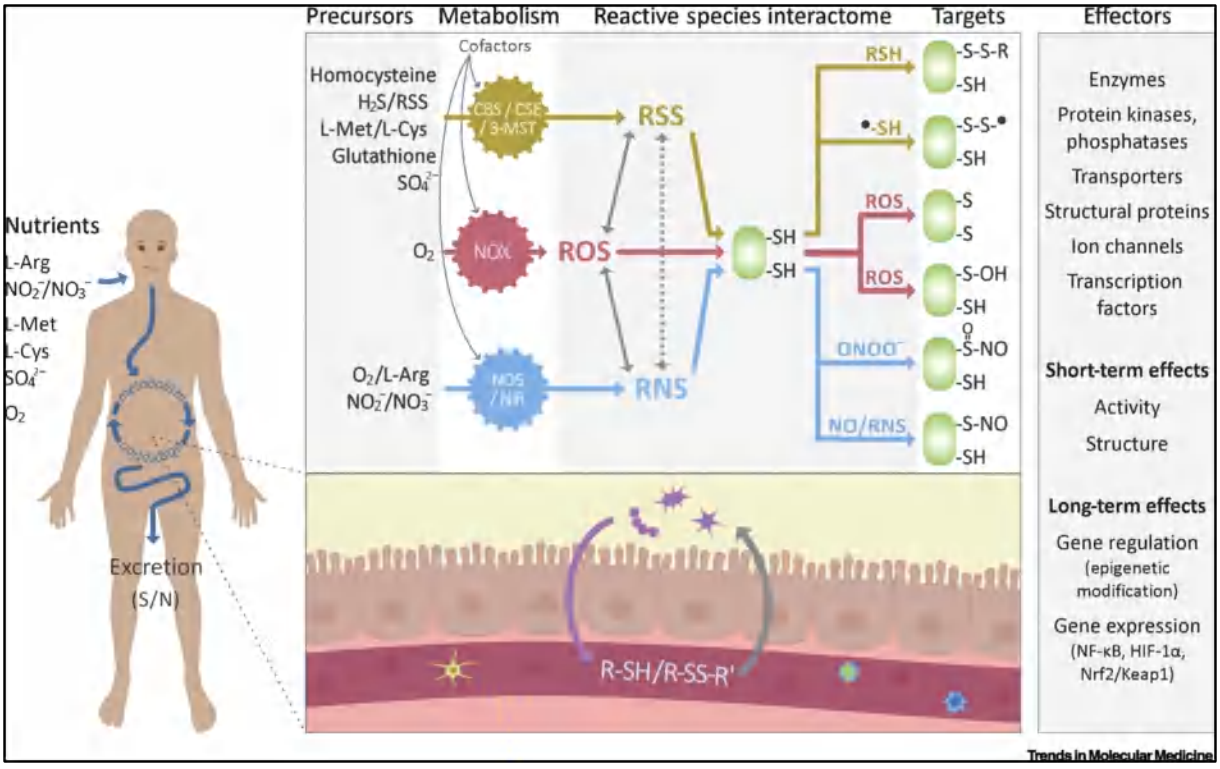

Figure 2. Main constituents of the reactive species interactome (RSI) and its intra- and extracellular role in human physiology (Bourgonje et al., 2020).

I. 1. 5. Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are generated by redox reactions or electronic excitation and are derived from molecular oxygen coming from the aerobic life of cells. Non-radical and free radical (at least one free electron) species can be distinguished:

Two-electron (non-radical) ROS include: hydrogen peroxide (H_2O_2), Organic hydroperoxides (ROOH), singlet molecular oxygen($^1O_2$), Ozone (O_3), and both hypochlorous acid and hypobromous acid HOCl and HOBr. Free radical ROS include: superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical (·OH), peroxyl radical (ROO·) and alkoxyl radical (RO·) (Sies and Jones, 2020). A great body of data has been accumulated on the chemistry of the different ROS molecules, and we can now better appreciate their biological significance. Specifically, it is

now clear that ROS are fundamentally important entities as physiological players in functional signaling.

Hydrogen peroxide (H_2O_2) is known as one of the main ROS in redox regulation of biological functions (Rhee, 1999; Winterbourn, 2018). H_2O_2 was first shown by the end of the last century, to occur physiologically at a low steady-state level in regularly respiring eukaryotic cells at (Sies and Chance, 1970). Like Ca_2^+, the intracellular levels of H_2O_2 are maintained in a low nanomolar range (approximately 1–100nM) under a rigorous control: the generation of H_2O_2 is induced by metabolic cues or by different stress starters, such as growth factors, chemokines or physical stressors, while its elimination is achieved by an efficient antioxidant system.

Homeostatic physiological flux of H_2O_2 towards specific proteic targets leads to their reversible oxidation, thus, an impairment of their activity, localization and interactions, which participates in the orchestration of several cellular and tissue processes, such as cell proliferation, differentiation, migration and angiogenesis (Jones and Sies, 2015; Berndt and Jones, 2017; Zhang *et al.*, 2019). During oxidative eustress state a low-level of H_2O_2 is maintained associated with a physiological redox signaling (Sies, 2017) (Figure 3)

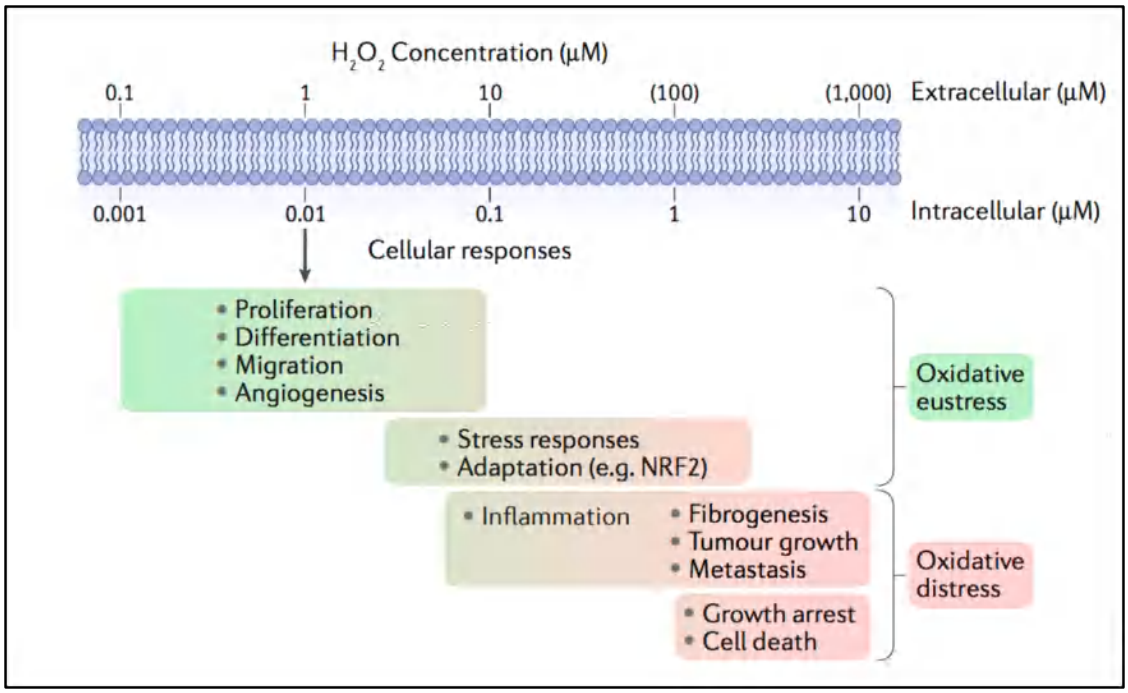

Figure 3. Estimated ranges of H_2O_2 concentration with respect to cellular responses: oxidative eustress and oxidative distress (Sies, 2017).

The physiological intracellular range spans up to approximately 100 nM. Stress responses and adaptation occur at higher concentrations. Even higher exposure induces inflammatory response, growth arrest and cell death by several mechanisms (Sies, 2017). At the contrary of physiological levels of H_2O_2 that are essential for redox cell signaling, supra physiological concentration of H_2O_2 (excessively estimated to overcome 100 nM) induces unspecific oxidation of proteins and impaired response patterns as well as reversible and irreversible damage of biomolecules, provoking growth arrest and cell death, in relation with pathological conditions, previously described as 'oxidative distress' (Figure 1). This latter is responsible for the damage to all classes of macromolecules, thus, impairing their bioactivity (Sies, 1986), mainly, lipid peroxidation, protein oxidation, oxidative DNA methylation and chromatin structure changes and RNA damage.

Therefore, oxidants possess inherent duality of purposeful beneficial functions, in part , and deleterious products of oxidants tend to accumulate over time, from another part , Thus, this ''pleiotropic'' character of oxidants impacts numerous fundamental processes simultaneously, with widespread consequences in health and disease (Santolini *et al.*, 2019).

I. 1. 6. Endogenous and exogenous ROS sources

Generally talking, the major endogenous enzymatic sources of $O_2^{\cdot-}$ and H_2O_2 in human cells are transmembrane NADPH oxidases (NOXs) and the mitochondrial electron transport chain (ETC). A total of 50 H_2O_2- and/or $O_2^{\cdot-}$generating enzymes have been characterized (Go *et al*, 2015), along with enzymes generating other ROS such as lipid hydroperoxides or nitric oxide (NO) and hypochlorous acid. (Mishina *et al.*, 2013; Spencer and Engelhardt, 2014). In the mitochondrial compartiment, ETC, complex I and in part complex II generate $O_2^{\cdot-}$/H_2O_2 towards the mitochondrial matrix, whereas release from complex III is towards the cristae lumen and the intermembrane space (Bleier *et al.*, 2015; Brand, 2016).

On the other hand, H_2O_2 is also generated by various other oxidases including the endoplasmic reticulum (ER) and peroxisomes, as well as by several superoxide dismutases (SOD1–SOD3). Additionally, an important area of ROS research focus on lipid-derived ROS, whereby polyunsaturated fatty acids are oxidized, releasing lipid hydroperoxides and derived radicals, peroxyl and alkoxyl. Such oxidized lipids have a great impact in immune system signaling modulation (Hitzel *et al.*, 2018; Tyurina *et al.*, 2018). Parallelly, lipoxygenases and prostaglandin synthases (cyclooxygenases) release reactive oxidants as intermediates in the

activation and control of inflammatory responses (Czapski *et al.*, 2016; Niedzwiecki *et al.*, 2019).

A recent estimate of the relative contribution from NOXs and mitochondrial ETC sites in resting myoblasts reported that around 40% of net cellular H_2O_2 production was generated by NOXs and approximately 45% was from the ETC, while the rest, deriving from other enzymatic origin (Wong *et al*, 2019). Apart from intracellular sources, oxidants are also generated as a result of exposure to the so called the 'exposome', a cumulative environmental, including molecular factors like nutrients, drugs, toxicants and pollutants as well as physical stressors (UV, X-ray and other ionizing radiation) and psychological stressors (lifestyle) (Sies, 2017).

I. 1. 7. ROS and mitochondria

Mitochondria, serve as the prime source of ROS in normal physiological conditions as well as a target for their damaging effects if pathophysiological conditions occur. Actually, the respiratory chain generates the majority of cellular ROS, which can then interact directly with mitochondrial proteins, lipids and DNA, obscuring their functions and the operation of the organelles. In addition to their role in their host cells' biosynthesis, metabolism, and cell death or viability functions, recent growing evidence has reported other roles of mitochondria in regulating Oxidative stress as well as innate immune responses. (Chen *et al*., 2018).

This is believed to be tightly related to their role as the major endogenous resource of ROS, which are essentially generated at Complex I and III of the electron transport chain. On the other hand, excessive ROS production has been implicated in mtDNA mutations, ageing, and cell death. Nearly, 1–2% of the molecular oxygen consumed during normal physiological respiration is converted into superoxide radicals. The one-electron reduction of molecular oxygen produces a relatively stable intermediate, the superoxide anion ($O^{\cdot -}_2$), which serves as the precursor of most ROS, and can later generate the highly reactive hydroxyl radical (OH) through a series of H_2O_2 and O^{-}_2 subsequent interactions (Ott *et al*., 2007), (Figure 4). To prevent eukaryotic cells oxidative impairment, mitochondria interplay several antioxidant defense systems and it has been fully demonstrated that, regulation of these systems directly affects the susceptibility of cells to undergo apoptosis (Ott *et al*., 2007).

I. 1. 8. Redox signaling

It is a cell response to an oxidant or reductant, or a change in the redox status of a cellular component, which has a variety of downstream effects on cell state, either directly or through

a necessary redox relay from a source to a target (Sies and Jones, 2020). The major mechanism by which some oxidants attains specificity to mediate biological signaling effects is through oxidation of sulfur (thiolate groups) in target proteins. Redox signaling can also occur through reversible methionine oxidation (Kaya, *et al.,* 2015), through seleno proteins (Brigelius-Flohé, 2017), through oxidation of protein metal centers (Santos *et al.*, 2016) and through oxidized lipids (Poli *et al.*, 2004).

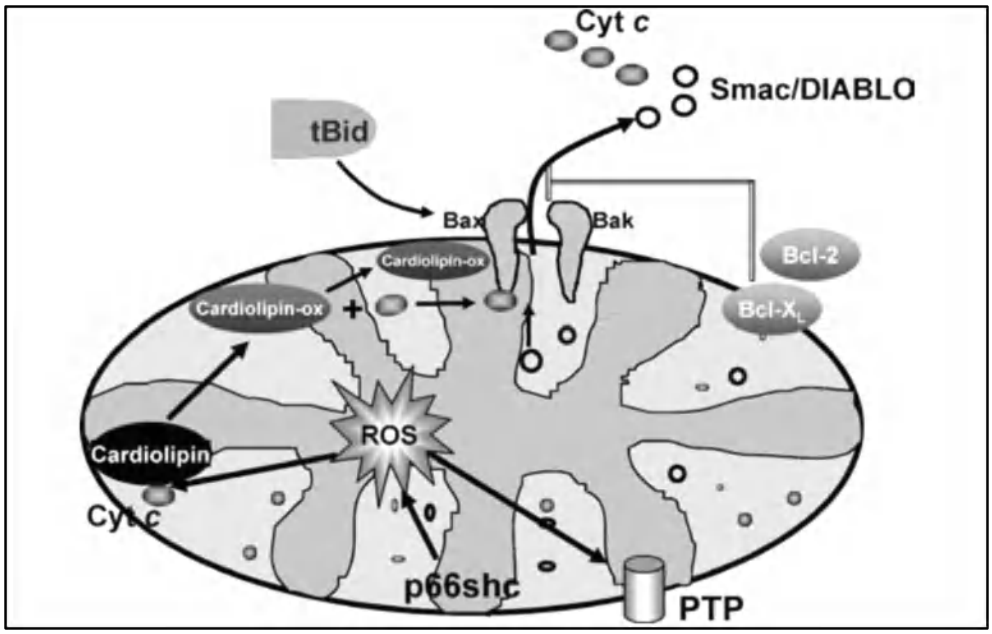

Figure 4. Role of (ROS) in mitochondrial control of cell death (Ott *et al*., 2007).

It also depends on particular interactions of signaling proteins towards hydrogen peroxide (H_2O_2) or other electrophiles that behave as second messengers; however, the regulation is necessary for redox signaling to be physiological rather than pathological (Forman and Zhang, 2021). The redox homeostasis is needed for maintaining normal physiology functions (Ursini *et al,* 2016). Consequently, diseases that involve oxidative stress can be due to the impairment of this homeostasis, However, redox signaling may also occur under pathological conditions, since the oxidative stress is able to activate the same pathways as redox signaling under physiological conditions, with the difference in this context is that the signaling regulation becomes chaotic and accompanied with undifferentiated damage (Forman and Zhang, 2021).

I. 1. 9. Oxidative stress in diseases

Oxidative stress has the ability to damage different molecules and cellular structures, altering the correct function of organs and systems. Increasing evidence points to the involvement of oxidative stress in the physiopathology of various chronic diseases that require prolonged

periods of pharmacological treatment. Long-term treatments may contribute to changes in systemic oxidative stress (García-Sánchez *et al.,* 2020).

Oxidative stress contributes to diseases through two major mechanisms. First, by producing reactive species particularly •OH, $ONOO^-$ and HOCl that directly interact with macromolecules and oxidize them, including membrane lipids, structural proteins, enzymes and nucleic acids, which leads to impaired cell function and death. Second, oxidative stress can impair the redox signaling pathways. Oxidants, specifically H_2O_2 released by cells during physiological interactions, is able to act as second messengers. Besides, non-physiological production of H_2O_2 can also interfere with redox signaling negatively (Sies *et al.,* 2017).

Based on the implication of oxidative stress to the aetiology of these pathologies, they have been divided into two categories: first, Oxidative stress as the primary cause of pathology (including toxicities caused by radiation and paraquat, and in atherosclerosis); second, Oxidative stress as the secondary contributor to disease development (such as in COPD, hypertension and Alzheimer disease). However, this categorization is tentative as the role of Oxidative stress in many diseases is still incompletely understood, (Forman and Zhang, 2021).

I. 1. 10. Antioxidant defense system

The human antioxidant system is constituted of endogenous (enzymatic and non-enzymatic) antioxidants like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione (GSH) and their substrates, and exogenous antioxidants such as vitamin C, vitamin E, carotenoids and polyphenols, with the diet being the main source (Rojo *et al.*, 2004). SODs and enzymes responsible for H_2O_2 removal and lipid hydroperoxidation presents the first line of defense against redox status impairment (Forman and Zhang, 2021). Extracellular SOD (EC-SOD, SOD3) is generally linked to the outer extracellular membrane fluids of some cells. (Ursini *et al*., 2016).

The second line of antioxidant defense system is constituted of thioredoxin (TRX) synthesis, GCL and glutathione synthetase responsible for the synthesis of GSH, glutathione reductase and thioredoxin reductase which use NADPH to reduce GSSG and TrxS2. (Ursini et al., 2016). While the third line of antioxidant defense is based on the repair or the removal of oxidized macromolecules, the enzymatic systems for removal of oxidized damaged molecules are generated by oxidants (Forman and Zhang, 2021).

Endogenous and exogenous antioxidants interact synergistically to maintain or re-establish redox homeostasis, for e.g during the regeneration of vitamin E by glutathione (GSH) or

vitamin C to prevent lipid peroxidation processes (Valko *et al.,* 2007) which can affect membrane fluidity and damage membrane proteins by e.g., inactivating receptors, enzymes and ion channels, even disrupting membrane integrity leading eventually cell death (Ratnam *et al.*, 2006; Bouayed *et al.,* 2009), (Table 1).

I. 2. Inflammation and inflammatory disorders

Inflammation has a crucial role in the host's defense against infectious agents and injury, but it also participates in the pathophysiology of a variety of chronic disorders. Aspects of acute and chronic inflammation that underpin disorders of many organs are orchestrated by interactions between cells in the innate immune system, adaptive immune system, and inflammatory mediators (Libby, 2007).

I. 2. 1. Inflammation

Inflammation is the immune system's reaction to harmful stimuli such pathogens, damaged cells, poisonous substances, or irradiation (Medzhitov, 2010), and it works by eliminating injurious stimuli and commencing the healing process (Ferrero-Miliani *et al.*, 2007). Within tissues, redness, swelling, heat, discomfort, and loss of tissue function are all symptoms of inflammation, which are caused by local immunological, vascular, and inflammatory cell responses to infection or injury (Takeuchi and Akira, 2010). Vascular permeability alterations, leukocyte recruitment and accumulation and inflammatory mediator release are all important microcirculatory events that occur during the inflammatory phase (Chertov *et al.*, 2000; Ferrero-Miliani *et al.*, 2007).

Inflammation can be induced by a variety of pathogenic factors by infected or non-infectious causes that are able to cause tissue damage, such as infection, tissue injury, or myocardial infarction. In reaction to tissue injury the body releases a chemical signaling cascade, which induces responses targeted at mending the injured tissues. These signals cause leukocytes to migrate from the overall circulation to damaged areas. The cytokines produced by these activated leukocytes cause inflammatory reactions (Jabbour *et al.*, 2009).

I. 2. 1. 1. Innate and adaptive immunity

The 21st century has extensive knowledge of the cells and mediators that cause the typical signs of inflammation noticed so clearly by ancients. The host defense systems are divided into two pathways that are intricately linked (Figure 5). The innate immune system responds rapidly to a stimuli. It recognizes a wide spectrum of pathogen-associated molecular

patterns (PAMPs), which are typically seen on pathogens but are not found in mammals, and thus lacks the fine structural specificity of recognition in the adaptive immune response (Medzhitov and Janeway, 2000; Janeway and Medzhitov, 2002).

Table 1. Human antioxidant defense systems include endogenous (enzymatic and non-enzymatic) and exogenous antioxidants, with the diet being the main exogenous source (Bouayed, 2010; Ratnam *et al*., 2006).

Antioxidant defense system	
Endogenous antioxidants	**Exogenous antioxidants**
Enzymatic antioxidants - Superoxide dismutase (SOD): enzyme detoxifying superoxide radical (O2•−) - Catalase (CAT) and glutathione peroxidase (GPx): enzymes involved in the detoxification of peroxides (CAT against H2O2, and GPx against both H2O2 and ROOH) - Glutathione reductase: enzyme involved in the regeneration of glutathione - Thioredoxin reductase: enzyme involved in the protection against protein oxidation - Glucose-6-phosphate dehydrogenase: enzyme involved in the regeneration of NADPH **Non enzymatic antioxidants** (principal intracellular reducing agents) Glutathione (GSH), uric acid, lipoic acid, NADPH, coenzyme Q, albumin, bilirubin	**Principal dietary antioxidants from fruits, vegetables and grains** **- Vitamins**: vitamin C, vitamin E **- Trace elements :** zinc, selenium - **Carotenoids**: ß-carotene, lycopene, lutein, zeaxanthin **- Phenolic acids:** chlorogenic acids, gallic acid, cafeic acid, etc. **- Flavonols:** quercetin*, kaempferol*, myricetin* **- Flavanols:** proanthocyanidins and catechins **- Anthocyanidins:** cyanidin* and pelagonidin* **- Isoflavones:** genistein*, daidzein* and glycitein* **- Flavanones:** naringenin*, eriodictyol* and hesperetin* **- Flavones:** luteolin* and apigenin*

* and their glucosides

This latter provides a more finely focused response mechanism that requires specific molecular structures detection and relies on blast cell somatic rearrangement mechanisms to generate huge numbers of antigen receptors, including as T-cell receptors and immunoglobulins (Hansson *et*

al., 2002). When T-cells recognize a foreign antigen, they activate reactions that specifically target that antigen, such as cytotoxic T-cells attacking the antigen-bearing cell, B-cells stimulating antibody production, and development of a local inflammatory response. T-cells can subtype into at least into two different types of T helper (Th) cells. Th1 cells produce a variety of cytokines, with interferon-gamma (IFN-γ) being one of the most important. It stimulates macrophages to produce a wide range of mediators, including autacoids, reactive oxygen species, lipid species, and pro-inflammatory cytokines, allowing crosstalk between the innate and adaptive limbs of the immune and inflammatory responses (Frostegård *et al.*, 1999; Robertson and Hansson, 2006).

Th2 cells can activate humoral immunity by releasing a variety of cytokines that stimulate B-cell maturation into antibody-producing plasma cells and promote B-cell class switching to boost IgE antibody production. Mast cells, another effector of allergic responses and contributor to chronic inflammation in a range of tissues and disease states. Th2 cells can decrease the inflammatory response by elaborating cytokines with anti-inflammatory properties such as interleukin-10 (IL-10) (Robertson and Hansson, 2006).

I. 2. 1. 2. Activation of inflammatory pathways

Inflammatory pathways, which involve common inflammatory mediators and regulatory mechanisms, impact the pathogenesis of a variety of chronic diseases. Intracellular signaling pathways are activated by inflammatory stimuli, which then activate the synthesis of inflammatory mediators. Primary inflammatory stimuli, such as microbial products and cytokines including interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor (TNF-α), mediate inflammation by interacting with TLRs, IL-1 receptors (IL-1R), IL-6 receptors (IL-6R), and TNF receptors (TNFR) (Kaminska, 2005). Important intracellular signaling pathways, including the mitogen-activated protein kinase (MAPK), nuclear factor kappa-B (NF-κB), Janus kinase (JAK)-sign transducer and activator of transcription (STAT) pathways (Henríquez-Olguín*et al.*, 2015; Hendrayani *et al.*, 2016).

I. 2. 2. Cell types in inflammatory responses

A complex network of different cell types is involved in the inflammatory response. Local responses to tissue injury and infection are mediated by activated macrophages, monocytes, and other cells. Damaged epithelium and endothelial cells release substances that initiate the

inflammatory cascade, as well as chemokines and growth factors that are cruit neutrophils and monocytes, at the injury location.

Neutrophils are the first cells to be drawn to an injury site, followed by monocytes, lymphocytes (natural killer cells [NK cells], T cells, and B cells), and mast cells (Stramer *et al.,* 2007; van Linthout *et al.,* 2014; Robb *et al.*, 2016). Many diseases, including asthma, cancer, chronic inflammatory disorders, atherosclerosis, diabetes, and autoimmune and degenerative diseases, are associated with inflammation-mediated immune cell changes. Neutrophils that essentially target microorganism are also able to impair host cells and tissues (Nathan, 2006).

I.2. 2. 1. Inflammatory markers

Markers are employed in clinical applications to distinguish between normal and pathological biological processes and to measure therapeutic interventions. Inflammatory markers have been considered as risk factors to predict several inflammatory disorders (Pai *et al.*, 2004; Bautista *et al.*, 2005; Carrero *et al.*, 2008) by correlating with the induction and the consequences of various inflammatory diseases, including cardiovascular disease, endothelial dysfunction, and infection (Carrero *et al.*, 2008b; Machowska *et al.*, 2016). Inflammatory cells, such as macrophages and adipocytes, are activated, and inflammatory cytokines, including as IL-1, IL-6, and TNF-α, as well as inflammatory proteins and enzymes, are produced in response to an inflammatory stimulus. (Shlipak *et al.*, 2003; Goldstein *et al.*, 2009; Gupta *et al.*, 2012).

I. 2. 2. 2. Inflammatory cytokines

The producers of cytokines are mainly the immune cells such as monocytes, macrophages, and lymphocytes, Inflammation is facilitated and inhibited by pro- and anti-inflammatory cytokines, respectively. ILs, colony stimulating factors (CSF), IFNs, TNFs, TGFs, and chemokines are inflammatory cytokines that are produced by cells to recruit leukocytes to the site of infection or injury (Turner *et al.*, 2014). Cytokines regulate inflammation and modulate the immune response to infection or inflammation through a complex network of interactions. Excessive inflammatory cytokine production, on the other hand, can cause tissue damage, hemodynamic abnormalities, organ failure, and extremely death (Czaja, 2014; Liu *et al.*, 2016).

I. 2. 2. 3. Inflammatory proteins and enzymes

During trauma, stress, or infection, inflammatory proteins, such as C-reactive protein (CRP), haptoglobin, serum amyloid A, fibrinogen, and alpha 1-acid glycoprotein (Eckersall and Bell,

2010), help restore homeostasis and inhibit microbial development independently of antibodies in the blood (Murata, Shimada and Yoshioka, 2004). The impaired activation of enzymes like High-mobility group box 1 (HMGB1), superoxide dismutase (SOD), glutathione peroxidase (GPx), NADPH oxidase (NOX), inducible nitric oxide synthase (iNOS), and cyclooxygenase (COX)-2 play a pivotal role in the development of inflammatory disorders such cardiovascular disease and cancer (Murakami and Ohigashi, 2007; Lopresti *et al.*, 2014).

I. 2. 2. 4. Other inflammatory markers

Oxidative stress is influenced by antioxidant defense mechanisms, which include antioxidant enzymes. Increased oxidative stress can result in the generation of (ROS), malon dialdehyde (MDA), 8-hydroxy-2-deoxyguanosine (8-OHDG), and isoprostanes (Lopresti *et al.*, 2014; Park *et al.*, 2015) , all of which can activate transcription factors such as NF-κB, AP-1, p53, and STAT. As a result, the expression of genes encoding growth factors, inflammatory cytokines, and chemokines can be increased (Reuter *et al.*, 2010).

I. 2. 3. Chronic inflammation

Chronic inflammation is a pathological condition characterized by continued active inflammation response and tissue destruction. Many of the immune cells including macrophages, neutrophils and eosinophils are involved directly or by production of inflammatory cytokine production in pathology of chronic inflammation. (Khansari *et al.,* 2009).Two instances of this syndrome include chronic cellular damage and persistent infection (Joris *et al.*, 1983; Serhan and Savill, 2005). It is worth noting that the stimulus that causes the bulk of inflammatory diseases has yet to be identified. Notably, chronic inflammation is the leading source of clinical conditions in which tissue damage is caused by the inflammatory reactions mentioned above, rather than by external infections or other invaders (Ahmed, 2011).

I. 2. 4. Inflammatory diseases

Inflammatory diseases encompass a wide range of conditions that are either caused by inflammation or involve inflammation as a major process. The existence of an impaired inflammatory response is a defining feature of this category of diseases. Chronic inflammation is so closely linked to the pathogenesis of inflammatory diseases that it can be difficult to distinguish between their effects and causes in most cases (Serhan and Savill, 2005). Inflammatory disorders have a wide range of implications on human health, affecting practically every physiological function of the human body. (Garrett *et al.,* 2010).

I. 2. 5. A generic model of chronic inflammatory disease

Diverse subspecialties have traditionally claimed chronic diseases of various organ systems as their own and investigated the pathogenesis in a reductionist approach. However, from a broader perspective, many of these diseases share more pathways than is commonly recognized or acknowledged. Indeed, a generic model of chronic inflammatory disease has been developed, emphasizing these common pathophysiologic pathways. The innate and adaptive immune systems interact and converge on two prototypic cell types: an epithelial cell and a mesenchymal cell of the damaged organs, according to this design. These signals control a wide range of tissue responses, including leukocyte recruitment for chronic inflammation, extracellular matrix remodeling, cellular proliferation and death, and angiogenesis (Figure 5) (Libby, 2007). While inflammatory chronic diseases can manifest diversely, the disease process is driven by the same fundamental mechanisms, pathways and mediators (Pasceri and Yeh, 1999; Ross, 1999).

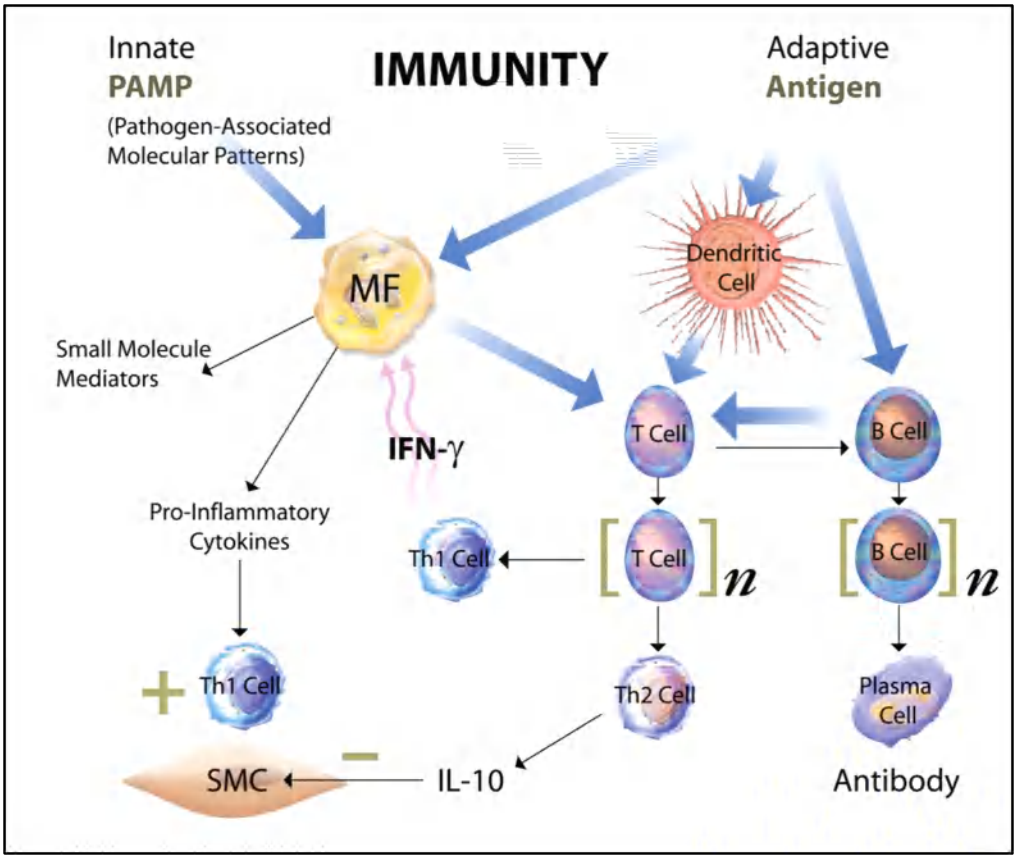

Figure 5. Interplay between adaptive and innate immunity during atherogenesis. (Hansson et *al.,* 2002).

T Helper cells are abundant in chronic inflammatory lesions in various organs. The mononuclear phagocyte, also known as a foam cell, osteoclast, histiocyte, microglia, or alveolar

macrophage, is also common in these conditions. In atherosclerosis, the epithelial cell implicated is the vascular endothelial cell, in inflammatory bowel disease, the enterocyte, and in renal illness, the glomerular or tubular epithelial cell. Similarly, depending on the organ, different types of mesenchymal cells are involved in inflammatory and immunological mechanisms—arterial smooth muscle cells, fibroblasts, myofibroblasts, mesangial cells, synoviocytes, or pericytes (Libby, 2007), (Figure 6).

Inflammation is characterized by the selective and sequential migration of blood cells into tissues, followed by local activation and interaction of these blood-derived cells with resident tissue cells. In some cases, only a few elements of typical inflammatory processes are displayed, whilst in others, major inflammatory mediators predominate but without the concept of classic inflammatory mechanisms (Libby, 2007). A persistent stimulation often induces an injurious response coaxed from a physiological response and necessary host defensive mechanism. In either situation, the resulting responses can damage the organ or tissue's function over time (Akira, Takeda and Kaisho, 2001).

I. 2. 6. Organ specific inflammatory responses

Inflammation has long been recognized as a significant cause to disease. Chronic infection and inflammation are thought to be responsible for 15% of all human cancers (He and Karin, 2011). Many organ systems, including the heart, pancreas, liver, kidney, lung, brain, digestive tract, and reproductive system, show acute and chronic inflammation-mediated tissue injury (Chen *et al.*, 2018).

Both acute and chronic inflammatory diseases of the intestinal tract, can cause a variety of health problems and reduce patient quality of life worldwide (Sanchez and Bercik, 2011; Hunt *et al.*, 2014). An example of chronic diseases in the intestinal tract are Inflammatory bowel disorders (IBDs) which are characterized by an overactive inflammatory response to the microbial flora in the gut lumen (McGuckin *et al.*, 2009), but also as noninfectious intestinal inflammation, but also linked to high non controlled production of a variety of cytokines (Cario and Podolsky, 2000; Strober et al., 2007).

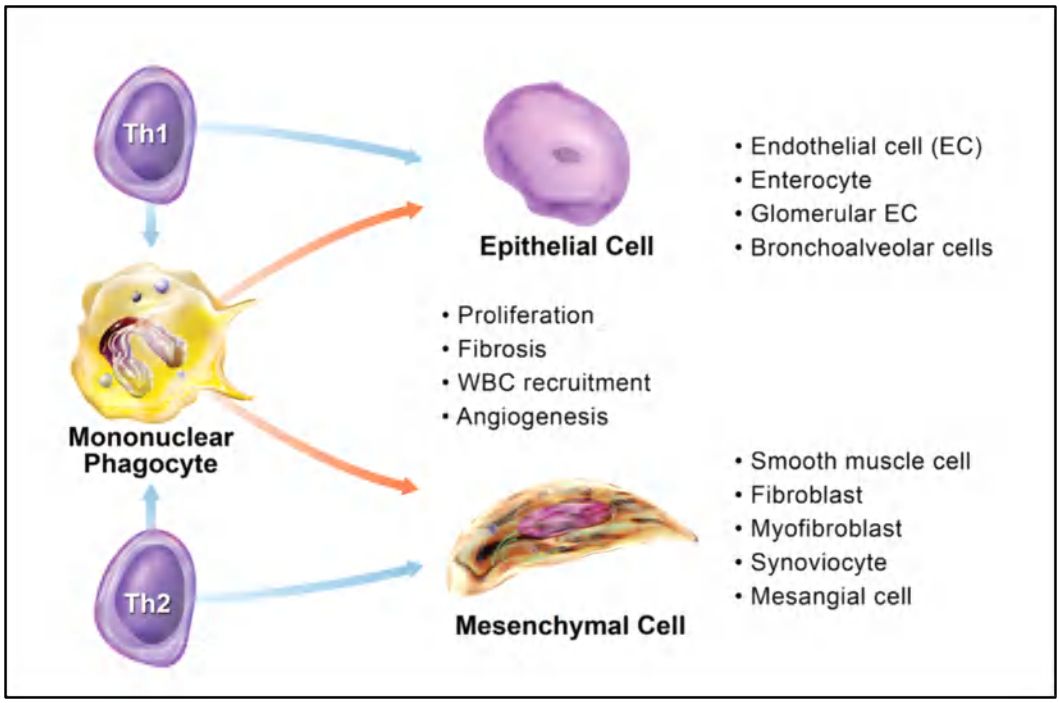

Figure 6. Common inflammatory and immune processes act on different cell types, leading to different disease states. (Libby, 2007).

I. 3. Association between Oxidative Stress and Inflammation

Oxidative stress and inflammation are closely related pathophysiological processes, one of which can be easily induced by another (Biswas, 2016). Indeed, increased free radical production, oxidative stress, and inflammation are all strongly linked. The tissue damage caused by oxidative stress can elicit an inflammatory response, which can be in return, a direct inducer of oxidative stress (Closa and Folch-Puy, 2004; Hulsmans and Holvoet, 2010).

Oxidative stress has been found to activate redox-sensitive transcription factors such as activator protein-1 (AP-1) and nuclear factor-kappa B (NF-κB) in this situation. These mediators can induce inflammation and activate the innate immune system, resulting in the recruitment and accumulation of inflammatory cells such macrophages, leukocytes, and neutrophils to the injury site (McDonald *et al*., 1997; Xanthoulea *et al.*, 2005). These inflammatory cells can then generate metabolites of soluble mediators such arachidonic acid, chemokines, and pro-inflammatory cytokines like interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF-α) (Davies *et al*., 1980; Duque and Descoteaux, 2014) (Figure 7).

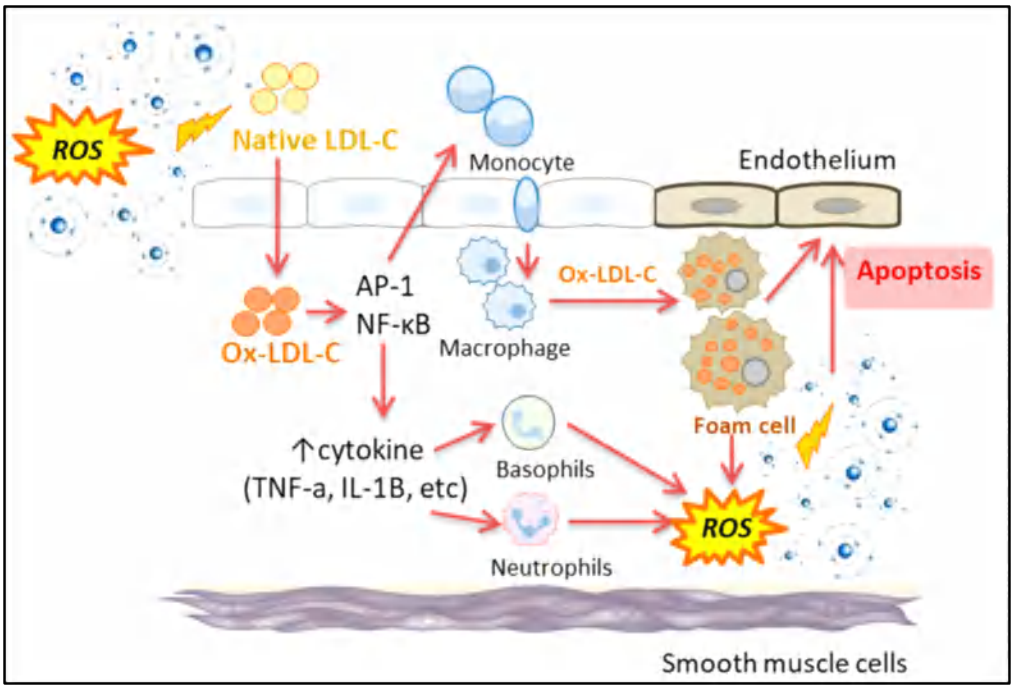

Figure 7. The interplay between oxidative stress and inflammatory processes (i.e., NFκB and cytokine activity) in the development of endothelial cell damage and apoptosis. (Seyedsadjadi and Grant, 2020).

Chapter II

The inflammatory bowel disease (IBD)

Highlights

- IBD is a part of set of inflammatory disorders of the gastrointestinal tract of varied symptoms and chronic character

-Oxidative stress key feature of inflammatory Bowel disease (IBD)

-Inflammatory bowel disease (IBD) is associated with the production of reactive species, thereby affecting gene regulation, DNA damage, ion transport, intermediary metabolism, and mitochondrial function.

II. 1. Presentation of IBD pathology

IBD comprises a group of idiopathic, chronic, relapsing, and remitting diseases of the gastrointestinal tract that are idiopathic, complex, and multifactorial. Complex interactions between genetic, immune, and environmental factors are involved (Rigoli and Caruso, 2014). IBD includes two chronic, progressive inflammatory disorders affecting the gastrointestinal tract (GI): Crohn's disease (CD) and ulcerative colitis (UC). Individuals with genetic susceptibility to both diseases are characterized by inflammation of the gut mucosa caused by a response to the gut microbiome that is inappropriate (Abraham and Cho, 2009).

IBD incidence is steadily increasing, equally among men and women, but varies with ethnicity (Loftus, 2004). The causes remain unclear, but recent emphasis has been placed on the exposome (the diversity and range of all exposures individuals experience during their lifetime, from conception on, highlighting a critical interface between chemistry and biology (Vermeulen *et al*., 2020). Exposome concept describes the biological consequences of exposure to environmental factors, such as physical factors and synthetic chemicals, but also dietary components and psychosocial stressors, which can lead to chronic metabolic inflammation (metaflammation) (Molodecky *et al*., 2012; Ananthakrishnan, 2015; Christ and Latz, 2019).

II. 2. IBD etiology

As shown in Figure 8, the etiology of IBD is complex and not fully understood, and may be related to the environment, heredity, infections, immunity, or intestinal microorganisms (Kim *et al*., 2019). All of these factors interact and contribute to the development and progression of IBD, resulting in a disruption of gut mucosal homeostasis and distinct immunological alterations. In IBD, the intestinal mucosa is typically infiltrated with a large number of inflammatory cells. As a net result of excessive immune activity of effector lymphocytes with increased production of proinflammatory cytokines a chronic uncontrolled immune response is

established, synchronized with a failure of regulatory immune cells and mediators f to maintain tissue homeostasis (Naito, Takagi and Yoshikawa, 2007), (Figure 8).

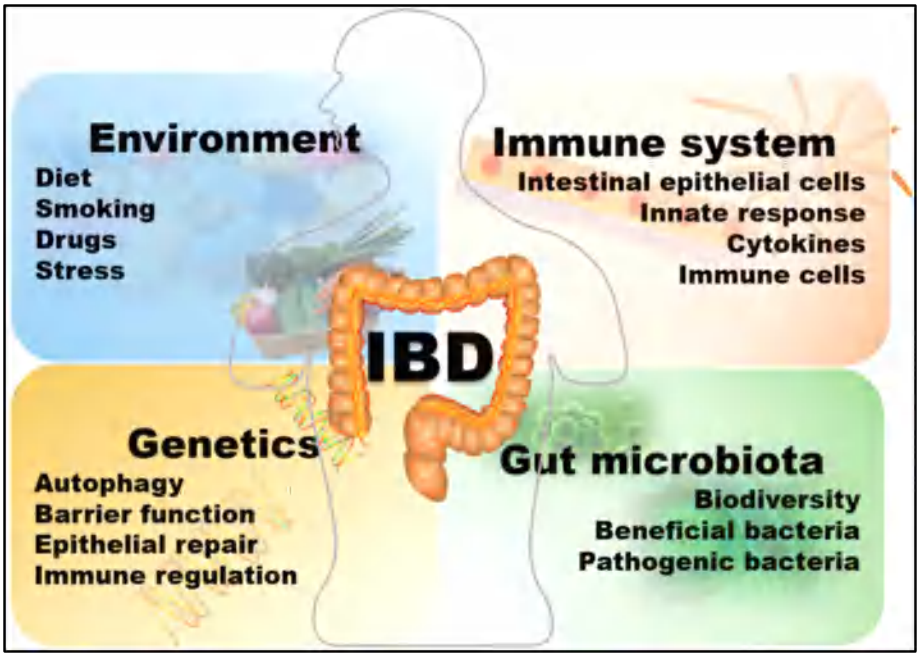

Figure 8. IBD etiology. (Naito *et al*., 2007).

II. 3. The intestinal barrier in IBD

A variety of defensive mechanisms including tightly adherent epithelial cells, antimicrobial peptides released by intestinal epithelial cells, and complex mucosal innate and adaptive immunological arms aimed at removing invasive infections; are employed to reduce infections caused by IBD (Swidsinski *et al*., 2002). The mucosa, submucosa, muscularis externa, and adventitia are the four layers of the gastrointestinal system that exhibit functional anatomical specialization (Sarriá *et al*., 2012).

The mucosa is the inner layer of the gastrointestinal tract and plays a very significant role in the digestion of food. It forms a barrier between the internal organs and the lumen of the gastrointestinal tract. This layer mainly consists of epithelial cells that are connected by tight junctions and play an important role in allowing the transport of nutrients across the epithelium, while preventing the passage of molecules that might be harmful to the host (Hawiger, 2001). These epithelial linings also contain goblet cells and endocrine cells (Moore *et al.*, 1998). External microorganisms that can breach into the epithelial wall have unrestricted access to the circulatory system, which is one of the leading causes of gastrointestinal pathologies. Indeed, murine and human research have shown that IBD disease, particularly, Crohn's disease, is

characterized by an epithelial barrier failure and altered mucus formation, resulting in increased intestinal permeability and toxicity adherence in intestinal cells (Swidsinski *et al*., 2002; Schreiber *et al*., 2013).

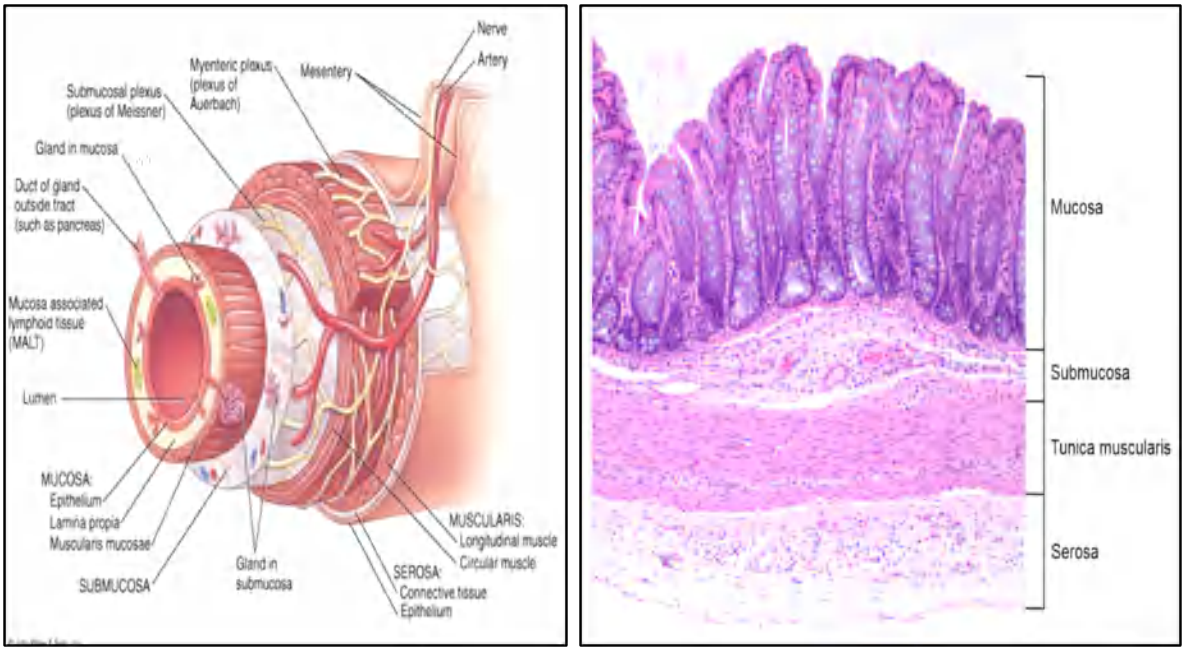

Figure 9. Histology of the Digestive System. source: https://www.docme.su (2022).

Crohn's disease is characterized by a disruption in the integrity of the epithelial barrier, which allows microbial antigens (Microbiota) and other exogenous factors to translocate (Cobrin and Abreu, 2005). Several studies have found an increase in adhering bacteria in Crohn's disease patients (Swidsinski *et al*., 2002). When the mucosal immune system is exposed to external luminal materials, it greatly increases the production of proinflammatory cytokines and promotes T-cell differentiation to T-helper (Th)1-type effector cells, which causes the release of proinflammatory cytokines (Fakhoury *et al*., 2014) (Figure 9).

II. 4. IBD types and symptoms

Although Crohn’s disease and ulcerative colitis share similar characteristics, they differ in terms of the location and nature of the inflammatory changes. The distinction between these two diseases is that in Crohn’s disease inflammation can affect any part of the gastrointestinal tract, while ulcerative colitis is characterized by inflammation localized to the large intestine. (Cassinotti *et al*., 2008). At the microscopic level, Crohn’s disease affects the entire bowel wall, while ulcerative colitis is restricted to the epithelial lining of the gut. Since the two diseases

share similar symptoms, the diagnosis of one form of IBD over the other is often very difficult (Fakhoury *et al.*, 2014).

The symptoms of IBD differ based on the type. Table 2 compares the essential characteristics of Crohn's disease with ulcerative colitis. Patients with ulcerative colitis frequently suffer of pain in the lower left part of their abdomen and diarrhea. As a result, they may lose weight and have blood on a rectal examination. In contrast, Crohn's disease patients have pain in the lower right abdomen, while rectum hemorrhage is less common in ulcerative colitis. The most common complication of Crohn's disease is intestine blockage caused by swelling, which results in intestine wall thickening (Fakhoury *et al*., 2014).

Furthermore, people affected by IBD frequently encounter nutritional deficits problems as a result of poor absorption. Colorectal and small bowel cancer research have revealed that subjects with Crohn's disease also have comorbidities such as colorectal cancer, cardiovascular illness, and respiratory disease (Jess *et al*., 2005; Canavan *et al*., 2006). Similarly, various investigations on the ulcerative colitis mortality rate have been conducted (Höie *et al*., 2007; Hutfless *et al*., 2007) (Table 2).

II. 5. Immunity interactions and inflammatory pathways in IBD

Recent studies on IBD is triggering T helper cells (Th) as former studies have confirmed that CD is a Th-1 mediated inflammation condition, accompanied by an overproduction of IFN-γ and TNFα after induction by IL-12, and UC is associated with the Th-2 cells, in which there is a higher production of IL-4, IL-5, and IL-13 while the level of IFN-γ was proven to be normal. (Alfen *et al*., 2018; Barnig *et al*., 2019; Bauché *et al*., 2020; Tindemans *et al*., 2020).

Chronic mucosal inflammation caused by Th1 or Th2 cells causes intestinal wall integrity to deteriorate. It inhibits epithelial barrier regeneration, allowing intestinal contents such as bacteria and food antigens to easily permeate the intestine and trigger the lamina propria-based immune response, which then leads to IBD. In addition to the typical Th1 and Th2 cell reactions, Th17 cells have been linked to inflammatory responses in IBD (Kim et *al*., 2011; Ctan et *al*., 2015; Salem et *al*., 2019).

Table 2. Clinical aspects of IBD (Fakhoury *et al*., 2014).

key features	Crohn's disease	Ulcerative colitis
Location:		
-Upper parts of the GIT	Rarely	Never
-Distal ileum	Very common	Never
-Colon	Common	Always
-Rectum	Rarely	Never
Signs and symptoms	Pain in the lower right abdomen, swelling. Thickening in the bowel wall	Pain in the lower left abdomen, diarrhea, weight loss, rectal bleeding

Dendritic cells (DCs) may trigger the first T cells to develop into Th1 cells by secreting IL-12, as well as an important amount of interferon IFN-γ to mediate the intestinal mucosal inflammation. IBD patients typically have much higher serum IL-23 expression than healthy participants, which is also strongly correlated with UC severity (Neurath, 2019).

In addition, toll-like receptors are strongly associated with the pathogenic mechanism of IBD. Consequently, various inflammatory cytokines are produced, leading to the activation of the NF-κB immune signal pathway for excessive immune stimulation. NOD2, a susceptibility gene, negatively regulates the TLRs and inhibits the over activation of the NF-κB immune signal pathway. Besides the NF-κB immune signal pathway, the JAK/STAT and the TGF-β1/SMADs signal pathways are involved in the pathogenic mechanism of IBD (Cordes *et al*., 2020; de Ceuninck *et al*., 2020) (Figure. 10).

Furthermore, toll-like receptors are strongly linked to the pathogenic process of IBD. As a result, numerous inflammatory cytokines are produced, which activates the NF-κB immune signal pathway, resulting in excessive immunological stimulation. NOD2, a susceptibility gene, suppresses the overactivation of the NF-κB immunological signal pathway by negatively regulating TLRs. The JAK/STAT and TGF-1/SMADs signal pathways, in addition to the NF-κB immune signal pathway, are involved in the pathogenic process of IBD (Cordes *et al*., 2020; de Ceuninck *et al*., 2020) (Figure. 10).

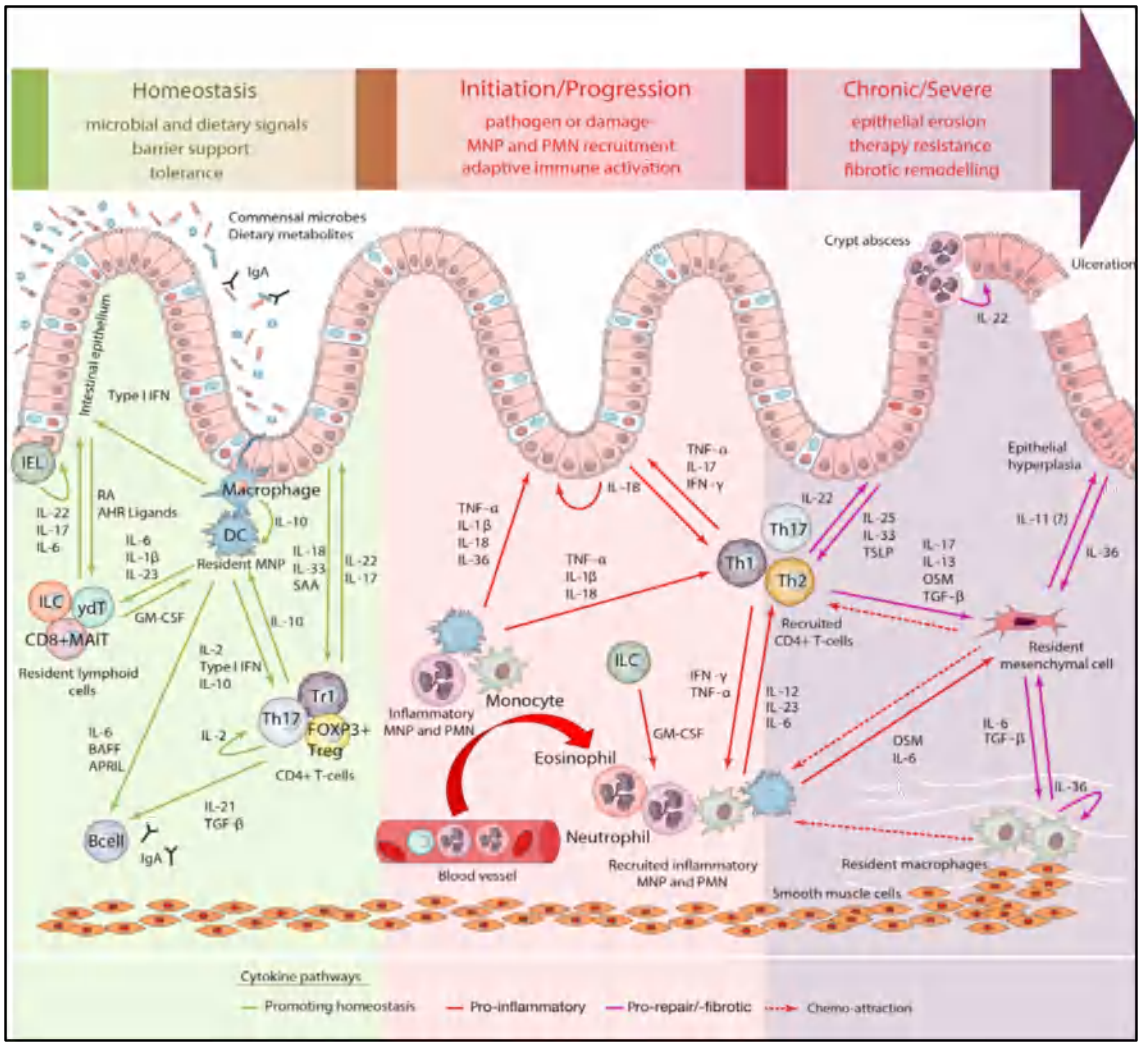

Figure 10. Inflammatory pathways activation and interactions networks during IBD Progression. (Friedrich *et al*., 2019).

II. 6. Oxidative stress and IBD

Oxidative stress occurs in IBD not only in the inflamed intestinal mucosa, but also in the deeper layers of the intestinal wall and mirrored in the systemic circulation (Piechota-Polanczyk and Fichna, 2014; Moura *et al*., 2015; Balmus *et al*., 2016; Guan and Lan, 2018; Bourgonje *et al*., 2019). ROS overproduction in IBD can be evaluated by detecting various systemic redox status components (Banne, Amiri and Pero, 2003). In addition to ROS, additional species, particularly reactive nitrogen species (RNS) and reactive sulfur species (RSS), interact in similar chemical processes (Bourgonje *et al*., 2020).

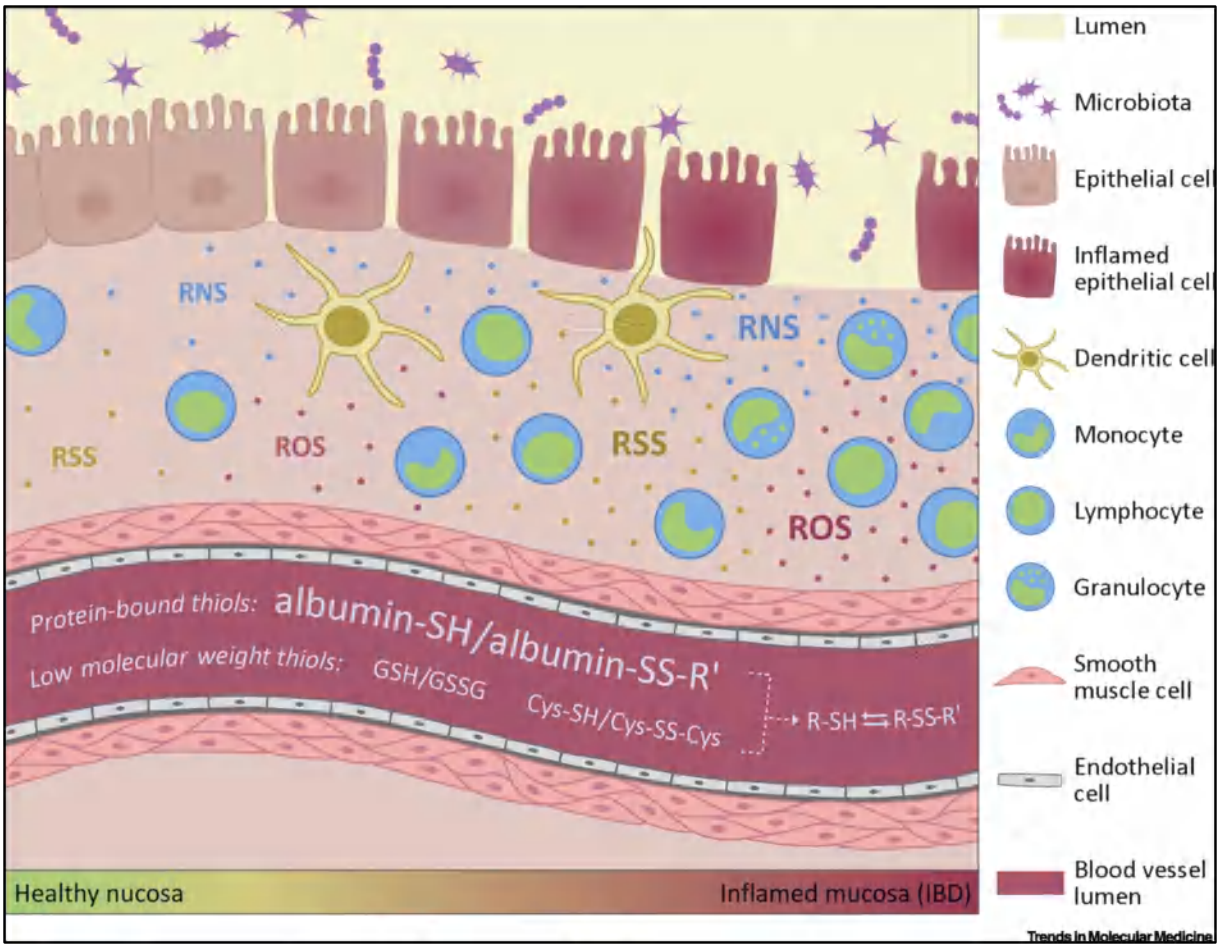

Figure 11. Interface between the intestinal immune system and the systemic redox status in the healthy and inflamed intestinal mucosa. (Bourgonje *et al*., 2020).

Reduced total free thiol levels are indicative of systemic oxidative stress in inflammatory bowel disease condition (IBD). Continuous exposure of the intestinal mucosa to reactive species, on the other hand, causes a pathogenic imbalance in the thiol/disulfide ratio (R-SH vs. R-SS-R′) as antioxidant defense mechanisms become overwhelmed. This is accompanied by collateral tissue damage (e.g., epithelial cell destruction and/or increased intestinal permeability) and gastrointestinal physiological disruptions (Bourgonje *et al*., 2020). (Figure 11).

II .7. Treatments in IBD disorders

Although there is no cure for IBD, a variety of medications (e.g., corticosteroids, immunomodulators, antibiotics, aminosalicylates, and biotherapy) are used to manage disease symptoms. Medical treatment side effects are highly diverse, and there is the possibility of considerable morbidity over long periods with disease medication (Novak and Mollen, 2015). Furthermore, immunosuppressive medications and anti-inflammatory chemicals are heavily used in the therapy of IBD (Ljung *et al*., 2004).

Despite the availability of immunosuppressive medications such as azathioprine, mercaptopurine, and methotrexate, attaining IBD remission in patients remains a therapeutic challenge (Bauditz *et al*., 2002). This class of drugs is particularly effective at reducing the severity of inflammation, but it has a wide range of side effects. Such pharmaceutical substances, for example, can cause fluid retention, sleeplessness, weight gain, drowsiness, hypertension, constipation, and vomiting. As a result, various studies in ongoing research for additional therapies have focused on the creation of novel formulations to treat IBD with minimum side effects, increased patient compliance, and hence better clinical outcomes (Fakhoury *et al*., 2014).

II .8. Therapeutic Targets and Drug-Use Strategy for IBD

Various therapeutic targets have been approved for IBD. Several attempts have been made to create the next generation of small molecules to be used as specific targets for IBD therapy. For example, a balanced intestinal immune dynamic relies on the regulation of TGF-β by specific intestinal microorganisms, and abnormal transforming growth factor-β signal has been reported in some instances of IBD, which suggests that TGF-β could be used as a therapeutic target in IBD therapy (Ihara *et al*., 2017).

In IBD condition, several treatment targets have been approved. Several efforts have been made to develop the next generation of small compounds that can be employed as particular targets for IBD therapy. A balanced intestinal immune dynamic, for example, is dependent on the regulation of TGF-β by specific intestinal microbes, and aberrant TGF-β signaling has been described in some cases of IBD, suggesting that TGF-β could be utilized as a therapeutic target in IBD therapy (Ihara *et al*., 2017).

However, numerous innovative techniques that successfully blocked TNFα in IBD were ineffective in treating CD (Friedrich *et al*., 2019), implying that additional processes other than neutralizing soluble TNFα may be involved (Levin *et al*., 2016). It is critical to uncover novel potential therapeutic targets and re-examine existing IBD targets in order to create an effective therapy map for IBD.

Chapter III

Phytotherapy in IBD, bioactive molecules and medicinal plants of interest

Highlights

In searching for novel pharmacologically active substances that could be used for treating IBD, natural ingredients from various sources, such as plants, animals, and microorganisms, are receiving great attention nowadays. Studies have demonstrated that natural products, including natural medicines, their extracts, and their metabolites, can effectively treat IBD.

III. 1. Phytotherapy in IBD

Side effects and acceptable safety of conventional therapies have caused an increased interest in herbal therapies for the management of IBD and other diseases. Herbal remedies have been shown to decrease histological damage, increase the disease activity index as well as the level of clinical response, improve the remission and decrease the relapse rates, improve stool consistency, and ameliorate rectal bleeding in IBD patients (Ganji-Arjenaki and Rafieian-Kopaei, 2019). Herbal medicines have shown to improve these parameters by several mechanisms, including antioxidant activity, inhibition of IKK or NF-κB, suppression of LTB4, inhibition of platelets, and iNOS activities (Ganji-Arjenaki and Rafieian-Kopaei, 2019).

III. 2. Mechanism action of herbal medicines

Herbal therapies exert their therapeutic benefit by different mechanisms including immune regulation, antioxidant activity, inhibition of leukotriene B4 and nuclear factor-kappa B, and antiplatelet activity (Triantafyllidi *et al.*, 2015). Factors the regulating T-cells and pro-inflammatory cytokines have the potential to decrease inflammation scores and then improve the patient's IBD (Ganji-Arjenaki and Rafieian-Kopaei, 2019). Therefore, in searching for novel pharmacologically active substances that could be used for treating IBD, natural ingredients from various sources, such as plants, animals, and microorganisms, are receiving great attention nowadays. Studies have demonstrated that natural products, including natural medicines, their extracts, and their metabolites, can effectively treat IBD (Ganji-Arjenaki and Rafieian-Kopaei, 2019).

III. 3. Anti-inflammatory, antioxidant secondary metabolites of medicinal plants

Substances of plant origin, belonging to the most diverse chemical classes, have already demonstrated proven anti-inflammatory activity (Fialho *et al.*, 2018). Among them, alkaloids, terpenes (Singh and Sharma, 2015; Bi *et al.*, 2016; Hussein and El-Anssary, 2019; Mondal *et al.*, 2019), and phenolic compounds such as tannins, lignans, coumarins, saponins, and especially the flavonoids stand out (Mitra *et al*., 2010; Singh and Sharma, 2015; Rex *et al*., 2018; Hussein and El-Anssary., 2019; Gorlenko *et al*., 2020) (Figure 12).

III. 3. 1. Polyphenols

Polyphenols are polyhydroxyphenols, with the structural class of compounds that are mostly composed of compounds that exist naturally. They are classified into phenolics, stilbenes, flavonoids, tannins and lignins. Polyphenols are mostly of plant origin and they are among the most widely studied class of phytochemicals and largely found in the human diet through various fruits, beverages, food and foodstuff. Polyphenols have high nutraceutical values and are antioxidant, anti-inflammatory, and antimicrobial compounds, with skin protection, blood pressure reduction, cardioprotective and anticancer properties (Tijjani *et al.*, 2020).

III. 3. 2. Catechins

Catechins are a kind of polyphenols whose chemical structure has a flavonoid parent nucleus structure, occurring in certain foods and medicinal plants. Catechins mainly include catechin, epicatechin (EC), epicatechin gallate (ECG), (2)-epigallocatechin-3-gallate (EGC) and its stereoisomer gallocatechin (GC), EGCG, and stereoisomer gallocatechin gallate (GCG) (Duan *et al.*, 2021). Recently, the role of catechins in the prevention and treatment of colitis has been gradually explored. As discovered by Xue et al. EGCG relieved the colitis induced by DSS in the experimental rats (Xue *et al.*, 2016).

III. 3. 3. Flavonoids

Represent a group of vegetal pigments with extensive distribution in nature, being available in fruits, seeds, flowers, and barks (Torres *et al.*, 2022). Their basic structure is the flavan nucleus, which consists of 15 carbon atoms arranged in 3 rings (C6-C3-C6) which are labeled as A, B, and C. Figure 11presents the structure of the flavonoids and their numbering to distinguish the position of the carbons around the molecule (Kite and Dewick, 1998).The structural variation in ring C subdivides flavonoids into six main subclasses: flavonols (e.g., quercetin, kaempferol, myricetin), flavones (e.g., luteolin, apigenin), flavanols (e.g., catechin), flavanones (e.g., hesperetin), anthocyanidins (e.g., cyanidin, pelargonidin), and isoflavones (e.g., genistein, daidzein) (Ara *et al.*, 2018).

Flavonoids have an anti-inflammatory capacity since they inhibit the production of inflammatory mediators by modulating the arachidonic acid pathway, inhibiting several enzymes such as ATPase, prostaglandin, cyclooxygenase, lipoxygenase, NADH oxidase, protein kinase, hydrolases, peroxidases, metallopeptidases, tyrosinases, and phospholipases.

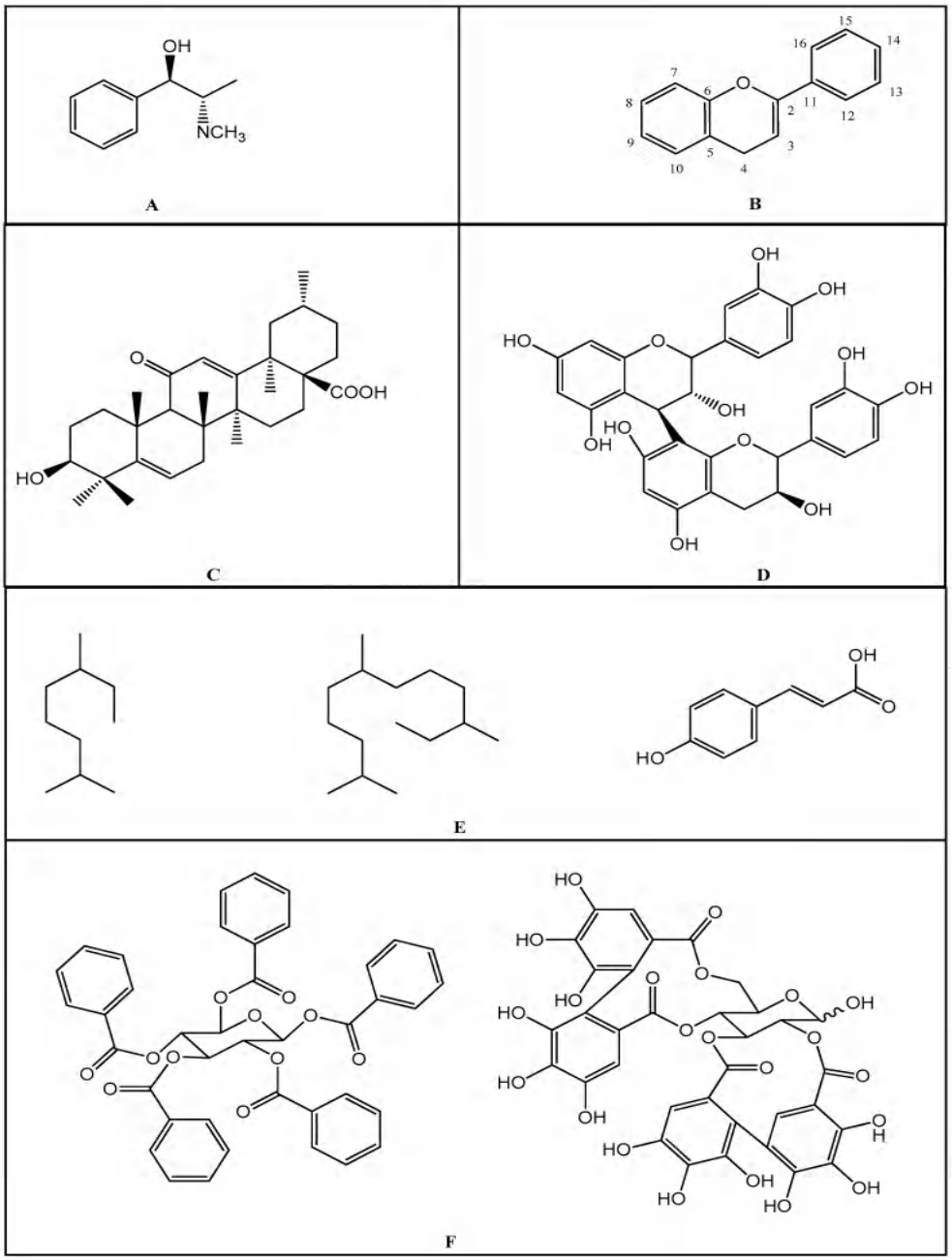

Figure 12. Secondary metabolites structure. a): Chemical structure of alkaloids: Ephedrine. b): Basic Flavonoids structure. c): Chemical structure of saponins: Glycyrrhizin, d): Structure of condensed tannin. e): Structure of terpenoids and phenylpropanoids, f): Structure of hydrolysable tannins: Galotannins and Ellagitanins (Kite and Dewick, 1998; Saxena *et al*., 2013).

Thus, flavonoids have been the target of increasing interest as a potential therapeutic drug in inhibiting or even decreasing inflammatory activity (Rex *et al.*, 2018). Although an expressive number of flavonoids are present in plants in the form of glycosides, consignments about the biological activities of flavonoids have been made mainly for aglycones. Some research on the structure-activity relationship and the inhibition of inflammatory mediators can

also be found in the scientific literature (Aravindaram and Yang, 2010). It has been reported that the presence of hydroxylations at the 3′ and 4′ positions of the B ring of the flavonoid structure enhances the modulatory action on the inhibitory activity of TNF-α, while the presence of only one hydroxyl minimizes inhibition and the absence of this group overrides the inhibitory effect. The anti-inflammatory potential of 9 flavonoid aglycones have been demonstrated in literature: kaempferol, quercetin, apigenin, chrysin, diosmetin, luteolin, daidzein, genistein, and hesperetin (Aravindaram and Yang, 2010). Investigations involving kaempferol, quercetin, and aromadendrene glycosides and their anti-inflammatory activity due to suppression of NO levels in LPS-stimulated microglial cells indicated that glycosylation attenuated the aglycone suppressor activity (Szeja *et al*., 2017).

- **Naringenin**

(4',5,7-trihydroxyflavanoneflavonoid) is the aglycon of naringin which is abundant in grapefruit (Zaidun *et al*, 2018; Hartogh and Tsiani, 2019). In recent studies, naringenin has demonstrated potential effects in different experimental models of IBD (Pinho-Ribeiro *et al.*, 2016; Zeng *et al.*, 2018) (Zobeiri *et al.*, 2018). By protecting against in a dextran sodium sulfate (DSS)-induced colitis mouse model (Alam *et al.*, 2014).

- **Quercetin**

Is a frequently used natural flavonoid compound, generally existing in glycosylated forms, such as rutoside (3-rhamnosy-glucosyl quercetin) and quercitrin (3-rhamnosylquercetin) (Tao *et al.*, 2013). Quercetin is reported to alter the intestinal host–microbial relationship through the recovery of the pro-inflammatory, anti-inflammatory, and bactericidal activities of intestinal macrophages, which leads to improvement in colitis (Nogata *et al.*, 2006). Comalada and colleagues observed that quercetin protected against DSS-induced colitis in the experimental rat model (Comalada *et al.*, 2005). Moreover, both *in vivo* or *in vitro* studies have revealed that quercetin inhibits cytokine production and induces NOS through the suppression of the NF-κB signaling pathway (Ju *et al.*, 2018).

- **Myricetin and kaempferol**

Are natural flavonol compounds that exist mainly in the form of aglycones in plants. Myricetin is a natural polyhydroxy flavonoid compound extracted from the bark, plant seeds and leaves of Myricarubra (Jones *et al.*, 2011; Imran, Salehi, *et al.*, 2019; Wang *et al.*, 2019). The

pharmacological activities of these two flavonols, such as antitumor, anti-inflammation, antibiosis, and antioxidation, have been studied extensively (Camuesco *et al.*, 2004).

It has been reported that kaempferol supplementation in diet mitigated the DSS-induced colitis through a decrease in the biochemical and clinical inflammatory factors, such as IL-6,IL-1b, COX-2, TNF-α, and iNOS, and reduction in the MPO, PGE2, and NO levels in colonic mucosa (Semwal *et al.*, 2016). In addition, kaempferol is reported to upregulate the expression of (TFF3) a critical gene regulating the function of goblet cells (Park et al., 2012), improve acute UC and upregulate the expressions of TGF-β and IL-10, the levels of regulatory T cells (Qu *et al.*, 2019).

- **Apigenin**

Is a flavonoid present naturally in several vegetables and fruits, particularly citrus fruits, with grapefruit being particularly rich in its content (Nabavi *et al.*, 2018; Yu *et al.*, 2020). Apigenin possesses excellent antioxidation and anti-inflammation activities and has been recently demonstrated to provide relief in inflammatory bowel disease. Marquez-Flores et al. demonstrated that apigenin protected against colitis induced by DSS in mice. Moreover, apigenin reduces COX-2, MMP-3, iNOS, TNF-α, and IL-1b expressions by suppressing the inflamasome pathway (Márquez-Flores *et al.*, 2016).

- **Luteolin**

(3',4',5,7-tetrahydroxyflavone) is a common flavonoid that is isolated from celery, honeysuckle, garden bitter melon stems, and other plants across the world (Lin *et al.*, 2008; Aziz *et al*, 2018; Imran *et al.*, 2019). Among all flavonoids, luteolin is the one that exerts a significant effect against IBD, as confirmed in various experimental models of IBD. For instance, as reported by Nunes et al., luteolin treatment modulated intracellular inflammatory signaling in HT-29 colonic epithelial cells by suppressing the JAK/STAT pathway (Nunes *et al.*, 2017).

- **Phenolic acids**

Are among the most widely distributed plant non-flavonoid phenolic compounds present in the free, conjugated-soluble and insoluble-bound forms. This review concentrates on the occurrence, extraction and identification, *in vitro* and *in vivo* antioxidant activities of hydroxybenzoic and hydroxycinnamic acids and their derivatives. Phenolic acids are found in

all food groups and they are abundant in cereals, legumes, oilseeds, fruits, vegetables, beverages and herbs. They can exert antioxidant activity by scavenging hydroxyl radical, superoxide radical anion, several organic radicals, peroxyl radical, peroxynitrite and singlet oxygen, among others. Furthermore, they act as chain breaking antioxidants and reducing agents. In addition, phenolic acids are important compounds to change cell signaling pathways. (Chandrasekara, 2019).

- **Cafoeylquinic acids and derivatives**

CQAs are among the most abundant polyphenols and are esters of caffeic acid with quinic acid and present a category of specialized bioactive molecules deriving from phenylpropanoid biosynthesis pathway. These compounds are ubiquitous among fruits and vegetables. Plant species from the *Coffea* genus, species from the Asteraceae family, *C. sinensis*, *Ilex paraguariensis*, and *Centella asiatica* are particularly rich in CQAs in both quantity and diversity. Besides CQAs, DiCQAs and triCQAs are often found in plants (Alcázar Magaña *et al.*, 2021).

In plants, in humans, CQAs have a wide range of potential benefits with therapeutic applications. These compounds have been reported to exhibit antioxidant (Hamed *et al.*, 2020; Nzekoue *et al.*, 2020; Trendafilova *et al.*, 2020), antibacterial (Hamed *et al.*, 2020; Nzekoue *et al.*, 2020) cancer-related (Taira *et al.*, 2014; Giorgio *et al.*, 2015; Murad *et al.*, 2015; Zheleva-Dimitrova *et al.*, 2017; Bulgakov *et al.*, 2018; Liu *et al.*, 2020) antiviral, anti-Alzheimer (Tsunoda *et al*, 2018; Matthews *et al.*, 2020), and neuroprotective activity (Gray *et al.*, 2014; Sasaki *et al.*, 2019; Metwally *et al.*, 2020), and antidiabetic (Imai *et al.*, 2020).

- **Tannins**

Are classified into two main groups, whose structures are very different from each other, although all of them have in the molecule polyhydroxyphenols or their derivatives. Those belonging to the first group are called hydrolyzable tannins, which include galotannins and ellagitannins, polymers derived from gallic and ellagic acids (He *et al.*, 2015).The constituents of the second group are called condensed tannins. They are found in greater proportions and importance in food (Jain, 2012). The pharmacological activities of tannins are due to their high complexity with metallic ions (iron, manganese, copper, and others), antioxidant activity, and the ability to complex with other molecules such as proteins and polysaccharides (Xie *et al*., 2012).

- **Alkaloids**

Are a secondary metabolites that contain nitrogen in a heterocyclic ring, in a state of negative oxidation, with an amine group that gives them a basic character (Carlos *et al.*, 2010). Alkaloids are derived from amino acids such as ornithine, lysine, tyrosine, and tryptophan. Ornithine is a precursor for pyrrolidine and tropic alkaloids, while lysine gives the piperidine alkaloids. Alkaloids are considered the most toxic among the several active principles. Some are known and used in scientific therapy, such as morphine, ergotamine, and ephedrine (Sandini *et al.*, 2013).

- **Saponins**

Such as glycyrrhizin, on the other hand, are steroid glycosides or polycyclic terpene used for the synthesis of cortisone (anti-inflammatory) and sex hormones. They increase the uptake and use of certain minerals (Smeriglio *et al.*, 2017) and can form complexes with steroids, proteins, and membrane phospholipids determining important biological properties such as changes in cell membrane permeability (Jesch and Carr, 2017).

- **Terpenes**

Terpenes, exhibit pharmacological properties, such as anti-inflammatory and antinociceptive, inhibit platelet aggregation; and interfere at an intracellular level in several steps of the transduction mechanism. The chemical components of volatile oils can be divided into two groups: terpenoid derivatives (representing the union of two, three, or more isoprene units) formed by the mevalonic acid-acetate route, and the phenylpropanoid derivatives, aromatic compounds formed by the shikimic acid pathway (Gupta, 2016). Triterpenes such as α/β-amyrin acetate, nimbin, filicene, and oleanolic acids are found in high concentrations in plants such as *Thymus serpyllum, Syzygium aromaticum, Salvia triloba, Rosmarinus officinalis, Origanum majorana, Ligustrum lucidum,* and *Lavandula latifolia* (Azab, 2016).

III. 4. Studied plants

III. 4. 1. *Ambrosia maritima*

Ambrosia maritima L. (Asteraceae) (Table 3) is a perennial herbaceous plant, broadly distributed over the Mediterranean basin and Africa (Abdelgaleil, 2011; Dirar *et al.*, 2014). The phytochemical composition of *A. maritima* includes a variety of secondary metabolites, such as

pseudoguaianolides, sesquiterpene lactones, coumarins, triterpenes, and sterols (Mohamed *et al.*, 2020) (Figure 13).

- **Therapeutic properties**

This plant has antispasmodic merits and is able to relief bronchial impairments and urinary tract issues (Abdelgaleil, 2011; Dirar *et al.*, 2014). Sesquiterpene lactones have been shown to prove diverse bioactivities, including anti-inflammatory, antimicrobial, and antitumor antispasmodic properties (Mohamed *et al.*, 2020).

Figure 13. *Ambrosia maritima*

III. 4. 2. *Bituminaria bituminosa*

Bituminaria bituminosa (L.) C. H. Stirton (Table 4) is a perennial wild legume plant, widely distributed throughout the Mediterranean basin, traditionally used in the treatment of spasms, fever, and epilepsy (Azzouzi *et al.*, 2014). It is also considered as a source of pharmaceutically active compounds with relevant bioactivities (Nelson *et al.*, 2020) such as furanocoumarins (psoralen, angelicin), pterocarpans (erybraedin C, bitucarpin A), and flavonoids (daidzin, isoorientin) (Maurich *et al.*, 2006; Walker *et al.,* 2006; Azzouzi *et al.*, 2014) (Figure 14).

Table 3. Systemic classification of Ambrosia maritima (Ambrosia maritima, EPPO)

kingdom	**Plantae**
phylum	Tracheophyte
Class	Magnoliopsida
Order	Asterales
Family	Asteraceae
Genus	*Ambrosia*
Species	*Ambrosia maritima L.,1753*

- **Therapeutic properties**

The phytoconstituents of *A. maritima* have shown antimicrobial, anti-HIV activities, and were able to prevent and treat solid organ transplantation rejection, besides other anti-proliferative, estrogenic, hepatic-protective, anti-allergy, anti-inflammatory, apoptotic, and anti-tumor properties (Zhou *et al.*, 2000; Shikishima *et al.*, 2001; Maurich *et al*., 2004; Maurich *et al.*, 2006; Pazos-Navarro *et al.*, 2011; Wolff *et al.*, 2011; Río *et al.*, 2014; Pistelli *et al.*, 2017).

Figure 14. *Bituminaria bituminosa L.*

Table 4. Classification of *Bituminaria bituminosa* (*Bituminaria bituminosa (L.) C.H.Stirt., 1981)*

kingdom	**Plantae**
phylum	Tracheophyte
Class	Magnoliopsida
Order	Fabales
Family	fabaceae
Genus	*Bituminaria*
Species	*Bituminaria bituminosa* L.C.H. Stirt 1981

Experimental part

IV. Materials and Methods

IV. 1. Materials

IV. 1. 1. Chemical reagents

Unless stated in the text all the reagents were purchased from Sigma (Sigma, St Louis, MO, USA).

IV. 1. 2. Plants material collection

The aerial parts of *A. maritima* were collected in North-East of Algeria Ighzer Amokrane, Ouzellaguen- Bejaia (36°33'4.51"N - 4°36'29.19"E), while *B. bituminosa* aerial parts were collected in May 2016 from Taza National Park in the region of Ziama Mansouria, Jijel in the North-East of Algeria (36°42'17.37"N - 5°33'23.70"E) (Figure15). Both plants were identified by Pr. K. Rebbas from Nature and Life Department (University of M'sila, Algeria), and vouchers were deposited in his personal herbarium. Plants' material was air-dried at room temperature for three weeks in the shade, then ground into a fine powder using an electric mill, then stored until use.

Figure 15. Plant material collection

IV. 1. 3. HUVECs Cells and CD sera from patients

IV. 1. 3. 1. Culture

As described by (Pasciu *et al*., 2009; Sanna *et al*., 2002) HUVECs cells were isolated from the interior human umbilical vein cord after incubation for 10 min at 37°C with 0.05% (w/v) *Clostridium hystolyticum* collagenase type II enzyme solution which was prepared in M199 medium (Invitrogen), additioned with 100 U/ml of penicillin G sodium salt and 100 µg/mL streptomycin sulfate.

Then, the solution was centrifuged at 1000×g for 10 min and the pellet was resuspended in 5 mL medium M199 supplemented with 10% (v/v) foetal calf serum (FCS), 10 % (v/v) newborn-calf serum (FBS) (Invitrogen, Carlsbad, CA), 2 mM glutamine (1%) and antibiotics (1% of Streptomycin and Penicillin). Cells were then transferred into 25 cm^2 tissue culture flasks (Falcon, Oxnard, CA) pre-coated with 0.1 % gelatin and cultured in an atmosphere of 5 % CO_2 /95 % air.

Cultured cells were characterized as endothelial by their typical cobblestone appearance and production of Von Willebrand factor and were used within three passages. All experiments were performed using cells grown until confluency rate of 50% in order to reach contact-dependent growth inhibition and to mimic physiological vessel wall conditions (Figure 16). Cells were plated at a concentration of 7 to 8 $x10^4$ cell per well for 48h then processed for experiments in basal medium in 96 well black plates (BD Falcon) for ROS levels measurement and for 24 h in transparent 96 wells microplate for MTT assays.

The same later medium was used in MTT assays and to prepare *A. maritima* different concentrations for this assay. Hanks' medium prepared with Hanks' Balanced Salt Solution (HBSS), 1.5 mM of HEPES buffer solution and 2.5% of FCS was used in the experiments of ROS intracellular measurement and to prepare both the extracts stock solutions and H_2DCFDA probe (5 µM). The tested extracts were added to the cell culture with concentrations of 1, 10, 50 and 100 µg/ml.

This range was selected according to literature where different ranges used in previous studies from the same plant extracts or leads were applied at concentrations range of (0.001 µM- 1000 µM) in the case of pure compounds or the equivalence for extracts or leads of (0,1- 1000 µg)

(Kuete and Efferth, 2013; Saeed *et al*., 2015; Said *et al*., 2018). Notably, The concentration of compounds applied in cell-based screening assays are defined according to their practicability and not by their efficiency or maximal toxic concentrations (Gerő, 2018).

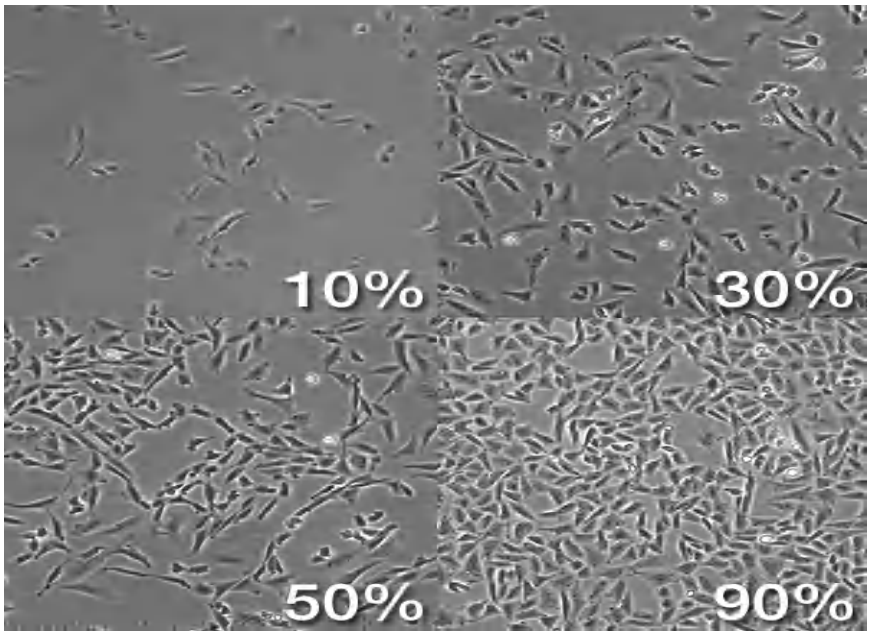

Figure 16. HUVECs different densities of confluency (expressed in percentage %).

IV. 1. 3. 2. Tests for cell culture

The following tests were used as checkpoints (Cell viability and intracellular ROS) in the different cell culture experiments:

- **MTT test (Cell viability)**

To assess the cytotoxicity activity, MTT test was performed as previously described by (Posadino *et al*., 2017) in 96-well plates (BD Falcon) (Promega, Madison, WI). This test measures the reduction of the yellow compound 3-(4,5-dimethythiazol2-yl)- 2,5-diphenyl tetrazolium bromide by mitochondrial succinate dehydrogenase at the ubiquinone and cytochrome b and c sites of the mitochondrial electron transport system in living cells inside the mitochondria (Supino, 1995). The reduction of MTT is considered as an indicator of cell growth and mitochondrial metabolic activity (Rai *et al*., 2018).

Briefly, cells were cultured as described above (cell culture section). 20 µl of MTT solution (5 mg/ml) were added in each well 3 hours before the end of the experiment. Next, the medium was removed and the crystals of formazan were solubilized with acidic isopropanol (0.04 N

HCl in absolute isopropanol), and absorbance was read at 570 nm wavelength using a Tecan GENios Plus microplate reader (Tecan, Switzerland) with background subtraction at 650 nm. In different experiments cells were preincubated in the presence and absence of different treatments of H_2O_2 AM and CD sera.

$$Percentage\ of\ cell\ viabillity(\%) = \frac{(\text{A treated cells} - \text{A control}) \times 100}{\text{Acontrol}}$$

Where :
A = absorbance.
Control : Untreated cells.

- **H_2DCFDA probe test (ROS levels measurement)**

Intracellular ROS levels were investigated using the redox sensing probe 2',7'-dichlorodihydrofluorescein diacetate H_2DCFDA (Fois *et al*., 2018; Posadino *et al*., 2019). Within the cell, esterase enzymes are able to cleave the acetate groups on H_2DCF-DA, thus trapping the reduced form of the probe (H_2DCF). Intracellular ROS oxidize H_2DCF, yielding the fluorescent product, DCF. After pretreatment cells were incubated for 15 min with HBSS containing 5 µM of H_2DCFDA (Invitrogen), then washed with PBS.

Fluorescence variation induced by treatments was kinetically measured in cell culture medium using a Tecan GENios Plus microplate reader (Tecan, Switzerland) every 20 min for 14 hours (Total of 900 min) in 485 nm and 535 nm wavelengths respectively in light-protected conditions. All fluorescence measurements were corrected for background fluorescence and protein concentration. Data of intracellular ROS levels were expressed as means ± SD of three experiments of measurements repeated in triplicate of the relative fluorescence unit (RFU) values. To investigate the potential (pro/antioxidant) effect of *A. maritima* and *B. Bituminaria* extracts on the intracellular redox state, cells received different treatments in the presence of AM, H_2O_2 and CD sera. Results were presented in histograms taking in consideration the recorded levels during the last 20 min of measurements.

IV. 1. 3.3. Sera from patients

14 sera from CD patients and healthy controls were provided from a previous CD sera study compilation (di Sabatino *et al*., 2011), 7 patients and 7 healthy controls. The study diagnosis of CD was based on a convention of clinical, endoscopic, and histological criteria and clinical phenotypes of the disease were classified as: fistulizing, stricturing and luminal according to (Gasche et al., 2000). In this study, 7 CD sera from patients were used (4 males and 3 females; mean age 32.6 years, range 21–55 years) and 7 healthy controls (4 males and 3 females; mean

age 25.3 years, range 21–29 years) were also selected as controls. Sera were stored at -80 °C before their use (Table 5).

IV. 1. 4. Animals

120 Adult male albino mice (*Mus musculus,* 2-2.5 months old) with a weight range between 20–33 g were selected to carry out *in vivo* experiments. The animals were randomly selected and marked for individual identification, and kept in cages before dosing for at least 5 days (to allow adaptation to the lab's conditions. The temperature of the animal room was about 22°C (+3°C), with a relative humidity of 30 %, and artificial lighting (12h light/12h dark). Water was available ad libitum, and food was provided in the form of dry pellets (Bouzaréah, Algeria)(OECD/OCDE, 2002) (Figure 17).

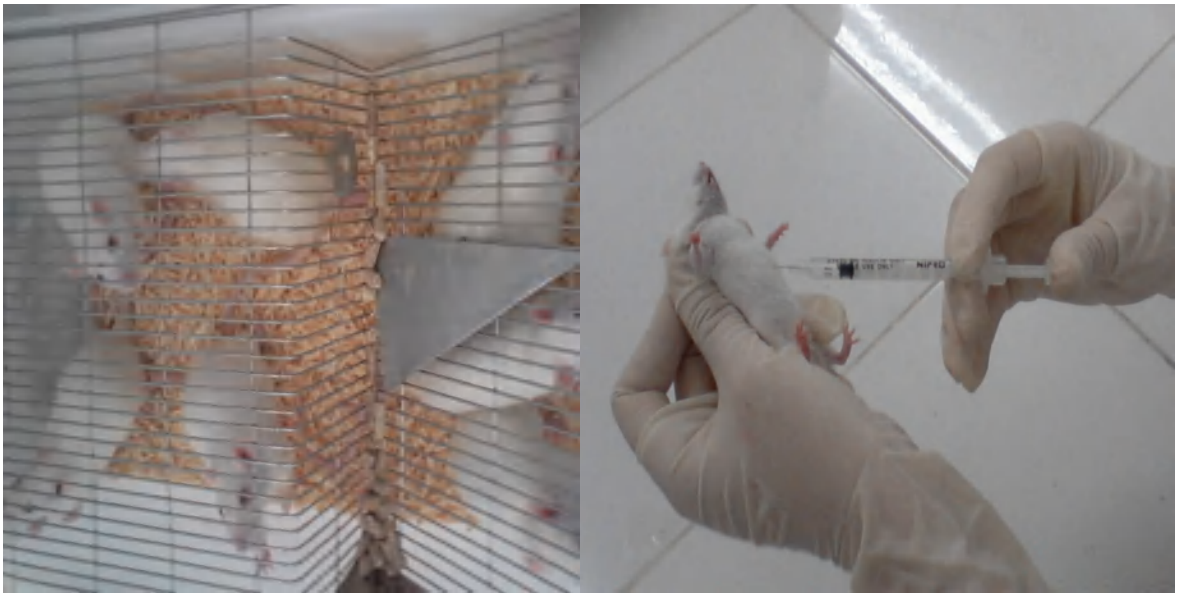

Figure 17. Animals' experiments.

IV. 2. Methods

IV. 2. 1. Phytochemical screening of *A. maritima* and *B. bituminosa*

IV. 2. 1. 1. Extraction procedures

Two different methods of extractions were realized**:**

IV. 2. 1. 1. 1. Maceration and liquid-liquid extraction

The extraction process was performed according to Lemoui *et al.* (2018). Briefly, air-dried and powdered aerial parts (1 kg) from each plant were macerated at room temperature in ethanol and water ($EtOH/H_2O$) (80/20: v/v) mixture repeated 3 times every 24h. The macerate was then filtered and concentrated using a rotative evaporator. The obtained residues were dissolved in

warm distilled water. The resulting solutions were extracted using the liquid-liquid method by the affront to different organic solvents with a decreased polarity in the following order: chloroform (CHL), ethyl acetate (EtOAc), and n-butanol (n-BuOH). The extracts were referred to as. AM for hydroethanolic extract of *A. maritima*, and BB for hydroethanolic extract of *B. bituminosa*; AMC, AMA and AMB were the chloroform, ethyl acetate and n-butanol extracts of *A. maritima*; BBC, BBA and BBB were the chloroform, ethyl acetate and n-butanol extracts of *B. bituminosa*. Extracts were then condensed and conserved at +4°C until next use. These extracts were used in the following tests: Total phenols, total flavonoids, anti-inflammatory activity *in vitro*, acute toxicity and immunostimulatory activity (Figure 18).

Table 5. Clinical features of selected patients with Crohn's disease (n=7)

Criteria	**Means (years)**	**Range (years)**	**Number of patients**
Age	32.6	21-55	7
Gender		/	
Male			4
Female			3
Clinical phenotype		/	
	Fistulizing		1
			3
	Stricturing		3
	Luminal		
Duration of disease	6.46	0 - 16	/

IV. 2. 1. 1. 2. Phenolic low temperature extraction (LTE)

The extraction was realized as described by (Barberis *et al*., 2015) with few modifications. LTE was performed in the possibly lowest temperature to preserve the phenols structure intact (<42°C). Briefly, Aerial parts of *A. maritima* and *B.bituminosa* were air dried at room temperature (27 ± 3 °C), and then grinded up-to fine powder with a planetary ball-mill (RETSH PM100, Haan, Germany) employing a 500 mL porcelain container with 3 balls (3 cm ø) (rotation speed 0.48g / 20 min) Liquid nitrogen was added to avoid sample heating.

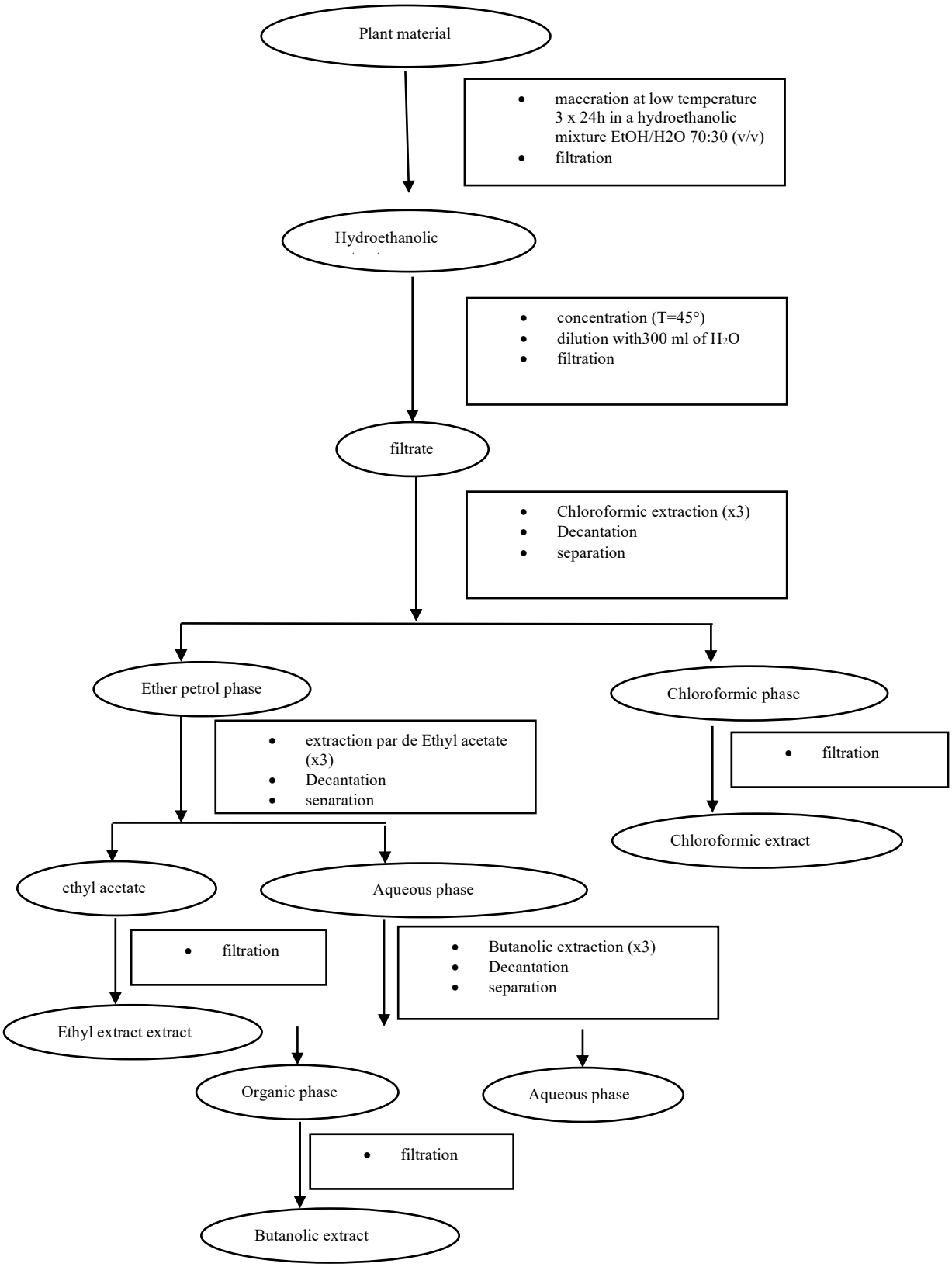

Figure 18. Maceration and liquid-liquid extraction method

The resulting powder was split into six falcon vails of 50 mL containing approximately 5 g each and 25 mL of a hydro-alcoholic (60:40%) solution was added and extraction took place overnight at 25°C by employing a planetary agitator. In order to separate the pellet and recover the supernatant, the vails were centrifuged for 15 min at 4°C and 4627G. The supernatants were pooled together (140 mL) and filtered with Whatman 1 filter paper.

The filtrate was immediately stored at -80°C and once frozen put under a nitrogen flow to evaporate the ethanol and avoid oxidation, the defrosted sample was moved to the rotavapor (IKA-Werke GmbH, Staufen, Germany) operating at 38 - 40°C for 10 min under vacuum for complete removal of ethanol. Once ethanol was removed, the sample was frozen again and water was removed by lyophilization allowing the obtain of a fine powder (Figure 19). The hydrophilic powder was put into a capped vail, kept into a desiccator at 25 °C, and finally stored at 4 °C until use. The yield of extraction was calculated using the following equation:

$$Yeild\ of\ extraction\ (\%) = \frac{(The\ weight\ of\ freeze\ dried\ recover) \times 100}{Initial\ weight\ of\ plant\ powder}$$

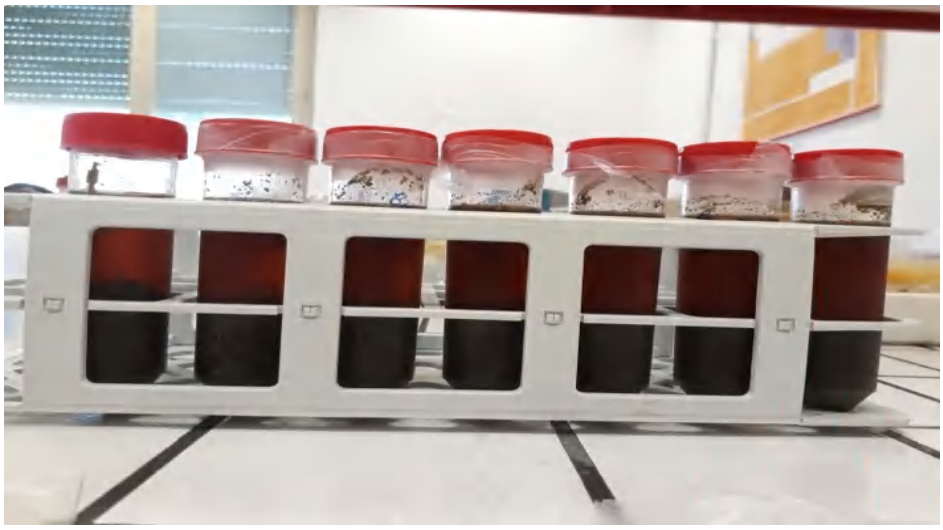

Figure 19. Total phenols low temperature extraction (LTE).

The resulting extracts were referred to as AMp for *A. maritima* phenolic extract and BBp for *B. bituminosa* were used to perform the following activities: Total phenols, LC/MS analysis, cell culture experiments.

IV. 2. 1. 2. Total phenolic content (TPC)

The total phenols content was assessed using Folin-Ciocalteu method (Fadda *et al*., 2016; Singleton and Rossi, 1965) slightly modified. A mixture of 1 mg of lyophilized extract and 9 mL of cold ethanol (80%) was prepared (1:10 w/v), vortexed (Stuart, U.K. model SA8.) at 1600 rpm for 2 min and centrifuged (ALC-Centrifuge 4227R, Milan, Italy) at 16,000× g for 15 min at 4 ◦C. 200 µL of each extract mixture were mixed with a 10% solution of Folin-Ciocalteu reagent (1 mL) and left to react for 8 min, then 800 µL of sodium carbonate solution 0.075 % was added before incubating in the dark for one hour at room temperature (20 ± 3 ◦C) followed by an additional hour at 0 ◦C. The absorbance was read at 760 nm with a spectrophotometer (8453 Agilent Technologies, Santa Clara, CA, USA) and results were expressed as milligrams of gallic acid equivalent/g of dry weight on the basis of a gallic acid calibration curve (40 to 80 mg/L).

IV. 2. 1. 3. Flavonoids content (TFC)

Total flavonoid content (TFC) was evaluated using $AlCl_3$ (aluminum chloride), with minor modifications for a 96 wells microplate use. Briefly, an aliquot of 50 µL of each extract (0.25 mg/mL) was added to 10 µL of $Al(NO_3)_3$ (aluminum nitrate) solution (10%), 10 mL of 1M CH_3COOK (potassium acetate), and 130 µL of methanol. The mixture was thoroughly stirred and incubated for 10 min at room temperature. The absorbance was recorded at 430 nm. All samples were analyzed 3 times, and the content of flavonoids was determined using a quercetin calibration curve. Results were expressed as microgram of quercetin equivalent in mg of dry plant extract weight (µg QE/mg) (Topçu *et al*., 2007).

IV. 2. 1. 4. LC-DAD/ ESI-MS analysis

IV. 2. 1. 4. 1. LC/DAD

LC-DAD/ ESI-MS analysis was performed as described by (Barberis *et al*., 2015). Briefly, Chromatographic separation was performed on an Agilent 1100 LC System that included a binary pump, Diode-Array Detector, column thermostat, degaser and HTS-PAL autosampler (Agilent Technologies, Palo Alto CA USA). The HPLC column was a Gemini C18 (100 x 2.1 mm, 5 µm) from Phenomenex (Torrance, CA, USA) with a security guard cartridge (4 × 2 mm). Briefly, two mobile phases were employed for elution consisting of Eluent A water (mixed with 0.2 % acetic acid - 0.01% trifluoracetic acid) and Eluent B acetonitrile. The flow rate was set at 0.2 mL/min with an injection volume of 5 µL, and the column temperature was 35 °C. Total

run time was 50 min. The gradient profile was 0–20 min, 95% to 85% A; 20–40 min, 85% to 70% A; 40–50 min, and 70% to 40% A. The flow rate was 0.2 mL/min from the time 0 to 50 min. The Diode Array Detector was set at 270 and 520 nm Bw=4 Reference 800 (Table 6).

Table 6. HPLC/DAD Gradient program.

Time (min)	Phase A (%)	Phase B (%)	Flow (mL/min)
0	95	5	0.2
20	85	20	0.2
40	70	32	0.2
50	40	60	0.2

IV. 2. 1. 4. 2. Mass spectrometry (MS)

The mass spectrometer was an Agilent G1946 (MSD 1100) single stage quadrupole. The instrument was interfaced with an Electrospray Atmospheric Pressure Ionization ESI used in the positive ion mode $[M+H]^+$. The mass spectrometer was programmed to admit protonated molecules at a mass range 150-850 m/z. The positive ion spray voltage was 3200 mV and the Fragmentor was 85 V. After optimization, the heated nebulizer parameter was set as follow: Temperature 35°C, Nebulizer pressure 42 psi and flow rate of drying gas 9.8 L/min. Peaks were identified on the basis of their retention time relative to external standards (t_r), UV-VIS spectra, mass spectra, phytochemical database, and their corresponding molecular masse range m/z.

IV. 2. 2. Evaluation of *A. maritima* and *B. bituminosa* toxicity

IV. 2. 2. 1. Cytotoxicity effect

We assessed the cytotoxicity and cytoprotection effect of AMp and BBp, by analyzing HUVECs mitochondrial metabolic activity (MMA) using the MTT test, which indirectly expresses the cell viability. MTT test was performed as described above. Cells were preincubated with AMp and BBp extract (t=0h) at concentrations of 1, 10, 50 and 100 µg/mL.

IV. 2. 2. 2. Acute toxicity test *in vivo*

The acute toxicity of AM and BB extracts was assessed as described in (OECD/OCDE, 2002) with slight modifications. Briefly, groups of 5 animals were dosed using the fixed concentrations of 10, 100, 1000, and 2000 mg/Kg of body weight by injection via intraperitoneal route. Clinical signs of mortality or toxicity linked to pain, suffering, and impending or death were recorded for each animal after dosing at least once during the first 30

minutes, periodically for the first 24 h, with special consideration during the first 4 hours, and daily thereafter, for a total of 14 days (except where they needed to be removed from the study, and humanely killed for animal welfare reasons; or were found dead). Body weight of each individual was recorded on a daily basis for 14 days.

IV. 2. 3. Evaluation of antioxidant potential *in vitro* of *A. maritima* and *B. bituminosa*

IV. 2. 3. 1. Chemical methods

Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), Ascorbic acid and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were used as reference standards in all the following antioxidant activities *in vitro*:

IV. 2. 3. 1. 1. Cupric ion reducing power (CUPRAC)

The cupric acid-reducing activity was evaluated following the method of (Apak *et al*., 2004) slightly modified. In each well of a 96 microplate, 50 µL of 10 mM of Cu (II), 50 µL of 7.5 mM neocuprine, and 60 µL of NH_4Ac buffer (1 M, pH 7.0) were added. Finally, 40µL of plant extract with different concentrations were added and the plate was incubated for 60 min. The absorbance was read at 450 nm and results were expressed as 0.50 absorbance ($A_{0.5}$).

IV. 2. 3. 1. 2. DPPH radical scavenging assay

The free radical scavenging potential was assessed using the DPPH method according to (Blois, 1958). Briefly, 160 µL of DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) solution (60 µM) was added to 40 µL of different extract concentrations then left to react for 30 min. The reducing power was measured using a 96 microplate spectrophotometer and the absorbance was recorded at 517 nm. The results were expressed as the inhibitory concentration of 50% of free radicals (µg/mL).

IV. 2. 3. 1. 3. O-Phenanthroline free radical reducing activity

This assay was performed according to (Szydłowska-Czerniak *et al*., 2008) method. A mixture of 30 µL of O-phenanthroline (0.5%), 50µL $FeCl_3$ (0.2%), 110 µL Methanol. This mixture was added to 10 µL of different concentrations of plant extract and incubated for 20 min at 30°C. Then the absorbance was measured at 510 nm. The results were expressed as the percentage of inhibition.

IV. 2. 3. 1. 4. ABTS cation radical decolorization assay

ABTS radical scavenging activity was determined following (Re *et al*., 1999) method. Briefly, 7 mM of ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radical *cation*and 2.45 mM of potassium of persulfate solutions were mixed and incubated for 16 hours at room temperature in obscurity. Later, the activated solution of $ABTS^{\cdot+}$ was gradually diluted until having a solution with an absorbance of 0.70 ± 0.2 at 734 nm. Then, 160 µL of $ABTS^{\cdot+}$ was added to 40 µL of the prepared concentrations of AM extract and left to react for 10 min. The absorbance measurement was realized using a 96 microplate reader at 734 nm. The results were presented as IC_{50} expressed in (µg/mL).

IV. 2. 3. 1. 5. Galvinoxyl free radicals (GOR) scavenging assay

Free radicals scavenging activity was assessed according to (Shi *et al*., 2001)assays. Briefly a 160 µL of methanolic solution of Galvinoxyl (0.1 mM) was added to 40µL of each concentration of AM extract, then, incubated for 120 min in obscurity at room temperature. The absorbance was read at 428 nm and Galvinoxyl solution was used as control.

IV. 2. 3. 1. 6. *β*-carotene/linoleic acid assay

β-carotene-linoleic acid system was used to evaluate the capacity of extracts to inhibit lipid peroxidation as described by (Miller, 1971; Sabudak *et al*., 2013). Briefly, an emulsifying agent was obtained from mixing 0.5 mg of *β*-carotene in 1 mL of chloroform to which 25 µL of linoleic acid and 200 mg of Tween 40. Chloroform was then evaporated under vacuum (Buchi, R215, Switzerland), and the residue was taken in 50 mL of hydrogen peroxide (H_2O_2). Next, 40 µL of extracts with different concentrations ranging from 12.5 to 800 µg/mL were mixed with 160 µL of the previous emulsion. The microplate was incubated for 2 h at 50°C. Ethanol was used as a negative control, while butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) were used as antioxidant standards. The absorbance was read at 470 nm, at t = 0 min and t = 120 min. The results were expressed as the inhibitory concentration at 50 % (IC_{50}).

The inhibition percentage of *β*-carotene bleaching was calculated using the following formula:

$$I(\%) = \left(\frac{As[t=0] - As[t=120]}{Ac[t=0] - Ac[t=120]}\right) \times 100$$

With I (%) = percentage of inhibition.

As [t = 0] = the absorbance at 470 nm of the sample at 0 min.

As [t = 120] = are the absorbance at 470 nm of the sample at 120 min.

Ac [t = 0] = the absorbance at 470 nm of the positive control at 0 min.

Ac [t = 120] = the absorbance at 470 nm of the positive control at 120 min.

IV. 2. 3. 2. Evaluation of the cytoprotective effect of *A. maritima* and *B. bituminosa* towards the oxidative endothelial cell death induced by H_2O_2

- **Cell viability (MAA)**

To investigate the potential cytoprotective effect of AMp and BBp and to assess the mitochondrial metabolic activity (MAA) of HUVECs in the presence of H_2O_2; MTT test was performed as previously described. Briefly, Cells were preincubated with plants' extracts (t=0h) at the concentrations of 1, 10, 50 and 100 µg/mL and 2 µL of H_2O_2 were added at a concentration of 25 µM 2 h before the end of the experiment (t=22h). In selected experiments cells were pretreated with 2µL of different concentrations of H_2O_2 only, ranging from 5 µM to 600 µM added 2 h before the end of the experiment (t=22h) in order to set a range of concentrations able to provoke mild cell damage with 50 to 60% viability. Results were expressed as percent of untreated control cells and calculated as means ± SD of measurements repeated in triplicate for three different experiments.

- **Intracellular ROS measurement**

AMp extract with the concentrations' range of 1, 10, 50 and 100 µg/mL (prepared in Hanks' medium) was added to the medium and plates were persuaded to ROS measurement. H_2O_2 was added at a final concentration of 25µM with a volume of 2µl per well, 2 hours before the end of the reading. Variation of fluorescence was kinetically measured every 20 min for 14 h and expressed relative fluorescence units, results were presented in histograms taking in consideration the recorded levels during the last 20 min of measurements.

IV. 2. 3. 3. Evaluation of the cytoprotective effect of *A. maritima* towards the oxidative endothelial cell death induced by CD sera

- **Intracellular ROS measurement**

Cells were pre-incubated with a single concentration of 10 µg/mL of AMp and extracts in basal medium for 24 h. Medium was aspired and plates were washed with PBS. After incubation with the probe for 15 min, plates were washed, then, Hanks' medium and 5% (v/v) of the subjects'

sera Crohn's disease patients (CD) were added to treated cells and only Healthy donors (HD) for untreated cells. Fluorescence variation was kinetically followed in cultured HUVECs every 20 min during a time course interval of 14 h. Results were expressed as RFU.

- **Cell viability (MAA)**

HUVEC's were preincubated 24h with a single dose of AMp (10 µg/mL) 5% (v/v) of the subjects' sera (CD sera for treated sera and HD for untreated cells). MTT assay was performed to measure the viability of cells as described in materials and methods section in a time course of 14h. Results are expressed as a percent of untreated control cells.

IV. 2. 4. Evaluation of the anti-inflammatory activity of *A. maritima* and *B. bituminosa in vitro* (Protein denaturation method)

The anti-inflammatory potential of the studied plants' extracts was assessed *in vitro* by measuring their capacity to counteract heat-induced denaturation of bovine albumin serum (BSA) as described by (Karthik *et al*., 2013). Briefly, a solution of BSA (0.2 % w/v) was prepared in a tris-HCl buffer with an adjusted pH at 6.8. Solutions of standards and extracts were prepared in distilled water, with concentrations ranging from 32.5 µg/mL to 250 µg/mL. 500 µL of each extract was added to 500 µL of 0.2 % w/v BSA.

Ketoprofen® and Diclofenac sodium (Voltaren®) were used as positive controls. The test tubes were incubated for 15 min at 37°C and then heated at 72°C for 5 minutes. The absorbance at 660 nm was taken after cooling to the room temperature using a UV-VIS spectrophotometer (U-2810 Spectrophotometer Digilab Hitachi). Each experiment was achieved in triplicate, and values were expressed as mean ± SD. The percentage of denaturation inhibition was expressed as the % basis relative to control, using the following formula:

$$\text{Inhibition Percentage of Denaturation} = \frac{\text{Absorbance of control x Absorbance of extract}}{\text{Absorbance of control}} \text{ x } 100$$

IV. 2. 5. Evaluation of the immunostimulatory activity of *A. maritima* and *B. bituminosa in vivo* (Phagocytic activity)

The immunostimulatory capacity was tested *in vivo* on *Mus musculus* using the carbon clearance method from the institutional phagocytic activity of reticuloendothelial systems

(RES) as reported in (Biozzi *et al*., 1970) . Briefly, concentrations range of 25, 50, 100, and 200 mg/Kg of AM and BB extracts were prepared in 10 mL 0.9 % NaCl.

Then, mice divided into five groups: I, II, III, IV and V; received different treatments, set as follow: group I (control) was administered (via intraperitoneal route) a 0.5 ml of 0.9 % NaCl solution, while groups II, III, IV and V received plants extracts of various concentrations: 25, 50, 100, and 200 mg/Kg of body weight (respectively). 48h later, 0.1 mL/10g of carbon ink solution was injected into the tail vein of each animal. This mixture was prepared with 4 ml of saline, 3 mL of black carbon ink, and 4 ml of 3% gelatin solution. Blood samples (14 drops ≈ 25µl) were subsequently withdrawn from the retro-orbital plexus using heparin glass capillaries at time = 5 and 15 min, then lysed with 4 mL of a 0.1 % sodium carbonate solution.

Then, the absorbance of mixture was measured at 676 nm. The phagocytic activity is expressed by the phagocytic index K: the rate of reticuloendothelial clearance of the antigen (the carbon ink), which measures all the reticuloendothelial system function in contact with the circulating blood; and by corrected phagocytic index α: unit of active weight organs (liver and spleen); while the clearance rate is expressed as the half-lifetime of the antigen in the blood (t 1/2) expressed in minutes (min).
These three parameters are calculated using the equations 1, 2 and 3:

$$(1) \qquad K = \frac{logOD\ 1 - logOD\ 2}{t1 - t2}$$

$$(3) \qquad \alpha = \sqrt[3]{K} \times \frac{Body\ weight}{liver\ wt + spleen\ wt}$$

$$(2) \qquad t1/2 = \frac{0.693}{K}$$

IV. 2.6. Statistical analysis

Data for all tests were expressed as mean ± SD of measurements repeated in triplicate. One-way analysis of variance (ANOVA) followed by post-hoc Newman-Keuls test were performed in all experiments, accept for the acute toxicity test which was analyzed using two-way ANOVA for the body-weight measurement. The p value significance was determined at (p <

0.05). Statistical analysis was performed using GraphPad Prism version 8.00 (GraphPad Software, San Diego California USA).

V. Results and discussion

Results

V. 1. Phytochemical screening of *A. maritima* and *B. bituminosa*

V. 1. 1. Extraction yields

V. 1. 1. 1. Maceration and Liquid-liquid extraction

The obtained quantities of each extract (g) are shown in table 7:

Table 7. Maceration and Liquid-liquid extraction yield of *A. maritima* and *B. bituminosa.*

Plant	Extract	Weight (g)
A. maritima	AM	8.9
	AMC	3.41
	AMA	2.9
	AMB	2.63
B. bituminosa	BB	10.06
	BBC	5.12
	BBA	4.3
	BBB	3.21

AM: Hydroethanolic extract AMC: Chloroform extract, AMA: Ethyl acetate extract, AMB: Butanolic extract of *A. maritima*. BB: Hydroethanolic extract. BBC: Chloroform extract, BBA: Ethyl acetate extract, BBB: Butanolic extract of *B. bituminosa*.

V. 1. 1. 2 Low-temperature phenolic extraction (LTPE)

The extraction method employed (Freeze drying and low-temperature conditions) allowed to obtain a 17,66% and 18,25% extraction yield of the desolvented hydroethanolic extracts of AMp and BBp respectively suitable for cell culture applications (table 8).

Table 8. Total phenols low temperature extraction yield (%)

Sample	Extraction yield (%±SD)
AMp	17.66±3.67
BBp	18.25±1.34

AMp: *A. maritima* phenolic extract, BBp: *B. bituminosa* phenolic extract. SD: standard deviation.

V.1. 2. Total phenols content (TPC)

The evaluation of total phenols revealed that both *A. maritima* and *B. bituminosa* demonstrated

an important phenolic content, with BBA and BBB showing the highest amounts (323.11±0.76 and 198.31±0.76 µg QE/mg, respectively), while the AMC extract was the richest in AM plant, with an IC_{50} value of 138.28±6.61 (µg QE/mg) (Table 9).

V.1. 3. Flavonoids content (TFC)

The evaluation of total flavonoids demonstrated that *B. bituminosa* was the richest in term of flavonoids content, with BBA and BBC showing the highest amounts (193.39 ± 24.15 and 158.15 ± 3.8 µg QE/mg, respectively, while the AMC extract was the richest for *A. maritima* plant, with an IC_{50} value of 117.54 ± 10.33 (µg QE/mg) (Table 9).

Table 9. Total phenols content of *A. maritima* and *B. bituminosa.*

Plants	Extract	Total phenols content (mgGAE/g±SD)	Total flavonoids content (µg QE/mg±SD)
A. maritima	AM	86.79±1.90	18.83±2.34
	AMC	138.28±6.61	117.54±10.33
	AMA	110.19±4.64	61.89±1.93
	AMB	128.80±1.38	26.28±1.38
	AMp	110.74±1.06	/
B. bituminosa	BB	65.020±1.04	56.31±3.58
	BBC	31.86±0,76	17.94±0.38
	BBA	323.11±0.76	193.39 ±4.15
	BBB	198.31±0.76	158.15±3.80
	BBp	59.002±4.155	/

AM: Hydroethanolic extract AMC: Chloroform extract, AMA: Ethyl acetate extract, AMB: Butanolic extract of *A. maritima*. BB: Hydroethanolic extract. BBC: Chloroform extract, BBA: Ethyl acetate extract, BBB: Butanolic extract of *B. bituminosa.* AMp: Phenolic extract of *A. maritima,* BBp: Phenolic extract of *B. bituminosa*. mgGAE/g: mg of gallic acid equivalent /g of dry plant extract weight. µg QE/mg: µg of quercetin equivalent/mg of dry plant extract weight. SD: Standard of deviation. /: non-defined. Values are expressed as means ± SD of three parallel measurements.

V.1.4. LC-DAD/ ESI-MS analysis

Out of 27 detected compounds, 7 polyphenols from different classes were identified in the AM extract. Three known phenolic acids were detected for the first time in AMp (3,4 Dicaffeoylquinic acid, 3,5 Dicaffeoylquinic acid and 4,5 Dicaffeoylquinic acid), some flavonoids were also detected including an acylated-*C*-glycosyl flavone (Apigenin 8C-(-6-*O*-Feruloyl)-glucopyranoside), an *O*-glycosyl flavone (Kaempferol -7- *O*-glucoronide) and a dihydroflavonol. Finally, sesquiterpene lactone has also been identified in the extract profile (4,5 Dihydrolychnopholide). (Fig 20) and (Table 10).

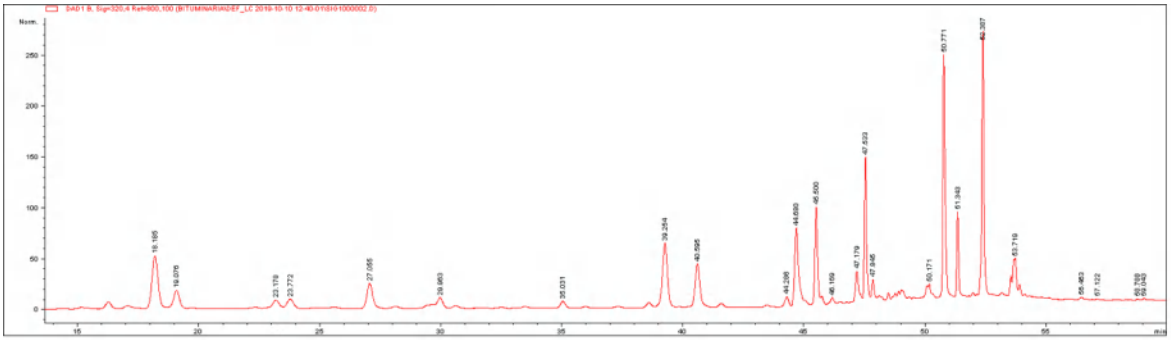

Figure 20. HPLC-DAD/ESI-MS chromatogram of AMp extract

Table 10. Phenols identification using HPLC-DAD/ESI-MS from AMp extract.

peak	Compound	t_R	(m/z)	Species
1	Dihydroflavonol	44.236	271	$[M+H]^+$
2	3,4 Dicaffeoylquinic acid	45.5	517	$[M+H]^+$
3	Kaempferol -7- *O*-glucoronide	47.119	461	$[M+H]^+$
4	3,5 Dicaffeoylquinic acid	47.533	517	$[M+H]^+$
5	4,5 Dicaffeoylquinic acid	47.84	517	$[M+H]^+$
6	Apigenin 8C-(-6-*O*-Feruloyl)-glucopyranoside	51.343	609	$[M+H]^+$
7	4,5 Dihydrolychnopholide	52.387	361	$[M+H]^+$

t_R: Retention time, (m/z): molecular masse range, Species: Operating ion mode.

For BBp extract, the HPLC-DAD/ESI-MS fingerprint revealed the presence of 11 polyphenols from different classes that correspond to glycosylated flavonoids, especially from Apigenin derivatives, Luteolin and Kaempferol derivatives besides Isoorientin. (Figure 21), (Table 11).

V. 2. Evaluation of *A. maritima* and *B. bituminosa* toxicity

V. 2. 1. Cytotoxicity effect

To investigate AMp and BBp extract biological activities, we assessed their cytotoxicity and cytoprotection, by analysing the cell mitochondrial metabolic activity (MMA) using the MTT test, which indirectly expresses the cell viability (Green, 198. Each extract potential toxicity was investigated by exposing the cells to increasing extract concentrations.

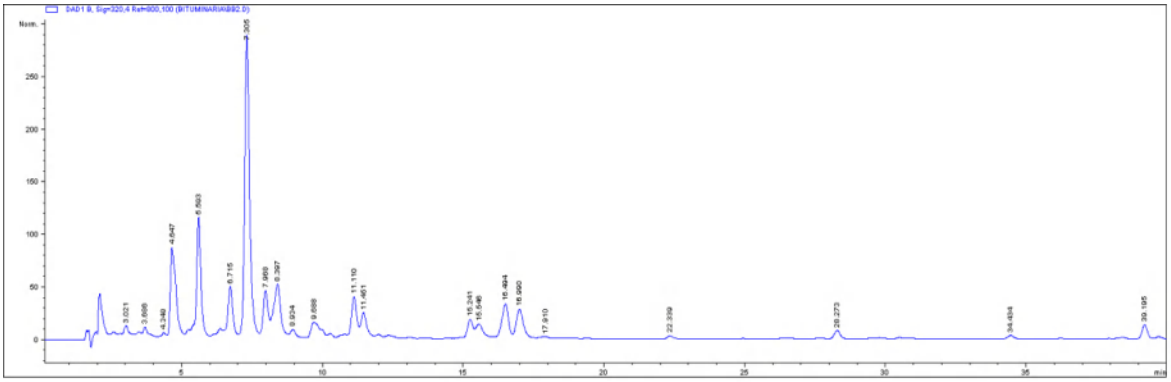

Figure 21. HPLC-DAD/ESI-MS chromatogram of BBp extract

Table 11. Phenols identification using HPLC-DAD/ESI-MS from BBp extract

peak	Compound	t_R	(m/z)	Species
1	Apigenin 8 glucoside	3.3	433	$[M+H]^+$
2	Phloretin C glucoside (Notofagin)	4.54	437	$[M+H]^+$
3	Apigenin 6,8 di glucoside (Vicenin-2)	4.74	595	$[M+H]^+$
4	Medioresinol	5.75	389	$[M+H]^+$
5	Apigenin 6 Hexoside-8-Pentoside	7.45	563	$[M+H]^+$
6	Isoorientin	8.1	449	$[M+H]^+$
7	Kaempferol-*O*-rutinoside	8.46	595	$[M+H]^+$
8	Apigenin 7 Glucoside	11.26	433	$[M+H]^+$
9	Apigenin 7 Galactoside	12.66	433	$[M+H]^+$
10	Kaempferol rhamnoside	15.41	433	$[M+H]^+$
11	Luteolin Rutinoside	16.6	433	$[M+H]^+$

t_R: Retention time, (m/z): molecular masse range, Species: Operating ion mode.

Data reported in figure (Figure 22a) show that the extract has no significant cytotoxic effect at the tested concentrations of 1 and 10 µg/mL ($p > 0.05$), while a significant MMA reduction was observed at the two highest doses (50 and 100 µg/ml) when data were compared to untreated cells ($p < 0.0001$). Whereas no significant cytotoxicity was shown by BBp extract for all the tested doses and cells had a normal viability in comparison with the control ($p > 0.05$) (Figure 22b).

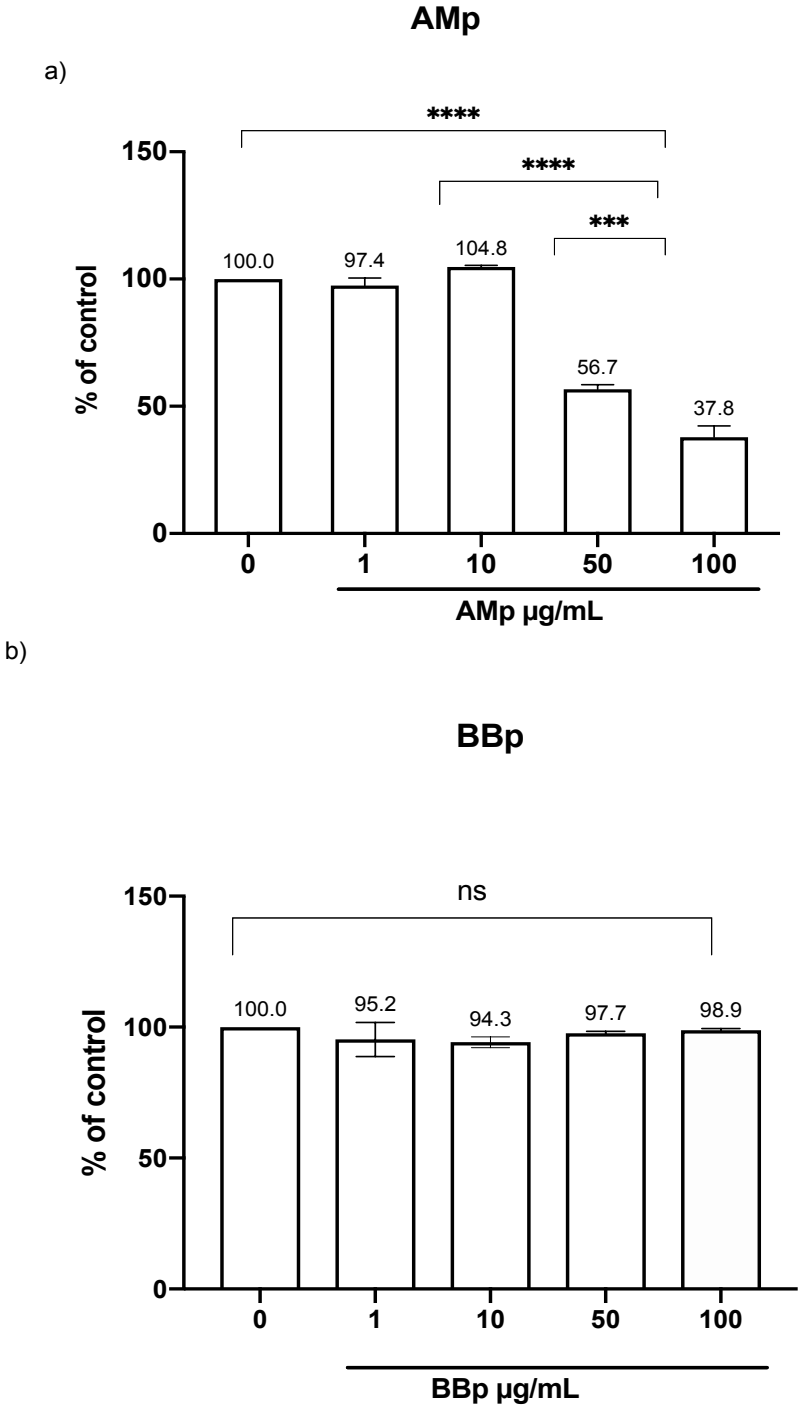

Figure 22. Cytotoxicity effect of *A. maritima* and *B. bituminosa*. a): The effect of AMp on the mitochondrial metabolic activity (MMA). b): The effect of BBp on the mitochondrial metabolic activity (MMA). AMp: phenolic extract of *A. maritima*. BBp: Phenolic extract of *B. bituminosa*. Values are expressed as means ± SD of three parallel measurements ($p < 0.05$ for one way ANOVA and Newman-Keuls multiple comparison tests).ns: none significant.

V. 2. 2. Acute toxicity test *in vivo*

In the assay of acute toxicity, no mortality was recorded in all the tested groups, in both AM and BB extracts up to 2000 mg/Kg during the 14 day-assessment times. The animals showed no abnormal behavior or clinical signs, including toxicity pain suffering or impeding. Parallelly, no significant body weight fluctuation was registered during the period of experimentation ($p > 0.05$). This result stands up for an LD_{50} higher than 2000 mg/Kg (Figure 23a, b).

V. 3. Evaluation of the antioxidant potential *in vitro* of *A. maritima* and *B. bituminosa*

V. 3. 1. Chemical methods

To gain broad information about the potential AMp and BBp phenolic extract antioxidant capacity, five *in vitro* antioxidant assays were employed (GOR, ABTS, DPPH, CUPRAC and Phenanthroline assays with vitamin C, BHA, BHT and Trolox) since they are considered as some of the standard and strongest non enzymatic antioxidant tests (Carocho et al., 2013). Results were depicted as IC_{50} for DPPH, GOR, ABTS, β-carotene assays and as A0.5 for CUPRAC and Phenanthroline tests. For AMp extract The scavenging efficacy in all performed tests was inversely proportional to the recorded IC_{50} (Table 12), and all the obtained values fluctuated in a tight interval [14.33 ± 3.86 – 43.02 ± 3.44].

The lowest IC_{50} was recorded in the GOR method and the highest in *β*-carotene assay, while the following increasing order was observed considering all the employed methods: GOR (14.33 ± 3.86) Phe (25.79 ± 1.63) < CUPRAC (26.2 ± 2.56) < ABTS (29.88 ± 2.69) < DPPH (34.26 ± 4.03) < *β*-carotene (43.02 ± 3.44) µg/mL. For BBp extract no apparent scavenging activity was shown in the different analysed activities accept for the Phen (25.13 ± 1,49) and *β*-carotene (43.02 ± 3.44) which were the lowest, and the following order for the employed methods was observed: Phen (25.13 ± 1.49) < *β*-carotene (43.02 ± 3.44) < CUPRAC (76.75 ± 3.77) < GOR (81.09 ± 1.03) < ABTS (95.23 ± 3.54) < DPPH (145.86 ± 16.97).

Overall, the AMp extract displayed an efficient antioxidant activity compared to BBp and to the different standards despite of the significant difference ($p < 0.05$). Among standards, BHA was the most efficient in free radicals quenching (0.93 ± 0.07) in Phe assay while BHT was the less efficient (12.99 ± 0.4) in DPPH method (Table 12).

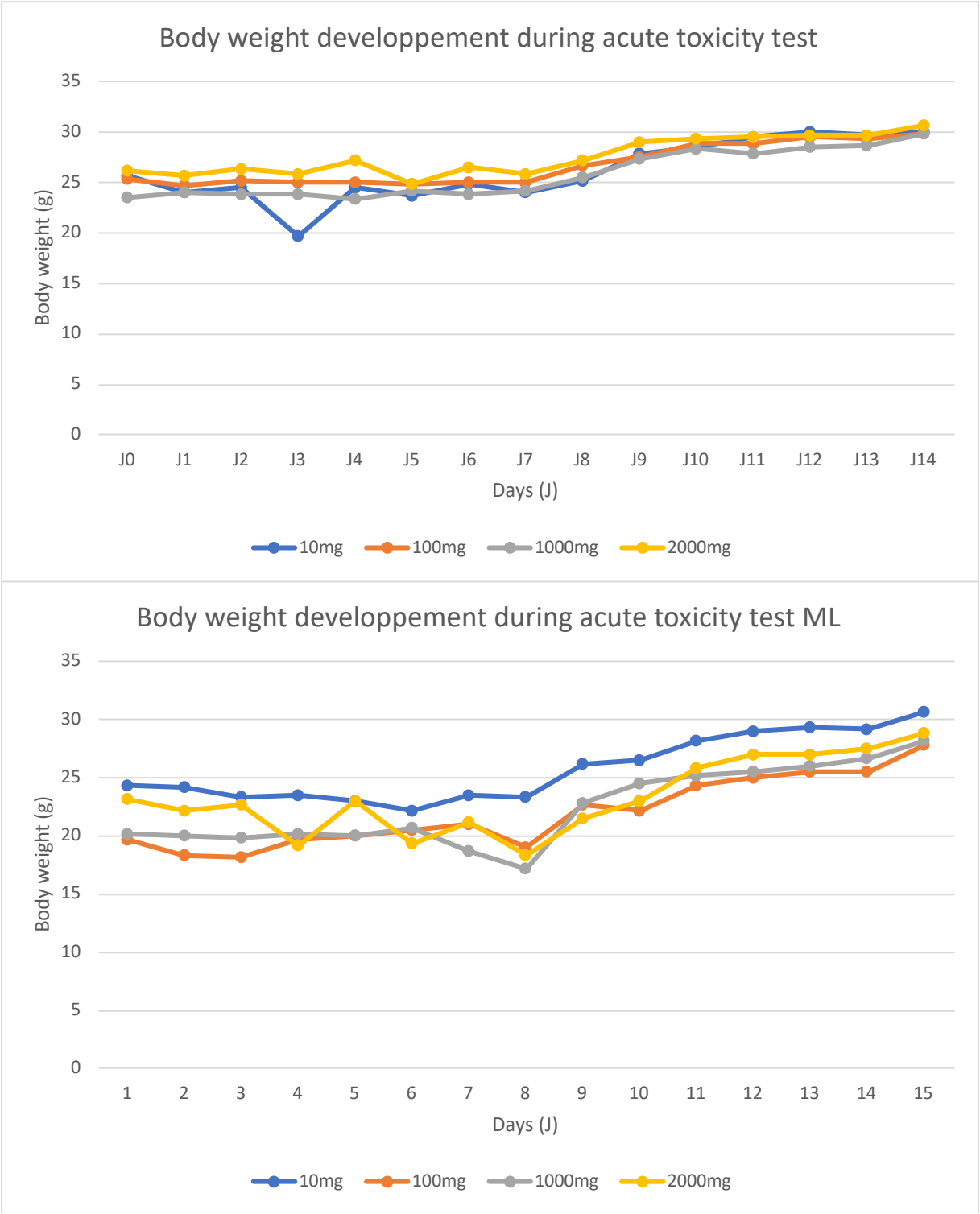

Figure 23. The acute toxicity effect of *A. maritima* and *B.bituminosa*. a): The effect of AM extract on the body weight kinetics. b): The effect of BB on the Body weight kinetics. AM: The hydroethanolic extract of A. maritima. BB: the hydroethanolic extract of *B.bituminosa*. Results are expressed as (mean±SD)

Table 12. Antioxidant activity of *A. maritima* and *B. bituminosa*.

SAMPLES	IC_{50} GOR (µg/mL±SD)	IC_{50} ABTS (µg/mL±SD)	IC_{50} DPPH (µg/mL±SD)	$A_{0.5}$ CUPRAC (µg/mL±SD)	$A_{0.5}$ Phen (µg/mL±SD	IC_{50} (β-carotene) (µg/mL±SD)
AMp	14.33±3.86 [a]	29.88±2.69[a]	34.26±4.03 [a]	26.2±2.56 [a]	25.79±1.63 [a]	43.02±3.44 [a]
BBp	81.09±1.03 [b]	95.23±3.54[b]	145.86±16.97 [b]	76.75±3.77 [b]	25.13±1.49 [b]	43.87 ± 2.19 [b]
BHA	5.38 ±0.06[c]	1.81±0.10[c]	6.14±0.41[c]	3.64±0.19[c]	0,93±0,07[c]	1.05 ± 0.01 [c]
BHT	3.32±0.18[d]	1.29±0.30[c]	12.99±0.4[d]	9.62±0.87[d]	2.24±0,17[d]	0.90 ± 0.02 [c]
Ascorbic acid	5.02±0.0[b]	3.04±0.05[d]	4.39±0.01[e]	8.31±0.15[e]	3.08±0.02[d]	/
Trolox	4.31±0.05[e]	3.21±0.06[d]	5.12±0.21[f]	8.69±0.14[e]	5.21±0.27[e]	/

SD: standard deviation; IC_{50}: sample concentration at which 50% of the free radicals activity is inhibited. AMp: *A. maritima* hydroethanolic extract, BBp: *B. bituminosa* phenolic extract, BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene. Trolox: 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid. GOR: Galvinoxyl free radicals scavenging assay. ABTS: ABTS cation radical decolourisation assay. DPPH: DPPH radical scavenging assay. CUPRAC: Cupric ion reducing power. Phen: O-Phenanthroline free radical reducing activity. Values are expressed as means ± SD of three parallel measurements. Values that do not share the same letters are significantly different ($p < 0.05$ for one way ANOVA and Newman-Keuls multiple comparison tests).

V. 3. 2. The cytoprotective effect of *A. maritima* and *B. bituminosa* towards the oxidative endothelial cell death induced by H_2O_2

- **Cell viability (MMA)**

To study the Cytoprotective effect of both AMp and BBp extract, we first tried to detect the H_2O_2 concentration capable of eliciting 50% of cell death, which was then employed in the successive experiments. As depicted in (Figure 24a), cells treated with increasing H_2O_2 concentrations showed a dose-dependent MMA decline indicating 25mM as the dosage capable to induce 50% of cell mortality. We then investigated whether the extract would possess antioxidant properties by testing its ability to protect cells from H_2O_2-induced oxidative stress and cell death.

To this end, cells were pre-treated for 24 hrs in both absence and presence of increasing extract concentrations and then exposed to 25µM H_2O_2 for 2 hrs before the end of the experimental time. For AMp extract, low extract concentrations (1-10µg/ml) were able to counteract H_2O_2-induced cell death keeping the MMA similar to the untreated cells. On the contrary, a reverse extract action was observed at high concentrations (50-100µg/mL), which was able to significantly reduce the cell MMA to levels similar to H_2O_2-treated cells (Figure 24b). The

current findings indicate a dose-dependent extract behaviour for AMp, respectively protective at low concentrations and cytotoxic at high concentrations. For BBp extract, all the tested concentrations (both high and low doses) were able to counteract H_2O_2-induced cell death keeping the MMA similar to the untreated cells. The current findings indicate the absence of a dose-dependent extract behaviour for BBp in its protective effect for the tested doses (Figure 24c).

- **Intracellular ROS measurement**

To better understand AMp and BBp pro/antioxidant behaviour towards the cellular redox state, we investigated their capability to influence the intracellular redox state in untreated and H_2O_2-treated (25mM) cell using the same experimental conditions employed in MMA experiments. Parallelly, we aimed to set a convenient AMp extract concentration with antioxidant potential to be safely employed in the next experiments. For this purpose, we challenged the cell with increasing AMp and BBp concentrations and measured their effect on the ROS intracellular levels. Cells treated with low doses of AMp showed steady levels of ROS, similar to those detected in untreated cells, while higher AMp concentrations significantly increased ROS levels when compared to untreated cells (Figure 25a).

However, in the presence of H_2O_2, as previously observed for MMA, low doses of AMp (1-10µg/ml) elicited an antioxidant effect with the best effect observed at 10µg/mL, while the higher doses (50-100µg/mL) generated increased ROS levels similar to the one detected in H_2O_2 treated cells (Figure 25b). while BBp extract showed increasing ROS levels in a dose-dependant manner and failed to demonstrate an antioxidant behaviour when challenged with primary HUVECs H_2O_2-treated or untreated cells with all the tested concentrations (Figure 26 a,b).

Giving the observed findings, the prepared AMp extract, when challenged with primary HUVECs, can elicit both antioxidant and prooxidant effects depending on the employed dosages. Taken together, our data indicate that AMp has dose-dependent dual behavior, being able to counteract oxidative stress and oxidative-mediated cell death when employed at a relatively low dosage while becoming pro-oxidant and harmful to the cells at relatively high concentrations. Our observations are in agreements with previous results concerning the natural occurring antioxidant Resveratrol and other phenolic compounds with antioxidant characteristics (REF). According to these results, we selected the concentration of 10µg/mL for the next experiments.

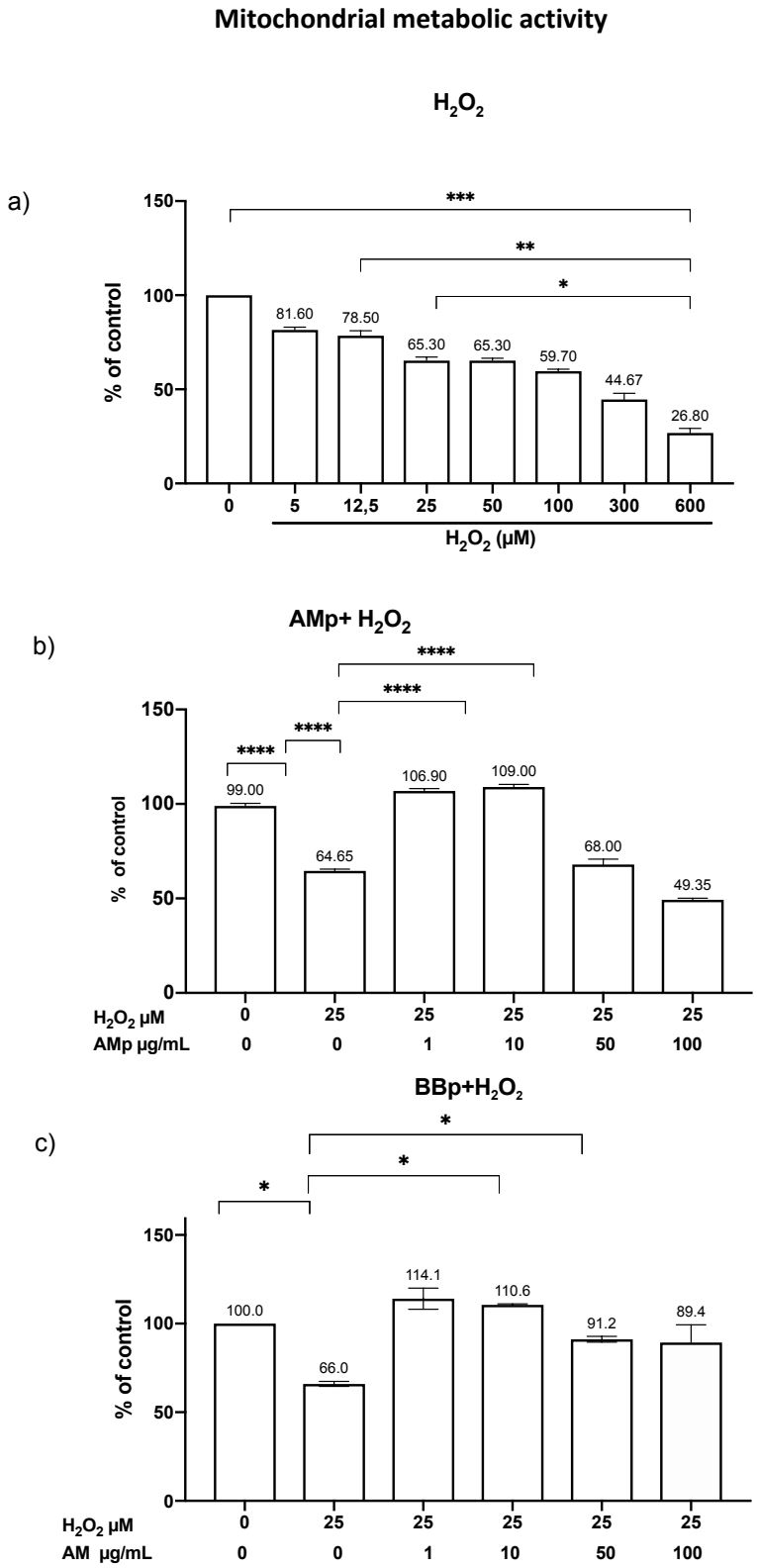

Figure 24. The cytoprotective ability of *A. maritima* and *B. Bituminosa* against HUVECs cell death induced by H_2O_2. a): Different doses cytotoxic effect of H_2O_2 on HUVECs in different doses. b): Cytoprotective effect of AMp against HUVECs H_2O_2-induced cell death. c): Cytoprotective effect of BBp against HUVECs H_2O_2-induced cell death. AMp: *A. maritima* phenolic extract. *B. bituminaria* phenolic extract. Values are expressed as a percentage of control for mitochondrial metabolic activity and as means ± SD of three parallel measurements ($p < 0.05$ for one-way ANOVA and Newman-Keuls multiple comparison tests).

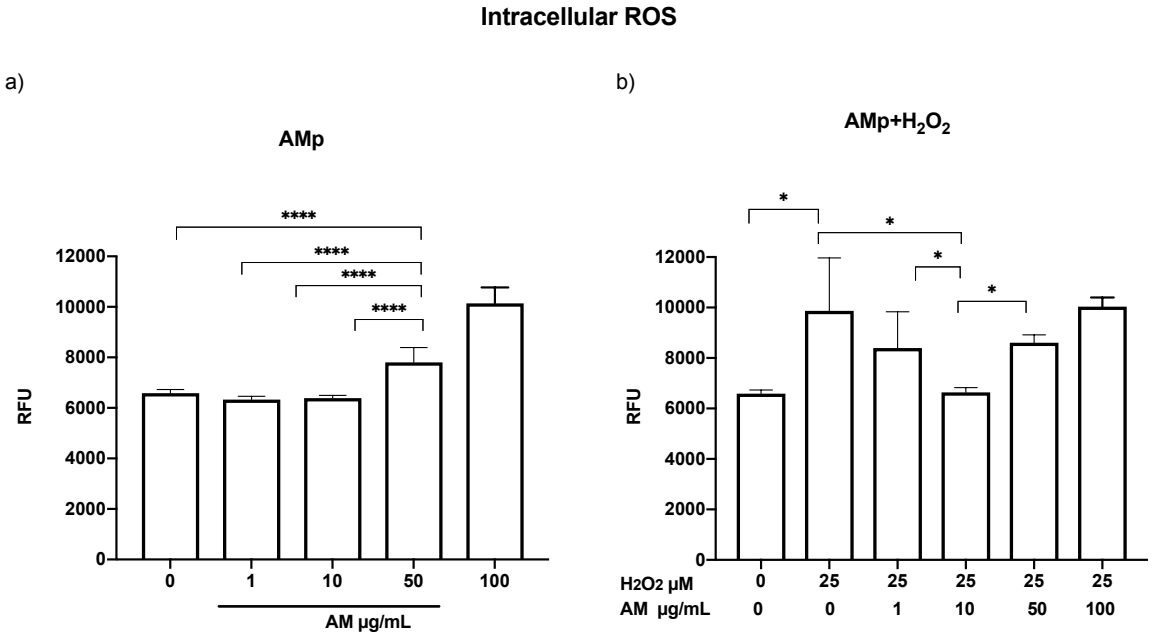

Figure 25. The pro/antioxidant effect of *A. maritima* and *B. bituminosa* on the intracellular ROS levels. a): The effect of AMp extract on the cell redox state of HUVECs. b) The effect of AMp extract on the cellular redox state of HUVECs in the presence of H_2O_2. AMp: *A. maritima* phenolic extract. Results were expressed as RFU for ROS levels and were expressed as means ± SD of measurements repeated in triplicate ($p < 0.05$ for one-way ANOVA and Newman-Keuls multiple comparison tests).

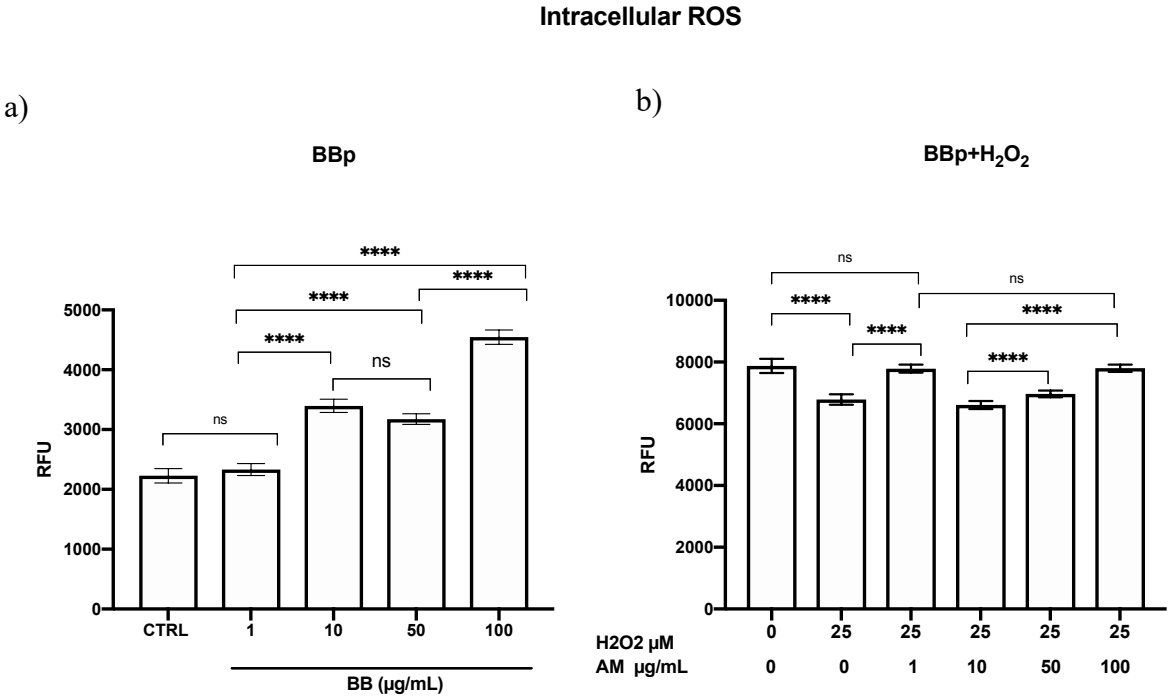

Figure 26. The pro/antioxidant effect of *A. maritima* and *B. bituminosa* on the intracellular ROS levels. a): The effect of BBp extract on the cell redox state of HUVECs. b): The effect of BBp extract on the cell redox state of HUVECs in the presence of H_2O_2. BBp: *B. Bituminaria* phenolic extract. Results were expressed as RFU for ROS levels and were expressed as means± SD of measurements repeated in triplicate ($p < 0.05$ for one-way ANOVA and Newman-Keuls multiple comparison tests).

V. 4. Evaluation of the cytoprotective effect of *A. maritima* towards the oxidative endothelial cell death induced by CD sera

We next hypothesized that sera from Crohn's disease patients (CD) may generate an imbalanced intracellular redox state and may ultimately lead to cell damage and death. To gain insight into this hypothesis, we tested the ability of CD sera to promote oxidative stress and cell death, along with the capacity of AM to counteract these two phenomena.

- **Intracellular ROS measurement**

To this end, HUVECs were challenged with CD sera in the presence or absence of a pre-treatment with a single dose of AM (10 mg/mL). Cells treated with healthy donors (HD) were utilized as a control. As expected, CD sera were able to generate an increase in ROS levels compared to HD sera, a phenomenon that was significantly counteracted by the AM pre-treatment (Figure 27a).

- **Cell viability (MMA)**

In line with the observed CD-induced oxidative stress, cell treatment with the pathological sera also significantly increased cell mortality (Figure 27b). Also in this case, AM pre-treatment significantly reduced CD-induced cell mortality to values close to HD (Figure 27b). The current findings indicate that AM extract (10µg/mL) can counteract CD sera-induced oxidative stress and associated cell death providing a protective effect against pro-oxidant factors present in the sera of CD patients.

V. 5. Evaluation of the anti-inflammatory activity of *A. maritima* and *B. bituminosa in vitro*

An overall anti-inflammatory effect was displayed by both plants' extracts in a dose-dependent manner. Interestingly; AM, BB, and BBC extracts exerted a denaturation inhibition ability with the lowest IC_{50} values of 131.07 ± 0.027, 148.5 ± 0.034, and 151 ± 0.024 µg/mL respectively, whereas the concentration of 250 µg/mL was the most effective for these extracts with inhibition percentages of 81.4 ± 1.38, 73.09 ± 1.24, and 79.27 ± 0.96 respectively (Figure 28a) and (Figure 29a, b). These values represent two folds of the Voltaren® IC_{50} (63.87 ± 1.2 µg/mL, $p < 0.0001$) with a percentage of inhibition at 250 µg/mL of 99.07% ± 0.44, but remained close to the Ketoprofen® IC_{50} (165.96 ± 1.39 µg/mL) with 73.57% ± 0.11 ($p > 0.05$), with the Voltaren® being more effective than the Ketoprofen® ($p < 0.0001$) (Figure 28e, f). Moreover, a moderate inhibition was exerted by BBA and AMB extracts with respective values of 241.7 ± 0.038 and 177.4 ± 0.011 µg/mL (Figure 29c) and (Figure 28d) whereas no significant activity was induced by AMC, AMA extracts (Figure 28b, c), and BBB extract (Figure 29d) (Table 13).

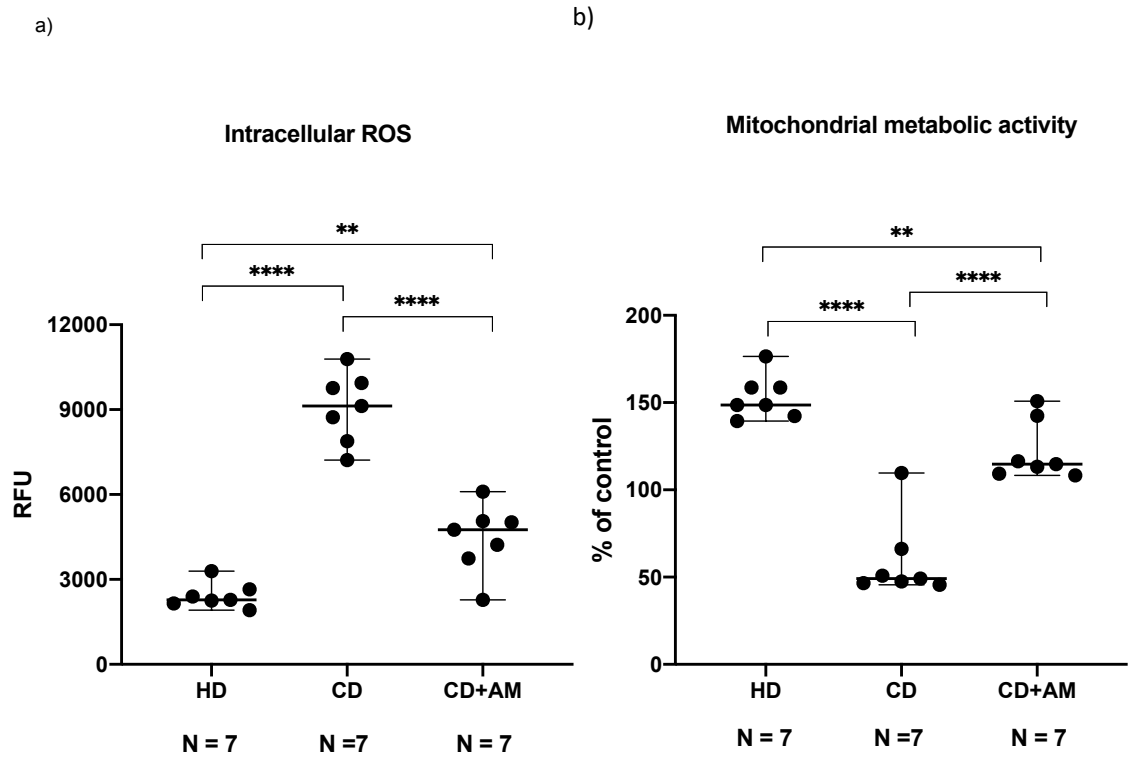

Figure 27. The pro/antioxidant effect of *A. maritima* and *B. bituminosa* on the intracellular ROS levels. a):Evaluation of the cytoprotective effect of *A. maritima* towards the oxidative endothelial cell death induced by CD sera. a): Intracellular ROS levels. b): Mitochondrial metabolic activity. HD: Cells incubated with healthy donors' sera, CD : Cells incubated with sera from Crohn's disease patients. CD+AM: Cells pretreated with AMp then incubated with CD sera. AMp: *A. maritima* phenolic extract. Results were expressed as percentage of control for mitochondrial metabolic activity and were expressed as means ± SD of measurements repeated in triplicate. Groups that do not share a letter are significantly different ($p < 0.05$ for one-way ANOVA and Newman-Keuls multiple comparison tests).

Extract	Anti-inflammatory activity (IC_{50}: µg/mL)
Voltaren®	63.87 ± 1.2 [a]
Ketoprofen®	165.96 ± 1.39 [b]
AM	131.07 ± 0.027 [c]
AMC	>250
AMA	>250
AMB	241.7 ± 0.038 [d]
BB	148.5 ± 0.034 [e]
BBC	151 ± 0.024 [f]
BBA	177.4 ± 0.011[g]
BBB	>250

Table 13. The anti-inflammatory activity of *A. maritima* and *B. bituminosa in vitro*
IC_{50}: sample concentration at which 50% of the protein denaturation was inhibited. AM: Hydroethanolic extract, AMC: Chloroform extract, AMA: Ethyl acetate extract, AMB: Butanolic extract of *A. maritima*. BB: Hydroethanolic extract. BBC: Chloroform extract, BBA: Ethyl acetate extract, BBB: Butanolic extract for *B. bituminosa*

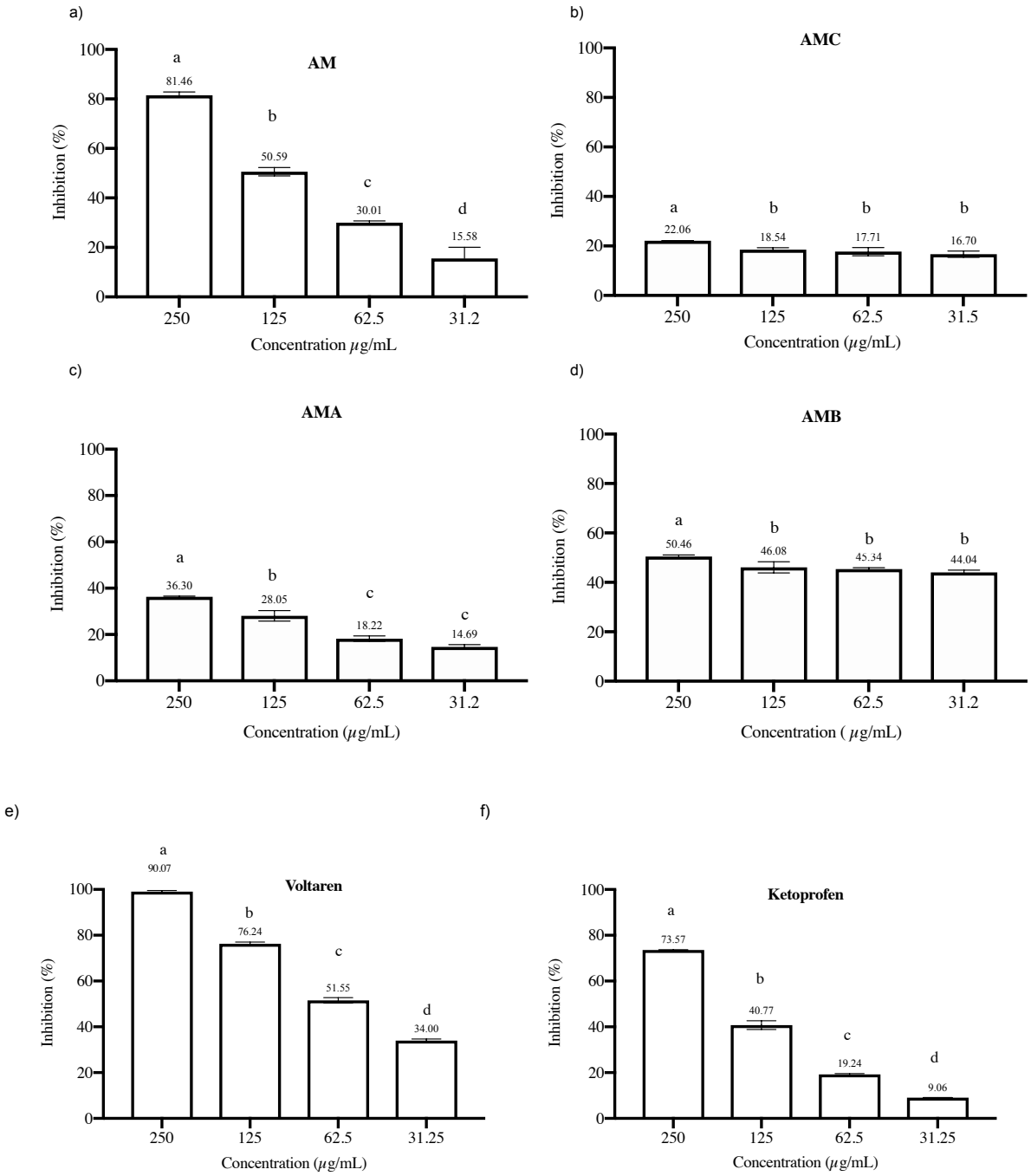

Figure 28. The pro/antioxidant effect of *A. maritima* and *B. bituminosa* on the intracellular ROS levels. a):Anti-inflammatory effect of *A. maritima*. a, b c, d, e, f, represent the anti-inflammatory activity of AM, AMC, AMA, AMB, Voltaren, Ketoprofen, respectively. AM: Hydroethanolic extract, AMC: Chloroform extract, AMA: Ethyl acetate extract, AMB: Butanolic extract of *A. maritima*. Values are expressed as a percentage of inhibition and as means ± SD of three parallel measurements. Values that do not share a letter are significantly different ($p < 0.05$ for one-way ANOVA and Newman-Keuls multiple comparison tests).

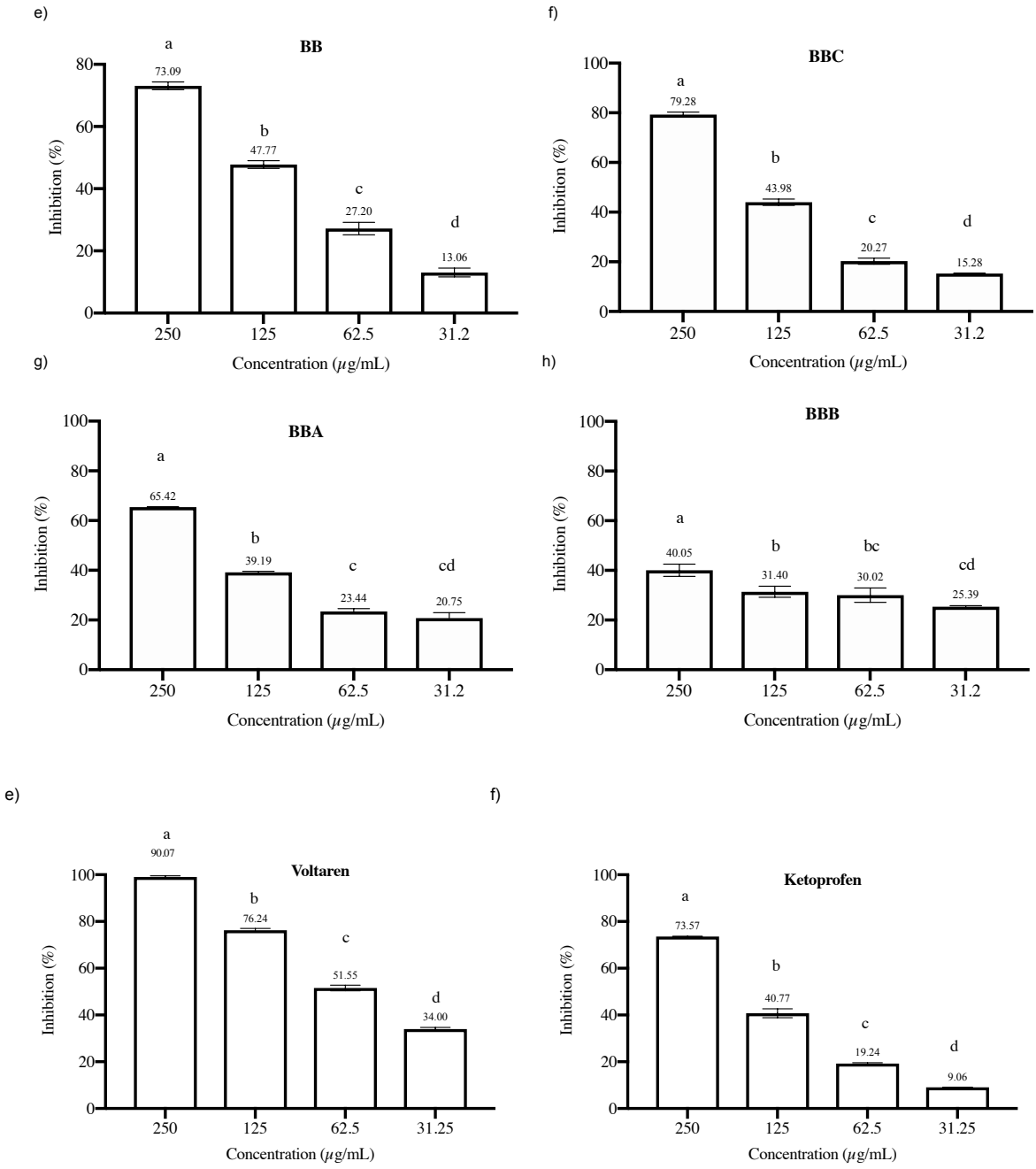

Figure 29. Anti-inflammatory effect of *A. maritima*. a, b, c, d, e, f, represent the anti-inflammatory activity of BB, BBC, BBA, BBB, Voltaren, and Ketoprofen, respectively. BB: Hydroethanolic extract. BBC: Chloroform extract, BBA: Ethyl acetate extract, BBB: Butanolic extract for *B. bituminosa*. Values are expressed as percentage of inhibition and as means ± SD of three parallel measurements. Values that do not share a letter are significantly different ($p < 0.05$ for one-way ANOVA and Newman-Keuls multiple comparison tests).

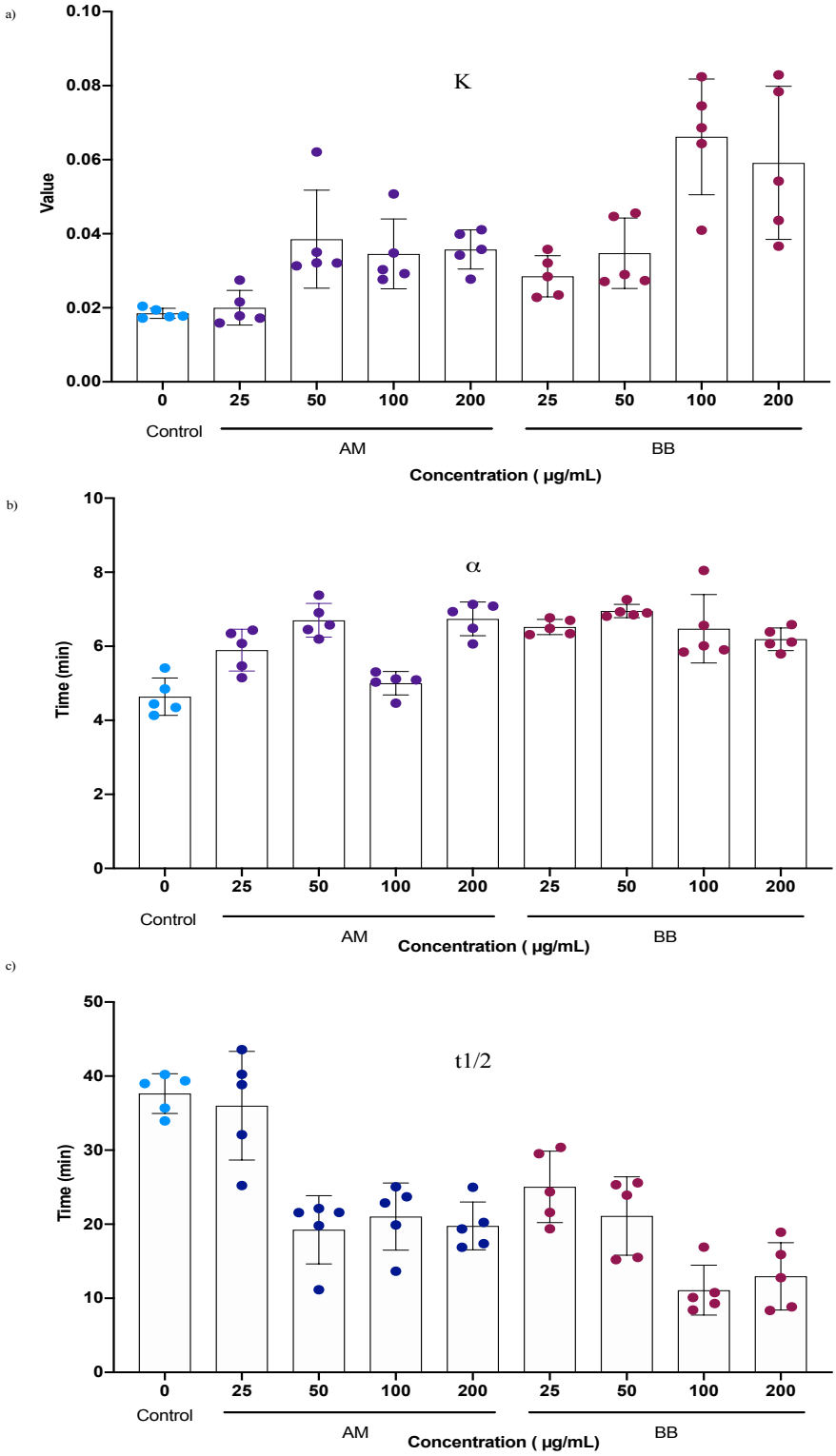

Figure 30. Aa): The phagocytic index K measurement in presence of AM and BB hydroethanolic extracts, b): corrected phagocytic index α measurement in presence of AM and BB hydroethanolic extracts. c): The half-life time of the antigen in the blood (t1/2) expressed in minutes (min) measured in presence of AM and BB hydroethanolic extracts. Groups that do not share the letter are significantly different ($p < 0.05$ for one-way ANOVA and Newman-Keuls multiple comparison tests).

Discussion

Plants and herbs' secondary metabolites are increasingly captioning a great interest as potent beneficial components of the human diet (Crozier *et al*., 2007) with a broad of traditional applications encompassing a variety of therapeutic properties related to their phytochemical composition (Dirar *et al*., 2014). Accordingly, It is well recognized that CD is a non-specific and a diffuse inflammatory pathology with a high disability rate. Its complex therapy includes conventional treatments based on steroids and salicylic acid derivatives besides other biological agents that lack effectiveness and their side effects for CD patients are well established (Bourgonje *et al*., 2019).

To date, a positive correlation between oxidative stress and Crohn's disease is believed to exist. Indeed, tissue injury and fibrosis formation associated with CD have been proven to be accompanied by an imbalance between increased ROS levels and decreased antioxidant defenses within the affected tissues (Alemany-Cosme *et al*., 2021; Alzoghaibi, 2013; Basílio *et al*., 2021). Nowadays there is a rising demand for alternative therapies with better induction and maintenance of CD remission, and the implementation of antioxidant therapy is one of many promising approaches.

In this context, alternative medicines, particularly herbal therapies, are attracting attention. In fact, up to 21%–60% of IBD patients resort to medicinal plants due to their remarkable natural efficiency in managing the disease by means of various mechanisms including antioxidant activity and immune system regulation (Ganji-Arjenaki and Rafieian-Kopaei, 2019). In this regard, several species from the Asteraceae and the fabaceae families have been extensively investigated for their antioxidant potential (Fraisse *et al*., 2011; Macêdo *et al*., 2018).

In the present work, we evaluated the phenolic profile of *A. maritima* and *B. bituminosa*, two medicinal plants from the *Asteraceae* and the *Fabaceae* families respectively, by assessing their antioxidant behaviour and capability to counteract oxidative stress impairment elicited by H_2O_2 and CD sera on HUVECs cellular model besides their anti-inflammatory *in vitro*, immunostimulatory and acute toxicity effects *in vivo*. Many studies emphasized the importance of extraction methods and their procedures as a pivotal key in the effectiveness and the quality of natural antioxidants' extracts, hence, influencing their potential bioactivity (Sasidharan *et al.*, 2011; Altemimi *et al.*, 2017; Zhang *et al.*, 2018). Temperature, type and concentration of the solvent are influential factors playing an important role on the extraction efficiency (Xu *et*

al., 2017). In this work we used both low pressure and temperature extraction carried out in a hydroethanolic mixture (60%,40%) to prevent the degradation of thermolabile molecules.

Phenolic extracts were obtained with a yield of extraction of 17,66% and 18% respectively for *A. maritima* and *B. bituminosa*. The majority of phenolic compounds are hydro-soluble antioxidants in polar and medium polar solvents, such as water, ethanol, methanol, and their aqueous mixtures and are commonly used in the extraction (Xu *et al*., 2017). In the other side, polyphenols and their phenolic acids, have been considered as a focal point of a great deal in research because of their broad distribution in plants, their biological activities and their positive impact on health (Havsteen, 2002; Posadino *et al*., 2019, 2018, 2013). In the Asteraceae and fabaceae families, these compounds have long been characterized and studied for their antioxidant capacity.

Globally, Both phenolic and flavonoids profiles of extracts revealed a considerable levels of phenols and flavonoids content with the highest amount of phenolic content recorded by BBA extract with a value of 323,11g ±1,06 mgGAE/g our findings are in concordance with the works of (Ventura *et al*., 2019) which detected important amounts of total phenols in *B. bituminosa* and other varieties of the same plant. Whereas for *A. maritima,* AMC extract showed the highest value of 138,28 ± 6.61 in term of total phenols. Close values were shown in (Said *et al*., 2018) study with an amount of 188,06 mg gallic acid equivalent from the leaf powder of the same plant suggesting it as potential source of phenols. For the TFC values *B. bituminosa* was found to be the richest with the highest values of 193,39 ± 4,15 and 158,15 ± 3,80 µg QE/mg demonstrated by BBA and BBC respectively while *A. maritima*. Indeed many flavonoids have been identified from *B. Bituminosa* (Pistelli *et al*., 2003; Sarikurkcu *et al*., 2016).

The use of high performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry to establish a fingerprint analysis of bioactive molecules from plant extracts has captured a great interest widespread especially in the separation and the isolation of flavonoids and their glycosides with a great ability to distinguish their *O*-glycosides, *C*-glycosides and *O,C*-glycosides as well as the substitution position even without a previous separation or purification from the complex (Chen *et al*., 2016).

We reported for the first time the detection of three dicaffeoylquinic acid isomers in AM: 3,4 Dicaffeoylquinic acid, 3,5 Dicaffeoylquinic acid and 4,5 Dicaffeoylquinic acid. A great body of literature reported that plants belonging to the Asteraceae family contain important amounts of caffeoylquinic acid derivatives and flavonoids (Fraisse *et al*., 2011; Imran *et al*., 2019;

Marksa *et al*., 2020). Our findings are in a line with those reported in Martinez et al study stating that 3,5 Dicaffeoylquinic acid is an abundant compound in *Ambrosia artemisiifolia L*. a species from the same genus as AM.

Two flavonoids were also detected from AMp extract Apigenin 8*C*-(-6-*O*-Feruloyl)-glucopyranoside and Kaempferol -7- *O*-glucoronide. For *B. bituminosa* different compounds corresponding to glycosylated flavonoids, especially from Apigenin, Luteolin and Kaempferol besides Isoorientin and other phenols were characterized, which is in a line with (Llorent-Martinez *et al*., 2015) who identified more than 40 phenolic compounds from the same species that corresponds majorly to glycosylated flavonoids and Isoorientin was identified for the first time in their profile. Kaempferol and its derivatives have commonly been isolated from many species belonging to the Asteraceae family (Calderon-Montano *et al*., 2011).

Accordingly, numerous biological activities and a variety of health benefits of ferulic acid, especially those against chronic human diseases and oxidative damage, are undoubtedly correlated to its antioxidant properties, which are due to its phenolic nucleus and the conjugated C3-side chain (Shahidi and Chandrasekara, 2010). Several *in vitro*, *in vivo* and epidemiological studies reported that the bioactivities and health benefits of ferulic acid, particularly against chronic diseases related to oxidative damage are undeniably linked to its higher antioxidant capacities in comparison with other related compounds, strongly associated to its phenolic nucleus, the conjugated C3 side chain and the hydroxyl group (Fraisse *et al*., 2011).

It is also common for naturally occurring components to bear 1 or more feruloyl moieties in their skeleton easily recognizable by mass spectrometry (MS) data, and they are found to display relevant biological activities, It has been also stated that ferulated compounds may be even stronger antioxidants that the acid itself. To note, a wide range of sugar monomers substitutions linked to oxygen at carbons in various positions of the skeleton including glycosyl also occur in ferulated flavonoids (Fraisse *et al*., 2011; de Oliveira Silva and Batista, 2017). Sugar and their acids substitution is common in plants derived flavonoids and attribute them a health promoting properties. This conjugation is also found to influence their cellular uptake but also their anti/pro-oxidant properties since the conjugated forms of some flavonoids exhibit a stronger antioxidant activity than the parent flavonoid (Boersma *et al*., 2002), and our LC/MS fingerprint analysis showed the presence of both glycosylated and glucuronated flavonoids.

Though the cytotoxic effect of leads from AM have been reported in the literature (Dirar *et al*., 2014a; Dirar *et al*., 2014b). AMp extract showed a moderate cytotoxicity in high concentrations and seems to be harmless in low doses. Despite of the safety of BBp extract in the tested doses many studies demonstrated the toxicity of particular compounds from the same plant particularly pterocarpans, furanocoumarins and isoflavones (D'Angiolillo *et al*., 2014).

Furthermore, we realized *in vivo* acute toxicity test to evaluate the safety of our plant extract. Both of our plants showed no toxicity or mortality towards mice. Overall, herbal medicines are considered as nontoxic and effective if used rationally (Ardalan and Rafieian-Kopaei, 2013). It has been estimated that 25% of modern drugs are based on molecules from plants; first used traditionally, such as Aspirin (Ephedrine), and Paclitaxel (Artemisinin). However, an inappropriate or abused use of herbal medicines can cause deleterious and dangerous effects (Zhang *et al*., 2015).

The antioxidant activity of our plants was chemically tested *in vitro* using five different methods with the lowest IC_{50} recorded for the GOR test with a value of 14.33±3.86 µg/mL for *A. maritima*. Similar results have been shown in Nazik's works showing that some fractions purified from AM demonstrated a remarkable antioxidant activity fluctuating in a range of (80% - 86%) using DPPH test (Nazik *et al*., 2020). It is widely accepted that applying several tests allow the assessment of the antioxidant activity with different targets within the matrix. For example, ABTS technique, is based on the decolorization in presence of antioxidants (like carotenoids, phenolic compounds and derivatives). While the DPPH technique is based on the fact that the antioxidant behave as a hydrogen donor. Cu (II) can also be used as an oxidant in the determination of the antioxidant potential in the CUPRAC assay (Carocho and Ferreira, 2013).

Accumulated data from literature reported polyphenols such as caffeoylquinic acid and derivatives for being major antioxidant metabolites (Fraisse *et al*., 2011; Kim and Lee, 2005; Mijangos-Ramos *et al*., 2018). In the other side, antioxidant properties are widely linked to flavonoids as well and have been identified to fulfil the main criteria for possessing an antioxidant properties mainly including the protection of the antioxidant system and the ROS scavenging (Fraisse *et al*., 2011; Kim and Lee, 2005; Mijangos-Ramos *et al*., 2018). Accordingly, several studies related the variation of antioxidant properties of flavonoids to their degree of hydroxylation, methoxylation, and the glycosylation of A, B and C rings which results

in derivative structures of the parent flavonoid (Pietta, 2000). Interestingly, a great body of data reported the antioxidant activity of Apigenin and derivatives mainly related to their capacity to upregulate the expression of antioxidant enzymes (Chen *et al*., 2016). Besides, several studies reported that the antioxidant activity of Kaempferol is essentially attributed to the degree of conjugation and hydroxylation that is responsible for the electrons delocalization (Dirar *et al*., 2014; Pietta, 2000).

Effectively, the recent interest in polyphenols as potent antioxidants, due to their widespread consumption in foods, was controverted by accumulating evidence showing that they exhibit a biphasic concentration-dependent effects, acting as antioxidants at low concentrations and as prooxidants at high concentrations. Such an aspect seems associated and influenced by the antioxidant type, dosage, and redox environment, which are emerging pivotal determinants in affecting the equilibrium between their beneficial and deleterious effect by impacting the intracellular redox homeostasis. Particularly, flavonoids and phenolic acids are a good example of such dual behaviour, noticeably, quercetin, myricetin, kaempferol, resveratrol and caffeic, chlorogenic and ferulic acids (Bouayed, 2010; Kung *et al*., 2021).

Moreover, Galati and O'Brien works reported the ability of some individual dietary polyphenols to induce mitochondrial mediated cell death and apoptosis in a dose dependent manner (Galati and O'Brien, 2004). In line with the literature, our current results revealed a dual behaviour of the AMp phenolic extract, both cytoprotective and cytotoxic, on primary HUVECs. This peculiar extract behaviour has been further confirmed by assessing its effect on the intracellular ROS levels under the same experimental conditions confirming its low concentration-associated antioxidant effect and its high dosage-associated pro-oxidant properties.

Such extract peculiar aspect can be preassembly justified by the presence of chlorogenic acid and kaempferol derivatives already recognized as polyphenols with a dose-dependent dual redox behaviour (Bouayed, 2010). Whereas for kaempferol, its dose related dual behaviour as an individual compound has been well established (Calderon-Montano *et al.,* 2011).
On the other hand, Apigenin was found to be safe exerting no toxicity even in high doses (Salehi *et al*., 2019) which may explain the fact that BBp exerted no toxicity on cells or appearing prooxidant effect since its HPLC-DAD fingerprint proved its richness in Apigenin derivatives. Several studies have reported the implication of oxidative stress in the onset and development

of IBD (Bourgonje *et al*., 2020, 2019; Khaloian *et al*., 2020; Luceri *et al*., 2019). In our study, low doses of AMp phenolic extract were able to counteract oxidative damage and cell death induced by sera from CD patients. The protective effect of several polyphenols and their derivatives against gastrointestinal IBD disorders like Crohn's disease (CD), ulcerative colitis (UC), and irritable bowel syndrome (IBS) have been investigated, and their antioxidant, anti-inflammatory and immunomodulatory properties (Chiu *et al*., 2021) have been demonstrated *in vivo* and *in vitro* (cell culture) experiments (Kaulmann and Bohn, 2016; Martin and Bolling, 2015).

Interestingly, experiments performed with kaempferol, hydroxycinnamic acids and derivatives showed that their consumption in a *in vivo* colitis model was able to significantly lower the disease index scores (Larrosa *et al*., 2009; Park *et al*., 2012). However, in respect to the phenolic compounds dose dependent dual redox behaviour, many studies highlighted that their adverse effect may be enhanced in the presence of free metal ions like copper or iron when the tissue is damaged, common aspects of the IBD pathology (Pallone and Monteleone, 2001). Conversely, when applied in considerable yet physiologically relevant doses, phenolic compounds are capable of reducing oxidative stress in cells oxidatively stressed or inflamed (Kaulmann and Bohn, 2016).

Parallelly, we tried to evaluate the immunostimulatory ability of both plants using the carbon clearance test *in vivo.* An overall immunostimulatory effect was induced in a dose-dependent manner by both tested extracts AM and BB. Our results are in line with other studies which reported that different plants' extracts rich in polyphenols and flavonoids exerted a considerable immunostimulatory effect (Sakhri *et al*., 2021; Slimani *et al*., 2020). Secondary metabolites such as flavonoids, polysaccharides, lactones, alkaloids, diterpenoids, and glycosides are present in several plants, and have been reported to be responsible for the plants immunomodulating properties. Several pathologies can be alternatively counteracted by immunomodulators using medicinal herbs, instead of chemotherapy. The discovery and purification of more specific immunomodulatory components from plant origin is increasingly becoming a tempting lead (Jantan *et al*., 2015).

Proteins denaturation is a well-documented effect of alterations in the biological, chemical, and physical properties of cellular proteins by a slight disruption of their structure, which is also known to be a net aspect of macromolecules damage during inflammation (Eckersall and Bell, 2010; Reuter *et al*., 2010). In this regard, we assessed the ability of our extracts to inhibit the

thermal denaturation of serum bovine albumin protein (BSA). AM, BB, and BBC extracts showed the best anti-denaturation effect in a dose-dependent manner with the lowest IC_{50} values. Accordingly, many flavonoids' rich plants proved an anti-inflammatory activity by exhibiting an anti-denaturation capacity (Bakhouche *et al*., 2021; Djuichou-Nguemnang *et al*., 2019; Lekouaghet *et al*., 2020; Naz *et al*., 2017; Pradhan *et al*., 2021). Previous studies have also discussed the anti-inflammatory effects of both *A. maritima* and *B. bituminosa,* in relation to their phytochemical profile (Mohamed *et al*., 2020; Pistelli *et al*., 2003).

We can suggest here that the anti-inflammatory activity of our plant could be attributed to their rich flavonoids contents, since these latter are known to exert antioxidative and anti-inflammatory properties, mainly due to their capacity to modulate key cellular protein functions (Panche *et al*., 2016).

Since both inflammation and oxidative stress are the main self-defense strategies of our bodies to counteract pathogens, plants with anti-inflammatory and/or radical scavenger properties are considered as a potential source of new alternative drugs with less side effects and low cost. In this paper, antioxidant, anti-inflammatory and immunomodulatory effects have been demonstrated for *A. maritima* and *B. bituminosa,* by elucidating lipids anti-peroxidation and protein anti-denaturation effects, and were suggested to be linked to their flavonoids rich content. Along with their ability to stimulate the phagocytic system, their safety as phytochemical components source was proven *in vivo*. Further investigations should be conducted for adequate applications of these plant-derived extracts as alternative drugs for conventional treatments of immune system disorders an related infectious pathologies.

Finally, it is important to state that experimental trials (*in vitro* and *in vivo*) have their own limitations concerning CD investigation since they have been performed with native components without taking in consideration the effect of the digestion and the eventual interference with microbial metabolism which may reduce their bioavailability in the gastrointestinal system. Besides, the monolayer cell culture model show limitations like the absence of immune cells and microbiota interactions, short term exposure to stress conditions and the possible changes of the phenolic profile due to digestion process (Kaulmann and Bohn, 2016). Hence, a well-established and designed complexed model is required to better study the effect of natural compounds, especially polyphenols, on the amelioration of the CD redox state. In particular the environmental redox state, the microbial interactions and immune system

modulation aspects need to be taken into consideration to better understand the bioactivity of phenolic compounds in the context of this pathology.

Conclusions and perspectives

Since both inflammation and oxidative stress are the main self-defense strategies of our bodies to counteract pathogens, plants with anti-inflammatory and/or radical scavenger properties are considered as a potential source of new alternative drugs with less side effects and low cost.
Our work concerning the impact of the *A.maritima* and *B. bituminosa* on different *in vitro* and *in vivo* experimental models regarding oxidative stress, inflammation and immunomodulation show relevant findings. *A.maritima* and *B. bituminosa* extracts displayed a remarkable levels of antioxidant activity *in vitro* and *A.maritima* and was able to counteract cell death and oxidative stress elicited by both H_2O_2 and sera from CD patients in cultured HUVECs. interestingly, low doses of AMp extract exhibited an antioxidant beneficial effect, while high doses showed an adverse effect as the extract acted as a prooxidant inducing ROS increase and cell death.

We hypothesized that this comportment may explained the extract phytochemical composition. Some interesting antioxidant phenolic compounds were detected in this plant extract for the first time di-caffeyolquinic acid isomers, besides apigenin and kaempferol two well-established antioxidant compounds, While a range of Apigenin glycosylated derivatives, commonly found in *Bituminaria* genus, were characterized in *B. bituminosa*.

Parallelly, anti-inflammatory and immunomodulatory effects have been also demonstrated for our studied plants by elucidating protein anti-denaturation effects and the immunostimulant ability in mice. Globally these bioactivities were suggested to be linked to their Phenols' flavonoids' rich content. Along with their ability to stimulate the phagocytic system, their safety as phytochemical components source was proven *in vivo*. Take together our data indicate that *Ambrosia maritima* extract is a potential source of antioxidants that can have applications in ameliorating the redox state impairment associated to the CD pathology.

Nevertheless, further large-scale trials are required to better understand the mechanism of action of this extract including *In vitro, In vivo* and clinical based experimentation to better understand their bioavailability and their interference with this pathology's different stages; and Further investigations should be conducted for adequate applications of these plant-derived extracts as alternative drugs for conventional treatments of immune system chronic disorders an related infectious pathologies. The following techniques can be realized in the future:

- Purification of respective characterized compounds also applied to other extracts.

- Multiplying the number of *in vivo* and *in vitro* experimental models to also include assays on pure compounds.

- Realize analyses of other biomarkers and checkpoints including Cytokines immune cell lines, epithelial cells, gene expression, metabolites derivatives …..etc.

- Increase the number of patients and enlarge the study to include ulcerative colitis patients to gain broad information about our extracts' efficiency.

- Propose a pharmacological form of the possible active molecules.

- Realize other tests to include clinical assays to reach more evident results about the efficiency of our plants regarding IBD disease.

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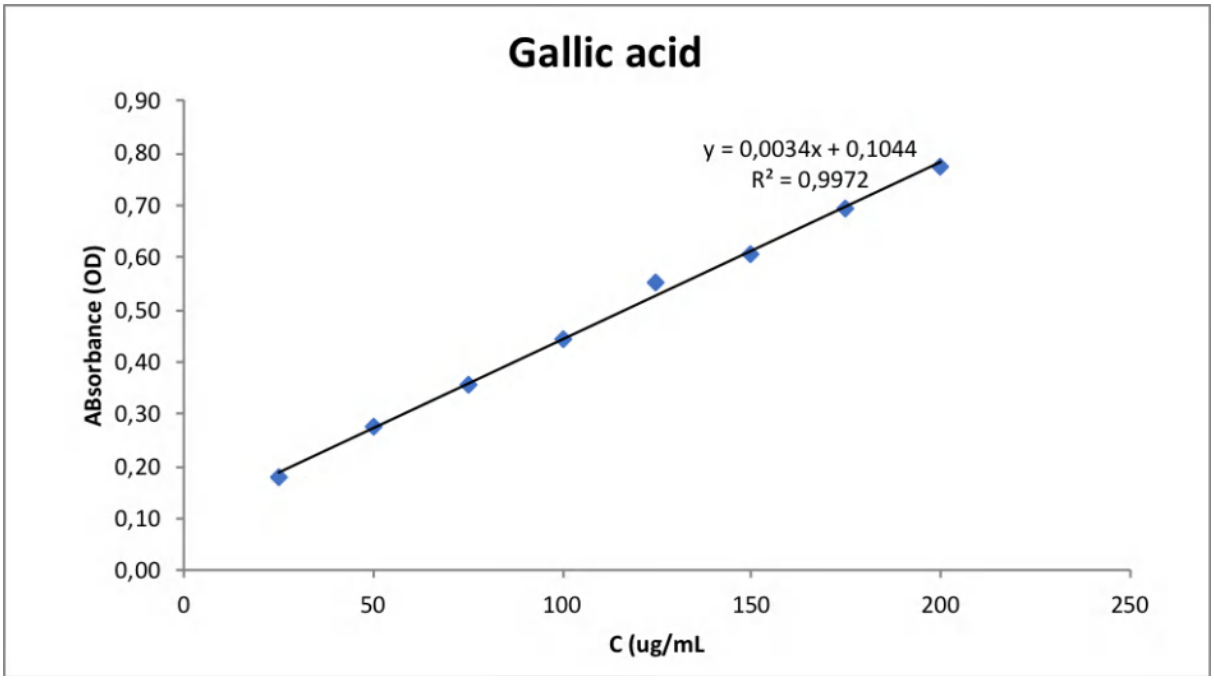
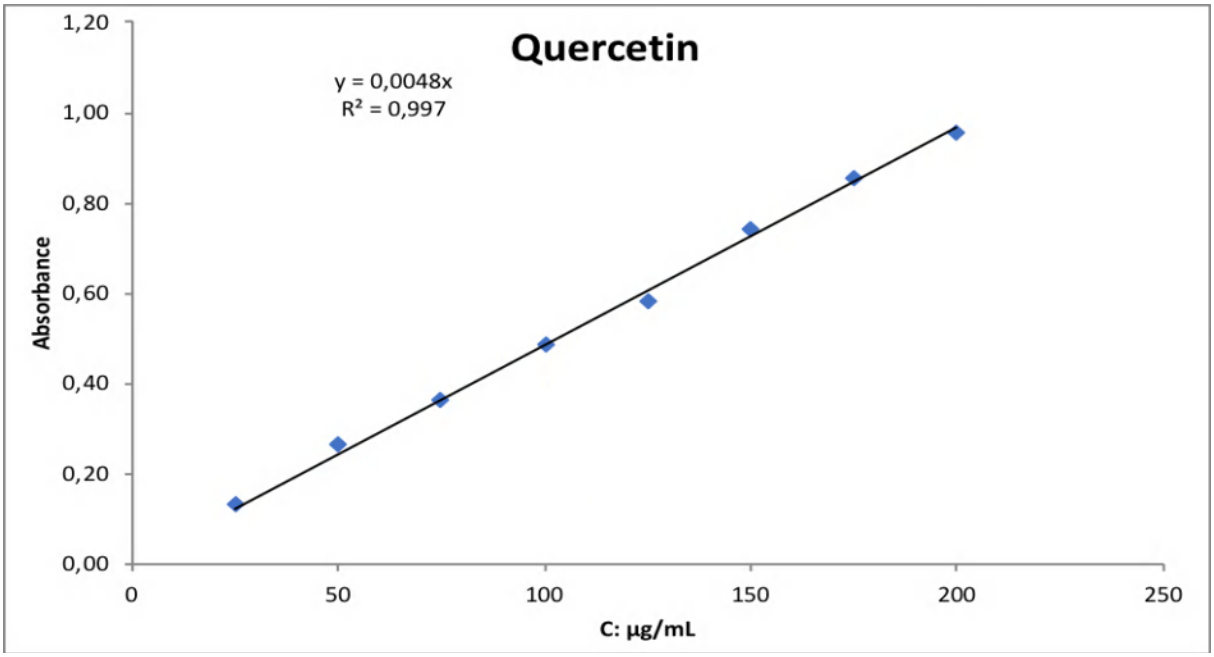
Appendices

Appendices

I. Calbiration curves



1. Calibration curve of Gallic acid



1. Calibration curve of Quercetin

Abstracts

Résumé

Dans ce présent travail, *Ambrosia Maritima* et *Bituminaria bituminosa* ont été évaluées pour leur composition phénolique, leur activité antioxydante *in vitro* et leur effet cytoprotecteur dans la culture des cellules endothéliales primaires de la veine ombilicale humaine (HUVEC) traitées avec du peroxyde d'hydrogène (H_2O_2) et du sérum des malades atteints de la maladie de Crohn (CD), en plus de l'évaluation de leur potentiel anti-inflammatoire et immunostimulant. L'extraction a été réalisée selon deux méthodes: Macération suivie d'une extraction liquide-liquide et d'une extraction à basse température. L'activité antioxydante a été évaluée par des méthodes chimiques et par la méthode HDCFDA (Indicateur de stress oxydatif) dans la culture des cellules HUVECs. L'activité anti-inflammatoire a été testée par la méthode d'anti-dénaturation de protéine. La toxicité aiguë et l'effet immunostimulant ont été réalisés chez la souris. Les extraits BBA, l'AMA et l'AMC étaient les plus riches en terme de teneur en flavonoïdes (respectivement 193,39±24,15 et 158,15±3,8 µg QE/mg). L'évaluation des phénols totaux a révélé que *A. maritima* et *B. bituminosa* présentaient une importante teneur phénolique, en particulier BBA et BBB (323,11±0,76 et 198,31±0,76 µg QE/mg, respectivement). Trois isomères de l'acide di-caféoylquinique ont été détectés pour la première fois dans l'extrait AMp tandis qu'une gamme commune de dérivés d'apigénine caractérisait l'extrait BBp. Une activité antioxydante *in vitro* significative a été rapportée pour les extraits AMA, AMC et AMB avec des valeurs de IC_{50} de 19,90±0,01, 11,72±0,79 et 19,90±0,01 µg/mL, respectivement, prouvant une capacité antioxydante pertinente de notre plante *A. maritima*. Les résultats de viabilité et de niveaux de ROS obtenus respectivement avec le MTT et le H2DCFDA ont démontré l'effet prooxydant et cytotoxique des sérums CD sur les HUVEC. En revanche, l'extrait AM a exercé un effet biphasique dose-dépendant sur les cellules traitées au H2O2, respectivement cytoprotecteur et antioxydant à faibles doses et cytotoxique et prooxydant à fortes doses, tandis que l'extrait de BBp n'a montré aucune cytotoxicité ou comportement oxydatif apparent. De plus, à la concentration de 10 µg/mL, l'extrait AMp a pu contrecarrer à la fois le stress oxydatif induit par les sérums des CD et la mort cellulaire. Les plantes étudiées n'ont indiqué aucun signe de toxicité ou de mortalité *in vivo*. Globalement Un effet immunostimulant dose-dépendant global a été induit. Nous suggérons *A. maritima* et *B. bituminosa* comme source puissante d'agents antioxydants. D'autres essais sont requis afin de mieux élucider leurs actions sur le système immunitaire et sur la pathologie de la maladie de Crohn.

Mots clés : *A. maritima*, *B. Bituminosa*, Maladie de Crohn, Stress Oxidatif, Inflammation, Phénol.

ملخص

في هذا العمل قمنا بتقييم كل من *Ambrosia Maritima* و *Bituminaria bituminosa* وذلك عن طريق دراسة التركيب الفينولي لكليهما، بالإضافة لنشاطهما المضاد للأكسدة مخبريا، وتأثيرهما الواقي للخلايا في الخلايا البطانية الأولية للوريد السري البشري المستزرعة (HUVECs) المعالجة ببيروكسيد الهيدروجين (H_2O_2) والمصل من مرضى كرون ، إلى جانب قدرتها المضادة للالتهابات وتنشيط المناعة. تم إجراء الاستخراج بطريقتين: التعطين يليه الاستخلاص السائل والسائل والاستخلاص تحت درجة حرارة منخفضة. تم تقييم النشاط المضاد للأكسدة باستخدام الطرق الكيميائية وفي الوسط الزراعي لخلايا HUVEC باستخدام الكاشف HDCFDA .تم اختبار النشاط المضاد للالتهابات عن طريق تقييم قدرة المستخلصات على حماية البروتينات من التمسخ . تم فحص السمية الحادة والتأثير المناعي على الفئران. وجدنا أن BBA و AMA و AMC كانت الأغنى من حيث الفينولات خاصة الأغنى من حيث محتوى الفلافونويد (193.39±24.15 ، و 158.15± 3.8 ميكروجرام من / QE مجم ، على التوالي). أظهر تقييم إجمالي الفينولات أن كلا من *A. maritima* و *bituminosa* أظهروا محتوى فينوليًا مهمًا ، حيث أظهر BBA و BBB أعلى الكميات (323،11±0،76 و 198،31± 0،76 ميكروغرام / مجم).من جهة أخرى تم اكتشاف ثلاثة أيزومرات حمض الكافويلكوينيك لأول مرة في AMp بينما وجدنا مجموعة من مشتقات الأبجينين بخلاصة BBp.باستخدام طرق تحليلية مختلفة ، قمنا بتحديد النشاط المضاد للأكسدة مخبريا لـ AMA و AMC و AMB بقيم 19.90±0.01 IC50 و 11.72±0.79 و 19.90±0.01 ميكروجرام / مل ، على التوالي ، مما يثبت قدرة مضادات الأكسدة ذات الصلة لـ *A. maritima*. أظهرت نتائج الجدوى ومستويات ROS التي تم الحصول عليها على التوالي باستخدام MTT و H2DCFDA تأثير بروكسيد مصل CD وتأثير سام للخلايا علىHUVECs . في المقابل ، أظهر مستخلص AM تأثيرًا ثنائي الطور يعتمد على الجرعة على الخلايا المعالجة بـH_2O_2 ، مضادا للخلايا ومضادا للأكسدة على التوالي بجرعات منخفضة وسام للخلايا ومضاد للأكسدة بجرعات عالية بينما لم يظهر مستخلص BBp أي سمية خلوية أو سلوك مؤكسد. بالإضافة إلى ذلك ، عند تركيز 10 ميكروغرام / مل ، كان AMp قادرًا على مواجهة كل من الإجهاد التأكسدي الناجم عن مصل CD وموت الخلايا. المستخلصات AM و BB و BBB أظهرت أفضل تأثير مضاد للتمسخ بطريقة تعتمد على الجرعة بأقل قيم 131.07±0.027 IC50 و 148.5±0.034 و 151±0.024 ميكروغرام / مل على التوالي. لم تظهر النباتات المدروسة أي سمية أو موت حيويا. كما تم إثبات إحداث تأثير مناعي شامل بطريقة تعتمد على الجرعة. إذن نقترح *A. maritima* و *B. bituminosa* كمصدر فعال لعوامل مضادات الأكسدة لمزيد من التجارب لتوضيح تأثيرها بشكل أفضل على جهاز المناعة وعلى مرض كرون.

الكلمات المفتاحية: *A. maritima*، *B. Bituminosa* ، مرض كرون ، الإجهاد التأكسدي ، الالتهاب ، الفينولات

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Related-oxidative stress effect and biological activities of biomolecules from Algerian medicinal plants on the model of sera from Crohn's disease patients

Thesis submitted for the obtention of Doctorat degree in Biochemistry and pharmacotoxicology

Abstract

Here, an *Ambrosia maritima* and *Bituminaria bituminosa* were assessed for their phenolic profile composition, their potential antioxidant activity *in vitro,* and their cytoprotective effect in primary cultured human umbilical vein endothelial cells (HUVECs) treated with hydrogen peroxide (H_2O_2) and sera from CD patients, besides their anti-inflammatory and immunostimulatory potential. Extraction was performed using two methods: Maceration followed by liquid-liquid extraction and low temperature extraction. The anti-antioxidant activity was assessed using chemical methods and in HUVECs cell culture using HDCFDA probe method. The anti-inflammatory activity was tested by performing the protein anti-denaturation assay. Acute toxicity and immunostimulatory effect were performed in mice. BBA, AMA and AMC while were the richest in term of phenols were the richest in terms of flavonoids content (193.39 ± 24.15, and 158.15 ± 3.8 µg QE/mg, respectively). The evaluation of total phenols revealed that both *A. maritima* and *B. bituminosa* demonstrated an important phenolic content, with BBA and BBB showing the highest amounts (323,11±0,76 and 198,31±0,76 µg QE/mg, respectively) Three di-caffeoylquinic acid isomers detected for the first time in AMp while a common range of apigenin derivatives characterized the BBp extract. Using different analytical methods, a significant *in vitro* antioxidant activity was reported for AMA, AMC and AMB with IC_{50} values of 19.90 ± 0.01, 11.72 ± 0.79 and 19.90 ± 0.01 µg/mL, respectively, proving a relevant antioxidant ability of *A. maritima.* Viability and ROS levels result obtained respectively with MTT and H_2DCFDA demonstrated CD sera's prooxidant and cytotoxic effect on HUVECs. In contrast, the AM extract showed a biphasic dose-dependent effect on H_2O_2-treated cells, respectively cytoprotective and antioxidant at low doses and cytotoxic and prooxidant at high doses while BBp extract showed no appearing cytotoxicity or oxidative behaviour. Additionally, at the concentration of 10µg/mL, AMp was able to counteract both CD sera-induced oxidative stress and cell death.. AM and BB and BBB showed the best anti-denaturation effect in a dose-dependent manner with the lowest IC_{50} values of 131.07 ± 0.027, 148.5 ± 0.034, and 151 ± 0.024 µg/mL, respectively. The studied plants showed no toxicity or mortality *in vivo*. An overall immunostimulatory effect was induced in a dose-dependent manner. We suggest *A. maritima* and *B. bituminosa* as potent source of antioxidant agents for further assays to better elucidate their actions on the immune system and in Crohn's disease pathology.

Key words: *A. maritima*, *B.bituminosa*, Crohn's disease, Oxidtaive stress, Inflammation, Phenols