Universidad de Oriente
Facultad de Ciencias Naturales y Exactas
Departamento de Farmacia

University of Antwerp
Faculty of Pharmaceutical, Biomedical
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Anti-inflammatory potential of the aerial parts of *Adelia ricinella* L.

Dissertation submitted in fulfillment of the requirements for the degree of
Doctor in Health Sciences

Dissertation submitted in fulfillment of the requirements for the degree of ***Doctor in Pharmaceutical Sciences*** (Ph.D)

Clara Azalea Berenguer Rivas

Santiago de Cuba
2022

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Santiago de Cuba
2022

The secrets are in the plants, to elicit them
you have to love them enough

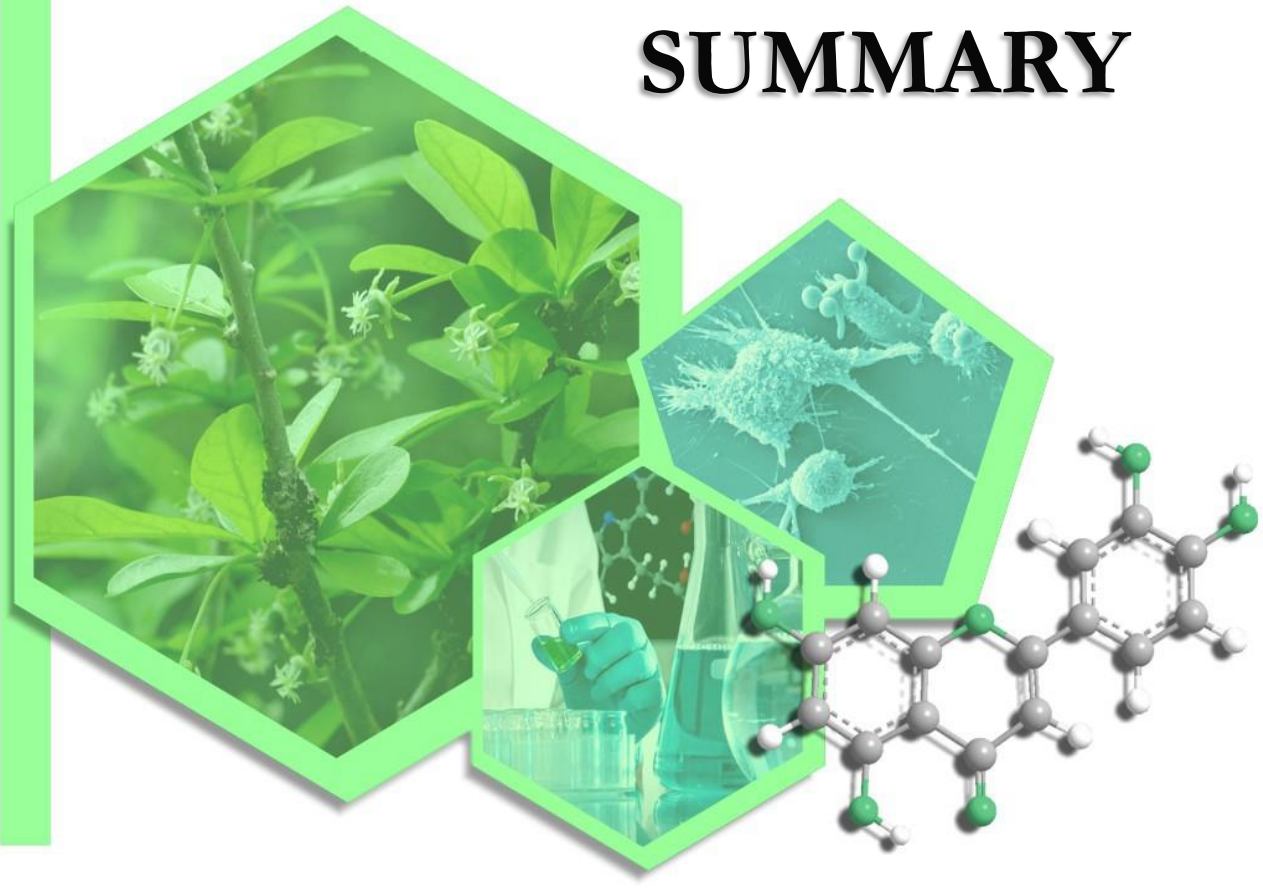
George Washington Carver

To my children: Sofía and Luis Andrés

To my parents

To my husband

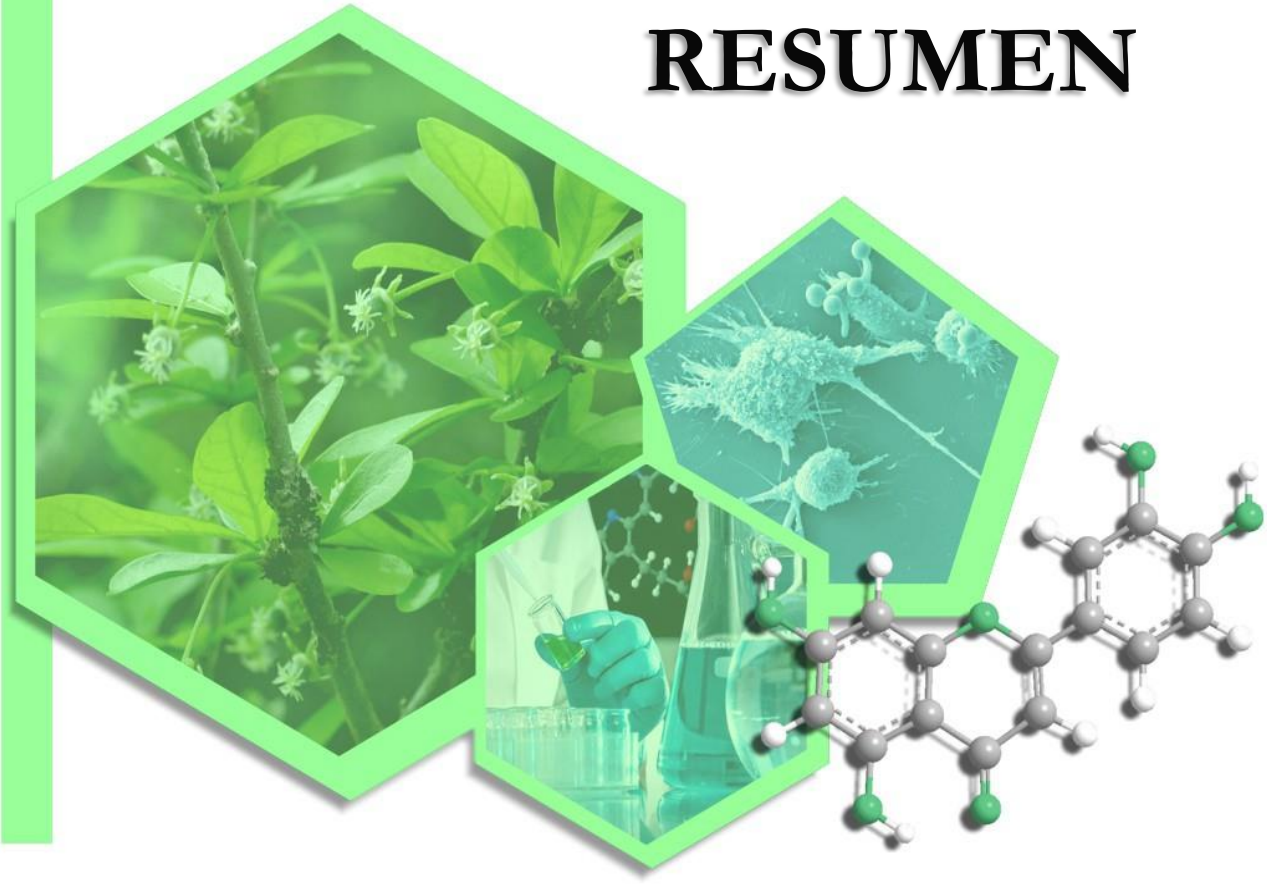
SUMMARY



SUMMARY

Adelia ricinella L is a species that belongs to the Euphorbiaceae family, traditionally used for its anti-inflammatory and analgesic properties. However, to the best of our knowledge there are not scientific evidences published which validate its therapeutic properties. That is why; the present study aimed to evaluate the anti-inflammatory activity of extracts from the aerial parts of this species. Three different extracts were prepared by soxhlet extraction using water, ethanol and an equal ratio mixture of both solvents. The pharmacognostic parameters of the raw material and the three extracts generated: 95% ethanol (AR1), 50% ethanol (AR2) and aqueous (AR3) were evaluated as the first reported quality control criterion. On the other hand, the three extracts phytochemical profiles were similar, with high concentration of polyphenols in particular flavonoids, specially in AR2. The UPLC-DAD-MS/MS analysis allowed to tentatively identify 13 flavonoids derived from luteolin and apigenin as basic structures (C- and O-glycosylated). Orientin and vitexin were the most abundant compounds and luteolin and apigenin were quantified by HPLC-DAD-MS/MS, resulting more abundant in extract AR2; all first reported for this species. From the pharmacological point of view, all the extracts evaluated, especially the AR2 extract, demonstrated anti-inflammatory potential. The ability to neutralise free radicals, contribution in the eritrocyte membrane stabilisation, cytoprotective effect in 5 cell lines (RAW264.7, THP-1, Vero, RBC y HUVECs), and the capacity to inhibit the release of important inflammatory mediators such as: nitric oxide, pro-inflammatory cytokines, lipid mediators, especially PGE2 by inhibition of the enzyme cyclooxygenase (COX-1 and COX-2) was demonstrated. The extracts also inhibit leukocyte adhesion to endothelial cells and extravasation to the site of inflammation, leading to a reduction of oedema in BALB/c mice using the acute paw oedema model. These results constitute the first *in vitro* and *in vivo* evidences of this species aerial parts and reveal its anti-inflammatory potential for the future development of herbal products.

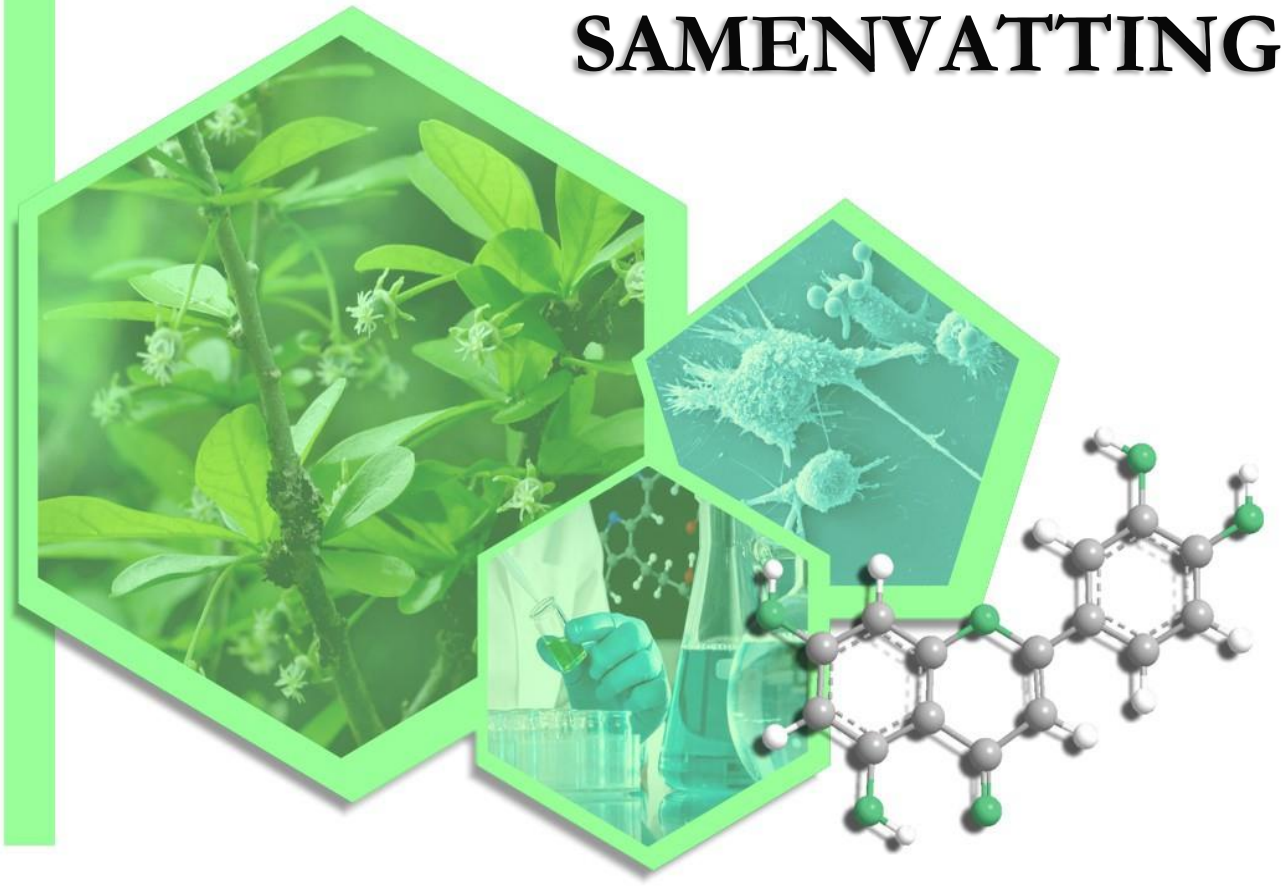
RESUMEN



RESUMEN

Adelia ricinella L es una especie perteneciente a la familia Euphorbiaceae, utilizada tradicionalmente por sus propiedades antiinflamatorias y analgésicas. Sin embargo, no se han publicado evidencias científicas que validen sus propiedades terapéuticas. Por ello, el presente estudio tuvo como objetivo evaluar la actividad antiinflamatoria de los extractos de las partes aéreas de esta especie. Para ello se prepararon tres extractos diferentes mediante extracción soxhlet utilizando agua, etanol y una mezcla en igual proporción de ambos disolventes. Al material vegetal y a los extractos obtenidos: etanol 95% (AR1), etanol 50% (AR2) y acuoso (AR3) se le determinaron por primera vez sus principales parámetros farmacognósticos como criterio de control de calidad. Los perfiles fitoquímicos de los tres extractos evaluados fueron similares con una alta concentración de polifenoles en particular de flavonoides, especialmente en el AR2. El análisis UPLC-DAD-MS/MS permitió identificar tentativamente 13 flavonoides derivados de la luteolina y la apigenina como estructuras básicas (C- y O-glicosiladas). La orientina y la vitexina fueron los compuestos más abundantes y la luteolina y la apigenina se cuantificaron por HPLC-DAD-MS/MS, siendo más abundantes en el extracto AR2. Todos ellos, constituyen los primeros reportes para esta especie. Desde el punto de vista farmacológico, todos los extractos evaluados demostraron su potencial antiinflamatorio, en especial el AR2. Demostrándose su capacidad para neutralizar los radicales libres, su contribución a la estabilización de la membrana eritrocitaria, su efecto citoprotector en 5 líneas celulares (RAW 264.7, THP-1, Vero, RBC y HUVECs), y su capacidad para inhibir la liberación de importantes mediadores inflamatorios como: óxido nítrico, citoquinas proinflamatorias, mediadores lipídicos, especialmente PGE2 por inhibición de la enzima ciclooxigenasa (COX-1 y COX-2). Además, fueron capaces de inhibir la adhesión de los leucocitos a las células endoteliales y la extravasación al lugar de la inflamación, lo que conduce a una reducción del edema en ratones BALB/c utilizando el modelo de edema agudo de la pata. Estos resultados constituyen las primeras evidencias *in vitro* e *in vivo* de las partes aéreas de la especie y revelan su potencial antiinflamatorio para el futuro desarrollo de un producto herbarios.

SAMENVATTING



SAMENVATTING

Adelia ricinella L. is een soort die behoort tot de familie van de Euphorbiaceae, die van oudsher wordt gebruikt om haar ontstekingsremmende en pijnstillende eigenschappen. Voor zover wij weten zijn er echter geen wetenschappelijke bewijzen gepubliceerd die de therapeutische eigenschappen ervan valideren. Daarom, werd in de huidige studie de ontstekingsremmende werking van extracten van de bovengrondse delen van deze soort geëvalueerd. Drie verschillende extracten werden bereid door soxhletextractie met water, ethanol en een mengsel in gelijke verhouding van beide oplosmiddelen. De farmacognostische parameters van de grondstof en de drie gegenereerde extracten: 95% ethanol (AR1), 50% ethanol (AR2) en waterig (AR3) werden geëvalueerd als het eerste gerapporteerde kwaliteitscontrolecriterium. Anderzijds vertoonden de drie extracten soortgelijke fytochemische profielen, met een hoge concentratie polyfenolen, met name flavonoïden, vooral in AR2. Met de UPLC-DAD-MS/MS-analyse konden 13 van luteoline en apigenine afgeleide flavonoïden voorlopig als basisstructuren worden geïdentificeerd (C- en O-geglycosyleerd). Orientin en vitexin waren de meest overvloedige verbindingen en luteolin en apigenin werden gekwantificeerd door HPLC-DAD-MS/MS, wat resulteerde in een grotere overvloed in extract AR2; alle voor het eerst gerapporteerd voor deze soort. Vanuit farmacologisch oogpunt vertoonden alle geëvalueerde extracten, met name het AR2-extract, een ontstekingsremmend potentieel. Aangetoond werd het vermogen om vrije radicalen te neutraliseren, een bijdrage tot de stabilisatie van de eritrocytmembraan, een cytoprotectief effect bij 5 cellijnen (RAW264.7, THP-1, Vero, RBC y HUVECs), en het vermogen om het vrijkomen van belangrijke ontstekingsmediatoren te remmen, zoals: stikstofmonoxide, pro-inflammatoire cytokinen, lipide mediatoren, met name PGE2 door remming van het enzym cyclooxygenase (COX-1 en COX-2). De extracten remmen ook de aanhechting van leukocyten aan endotheelcellen en de extravasatie naar de plaats van de ontsteking, wat leidt tot een vermindering van het oedeem bij BALB/c muizen met het acute pootoedeemmodel. Deze resultaten vormen het eerste in vitro en in vivo bewijs van de bovengrondse delen van deze soort en onthullen het ontstekingsremmend potentieel ervan voor de toekomstige ontwikkeling van kruidenproducten.

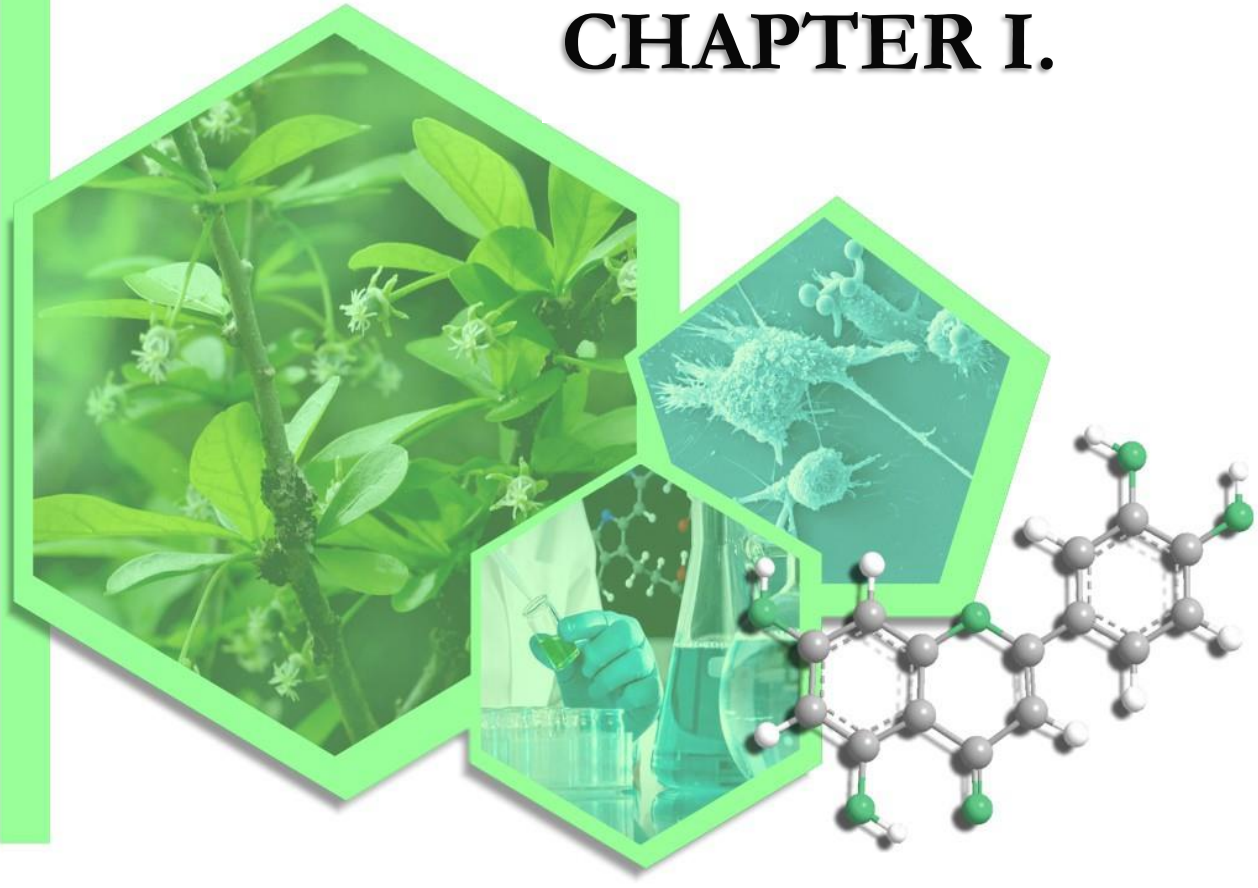
TABLE OF CONTENT

ABBREVIATION'S LIST

- ABTS:2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonicacid
- ATCC: American type culture collection
- BSA: Bovine serum albumin
- COX: Cyclooxygenase
- DAMP: Damage-associated molecular pattern
- DMSO: Dimethylsulfoxide
- DMEM:Dulbecco'sMediumEagle Modified
- DNA: Deoxyribonucleic acid
- DPPH:2,2-Diphenyl-1-picrylhydrazyl
- EC_{50}:Half maximal effective concentration
- ELISA: enzyme-linked immunosorbent assay
- ERK: extracellular signal-regulated kinase
- ESI: Electro spray ionization
- FBS: Fetal bovine serum
- GC-MS: Gas chromatography-mass spectrometry
- HETE : Hydroxyeicosatetraenoic acid
- HPETE: Hydroperoxyeicosatetraenoic acid
- HPLC: High Performance Liquid Chromatography
- HPLC-DAD: High-performance liquid chromatography-diode array detector
- HUVECs: Primary human umbilical vein endothelial cells
- IC_{50}: Inhibition Concentration-50 (Concentration of substance that produces 50% inhibition of certain process)
- ICAM: Intercellular adhesion molecule
- IKK: Inhibitor of κB kinase
- IL:Interleukins
- INF:Interferon
- JAK: Janus kinase
- JNK:JUN N-terminal kinase
- L-NAME: N5-(imino(nitroamino)methyl)-L-ornithinemethyl ester, monohydrochloride
- LMPH: Laboratory for Microbiology, Parasitology and Hygiene
- LFA-1: Lymphocyte function-associated antigen
- LOX: Lipoxygenase
- LPS: Lipopolysaccharide
- LSD: Low significant differences

- LT: Leukotriene
- MAPK: Mitogen-activated protein kinase
- MHC: Major histocompatibility complex
- MyD88:Myeloid differentiation primary response protein 88
- NADPH-oxidase: Reduced nicotinamide adenine dinucleotide phosphate-oxidase
- Nf-kB: Nuclear factor-κB
- NO●:Nitric oxide radical
- NOS: Nitric oxide synthase
- NSAIDs: Non Steroidal Anti-Inflammatory Drugs
- O_2●−: Superoxide radical
- OH●: Hydroxyl radical
- PAMP: Pathogen-associated molecular pattern
- PBS: phosphate buffer saline
- PG: Prostaglandin
- PLA2: Phospholipase A2
- PRR: Pattern recognition receptor
- RBC: Red Blood Cell
- RNS: Reactive nitrogen species
- ROS: Reactive oxygen species
- RPMI: Roswell Park Memorial Institute
- SD: Standard deviation
- TGF-β:Transforming growth factor beta
- TIR:Toll/interleukin-1 receptor
- TIRAP:TIR-domain adaptor protein
- TLR: Toll-like receptors
- TNF-α:Tumor necrosis factor alfa
- TRAM:TRIF-related adaptor molecule
- TRIF:TIR domain-containing adaptor protein inducing interferon beta
- TIC: total ion chromatogram
- UPLC-DAD-MS: Ultrahigh-performance liquid chromatography-diode array detector-mass spectrometry
- UV-VIS:Ultraviolet-visible light
- VCAM: Vascular cell adhesion molecule
- VEGF: Vasculoendothelial growth factor
- VLA-4:Integrin receptor for VCAM
- WHO: World Health Organization

CHAPTER I.



CHAPTER I. Introduction and Research Objective

I.1- Introduction

Inflammation is recognized as a complex, dynamic and necessary process for the restoration of the body's homeostasis, and can be characterized as a protective response of the immune system[1]. Its main objective is the protection against microbial invasions or antigens of various types and / or any injury or damage to cells / tissues, including those caused by the imbalance in the REDOX equilibrium. Considerable progress has been made in understanding the events involved in the acute and chronic inflammatory response to infection and tissue injury[2]. However, inflammation remains the subject of numerous studies at the cellular and molecular level because this process is involved in multiple pathologies affecting human health.

Cancer, osteoarthritis, rheumatoid arthritis, gastric ulcers, Crohn's disease, disorders associated with metabolic syndrome, atherosclerosis, Parkinson's disease, Alzheimer's disease, asthma are some of the diseases associated with chronic inflammatory processes[3, 4]. These constitute one of the main reasons for medical consultation in Cuba and in the world. According to WHO data, chronic inflammatory diseases are the most important cause of death worldwide. In their report, they qualified them as the greatest threat to human health, predicting that its prevalence will persistently increase over the next 30 years[5-9].

During the last decade, pharmaceutical companies have been confronted with a reduction in new drugs, mainly those prepared by chemical synthesis. Serious side effects, low efficacy and lack of specificity in their action have prevailed. The group of drugs known as non-steroidal anti-inflammatory drugs (NSAIDs) has not been exempt from this problem, manifesting adverse effects on the gastrointestinal and renal tracts[10]. This has led the pharmaceutical industry to focus its research on the search for new products, where medicinal plants have emerged as a viable solution.

Phytotherapy has been and will continue to be a rich resource in the development of new drugs, which at the moment has captured the attention of scientists around the world[11]. However, for the development of new alternatives derived from this practice, solid scientific evidence is needed. Therefore, demonstrating on a scientific basis the usefulness of a medicinal plant with the requirements of efficacy, safety and quality (even with wide

ethnomedicinal use), is essential for compliance with international regulations in the evaluation and quality control of herbal medicines.

Cuba has a wide wealth of flora from the taxonomic, ethnomedical and chemical point of view. In recent years, the use of medicinal plants has been promoted and rescued, by the population and the Ministry of Public Health (MINSAP), with the aim of complementing conventional treatments for numerous pathologies, included those associated with inflammatory processes. At the same time, scientific research that characterizes the pharmacological mechanisms and safety of medicines derived from medicinal plants has been stimulated.

Euphorbiaceae is one of the largest botanical families in the world and ranks sixth for its diversity. It consists of approximately 432 genera and 8000 species[12]. It is considered as one of the top 25 economically important plant family[13]. Chemical and pharmacological aspects of this family are among the most diverse and interesting of the flowering plants[14]. From the biochemical point of view, many species of this family have been investigated, determining secondary metabolites such as: triterpenes, flavonoids, alkaloids, coumarins, cyanogenic glycosides, tannins, among others. This confers a wide range of therapeutic applications that include the treatment of some types of cancer, asthma, fever, skin diseases, pneumonia, rheumatic pain and as insecticide, just to mention the most important and frequently reported.

Adelia ricinella L. (Jía blanca), is a shrub belonging to the Euphorbiaceae family, that is native to the Caribbean. In Cuba, it is located throughout the archipelago, and its presence is also reported in Antilles's islands, in regions of Venezuela and Colombia. Medicinal properties are attributed to it such as: antipyretic, analgesic and anti-inflammatory activity. The scarse scientific information available for the species in the databases consulted demonstrates the need for a more in-depth study of its phytochemical and pharmacological potential.

For this reason, this research sets out the following **Scientific Problem**: The aerial parts of *Adelia ricinella* L. species are empirically used by the Caribbean inhabitants for their anti-inflammatory properties, but no scientific evidence is available; that is why the identification of their main compounds as well as it activity associated with the ethnomedicinal use of this natural resource results in a problem to be scientifically elucidated.

Hypothesis:

The identification of the *Adelia ricinella* L. main compounds as well as it anti-inflammatory activity will provide the first scientific evidences to support the empirical use reported by the Caribbean inhabitants.

Therefore, according to these facts the **General Objective** of this thesis was: to evaluate the anti-inflammatory activity of well-characterized extracts from the aerial parts of *Adelia ricinella* L.

In order to fulfill this general objective, four **Specific Objectives** were defined:

1. To characterize the pharmacognostic parameters, and the ability to capture radicals of *Adelia ricinella* L. aerial parts and extracts (**Chapter VI)**.
2. To assess the cytoprotective potential of *Adelia ricinella* L. extracts on red blood cell membrane stability with and without hypotonic/oxidant medium (**Chapter V)**.
3. To determine the main chemical constituents and the "*in vitro"* anti-inflammatory effect of *Adelia ricinella* L. extracts by measuring COX-1 and COX-2 enzyme suppression, PGE_2 release in lipopolysaccharide (LPS)-activated RAW 264.7 murine macrophages (**Chapter VI**).
4. To assess the *in vitro* and *in vivo* anti-inflammatory activity from *Adelia ricinella* L. aerial parts extracts (**Chapter VII**).

I.2- Novelty

- Pharmacognostic parameters of the aerial parts and their extracts (water, ethanol 95% and 50%) are for first time reported, constituting an initial approach of physical and chemical quality control standardization; valuable information for their future use as raw material with pharmaceutical interest.
- The determination by UPLC-DAD-MS/MS of the thirteen new chemical constituents in the three extracts of *A. ricinella* L., as well as luteolin and apigenin concentrations is informed for the first time in this species.
- The first preliminary scientific evidences on cytoprotective activity of this medicinal species are informed, based on the *in vitro* non-cytotoxicity effects on five cell lines.
- Anti-inflammatory activity of *Adelia ricinella* L. aerial parts was demonstrated for the first time with the *in vitro* determination of important key enzymes, inflammation mediators and using the *in vivo* model: Formalin-induced rodent hind paw oedema model; which together allowed to suggest a molecular mechanism of

action. In this way, these results contribute to the validation of the ethnopharmacological use by Caribbean inhabitants.

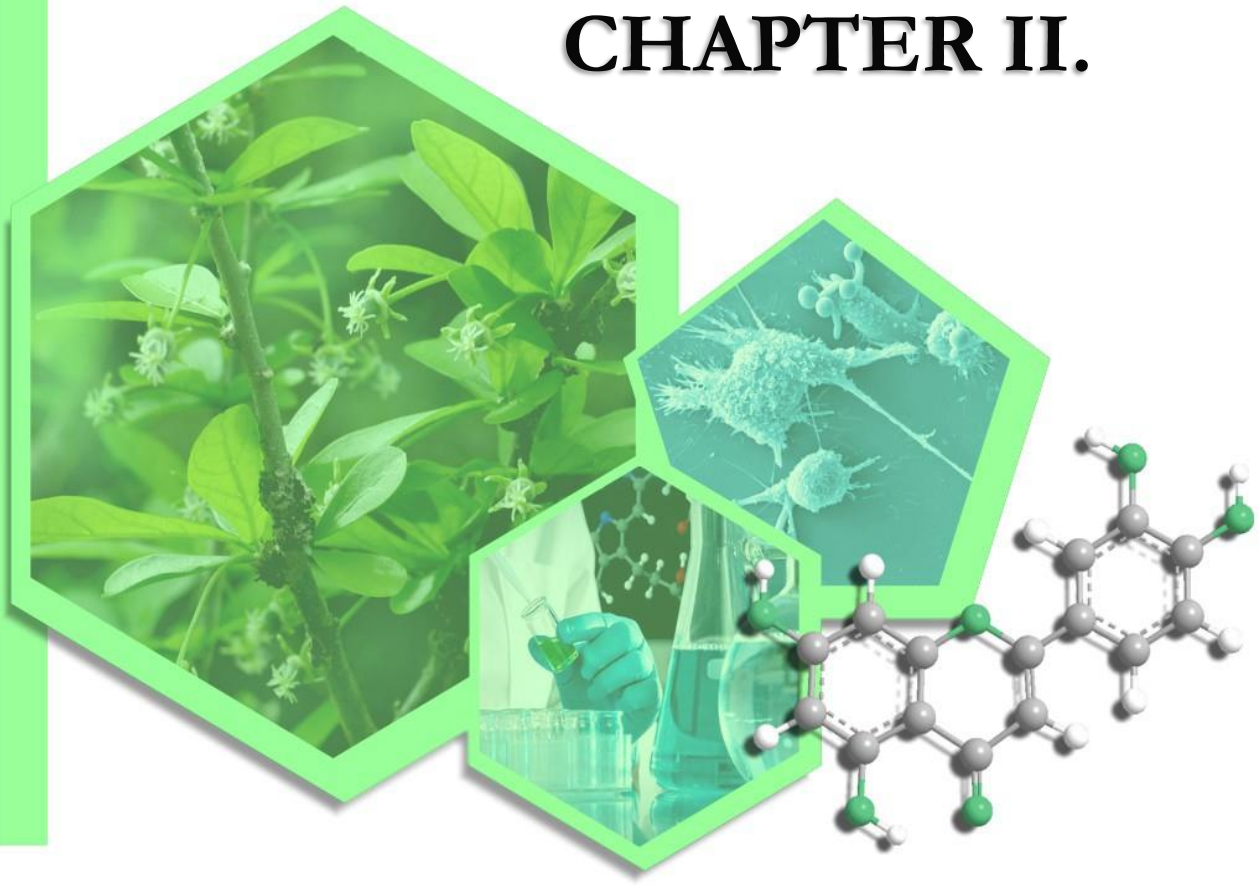
I.3- Practical relevance

Adelia ricinella L. aerial parts are commonly used by Caribbean inhabitants to treat inflammatory diseases without scientific evidences. With this work, it was demonstrated by "*in vivo*" and "*in vitro*" experiments that these uses can be associated to the high diversity and concentration of flavones derivates from luteolin and apigenin. Those compounds have proved it inhibition of: 1) different inflammatory mediators, 2) key enzymes and, 3) migration of cellular mediators to the inflammatory focus; as well by the *A. ricinella* L. extracts done. Additionally, the work offers preliminary elements of it safe use considering the cytoprotective effect shown using a wide range of tests. Altogether, this research offer valuable information about this species used by the inhabitants of the Caribbean, highlighting it promising value as source of bioactive metabolites with therapeutic interest for the development of phytomedicines with potential clinical use as anti-inflammatory.

I.4- Social relevance

Inhabitants of low incoming countries are common users of their natural recourses. This is a proven fact even recognized by the WHO. *Adelia ricinella* L. is a Caribbean shrub used to treat different diseases associate to inflammatory disorders. That is why, investigations focused to demonstrate scientifically the effectiveness and safety of such practices offers outputs that reaches a huge impact in the behavior of the inhabitants, supporting it empirical use and contributing to enhance the cultural heritage of those regions.

CHAPTER II.



CHAPTER II. Inflammation

II.1.-General description of the inflammatory process

The human body is subjected to constant damage by certain stimuli, including infections, traumatic injuries, exposure to toxic substances, autoimmune disorders, among others. This causes an essential immune response in the body that initiates a process defined as: inflammation, the main objective of which is the maintenance of tissue homeostasis[15]. This process has been known to humanity since immemorial time because it accompanied two great problems of the past: wounds and infections. The first to define its clinical symptoms was the Roman doctor Cornelius Celsus, in the 1st century AD. These symptoms came to be known as the four classical signs of inflammation: redness, warmth, swelling and pain. The fifth sign was described by Rudolph Virchow in 1858 as loss of function.

Nowadays, research in this field has gained attention as inflammation has been recognized as a key component of aging and various neurodegenerative, vascular as well as other diseases and cancers. Important advances include: the identification of different types of molecular and cellular mediators as well as the complex network that is established between them; how they act depending on the inducing agent and the pathways that control their production; and how the regulation and resolution processes occur to facilitate tissue recovery[16]. However, despite the complexity of the inflammatory process, several fundamental components and stages have been identified. The main components are classified as inducers, sensors, mediators and effectors. Each component can be found in multiple forms and their combinations allow the activation of different pathways determining the type of inflammatory response. Five stages are labelled: 1) recognition of the infection or tissue damage by the Pattern Recognition Receptors (PRR); 2) activation of the signalling pathways; 3) synthesis and liberation of pro-inflammatory mediators; 4) regulation; and 5) solution of the problem, restoring homeostasis.

An overview of the acute inflammatory process (Figure II.1) due to infection, tissue injury or even an allergic stimulus requires the involvement of different blood components (plasma and leukocytes) and molecular mediators at the damaged site. How it starts depends partially on the nature and entry route of the inducing agent, followed by a complex but highly coordinated sequence of events involving molecular, cellular and physiological alterations. These damages are detected by pathogen-associated molecular patterns (PAMPs) and by damage-associated molecular patterns (DAMPs), which indicate

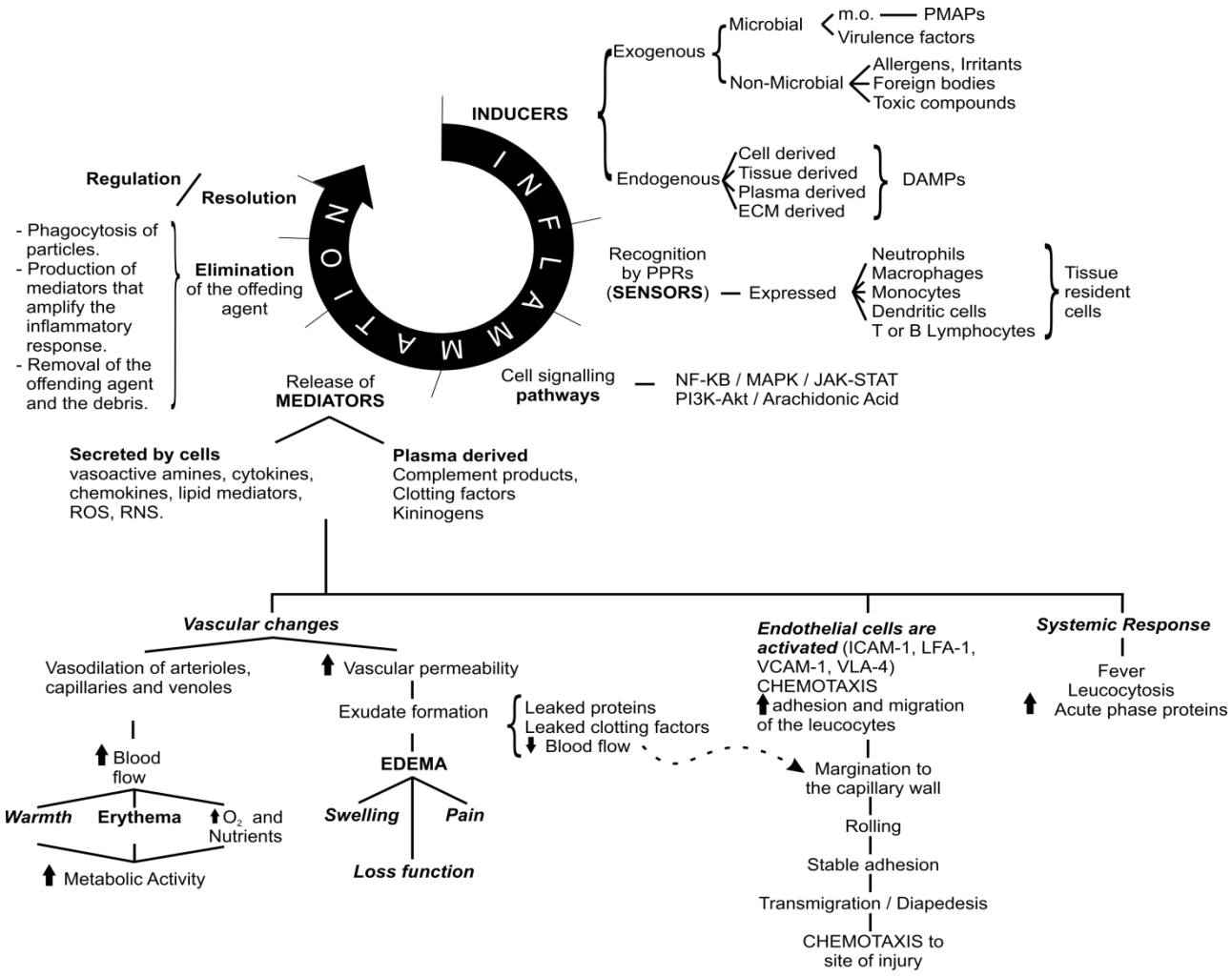


Figure II.1. Schematic representation of the inflammatory process.

Abbreviations: Pathogen-associated molecular patterns (PMAPs); Damage-associated molecular patterns (DAMPs); Pattern recognition receptors (PRRs); extracellular matrix (ECM); transcription factor (NF-κB); Mitogen-activated protein kinase (MAPK); Janus kinase (JAK)/signal transducer and activator of transcription (STAT) proteins; Phosphatidylinositol-3-kinase (PI3K); Protein Kinase B (Akt); reactive oxygen species (ROS); reactive nitrogen species (RNS); intercellular adhesion molecule 1 (ICAM-1); Lymphocyte function-associated antigen (LFA-1); vascular cell adhesion molecule (VCAM); very late antigen-4 (VLA-4)

damage / necrosis recognized by the PRRs of the innate immune system[17]. Within the PRRs are Toll-like receptors (TLRs) and NOD-like receptors (nucleotide binding oligomerization domain containing protein 2 (NLR))[18].

This initial recognition of the infection is mediated by tissue resident cells (macrophages and mast cells) triggering the production and release of a variety of inflammatory/pain mediators, including vasoactive amines, inflammatory cytokines (IL-1, IL-6, tumor necrosis factor α (TNF-α)), chemokines, eicosanoids and proteolytic cascade products. In turn, these mediators that are released into the medium together with the activated PRRs induce different signalling pathways[19]. Among the most studied mediators are those induced by the transcription factor NF-κB, mitogen-activated kinases (MAPK) / Jun kinase (JNK)/p38 and others that promote the transcription of different genes involved in inflammation and the immune response.

The main and most immediate effects of these mediators are accompanied by microvascular changes. These changes are characterized by vasodilation of the arteries, capillaries and blood vessels; increased permeability and decreased blood flow. All as one, lead to the formation of oedema and increased local temperature. These alterations, together with the consequent activation of endothelial cells, result in marginalization of the leukocytes (mainly neutrophils or eosinophil's depending on the nature of the stimulus and subsequently monocytes) which under normal conditions circulate through the centre of the vessels. This process of selective migration towards the inflammation focus known as diapedesis, occurs in four well-defined sequential stages: marginalization, rolling, stable adherence and migration[17].

Margination is the process of neutrophil movement from the central bloodstream to the periphery of the vessel. This phenomenon is facilitated by stasis following fluid exudation at the site of inflammation and physical interactions between erythrocytes and neutrophils[18]. After margination, the rolling and stable adherence stages are produced by interactions between leukocytes and endothelial adhesion molecules by inducible binding of endothelial selectins (E and P-selectins) with integrins and chemokine receptors on leukocytes. These interactions allow the formation of stable bonds that cause immobilization and trans-endothelial migration of leukocytes that will be accumulated at the inflammation site[20, 21]. The expression of these adhesion molecules is stimulated by pro-inflammatory cytokines (particularly IL-1ß). Once they migrate into the affected tissue, neutrophils are activated either by direct contact with the pathogen or by the action of

cytokines secreted by tissue resident cells. These neutrophils destroy the aggressive agents by releasing proteolytic enzymes, reactive oxygen and nitrogen species (ROS and RNS, respectively), among others agents which assist in the maintenance of the inflammatory and nociceptive process. This entire process might be accompanied by a systemic corporal response characterized by fever, leukocytosis and an increment of proteins during the acute inflammation phase[22].

A successful inflammatory response results in a removal of the inducing agent, followed by an inflammation resolution/tissue repair, mediated mainly by resident tissue and macrophage recruitment. Resolution is not a simply and passive endpoint of the inflammation, but rather an active biochemical and metabolic process. This allows the transition from the inflammatory stage to the homeostasis[10]. This process begins quickly in the affected site by cellular mechanisms that synthesize dual lipid mediators with anti-inflammatory and pro-resolution activity, such as lipoxins, resolvins, protectins and maresins. If this process fails and persists over time, it acquires new characteristics: losing its adaptive value and acquiring a harmful character associated with permanent tissue damage as a chronic process.

II.1.1- Inducers

The inflammatory response can be triggered by different stimuli from exogenous or endogenous origin, known as inflammatory inducers (Figure II.1). These stimuli include: infections (caused by viruses, bacteria, fungi), injuries, exposure to different xenobiotics, signals produced by stress, damage or malfunction of tissues cells, and products of aging. These stimuli are detected by PAMPs and DAMPs, which are recognized by specialized receptors activating different signal pathways that allow the release of different mediators of the inflammation[17].

II.1.2- Sensors

The PAMPs constitute a limited and a well-defined series of conserved molecular patterns for all pathogens in general. These are recognized in the host by a specific receptors group PRRs (Pattern Recognition Receptors) that detect their presence. In the other hand, some PRRs also recognize various endogenous signals activated during cell and tissue damage (DAMPs), which are capable of initiating and perpetuating a non-infectious inflammatory response[23].

There are different classes of PRRs which include: Toll-like receptors (TLRs), NOD-like receptors (nucleotide-binding and oligomerization domain-like receptor, NLR), C-type lectin receptors (CLRs), and RIG-I-like receptor (retinoic acid-inducible gene I like receptors). These are strategically located throughout the cell: attached to cell membranes, in endosomes and in the cytoplasm. PRRs are expressed by many cell types of the innate immune system including macrophages, monocytes, dendritic cells, neutrophils, and epithelial cells[24].

Membrane-bound PRRs are one of the most studied signalling receptors. They are linked to the microbial recognition and activate transductional pathways that induce the expression of genes coded to synthesize antimicrobial peptides, pro-inflammatory cytokines (TNF-α, IL-1, IL-6), chemokines, and adhesion molecules. The so-called TLRs, which were discovered in the 1990s, belong to this group and are known to be a family of highly conserved PRRs, being one of the most studied.

More than ten members of the TLR family have been identified at cellular and in intracellular spaces. They are mainly expressed as antigen presenting cells, such as: dendritic cells, macrophages, mast cells, among others. These receptors are transmembrane proteins that present three fundamental structural features: 1) an extracellular domain rich in leucine that mediates the recognition of PAMPs, 2) a binding transmembrane domain and 3) a cytoplasmic domain, or Toll/interleukin-1–receptor (TIR). The latter one, homologous to the IL-1 receptor, is essential for initiating signalling pathways and recruits different signalling molecules that promote the transcription of genes involved in inflammation and the immune response generated by the pathogenic agent[25].

Each TLR adapts the immune response to the type of pathogen that it recognizes: TLRs 1, 2, 4 and 6 bind to bacterial lipids such as lipopolysaccharides from the wall of Gram-negative bacteria (LPS), lipoteichoic acid, peptidoglycan and zymosan (β-glycan); TLR 3, 7 and 8 detect viral RNA; TLR 9 recognizes bacterial DNA and TLR 5 and 10 detect bacterial or parasite proteins.

The activation starts with a signalling cascade that runs through two pathways: MyD88-dependent (myeloid differentiation primary response 88) or TRIF-dependent (TIR domain – containing adapter-inducing IFN-β). Depending of the type of receptor activated, one or another pathway is triggered: TLR1, 2, 4, 6, 7, 8 and 9 exclusively activate the MyD88-dependent pathway; while TLR3 and 4 activate the TRIF-dependent signalling cascade. So far, only TLR4 is capable of activating both cascades. In addition, TLRs 1, 2, 4 and 6 require

a second adapter for MYD88 binding, called TIRAP (TIR-containing adapter protein), and the TLR4 receptor requires TRAM (TRIF-related adapter molecule) for the union of TRIF. These adapters recruit different proteins, such as kinases, which initiate different intracellular signalling pathways, as will be later discussed[26].

Endogenous inducers produced by stress, damage, or tissue malfunction constitute another large and diverse group of signals. These are recognized by various PRRs, especially NLR-like receptors. Members of this family (NOD1 and NOD2) activate signalling pathways similar to those of Toll-like receptors (TLR4), where NF-κB is activated and at the same time, the expression of pro-inflammatory cytokines is induced[27]. NLRs share a common structure: 1) central NOD domain (also called NACHT) that participates in dNTPase activity and in the oligomerization process; 2) an N-terminal effector domain that interacts with other proteins; 3) a C-terminal domain rich in leucine units (LRR), which mediates ligand binding or activator detection[28]. At least 22 members of this family are described in humans and 34 in mice[29].

The function of other members of the NLR family is different from NOD 1 and 2, such as NLRP1, NLRP3 and NLRC4 that control the activation of inflammation. The inflammasome is a multiproteic complex connected to the activation of caspase-1, with the consequent mellowing and release of the pro-inflammatory cytokines IL-18 and IL-1β, as well as a type of programmed cell death called pyroptosis, which activates the innate immune defence[29]. The ligands involved in the activation of these receptors are quite diverse including bacterial RNA, uric acid crystals, bacterial toxins and flagellins[30]. However, unlike TLRs, these ligands reach the cytosol.

II.1.3- Cell signalling pathways involved in inflammation

The binding and recognition of the different PAMPs and DAMPs by the several PRRs activate (in a coordinated way), different signalling pathways that regulate the expression of multiple mediators of the inflammation. These pathways include: 1) NF-κB, 2) Mitogen-activated protein kinase (MAPK), 3) Janus kinase (JAK)/signal transducer and activator of transcription (STAT) proteins, 4) Phosphatidylinositol-3-kinase (PI3K) / Protein Kinase B (Akt), 5) Arachidonic acid cascade, among others.

The NF- κB pathway

The transcription factor NF-κB plays an important role in processes related to inflammation, immunity, cell proliferation, differentiation and apoptosis. The NF-κB family includes five related transcription factors: P50, p52, RelA (p65), RelB, and c-Rel. Under

physiological conditions, NF-κB remains inactive in the cytoplasm together with its inhibitor, a protein complex called IκB kinase (IKK).

After its binding with ligands such as cytokines, growth factors or microbial products to their respective cell surface receptors, the activation of the IKK complex occurs. PRRs use similar signal transduction mechanisms to activate the inhibitor (IKK). On its side, IKK is composed by two kinase subunits, IKKα and IKKβ, as well as a regulatory subunit IKKγ. This complex regulates the activation of the NF-κB pathway through the phosphorylation of IκB. Once IκB is phosphorylated, it undergoes proteasomal degradation, which releases NF-κB for nuclear translocation and the activation of the transcription of specific target genes. This pathway induces the expression of different mediators such as: pro-inflammatory cytokines, chemokines, different enzymes (Cyclooxigenase-2 (COX-2), inducible nitric oxide syntase (iNOS), phospholipase A2, 5-lipoxygenase), promoters of DNA damage (ROS, RNS), adhesion molecules, angiogenic factors (VEGF, angiopoietin), among others[31]. This transcription factor has been found to be actively involved in chronic inflammatory diseases such as Crohn's disease, inflammatory bowel disease, and inflammatory lung and kidney diseases[32].

Mitogen-Activated kinase protein (MAPK) pathway

The MAPKs constitute one of the main signal transduction pathways: MAPKs are a family of serine/threonine protein kinases that include different kinases such as: 1) Extracellular signal-regulated kinase (ERK1 and ERK2), 2) C-Jun N-terminal kinase (JNK) and 3) p38 MAPK. Mitogens and growth factors frequently activate the ERK 1 and ERK2 pathways, while stress and inflammatory processes are the main trigger for the JNK and p38 cascade. In consequence, they mediate the physiological responses to osmotic shocks, inflammatory cytokines, and other injuries associated to survival and apoptosis decisions[33].

The binding of various pro-inflammatory stimuli to G proteins or tyrosine kinase receptors by conversion of GDP to GTP, activates MAP3K. MAP3K in turn phosphorylates and activates MAP2K, which activates MAPK (ERK or p38 or JNK). Activation of ERK promotes the activation of transcription factors such as: cFos, c-Jun, activating transcription factor 2 (ATF-2). Likewise, JNK activation further activates ATF-2, c-Jun and the activating protein (AP-1), a redox-sensitive transcriptional factor that turn on the transcription of numerous genes in the nucleus, such as: growth factors, antioxidant enzymes, adhesion molecules and cytokines. The p38 MAPK mediates both anti-inflammatory and pro-inflammatory

processes, and its action involves different transcription factors that induce the synthesis of various genes related to inflammation, including iNOS, IL-6, and TNF-α[34].

The JAK / STAT pathway

The JAK / STAT pathway is an essential intracellular cytokine signalling mechanism that regulates the expression of multiple genes involved in proliferation, cell differentiation and inflammation. This pathway engrosses various cytokines, growth factors, interferons, and others components.

The cell response to the external cytokine stimuli implicates the binding of the ligand to it transmembrane receptor. When this occurs, two or more receptor-associated JAKs are brought into a close proximity through the oligomerization of the receptor, promoting its phosphorylation. Once activated, JAK phosphorylates tyrosine residues in the cytoplasmic region of the receptors, creating SH2 (Src-2 homology domain) recognition sites for members of the STAT family. Once bound to the receptor by its SH2 domains, STAT proteins are phosphorylated by JAK, which favours their dimerization and subsequent translocation to the nucleus, where they initiate the transcription of specific genes that respond to cytokines[35].

The phosphatidylinositol-3-kinase (PI3K)/Akt pathway

The PI3K/Akt pathway is another pathway which plays a role in the pathogenesis of immune-mediated disorders. PI3K is induced under physiological conditions by various stimuli, including growth factors, cytokines, and hormones that bind to receptor tyrosine kinase (RTK). After binding of the ligand to its receptor, PI3K is recruited into the cell membrane and activated. PI3K induces a downstream signalling cascade activating PIP3 that further activates Akt and PKB. Furthermore, Akt plays a crucial role in inflammation by activating the transcription factor NF-κB, because it activates IKK and induces the degradation of the inhibitor IκB[36].

The arachidonic acid pathway

Eicosanoids such as prostaglandins, thromboxanes, and leukotrienes are mediators that induce inflammation. Arachidonic acid (AA) present in membrane phospholipids is released by the action of activated phospholipase A2 (PLA2). The released AA is then transformed into prostaglandins and thromboxanes by a series of enzymatic reactions following the action of cyclooxygenases (COX). Similarly, 5-lipoxygenase (5-LOX) activity results in the transformation of arachidonic acid into leukotrienes[37].

II.1.4- Cellular mediators of the inflammatory response

Different types of effector cells are involved in the inflammatory process, and vary as the inflammatory response evolves. The inflammatory cascade includes the following cells: neutrophils, eosinophils, basophils, mast cells, monocytes, macrophages, dendritic cell, fibroblasts, platelets, and B or T lymphocytes.

One of the most important cellular mediators in the immune and inflammatory responses is the mononuclear phagocytic system (composed by monocytes and macrophages). This system plays a key role in the initiation and perpetuation of the inflammatory response due to its ability to synthesize mediators such as NO, cytokines, prostaglandins, among others. Furthermore, they constitute an essential element in the maintenance of tissue homeostasis, as they are able to phagocyte pathogenic organisms or eliminating necrotic or apoptotic cell debris[4]. Due to these characteristics, they have been used as a model cell system in the evaluation of substances with anti-inflammatory activity.

Monocytes are 12 to 20 μm cells, with a kidney-shaped nucleus, without chromatin, with an abundant cytoplasm that contains organelles necessary to synthesize secretory and membrane proteins. The lysosome is one of the most important, serving as a warehouse for multiple enzymatic components. The monocyte population in blood represents 1-6% of circulating nucleated cells. Their half-life is around 24 hours. After that time, they penetrate into various types of tissues where they undergo changes in their morphology[38]. This implies a cellular differentiation into macrophages, taking names and specific functions depending to the tissue in which they are found. According to their location, they are known as adipose tissue macrophages, Kupffer cells (liver), microglia (central nervous system), osteoclasts (bone), histiocytes (connective tissue), alveolar macrophages (lung), epithelioid cells (granulomas), all with different effector functions and individual capacities[18, 39].

Macrophages are long-lived effector cells with a high reactive capacity, as they respond to endogenous and exogenous stimuli, but are also proactive, as they produce mediators that modulate the action of the surrounding cells. They remain inactive in the tissues and are activated upon recognition of various stimuli. Among the most common are: 1) lipopolysaccharides from the wall of Gram-negative bacteria (LPS), 2) cytokines or sequences of small pathogen-associated sequences (PAMPs) and 3) transmembrane receptors known as Toll-like receptors (TLRs), triggering thus the immune response[39].

After this activation, they can perform any of their three main functions: antigen presentation, phagocytosis and immunomodulation.

Macrophages have been classified according to the pattern of cytokines they secrete: "classically activated" macrophages (M1) or "alternatively activated" macrophages (M2). Its classification translates into a functional and interchangeable state according to the microenvironment in which the macrophages are found, due to the high plasticity of these cells[40]. Activation of the M1 phenotype can occur by LPS, IFN-γ, TNF-α and GM-CSF and the M2 phenotype with IL-4, IL-13 and MC-SF. From a functional and phenotypic point of view, both populations behave differently. M1 macrophages have a greater capacity for antigen presentation and are considered pro-inflammatory, producing cytokines such as IL-1β, IL-6, IL-8, IL-12, IL-23. On the other hand, M2 macrophages have a greater capacity for phagocytosis and greater immune-regulatory activity; producing large amounts of IL-10 and TGF-β[41].

The M2 phenotype is further subdivided according to the inducing stimulus: M2a activated by Th2 cytokines IL-4 / IL / 13; M2b induced by immune complexes and TLR and M2c ligands stimulated in the presence of IL-10. M2, in general, does not express pro-inflammatory cytokines and are important in mucosal regeneration mechanisms[42, 43].

II.1.5 - Molecular mediators of the inflammatory response.

Vascular and cellular episodes of inflammation are mediated by cell- or plasma-derived molecules. Cell-derived mediators are either preformed (vasoactive amines) and released by granule exocytosis or are synthesized after a stimulus. The latter group of mediators includes cytokines, chemokines, lipid mediators (prostaglandins, thromboxanes and leukotrienes), platelet activating factor, reactive oxygen and nitrogen species (ROS and RNS). Plasma-derived mediators are characteristically synthesized in the liver and circulate as inactive precursors, activated by proteolysis such as complement products (C5a, C3a, and C4a), activated kinins and proteases during coagulation.

Some of these mediators exist in an inactive form or are activated as a product of inflammation. Besides, most of them act by binding to specific receptors, although some have direct enzymatic activity like proteases or mediate oxidative damage, promoting the body's inflammatory response[22].

A brief description of the main characteristics of these mediators is below detailed:

II.1.5.1- Vasoactive amines

Vasoactive amines are low molecular weight compounds that are stored in intracellular granules. Histamine is contained in mast cells and basophils granules, while platelets are the main source of serotonin. Localized histamine release results in wheal formation due to increased vascular permeability. It induces the formation of reversible openings in the endothelial tight junctions, triggers the formation of prostacyclins, and induces the release of NO from the endothelium. Serotonin activates receptors in vascular smooth muscle cells where it produces vasoconstriction, while the interaction with endothelial receptors produces vasodilation (through NO release) and increased permeability.

II.1.5.2- Cytokines

Cytokines are signalling proteins produced by immune cells and play an important role both in the immune response to infection and in the regulation of the inflammatory response. At a physiological level, they regulate important biological processes such as homeostasis, cell growth and activation and the repair of damaged tissue. However, its excessive production leads to pathological situations, hence its involvement in inflammatory, immune and infectious diseases. They are secreted by a wide variety of cells including monocytes, macrophages, granulocytes, endothelial cells, fibroblasts, dendritic cells, keratinocytes, B and T lymphocytes, mast cells, basophils, tumor cell lines, among others. In addition, they are able to bind to specific receptors on their target cells, triggering the synthesis and release of other secondary mediators, such as other cytokines, nitric oxide, or arachidonic acid metabolites (prostaglandins and leukotrienes)[44].

They are classified as interleukins, chemokines, interferons, tumor necrosis factors, growth factors, and colony-stimulating factors. They are further subdivided into pro-inflammatory cytokines (IL-1, IL-6, IL-15, IL-17, IL-23, TNF-α); anti-inflammatory cytokines (IL-4, IL-10, IL- 13, transforming growth factor β (TGF-β) and interferon γ (IFN-γ)[45].

Among the pro-inflammatory cytokines most involved in the inflammatory process are:

Tumor necrosis factor α (TNF-α) is an important mediator of inflammation among cytokines with numerous effects on various cell types, including inflammatory cells, endothelial cells and fibroblasts. This non-glycosylated 17 kDa protein is a mediator of the acute immune response. It is secreted in response to infections and tissue damage, activating coagulation, inducing fever, increasing the adhesion of cells to the endothelium or activating the secretion of cytokines (IL-1 and IL-6) in macrophages. This cytokine is released as a preformed mediator in some types of cells such as monocytes and macrophages, although

its synthesis has also been reported by lymphocytes, neutrophils, microglia, keratinocytes, among others. It can act through a positive feedback mechanism, leading to increased secretion. However, uncontrolled or chronic secretion of TNFα is nocive, being common in a several diseases such as chronic inflammatory diseases (Crohn's disease, ulcerative colitis and inflammatory bowel disease), cancer and autoimmune diseases[46].

Two types of TNF receptors have been identified: TNF-RI present on most of the body cells and TNF-RII which is mainly expressed on hematopoietic cells[46]. Upon activation, TNF receptors intracellular adapter proteins are recruited and trigger multiple signalling pathways. Its most notable cellular effects are to activate the transcription of genes controlled by the transcription factor NF-κB and to induce activity of the enzyme phospholipase A2 with the consequent release of arachidonic acid and its metabolites. In addition, it also causes endothelial disruption, promotes thrombotic processes, has a chemo-attractant effect on neutrophils and stimulates the synthesis of chemokines such as IL-8[47]. Also, it enhances phagocytosis in macrophages and neutrophils; it induces the respiratory burst characterized by the production of ROS, RNS, IL-1, colony-stimulating factors, IFN-γ, activation of pro-inflammatory enzymes; and it stimulates the expression of MHC I and MHC II[48]. Additionally, TNF-α induce the release of the P substance, that transmit pain signals, regulate vessel tone, and modulate vascular permeability[18].

Interleukins are another important class of cytokines that play a pivotal role in immune and inflammatory modulation. Among them, Interleukin IL-1 is involved in the transformation of phagocyte infiltrates during inflammatory states or cancers for the production of ROS and RNS, as well as the synthesis of inflammatory molecules such as chemokines, integrins and MMPs. There are two forms of IL-1 agonists: IL-1α and IL-1β; and an antagonist form: IL-1 receptor antagonist (IL-1Ra). IL-1α is localized in the cytosol or cell membrane and acts in the intracellular environment. IL-1β is secreted extracellularly by activated macrophages and neutrophils, while IL-1Ra is produced by monocytes and macrophages and is released into the systemic circulation in > 100-fold excess than either IL-1α or IL-1β in response to inflammatory[49].

IL-1β is a classical endogenous pyrogen that acts on the central nervous system, inducing fever and leukocyte migration. In addition, it activates the hypothalamic-pituitary-adrenal axis, promoting cortisol secretion, exerting regulatory actions on the innate and adaptive response[50]. IL-1β also promotes the acute phase response, increasing C-reactive protein production. Furthermore, it stimulates the expression of adhesion molecules on both

immune and endothelial cells, the secretion of chemokines, enzymes such as COX-2 and iNOS, components of complement and regulates tissue damage. Similarly, IL-1 binding to its receptor together with IL-6 activates the kinase IKKβ, which promotes the degradation of IKBα and activates NF-κB and other signalling pathways[51].

Interleukin IL-6 is another major molecule during acute inflammation, and its uncontrolled production leads to many inflammatory diseases. Monocytes, macrophages and T cells at the site of inflammation are the main sources of its production in response to stimuli such as LPS, viruses, TNF-α and IL-1. Meanwhile, the production and release of IL-6 is dependent on several transcription factors, particularly NF-κB and AP-1[52]. It has dual effects on inflammation and despite being classified as a pro-inflammatory cytokine, there is experimental evidence indicating its anti-inflammatory effects. The fact that IL-6 production by lung dendritic cells promotes Th2 differentiation and inhibits the Th1 response is one of those evidences[53].

IL-11 is produced by bone marrow cells and by some fibroblasts. This cytokine is able to induce the release of acute phase proteins by the liver, to stimulate immunoglobulin secretion by T and B lymphocytes, and to induce IL-6 synthesis of by CD4+ T lymphocytes. Another important cytokine in the acute inflammatory response is IL-17, which is produced by activated T lymphocytes and is able to stimulate the synthesis of IL-6 and IL-8. It also enhances the expression of intercellular adhesion molecule 1 (ICAM-1) in human fibroblasts[46].

II.1.5.3- Chemokines

Chemokines are chemo-attractant cytokines which act in a coordinated manner and are involved in many biological processes like cell invasion, cell motility, cell survival and interactions with the extracellular matrix during immunological and inflammatory reactions. So far, more than 50 chemokines and at least 18 human chemokine receptors have been discovered. Chemokine receptors are coupled to G-proteins which contain seven transmembrane domains. IL-8 (CXCL8) is one of the major pro-inflammatory chemokines whose biosynthesis is controlled by TNFα, IL-1β, hypoxia and steroidal hormones (estrogens, androgens). IL-8 promotes chemotaxis and neutrophil activation, i.e. CCL2, CCL7 and CCL12 that attract macrophages; and CCL5 which attracts basophils[54]. They bind to widely distributed receptors with high affinity, control leukocyte extravasation, promote chemotaxis towards affected tissues and regulate cell proliferation and apoptosis.

II.1.5.4- Lipid mediators

Among the mediators involved in the inflammatory response, lipid mediators stand out. Most of them are derived from the phospholipids of the cell membranes where eicosanoids are generated. These are locally acting bioactive signalling molecules synthesized by the oxidation of arachidonic acid (AA) or other polyunsaturated fatty acids, which produce prostaglandins, thromboxane, leukotrienes, endocannabinoids and isoeicosanoids (figure II.2)[55].

Arachidonic acid is the most important element in the production of lipid mediators, because the vast majority is derived from it. Eicosanoid biosynthesis is initiated by the activation of the first enzyme, phospholipase A2 (PLA2) and with the release of AA from membrane phospholipids. These phospholipids are further metabolized by cyclooxygenase (COX), lipoxygenase (LOX) and the cytochrome p450 enzyme[56]. Cyclooxygenases are mainly found in three isoforms: COX-1, COX-2, and COX-3. COX-1 is constitutive, localised in almost all body tissues and the PGs derived from this enzyme participate in physiological responses such as vascular homeostasis, renal Na^+ re-absorption and gastrointestinal mucosal protection[57]. COX-2 is not expressed in most cells under normal conditions, it is located in macrophages, leucocytes and fibroblasts and its expression is inducible in response to pro-inflammatory cytokines, growth factors or endotoxins. The high production of COX-2 is considered the mechanism by which the levels of PGs increase during inflammation. The COX-1 and COX-2 isoforms are homodimers and share approximately 60% homology at both the cDNA and amino acid levels, with similar structural and kinetic properties[58]. COX-3 is the most recently discovered isoform. Although different pharmacological properties have been described for COX-3, many researchers consider it to be a variant of COX-1[59,60].

When arachidonic acid is metabolized by COXs, prostaglandins, prostacyclins, and thromboxanes are produced. Prostaglandins are molecules with a very short half-life that can act in an autocrine and/or paracrine way through specific receptors[61]. There are several types of PGs; the most important ones in the inflammatory response are PGE_1, PGE_2 (in the vascular system), PGD_2 (in the central nervous system), $PGF_2\alpha$ (in the smooth muscle), PGI_2 (in the vascular endothelium and gastric mucosa); and thromboxane A_2 (TXA2) in platelets. PGE_1 and PGE_2 have a potent vasodilator effect that allows an increased blood flow to the swollen areas. PGE_1 regulates neutrophil functions by reducing their activation and decreasing platelet or leukocyte aggregation; while PGE_2 can be considered

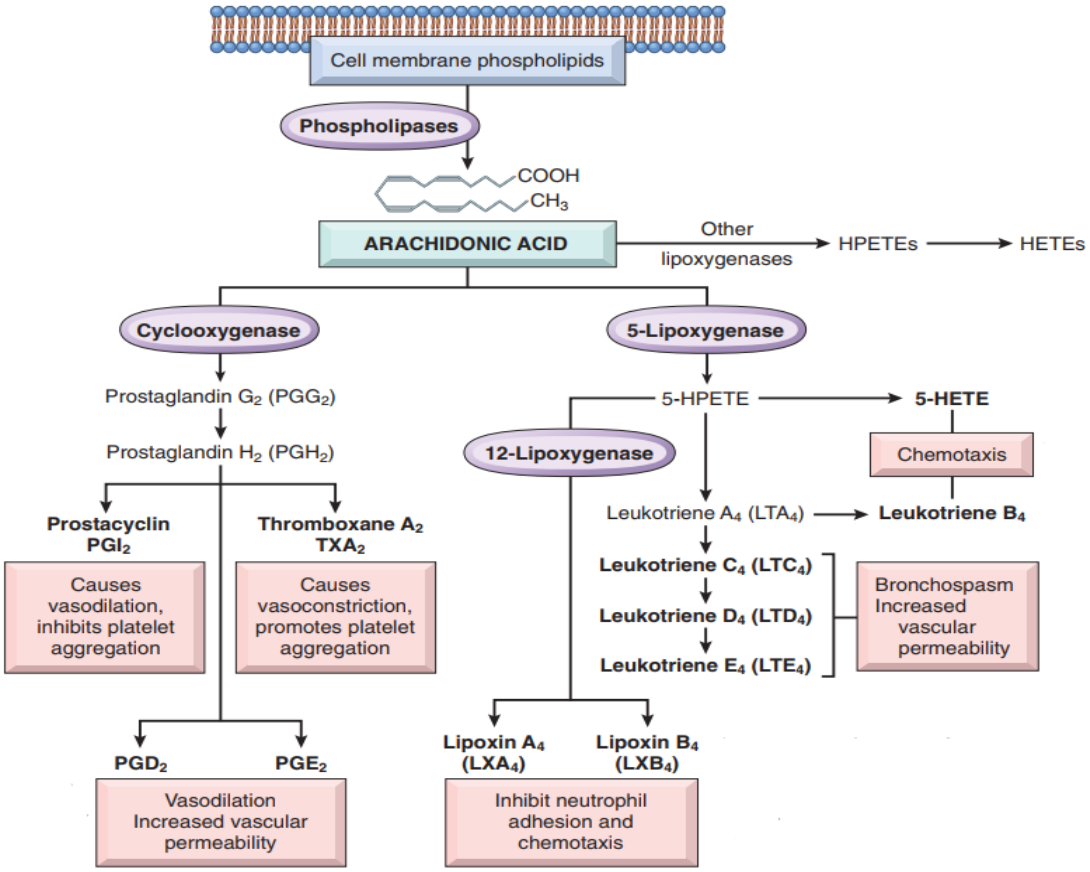


Figure II.2. Biosynthesis of prostanoids. Cellular mechanisms of oxidation of arachidonic acid and its products. From Kumar V et al., 2017.[43]

as a pro- and anti-inflammatory molecule depending on its location[62]. This lipid mediators acts synergically with others mediators that produce pain and is also a powerful pyretic agent. On one hand, it can increase neutrophil extravasation towards the damaged area, but on the other hand, it is able to inhibit the activation of the NF-κB pathway in macrophages, a fact that is pivotal in initiating the resolution of inflammation through lipid mediators[63,64].

The enzyme lipoxygenase (LOX) is found in different isoforms, i.e. 5-LOX, 12-LOX and 15-LOX (15-LOX 1 and 15-LOX 2), but the one that plays an important role in modulation of the inflammatory response is 5-LOX, expressed mainly in neutrophils. The main product of 5-LOX is 5-hydroperoxyeicosatetraenoic acid (5-HPETE), a chemotactic compound for neutrophils, which also serves as a precursor for leukotriene synthesis[45].

Leukotriene B4 (LTB4) is one of the most important lipid mediators of this pathway; it acts as a chemotactic agent for neutrophils, eosinophils and lymphocytes, increases leukocytes adhesion to the endothelium and enhances the production of ROS. The LOXs pathway also produces lipoxins, which are products of 15-LOX and are able to inhibit neutrophil transvasation[63,65], but in turn, they also increase superoxide anion production in these immune cells. 15-LOX-deficient mice are unable of producing lipoxins and at the same time have an overstated inflammatory response. However, Phillis JW et al. demonstrated that lipoxin treatment limits the inflammatory response and reduces the levels of pro-inflammatory cytokines[57]. Additionally, lipoxin A4 is able to increase the levels of IL-10, a cytokine with potent anti-inflammatory effects, thus participating in the resolution of inflammation[25].

In recent years, a bloom series of molecules derived from the oxidation of omega 3 fatty acids has been discovered that have a powerful effect on resolving inflammation. Among these molecules, the maresins, protectins and resolvins stand out. These molecules originate from the oxidation of docosahexaenoic (DHA) and eicosapentaenoic acids via LOXs. The D series resolvins, as well as maresin 1 and D1 protectin are derived from DHA, while the E series resolvins are derived from EPA[16]. Maresins are able to reduce leukotriene synthesis and promote tissue regeneration, thus qualifying as a potent anti-inflammatory agent[64]. Furthermore, the maresins induce the appearance of macrophages with the M2 phenotype with a more anti-inflammatory profile, while the protectins limit the infiltration of neutrophils into the tissues, reduce the levels of TNF-α and IFN-γ and promote apoptosis of Th lymphocytes. Resolvins are synthesized by LOX and COX-2, using

DHA or EPA as a substrate. There are D-series resolvins derived from DHA and E-series resolvins derived from EPA. The main function of resolvins is to stop the infiltration of neutrophils and monocytes into the affected tissue[10].

II.1.5.5- Reactive Oxygen Species (ROS) and Nitric Oxide

ROS are chemical species characterized by their high reactivity that are commonly present in biological systems. They are produced by the partial reduction of oxygen and the most known are the hydroxyl (•OH), superoxide (O_2•-) and hydroperoxy (HOO•) radicals, the peroxide ion (O_2^{2-}), hyperchlorous acid (HOCl) and hydrogen peroxide (H_2O_2)[18]. At low concentrations, these species constitute a secondary messenger system that transmits biological information through the specific modulation of intracellular signalling molecules, enzymes and proteins. However, when local levels of ROS are high, and are not neutralized by endogenous antioxidants (superoxide dismutase, catalase, glutathione peroxidase, thioredoxin and peroxyredoxins, etc.); oxidative stress occurs, which is detrimental to DNA, proteins and lipids. Oxidative stress plays a crucial rol in modulation of versatile immune response against inflammatory response[25,65].

The main source of these species are leukocytes, macrophages, mitochondria, lysosomes, peroxisomes, as well as the cytoplasmic nuclear membrane, the endoplasmic reticulum, among others, and can be generated by both enzymatic and non-enzymatic mechanisms. A large number of enzymes associated with pathological processes that generate ROS have been identified, as secondary products of their catalytic activity such as: xanthine oxidase, cyclooxygenase, lipoxygenase, myeloperoxidase, cytochrome P450 monooxygenase, nitric oxide synthase (NOS), peroxidases, NADPH oxidase (NOX), among others[66].

NOX is a family of enzymes that comprises of seven isoforms and is the main source of superoxide radicals during the respiratory burst in phagocytic and non-phagocytic cells. Certain evidence suggests that members of this enzyme family are the main source of ROS production during the acute and chronic inflammation. In this way, NOX plays an important role in the generation of ROS and defends the host against a variety of harmful stimuli[67].

There is a lof of scientific evidence for the successful role that antioxidant treatment plays in diseases associated with inflammatory processes and infectious diseases. Furthermore, many commercially available anti-inflammatory drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to have antioxidant properties and can achieve effective levels of antioxidant activity *in vivo*. Acetyl salicylic acid and other

NSAIDs inhibit the functions of cellular mediators of inflammation such as neutrophils, cells that when activated, are the main source of reactive oxygen species[68]. Ibuprofen and diclofenac inhibit the production of oxygen free radicals that are involved in tissue damage[69], while paracetamol is a superoxide radical scavenger[70]. Therefore, anti-inflammatory drugs exert at least part of their activity by interaction with reactive oxygen species or the systems that generate them.

Nitric oxide (NO) is a small pro-oxidant molecule that fulfils a dual function in the body, acting in pathological and physiological processes that are fundamental for the proper functioning and defence of the body. Its functions include smooth muscle relaxation, inhibition of platelet aggregation, neurotransmission and immune and inflammatory response[71].

It is synthesized from L-arginine and molecular oxygen by the action of the enzyme nitric oxide synthase (NOS), which requires NADPH as an electron donor, leading the formation of NO and citrulline. Once synthesized, NO can diffuse into the same or neighbouring cells, where it can bind to the heme group of guanylate cyclase, stimulating cGMP production. The activated cGMP can then bind to target proteins, transcription factors, kinases, and phosphodiesterases to cause various effects on blood pressure control, neurotransmission, and platelet functions[71]. NO can also act in a cGMP-independent manner, directly modifying proteins or contributing to the oxidation of proteins and lipids, increasing the number and complexity of potential NO roles[72].

Three NOS isoforms have been identified: NOS-1 or neuronal NOS, found in high concentrations in the brain, NOS-2 or inducible NOS (iNOS), which is expressed mainly in cells of the inflammatory immune system, where it is induced during inflammatory, and NOS-3 or endothelial cell NOS (eNOS) expressed in endothelial cells. The enzymes NOS-1 and NOS-3 are constitutively expressed in cells and are highly dependent on increases of intracellular Ca^{2+} for enzyme activation[45]. The NO concentrations produced by these enzymes are less than 1 μM, acting as a messenger in the Central Nervous System, inhibiting platelet adhesion and aggregation and inducing vasodilation. NOS-2 expression is induced by bacterial endotoxins and pro-inflammatory cytokines such as IL-1, IFN-γ and TNFα[73]. NO further regulates the functional activity, growth and death of various cells. In mast cells, NO suppresses antigen-induced degranulation, mediator release, and cytokine synthesis; in T cells it inhibits the production of Th1 cytokines and at the same time

promotes the Th2 response. It is considered a catabolic factor clearly involved in inflammatory diseases such as osteoarthritis[74].

II.2- Chronic inflammation

When the acute inflammatory response does not eliminate the inflammatory inducer, the resolution phase cannot unfold properly and a chronic inflammation state ensues, which depends on the persistence of the causative agent and host-specific factors. Hence, it is denominated as chronic inflammation, i.e. the one that persists for a long period of time, meaning from weeks to months, with signs of acute inflammation, tissue destruction and reparation.

Its onset can be overlapping and asymptomatic. During acute inflammation, hazard signals are produced, which will activate both fibroblasts and macrophages to produce a series of pro-inflammatory cytokines and chemokines[39]. These hazard signals will also activate immature dendritic cells, which will migrate towards the regional lymph nodes to initiate an adaptive immune response. The transition from an innate or nonspecific to an adaptive immune response is crucial in the chronic inflammation process. In the same model, lymphocytes, macrophages and fibroblasts infiltrate the tissue and persist there for a long time, prolonging the inflammatory process[25].

From a microscopic point of view, the chronic process is characterized by infiltration of mononuclear cells (lymphocytes and macrophages), evidence of tissue destruction caused by these cells, and repair attempts through angiogenesis and fibrosis. The tissue macrophage is the dominant cell in chronic inflammation. Their accumulation in the inflammatory focus persists by different mechanisms, including continuous recruitment from the circulation and local proliferation of macrophages. In addition, these cells have a prolonged survival in the inflammatory focus. Once activated, the macrophages cause tissue damage through four mechanisms: production of ROS and RNS, induction of metalloprotease synthesis, activation of clotting factors, and lastly, production of arachidonic acid metabolites. On the other hand, in the chronic inflammatory focus, macrophages participate in the genesis of fibrosis, through the production of growth factors involved in the proliferation of fibroblasts (PDGF, TGFb, FGF), production of angiogenic factors (FGF, VEGF) and stimulation of collagen deposition through IL-13 and TGFb[16].

II.3- Diseases associated with chronic inflammatory processes.

Several studies indicate that inflammatory processes, especially chronic ones, are associated with the appearance of multiple pathologies. Cancer, asthma, diabetes, rheumatoid arthritis, osteoarthritis, cardiovascular diseases, Alzheimer's disease, gout, multiple sclerosis, atherosclerosis among others, are diseases associated with chronic inflammation[3,4, 55, 62].

Chronic inflammation plays a determining role in carcinogenesis. In the tumour microenvironment this process stimulates the pro-inflammatory mediators that, together with the activation of oncogenes and the loss of activity of tumour suppressor genes, lead to the activation of transcription factors such as NF-κB and the inflammasome. They also increase the concentration of pro-inflammatory cytokines and promote the infiltration of macrophages type M1 and myeloid cells with suppressive activity[32]. In addition, in this process the release of free radicals is stimulated, which play a prominent role in the initiation, promotion and progression of tumours, directly damaging DNA, inhibiting its repair, blocking apoptosis and contributing to angiogenesis[75].

Cancer is the leading cause of death worldwide and is a major public health burden in both developed and developing countries. Different studies indicate that about 20 - 25% of cancers today are the result of a previous chronic inflammation, with an infectious or aseptic origin, which contributes to the malignant progression of the disease, neo-angiogenesis and generation of a possible metastasis[76]. It estimated that the number of new cancer cases worldwide may increase up to 70% in the coming decades, reaching 24 million cases per year by 2035[75, 77].

Obesity and associated diseases such as diabetes are consequences of poor eating habits and physical inactivity. Data from the WHO suggest that since 1975, obesity has tripled worldwide; with 39% of adults aged ≥18 years being overweight and 13% obese. There are different theories about the mechanisms involved in the development of these metabolic diseases and their complications, that converge in the existence of a chronic pro-inflammatory state[28]. These metabolic alterations are elements that interact with the immune system and in this way affecting different anti-inflammatory, antithrombotic and anti-apoptotic pathways. The presence of excessive fat activates the cellular blockade of insulin receptor substrate proteins (IRS) and the production of pro-inflammatory cytokines. Based on these mechanisms, new therapeutic targets have been developed with the objective of reducing the pro-inflammatory cascade, such as IL-1β receptor

antagonists, TLRs antagonists and thioredoxins (proteins related to the redox state and resistance to insulin)[78]. However, we consider that among all the therapeutic approaches, IL-1β antagonists seem to be of special importance, given the role that this cytokine plays in the inflammosome, secondary to hyperglycaemia and reactive oxygen species leading to the production and secretion of IL -1β from pancreatic cells[79].

In brain aging, a group of changes occurs that leads to a decline in adaptive and responsive processes. These transformations can lead to cognitive impairment (CI) and Alzheimer's disease (AD). Although the origin of those changes is diverse, chronic inflammation and oxidative stress explain part of the pathophysiological mechanisms of these abnormalities of brain function. Neuroinflammation triggers damage at the neuronal level through the presence of inflammatory cytokines and the activation of microglia, via membrane receptors and nuclear activation factors. In this case and to counteract these phenomena, neuronal biology is proposing the concomitant use of drugs for the classic targets of AD and anti-inflammatory drugs, immunomodulators and antioxidant substances to slow down or stop the progression of the disease[80]. It is important to note that some research shows that the use of non-steroidal anti-inflammatory drugs may decrease amyloid aggregation and delay the conversion of CI to dementia[81].

Asthma is a chronic respiratory disease with an inflammatory basis and multifactorial aetiology. It is considered the most frequent chronic disease in children, affecting 8-10% of them in developed nations. The WHO estimates that are about 150 million people with asthma in the world, with an annual direct mortality of 2 million people. In addition, it is estimated that each year there are 1.5-2 million asthma-related visits in the emergency services of United States, generating a direct and indirect costs in excess of US$ 12 billion dollars annually[82]. In asthma inflammatory cells, especially eosinophils, infiltrate in the airways submucosa, where pro-inflammatory cytokines, interferon gamma (IFN-γ), IL-4 and IL-2, epidermal growth factor (EGF) receptor and NF-κB seem to play an important role. A key observation regarding treatment is that a premature or early intervention with inhaled or oral corticosteroid-type drugs (steroidal anti-inflammatory drugs) seems to reduce the progression of the disease, both chronic airflow obstruction and bronchial hyper-responsiveness[83].

Chronic obstructive pulmonary disease (COPD) involves chronic inflammation of the small airways and the lung parenchyma, with the presence of neutrophils, macrophages, cytotoxic T lymphocytes and increased production of pro-inflammatory cytokines. This

inflammation leads to fibrosis with stenosis of the small airways (chronic obstructive bronchitis) and destruction of the lung parenchyma due to the action of various proteases (emphysema)[38].

Rheumatoid arthritis, suggested as one of the most prevalent autoimmune diseases, affects around 1% of the world's population. Its prevalence varies between countries, but women are three times more likely to suffer from this disease than men, and although it can occur at any age, it has a peak incidence between 40 and 60 years old[84]. In Cuba, morbidity due to diseases of the osteomyoarticular system constitutes an important health problem. About 21% of the Cuban population is over 60 years of age, which makes it an aging population with a high prevalence of rheumatic diseases, of which arthritis with 43.9% is the most frequent cause, located among the 35 leading causes of death in 2019[9]. The use of anti-inflammatory drugs such as NSAIDs in the treatment of this disease is recommended, because in addition to their anti-inflammatory activity, they have a little effect on the progression of joint deformity. In this pathology, disease-modifying antirheumatic drugs (DMARDs) are the first therapeutic choice as they reduce clinical manifestations and slow the progression of arthritic tissue destruction. However, its use is well controlled due to the high rate of reported adverse reactions[35].

According to the data provided by the latest Statistical Yearbook of Health published in Cuba in 2019, a total of 109,085 deaths was reported, grouped according to the cause of death. In the top ten, seven were associated with diseases that occur due to inflammatory processes such as malignant tumours, cerebrovascular diseases, influenza/pneumonia, chronic diseases of the lower respiratory tract, diabetes and chronic liver diseases/cirrhosis with incidents of 25,035, 10,008, 8,923, 4,310, 2,313 and 1,939 deaths respectively. This represents 48% of all deaths in 2019[83].

Given the high prevalence of these pathologies, the consumption of drugs with anti-inflammatory activity is high; as well as the duration of treatment, which implies an increase in the appearance of adverse reactions associated with them. That is why the population turns to alternative treatments with medicinal plants, being a very common practice not only in Cuba but also in the rest of the world[85,86].

II.4- Effectiveness and safety of anti-inflammatory drugs

As described above, in many diseases associated with chronic inflammatory processes, the inflammatory response is disproportionate and causes damage to the body. As consequence, non-steroidal anti-inflammatory drugs (NSAIDs) are used for the treatment

of inflammation and pain becoming in one of the most worldwide studied medicines nowadays. Despite the frequency of the adverse effects, this group of drugs are widely used/abused, especially through self-medication. NSAIDs are responsible from 21% to 25% of adverse events related to drug consumption, and constitute the second worldwide cause of hypersensitivity to drugs and the first in Latin America region[87, 88].

Ibuprofen, diclofenac, indomethacin and naproxen are the most common prescribed drugs. Prolonged use of these NSAIDs can cause severe gastrointestinal (GI) damage and renal dysfunction, related to their relative selectivity for COX-1 and/or COX-2, limiting the use of these drugs. Drugs with inhibitory activity over COX-1 trend to cause adverse effects on the GI tract. That is why, selective COX-2 inhibitors such as celecoxib, developed and marketed by Pfizer, produces fewer GI adverse effects than non-selective NSAIDs. However, its use in the long-term is also limited due to its severe cardiovascular effects and renal failure, this time because it selective COX-2 condition. Therefore, a better understanding of the inhibitory activity and COX-1/COX-2 selectivity of an NSAID at therapeutic doses becomes necessary. Meaningful researches based on pharmacokinetic and pharmacodynamic properties (eg, inhibitory dose, absorption, plasma versus tissue distribution, and elimination), and the impact on drug tolerability and safety; can guide the selection of appropriate NSAIDs for pain and inflammation management[89].

II.5- Methods to determine anti-inflammatory activity

The determination of the potential anti-inflammatory activity of new compounds and plant extracts can be investigated through different experimental models. A pharmacological model can be considered relevant when the effects obtained in the preclinical model are related to the results in the clinical setting. There are many *in vitro* and *in vivo* methods, based on the pathophysiology and apparent signs of inflammation. *In vitro* methods to assess anti-inflammatory activity are important tools in the pharmacological evaluation of natural products. This type of assay allows pharmacological and biological characterization, identification of the mode of action of the compounds through the simulation or activation of the inflammatory process using cells, outside of a living organism.

Murine macrophage cell lines (RAW 264.7) and human monocytic leukemia cells (THP-1) are generally used to evaluate the anti-inflammatory effect of products derived from medicinal plants. Stimulation of these cells with LPS or IFN-γ induces the release of pro-inflammatory mediators such as NF-κB, TNF-α, IL-6, reactive oxygen species (ROS),

superoxide anions and proteases. Thus, the intensity of the inflammatory response can be assessed through the determination of pro-inflammatory mediators (TNFα, IL-6, IL-1, NO, cyclooxygenase and others) in the supernatants. All these inflammatory molecules above mentioned are considered potential targets for anti-inflammatory drugs[90, 91].

ELISA enzyme immunoassay techniques can be used to quantify mediators such as prostaglandin E (PGE_2), interleukins, TNF-α, among others. The expression of these pro-inflammatory mediators is controlled by different signalling pathways such as NF-κB, mitogen-activated protein kinase (MAPK), JUN N-terminal kinase (JNK)/ Signal transducers and activator of transcription (STAT), among others. Western blot assay and reverse transcription-polymerase chain reaction (RT-PCR) are valuable tools to measure the expression of genes of transcription factors such as p38 MAPK, JNK, ERK, IKKα/β and many more[92].

Mammalian erythrocytes have been widely used to study the mechanisms of oxidative damage and toxicity in biomembranes. They represent a very simple and useful cell model[93]. They are easy to manipulate and do not contain a nucleus or internal membranes; being very susceptible to attack by reactive oxygen species (ROS) in situations of tissue deterioration or when exposed to oxidative events[94]. That is why it is a widely used model to evaluate membrane cytoprotection after exposure to oxidizing agents and anti-inflammatory activity, due to its similarity to lysosome membranes[95]. On the other hand, inflammation is also associated with protein denaturation and under this concept; models such as the inhibition of denaturation of egg albumin or bovine serum albumin (BSA) are also commonly used.

In addition, techniques to assess the *in vivo* anti-inflammatory activity are used as a tool to simulate biological models of inflammation where the effect occurs acutely or chronically[96, 97]. Generally, these studies are carried out in rodents (rats and mice) in which many suitable experimental models have been described, due to their similarity to humans with regard to pharmacokinetic processes. Furthermore, it is always important to comply with Russel and Burch's 3-RRR (reduction, refinement and replacement) principle[98].

Traditionally, different models of acute inflammation have been used, but the most common ones are plantar and atrial oedema. Paw oedema is the hallmark pattern of acute inflammation that has the highest reproducibility. Carrageenan is a non-antigenic phlogistic agent which is devoid of any visible systemic effects[99].Carrageenan stimulates

the early inflammatory phase by activating phospholipase A2 and the cyclooxygenase pathway, which triggers the release of pro-inflammatory mediators causing oedema However, in this model other substances such as dextran, formalin, histamine, bradykinin, LPS, and others, have been used as a phlogistic agent administered into the plantar aponeurosis of the biomodel. Glucocorticoids and NSAIDs show anti-inflammatory activity in this preclinical model and are used as positive control groups. This model is widely used and is a very practical method for screening drugs that are evaluated for anti-inflammatory activity. It is a sensitive method to detect drugs that act by inhibiting cyclooxygenase.

In the ear oedema test, croton oil is used as an irritant and represents a useful model for skin inflammation in the evaluation of systemic and local anti-inflammatory compounds. Topical application of croton oil produces vasodilation, promotes vascular permeability, induction of neutrophils, synthesis of eicosanoids, and release of serotonin and histamine. PGI_2 and LTB4 are mainly responsible for the observed effects. The model has been also widely used to evaluate the anti-inflammatory activity of steroidal and non-steroidal drugs[100,101].

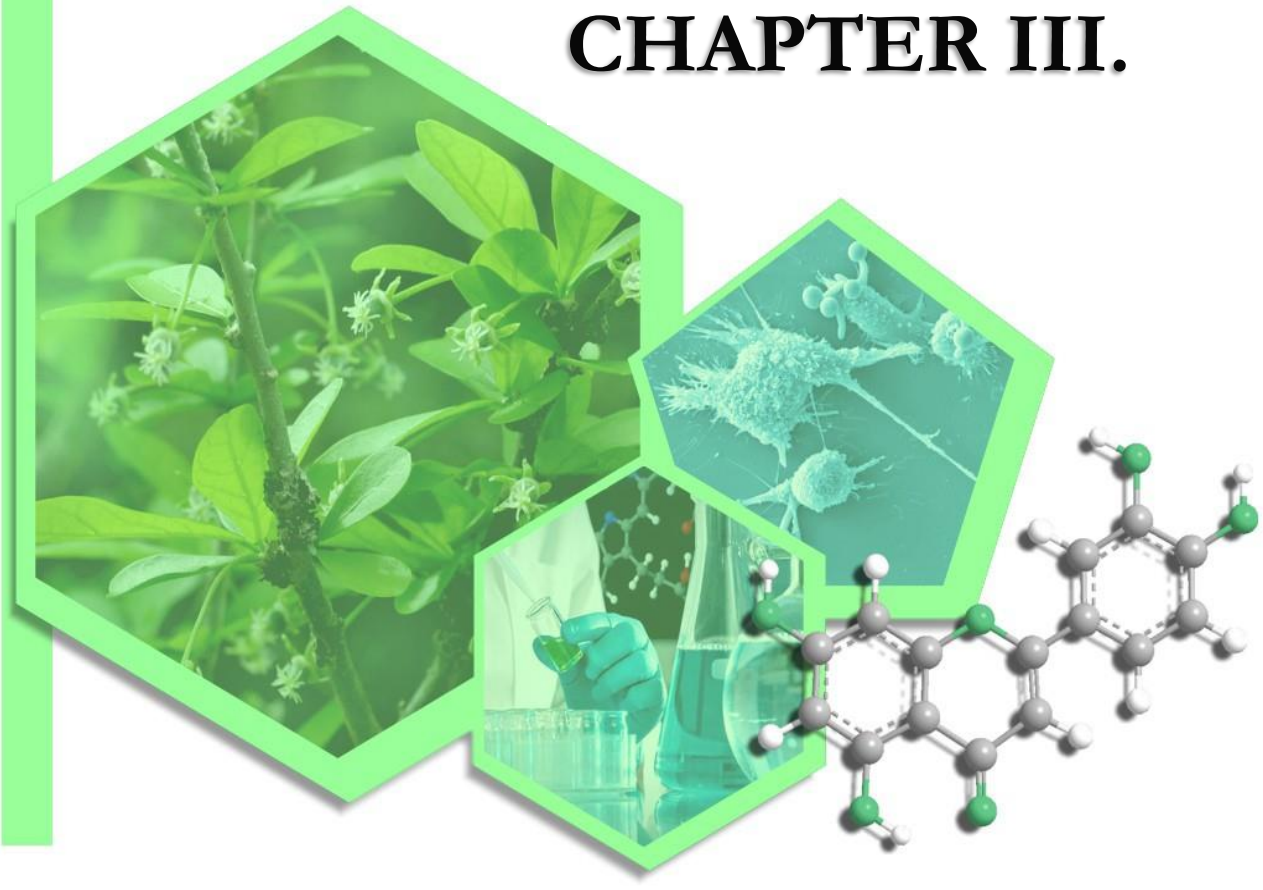
The air bag model is used as a way to test sub-chronic inflammation. The administration of irritants into the subcutaneous air bag produces proliferation of granulation tissues that are mainly composed of endothelial cells and fibroblasts. In addition, the administration of an irritant substance causes the infiltration of macrophages and PMN[102]. This method allows test substances to come into contact with the target cells by being placed directly into the air bag.

For chronic inflammation screening is mainly used in the preclinical practice the cotton disc-induced granuloma[97]. This model represents the pathological events of chronic inflammation such as monocyte infiltration, fibroblast proliferation, angiogenesis and exudation. The transudative and proliferative elements of chronic inflammation are evaluated through this model with the formation of the granuloma. The fluid absorbed by the disc greatly affects the wet weight of the granuloma. The difference in weight between the wet/dry cotton pads is related to the formation of transudate and granulomatous tissue, respectively. Another widely used technique is the induction of arthritis in rodents where limb dysfunction is analysed. Although the information obtained is not absolutely equal to the response on the human organism, it is closer to the expected response in living organisms where ADME processes occur and can be evaluated for long periods of

time. However, many factors can influence the results obtained due to the complexity of each organism[103].

Recently, zebrafish was used as an animal model to study anti-inflammatory activity due to its morphological and physiological similarities with mammals and its high genetic similarities with humans[104]. Similarly, biomodels with genetically manipulated experimental animals have been introduced to evaluate specific mediators of inflammation and how their behave in an *in vivo* model. This is the case of transgenic mice used in intestinal inflammation models that overexpress different proteins such as STAT, Il-17 and others that show high susceptibility to changes in the bacterial ecology of the intestine. Another example are mice with a deficit in one or more adhesion molecules that, together with the development of specific antibodies for many of them, have made it possible to evaluate the role they play in the cell recruitment process in different inflammation models[105].

CHAPTER III.



CHAPTER III. Medicinal Plants: *Adelia ricinella* L.

Nature, as source of medicines, has always been of paramount importance for humans in the treatment of their diseases. In fact, the use of medicinal plants dates back to ancient times evolving together with the human being on the basis of trial and error practices. Therefore, phytotherapy is worldwide the oldest form of therapy. Nowadays and according to WHO´s records, an estimated 65% of the World´s population relies on plant-derived medicines for their primary health care[106].Consequently, plants continue to be in the focus of modern research due to their large chemical and biological diversity and because they produce a variety of compounds with promising biological activities[86]

III.1- Use of medicinal plants in inflammation

Medicinal plants are a valuable resource that human gets from the nature. A recent bibliometric study (2020) on Scopus database using "Medicinal Plant" as a keyword reveals that more than 110000 papers have been published in the period from 1960 to 2019[107]. A total of 159 countries contribute with at least one paper, standing out China and India with more than 10,000, influenced by their extensive knowledge of traditional medicine. They most referred works are linked to antioxidant activity, but in India also highlight the antidiabetic properties. United States of America and Brazil, with more than 5000 publications, followed the above mentioned countries.

Cuba, a country with a high level of plant endemism, also focuses its efforts on the studies of its medicinal plants[108, 109]. These analyzes show that most of the studies focus on traditional medicine and *in vivo* studies aimed at revealing anti-cancer, anti-diabetic and anti-inflammatory activities. It was also observed that research is focused on finding new drugs or active compounds[108].

The use of medicinal plants for the treatment of inflammatory diseases is generally based on ethnobotanic information inherited from ancestral cultures, although extensive prior scientific knowledge is currently available for some species. The impact of these investigations is reflected in scientific progress and in the development of new compounds used in the treatment of diseases associated with inflammatory processes. In turn, they can be monitored through the number of scientific-technical articles that link the use of medicinal plants in research related to diseases or inflammatory processes. With this purpose, an extensive bibliometric study of the papers indexed in the database Scopus

that contains the words "Plant and Anti-inflammatory" within the Title, Abstract or Keywords sections.

Results show that since 1956, the date on which was published the first work recorded, a total of 29553 papers have been published relating any plant species with its anti-inflammatory activity. In general, during the 50s and 60s, scientific production in this field was quite low with a total of 61 hits. A slight increase was observed during the 70s and 80s (133 and 226 hits), but the real growth in interest on this topic appears from the 90s. In this sense, during the 90s, 2000s, and 2010s, the accumulated hits reached values of 1234, 5292 and 18085 respectively. Year 2020 scores the highest value with 2,920 published papers (Figure III.1).

This accelerated growth could be due to the incessant search of the scientific community to solve the serious problem of the side effects observed in synthetic drugs such as NSAIDs that include gastrointestinal, cardiovascular, liver and kidney disorders, where medicinal plants products appear as a more feasible and safe therapeutic alternative.

Most of these 29617 studies were classified into the Scopus subject area of "Pharmacology, Toxicology and Pharmacy categories, representing 57.6%. Other subject areas classified as main were Medicine (38.36%), Biochemistry, genetics and Molecular biology (31.6%), and chemistry (15.5%). Others such as Immunology and Microbiology and nursing scored less than 10%. (Figure III.2)

Authors from China, India, South Korea, United States of America and Brazil were the most active in number of contributions, but as is characteristic of the journals indexed in this database; English language prevails representing 94.3%. Chinese was the only language that goes over the 2%, while German, Spanish, French, Portuguese barely exceeds the hounded hits.

In order to explore how often Cuban plants have been studied in their potential anti-inflammatory activity, a similar analysis was performed but added the keyword Cuba. Only 26 articles belonging mainly to the subject areas of Pharmacology, Toxicology, and Pharmacy (88.5%), Medicine (11.5%), Biochemistry, genetics and Molecular biology (15.4%), chemistry (3.8%), and agriculture and Biological sciences (23.1%) (see Figure III.3) were scored. Similarly, the English language represents the two/thirds parts of the paper hits, leaving to the Spanish language a total of 9 hits, thus limiting its visibility.

Among the most studied Cuban species, *Mangifera indica* L. stands out, with a pharmaceutical product registered under the VIMANG® Trade mark in Cuba. The wide

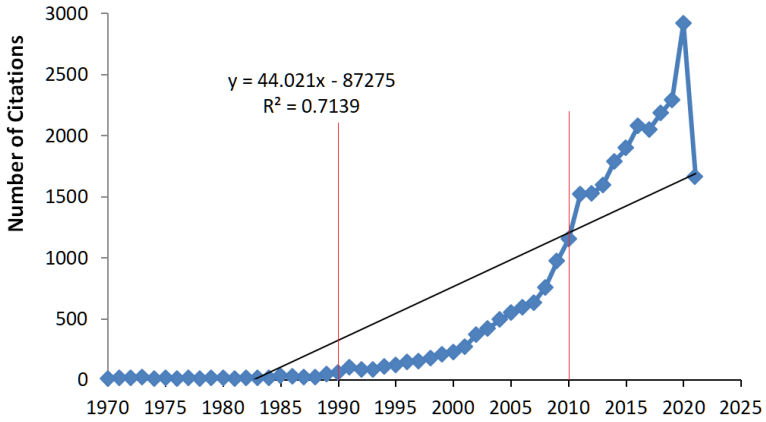


Figure III.1 Scopus database papers focused on plants with anti-inflammatory properties (Period 1970-2021).

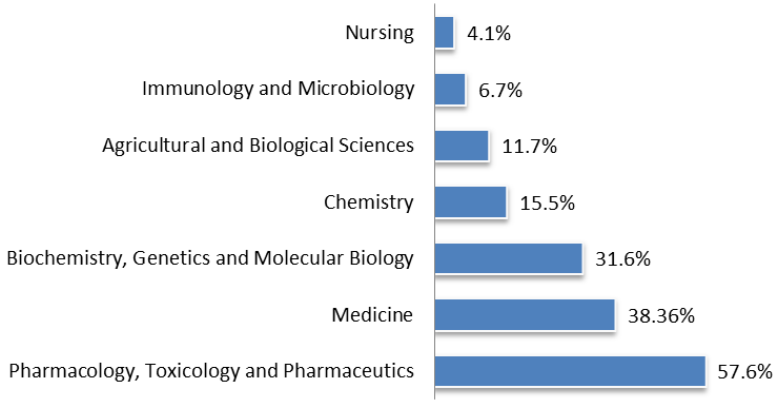


Figure III.2 Scopus subject areas holding the papers focused on plants with anti-inflammatory properties

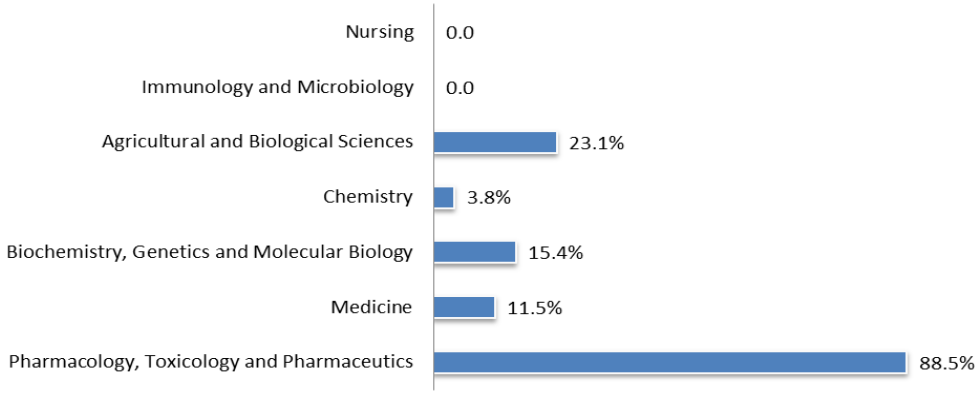


Figure III.3 Publications from Cuba by scientific categories indexed in Scopus

number of studies made it possible to determine for the first time the antioxidant, antinociceptive and anti-inflammatory actions of the aqueous extract of *Mangifera indica* L (VIMANG®) bark ; allowed the development of a new therapeutic resource with a great impact on health[110,111].

In a recent review (2020), Nunez and *et al*. underlined the elevated number of research and review articles published on medicinal plants with anti-inflammatory activity. They highlighted 42 species belonging to the families Anacardiaceae, Euphorbiaceae, Acanthaceae, Amaranthaceae, Apocynaceae, Asteraceae, Cucurbitaceae among others. At the same time, they stood out that leaves, roots, bark, and whole plants are the plant parts more used for those purposes. Terpenes, alkaloids, saponins, and phenolic compounds (tannins, lignans, coumarins, and especially flavonoids) were suggested as the secondary metabolites responsible for the anti-inflammatory activity observed. In addition, the relationship between the ability to scavenge and neutralize reactive oxygen and nitrogen species (which cause oxidative stress) and anti-inflammatory activity was highlighted. This confirms that, in many cases, plant extracts containing substances with antioxidant capacity such as flavonoids, polyphenols, and tocopherol, in turn, have an anti-inflammatory effect[112].

Maione *et al*. for their part, state that in the last decade there has been a notable increase in research on medicinal plants, the identification of their main components, and the *in vivo* and *in vitro* evaluation of their anti-inflammatory activity. Those results were obtained through a search in Medline-PubMed, using different combinations of terms or keywords such as anti-inflammatory, medicinal plants and natural products during the period from 2005 to 2015. From a total of 680 articles, they selected 50 that addressed preclinical studies of compounds isolated from medicinal plants and that were well characterized. The results showed 47 medicinal plants with promising anti-inflammatory activity and 52 isolated compounds that can be chemically classified as phenols/ flavonoids (36.30%), terpenoids (32.70%), glycosides (11.60%) and others (19.40%). They also acknowledge the progress of the scientific community in the last two decades for the identification and characterization of a wide variety of compounds with anti-inflammatory properties[113]. However, setting a number of plant species with potential anti-inflammatory activity is still haphazard. Studies developed in Brazil (one of the countries with the greatest biodiversity and the fifth in a number of contributions on the subject addressed) raise the number of plant candidates. This research used more or less

the same protocol that the previous one; by searching in databases like Web of Science, SciFinder, Pub-Med, and Science Direct. The terms "anti-inflammatory" and "Brazilian medicinal plants" were used as keywords in the search engine, in turn, the origin of the plants was verified, in order to include only those native to Brazil. The data collected showed that 70 plants used in the form of crude extract and/or isolated compounds, showed anti-inflammatory activity. The main mechanisms involved were the inhibition of the release of inflammatory mediators, such as interleukins (IL), nuclear factor kappa B (NF-κB), prostaglandin E_2 (PGE_2), cyclooxygenase (COX), and reactive oxygen species (ROS)[86].

In Cuba, Regalado A and Sánchez LM carried out an extensive bibliographic review on plant species that grow in Cuba that report anti-inflammatory properties. To do this, they conducted a search in Medline / PubMed), as well as national journals in the period 2000-2015, with the keywords "inflammation" and "Cuban anti-inflammatory plants" or "anti-inflammatory activity" and "medicinal plants"[114]. Among the most outstanding anti-inflammatory species from Cuba include *Arnica Montana* L.[115], *Salvia officinalis* L.[116], *Pedilanthus tithymaloides* Poit[117], *Aloe vera*(L.) Burm. f. [118], *Annona squamosal* L.[119], *Bauhinia kalbreyeri* Harms[120], *Boldoa purpurascens* Cav. ex Lag.[121], *Morinda citrifolia*L.[122], *Zanthoxylum elephantiasis* Macfad.[123], *Plantago lanceolata* L.,[124]*Capraria biflora* L.[125] among others. All these plant species are used by the Cuban population in the form of decoctions, infusions, fomentations and creams. Similar result was exposed by Rodríguez *et al* in 2020, revealing 43 species with potential anti-inflammatory activity, 23 of them corresponding to Cuban medicinal plants[126].

III.2- Secondary metabolites. Phenolic compounds

Plants produce a wide range of organic compounds that are divided into two large groups. The "primary metabolites", directly involved in the primary processes related to plant life, play recognized roles in the processes of assimilation, respiration, transport and differentiation. The second group corresponds to the "secondary metabolites" which are chemical compounds synthesized to fulfil non-essential plant life functions, but which are involved in ecological interactions and the adaptability of the plant to its environment[127]. Therefore, the main metabolic role attributed to these secondary compounds is protection against attack by herbivores and microbial or viral infections. Unlike the primary metabolites, they have a restricted distribution, they can be found only in one species or in a group of taxonomically related species[128].

The chemical nature of the secondary metabolites allows their classification in essential oils, alkaloids, coumarins, steroids, phytosterols, phenolic and flavonoid compounds, glycosides, sesquiterpene lactones, mucilages, quinones, saponins, tannins, terpenes (mono, di, tri, tetra, sesquiterpenes), and so on. This structural diversity allows equal diversity of pharmacological properties, particularly because a single class of secondary metabolites can show multiple pharmacologic effects.

Phenolic or polyphenolic compounds constitute one of the most studied groups of secondary metabolites. They are involved in different processes such as growth, lignification, pigmentation, pollination, resistance against pathogens and predators, and they are involved in defence mechanisms against external aggressions[129]. This group ranges from compounds with a single phenolic ring in their structure up to highly polymerized compounds. There are more than eight thousand different phenolic compounds distributed in plants, classified into various classes and subclasses. These are defined based on chemical structure as: phenolic acids, quinones, stilbenes, lignans, flavonoids among others; occurring as aglycones or as glycosides (Figure III. 4)[130].

III.2.1- Flavonoids

Flavonoids (from the Latin flavus, "yellow") constitute one of the most abundant groups of natural products with variable phenolic structures and are found mainly in fruits, seeds, roots, leaves but also in other plant organs. They are responsible for the attractive colours of many flowers, fruits and leaves[131]. In addition, they are present in photosynthetic organisms, especially in higher plants, but they are not very abundant in algae and fungi. Flavonoid concentrations vary according to the plant species, and also depending on the plant's environment. They are responsible for the organoleptic characteristics of fruits and vegetables, contributing to the colour and flavour (bitter and astringent)[132, 133].

Flavonoids are involved in a multitude of physiological functions within plant cells, functioning as essential metabolites for plant growth and reproduction. In addition, they provide protection against pathogens and herbivores, and in turn attract pollination animals. They are ubiquitously distributed among the higher vascular plants, being more abundant in the young aerial parts more exposed to the sun, such as leaves, fruits and flowers, since sunlight favours their synthesis[134].

Over 9000 compounds of this group have been identified[131]. They have a common backbone consisting of fifteen carbon atoms, arranged as a C6-C3-C6 system (Figure III.5). The two aromatic rings, denoted as A and B, are linked to a three-carbon unit, which may or

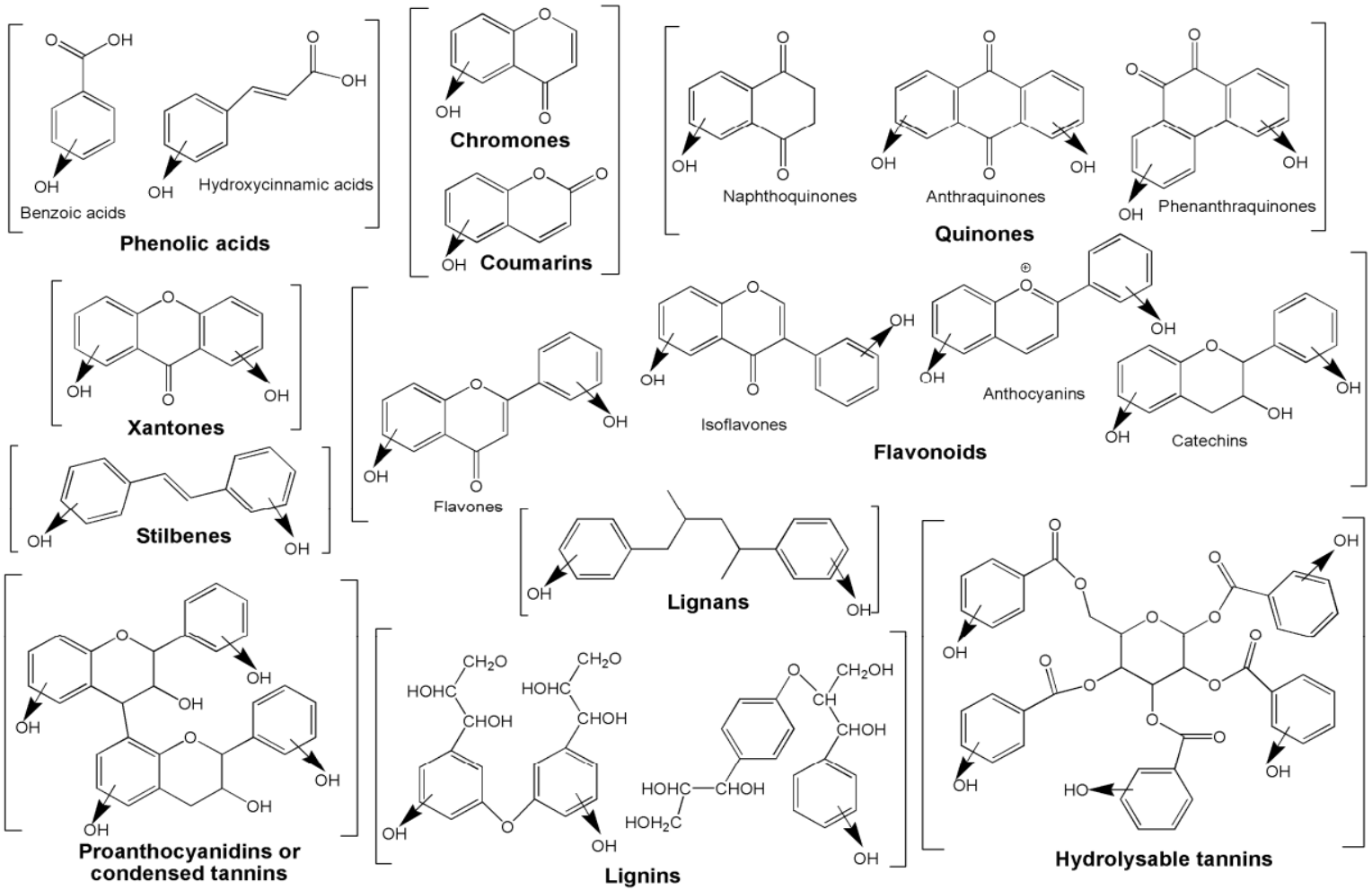


Figure III.4. Typical phenolic compounds and their derivatives. From Llauradó MG *et al.*, 2020 [130].

may not form a third ring, which if present is denoted as ring C. This carbon skeleton explains the chemical diversity of this class of compounds. They are synthesized in the phenylpropanoid pathway. This route begins with the condensation of one molecule of *p*-coumaroyl-CoA with three molecules of malonyl-CoA to produce the chalcone backbone catalyzed by chalcone synthase. The next step is isomerization of chalcone to flavanone by chalcone isomerase. From this step onwards, the pathway branches to different flavonoid classes, including aurones, dihydrochalcones, flavanonols (dihydroflavonols), isoflavones, flavones, flavonols, leucoanthocyanidins, anthocyanidins and proanthocyanidins (Figure III.6)[135].

Flavonoids are found primarily as glycosides, but they can also occur in their free form as aglycones. Glycosides can be found in two forms: as *O*-glycosides, with carbohydrates linked through oxygen atoms, or as *C*-glycosides, with carbohydrates linked through carbon-carbon bonds. The most abundant natural form is *O*-glycoside, usually at carbons 3 and/or 7, with the most common sugars being glucose, galactose, rhamnose, xylose and arabinose[134, 135].

From a pharmacological point of view, flavonoids stand out for their multiple healing applications and their low toxicity. The best-known biological activities and therapeutic effects of flavonoids include anti-inflammatory, analgesic, antioxidant, vascular protective, antianginal, antitumor, antiallergic, hepatoprotective and antiulcer activities, among others. Despite of the abundance of flavonoids that have beneficial properties, the molecular mechanisms of their actions are very often unknown[133, 134, 136].Flavonoids with anti-inflammatory properties include quercetin and myricetin which inhibit COX and LOX[137, 138]. Quercetin, hesperidin and apigenin are able of inhibiting prostaglandin biosynthesis and reducing monocyte adhesion to endothelial cells by regulating adhesion molecules located on the monocyte surface[139, 140, 141]. At the same time, quercetin decreased IL-1β and TNF-α levels in blood serum as well as the transcriptional activity of NF-κB in blood mononuclear cells[142]. Fisetin regulated the balance between pro- or anti-oxidants and pro- or anti-apoptotic proteins in the myocardial tissue. These protective effects of fisetin are attributed to the down regulation of the receptor for advanced glycation end products (RAGE) and NF-κB[143].

Xagorari et al. reported that the flavonoids luteolin, luteolin-7-glucoside, quercetin and genistein inhibited the release of TNF-α and IL-6 in LPS-stimulated RAW 264.7 cells, while eriodictyol and hesperetin only inhibited the production of TNF-α. These effects are related

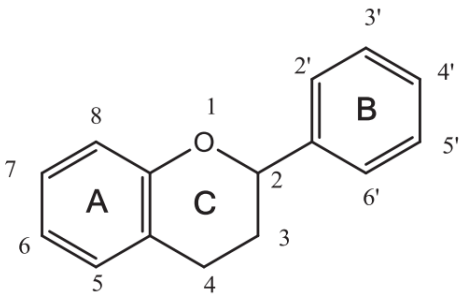


Figure III.5 Backbone of flavonoids.

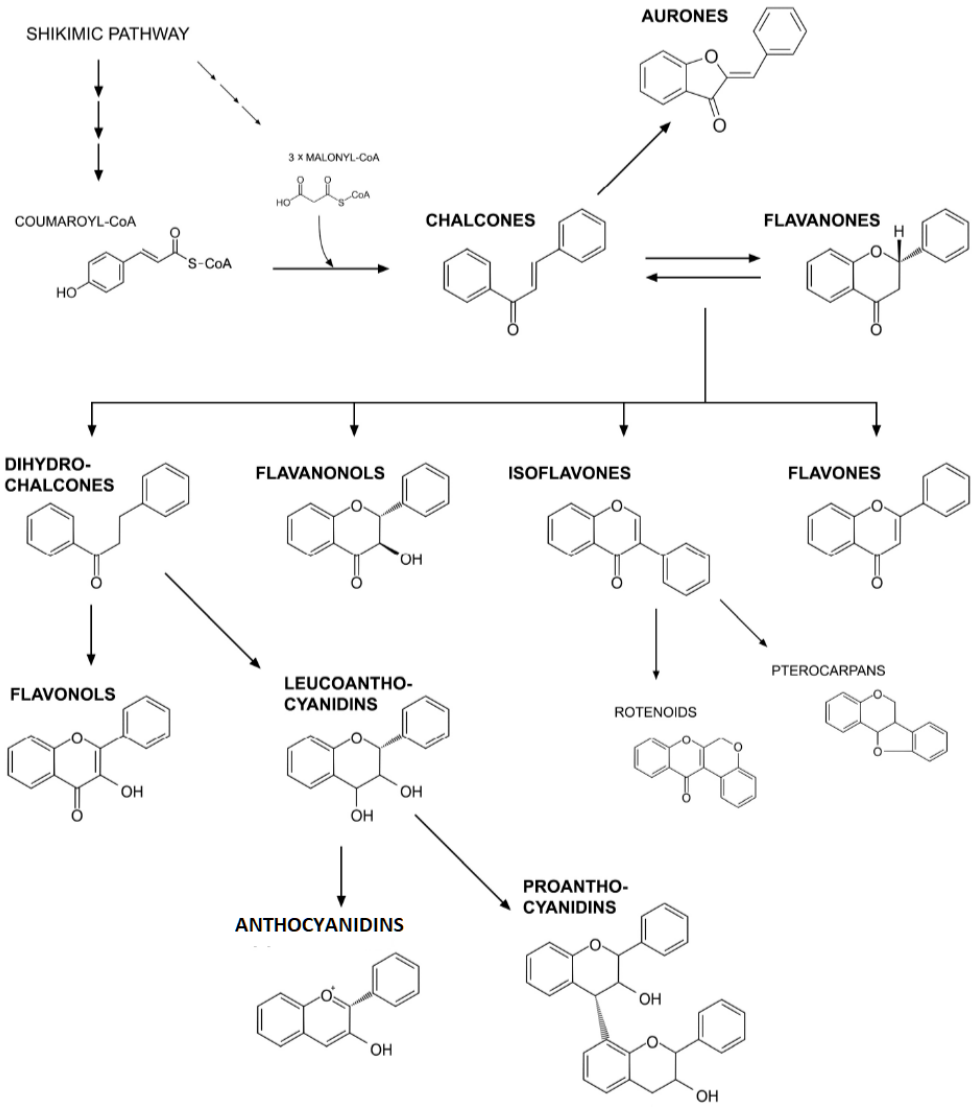


Figure III.6 Structures of various classes of flavonoids. From Hu M et al., 2017 (133).

to their ability to inhibit the activity of IκB kinases through phosphorylation of tyrosine residues in proteins, and consequently the gene expression of the transcription factor NF-κB and the production of pro-inflammatory cytokines[144].

III.3- The Euphorbiaceae family

Euphorbiaceae is one of the largest plant families in terms of the number of species and worldwide distribution[12]. This family is considered cosmopolitan and is composed of approximately 432 genera and 8000 species. It is divided into five subfamilies: Acalyphoideae, Crotonoideae, Euphorbioideae, Oldfieldioideae and Phyllanthoideae, with the Acalyphoideae subfamily as the largest and most complexone[145].It is the sixth most diverse family of flowering plants, with varied chemical characteristics interesting to society as food, medicine, oils, waxes, paint components and other industrial materials[146].

Its species are distributed throughout the world, although its greatest diversity is found in the tropical and subtropical regions, mainly in the Indo-Malaya region and tropical America. There is also a wide variety in tropical Africa, although not as abundant and varied as in the other two regions[147]. In the Americas there are about 2,500 species distributed in 92 genera[14].

The Euphorbiaceae family is represented in Cuba by 48 genera and 330 species. Some of the most represented genera are: Jatropha, Croton, Euphorbia, Pera, Hippomane, Aleurites, Sapium, Gymnanthes and Adelia[148, 149]. The species belonging to these genera have a great diversity of growth forms, ranging from tall trees, shrubs, annual and perennial herbs, to succulents and floating aquatic plants. Among vegetative adaptation niches, only the epiphytic habitat is scarce.

A review of phytochemical studies of this family indicated the presence of many secondary metabolites including alkaloids, coumarins, cyanogenic glycosides and tannins. In addition, many triterpenoids (tetra and pentacyclic) have been identified, isolated from the latex and from different parts (bark, trunk, flowers, leaves, roots and stems) in their free form or as their esters or glycosides. This family is also rich in flavonoids, particularly flavones and flavanols, which have been identified in many genera. These occur as *O*- and *C*-glycosides and as methyl ethers. Flavanones are also present but in relatively few species. Flavonoids have been detected in different parts of the plants, except in the roots[143].

III.3.1- Euphorbiaceae and inflammation

Given the great diversity of the Euphorbiaceae family and the large number of secondary metabolites with relevant therapeutic applications, many researchers have set themselves the task of further studying its species. A useful strategy for the selection of new species to be studied is to use ethno-botanical and chemotaxonomic criteria which, together with modern analytical tools from the fields of chemistry, biology and pharmacy, increase the possibility of a successful screening. In this regard, numerous species of the Euphorbiaceae family have been evaluated, and have shown to contain a wealth of phytochemicals with anti-inflammatory and immuno-modulatory properties.

Numerous species of the genus Croton have been characterized in which terpene-type compounds (monoterpenes, diterpenes), unsaturated fatty acids, aporphine-type, quinoline and tropane alkaloids have been isolated. Other isolated compounds are phenolic constituents such as derivatives of cinnamic acid and flavonoids, including catechin, quercetin, epicatechin, gallocatechins, proanthocyanidins, etc[150]. The aqueous extract of *Croton cajucara* Benth showed anti-inflammatory activity in the paw oedema model using Swiss mice. A 59% inhibition of inflammation was achieved, similar to that obtained for dexamethasone, the drug used as a positive control. Other species of this genus such as *Croton celtidifolius* Baill and *C. cuneatus* Klotzsch also showed antioxidant and anti-inflammatory activity[151, 152]. The essential oil from the leaves of *C. rhamnifolioides* and its main component 1,8-cineole (41.33%) showed anti-inflammatory potential by reducing the oedema induced by various pro-inflammatory agents[153].

Species belonging to the genus *Alchornea* have been analyzed using *in vitro* and *in vivo* models, correlating their anti-inflammatory properties with the presence of numerous flavonoids such as rutin, quercetin and kaempferol, among others. These metabolites are able to inhibit the release of pro-inflammatory cytokines, chemokines, adhesion molecules, arachidonic acid metabolites, iNOS, and the expression of NF-κB[154, 155].

In the same way, species of the genus *Euphorbia* have been studied for their wide ethnomedicinal use as an anti-inflammatory agent. Such is the case for *Euphorbia wallichii*, which demonstrated antioxidant activity against the DPPH (2,2-diphenyl-1-picrylhydrazyl), superoxide and hydroxyl radicals and in the reducing power test by measuring absorbance of Pearl's Prussian blue complex. In addition, the anti-inflammatory activity was evaluated using the human erythrocytes membrane stabilization method, showing that the different extracts under study (methanolic, ethyl acetate and aqueous) revealed activity in a dose-

dependent manner[93]. Extracts obtained from *Euphorbia lathyris* showed anti-inflammatory and anti-cancer activity in mice in a model of acute lung inflammation induced by LPS. The experimental group showed a decreased in the number of anatomical-pathological alterations and inhibited the release of pro-inflammatory cytokines and the migration of neutrophils to the site of inflammation, both effects are consequence of the suppression of the NF-κB activation[156].

The phytochemical analysis of *Excoecaria agallocha* L. allowed the isolation and identification of different diterpenes. These compounds showed an anti-inflammatory potential by inhibiting the expression of genes targeting the activation of NF-κB and AP-1 (activation protein 1) and to the cytokines IL-6 and TNF-α in LPS-stimulated RAW 264.7 macrophages[157].

Other species such as *Cnisdoscolus aconitifolius* (Mill.) I.M Johnst better known as Chaya, have a rich composition in phenolic compounds and flavonoids such as: gallic acid, vanillic acid, vanillin, chlorogenic acid, caffeic acid, ferulic acid, rosmarinic acid, p-coumaric acid, resveratrol, luteolin and apigenin[158]. The aqueous and ethanol extracts obtained from this species showed antioxidant activity against the radicals DPPH and ABTS (2,2-azino-bis- (3-ethyl benzothiazolin-6-sulfonic acid). In turn, both extracts showed anti-inflammatory activity, with the ethanol one the most active, by inhibiting the expression and production of pro-inflammatory cytokines such as TNF-α and IL-6 in THP-1 cells stimulated with LPS[159]. Likewise, the $CHCl_3$:EtOH (1:1) extract: of *Cnisdoscolus tehuacanensis* Breck on showed a significant topical anti-inflammatory effect in the acute ear oedema model and upon chemical fractionation, the presence of terpenes and sterols was detected[160].

III.3.2- Subfamily Acalyphoideae

Acalyphoideae is the largest subfamily of the Euphorbiaceae with 20 tribes, 16 genera and 2584 species[161, 162]. The region with the greatest diversity of species is in Asia, where 44.82% are located. The most diverse genera are Tragia, with 125 species, Dalechampia, which has 100, and Acalypha, with 450, distributed across all continents, and Macaranga with 300 species distributed in Africa, Asia and Madagascar. It is the most diverse subfamily with 36.59% of the genera in the family and a total endemism of 73%, with America being the region with the most accentuated generic endemism (72.22%)[128].

III.3.3- Adelia ricinella L.

III.3.3.1- Taxonomic classification

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Subclass: Rosidae
Order: Malpighiales
Family: Euphorbiaceae
Subfamily: Acalyphoideae
Tribe: Adelieae
Species: *Adelia ricinella* L.

Adelia ricinella L

III.3.3.2- Geographical distribution and common names

Adelia ricinella L. is a shrub native from the Caribbean belonging to the family Euphorbiaceae. It is distributed throughout all regions of Cuba, Jamaica and the Cayman Islands, as well as in coastal areas of Colombia and Venezuela (see Figure III.7)[163, 164].

This plant is known as Yellow Jía, White Jía, Spiny White Jía, Male Jía, Jín without thorns, Bramble white (Cuba), Trejo (Dominican Republic), Citroinmarron, Grenademarron (Haiti), Wild lime (Jamaica), Parakeet, Escambrón, Espinillo (Puerto Rico)[165, 166].

III.3.2.3- Botanical description

Adelia ricinella L. is a shrub that grows up to 10 m in height; branches of whitish bark, hairless and the apex ± spiny. The leaves are oblong to obovate, rounded, obtuse to acute at the apex, the base narrow, 7.5-1 mm, and 4-12 mm wide, hairless on the upper surface, pubescent on the underside in the axils of the veins. The flowers male with pedicel short, sepals 2-3 mm, 8-15 stamens on a central ridge; female pedicels 1-5cm, sepals 3 mm ±, ovary tormentose, hairy disc; capsule 3-lobed, 6-8 mm; seeds brown, globose[167].

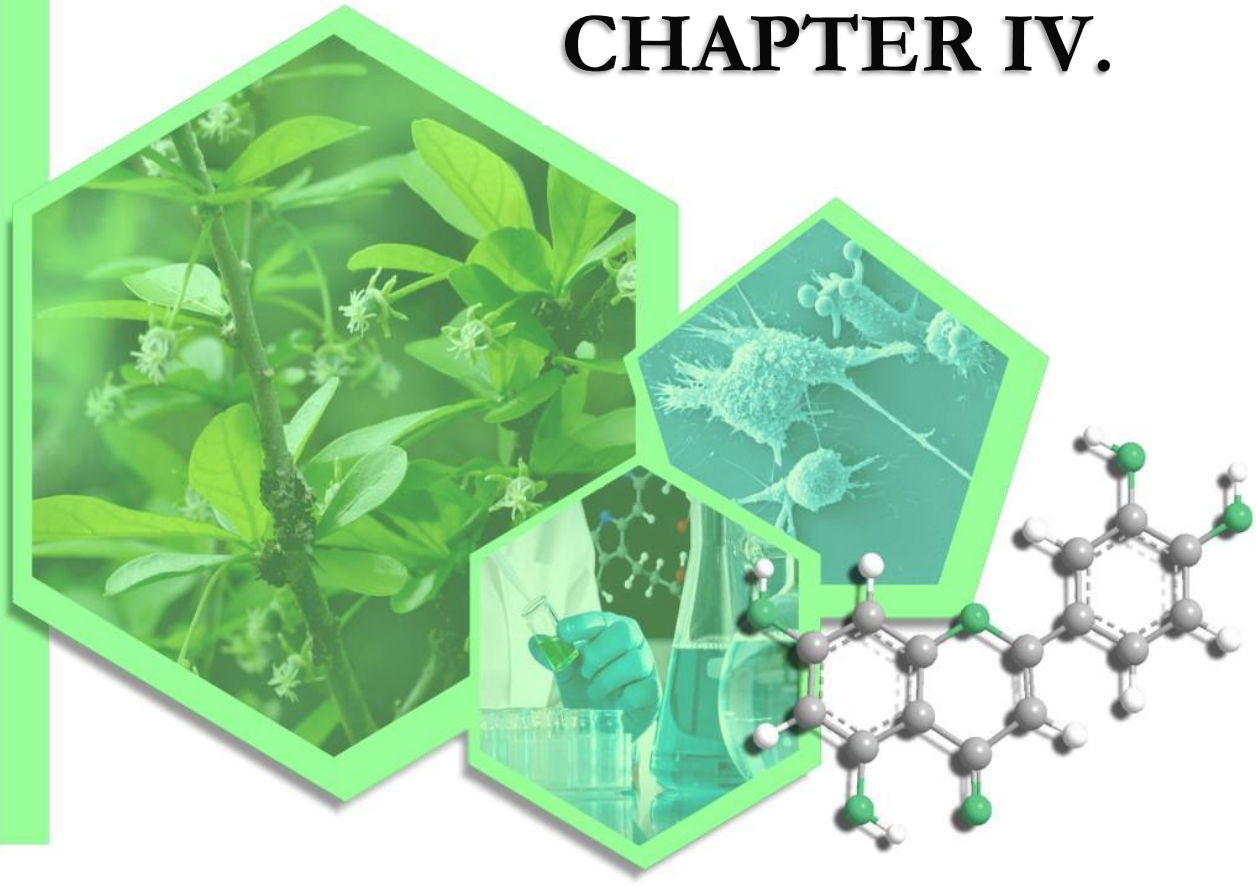
III.3.3.4- Ethnopharmacological uses

The use of the decoction of the aerial parts is reported to treat pain, fever and inflammation[168, 169]. The infusion of the root is used as an emmenagogue in some of the islands of the West Indies, where the plant is native. On the other hand, ethnopharmacological information refers to the use of the aerial parts as analgesic and anti-inflammatory (coastal rural areas of Santiago de Cuba).

The bibliographic review carried out did not reveal the existence of chemical, pharmacological and / or toxicological studies on *Adelia ricinella* L., being a practically unexplored species.

Figure III.7 Geographical distribution of *Adelia ricinella* L. From Taxonomy. Checklist dataset , 2021[164].

CHAPTER IV.



CHAPTER IV. Chemical composition and *in vitro* antioxidant activity of extracts of *Adelia ricinella* L.

Modified version from the published article: Berenguer-Rivas CA, Mas-Ortiz M, Batista-Corbal PL, Costa-Acosta J, Escalona-Arranz JC. Chemical composition and *in vitro* antioxidant activity of extracts of *Adelia ricinella* L. Revista Cubana de Química. 2018; 30(2), 191–209.

IV.1- Introduction

In the progressive development of medicine as a science, there has been a resurgence of Natural and Traditional Medicine (NTM). The use of herbal medicines and supplements has increased enormously in recent years, especially since the late 80's, based on a preference for natural products in detriment of synthetic products in all market segments[170, 171].

Various ethnopharmacological studies have shown a large number of plant species used by the population to treat their diseases, particularly the family Euphorbiaceae, which has a complex phythochemical composition. This family is one of the most representative group of the Magnoliophyta division which is reported with thousands species in a cosmopolitan distribution, with a great architecture diversity predominating trees and shrubs, but also herbs[12].

From the chemical point of view this family in the scientific literature reports the presence of many bioactive compounds such as terpenes, saponins, alkaloids and phenolic compounds (flavonoids, lignans, coumarins, tannins, quinones, phenolic acids, etc.)[12, 147, 172, 173]. Terpenoid compounds such as angelic acid, 7-hydroxy-1,6 cyclo-2,10,14-phytatrien-4-one, vomifoliol among others are reported for this family. Alkaloids of the aporphin, quinoline and tropane type (crotonosine, hernovine, Jacularine, nuciferine, salutaridine, wilsonirine, crotonoside and linearisine) and phenolic compounds like flavones (chrysin, luteolin), flavonol (galangin, quercetin, kaempferol, rutin, myricetin), flavanone (naringenin) isoflavone (genistein, daidzein), flavonol (catechin) are also some of the isolated compounds in this family[149, 174]. This diverse chemical composition has conferred a wide range of therapeutic applications such as immunomodulatory, anti-inflammatory, analgesic and antimicrobial activity, and the treatment of tumours, inflammations, asthma, fever, skin diseases, pneumonic and rheumatic pain, and as insecticide, among others[12, 149, 172-174].

Other researchers have emphasized the evaluation of the antioxidant activity of different species belonging to the family Euphorbiaceae, since the search for obtaining natural

antioxidants to replace the synthetic ones, has become a subject of high interest in the biological, medicinal, nutritional and agrochemical fields[12, 175-178].

Adelia ricinella L. better known as "Jía Blanca", is a shrub belonging to the family Euphorbiaceae[163]. It is native to the Caribbean, and in Cuba it is located throughout the archipelago and Isla de la Juventud. It also inhabits regions of Venezuela and Colombia, where it is claimed to have medicinal properties such as antipyretic, analgesic and anti-inflammatory[168,169 179]. From the scientific point of view, there is only few information about this plant species; however, several ethno-pharmacological uses are reported by the population. This fact demonstrates the need to carry out a deeper study of its chemical composition and pharmacological potential. Therefore, the following work was aimed to characterize the pharmacognostic parameters, and the ability to capture radicals of *Adelia ricinella* L aerial parts and extracts as first approach that support their ethno-pharmacologic values.

IV.2- Material and methods

The aerial parts of the *Adelia ricinella* L. plant were collected in Siboney-Juticí Ecological Reserve, in the municipality of Santiago de Cuba. A plant sample was taxonomically identified by a specialist at the Eastern Center for Ecosystems and Biodiversity (BIOECO) in the province of Santiago de Cuba and a voucher specimen was stored at the herbarium of said institution with the registration number 14 780 (see Annex 1). The plant material was divided in two batches: fresh and dried at room temperature until constant weight. Fresh leaves were used to determine the micro and macromorphological aspects as well as essential oils determination, while dried ones to determine their residual moisture, soluble substances, and total ash. This batch was also used to prepare the extracts after milled (MRC KM 700 blade mill, Germany) and sieved (particle size bellow 250 μm).

IV.2.1- Extracts Preparation

Three extracts of *Adelia ricinella* L. aerial parts were prepared, taking into account the ethno-botanic procedure made by the Caribbean inhabitants that uses hot water as extraction method. Ethanol at two concentrations was also used with the objective to decrease the polarity of the extracted compounds, keeping the heat application. The extracts were prepared by Soxhlet extraction, during four hours after the first reflux using 100g of dry plant material and water, ethanol 50% and ethanol 95% as a solvent, obtaining three extracts. The extracts were filtered using a Buchner funnel and filter paper, and

concentrated in a KIRKA - WERKE Rotary Evaporator (Germany) reducing the final volume to 100 ml, obtaining a final concentration equivalent to 10 mg/ml (dry plant material weight). From now on, extracts prepared with 95% and 50% ethanol will be identified as AR1 and AR2, while AR3 will be used to represent the extract prepared with water.

IV.2.2- Determination of the quality control parameters of plant material

As the species has no previous pharmacognostic studies, classified as an unofficial drug; consequently, pharmacognostic parameters were determined to establish the authenticity and quality of the plant material collected.

IV.2.2.1- Macro-morphological analysis

To determine this parameter a sample of 100 leaves from 10 plants was taken, and the mean dimensions of the width and length were calculated to describe the external morphology (shape), the disposal of the stem, venation, shape of the vertex and base, by margin or edge, consistency and by the color and nature of the upper and lower surfaces.

IV.2.2.2- Micro-morphological analysis

Five adult leaves were taken from four different individuals. The leaves were hydrated with distilled water and glycerine (ratio 1:1, v/v) due to their subcoriaceous texture. Subsequently, manual transversal cuts to the entire leaf lamina were made, as well as longitudinal and transversal cuts to the median vein on a paraffin support. Afterward were stained with safranin and differentiated with 70% alcohol, to be later washed with distilled water[180]. In the adaxial epidermis were determined the number of cell layers, thickness of the cuticle in relation to the epidermis, thickening or not of the anticlinal and periclinal cell walls and shape of the anticlinal walls. For the abaxial shape the paradermal surface, types of stomata on both leaf surfaces and the nature of the mesophyll were considered. Barthlott's terminology was used to determine the presence of waxes and the way they are arranged[181]. Observations were made with a bright field microscope (NOVEL N-220M/China) at 400X magnification and a stereo microscope (NOVEL NSZ-606/China) at 40X magnification.

IV.2.2.3- Determination of essential oils

Two hundred grams of fresh leaves were extracted by hydro-distillation in a Clevenger-type apparatus in order to quantify the presence/absence of essential oils. The experiment was performed in triplicate. The oil yield was calculated relative to the dry matter, the average of three determinations being reported.

IV.2.2.4- Residual moisture

A batch of leaves were dried in shadow conditions and weighted every 24 hours until constant weight. Afterward, residual moisture was determined by the infrared gravimetric method (Sartorius MA 35, Göttingen, Germany) according to the methodology described in the British Pharmacopeia[182]. Determinations were made in triplicate and the mean of the three replicates was reported. Equation 1 was used for the calculations.

$$Hr = \frac{(V2 - V1)}{M} \times 100 \qquad [1]$$

Where:

V2: Final volume of water (ml), V1: Initial volume of water (ml).

M: Mass of the sample (10 g)

IV.2.2.5- Soluble substances

Five grams of the dry plant material were placed in a 250 ml Erlenmeyer flask with frosted top lid and 100 ml of the solvent (water or ethanol) was added. After 12 h of stirring maceration and 6 h repose, the content was filtered and 25 ml of the filtrate was transferred to flat-bottomed dishes to evaporate the solvents at 105 °C in a Carl Roth SC 150 thermostatted bath (Karlsruhe, Germany). The cooled residue was weighed, expressing the values (w/v) in percent according to the follow equation[183]:

$$Ss = (R \times 500 \times 100) / (M \times (100-H)) \qquad [2]$$

Where: H: moisture content of the sample in %. 500 and 100: mathematical factors for the calculations. R: residue of the sample (g). M: mass of the sample (g).

IV.2.2.6- Determination of total ash

Total ashes were determined according to the NRSP 309/911 with slight modifications[183]. Two milliliters of the extracts were added in a porcelain crucible and incinerated at 700 ˚C in a ML.-12 muffle oven (Germany) for 2 hours. The crucible was cooled in a desiccator and weighed. The process was repeated until a constant mass was obtained (two consecutive weighing did not differ by more than 0.5 mg/g). The results were reported based on the percentage of ashes in anhydrous base, using equation 2 and 3 replicates were determined.

$$C_t = \frac{(M_2 - M_1) \times 100}{M} \times \frac{100}{100 - H} \qquad [3]$$

Where:

C_t: Total ashes in anhydrous base M: Mass of the sample (2 g).

M_1: Mass of the empty crucible (g). M_2: Mass of the crucible containing ashes (g).

H: % Residual Moisture.

IV.2.3- Physico-chemical characterization of the extracts obtained

The Physico-chemical characterization of the extracts was carried out according to the Cuban National Standards defined by the Health Ministry[183, 184]. The parameters determined were:

-Organoleptic characteristics: organoleptic properties were evaluated by simple inspection through the senses. Color, texture and smell were evaluated.

-pH: A direct pH value was obtained with a pH-meter (Hanna Instruments, Spain). The pH-meter was calibrated using buffer solutions at pH 4 and 7. Three measurements were carried out and the mean was reported.

-Relative density: was determined using a 25 ml pycnometer. Three measurements were carried out and the mean was reported.

-Total solids: were determined using a gravimetric method with 5 ml in a porcelain capsule.

IV.2.2.1-Qualitative chemical composition

Chemical reactions established as phytochemical screening techniques described in the literature were performed to define the metabolites or groups of metabolites present[185]. The metabolites to be determined were alkaloids, amino acids and amines, carbohydrates, saponins, phenols and tannins, reducing sugars, triterpenes and steroids, quinones, coumarins and flavonoids. In brief:

1. Test for alkaloids

a) Mayer's test: add to one ml of plant sample extract two drops of Mayer's reagent. Appearance of a white creamy precipitate indicates the presence of alkaloids.

b).Wagner's test: a few drops of Wagner's reagent are added to one ml of plant extract. A reddish- brown precipitate confirms the test as positive.

2. Test for amino acids and amines

a) Ninhydrin test: two drops of ninhydrin solution (10 mg of ninhydrin in 200 ml of acetone) are added to 2 ml of aqueous filtrate extract. The appearance of a purple color indicates the presence of amino acids and amines.

3. Test for carbohydrates

a) Molish' s test: to 2 ml of plant sample extract, two drops of alcoholic solution of α-naphthol are added. The mixture is shaken well and a few drops of concentrated sulphuric acid are gently added. A violet ring indicates the presence of carbohydrates.

4. Test for saponins

a) Foam test: the extract (10 ml) was diluted with 10 ml of distilled water. The solution was shaken for 15 min. A two cm layer of foam indicates the presence of saponins.

5. Test for phenolic compounds and tannins

a) Ferric chloride test: a few drops of neutral 5% ferric chloride solution were added to 1 ml of the extract. A dark green color indicates the presence of phenolic compounds.

6. Test for reducing sugars

a) Benedict's test: two ml of the extract were treated with Benedict's reagent and heated gently. An orange red precipitate indicates the presence of reducing sugars.

b) Fehling's test: two ml of the extract were hydrolyzed with dil. HCl, neutralized with alkali and heated with Fehling's A & B solutions. Formation of a red precipitate indicates the presence of reducing sugars.

7. Test for triterpenoids

a) Salkowski's Test: two ml of the extract were dried and re-dissolved in chloroform and filtered. The filtrates were treated with a few drops of concentrated sulphuric acid, shaken and allowed to stand. Appearance of a golden yellow color indicates the presence of triterpenes.

b) Liebermann Burchard's test: two ml of the extract are dried and re-dissolved in chloroform and filtered. The filtrates are treated with a few drops of acetic anhydride, boiled and cooled. Concentrated sulphuric acid is added along the sides of the test tube. The color changes from violet to blue-green indicated the presence of triterpenoids.

8. Test for quinones

a) Borntrager's test: take 1 ml of the extract, concentrate until dryness and re-dissolve in 1 ml of chloroform. One millilitre of a 10% ammonia solution was then added and shaked. Appearance of a pink, red or violet color in the ammoniacal (lower) phase indicates the presence of free quinones.

b) Variant with benzene: two milliliters of the extract are dried and re-dissolve in 2 ml of benzene. Two milliliters of a 5% NaOH solution are added and shaken. The formation of a red or yellow color is evidenced of the presence of quinone.

9. Coumarins test

a) Baljet test: one milliliter of the extract was treated with a solution of sodium picrate. The formation of a yellow color indicates the presence of lactones.

10. Test for flavonoids

a) Concentrated sulfuric acid: to one milliliter of the extract 2 ml of dilute ammonia solution were added in a test tube. After this, a few drops of concentrated H_2SO_4 were added. A yellow color indicates the presence of flavonoids.

b) Shinoda's test for flavonoids: one mililiter of the extract is dried and re-disolved in 1 ml ethanol. Three pieces of magnesium chips were then added to the filtrate followed by a few drops of conc. HCl. A pink, orange, or red to purple color indicates the presence of flavonoids.

c) Alkaline reagent test: extracts were treated with a few drops of sodium hydroxide solution. Formation of an intense yellow color, which becomes colorless on addition of dilute acid, indicates the presence of flavonoids.

IV.2.2.2- Quantification of Total Phenolic content (TPC)

The total phenolic content was determined by the colorimetric method using Folin-Ciocalteu's reagent (Sigma, USA)[186]. The reagent reacts with phenol groups by a REDOX reaction getting a blue color. The color produced is proportional to the amount of polyphenols present in the extract analyzed, absorbing from 750 to 770 nm. Briefly, 500 µL of sample (1 mg/ml solution based on total solids) was mixed with 1 ml of 50% Folin-Ciocalteu's and incubated at room temperature in a dark room. Subsequently, 2 ml of saturated sodium carbonate solution (Riedel-de Haën, 99.5% pure, Germany) was added. Finally, the solution was incubated at room temperature in the darkness for one hour. The absorbance was measured at 765 nm on a UV/VIS spectrophotometer from PG Instruments, model T60 (China). A standard curve using gallic acid (GA) (Sigma, USA) was created with 7 points (from 9.7 to 625 µg/ml). The mathematical equation used to calculate the concentration was:

$$y = 0.001x - 0.006; \quad R2=99.7\% \qquad [4]$$

The results were expressed as mg in gallic acid equivalents/g extract. All measurements are repeated 3 times. Based on findings of Dudonne et al., TPC were categorized as very high (> 300 mg GAE/g), high (200 - 300 mg GAE/g), moderate (50 - 200 mg GAE/g), low (15 - 50 mg GAE/g), very low (< 15 mg GAE/g)[187].

IV.2.2.3- Quantification of Total Flavonoid Content (TFC)

The quantification of the total flavonoid content was assessed through reaction with aluminum trichloride ($AlCl_3$, Riedel-de Haën, 99.9% pure, Germany)[188]. Briefly, 250 µL of the extracts (1 mg/ml solution based on total solids) were mixed with 1.25 ml of distilled water and 75 µL of a 5% $NaNO_2$ solution. After five min, 150 µL of a 10% $AlCl_3$ aqueous solution was added. After six min, 500 µL of 1M NaOH and 275 µL of distilled water were added. The solution was mixed well and read at 510 nm in the mentioned spectrophotometer.

Values were determined from a calibration curve prepared with quercetin (Q) (Sigma, 95% pure, USA) (ranging from 6.25 to 100 µg/ml) and expressed as mg of quercetin equivalent/g extract. The mathematical equation used to calculate the concentration of the sample expressed as quercetin was:

$$y = 0.008\ x - 0.060; \quad R2 = 99.3\%. \qquad [5]$$

Considering the previous criteria, TFC were categorized as very high (>300 mg QE/g), high (200-300 mg QE/g), moderate (50 - 200 mg QE/g), low (15 - 50 mg QE/g), very low (< 15 mg QE/g)[187].

IV.2.2.4- Quantification of Total Protein Content

The quantification of total proteins was performed following the Lowry methodology[189]. One milliliter of the samples (1 mg/ml solution based on total solids) were added to 5 ml of the Lowry reagent, which is composed of three solutions mixed at the time of use: 2% sodium carbonate in 0.1 M NaOH (50 ml), 1% cupric sulfate (0.5 ml) and 2% sodium potassium tartrate (0.5 ml). After 15 min, 0.5 ml of Folin-Ciocalteu reagent was added, allowing the mixture to stand for another 30 min. At the end, absorbance was measured at 595 nm. A standard curve was prepared using bovine serum albumin (BSA) in a concentration range between 5 - 200 µg/ml. The mathematical equation that describes it behavior is given as equation 3. The results were expressed as g BSA equivalent /100 g extract.

$$y = 0.001\ 8\ x + 0.024\ 9 \qquad R2 = 99.27\ \% \qquad [6]$$

IV.2.2.5-Quantification of Carbohydrates

The quantification of carbohydrates was realized by the phenol-sulfuric method, according to the methodology described by Dubois[190]. To milliliter of the samples (1 mg/ml solution based on total solids) were mixed with 2 ml of 5% phenol in test tubes and placed in a rack

submerged in a cold water bath. Five milliliters of H_2SO_4 were added letting the tubes for 15 min to measure the absorbance at 490 nm in a UV/VIS spectrophotometer. Glucose (Riedel-de Haën, 99.5% pure, Germany) was used for the calibration curve in concentrations from 10 to 200 µg/ml expressed by the following equation:

$$y = 0.003\,3x - 0.040\,8; \qquad R^2 = 99.5\% \qquad [7]$$

All measurements are repeated 3 times. The results were expressed as g of glucose equivalent /100 g extract.

IV.2.2.6-Quantification of Total Reducing Sugars

The determination of total reducing sugars was done using 3,5-dinitrosalicylic acid (DNS, Sigma-Aldrich, 98% pure, USA), following the methodology described by Miller[191]. For the preparation of this reagent, 0.8 g of NaOH (Riedel-de Haen, 97% pure, Germany) was dissolved in distilled water, then 15 g of potassium sodium tartrate tetrahydrate (Fluka, 99% pure, Germany) was added as well as 0.5 g of DNS. This mixture was poured into 50 ml with distilled water and stored in an amber flask at 4 °C. Subsequently 0.5 ml of each sample (1 mg/ml solution based on total solids) and 0.5 ml of the DNS reagent were placed in a beaker, boiled for 5 min to further stop the reaction adding cold water/ice. Five milliliters of distilled water were added to the samples, shaken and rested for 15 min. The absorbance was determined at 540 nm in a spectrophotometer (T60 UV-Visible Spectrophotometer). The same treatment was performed for the blank with distilled water. A calibration curve was developed, using glucose as standard in concentrations from 0.1 to 1 mg/ml, obtaining the following calibration curve:

$$y = 0.000\,4x - 0.007; \; R^2 = 0.994\,2 \qquad [8]$$

All measurements are repeated 3 times. The results were expressed as g glucose equivalent /100 g extract.

IV.2.4- Evaluation of antioxidant activity

IV.2.4.1-Antioxidant activity against the radical 2,2-azino-bis- (3-ethyl benzothiazolin-6-sulfonic acid) (ABTS).

This assay was carried out according to the methodology described in the literature[192]. The assay is based on the ability of an antioxidant compound to quench the ABTS radical (Sigma-Aldrich, 98% pure, USA). To reach that goal, 50 µL of each extract (solutions of 62.5, 125, 250, 500 and 1 000 µg/ml based on total solids) were added to 3 ml of diluted ABTS solution and after 90 min the absorbance was measured at 734 nm. A solution of 50 µL of

distilled water and 3 ml of diluted ABTS was used as absorbance blank. Ascorbic acid (Fluka, 99% pure, Germany) at a concentration of 1 mg/ml was considered as positive control. The ability of radical quenching was determined by calculating the percent inhibition of the radical (IpABTS) according to the formula:

$$IpABTS\ \% = [(A0 - A)/A0] \times 100) \qquad [9]$$

where:

A0 is the adjusting absorbance of the ABTS + solution (blank)

A is the absorbance of the sample

The scavenging of ABTS radicals by the extracts of *Adelia ricinella* L. was estimated as a function of the concentrations of extracts capable of quenching 50% of the radical (IC_{50}) obtained by intrapolation of the curve constructed from the five concentrations evaluated. All the experiments were repeated three times.

IV.2.4.2-Antioxidant activity against the radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH)

The standard methods described in the literature were followed[193]. In brief: A solution of 0.1 mM of DPPH (Merck, pure for analysis, USA) was prepared using 0.003 94 g dissolved in 100 ml of ethanol. A total of 0.25 ml of the extracts (solutions of 62.5, 125, 250, 500 and 1 000 µg/ml based on total solids) were placed in test tubes, to which 1.5 ml of the DPPH solution was added. The mix was shaken in a vortex (Heidolph REAX 2000, Germany) and kept in the dark for 20 min. The absorbance was measured in a spectrophotometer (T60 UV-Visible spectrophotometer) at 517 nm. The absorbance blank was prepared with 0.25 ml of ethanol and 1.5 ml DPPH solution. The positive control was an ascorbic acid solution a concentration of 1 mg/ml. The radical sequestration ability was determined by calculating the percent inhibition of the radical (Ip DPPH) by the equation:

$$Ip\ DPPH\ \% = (A0\text{-}A)\ /A0\ *100 \qquad [10]$$

where:

A0: Absorbance of control at the wavelength 517 nm

A: Absorbance of the sample at the wavelength 517 nm

In addition, the antioxidant capacity against these radicals was estimated as a function of the concentrations of the extracts at 50% inhibition (IC_{50}) obtained by intrapolation of the calibration curve constructed with the five concentrations evaluated. All the experiments were repeated 3 times.

IV.2.5- Statistical analysis

For the statistical analysis, Microsoft Excel was used (Microsoft Office 2007 package) as well as STATGRAPHICS Plus Version 5.1. The results of total phenols, flavonoids, proteins, carbohydrates and reducing sugar content were expressed as mean ± standard deviation of each extract and their means were compared using an ANOVA Variance Analysis, aided by Statgraphic Centurion. The differences between extracts were determined by Tukey's Low Significant Differences Test (LSD). The same processing was used for the biological parameters determined: antioxidant activities against ABTS and DPPH radicals. In all cases, the 95% confidence limit was considered. Calibration curve equations were obtained using the Simple Linear Regression option of the STATGRAPHICS software used.

IV.3-Results and Discussion

IV.3.1- Pharmacognostic parameters of plant material

Macro-morphological and micro-morphological analyses

Leaves of the *A. ricinella* L: plant are simple, fascicled at the nodes, short petiolate, caedaceous, apex retuse or acute, rounded to acute in the base, pinnate ribbing, and membranous consistency with the water stress to which these plants are exposed. Lustrous-green upper surface leaf is less colored and pubescent than the lower surface which happens to be light green. The study of the dimension of the leaves indicated average values of length and width equivalent to 19.95 ± 4.20 mm and 10.35 ± 1.16 mm respectively. In general, this description coincides with the reported in with Flora de Cuba in 1953[194] and Nova in 2006[163] which perform an extensive taxonomic revision of the genus *Adelia*. However, the average values of leaf length and width determined in this study are close to the lower limit. This variability in leaf size could be related to the plant degree of development and the environmental conditions of the harvest zone: Siboney-Juticí Ecological Reserve. This protected area corresponds to a Coastal Xeromorphic Scrubland, where the geomorphological structure and ecosystem is largely determined by the effect of the natural disturbances, aridity of the soils, long periods of drought and high temperatures during the day[195].

In the micro-morphological study, translucent dots with a density of 5-16/mm^2 were observed on the leaf surface (Figure IV.1-A). The 10-19 µm mesophyll has an adaxial epidermis with isodiametric to rectangular cells approximately 2 µm in diameter; straight to convex periclinal and straight to concave anticlinal walls with a 0.375-0.5 µm cuticle of serous nature. The palisade parenchyma is uni-stratified and the cells are 3 µm long and

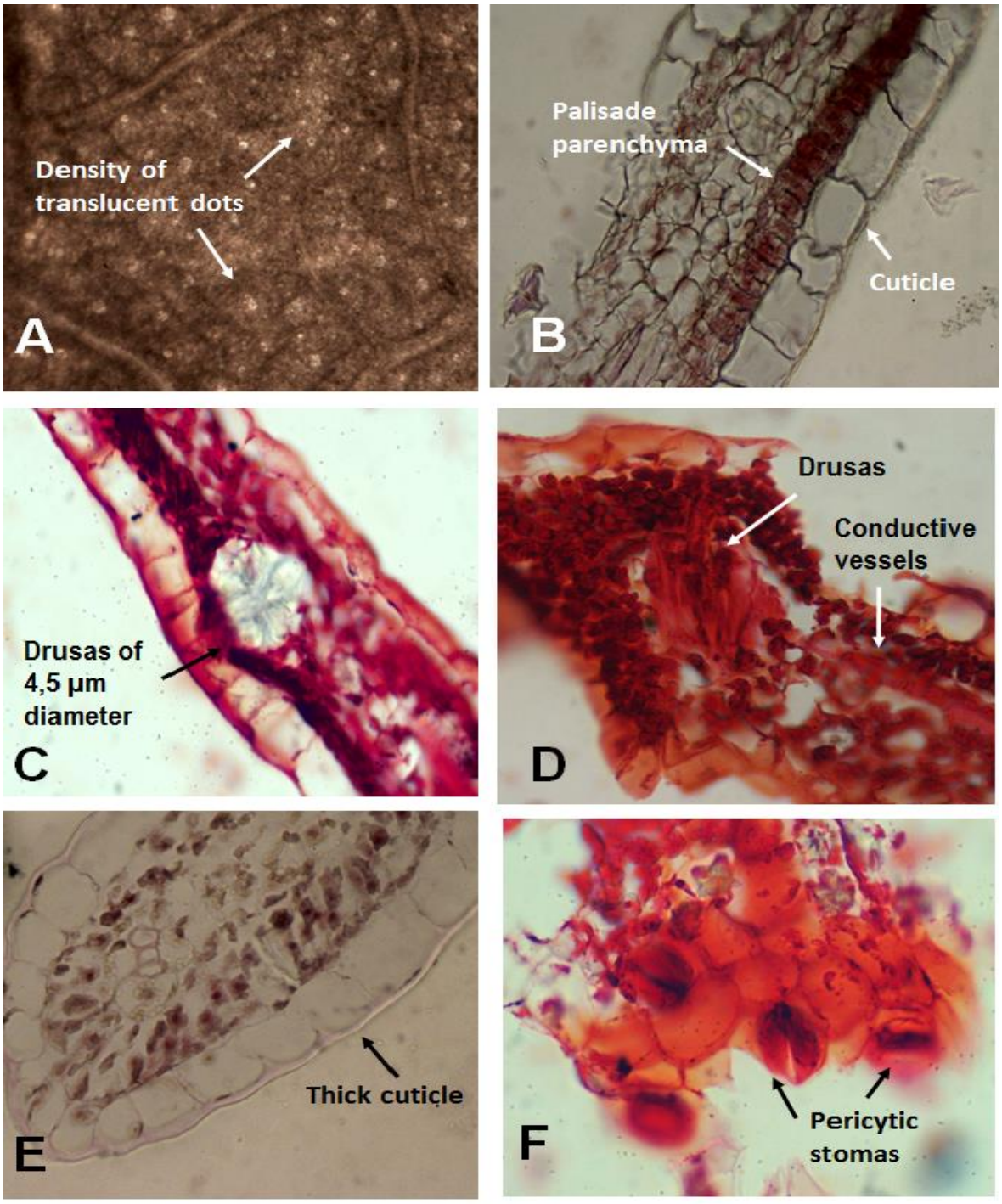


Figure IV. 1 Transverse and longitudinal sections of *Adelia ricinella* L. leaves.

A) Leaf observed under a stereo microscope 4X. (Leaf surface with translucent dots)

B, C, D, E, F) Transverse sections of mesophyll (100X): **A)** Leaf observed under a stereo microscope 4X; **B)** Periclinal walls (straight to convex), Anticlinal walls (straight to concave) **C, D)** Leaf stratum with abundant druses, **E)** Thick cuticle, **F)** Pericytic stomas

continued by several layers of aerial parenchyma that occupy 4.5-5 μm of the mesophyll (see Figure IV.1-B).

In the leaf stratum there are abundant druses of approximately 4.5 μm in diameter and density equal to 5 U in 438 μm^2. Also appears inclusions of solid or liquid materials of 7.3 μm which are coloured pink when treated with safranin (Figure IV.1-C). This material appears solid after fixation in Bouin's liquid and alcoholic dehydration and looks like a bundle fibre (Figure IV.1-D). There is also an inclusion on each side of the median nerve and in the terminal third of the leaf surface in cavities that show formation due to cell destruction and into which scalariform conducting vessels discharge. The abaxial epidermis (approximately 1.2 μm), has from straight to convex periclinal and from straight to concave anticlinal walls. It has a cuticle as thick as adaxial at least at the leaf margin (Figure IV.1-E) and pericytic type stomata (Figure IV.1-F). These characteristics are consistent with the xerophytic nature of the Coastal Xeromorphic Scrubland in which the living species under these conditions concentrates large amounts of cutin, suberin and waxes, which are able to prevent water loss and protect them from pathogen damage.

Essential oils

The fresh plant material batch was subjected to essential oils extraction by hydro-distillation; however, the extraction process was NEGATIVE (see Table IV.1).

Residual moisture

Two weeks were necessary to reach a constant weight when drying the *A. ricinella* L. leaves on shade. Residual moisture (RH) calculated was 11 ± 0.42% (see Table IV.1), classifying as acceptable for unofficial drugs[182], and ensuring that this drying process is adequate for a correct conservation of the plant material.

Soluble substances

This parameter is one of the most important numerical indices for selecting the best solvents for the extraction, especially when there are no previous reports in the literature. The results obtained (table IV.1), highlight ethanol as the most effective solvent to extract *A. ricinella* L. metabolites. Similar results are observed in numerous plants such as *Calendula officinalis* L. (marigold) (NRSP 325/2002)[196]; and *Mentha arvensis* L. (Japanese mint) (NRSP 348/97)[197]; in agreement with the medium polarity of ethanol solutions.

Determination of total ash

Table IV.2 shows the total ashes values obtained for the three *A. ricinella* L. extracts. The results revealed the following behaviour: AR1>AR3>AR2 and the values ranged between

3.86 ± 0.31 and 6.69 ± 0.26 obtained for the extracts 50% ethanol (AR2) and 95% ethanol (AR1) respectively. Those values exceed the established by the Pharmacopoeias for non-official drugs (up to 5%). This must be an expression of the arid condition of the soil within Siboney-Jutici area. Nevertheless, is not invalidating; considering many plant species show values higher than this pre-established 5%. The Cuban National Formulary of Phyto and Api phytomedicines[198] and Pharmacopoeias[199] records *Chamomilla recutita* L. (NRSP 317/91)[200], *Calendula officinalis* L. (NRSP 325/2002)[201], WHO 2002)[202]; *Plantago major* L., (NRSP 335/91)[203]; *Plectranthus amboinicus* (NRSP 352/97)[204]; and *Passiflora incarnate* L. (NRSP 328/91)[205] with total ash values over 10%.

IV.3.2- Physico-chemical characterization of the extracts

The plants of the family Euphorbiaceae are present in all types of habitats, adapting to abiotic stress factors such as: high temperature, salinity and long periods of drought. This induces them to produce a variety of secondary metabolites (polyphenols, flavonoids, tannins, alkaloids, coumarins, among others) to be used for the survival and/or defense against biotic and abiotic aggressions. This diverse secondary metabolism confers in turn a greater complexity and a high investigative potential, especially from the chemical and pharmacological point of view. Considering the above mentioned and according to the scarce information about *Adelia ricinella* L., it was decided to establish parameters for the quality control of the three extracts obtained in order to standardize the information (Table IV.2).

When analyzing the organoleptic properties, the colour of the extract in 95% ethanol resulted in an intense green tone. The extract obtained with ethanol 50% took a light green coloration, while the aqueous extract was light brown. The three extracts are slightly viscous and have a similar odor, like the original plant material.

The values of total solids obtained indicate that the extraction method and the solvents used are suitable for the extraction of the plant metabolites. Ethanol 95% (AR1) resulted as the extraction solvent with the highest amount of extracted substances expressed in mass units. This shows how solvents with medium polarity as ethanol, are suitable for the extraction of total metabolites of the species *Adelia ricinella* L.

Relative density results show a logic tendency with higher values for aqueous extract (AR3), followed ethanol 50% (AR2) and ethanol 95% (AR1) extracts. Those values are higher than the density of the pure solvents itself, corroborating the presence of metabolites extracted from the plant material; and are in agreement with the density of the

Table IV.1. Pharmacognostic quality control parameters for plant material (*Adelia ricinella* L.)

Quality control parameters	Fresh Drug	Dry Dug
Essential Oil (m/v)	Negative	-
Residual Moisture (%)	-	11.01 ± 0.42
Ethanol soluble substances (%)	-	12.96 ± 2.27
Water soluble substances (%)	-	7.30 ± 1.14

Table IV.2. Pharmacognostic quality control parameters for *Adelia ricinella* L. extracts

Quality control parameters	Ethanol 95% (AR1)	Ethanol 50% (AR2)	Water (AR3)
Organoleptic characteristics	Intense green, slightly viscous	Light green, slightly viscous	Light brown, slightly viscous
Total solids (g/100 mL)	12.01 ± 0.28^{a}	5.02 ± 0.08^{c}	8.21 ± 0.20^{b}
Relative density (g/mL)	0.951 ± 0.020^{c}	1.014 ± 0.008^{b}	1.039 ± 0.004^{a}
pH	4.47 ± 0.006^{b}	4.38 ± 0.006^{c}	4.70 ± 0.010^{a}
Total Ashes	6.69 ± 0.26^{a}	3.857 ± 0.31^{c}	4.42 ± 0.39^{b}

a, b, c: Different letters indicate significant differences ($p < 0.05$)

pure solvents used. The pH values ranged from 4.38 to 4.70, with AR2 extract being the more acidic one. All those physico-chemical parameters are statistically different for the three extracts tested.

In table IV.3 the presence of alkaloids, coumarins, reducing sugars, flavonoids, phenols and tannins, carbohydrates, amino acids and amines in the three prepared extracts is displayed. Saponins were only identified in the aqueous and 50% ethanol extracts. Many of those metabolites are phenols, probably causing the weakly acidic pH detected in the physico-chemical characterization.

The presence of alkaloids in the three extracts is quite interesting because they are compounds with a large structural variability and, in turn, a wide range of applications and biological activities[12, 193]. According to the colors developed in the different chemical tests, the flavonoids present in the extracts belong to two main subgroups: flavones and flavonols. In the ethanol 95% extract, the presence of triterpenes and steroids with unsaturated androstane nucleus in ring B and with a double bond between carbons 5 and 6 is detected, as judged by the dark green coloration obtained in the Lieberman Burchard test. In general, these results are in correspondence with those reported in the literature for plants belonging to the family Euphorbiaceae [12, 173].

The concentrations determined for each of the quantified classes of metabolites are shown in Table IV. 4. Significant differences ($p < 0.05$) can be observed in the content of total phenols, flavonoids, carbohydrates and reducing sugars when comparing ethanolic and aqueous extracts, noting that the highest concentrations of these metabolites are found in the ethanol 50% extract. The Total Phenol Content for the three extracts can be considered as very high, while the total flavonoids content qualifies as low (aqueous and ethanol 50% extracts) and very low (ethanol 95% extract) according to the criteria of Dudonne[187]. These values obtained for total phenols and flavonoids are higher than those obtained in other studies of plants belonging to the family Euphorbiaceae[206 – 208].

The protein content does not follow the same rule as observed for the other metabolites. In this case the highest concentrations are found in the aqueous extract, as can be expected when the high water solubility of this kind of compounds is considered.

Table IV.3 Qualitative chemical composition of extracts of the plant *A. ricinella* L.

Metabolites	Test	Ethanol extracts 95% (AR1)	Ethanol extracts 50% (AR2)	Aqueous extract (AR3)
Alkaloids	Mayer	+++	+++	+++
	Wagner	+	+	+
Triterpenes and steroids	Salkowski	+	+	NT
	Lieberman-Burchard	+	-	NT
Quinones	Borntrager	-	+	NT
	Variant with benzene	-	+	NT
Coumarins	Baljet	++	++	NT
Saponins	Foam	-	+	+
Reducing sugars	Fehling	+	++	+
	Benedict	+	++	+
Phenols and tannin	FeCl3	+	+	+
Free amino acids and amines	Ninhydrin.	+	+	+
Carbohydrate	Molisch	+	++	+
Flavonoids	H_2SO_4 conc.	+	+	+
	Shinoda	+	+	+
	Rosemheim	-	+	+

Legend: (+) indicates positive evidence, (+++) indicates markedly positive evidence, (-) indicates negative result; NT corresponds with not-tested.

Table IV.4. Quantification of total phenols, total flavonoids, proteins, carbohydrates and reducing sugars present in the extracts of *Adelia ricinella* L.

Extracts	Total Phenols (mg/g)	Total Flavonoids (mg /g)	Proteins (g/100 g)	Carbohydrates (g/100 g)	Reducing Sugar (g/100 g)
	Means ± D.E				
Ethanol 95% **(AR1)**	402.30 ± 19.50^{a}	10.00 ± 1.00^{a}	10.35 ± 0.12^{a}	38.75 ± 4.28^{a}	17.67 ± 2.40^{a}
Ethanol 50% **(AR2)**	967.90 ± 71.80^{c}	19.29 ± 1.09^{c}	24.87 ± 1.26^{b}	77.17 ± 18.05^{c}	80.91 ± 3.66^{c}
Aqueous **(AR3)**	523.10 ± 12.40^{b}	17.33 ± 2.85^{b}	34.55 ± 0.64^{c}	49.49 ± 4.53^{b}	52.58 ± 3.25^{b}

a, b, c: Different letters indicate significant differences ($p < 0.05$)

IV.3.3-Evaluation of antioxidant activity

Antioxidant activity against the radicals ABTS and DPPH

Figure IV.2 shows the percentage inhibition of the ABTS and DPPH radicals by the three extracts tested. All extracts were able to neutralize these radicals in a concentration-dependent manner. When facing the ABTS radical, the extracts had a similar statistic behavior that the reference antioxidant (ascorbic acid), but not in the case of the DPPH radical, where the extracts evaluated were less active than the positive control, exhibiting significant differences ($p < 0.05$).

Several authors have reported high antioxidant activity in different species belonging to the family Euphorbiaceae; associating this fact to the presence of a high content of phenolic compounds. However, it is possible that these antioxidant properties are due to the presence of other metabolites in the extracts such as alkaloids and carbohydrates[209 – 211].

Alkaloids derived from benzylisoquinoline have shown antioxidant capacity (AC) related to the presence of an adjacent benzylic hydrogen (ortho position) to a nitrogen atom with two unpaired electrons[212]. This type of alkaloid has been frequently identified in species of the family Euphorbiaceae[213]. Additionally, reducing sugars and carbohydrates are substances produced by the primary metabolism of plants, and are also reported to exhibit important pharmacological activities. They have a carbonyl group which can act as a reductant against other molecules. Recent studies have shown the ability of proteins and carbohydrates to neutralize superoxides and hydroxyl radicals, correlating with their concentrations[214].

Figure IV.3 shows the extract concentration required to decrease the initial concentration of the ABTS and DPPH radical by 50% (IC_{50}, mg/ml). In the case of ABTS, the results varied from 0.29 ± 0.01 to 0.45 ± 0.05 mg/ml. The 50% ethanol (AR2) extract exhibited the lowest IC_{50} with a value of 0.29 ± 0.01 mg/ml, less than the antioxidant reference compound (ascorbic acid: 0.36 ± 0.01 mg/ml). For the activity against the DPPH radical, once again the AR2 extract was the most active one with an IC_{50} value of 0.53 ± 0.02 mg/ml, followed by AR1 extract (0.82 ± 0.07 mg/ml). However, these values were higher when compared them with the reference substance showing statistically significant differences ($p < 0.05$).

Regarding to the evaluation of the antioxidant activity of the extracts, the best results are observed for the neutralization of the radical ABTS; a method which allows to measure the activity of hydrophilic and lipophilic compounds. This method is applicable to any substance with an oxido-reduction potential thermodynamically lower than ABTS (0.68 V)

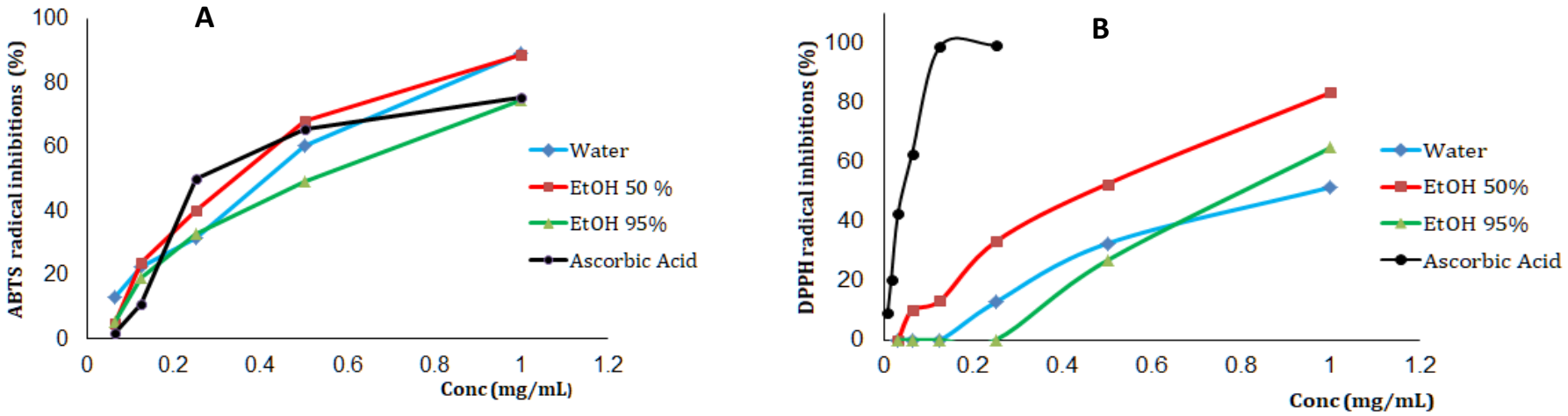


Figure IV.2. Inhibition of the ABTS and DPPH radicals by extracts obtained from *Adelia ricinella* L. A) ABTS, **B)** DPPH

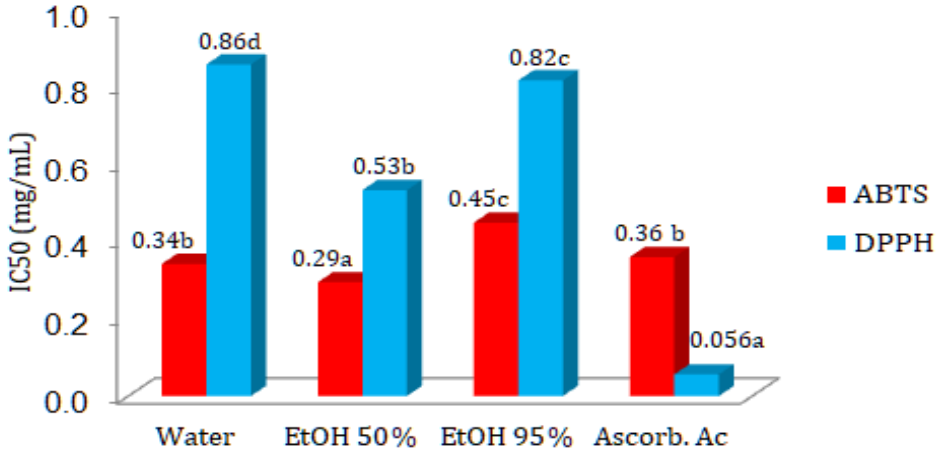


Figure IV. 3. Antioxidant activity of the extracts obtained against the radical ABTS and DPPH (IC50 Values)
(a, b, c, d) Different letters indicate significant differences for each assays ($p < 0.05$)

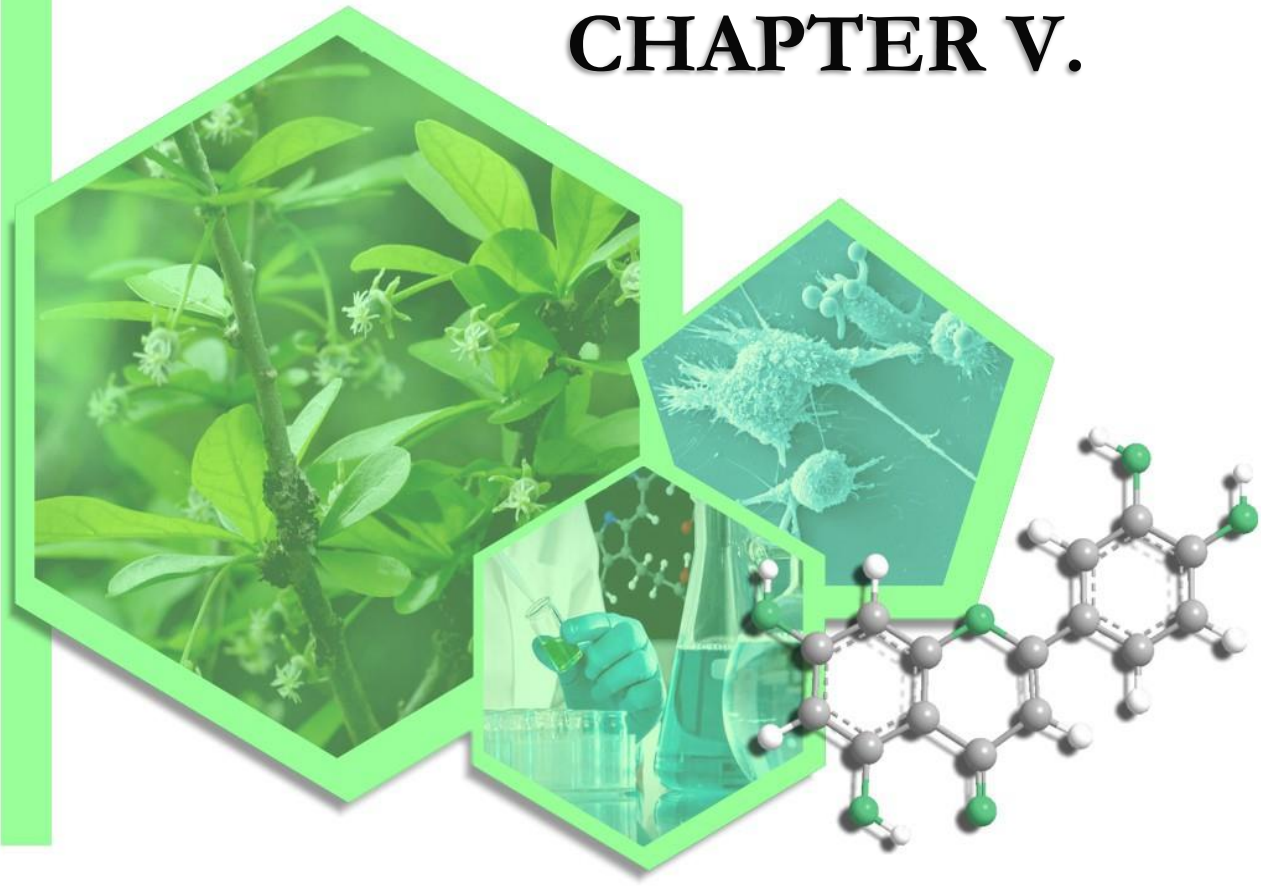
acting in this way as a reductant. Another difference between the two methods is related to the maximum absorbance peaks. The ABTS radical´s spectrum shows maximum absorbance at 414, 654, 754 and 815 nm in ethanol, while DPPH presents a single peak of absorbance at 515 nm, limiting in this way the radical detection[215, 216].

To establish a possible correlation between the antioxidant activities (ABTS, DPPH) versus the content of total phenols, flavonoids, carbohydrates, reducing sugars and proteins; linear regression analyses were performed. Results showed weak correlation between the chemical and pharmacological variables. However, between the ABTS and DPPH tests an acceptable correlation was observed (R^2=0.66). This fact demonstrates that, despite there is no correlation between the compounds quantified in the extracts; the antioxidant activity measured in both tests could be related to a synergistic effect between the different metabolites. In the following chapters this activity is evaluated for the three extracts in more depth using different *in vitro* methods, cell lines, and evaluating different variables.

IV.4- Conclusions

The experimental evidence obtained shows a high antioxidant potential for the extracts of *Adelia ricinella* L., especially the 50% ethanol extract; even when no effective correlations with the content of total phenols, flavonoids, carbohydrates, proteins and reducing sugars is found. In consequence, the alkaloids identified through qualitative tests in the extracts, are proposed as another type of metabolites which can contribute to the antioxidant activity evaluated. This suggestion is based on their ability to donate electrons and, therefore, to neutralize radicals. In this way, it is hypothesized that a synergistic action is involved to reach the antioxidant activity demonstrated by the extracts.

CHAPTER V.



CHAPTER V. Cytoprotective activity of extracts from *Adelia ricinella* L. aerial parts

Modified version from the published article: Berenguer-Rivas CA, Fong Lores O, Escalona-Arranz JC, de la Vega-Acosta J, Arro-Díaz DJ, Guisado-Bourzac F, Llauradó-Maury G, Morris-Quevedo HJ. Cytoprotective activity of extracts from *Adelia ricinella* L. aerial parts. Journal of Pharmacy & Pharmacognosy Research 2021; 9(6), 892-904.

V.1- Introduction

Several factors can affect cell integrity, thus leading to important pathological conditions in humans. In general, ionizing radiations, chemotherapeutic drugs and exposition to a wide range of toxicants may generate cell disturbances through the impairment of key biomolecules with the subsequent loss of functionality, e.g.: disruption of erythrocyte cells membrane[217, 218]. After these damaging factors, hematological alterations are common adverse effects that normally compromise the patient's quality of life. In addition to bone marrow failure, mechanisms involved in erythrocyte damage have been related to the oxidative stress and its accompanying deterioration of membrane lipids and proteins[219-221]. Therefore, cytoprotection by increasing erythrocyte membrane stabilization and functionality has become an interesting approach to reduce the risk of abnormal hematological changes.

Plant-based complementary therapies have gained an upsurge of interest because of their promising healing potential, cyto-compatibility and safety[222]. Many phytochemicals reveal powerful pharmacological activities mitigating the harmful effects in cellular functions produced by chemotherapeutic drugs and/or other factors[223]. The cytoprotective effects of some plant compounds have been mainly associated to polyphenol-like metabolites, which are recognized as good antioxidants[224, 225]. The antioxidative potential of natural antioxidants (such as flavonoids) results from the combination of their radical scavenging and metal-chelating abilities. Recent research in membrane science has been suggested to exert beneficial and protective effects on red blood cells. They act primarily by altering the surface charges of cells after scavenging cations, thus preventing the breakdown of the lipid membrane and increasing resistance to haemolysis[226,227].

Erythrocytes are critical targets for natural products and many other drugs. Indeed, human erythrocytes have been used as a model for studies of biological effects of reactive oxygen species induced oxidative stress for several reasons. They are continuously exposed to high oxygen tensions; the membrane lipids contain polyunsaturated fatty acid side chains that are vulnerable to peroxidation, and they have antioxidant enzyme systems. Moreover, they

are structurally simple and can easily be obtained[228]. In this context, several studies have demonstrated the usefulness of the *in vitro* human red blood cell membrane stability method as an excellent strategy to explore the cytoprotective role of natural products. Some investigations comprised a flavonoid and steroid-enriched methanolic extracts from *Millettia pachycarpa* Benth leaves[229], different extracts obtained from *Sargassum muticum* and *Ulva lactuca* algae and the plant species *Castanea sativa* Mill[217], and a methanolic extract from the marine brown alga *Turbinata ornata*[227].

Adelia ricinella L. (Euphorbiaceae) is a shrub that grows in the Caribbean area and empirically used by Cuban inhabitants, but scarcely scientifically studied for its medicinal properties and safety. It was recently reported that aqueous and ethanol preparations from the aerial parts consist of a rich matrix of active compounds mainly identified as polyphenols and flavonoids, which were probably responsible for the scavenging activity against DPPH and ABTS radicals found in cell-free assays, as analysed in the chapter IV. However, their biological properties are not completely elucidated, including the antioxidant-mediated cytoprotective mechanisms. That is why this work is aimed to evaluate the cytoprotective potential of *Adelia ricinella* L. extracts on red blood cell membrane stability after treatment with a hypotonic solution, or hydrogen peroxide. In this sense, *Adelia ricinella* L. extracts may prove their capacity of reducing the haematological alterations and cell toxicity by participating in the prevention of oxidative damage and inflammation-related processes, thus supporting its traditional use.

V.2- Material and methods

V.2.1-Plant material and extract preparation

The plant collection/taxonomic identification, and extract preparation were performed in the same way that sections IV.2, and IV.2.1. Briefly, 10 g of shadow-dried leaves were extracted with a Soxhlet apparatus. Extraction was done using 95% ethanol (commercial grade, for the AR1 extract), a mixture of ethanol/water (50%) (for the AR2 extract) and water (for the AR3 extract), and stopped after four hours of refluxing. The extracts were filtered, evaporated to dryness in a rotary evaporator at 45 °C (Kika Werke RV 05, Germany), and then stored at -20 °C until use.

V.2.2- HPLC-DAD quantification of luteolin and apigenin

HPLC-DAD quantification of luteolin and apigenin was carried out using the standard addition methodology[230]. Briefly, 20 µL of each sample was injected onto a Phenomenex

Luna C18 column (250 × 10 mm × 5 µm, Utrecht, The Netherlands) on an Agilent 1200 system coupled with a DAD detector (Agilent Technologies, Santa Clara, CA, USA). An aqueous formic acid (0.1%, v/v) solution was used as mobile phase A, while HPLC grade acetonitrile (Fisher Chemical, UK) was used as mobile phase B. The gradient program was: 5% B (0–5 min), 5–95% B (5–25 min), 100% B (25–30 min) at a flow rate of 1 ml/min. The column temperature was maintained at 26°C while the DAD signal was monitored and recorded at 210, 235, 254, 260, 270, 280 and 366 nm. Samples (AR1, AR2 and AR3) were prepared at a concentration of 1 mg/ml in methanol 50% enriched with a standard solution containing 20 µg/ml of both standards (Sigma Aldrich, St. Louis, MO, USA).

V.2.3-Cytoprotection and antioxidant assessment

V.2.3.1- Erythrocyte suspension

Fresh blood samples (12-15 ml) were collected from healthy human volunteers who did not consume any anti-inflammatory drugs in the previous 15 days. Samples were added to tests tubes with sodium citrate (1:10), and plasma was separated by centrifugation (2 500 rpm × 15 min) and then discarded. Afterward, the erythrocyte suspension was washed with a sterile phosphate buffer solution (PBS: 0.2 g of K_2HPO_4, 1.8 g of glucose, and 9 g of NaCl, pH 7.4) four times till the supernatant was colorless and the pellet was reconstituted (1:40 v/v, 8 × 109 cells/ml). The erythrocyte suspensions (RBC: Red Blood Cells) were always prepared prior to the *in vitro* assessment.

V.2.3.2-Haemolysis assay

The haemolysis assay was performed following the INVITOX protocol No. 37[231]. Plant extract concentrations (8–256 µg/ml) were chosen taking into account the standardized protocols of the Center for Toxicology and Biomedicine. Samples were diluted in PBS, added to 25 µL of RBC, and incubated at room temperature with stirring (100 rpm) for 10 min. The suspension was centrifuged (3500 rpm × 10 min) and the supernatant (hemoglobin content) was measured at 530 nm (Genesys UV/VIS spectrophotometer, Switzerland). The haemolysis value of 100% was set by measuring the absorbance of RBC incubated with distilled water, while the 0% value was defined by determining the spontaneous haemolysis of RBC incubated with PBS. All the samples were prepared in triplicate.

V.2.3.3-Hypotonic erythrocyte membrane stabilizing activity

The human red blood cell membrane stabilization *in vitro* assay has often been considered as a technique to evaluate the *in vitro* anti-inflammatory effect of phytochemicals[93]. Briefly, 1 ml of each plant extract (ranging 8-256 µg/ml) or positive control (sodium diclofenac, 98%, abcr GmbH, Karlsruhe, Germany, 8- 256 µg/ml) were mixed with 0.5 ml of erythrocytes suspension in PBS (1 ml of phosphate buffer and 2 ml of hypotonic NaCl solution 0.45%). Hypotonic NaCl 0.45% (1 ml) was used as negative control instead of plant samples. After incubation (37 °C for 30 min), samples were centrifuged at 3000 rpm, 10 min. Then, haemoglobin concentrations were recorded spectrophotometrically at 560 nm (Genesys UV/VIS spectrophotometer, Switzerland). The percentage of haemolysis was assessed by assuming the haemolysis produced in the appropriate control as 100%. The erythrocyte membrane stabilization in a hypotonic medium was estimated by equation [3]. The concentration in which extracts protect 50% of the RBC membrane integrity (HMP_{50}) was estimated when possible using a linear regression approach. All the samples were prepared in triplicate.

$$HMP\ (\%) = (100 - (A1\text{-}A2/A1)) \times 100 \qquad [3]$$

where: HMP (%) = Hypotonic Medium Protection percentage, A1 = Absorbance of negative control and A2 = Absorbance of test sample in hypotonic saline solution.

V.2.3.4- Antioxidant erythrocyte membrane stabilizing activity

The assay was performed by the measuring the inhibition of oxidative haemolysis induced by hydrogen peroxide (H_2O_2) in RBC, following the method reported by Escalona-Arranz et al., 2014[232] with a modification. Erythrocyte suspensions were treated with PBS, H_2O_2 (10 mM) (negative control), ascorbic acid (positive control), and plant extracts (AR1, AR2 and AR3) ranging 8-256 µg/ml. All experimental groups were incubated at 37 °C during 1 h and stirring with a vortex each 10 min (Heidolph REAX 2000, Germany). Afterwards, the samples were centrifuged, the supernatants were collected, and then absorbances were measured at 530 nm (Genesys UV/VIS spectrophotometer, Switzerland). The percentage of haemolysis was calculated assuming 100% of haemolysis in the negative control (H_2O_2). The stability of the erythrocyte membrane facing the oxidant agent was estimated by equation [4]. The concentration in which extracts protect 50% of the RBC membrane integrity (AP_{50}) was estimated when possible using the logarithm of the concentration using a linear regression approach. All the samples were prepared in triplicate.

$$AP\ (\%) = (100 - (A1\text{-}A2/A1)) \times 100 \qquad [4]$$

where: AP (%) = Antioxidant Protection percentage, A1 = Optical density of negative control and A2 = Optical density of test sample in the oxidant medium.

V.2.3.5- Erythrocytes morphological changes

To determine the mechanism by which *A. ricinella* L. extracts protect the erythrocyte membrane, the morphological changes induced by H_2O_2 as an oxidizing agent were observed in RBCs. A volume of 50 µL of RBC from the pellets of each experiment was added to a Neubauer chamber and visualized in a Transmitted Light Microscope (Leica DM 1000, Switzerland) coupled with a digital camera (Leica MC 170 HD, 400×, 2592 × 1944 pixels, Switzerland). Membrane integrity; erythrocyte form; presence/absence of deformations such as spherocytes, equinocytes, and other morphological alterations were considered for all test samples and controls [233].

V.2.4- Statistical analysis

Statistical analysis was performed using the statistical software package GraphPad Prism 7 (Windows, V. 7.04, 2017). All results obtained were statistically analyzed and expressed as the arithmetic means ± standard deviation (SD). One-way analysis of variance by ranks of Kruskal-Wallis followed by the *post hoc* Dunn's test was applied to determine the significance of differences between groups. Differences at $p \leq 0.05$ were accepted as significant.

V.3-Results

V.3.1-HPLC-DAD quantification of luteolin and apigenin

HPLC-DAD chromatograms recorded with UV detection at 366 nm of the *A. ricinella* L. extracts are shown in Figure V.1. Peaks with retention times from 25.136 to 25.148 min and labeled as (1) correspond to luteolin, while peaks with retention times from 27.347 to 27.361 min and labeled as (2) are due to apigenin. The concentration of these compounds in each extract was determined using standards addition, and the values estimated for luteolin (µg/ml) were 22.6 µg/ml, 207.5 µg/ml and traces in AR1, AR2 and AR3, respectively.; while for apigenin traces, 1.86 µg/ml and traces, respectively.

V.3.2- Cytoprotection and antioxidative assessment of Adelia ricinella L. extracts

V.3.2.1- Haemolysis assay

Firstly, the potential cytotoxic/destabilizing effect of *A. ricinella* L. aqueous and ethanol extracts at different concentrations on the membrane of RBCs was evaluated. Results

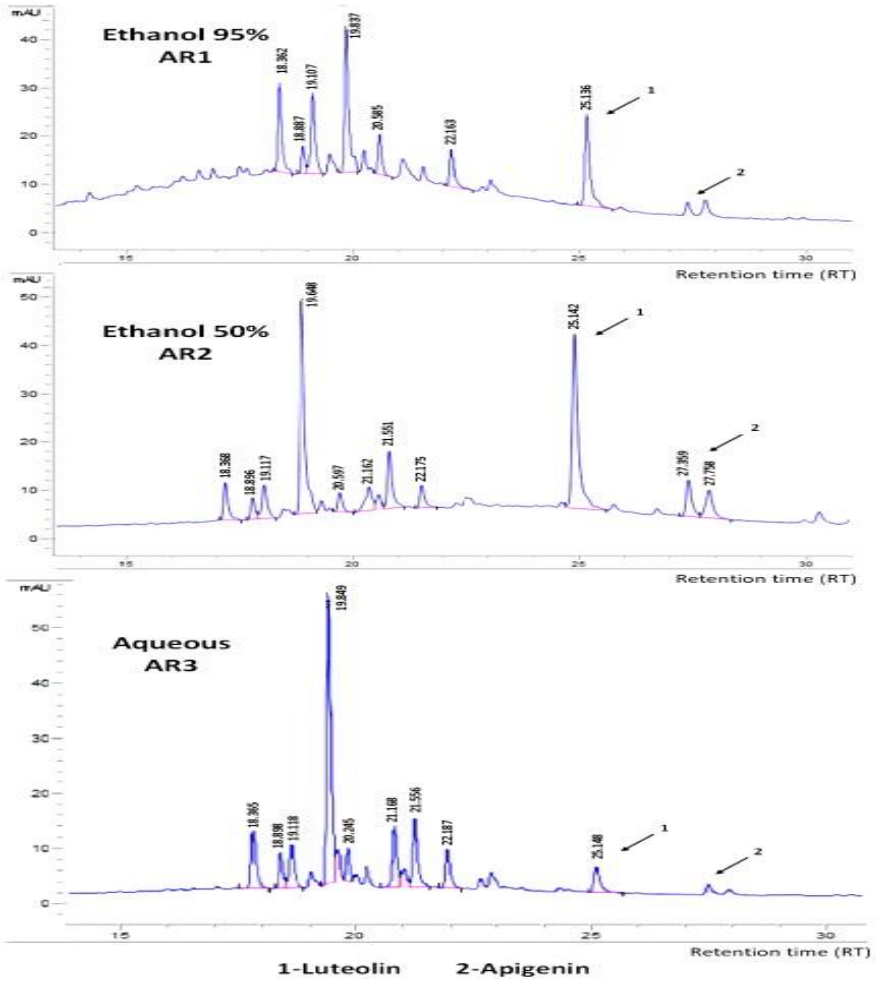


Figure V.1. HPLC-DAD chromatograms recorded at 366 nm of *Adelia ricinella* L. extracts.

Samples (AR1, AR2 and AR3) were prepared at a concentration of 1 mg/mL in methanol 50%. AR1: ethanolic extract; AR2: ethanol: water (1:1) extract; AR3: aqueous extract

revealed that the extracts acted as protectors against direct haemolysis when the concentration increases (Figure V.2). Some degree of haemolysis was observed only for the lower concentration of the three extracts (8 µg/ml) with no statistical differences between them. In the same way, the AR1 extract (16 µg/ml) caused a haemolysis ratio of 19.21%, in contrast with the other two extracts (AR2 and AR3) that reached 2 and 4%. Higher concentrations did not produce haemolysis, suggesting any erythrocyte membrane rupture.

V.3.2.2-Hypotonic erythrocyte membrane stabilizing activity

In general, *A. ricinella* L. extracts were able to protect the RBCs membrane from disruption caused by a NaCl hypotonic solution. Ethanol extracts at higher concentrations significantly demonstrated a stabilizing activity on red blood cells membrane with values of HMP over 70%. This activity slightly exceeds the protective effect exerted by diclofenac as a positive control (Figure V 3). In the three test samples and in the diclofenac group, a concentration-dependent response was observed, allowing the estimation of the HMP_{50}. Results revealed the followed behaviour in their protective capacity: AR1>AR2>diclofenac>AR3 with HMP_{50} values of 16.46, 30.05, 37.10 and 47.40 µg/ml, respectively. Significant statistical differences were found between all experimental groups.

V.3.2.3-Antioxidant erythrocyte membrane stabilizing activity

The evaluation of the cytoprotective effect of plant extracts on erythrocyte membranes was also explored using H_2O_2 as an oxidative agent. Many studies promote RBC-related models to assess the action of antioxidants for membrane protection because they are highly susceptible to oxidative damage. The AR2 extract showed a statistically significant protecting effect (>90% at 64 µg/ml) in erythrocyte membranes after H_2O_2 exposure in comparison with AR1 and AR3 extracts, which were less active (Figure V.4). The effect exerted by AR2 was similar to that of ascorbic acid (control group). As a result, the statistical analysis reveals significant differences between the experimental groups favouring AR2 (AP_{50} 17.49 µg/ml) as the most active extract followed by AR1 and AR3 with AP_{50} = 37.937 and 57.720 µg/ml, respectively. None of the extracts was more active than the ascorbic acid control (AP_{50} = 10.358 µg/ml).

V.3.2.4-Morphological changes of erythrocytes

Normally, erythrocytes are biconcave, non-nucleated, and discoid structures containing haemoglobin. Erythrocyte susceptibility to chemicals, osmotic media, and magnetic/electric radiation is well known: it leads to shape deformation and, subsequently, the loss of

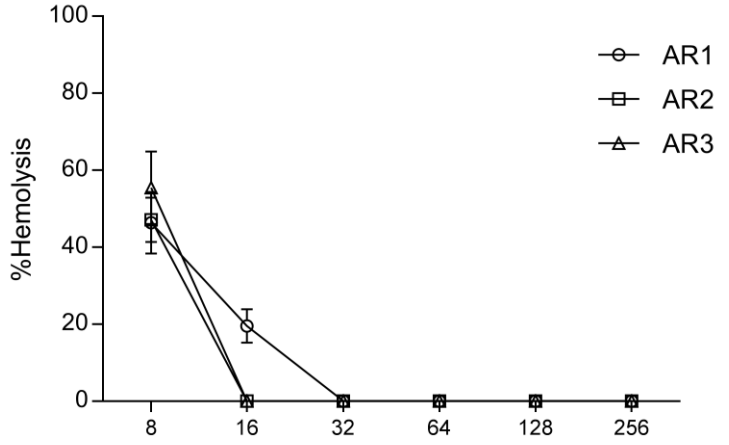


Figure V.2. Haemolysis percentage induced by different extract concentrations of *Adelia ricinella* L. (8–256 µg/mL).

Results are expressed as the arithmetic means ± standard deviation (SD) of three replicates. AR1: ethanolic extract; AR2: ethanol:water (1:1) extract; AR3: aqueous extract

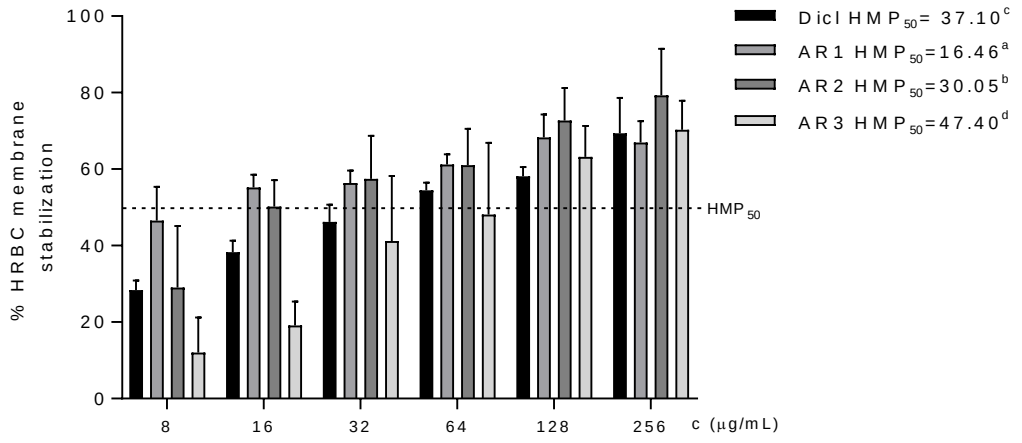


Figure V.3. Red blood cell membrane stabilization induced by different extract concentrations of *Adelia ricinella* L. in a NaCl hypotonic medium.

Results are expressed as the arithmetic means ± standard deviation (SD) of three replicates. Diclofenac (Dicl) was used as a positive control. Distinct letters indicate significant differences in Kruskal-Wallis coupled to Dunn's test, $p<0.05$. AR1: ethanolic extract; AR2: ethanol:water (1:1) extract; AR3: aqueous extract.

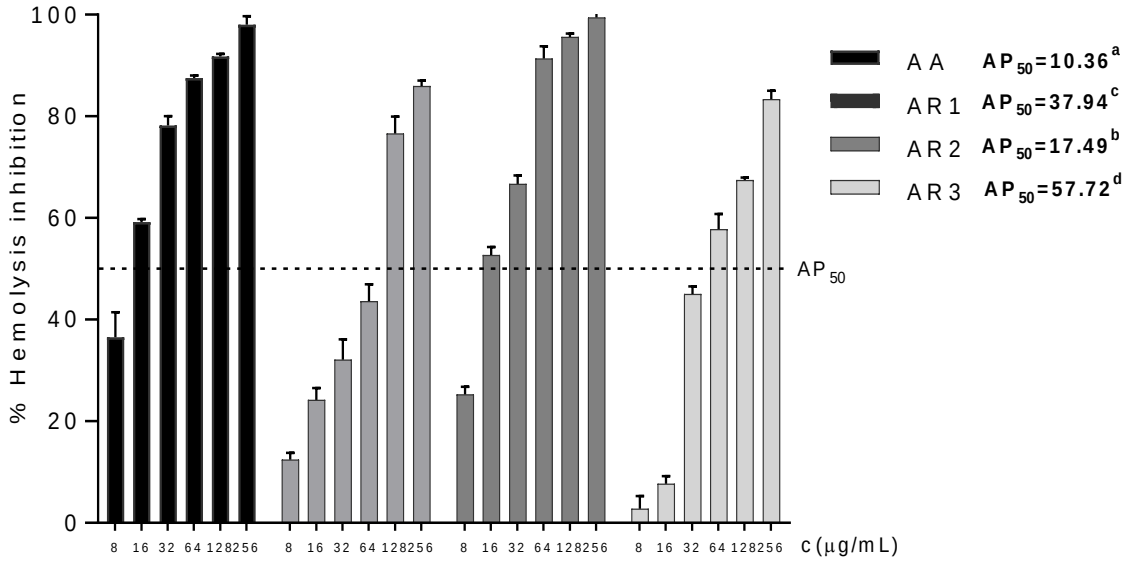


Figure V.4. Cytoprotection exerted by different extract concentrations of *Adelia ricinella* L. on red blood cells treated with hydrogen peroxide as an oxidant agent.

Results are expressed as the arithmetic means ± standard deviation (SD) of three replicates. Ascorbic acid (AA) was used as a positive control. Distinct letters indicate significant differences in Kruskal-Wallis coupled to Dunn's test, $p<0.05$. AR1: ethanolic extract; AR2: ethanol:water (1:1) extract; AR3: aqueous extract

functionality. Therefore, monitoring the external morphology can be considered a valuable tool for measuring the degree of damage degree caused by harmful agents. At the same time, substances acting as erythrocyte protective agents can prevent such deformations. The erythrocyte membrane stabilizing activity of three *A. ricinella* L. extracts (AR1-AR3) was explored with H_2O_2 as an oxidizing agent. The results were in agreement with the spectrophotometric determinations, indicating that AR2 was the most active extract. Main deformations in both experiments were associated with the presence of spheroechinocytes, echinocytes, and spherocyte forms (Figure V.5).

V.3.2.5-Cytotoxicity of A. ricinella L. extracts in Vero cells

Vero cells are a helpful cell system to explore the cytotoxic potential of toxicants, synthetic and natural agents, like phytochemicals[234,235]. All the tested *A. ricinella* L. extracts were not cytotoxic to mammalian Vero cells (Fig. V.6). Based on this finding, the estimated IC_{50} values of the three *A. ricinella* L. extracts were higher than 256 µg/ml, the highest test concentration, and classified as non-toxic at the concentrations assessed in this study. It is also noticed that all extracts at lower concentrations, slightly promoted the metabolic activity of Vero cells, as shown in the resazurin dye reduction test.

V.4-Discussion

Several factors can strongly damage cell membrane integrity by the impairment of key biomolecules like lipids and proteins[220]. Red blood cells possess a very sensitive cell membrane that is susceptible to abnormal changes, leading to important haematological alterations. For this reason, RBC membranes with their mosaic of proteins distributed in lipid bilayers serve as an excellent *in vitro* tool for understanding membrane destabilization/toxicity induced by synthetic or natural products. In this sense, some authors suggest the RBC model as an alternative but a reliable assay to explore the cytoprotective effect of antioxidants and anti-inflammatory natural drugs and/or synthetic analogous on lysosomal organelles, because both membrane components are structurally similar[236, 237].

From the structural point of view, it is well recognized that while the outer side of the erythrocyte membrane contains high quantities of phosphatidyl choline and sphingomyelin, the inner side holds the charged phospholipids phosphatidyl ethanolamine and phosphatidylserine. This membrane configuration with an asymmetric distribution of

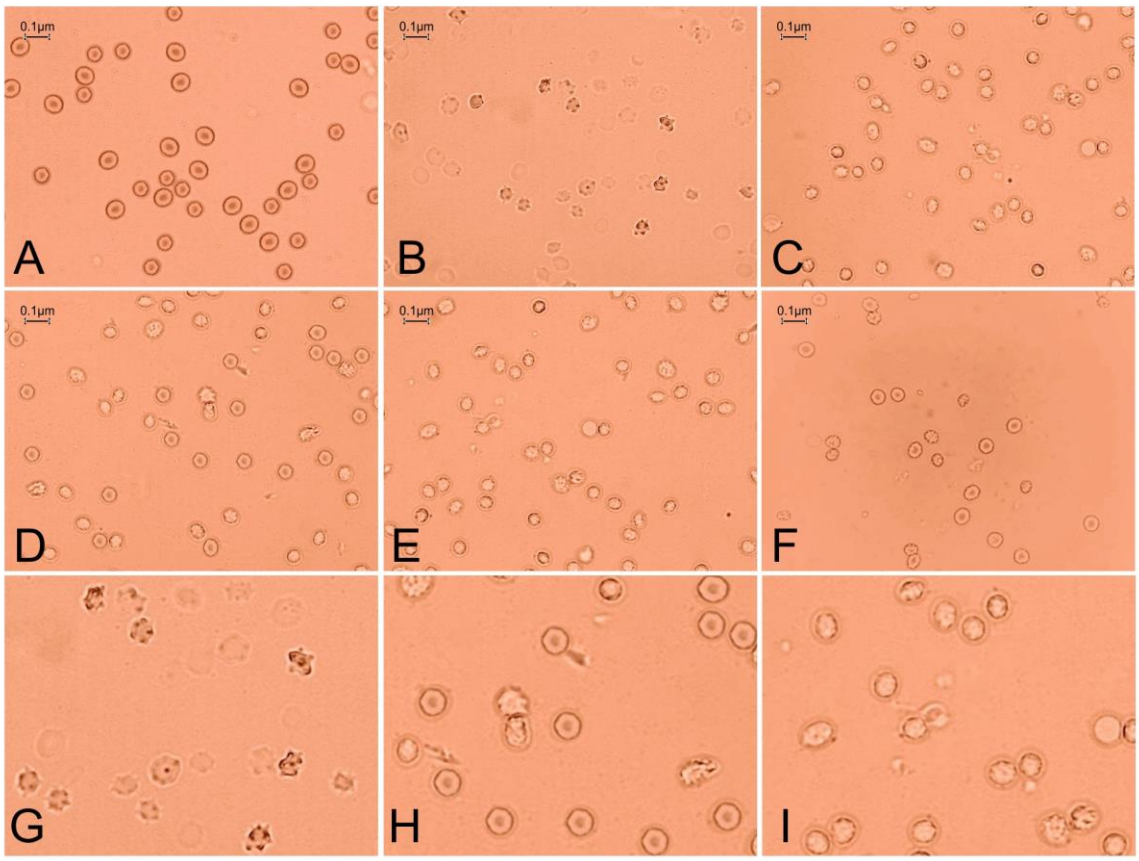


Figure V.5. Optical micrographs (400×) showing erythrocyte protection by *A. ricinella* extracts at 32 µg/mL to H_2O_2-induced oxidative damage.

(A)Normal cells, (B) Negative control (H_2O_2), (C) AR1, (D) AR2, (E) AR3, (F) Positive control (Ascorbic acid, 32 µg/mL). Pictures G, H and I are digital magnifications (zoom 3×) to highlight membrane changes: (G) Negative control (H_2O_2), (H) AR2, (I) AR3. AR1: ethanolic extract; AR2: ethanol:water (1:1) extract; AR3: aqueous extract

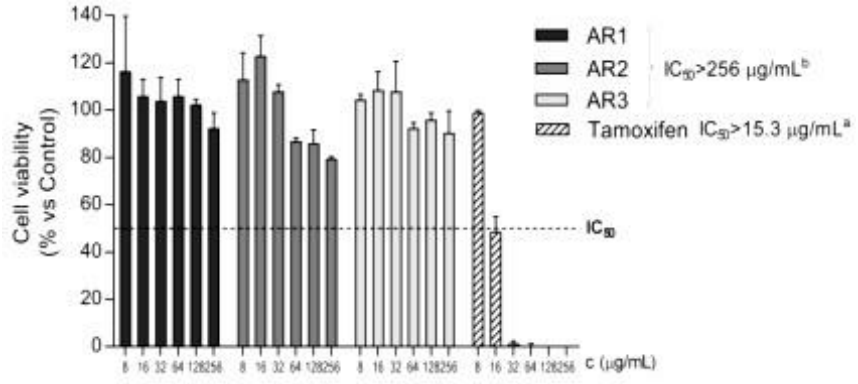


Figure V. 6. Cell viability of Vero cells incubated with different concentrations of *Adelia ricinella* L. extracts.

The potential cytotoxic effect of plant extracts on Vero cells was evaluated by the resazurin dye reduction test. Results are expressed as the arithmetic means ± standard deviation (SD) of three replicates. Distinct letters indicate significant differences in Kruskal-Wallis coupled to Dunn's test, $p<0.05$. AR1: ethanolic extract; AR2: ethanol:water (1:1) extract; AR3: aqueous extract.

molecules across the lipid bilayer determines not only their curved shape but also the way in which interact with the external medium.

Thus, drugs/extracts interacting with the outer side generate different morphological types (mainly echinocyte type) compared with inner leaflet[238]. In this interaction, the diameter, height, and density of the echinocyte type spicules are proportional to the number of molecules penetrating and interacting with the outer leaflet. On the other hand, it is also accepted that substances that interact with the membrane inner side generate shape changes leading to oval or elliptical erythrocyte forms by "affecting" the cytoskeletal network[239].

The use of plant-derived products emerges as an interesting and, in many cases, a safer than conventional drugs to prevent cellular membrane disruption. Flavonoid-type compounds and other polyphenols are probably the most explored plant metabolites in the last decade due to their wide range of medicinal activities primarily associated with their antioxidant capacity[240; 227]. In this context, the results shown in this chapter highlight that: i) flavonoid enriched extracts derived from aerial parts of *A. ricinella* L. exhibited a cytoprotective action on red blood cells by membrane stabilization when exposed to hypotonic and oxidative environments, ii) they were not cytotoxic to RBC and Vero cells.

Based on the polyphenol and flavonoid composition described previously (IV. 4. section IV.3.1); and the signal intensity showed in Figure V.1, it can be noticed that in AR2 and AR1 extracts, the peak corresponding to luteolin was the second most abundant, in agreement with this cytoprotective activity. According to the micrographs, H_2O_2 oxidative damage must have impaired both erythrocyte layers, because most of the "modified erythrocytes" are echinocyte or spheroechinocyte types (Figure V.5B and V.5G). On the other hand, *A. ricinella* L. extract components are able to protect both sides of the erythrocyte membrane against the damage (Figure V.5C –AR1, V.5E and V.5I -AR3, and mainly V.5D and V.5H – AR2). Therefore, compounds present in *A. ricinella* L. extracts must be able to penetrate across the membrane, also acting at its inner side.

The transport of flavonoids across the membranes is not yet fully understood. However, some evidence has already been accepted, such as: i) the relatively planar structure of flavones and flavonols boosts the penetration through biological membranes by increasing their affinity towards the lipid membranes[241], ii) glycosidic substitutions with more than one carbohydrate monomer unit increases molar volume and decreases hydrophobicity, affecting the flavonoid membrane penetration[233]. Considering that the 13 flavonoids

identified in the extracts of *A. ricinella* L. are mono- or disaccharides of luteolin and apigenin, we hold the hypothesis that they could interact on both sides of the erythrocyte membrane, exerting their cytoprotective activity in such layers.

However, a prerequisite to exert their effect at that level is that flavonoids and other polyphenols (mainly glycosides) need to be bioavailable to reach the experimental concentrations in blood. The absorption and metabolism of polyphenolic compounds is determined primarily by their physicochemical characteristics. For example, molecular size, basic structure, degree of polymerization or glycosylation, solubility, and conjugation with other phenolics can be considered critical[242]. As mentioned before, the physicochemical characteristics of flavonoids identified in *A. ricinella* L. extracts derived from luteolin and apigenin (planar structure of flavones and low degree of glycosylation) could favor their absorption and bioavailability.

Until now, *in vivo* investigations of the absorption, bioavailability, and metabolism of these flavonoids are limited[243]. In agreement with structural features favoring bioaccessibility of flavones,[244] it has been reported that after oral administration of *Chrysanthemum morifolium* extract (1.7 g/kg body weight, equivalent to 22.8 and 58.3 μmol/kg of luteolin and luteolin-7-O-glucoside, respectively) to rats, luteolin and its glycosides were detected in the plasma. Their levels were highest one hour after administration, for luteolin and its glucoside (0.76 ± 0.27 μM; 9.88 ± 1.35 μM, respectively). These compounds were also detected in media on the basolateral side from Caco- 2 cells treated with the *C. morifolium* extract. These results suggest that luteolin and luteolin monoglucoside are rapidly absorbed after oral administration of *C. morifolium* flower extract. The concentrations of these compounds in plasma also showed a secondary increase, meaning that intake of the *C. morifolium* extract led to the retention of these compounds in the circulatory system for a long time.

Thus, we can hypothesize that the major flavone-type flavonoid presented in *A. ricinella* L. extracts may also exert the observed beneficial effects *in vivo*. Erythrocytes-based assays are one step further in extrapolating to living models. Nevertheless, the combination of different assays, including some *in vitro* analytical methods for determining bioaccessibility and bioavailability of bioactive compounds (e.g., pre-digestion simulations), cell models "closer" to what could happen *in vivo*, and studies with animal models[245] is a major recommendation to confirm the medicinal value of *A. ricinella* L. extracts.

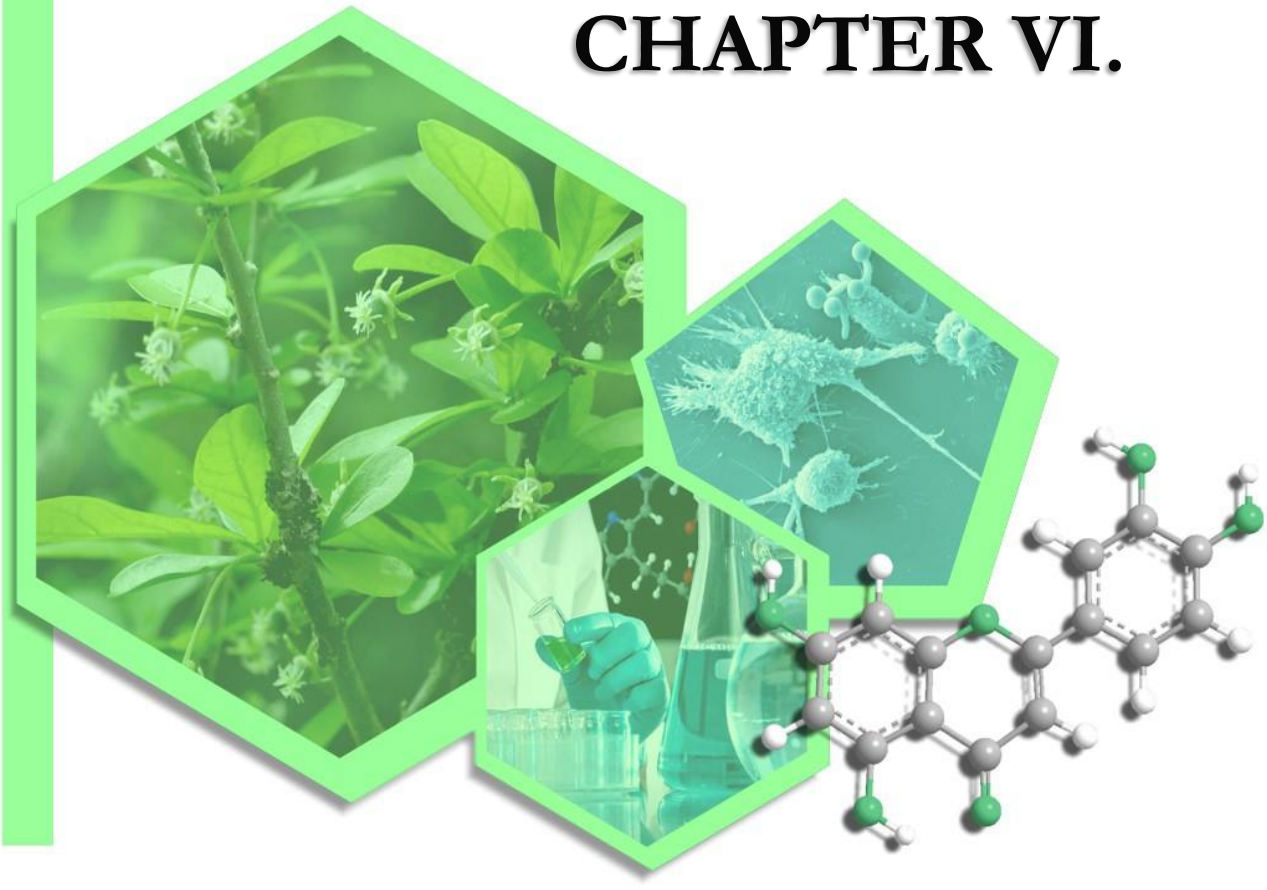
On the other hand, previous reports on *A. ricinella* L. extracts revealed a high antioxidant capacity to neutralize radicals such as ABTS and DPPH. The AR2 extract showed a lower IC_{50} in the ABTS assay than ascorbic acid and higher levels of antioxidant metabolites (total phenols and flavonoids) compared to its analogous AR1 and AR3. It is fully accepted that the harmful action of reactive oxygen species on the erythrocyte membrane reduces their cytoskeletal protein content producing high molecular weight proteins aggregates. These changes triggered abnormalities in RBC structure and in consequence to their shape, membrane-related functions, rheological properties, and ultimately leading to reduced erythrocytes survival[246]. Here after and in agreement with the aforementioned results, it can be suggested that, *A. ricinella* L. extracts can protect RBC membranes by scavenging oxidant compounds.

The relationship between the content of phenols/flavonoids and the cytoprotective action of plant extracts has been associated with the antioxidant activity of these metabolites. For example, the cytoprotective capacity of *Piper betle* L., *Areca catechu* L., and *Uncaria gambir* Roxb., in human gingival fibroblasts after inducing H_2O_2 damage, was associated with a high concentration of polyphenols[224]. Moreover, the safety of *A. ricinella* L. extracts was confirmed on Vero cells, without impairment of cell viability and metabolic activity.

V.5- Conclusions

Adelia ricinella L. flavonoids-enriched extracts, particularly the ethanol AR1 and AR2 extracts, were able to promote erythrocyte cytoprotection by stabilization of both membrane layers in an oxidative environment. Thus, *A. ricinella* L. extracts have proven their capacity of reducing haematological alterations and cell toxicity by participating in the prevention of oxidizing damage. This plant species therefore could be regarded as a natural source of membrane stabilizers, and was capable of providing an alternative remedy for the management and treatment of inflammatory related disorders and diseases.

CHAPTER VI.



CHAPTER VI. Anti-inflammatory effect of *Adelia ricinella* L. aerial parts.

Modified version from the published article: Berenguer CA, Escalona-Arranz JC, Llauradó G, Van der Auwera A, Piazza S, Méndez D, Foubert K, Cos P, Pieters L. Anti-inflammatory effect of *Adelia ricinella* L. aerial parts. J Pharm Pharmacol 2021; 73 (4): 553–559

VI.1- Introduction

Adelia ricinella L., a shrub native from the Caribbean; has been described in Cuba, Jamaica, Cayman Islands, Colombia and Venezuela by its use as antipyretic, analgesic and anti-inflammatory activity [169]. In spite of this strong ethno-pharmacological evidence, from the scientific point of view, only few information about the chemical or pharmacological potential is available. In the previous chapters it was demonstrated that *A*. *ricinella* L. produces high quantities of polyphenols and flavonoids, being responsible for the *in vitro* antioxidant activity. This characteristic was also revealed when facing red blood cells (RBC) promoting the erythrocyte cytoprotection by stabilization of both membrane layers in an oxidative environment especially by the ethanol AR1 and AR2 extracts. Were in fact those ethanol extracts which present higher levels of polyphenols and flavonoids and luteolin/apigenin concentration when analyzed by HPLC-DAD technique. By this way, *A. ricinella* L. extracts prove their capacity of reducing haematological alterations and cell toxicity by participating in the prevention of oxidizing damage and inflammation-related processes characteristic of many inflammatory diseases. In spite of such observations, inflammation is a quite complex process wich needs to be deepeds explored to proposs any substance as suitable for the use in the inflammatory diseases.

Macrophages actively participate in the development of inflammation-related pathological processes such as auto-immune diseases, arthritis and cancer[247, 248] These cells can be activated by various stimuli like bacterial and fungal components and chemical mediators[249]. Afterward, they release pro-inflammatory proteins (IL-1β, IL-6 and TNF-α) and other inflammatory mediators such as nitric oxide and prostaglandin E_2 (PGE_2), etc. which are responsible for several biological functions, although resulting in damage in case of overproduction of these mediators[86]. One of the main targets to reduce the negative effect of an intensified inflammatory reaction is to address the COX enzyme system. COX-1 and COX-2 mediate the eicosanoids release like PGE_2 and thromboxanes, which are well known to participate in chronic inflammation and tumour formation[250]. In this sense, the use of plant-based active products with anti-inflammatory potential, able to decrease COX

activities, is considered as an important goal for the development of new biopharmaceuticals lacking adverse effects[251].

This research was aimed to determine the main chemical constituents and the "*in vitro*" anti-inflammatory effect of *Adelia ricinella* L. extracts by measuring COX-1 and COX-2 enzyme suppression, PGE_2 release in lipopolysaccharide (LPS)-activated RAW 264.7 murine macrophages. This study may allow a better understanding of the traditional uses of this plant species by the local population against several inflammation-related diseases, establishing for first time a relationship between the pharmacological activity measured and the phytochemical composition.

VI.2- Material and methods

VI.2.1- Materials and reagents

Acetonitrile (HPLC grade) (ACN) was acquired from Fisher Chemical UK Ltd. Formic acid (FA) (98+%, analytical grade) was obtained from Acros Organics (Belgium). The external standards isovitexin, orientin and isoorientin (all with purity ≥ 99%) were from Extrasynthese (Lyon, France), while vitexin (99.7%) was purchased from Adipogen (Liestal, Switzerland).

Lipopolysaccharide (LPS) from *Escherichia coli* (0128:B12), dimethylsulfoxide (DMSO) (Uvasol), porcine hematin, L-epinephrine, Na_2EDTA, celecoxib, arachidonic acid, tamoxifen and resazurin sodium salt (7-hydroxy-3H-phenoxazin-3-one-10-oxide) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tris-buffer was purchase from Biorad. Dulbecco's Medium Eagle Modified (DMEM), Dulbecco's phosphate-buffered saline (DPBS) and 5 ml of ram seminal vesicle COX-1 (1 U) or human recombinant COX-2 (0.5 U) were also from Sigma-Aldrich (St. Louis, MO, USA). Indomethacin was from MP Biomedicals. Na_2EDTA (Titriplex III) was purchased from VWR International. Arachidonic acid, purified COX-1 from ram seminal vesicles and human recombinant COX-2 were from Cayman Chemical. The competitive PGE_2 EIA kit was purchased from Enzo Life Science.

VI.2.2- Plant material and extract preparation

The aerial parts of *Adelia ricinella* L. were collected in the Siboney Juticí Ecological Reserve (latitude: 19.9603 and longitude: −75.7081), located outside the city of Santiago de Cuba. The plant was also taxonomically identified at different moments by specialists at the Eastern Centre for Ecosystems and Biodiversity (BIOECO, Santiago de Cuba) and a vegetal

sample is already settled at the herbarium of the institution with the registration number 14 780.

The extracts were prepared by Soxhlet extraction during 4 h after the first reflux using either water, ethanol 50% and ethanol 95% as described in the chapter IV. Afterward, the extracts AR1 (EtOH 95%), AR2 (EtOH 50%) and AR3 (aqueous extract) were filtered using a Büchner funnel and filter paper, and finally evaporated to dryness under reduced pressure in a rotary evaporator (Kika Werke RV 05, Germany) below 45 °C.

VI.2.3- Analysis of chemical composition by UPLC-DAD-MS/MS

Chemical composition analysis was performed in an UPLC-DAD-MS/MS system using a Xevo G2-XS QTof spectrometer (Waters, Milford, MA, USA) coupled with an ACQUITY Lcsystem equipped with MassLynx version 4.1 software. For analysis, 5 µl of each extract (AR1-AR3) at 100 µg/ml were injected on a BEH Shield RP18 column (100 mm × 2.10 mm, 1.7 µm, Waters, Milford, MA, USA). The mobile phase solvents consisted of H_2O + 0.1% FA (A) and ACN + 0.1% FA(B), and the gradient was set as follows (min/B%): 0.0/10, 5.0/10, 20.0/15, 30/15, 40.0/25, 45.0/25, 55.0/40, 60.0/40, 65.0/100, 70.0/100, 75.0/10 and 85.0/10. Full scan data were recorded in ESI (−) and ESI (+) mode from *m/z* 100 to 1500 and the analyzer was set in sensitivity mode (approximate resolution: 22 000 FWHM). The spray voltage was set at either +1.5 kV and −1.0 kV; cone gas flow and desolvation gas flow at 50.0 L/h and 1000.0 L/h, respectively; and source temperature and desolvation temperature at 120 °C and 550 °C, respectively. Data were also recorded using MS^E in the positive and negative ionization modes (two analyses per mode), and a ramp collision energy from 20 till 30 V was applied to obtain additional structural information. Leucineencephalin was used as lock mass. DAD spectra were recorded between 190 and 500 nm.

VI.2.4- COX-1 and COX-2 enzymatic inhibition assay

COX-1 and COX-2 inhibition assays were performed in a 96-well plate as previously described the literature[252]. The incubation mixture contained 180 µl of 0.1 M Tris HCl-buffer (pH 8.0), 5 µm hematin, 18 mm epinephrine bitartrate, 0.2 U of enzyme preparation and 50 µm Na_2EDTA (only for COX-2 assay). Each sample solution (10 µl) was added and the mixture was preincubated for 5 min at room temperature. The extracts were dissolved in DMSO at different concentrations (2–256 µg/ml, DMSO concentration did not exceed 0.1%. Indomethacin (1.25 µM in EtOH abs.) and celecoxib (2.5 µM in DMSO) were used as

positive controls. The reaction was started by adding 10 µl of 5 µM arachidonic acid in ethanol p.a. and incubated at 37 °C. After 20 min the reaction was stopped by the addition of 10 µl of 10% formic acid.

VI.2.5-Cell-Based COX Assays

VI.2.5.1-Cell culture and reagents

RAW264.7 macrophages from ATCC (American Type Tissue Culture Collection, USA) were maintained at 37 °C, 5% CO_2 atmosphere in DMEM medium supplemented with 10% FCS, 2% L-glutamine and 4.5 g/L D-glucose.

Macrophage cell suspension was seeded in a six-well plate (5×10^5 cells/ml) and incubated at 37 °C in a humidified atmosphere with 5%. After 48 h, the old medium was discarded and the cells were pre-incubated with and without *Adelia ricinella* L. extracts (ranging 8–64 µg/ml) and LPS (100 ng/ml) for 24 h.

VI.2.5.2-COX-2 Protein Expression in Macrophages

Western blot analysis

Cells were washed twice with PBS, scraped into 0.5 ml of ice-cold extraction lysis buffer (50 mm Tris pH 8.0, 150 mm NaCl, 1% Nonidet P-40) and maintained for 30 min at 4 °C in constant agitation. Cell debris was removed by micro-centrifugation at 15.000×g during 30 min at 4 °C and supernatants were rapidly frozen. Protein concentrations in the supernatant were evaluated using the BCA Protein Assay Kit (Termo Fisher, Montluçon, France) as suggested by the manufacturer.

Total proteins (15 µg) were separated by electrophoresis on 12.5% polyacrylamide gels and transferred for 1 h onto nitrocellulose membranes. The membranes were then blocked for 1 h with 5% BSA Tris-buffered saline/0.1% Tween 20 and incubated with primary antibodies against COX-2 (1/1000) from Cell Signalling (Ozyme, Saint Quentin Yvelines, France). Then, membranes were incubated with secondary fluorescent goat anti-rabbit and goat anti-mouse IgG (H + L) antibodies (1/20 000) from Cell Signaling (Ozyme, Saint Quentin Yvelines, France). Proteins were visualized by enhanced chemiluminescence and imaged using a Syngene G:BOXChemi XR system and GeneSnap software (Version 7.08.11; Syngene USA) and the signals was quantified using Image J software. Samples were normalized by GAPDH housekeeping protein.

VI.2.5.3- PGE_2 measurement

After collecting the supernatants, the PGE_2 concentration in the culture medium was quantified using the EIA kit for PGE_2 according to the manufacturer's protocol. The production of PGE_2 was measured relative to the control treatment. All experiments were performed in triplicate.

VI.2.5.4-Cell viability assay

The effect *Adelia ricinella* L. extracts on the RAW 264.7 cell viability was determined by the resazurin dye reduction test. Briefly, 200 µl of cell suspension (5×10^5 cells/ml) was added in a 96-well plate and incubated in the same conditions mentioned above. After 24 h, the cells were washed twice with 200 µl of DPBS and then fresh DMEM without FBS was added. The plant extract (8, 16, 32, 64, 128 and 256 µg/ml) were added into each well and incubated at 37 °C, 5% CO_2. Subsequently, 50 µl of resazurin solution (2.2 µg/ml) was added and the fluorescence was measured after 4 h (λexcitation 550 nm, λemission 590 nm) using a TECAN GENios microplate reader (Männedorf, Switzerland). Two independent experiments were performed and the samples were tested in triplicate. Tamoxifen was included as a reference of cytotoxicity.

VI.2.5- Statistical analysis

Statistical analysis was performed using the statistical software package GraphPad Prism 7 (Windows, V. 7.04, 2017). All results were statistically analysed and expressed as the arithmetic means ± standard deviation (SD). One-way ANOVA test followed by the Tukey test was applied to determine the significance of differences between groups. Differences at $P \leq 0.05$ were accepted as significant.

VI.3-Results and Discussion

VI.3.1- Phytochemical analysis.

Three extracts of *Adelia ricinella* L., that is AR1 (ethanol/water 95:5% v:v), AR2 (ethanol/water 50:50% v:v) and AR3 (water) were analysed by UPLC-DAD-MS/MS. The total ion chromatogram (TIC) of all extracts (Figure VI.1) showed that most of the peaks are present in these three extracts, being only different by the intensity of the signal. The alcoholic extracts showed more defined and higher peak areas than the aqueous extract. Thirteen peaks were defined (peaks with an intensity level greater than 30% in any extract and at least one well-defined peak in one of the other two).

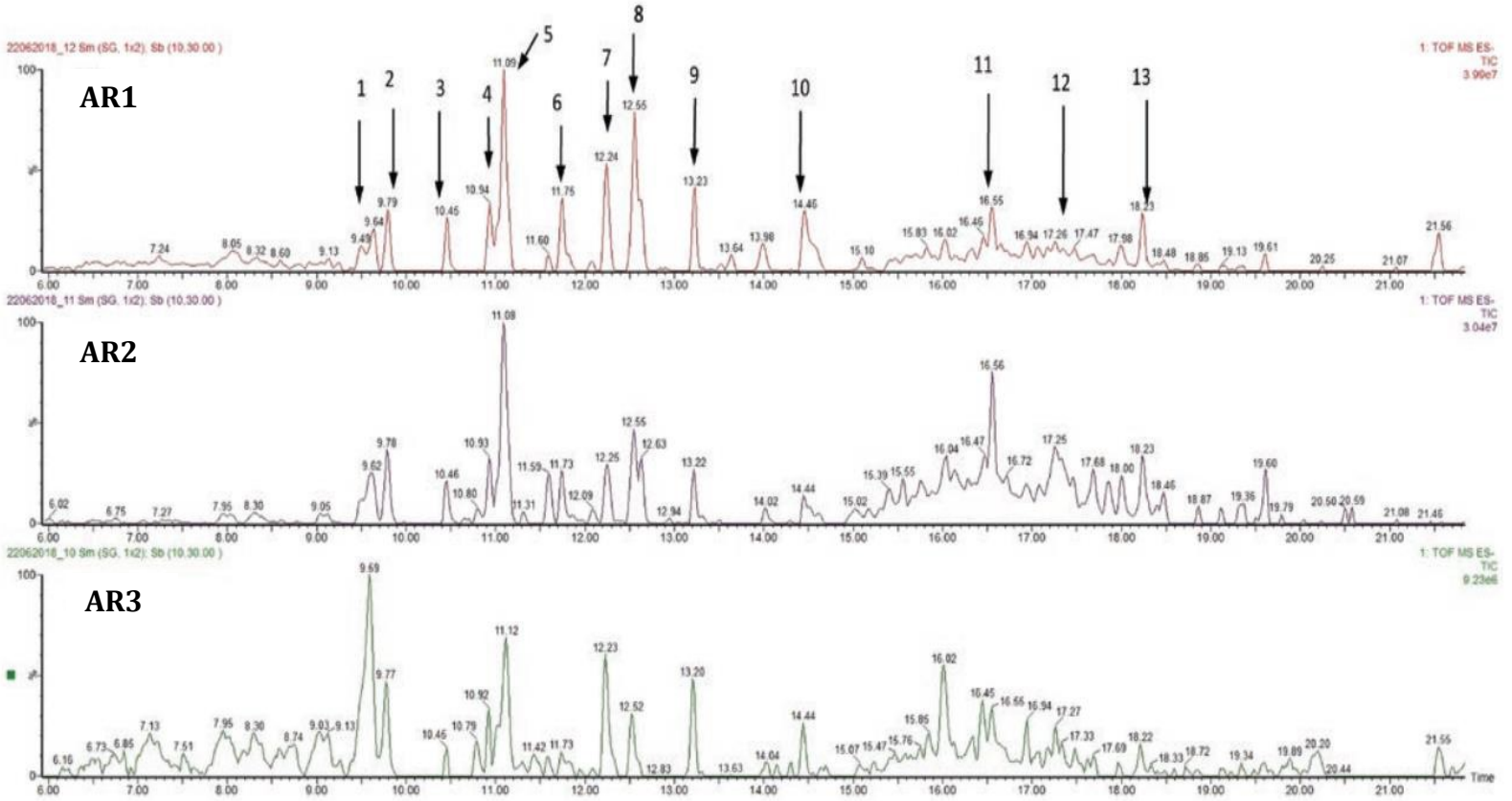


Figure VI.1. UPLC-DAD-MS/MS TIC profiles of *Adelia ricinella* L. extracts.

1) Luteolin-*C*,*O*-di-hexoside; **2)** Luteolin-di-*C*-hexoside; **3)** Luteolin-*O*-hexoside-pentoside; **4)** Luteolin-*O*-hexoside-deoxyhexoside; **5)** Orientin; **6)** Apigenin-*O*-hexoside-pentoside; **7)** Diosmin; **8)** Vitexin; **9)** (2-hydroxyethoxy)-*O*-hexoside- deoxyhexoside-4′-methyl-kaempferol; **10)** *O*-glucopyranuronosyl-deoxyhexoside-*O*-methylapigenin; **11)** Luteolin; **12)** Apigenin-*C*-(*O*-*p*-coumaroyl-hexoside); **13)** Apigenin

Table VI.1 shows the thirteen compounds identified with it most important signals in the ESI negative ion mode mass spectrum that helped mainly to elucidate the glycosidic type compounds. ESI positive ion mode was useful for determination of the nature of the aglycones. The thirteen peaks defined, indicated by a retention time range represented by the maximal signal appeared in each extract, were assigned as follows: The mass spectrum of peaks **1** (Rt from 9.59 to 9.64 min) and **2** (Rt from 9.77 to 9.79 min) both displayed a pseudo-molecular ion at *m/z* 609.1464 $[M-H]^-$ but with a different fragmentation pattern. While in peak **1** product ions were observed at *m/z* 447.0935, 327.0883 and 285.0407, peak **2** showed characteristic ions at *m/z* 461.1330, 327.0714 and 285.0405. In both cases, fragments from the ESI in the positive ion mode were observed at *m/z* 161.0227 and 179.0347, characteristic for the $^{0,4}B^+$-H_2O and $^{0,4}B^+$ cleavage of the C-ring of a flavone type aglycon, which was in agreement with luteolin and made it possible to make the distinction between kaempferol which has the same molecular weight. Clearly both compounds were di-glycosides of luteolin, but compound **1** corresponded to a luteolin-C,O-di-hexoside in which the O-hexoside unit was lost first; while compound **2** corresponded to a luteolin-di-C-hexoside, with each hexose linked at a different position, therefore C6 and C8. Compound **3**, eluted with Rt from 10.45 to 10.46 min, and the [M−H]− ion was observed at *m/z* 579.1365. It showed a characteristic *m/z* 285.0403 fragment ion in the MS^2 experiment in negative ion mode, corresponding to $[M-162-132-H]^-$ consistent with the loss of an O-linked-hexosidepentoside disaccharide residue. Once again, in the positive ion mode fragments at *m/z* 161.0227 and 179.0337 were observed, associated to the luteolin nucleus. Peak **4** (Rt from 10.92 to 10.94 min) showed an $[M-H]^-$ ion at *m/z* 593.1503 and a single product ion at *m/z* 285.0402, corresponding to [M-162-142-H]; therefore, it was deduced to be a luteolin-type-O-hexoside-deoxyhexoside (same behaviour in ESI positive ion mode MS). Peak **5**, based on the larger areas (UV detection at 256/366 nm) in the chromatograms of the alcoholic extracts, emerged with an Rt from 11.08 to 11.09 min as the main constituent of *A. ricinella* L.

Compound 5 showed a pseudo-molecular ion at *m/z* 447.0927 $[M-H]^-$ and product ions at *m/z* 327.0506 and 285.0402. This fragmentation pattern was consistent with a luteolin C-hexoside, which identity was confirmed to be orientin by analysing the standards orientin and isoorientin, displaying the same Rt and mass spectral data as orientin. The UV spectra of compounds 1–5 are all quite similar with maxima around 228 and 345 nm, being consistent with different luteolin glycosides.

Table VI.1 Assigned compounds, [M−H]− and ESI negative fragment ions of the thirteen flavonoids identified in *Adelia ricinella* L. extracts

Peak No.	[M-H]- (*m/z*)	Retention time range AR1–AR3 (min)	Molecular formula	Other fragments	Identification (Compound)
1	609.1464	9.59–9.64	$C_{27}H_{30}O_{16}$	447.0935, 327.0869, 285.0396	Luteolin-*C*,*O*-di-hexoside
2	609.1464	9.77–9.79	$C_{27}H_{30}O_{16}$	461.1330, 327.0714, 285.0405	Luteolin-di-*C*-hexoside
3	579.1365	10.45–10.46	$C_{26}H_{28}O_{15}$	285.0403	Luteolin-*O*-hexoside-pentoside
4	593.1503	10.92–10.94	$C_{27}H_{30}O_{15}$	285.0404	Luteolin-*O*-hexoside-deoxyhexoside
5	447.0927	11.08–11.09	$C_{21}H_{20}O_{11}$	327.0506, 285.0402	Orientin
6	563.1397	11.73–11.75	$C_{26}H_{28}O_{14}$	463.0877, 300.0275, 269.0451	Apigenin-*O*-hexoside-pentoside
7	607.1658	12.23–12.25	$C_{28}H_{32}O_{15}$	577.1551, 299.0554, 269.0451	Diosmin
8	431.0984	12.52–12.55	$C_{21}H_{20}O_{10}$	311.0580, 269.0449	Vitexin
9	653.1716	13.20–13.23	$C_{29}H_{34}O_{17}$	607.1671, 507.1183, 283.0614, 268.0375	(2-hydroxyethoxy)-*O*-hexoside-deoxyhexoside-4′-methyl-kaempferol
10	637.1764	14.44–14.46	$C_{29}H_{34}O_{16}$	591.1714, 283.0607, 268.0371	*O*-glucopyranuronosyl-deoxyhexoside-*O*-methylapigenin
11	285.0483	16.55–16.56	$C_{15}H_{10}O_{6}$	–	Luteolin
12	577.1349	17.25–17.27	$C_{30}H_{26}O_{12}$	431.0980, 329.2325, 269.0451	Apigenin-*C*-(*O*-*p*-coumaroyl-hexoside)
13	269.0454	18.22–18.23	$C_{15}H_{10}O_{5}$	–	Apigenin

Compound **6** (Rt from 11.73 to 11.75 min) was characterized by a pseudo-molecular ion at *m/z* 563.1397 $[M-H]^-$. The MS^2 experiment showed an ion at *m/z* 269.0454 corresponding to $[M-162-132-H]^-$. Fragments observed in ESI positive ion mode MS ($^{1-3}A^+$and $^{0-2}A^+$) at *m/z* 153.0119, characteristic of C^1—C^3 bond cleavage in the C-ring and an ion at *m/z* 149.0526 from C^0—C^2 bond cleavage respectively, were in agreement with an apigenin structure. Altogether, this suggested that compound **6** was an apigenin-O-linked hexoside-pentoside disaccharide. Peak **7** (Rt from 12.23 to 12.25 min) presented an $[M-H]^-$ ion at *m/z* 607.1658 and product ions at *m/z* 577.1551, 299.0554 and 269.0451. The ion at *m/z* 299.0554 suggested the loss of $[M-162-146-H]^-$, consistent with an O-hexoside-deoxyhexoside disaccharide, while ions at *m/z* 577.1571 [M-H-30.0087]$^-$ and 269.0451 represented the loss of an OCH_3 moiety. The same fragmentation pattern was observed in ESI positive ion mode MS, suggesting that compound **7** was the widespread flavonoid diosmin. Compound **8** (the third most abundant compound considering the peak area with UV detection 256/366 nm), assigned to the peak of the same number (Rt from 12.52 to 12.55 min), produced a pseudo-molecular $[M-H]^-$ ion at *m/z* 431.0984 and product ions at *m/z* 311.0580 and 269.0449. It was confirmed as being vitexin using an analytical standard. Compound 9 (Rt from 13.20 to 13.23 min) showed its $[M-H]^-$ ion at *m/z* 653.1716 and product ions at *m/z* 607.1671, 507.1183, 283.0614, and 268.0375 in ESI negative ion mode. The loss of 46 mass units can be associated with a 2-hydroxyethoxygroup, releasing the fragment ion at *m/z* 607.1671, while the fragment 507.1183 looks like the loss of a deoxyhexose $[M-H-146]^-$. The next fragment appeared at *m/z* 283.0614, representing the additional loss of a162 mass units related to an O-hexoside linkage. Finally, fragment ion at *m/z* 268.0375 represented the loss of 15 mass units ($-CH_3$) from *m/z* 283.0614. The fragmentation pattern in ESI positive ion mode MS shows fragments at 163.0703 and 153.0177 in a 2:3 peak intensity proportion, suggesting that compound **9** contains a 3-OH moiety (fragments $^{1-3}A^+$and $^{0-2}A^+$, respectively) and in consequence, indicating that the O–CH_3 substitution is placed at the 4′ position. Altogether, this allows to define compound **9** as (2-hydroxyethoxy)-O-hexoside-deoxyhexoside-4′-methyl-kaempferol. Compound **10** (Rt from 14.44 to 14.46 min) was characterized by an $[M-H]^-$ ion at *m/z* 637.1764. The MS^2 experiment showed ions at *m/z* 591.1714, 283.0607 and 268.0371. This compound looked quite similar to compound **9** with similar behavior in ESI positive ion mode, but with a deoxy sugar moiety. This allowed defining it as an O-glucopyranuronosyl-deoxyhexoside-O-methylapigenin derivative.

Compound **11** (Rt from 16.55 to 16.56 min, being the second most abundant constituent considering the peak area with UV detection at 256/366 nm) produced an [M−H]− ion at *m/z* 285.0483 without any other product ions with over 5% relative intensity. Fragments in ESI positive ion mode MS appeared at *m/z* 153.0181, 161.0231 and 179.0337, consistent with C1–C3 and C0–C4 cleavage (fragments $^{1-3}A^{+}$, $^{0-4}B^{+}$) being defined as luteolin. On the other hand, compound **13** (Rt from 18.22 to 18.23 min) showed an $[M-H]^{-}$ ion at *m/z* 269.0454 without any other product ion with over 5% relative intensity. Fragments observed in ESI positive ion mode MS suggested apigenin as compound 13. Both compounds (**11** and **13**) were confirmed by adding standards in others run. Compound **12** (Rt from 17.25 to 17.27 min) showed a $[M-H]^{-}$ ion at *m/z* 577.1349 and a product ion at *m/z* 431.0980 (low relative intensity), which suggested the loss of a coumaroyl group by cleavage at the carbonyl position, losing a charged fragment producing a peak at *m/z* 146.0369. Fragments at *m/z* 329.2325 and 269.0449 were assigned to a different pattern of a C-sugar moiety cleavage as is represented in Figure VI.2. Once again, fragments observed in ESI positive ion mode MS suggested the apigenin aglycone; therefore, the proposed structure is an apigenin-C-(O-p-coumaroyl-hexoside).

VI.3.2-COX-1 and COX-2 enzymatic inhibition assay

The *in vitro* effect of the *Adelia ricinella* L. extracts on the enzymatic activity of COX-1 and COX-2 (Figure VI.3A and B) was firstly evaluated using a cell-free experiment. This type of assay may offer prior information about the enzyme selectivity of bioactive compounds present in plant extracts. The ethanol extracts (AR1 and AR2) strongly inhibited COX-1 and COX-2 enzyme activity. Additionally, both extracts were, in a dose-dependent manner, significantly more active (up to ten times more) for COX-2 (IC_{50} 15.9 and 8.4 µg/ml, respectively) than for COX-1 (IC_{50} 52 and 86 µg/ml, respectively). On the contrary, the aqueous extract (AR3) did not exert any significant inhibitory effect on the enzymes.

Both COXs isoforms are well known to be targets for several nonsteroidal anti-inflammatory compounds. COX-1 is widely distributed and constitutively expressed in most tissues where it is involved in homeostatic functions, mainly in the gastrointestinal tract. However, an intensified inhibition of the enzyme activity by anti-inflammatory drugs may provoke several adverse effects on gastric functions. The COX-2 inducible isoform, more predominant at sites of inflammation, appears to play a key role in pathophysiologic conditions like inflammatory disorders, and has driven the therapeutic development of COX-2 inhibitors[253].

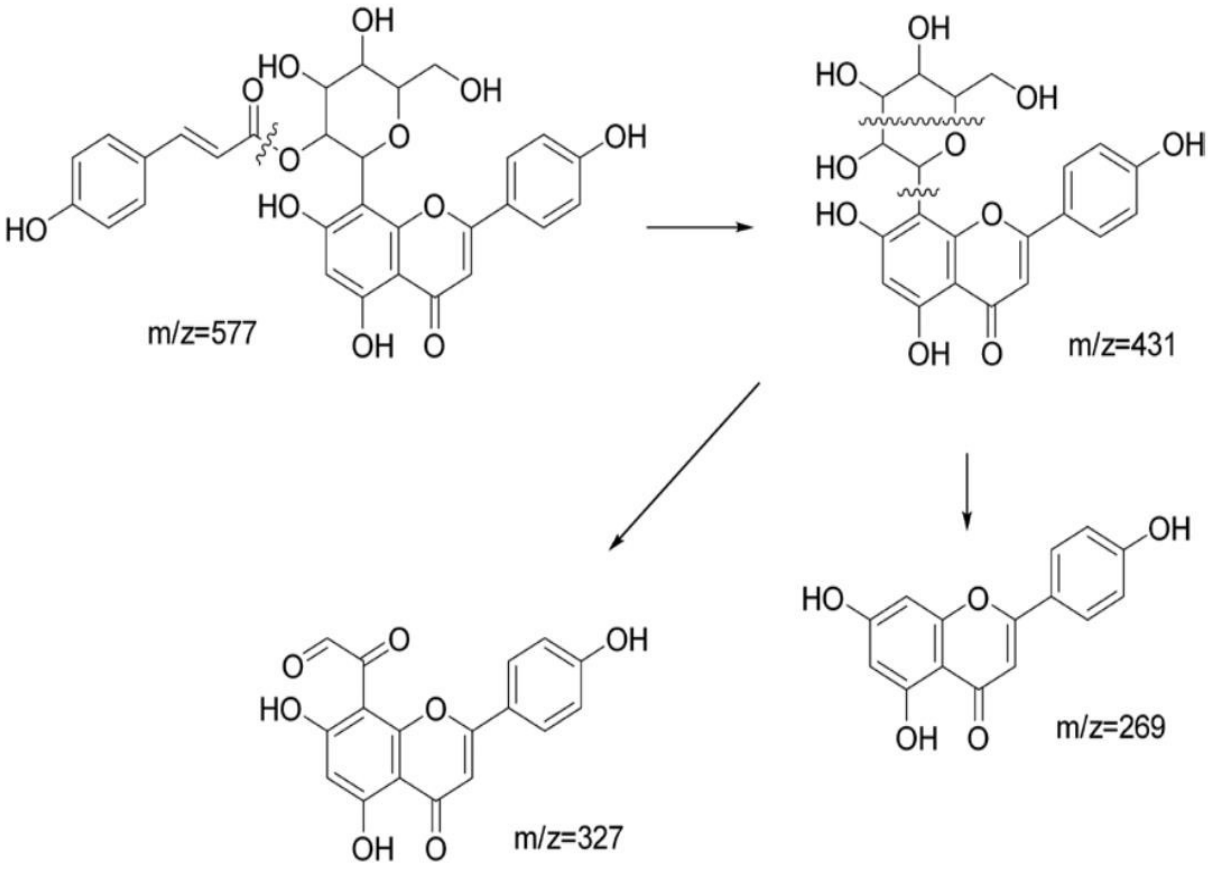


Figure VI.2. Fragmentation pathway of compound 12 (Positions of the C-linked sugar moiety and O-pcoumaroyl substituent are arbitrary).

VI.3.3-COX-2 protein expression in macrophages

In the case of COX-2 a cell-based assay was carried out. It was observed by Western blot analysis that the ethanol extracts (AR1 andAR2) significantly reduced COX-2 expression in LPS-stimulated RAW 264.7 macrophages at 64 µg/ml (Figure VI.3C). Additionally, the aqueous extract (AR3) also exhibited an important decrease of COX-2 expression at 64 µg/ml. When comparing the results of the cell-free enzyme inhibition assays with the cell-based COX-2 expression assays, it can be noted that the ethanol extracts (AR1 and AR2) are active in both models, whereas the aqueous extract is only active in the COX-2 expression assay. It can be hypothesized that the aqueous extract is richer in flavonoid glycosides than the ethanol extracts. Hence, the bulky and polar glycosidic substituents may hinder interaction with the active site of the enzyme.

It seems a contradiction that the most polar extract (AR3), more rich in glycosidic compounds, which are supposed to have decreased membrane permeability, also showed inhibitory activity in the COX-2 expression assay. However, it mainly concerns C-glycosides, and it has been reported that such compounds, for example orientin and vitexin, showed inhibition of NF-kB and COX-2 expression *in vitro*, indicating they are at least in part taken up by the cells[254, 255].

In vitro and *in vivo* reports confirm that orientin and vitexin, two of the most abundant compounds in *Adelia ricinella* L., contribute to reduction of inflammation through the decrease of the LPS-induced TNF-α/IL-6 release, leukocyte migration and reduction of the expression of pro-inflammatory enzymes (COX-2, iNOS) [256,257].

On the other hand, in 'in vivo' experiments O-glycosidic bonds can be cleaved, allowing absorption of the aglycones and interaction with the target[85]. Additionally, it is well known that flavonoids may also exert anti-inflammatory effects through their antioxidative mechanisms such as scavenging ROS (reactive oxygen species) and RNS (reactive nitrogen species), etc[250].

VI.3.4-PGE_2 measurement

Macrophages treated with the *A. ricinella* L. extracts were stimulated with LPS (100 ng/ml) for 24 h, and the PGE_2 concentration was estimated in the culture medium. As shown in Figure VI.3D, the ethanol extracts (AR1 and AR2) inhibited the LPS-induced PGE_2 release at 64 µg/ml. The results indicated that the inhibitory effects of *A. ricinella* L. extracts on LPS-induced PGE_2 production might be provoked by the down-regulation of the COX-2 enzyme, blocking the protein expression as previously described.

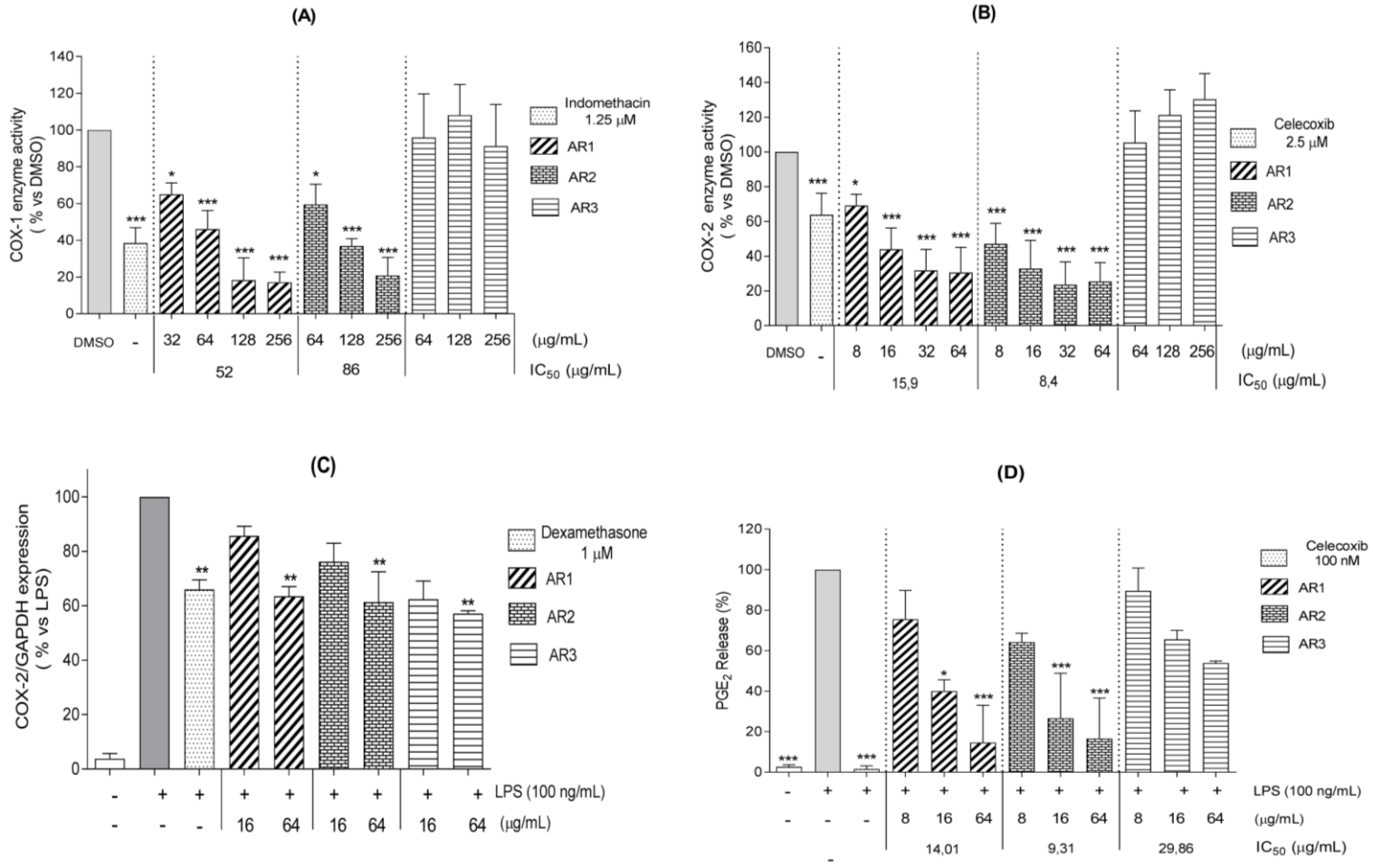


Figure VI.3 Inhibitory effect of *Adelia ricinella* L. extracts on COX-1 and COX-2 activities and PGE_2 release. A y B) COX-1 and COX-2 enzymatic inhibition assay (free cell experiment); **C)** COX-2 protein expression in macrophages; **D)** PGE_2 release.

Additionally, the inhibition of PGE_2 biosynthesis by the flavonoid enriched ethanol extracts (AR1 and AR2) could be closely related with the reduction of COX-2 enzyme inhibition evidenced in the previous cell-free experiment. The IC_{50} of both *in vitro* assays demonstrated that the inhibitory effect occurred at similar concentrations. It has been reported that flavonoids can inhibit the biosynthesis of prostaglandins, thromboxanes, leukotrienes by inhibition of the enzymes COX, PLA_2 or LOX[86, 250]. The absence of an additive or synergistic effect between inhibition of COX-2 expression and enzyme inhibition, may be related to the observation that, if the COX-2 enzyme is inhibited, the cell may paradoxically counterbalance with more protein expression[258].

On the other hand, the aqueous extract did not show a statistically significant inhibition on the LPS-induced PGE_2 production, despite a slight decrease observed at the highest concentration (64 µg/ml), which may due to the fact that the residual quantity of expressed COX-2 protein is sufficient to maintain PGE_2 production.

The extracts were not cytotoxic to RAW 264.7 macrophages cells (IC_{50} > 256 µg/ml) (Figure VI. 4) following the results obtained from the cell viability assay by Resazurin dye reduction test.

VI.4-Conclusion

The current study provided a new understanding regarding the main active constituents and the pharmacological potential of *Adelia ricinella* L. crude extracts. The extracts were mainly composed of glycosides of luteolin and apigenin. The C-glycosides orientin and vitexin, and the aglycone luteolin, are the three most abundant compounds in this plant species. The plant extracts demonstrated an inhibitory effect on COX-1 and COX-2 enzyme activities and COX-2 protein expression. The results also suggest that ethanol extracts mainly act on PGE_2 release probably by COX-2 enzymatic inhibition. This effect is in agreement with the biological properties attributed to the detected flavonoids. The evidence obtained in this work support in part the ethnopharmacological usage of aerial parts of *Adelia ricinella* L. as traditional remedy to treat inflammatory disorders.

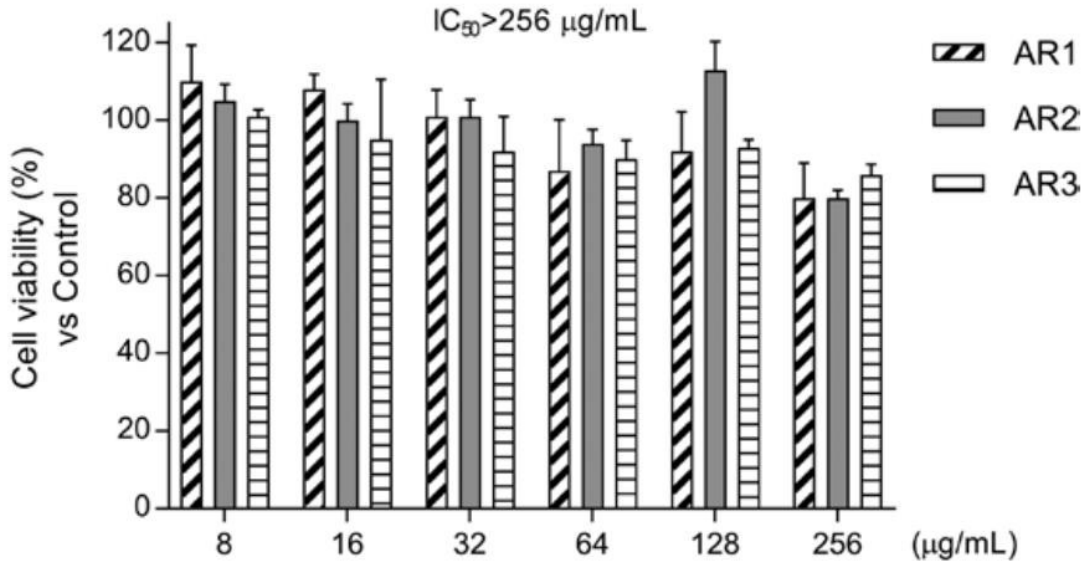
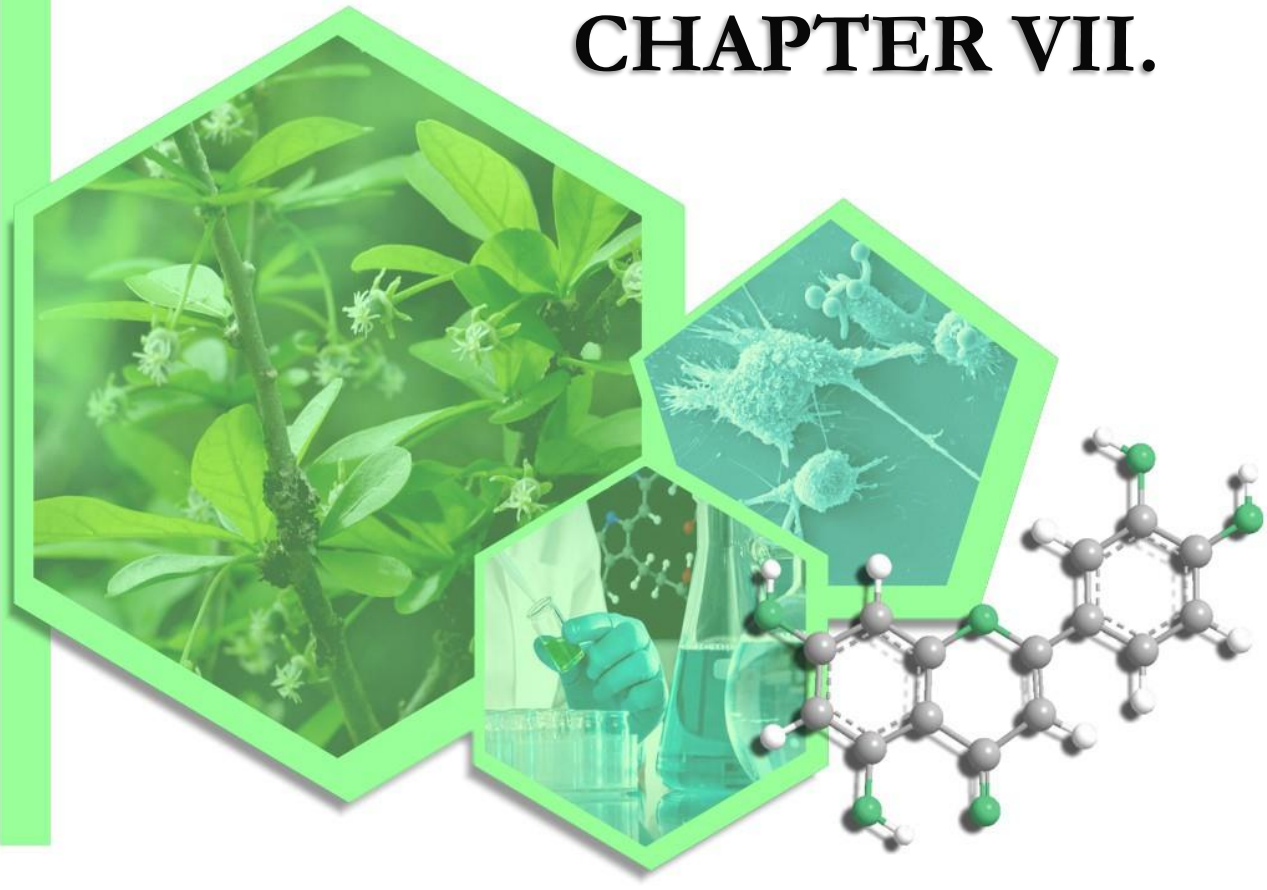


Figure VI. 4 Effect of *Adelia ricinella* L. extracts on RAW 264.7 murine macrophages cell viability (All values are expressed as the arithmetic mean ± SD of 6 replicates).

CHAPTER VII.



CHAPTER VII. Extracts from *Adelia ricinella* L. reduce paw oedema and inhibit release of pro-inflammatory cytokines and adhesion of leukocytes to endothelial cells.

Berenguer CA, Escalona-Arranz JC, Llauradó G, Puente ZE, Rodriguez EL , Suarez CJ, Cos P, Pieters L. Extracts from *Adelia ricinella* L. reduce paw oedema and inhibit release of pro-inflammatory cytokines and adhesion of leukocytes to endothelial cells. Phytotherapy research. (Article submitted)

VII.1- Introduction

Inflammation is a normal physiological response triggered by the organism to eliminate noxious stimuli, infections and to restore tissue damage [259]. Nevertheless, a prolonged inflammatory action often becomes pathogenic, and involves pro-inflammatory mediators like cytokines (IL-1β, IL-6 and TNFα) and reactive oxygen species (ROS), the induction of integrins and intercellular/vascular adhesion molecules (e.g.: LFA-1, L-selectin, ICAM-1, VCAM-1) promoting transmigration and adhesion of leukocytes to the endothelium, thus leading to several inflammation-related chronic diseases like cancer, cardiovascular and neurodegenerative disorders, etc[260-262]. Therefore, controlling the release of leukocytes-derived pro-inflammatory mediators and the inhibition of leukocyte extravasation by suppressing their interaction with endothelium has recently been established as a therapeutically valuable anti-inflammatory approach[260].

In this context, plants represent one of the main sources of pharmacologically active compounds [263, 250]. The anti-inflammatory effects exhibited by several natural substances have been scientifically validated. Among the molecular pathways studied, they can act by suppressing the release of pro-inflammatory cytokines as well as by inhibiting the induction of cell adhesion of particular molecules to leukocyte-like cell types[264; 265].

Ethnobotanical reports corroborate that many native Caribbean herbal medicines are often used for health maintenance and to treat inflammation-related diseases [263, 266]. *Adelia ricinella* L. (Euphorbiaceae) is one of those plants traditionally used as analgesic and anti-inflammatory remedy. In previous charpters it was demonstrated that aqueous and ethanol preparations from it aerial parts exhibited *in vitro* antioxidant and anti-inflammatory activities related to their radical scavenging properties and COX-1/COX-2 suppression in RAW 264.7 cells. These effects were attributed to the presence of active compounds like polyphenols and flavonoids identified in the plant extracts.

However, a comprehensive study of the effect of bioactive extracts on production and release of cytokines, the inhibitory effect on cell adhesion and the reduction of oedema in an *in vivo* model are still lacking. Therefore, this investigation has been designed to assess the *in vitro* and *in vivo* anti-inflammatory activity from *Adelia ricinella* L. aerial parts extracts.

VII.2- Material and methods

VII.2.1- Materials and reagents

Lipopolysaccharide (LPS) from *Escherichia coli* (0128:B12), DMSO, tamoxifen and resazurin sodium salt (7-hydroxy-3H-phenoxazin-3-one-10-oxide), Cell Titer-Blue® and Crystal violet powder were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Medium Eagle Modified (DMEM) and Dulbecco's phosphate-buffered saline (DPBS) were also from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 medium and fetal calf serum (FCS) was from Gibco (New York, NY, USA). Endothelial Cell Growth Medium (ECGM) was purchased from PELOBiotech GmbH (Planegg/Martinsried, Germany). Indomethacin was purchased from MP Biomedicals. L-NAME (N5-(imino(nitroamino)methyl)-L-ornithine methyl ester, monohydrochloride) was purchased from Cayman Chemicals (Ann Arbor, MI, USA). The Griess reagent kit was purchased from Molecular Probes® (Eugene, OR, USA).

VII.2.2- Plant material and extract preparation

The aerial parts of *Adelia ricinella* L. were collected and taxonomically identified at different moments by specialists at the Eastern Centre for Ecosystems and Biodiversity (BIOECO, Santiago de Cuba) as has been informed in previous charpters. A voucher specimen stored at the herbarium of this institution with the registration number 14 780 has been also informed.

The extracts were prepared by Soxhlet extraction during four hours after the first reflux using either water, ethanol 50% and ethanol 95% as described in chapter IV. Afterwards, the extracts AR1 (EtOH 95%), AR2 (EtOH 50%) and AR3 (aqueous extract) were filtered using a Buchner funnel and filter paper, and finally concentrated in a rotary evaporator. All extracts were dissolved in DMSO at different concentrations (ranging 256 - 8 μg/ml) not exceeding a final DMSO concentration of 0.1%.

VII.2.3- Cell cultures

RAW264.7 macrophages and THP-1 monocytes from ATCC (USA) were maintained at 37 °C, 5% CO_2 atmosphere in DMEM and RPMI medium, respectively, supplemented with 10% FCS, 2% L-glutamine and 4.5 g/L D-glucose.

Macrophages and monocyte cells suspensions was seeded in a 96-well plate ($5x10^5$ cells/ml) and incubated at 37 °C in a humidified atmosphere with 5% CO_2. After 48 h, the old medium was discarded and the cells were pre-incubated with and without *Adelia ricinella* L. extracts (ranging 256 - 8 µg/ml) and LPS (100 ng/ml) for 24 h.

Primary human umbilical vein endothelial cells (HUVECs) were seeded in T75 ml flasks containing Endothelial Cell Growth Medium supplemented with 10% FCS, 1% penicillin/streptomycin and 1% amphotericin B. After 48 h, cells were washed with pre-warmed phosphate buffer solution (PBS) and detached with trypsin/EDTA pre-warmed (3 ml) and incubated for 3-5 min. Stop medium M199 (10 ml) was added to re-suspend the cells which were transferred to 50 ml conical tubes to centrifuge (300xg, 5 min, room temperature). Cells were re-suspended in ECGM medium, transferred to 24-well plates (500 ml) previously treated with collagen (0.25% in PBS) for 30 min at 37 °C, 5% CO_2, and incubated in to aforementioned conditions during 48 h to reach confluence (>80%).

VII.2.4- Cell viability assay

VII.2.4.1-Metabolic activity (Cell Titer Blue®)

Plant extracts (300-1µg/ml) were added in triplicate to appropriate wells and incubated in the conditions mentioned above during 48 h at 37 °C, 5% CO_2. Cell Titer-Blue® Reagent was thawed (37 °C in a water bath) and placed at room temperature. Plates were removed from the incubator and 10 µl of Cell Titer-Blue® reagent was added per well. After the incubation period (4 h) the fluorescence was recorded at 560/590 nm. Controls: No cells, untreated cells and positive (tamoxifen and staurosporine) controls were included in the experiments.

VII.2.4.2- Cytotoxicity assay (Crystal violet assay)

Additionally, cell viability was evaluated in the Crystal violet (CV) cytotoxicity assay. Cells ($2x10^5$ cells/ml) were added to 96-well plates at 37 °C, 5% CO_2 during 24 h. Then, extracts (300 - 1 µg/ml) were added and incubated in the same conditions aforementioned. After 24 h, cells were washed once with DPBS and fixed by incubating with methanol/ethanol solution (2:1) for 10 min at room temperature. Then, cells were stained with crystal violet

solution (CV, 0.5%) during 10 min at room temperature and unbound crystal violet was removed by washing with distilled water. The plates were dried overnight before CV was reconstituted by incubation with 20% acetic acid for 30 min at room temperature. A volume (60 µL) of stained solution was transferred into a 96-well plate and optical density (OD) was recorder at 540 nm using GloMax® Promega equipment. In both assays, two independent experiments were performed (samples in duplicate) and cells treated with staurosporine were included as a reference control drugs for cytotoxicity. Untreated cells were considered as negative control.

VII.2.4.3- Apoptosis assay

HUVECs cell suspension (500 µL) was added to 24-well plates and incubated 48 h at 37 °C, 5% CO_2. Plant extracts (300-1µg/ml) were added in triplicate to appropriate wells and incubated during 48 h at 37 °C, 5% CO_2. Then, the supernatant of each well was collected and added to crystal FACS (Fluorescence Activated Cell Sorter) tubes. Cells were washed twice (PBS) and collected in the same crystal FACS tubes. Afterwards, trypsin-EDTA solution was added per well, incubated 3 min at 37 °C, 5% CO_2 to detach the cells. Stopping medium M199 (600 µL, cold) was added to the plate and collected in the same crystal FACS tubes. Samples were centrifuged at 300g x10 min x 4 °C. Subsequently, the supernatant was removed; the cells re-suspended in cold PBS (1 ml) with vortex (keeping in ice) and centrifuged 300g x10 min x 4 °C. The remaining cells pellets were permeabilized and stained using 500 µL of hypotonic fluorochrome solution (propidium iodide, PI, 50 µg/ml in 0.1% sodium citrate plus 0.1% Triton X-100, Sigma) and incubated at 4 °C overnight protected from the light. PI-staining was determined by flow cytometer analysis using a FACSVerse[267]

VII.2.5- Nitrite and cytokine determination

The extracellular nitrite accumulation was measured by the Griess reaction. RAW 264.7 macrophages ($5x10^5$ cells/ml) seeded in a 24-well plate, were previously stimulated with LPS (100 ng/ml) and IFN-γ (5 ng/ml); and then treated with plant extracts during 24 h. Afterwards, 150 µl of culture supernatant was collected and transferred to 96-well plates filled with 130 µl of demineralized water. After adding 20 µl of Griess reagent, samples were incubated for 30 min protected from light, and the absorbance was measured at 540 nm. A standard calibration curve was set up by diluting the nitrite standard solution of the kit.

The cytokine levels in supernatants were determined by mouse TNF-α, IL-1β and IL-6 immunoassay Quantikine® ELISA Kits (R and D Systems Inc., Minneapolis, MN, USA). Cells treated only with LPS (100 ng/ml) and IFN-γ (5 ng/ml) were used as control for stimulation.

VII.2.6- Cell adhesion assay

VII.2.6.1- THP-1 cell adhesion to TNF-stimulated HUVECs

HUVECs cells (500 µL) added to 24-well plate were pre-stimulated with plant extracts (300-1 µg/ml) for 30 min. Afterwards, the cells were stimulated with TNF-α (10 ng/ml) for 24 h and incubated under the same conditions described before. THP-1 human monocytes (10^4cells/ml) were centrifuged (300g x 5 min, room temperature) and re-suspended in serum-free RPMI. Cell tracker green (CTG) (5 µM) was added to an appropriate volume of THP-1 cells in RPMI and incubated 37 °C, 5% CO_2 for 30 min. Then, cells were centrifuged (300g x 5 min) and the resulting pellet washed with 10 ml of serum-free RPMI and centrifuged again (300g x 5 min). The supernatant was discarded and ECGM added. Thereafter, the culture medium was removed from the treated endothelial cells and the stained THP-1 cells were added to the HUVECs monolayer (500 µl of cell suspension per well). Fluorescence-labeled THP-1 cells were allowed to adhere for 5 min. Then, non-adherent cells were removed by washing (500 µl of PBS+, three times). After the last washing the plates were quantified by direct fluorescence measurements (λex: 485 nm; λem: 535 nm) using a microplate reader (Infinite F200, Tecan Trading-AG) to determine the cell adhesion.

VII.2.6.2-LPS-stimulated THP-1 adhesion to TNF-stimulated HUVECs

A second alternative of cell adhesion assay was performed using LPS-stimulated THP-1 monocytes. Briefly, THP-1 human monocytes stimulated with LPS (100 ng/ml, 30 min) were treated with plant extracts (300-1µg/ml) and the influence of adhesion to HUVECs previously activated with TNF was studied as we described above. The adhesion was quantified by direct fluorescence measurements (λex: 485 nm; λem: 535 nm) using a microplate reader (Infinite F200, Tecan Trading-AG).

VII.2.7- Formalin-induced rodent hind paw oedema model

The effect of *Adelia ricinella* L. extracts against formalin-induced rodent hind paw oedema was studied following the guidelines of the Institutional Animal Ethics Committee (Centre of Toxicology and Biomedicine) and was performed in accordance with Cuban legislation

and the EU Directive 2010/63/EU on the protection of animals used for scientific purposes. Female BALB/c mice (n=65) (8 weeks old) were purchased from the National Centre for the Laboratory Animal Production (Havana, Cuba) and maintained under controlled conditions of light (12 h/12 h), temperature (22± 3 °C) and humidity (60±10%). Animals were randomly distributed in plastic boxes (five animals each group) and fed ad libitum with commercial pelleted diet (ALYco®, Havana, Cuba) and distilled water (pH 6.8). Inflammation was induced in all groups of animals by sub-plantar injection of formalin 1% in the right hind paw of mice.

Group I, II, III and IV animals received the AR1 extract, and Group V, VI, VII and VIII animals received the AR2 extract (both at a dose of 25, 50, 100 and 200 mg/kg body weight). Group IX, X and XI animals received the AR3 extract (100, 200 and 400 mg/kg body weight). All plant extracts were administered one hour before formalin 1% injection. Animals (Group XII and XIII) treated with indomethacin (10 mg/kg body weight) and normal saline solution, were considered as positive and negative controls, respectively. Paw volume (swelling) of mice was measured using a Plethysmometer® (Ugo Basile, Italy) just before and then at 1, 3, 5, 7 and 24 h after formalin injection[97]. Percent inhibition in paw volume by plant extract/standard control drug was calculated by the following two steps:

% inflammation= (Vw-Vwo)/Vwo x 100, where Vw is the measure of swollen paw and Vwo is the normal paw.

% inhibition=(CTLneg-Tg)/CTLneg x 100, where CTLneg is the inflammation percentage of the negative control group and Tg is the inflammation percent of animals treated with plant extracts.

VII.2.8- Statistical analysis

Statistical analysis was performed using the statistical software package GraphPad Prism 7 (Windows, V. 7.04, 2017). All results were statistically analysed and expressed as the arithmetic means ± standard deviation (SD). One-way ANOVA test followed by the Tukey test was applied to determine the significance of differences between groups. Differences at $p \leq 0.05$ were accepted as significant.

VII.3-Results

VII.3.1- Effect of plant extracts on cell viability

Before actions on *in vitro* anti-inflammatory features were assessed in leukocytes and endothelial cell lines, potential plant extracts-derived effects on cell viability were

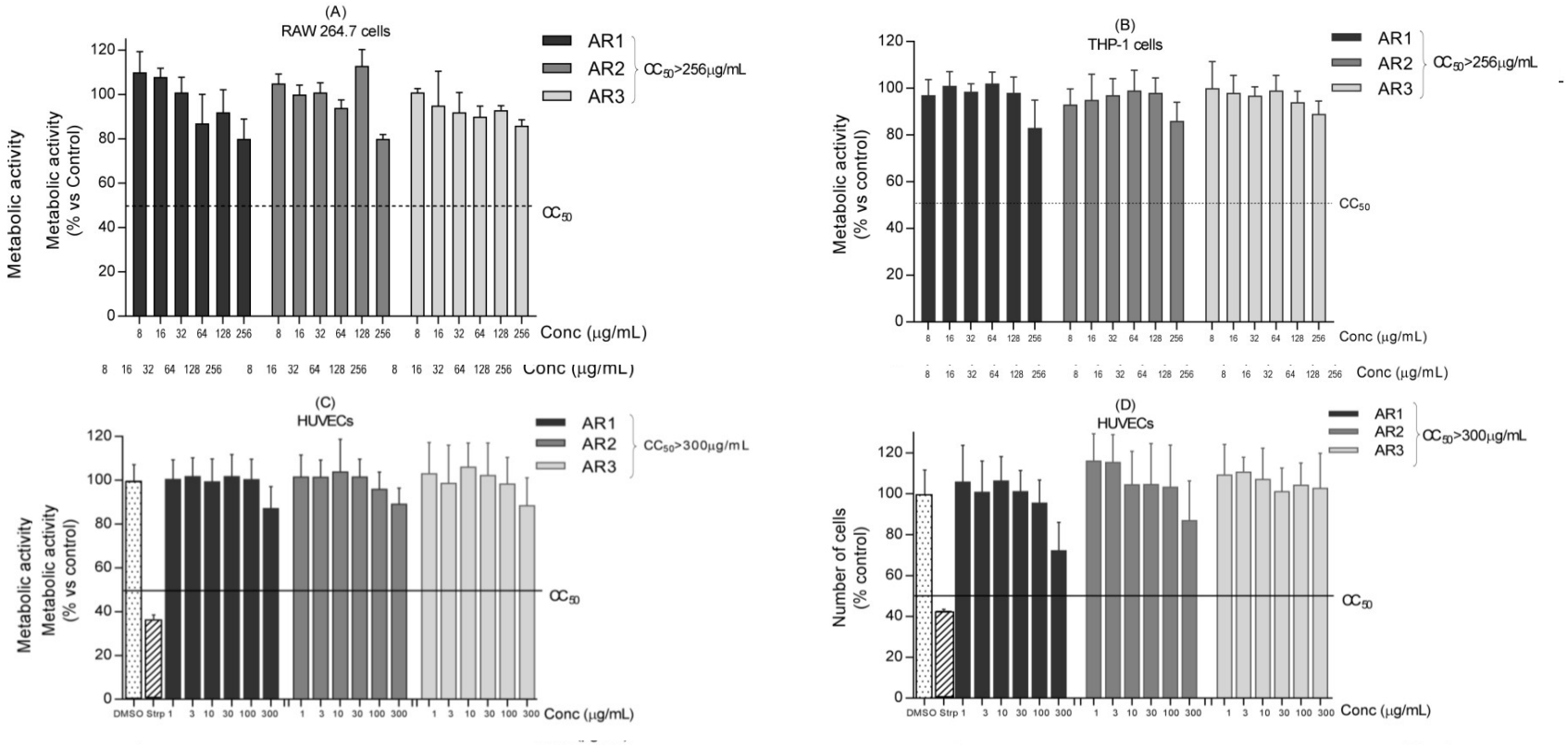


Figure VII.1. Effect of ethanolic and aqueous extracts of *Adelia ricinella* L. plant on the metabolic activity (A, B and C) and number of cells (D) of leukocytes and endothelial cells

Confluent leukocytes cell lines and HUVECs in 96-well plates were treated with plant extract for 24h. (A, B, C) Afterwards 10µl/well of Cell Titer-Blue® Reagent was added and incubated for 4h before recording fluorescence at (ex: 560 nm; em: 590 nm) for assessment of metabolic activity; (D) or cells were fixated with MetOH/EtOH (2:1), stained with Crystal Violet solution (20% MetOH), air dried overnight and acetic acid (20%) added for 30 min to record absorbance at 590 nm by a microplate reader. Staurosporine was used as positive control of cytotoxicity. The results are expressed as means ± SD ($p < 0.05$).

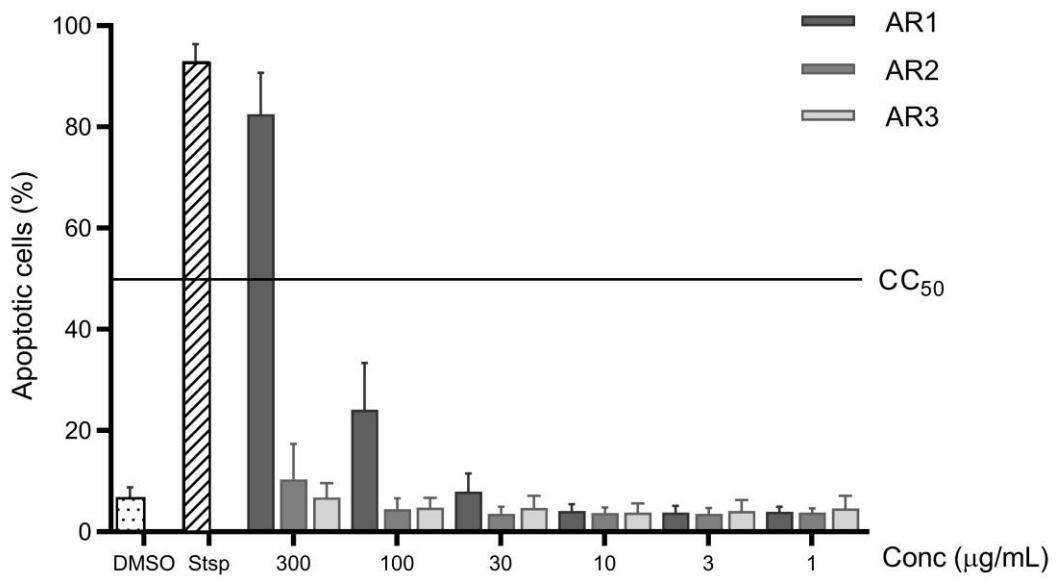


Figure VII.2A. Apoptotic effect of ethanolic and aqueous extracts of *A. ricinella* plant on endothelial cells.

Endothelial cells (in 24-well plates) were incubated with plant extracts (1-300 µg/mL) for 24 h. Propidium iodide staining and flow cytometric analysis was performed to determine the percentage of cells death [12]. Staurosporine was used as positive control of cell death. The results are expressed as means ± SD ($p < 0.05$)

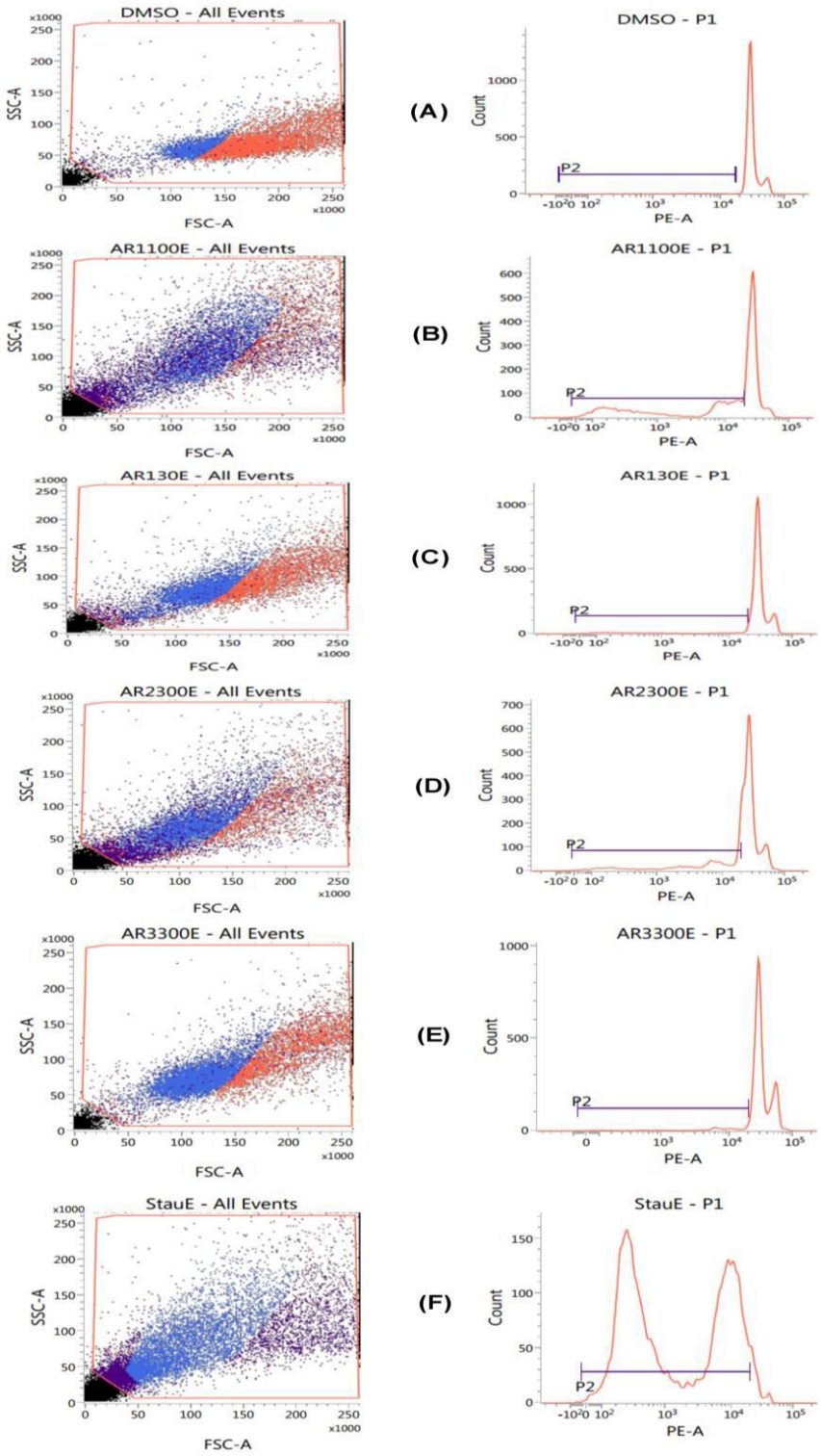


Figure VII.2B. Effect of ethanolic and aqueous extracts of *A. ricinella* plant on induction of apoptotic cell death by treating HUVEC cell line for 48 hours using flow cytometric analysis.

HUVECs cell suspension was treatment with different extracts of *A. ricinella* and incubated during 48 h. **A)** DMSO; **B** y **C)** AR1 (100 and 30µg/mL, respectively); **D** and **E)** AR2 and AR3 (300µg/mL) and **F)** Staurosporine was used as positive control of cytotoxicity.

determined. In general, we evidenced that after 24 h of incubation, the extracts did not significantly impair metabolic activity and membrane integrity in all cell lines (Figure VII.1).

However, when incubation was extended up to 72 h, apoptosis was significant in endothelial cells treated with AR1 at a concentration of 300 µg/ml (Figure VII.2A, VII.2B). On the contrary, both AR2 and AR3 did not exhibit any relevant cytotoxicity in HUVECs. Taking into account these findings, for *in vitro* experimental purposes concentrations lower than 300 µg/ml of the 95% ethanol extract were used

VII.3.2. Plant extracts reduced the production of nitrite and cytokine release in RAW 264.7 cells.

As shown in Figure VII.3, the nitrite production in LPS-stimulated macrophages was significantly decreased by all plant extracts at 12 and 24 h of incubation, which were statistically different in comparison with the negative control group (LPS-stimulated cells)($p<0.05$). Aqueous extract only showed statistical differences at 24 h. In the case of AR1 and AR2 (at 128 and 256 µg/ml), the inhibitory effect was similar to the cells exposed to the reference compound L-NAME (Figure VII.3, B). The inhibition of nitrite production on RAW264.7 macrophages by *Adelia ricinella* L. extracts followed a dose-dependent pattern.

The inhibitory effect of extracts on IL-1β, IL-6 and TNF-α pro-inflammatory cytokines release by LPS-stimulated macrophages was measured by ELISA assays (Figure VII.4). Ethanol extracts (at 128 and 256 µg/ml) exhibited the most interesting effect on the reduction of cytokines release ($p<0.05$). Particularly, AR1 and AR2 significantly decreased the IL-1β levels in a dose-dependent manner. The aqueous extract slightly reduced the protein levels, being significant only for the IL-1β cytokine. RAW264.7 cells treated with DMSO secreted a basal level of cytokines but barely detectable.

VII.3.3- Effects of plant extracts on THP-1 cell adhesion to HUVECs

In this experiment, it was analysed the influence of plant extracts on HUVECs on *in vitro* cell adhesion with THP-1 human monocytes by stimulating each cell partner with extracts. The plant extracts did not exhibit important effects on leukocyte adhesion to TNFα-stimulated endothelial cells (Figure VII.5) ($p<0.05$). Although a little reduction is observed by the higher concentrations, the adhesion of THP-1 cells onto the TNFα-stimulated endothelial monolayer was not significantly reduced by treatment of HUVECs with *Adelia ricinella* L.

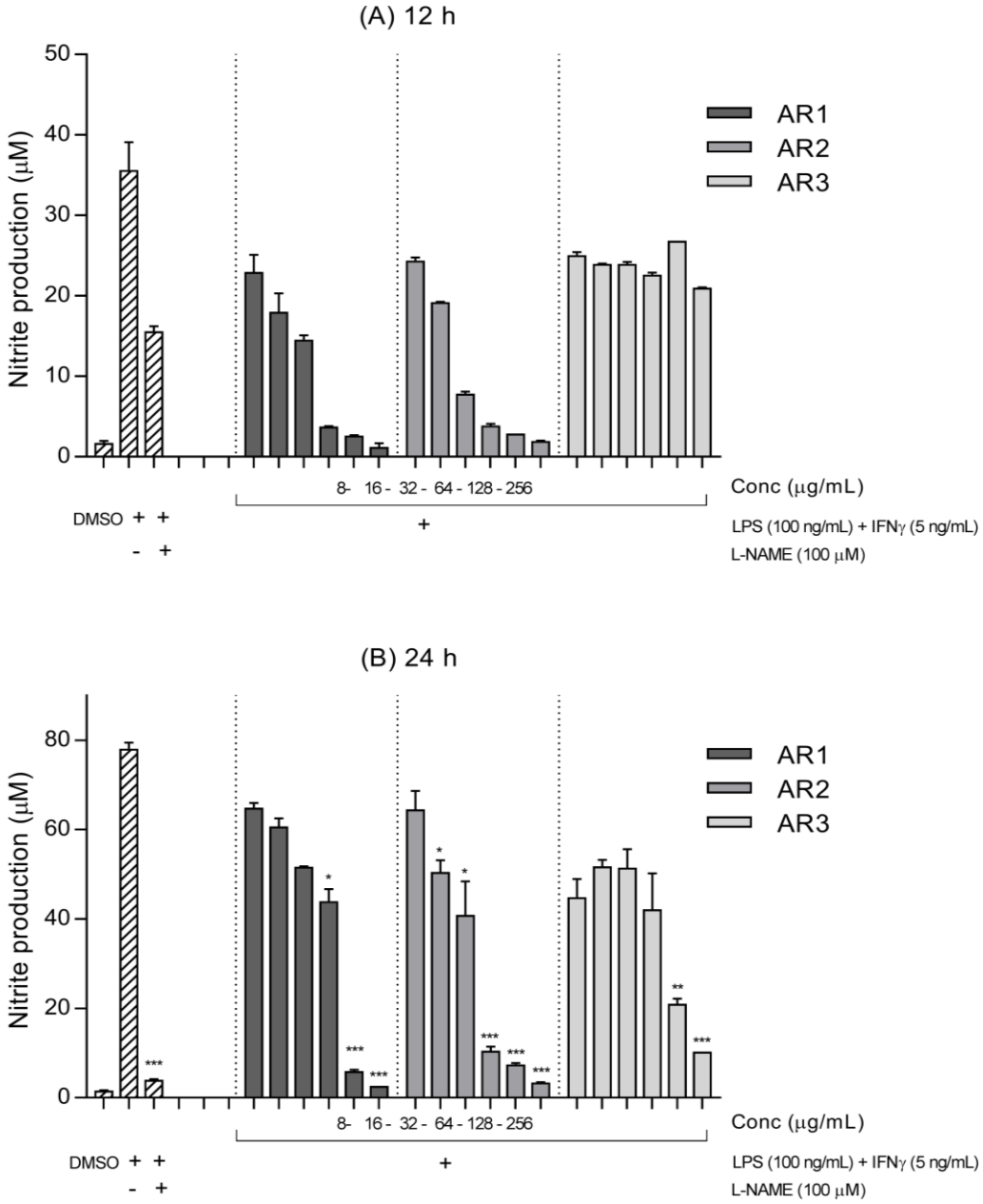


Figure VII.3. Effect of ethanolic and aqueous extracts on nitric oxide production by RAW 264.7 macrophages.

The nitric oxide production at 12h (A) and 24h (B) was measured by Griess reagent. LPS (100 ng/mL) and IFN-γ (5 ng/mL) were used as activator. L-NAME was used as anti-inflammatory control drug. The results are expressed as means ± SD. (*) and (**), differences between LPS- stimulated cells at $p<0.05$ or $p<0.01$, respectively

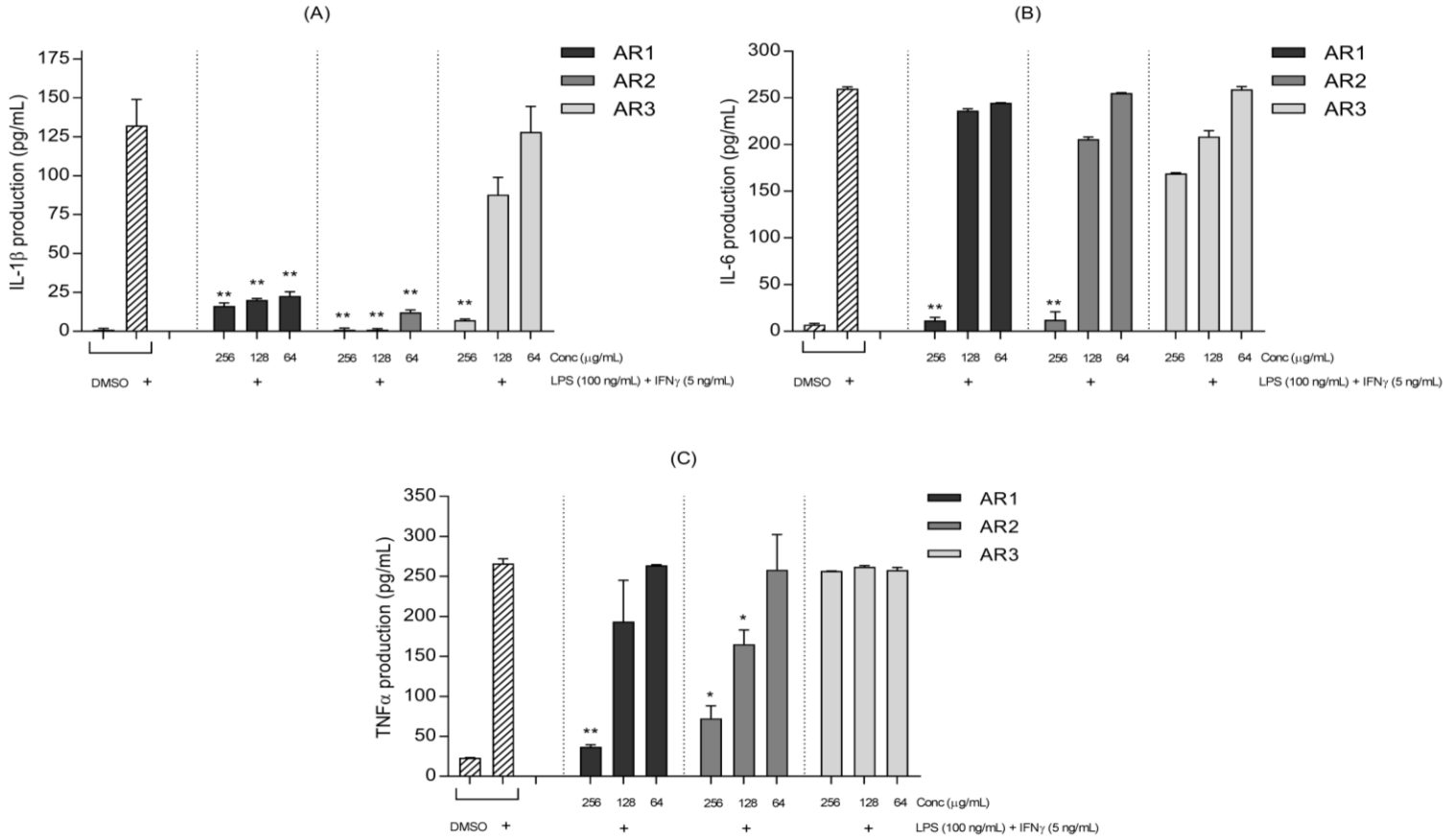


Figure VII.4. Effect of ethanolic and aqueous extracts on IL-1β (A), IL-6 (B) and TNF-α (C) cytokine release in RAW 264.7 macrophages.

The cytokine levels were determined by mouse IL-1β, IL-6 and TNF-α immunoassay Quantikine® ELISA kits. LPS (100 ng/mL) and IFN-γ (5 ng/mL) were used as activator. The results are expressed as means ± SD. (*) and (**), differences between LPS-stimulated cells at $p<0.05$ or $p<0.01$, respectively.

extracts. TNFα, which is known to activate endothelial cells, was used as a pro-inflammatory stimulus.

However, treatment with ethanol 50% (100 μg/ml) and aqueous extract (300 μg/ml) of LPS-stimulated THP-1 cells resulted in a reduction of adhered monocytes to TNFα-activated HUVECs (Figure VII.6) ($p<0.05$). Lipopolysaccharide, a potent monocyte-macrophage activator, can induce the release of pro-inflammatory cytokines (e.g. TNF-α) and the surface expression of adhesion molecules like L-selectin and integrins in leukocytes[268].

VII.3.4. Effect of plant extracts on percentage oedema inhibition

The measurement of oedema inhibition by different doses of ethanol and aqueous extracts obtained from *Adelia ricinella* L. aerial parts in the formalin-induced acute biomodel is shown in Figure VII.7 ($p<0.05$). Ethanol extracts at doses 50 mg/kg bw (body weight) showed 50% (AR1) and 72% (AR2) oedema inhibition at 7 h, which were significantly higher than that of the standard drug indomethacin. The inhibitory effect by ethanol extracts was maintained at 24 h (64% for AR1 and 81% for AR2). In the case of a lower concentration (25 mg/kg of bw), both extracts only significantly suppressed inflammation at 24 h after formalin exposition. On the contrary, the aqueous extract showed an inhibitory effect at higher concentration (35% of oedema inhibition at 200 mg/kg bw at 24 h).

VII.4-Discussion

Flavonoid-enriched extracts from *Adelia ricinella* L. aerial parts have previously been proven to possess bioactive effects on the resolution of inflammation by reducing COX-1 and COX-2 enzyme expression and PEG_2 release in macrophages cells (see in the chapter VI). However, its role on cytokine release, leukocytes adhesion onto the endothelium as well as the inhibition of the paw oedema in mice was not fully explored. Therefore, in the present study the suppression by *A. ricinella* L. extracts of pro-inflammatory markers of leukocytes and endothelial cells activated by an inflammatory stimulus was investigated.

Overall, plant extracts were not cytotoxic in the cell lines. Only the ethanol 95% extract at higher concentrations affected the cell viability of HUVECs. The percentage of cell death in the apoptosis assay demonstrated an intense toxic effect after 72 h of stimulation with plant extract. This cytotoxicity study on endothelial cells offers more evidences about the safety/toxicity of the traditional preparations derived from this plant.

Macrophages actively participate in immune responses by releasing pro-inflammatory cytokines (e.g.: IL-1β, IL-6 and TNFα) and inflammatory mediators such as nitric oxide[269].

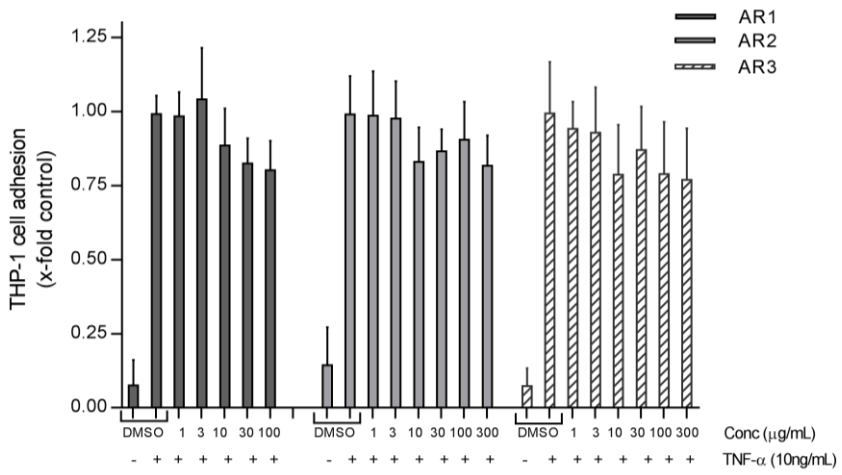


Figure VII.5. Ethanolic and aqueous plant extracts do not reduce the adhesion of THP-1 leukocytes onto TNFα-stimulated endothelial cells.

Confluent endothelial cells were treated with plant extracts (1-300 µg/mL) 30 min prior to TNFα (10 ng/ml) activation. After 24h fluorescence-labeled THP-1 cells (10^5 cells/well) were added and were allowed to adhere in HUVECs for 5 min. The amount of adherent THP-1 cells was determined by fluorescence measurements (ex: 485 nm; em: 535 nm) using a microplate reader. The results are expressed as means ± SD ($p< 0.05$).

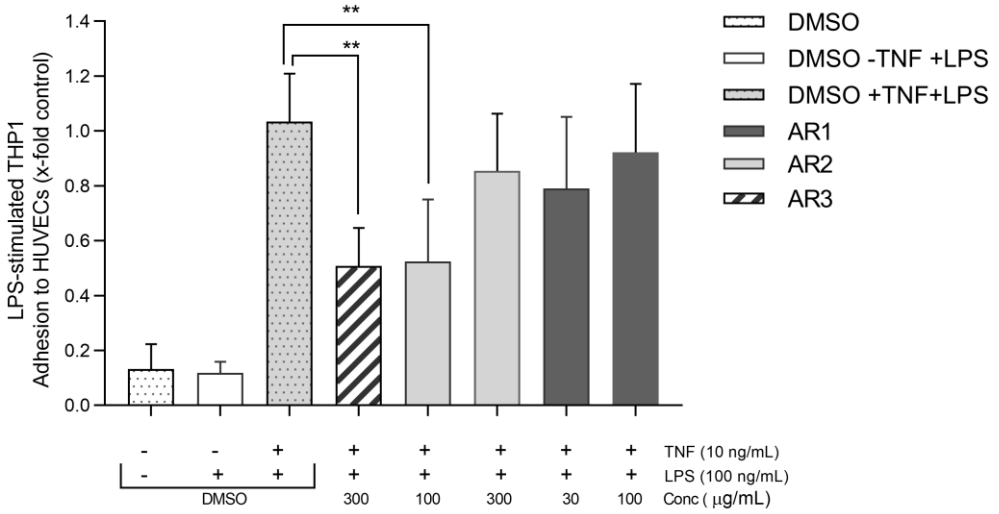


Figure VII.6. Effect of ethanolic and aqueous extracts on the adhesion of LPS- stimulated THP1 monocytes to endothelial cells.

THP-1 cells were treated with plant extracts (30-300µg/mL) 30 min before the LPS stimulation (100ng/mL) and HUVECs were activated with TNFα (10ng/mL). After 24h of incubation, fluorescence-labeled THP-1 monocytes (10^5 cells/well) were added and allowed to adhere to endothelial cells for 5 min. The amount of adherent THP-1 cells was determined by fluorescence measurements (ex: 485 nm; em: 535 nm) using a microplate reader. The results are expressed as means ± SD. (**) differences between LPS-stimulated cells at $p<0.01$.

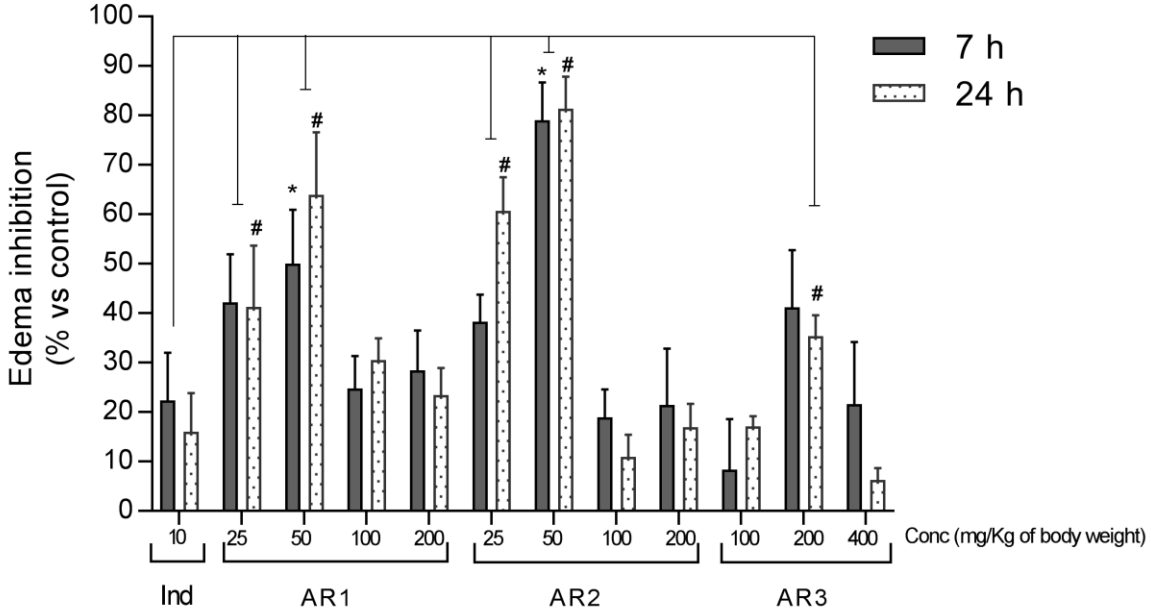


Figure VII.7. Inhibitory effect of ethanolic and aqueous extracts on paw oedema.

The results are expressed as means ± SD. (*) and (#), differences between indomethacin-treated mice and experimental groups ($p<0.05$), at 7h and 24 h, respectively.

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Here, it was proved that plant extract treatment decreased the pro-inflammatory cytokine levels as well as the nitrite production in LPS-stimulated macrophages. However, in those experiments, plants extracts had somewhat different effects on IL-1β, IL-6 and TNF-α cytokines. All extracts were more active to block the IL-1β protein release, and only ethanol extracts were able to decrease the IL-6 and TNF-α cytokine levels, but at higher concentrations. Additionally, all extracts reduced the nitrite production in RAW 264.7 cells, but mainly ethanol extracts. These results are in accordance with the previous results (Chapter VI), where the best effects in the PEG_2 release and COX-1 and COX-2 enzyme activities on RAW 264.7 macrophages were evidenced by the ethanol extracts (AR1 and AR2). It was hypothesized that the anti-inflammatory effects might be related to the presence of polyphenols, mainly of the flavonoid type.

The endothelium acts as a dynamic barrier that selectively controls the passage of plasma and cells from the blood into adjacent tissues[265]. During an inflammatory response, over-production of inflammatory mediators may irreversibly impair vascular integrity and cause decontrolled loss of fluid from the circulation. This harmful event may lead to prolonged tissue hypo-perfusion, organ dysfunction and death[265]. Then, prevention of vascular damage and maintenance of barrier integrity and functionality can contribute to improved survival of patients suffering from inflammation-related pathologies.

In this study it was observed that *A. ricinella* L. extracts did not affect leukocyte adhesion to endothelial cells. Both ethanol and aqueous extracts may not inhibit the expression of endothelial adhesion molecules in TNF-activated HUVECs. Nonetheless, EtOH 50% (AR2) and aqueous extracts (AR3) were able to reduce the adhesive interactions between LPS-stimulated THP-1 monocytes and endothelial cells, probably acting by diminishing the expression of LFA-1 and L-selectin integrins, thus avoiding the subsequent leukocyte extravasation. Several studies highlight the anti-inflammatory potential of phytochemicals by reducing the leukocyte endothelial interactions through inhibiting both integrins and/or adhesion molecules[250, 260, 264]. A study demonstrated that kaempferol, a flavonoid present in fruits and vegetables, may inhibit the expression of adhesion molecules like ICAM-1 and VCAM-1from LPS-induced rat intestinal microvascular endothelial cells[270].

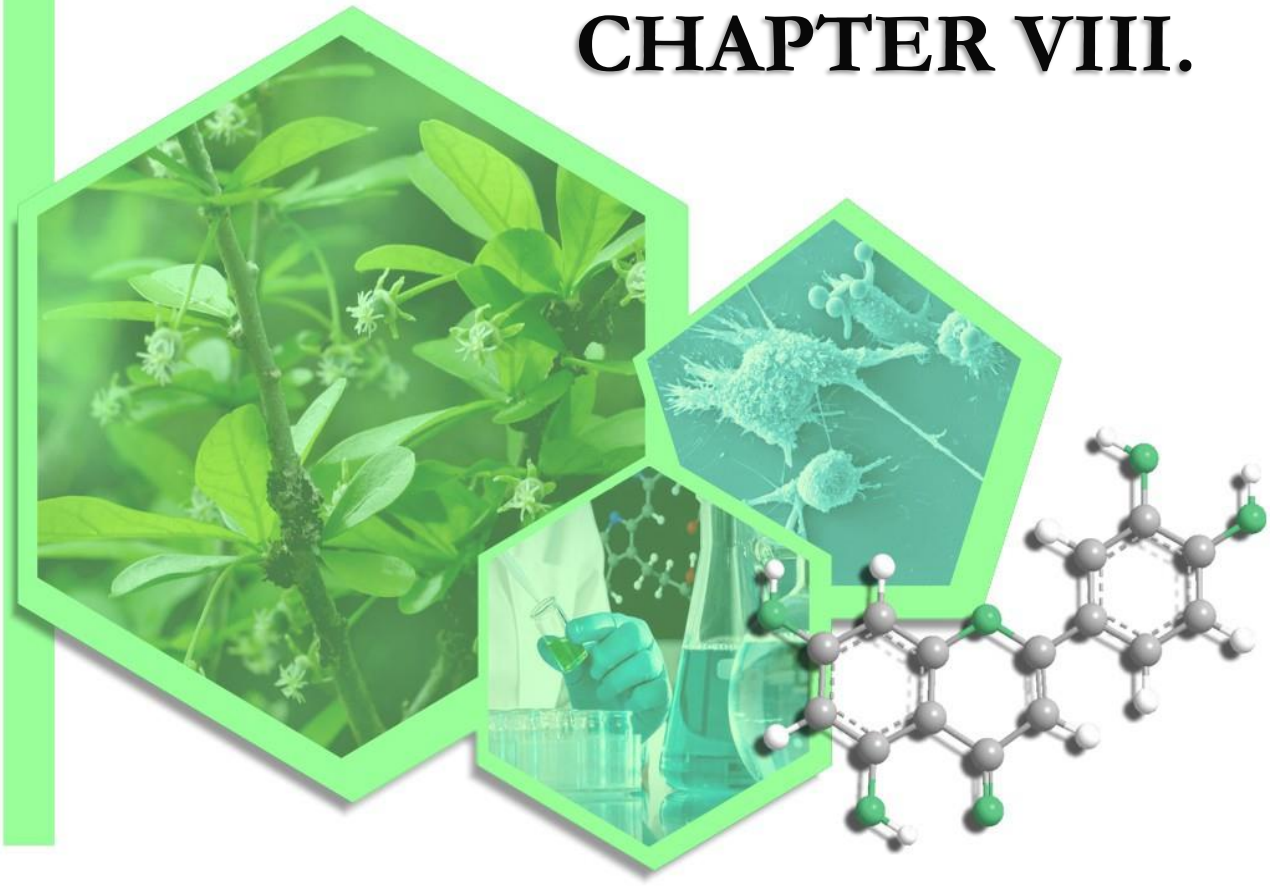
Excessive inflammatory mediators may lead to oedema, ulceration and carcinogenesis in tissue and vascular endothelium[271]. Therefore, effective regulation of the secretion of inflammatory factors and adhesion molecules becomes an essential step for treatment of inflammatory-related pathologies[269]. Both carrageenan- and formalin induced local

inflammation (paw oedema) are commonly used *in vivo* tests for assessing the efficacy of NSAIDs and also for determining the role of mediators involved in vascular changes associated with acute inflammation[272, 273]. In the present study, the paw oedema was reduced mainly in animals treated with AR1 and AR2 extracts at lower concentrations. This finding might be linked to the inhibition of pro-inflammatory factors produced by resident leukocytes such as IL-1β, IL-6, TNF-α and PGE_2 proteins and nitric oxide, which influence the vascular endothelium[86]. In addition, the extracts could be acting to down-regulate the overexpression of integrins like LFA-1 and L-selectin in leukocytes, or by blocking their adhesion onto endothelium, thus reducing the oedema.

VII.5- Conclusions

The current study provided new evidences regarding the anti-inflammatory potential of *Adelia ricinella* L. crude extracts. To the best of our knowledge, it was the first study using a robust *in vitro* platform of bioassays with endothelial cells to explore the possible effects of *A. ricinella* L. extracts on inflammation. As it was evidenced in the study, both ethanolic and aqueous extracts showed different pharmacological activities which can be related to the phytochemical profile. Taking together the protective effects of *Adelia ricinella* L. extracts on LPS-induced cellular disruption on THP-1 monocytes, the inhibition of inflammatory cytokines release and the suppression of paw oedema in mice suggest its potential use as a therapeutic agent against pro-inflammatory diseases like cancer, cardiovascular ailments and other chronic degenerative processes.

CHAPTER VIII.



CHAPTER VIII. General discussion

VIII.1- General discussion

Chronic inflammation is an important risk factor for many human diseases, therefore preventing the progression of inflammation in these pathologies turns into a good strategy. Studies on the anti-inflammatory potential of secondary metabolites derived from medicinal plants, their effectiveness, and safety arouse great interest at the present time. In fact, nowadays is common to see clinical trials with drugs derived from natural products and/or in other different stages of the medicinal plants' research route.

A fast review in the literature confirms that purified compounds isolated from Euphorbiaceae species and their base extracts prove a notable anti-inflammatory potential. *Adelia ricinella* L. is a species of this family that grows in Cuba and the Caribbean region. From an ethnopharmacological point of view, its use by the population as an analgesic and anti-inflammatory perfectly matches this observation. However, for this species, scarce scientific information is available; encouraging us to perform studies revealing the phytochemical composition as well as its pharmacological activity. In this way, it will be possible to offer elements that allow corroborating the traditional use done by the Caribbean inhabitants as well as to define a first profile of its effectiveness and safeties. In consequence, the present work combines two essential aspects in the research strategy on medicinal plants: 1) the phytochemical study of three extracts of the aerial parts of *A. ricinella* L. and 2) the evaluation of anti-inflammatory activity by *in vitro* and *in vivo* models; and their safety (cytotoxicity in RAW 264.7, Vero, HUVECs and human red blood cells).

VIII.1.1-Phytochemical study of the extracts of Adelia ricinella L.

The determinations of the chemical and chemical-physical parameters (organoleptic characteristics, pH, relative density, and total solids) of the three evaluated extracts from the aerial parts of the *Adelia ricinella* L. plant were determined as a quality control criterion. In general, these results constitute the first reports for this species and may contribute to the standardization of plant matter (aerial parts) in future medicinal preparations.

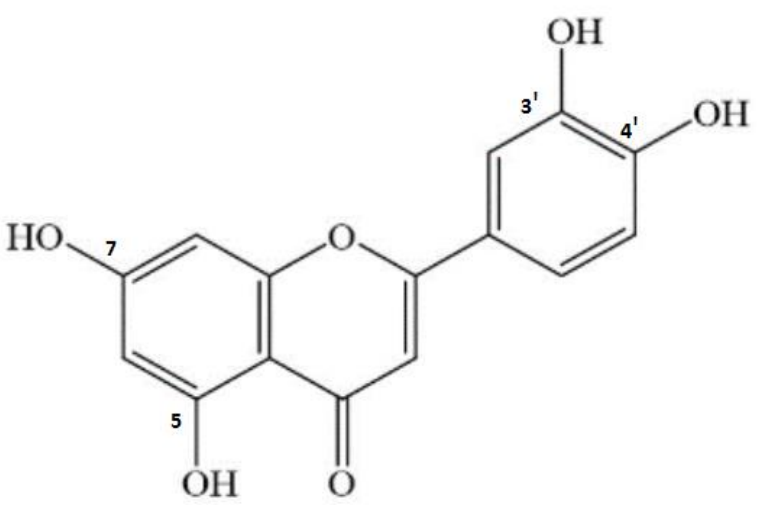
The main secondary metabolites were determined from qualitative and quantitative points of view. The qualitative chemical composition of the three evaluated extracts showed few differences in their composition, and strong evidence for phenolic compounds, such as

flavonoids. In general, these results are in correspondence with those reported in the literature for plants belonging to the family Euphorbiaceae[12, 173, 206].

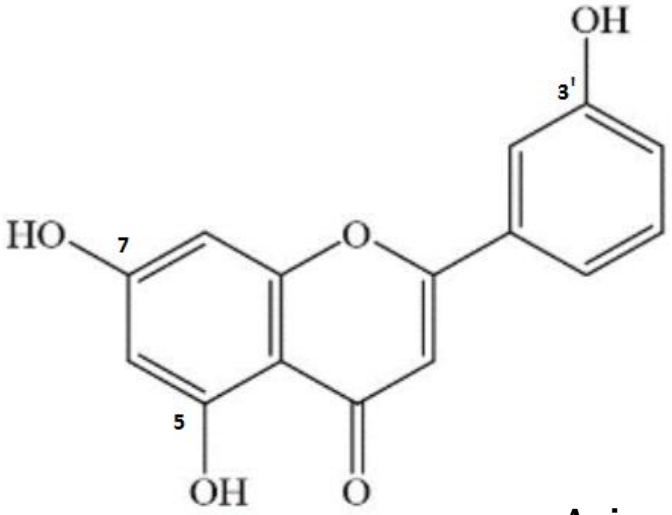
Using standard addition method, the concentrations of luteolin and apigenin were quantified by HPLC-DAD (Figure V.1), and it was found that the 50% ethanolic extract (AR2) contained the highest levels of those compounds with 207.5 µg / ml for luteolin and 1.86 µg / ml for apigenin respectively. At the same time a mixture of 13 flavonoids, mainly derived from luteolin and apigenin (C- and O- glycosides), were identified by UPLC-DAD-MS/MS, being orientin, vitexin and luteolin were the most abundant (Figure VI.1 and Table VI.1). Considering the scarce information about this plant species, these results emerge (to the best of our knowledge), as the first reports of the phytochemical characterization and of the main secondary metabolites present in its extracts.

Structurally, these flavonoids are very similar: luteolin has four hydroxyl groups (-OH) attached to positions 5, 7, 3' and 4', being the hydroxyl group at position 3´ that distinguishes this flavone from apigenin, which lacks the 3´-OH pattern (see figure VIII.1). Flavones and flavonols are characterized by the presence of a double bond between C2 and C3 and a ketone group in position 4 of the C ring, giving it a planar conformation and high electron delocalization. Several flavonoid QSAR studies highlight a strong structure-activity correlation of this flat backbone characteristic with the anti-inflammatory activity[274, 275]; particularly by the inhibition of specific pro-inflammatory enzymes as COX-1[276]. In the same way, Dash et al in 2015 revealed in a docking study that the structure of luteolin favours the inhibition of COX-2 due to the presence of the catechol moiety in ring B. That ring is oriented more towards the hydrophobic pocket, with 3'4'-dihydroxyl groups forming H-bonds with the Tyr^{385} and Ser^{530} residues[277]. This flavone is also a strong inhibitor of 5-LOX, a key component of an important pathway leading to the production of leukotriene B4, a mediator of many inflammatory diseases[278]. These results coincide with those obtained in this investigation, where luteolin and its derivatives (free and glycosylated) were found in higher concentration than apigenin derivatives in the three extracts. At the same time, the calculated luteolin concentration in the AR2 extracts was significantly higher than for the other extracts; thus generating a logical higher COX2 / COX1 selectivity ratio; with 10-fold selectivity in the cell-free assay.

In fact, there are many reports highlighting the antioxidant and anti-inflammatory activity of these flavones that also boast antimicrobial, chemopreventive, chemotherapeutic, cardioprotective, antidiabetic, neuroprotective, and antiallergic properties[279-281]. Similarly,



Luteolin



Apigenin

Figure VIII.1 Chemical structures of flavone compounds: luteolin and apigenin.

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plants with high production of these metabolites are widely used in traditional Iranian, Indian, Brazilian and Chinese folk medicine in the treatment of diseases related to inflammation[274, 282, 283].

For this reason, the present work focused on the evaluation of the anti-inflammatory potential of the extracts of the *A. ricinella* L. plant using *in vitro* and *in vivo* methods. These extracts exert their effects by regulating the expression and release of inflammatory mediators, where the following key points stand out:

1) Antioxidant activity (ABTS and DPPH assay) that indicates the ability of extracts to scavenge free radicals and inhibit ROS. This study reported for first time the preliminar composition of three extracts of aerial parts of *A. ricinella* L. which are responsible for the antioxidative potentialities(**Manuscript 1**).

2) Protection of the membranes (cytoprotective activity) of cells associated with the immune system or not, that participate in the acute phase of inflammation, preventing the release of mediators such as serotonin, bradykinin, histamine, among others (**Manuscript 2**).

3) COX-1 and COX-2 enzymatic inhibition

4) Inhibition of COX-2 protein expression and the release of lipid mediators such as prostaglandins in RAW264.7 macrophages (ATCC) (subsection c and d included in **Manuscript 3**).

5) Inhibition the release of NO and several pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α).

6) Inhibition of binding of THP-1 monocytes stimulated with LPS and IFN-γ to endothelial cells, preventing diapedesis and chemotaxis of cellular elements and mediators to the inflammatory focus.

7) Evaluation of the anti-inflammatory activity (*in vivo*) in formalin-induced paw oedema assays in BALB/c mice (subsection d, e and f included in **Manuscript 4**).

VIII.1.2- Antioxidant activity

Antioxidant activity is one of the most explored therapeutic applications of medicinal plants in recent times. This interest is related to the role played by many secondary metabolites (especially flavonoid-type compounds and other polyphenols) against oxidative stress and in the treatment of various pathologies that are associated with inflammatory processes[284]. Antioxidants can inhibit the initiation or propagation of oxidation processes, neutralizing reactive oxidative species that could damage DNA, RNA, modify proteins and

cause lipid peroxidation of cellular targets. Therefore, an antioxidant activity cannot be characterized by a single assay or assay type, but by its results in different test systems. For this reason, in the present work the antioxidant activity was evaluated in both: cell-free and cell-containing assays using different cell lines. By this way, the antioxidant capacity of the three extracts was explored using four methodologies that follow simple experimental procedures but based on different reaction mechanisms[285].

In cell-free assays, the extracts of *A. ricinella* L. (rich in flavonoids) were able to neutralize the radicals ABTS• and DPPH•. The AR2 extract showed a lower IC_{50} in both tests and a higher concentration of total phenols and flavonoids. In the test against the radical ABTS• the effect of the extract was greater than that shown by ascorbic acid used as a reference (see Figure IV.2 and IV.3). The ability of flavonoids to act as free radical scavengers, metal chelators and/or singlet oxygen suppressors in "*in vitro"* assays is well documented. In the specific case of these flavones and their respective glycosides, the ability to neutralize both radicals has also been reported, obtaining an effect similar to that observed in this study[284, 286].

On the other hand, in the cell-containing antioxidant tests, the extracts were evaluated against the oxidative damage induced by the hydrogen peroxide on red blood cells and by the determination of reactive nitrogen species in murine macrophages. In this last experiment, was evaluated nitric oxide, one of the key mediators of inflammation. In all four experiments, ethanol extracts were the most active, showing a concentration-dependent manner, especially AR2 extract.

It is known that among the most common sources of ROS generation within the body, are those related to enzymatic reactions that act at various levels such as: monoamine oxidase (MAO), cyclooxygenases (COX), lipoxygenases (LOX), and nitric oxide synthase (iNOS). These are all enzymes that intervene during the inflammatory process under the influence of a series of external factors or stimuli. Therefore, having antioxidant substances (rich flavonoids extracts) acting as reducing agents, hydrogen donors, and oxygen unpaired electron acceptors[287]; *A. ricinella* L. extracts would allow the suppression or prevention of inflammatory processes[240]. To achieve this, these metabolites must exert their effects by inducing or prolonging the half-life of antioxidant enzymes, such as superoxide dismutase, catalase or glutathione peroxidase, limiting in this way the generation of reactive oxygen species involved in the inflammatory process[222].

Zang J *et al*., and Wu N et al, reported that extracts rich in vitexin (apigenin C-glucoside) from the plant species *Mimosa pudica* L. and *Cajanus cajan* (L.) Mill sp. have been evaluated using DPPH• test obtaining IC_{50} values of 0.41 ± 0.04 mg/ml and 217.9 ± 2.7 µg/ml, respectively[288, 289]. In the case of the radical ABTS• the results obtained for extracts of *Colocasia esculenta* (L.), also with a high level of in vitexin were similar to those obtained for the extracts of *A. ricinella* L.[290]

Jae Sue Choi demonstrated that luteolin and its two C-glycosides orientin and isoorientin have a powerful antioxidant activity against the DPPH radical compared to ascorbic acid used as a positive control. Isoorientin was the one that shows the better activity in the inhibition of this radical with an IC_{50} of 1.64 ± 0.05 µM. However, luteolin showed the most potent inhibitory activity against the generation of ROS; estimating it IC_{50} value in 1.16 ± 0.03 µM, quite low when compared with Trolox (IC_{50} of 8.98 ± 0.06 µM), the positive control used as a in this study. This demonstrates that C-glycosylation at positions C-6/C-8, and specifically at position C-6, significantly increases the inhibitory activity towards the DPPH radical inhibition[291]. Likewise, Shazeli Che Zain et al in 2021 evaluated the antioxidant activity of different extracts of the *Elaeis guineensis* Jacq. plant against DPPH, and NO free radical-scavenging assays. Those extracts prove to be rich in luteolin and apigenin and their C-glycosides (orientin, isoorientin, vitexin and isovitexin). The freeze dried aqueous and methanol extracts showed the highest antioxidant activity which is correlated with the higher concentration of luteolin and apigenin, which were estimated in 2.04/56.30 and 1.84/160.38 g/mg, respectively[285].

On the other hand, studies in red blood cell shows that orientin, luteolin and apigenin decrease the erythrocyte haemolysis level in the H_2O_2-induced oxidative stress model within a dose-dependent manner. In addition, both compounds decrease the oxidative level of the products tested, protect the activities of antioxidant enzymes, and preserve the integrity of the morphology and structure of erythrocytes[292].

In other antioxidant reports, apigenin demonstrates it capacity to scavenge the hydroxyl (OH) radicals generated by the UV hydrogen peroxide photolysis, and to chelate iron cations. In addition, apigenin reduces the levels of myeloperoxidase (MPO) and malondialdehyde activity (MDA) in acetic acid-induced ulcerative colitis at such levels that are comparable to the corticoid, prednisolone[293]. This flavone also inhibit the NO radical production, the secretion of proinflammatory cytokines, the expression of the nitric oxide synthase (iNOS) and AP-1 proteins (c-Jun, c-Fos, and JunB) in human lung A549 cells

induced by LPS[294]. Also, prove to have a neuroprotective effect via suppressing the expression of the inducible form of nitric oxide synthase (iNOS) and nitric oxide liberation (NO) in microglial cells and macrophages[295].

Similarly, published reports shows the tumour growth reduction capacity of luteolin in the 1, 2-Dimethyl hydroxide-induced colon cancer biomodel in rats by inhibiting the lipid peroxidation and restoring the endogenous antioxidant enzymes[296]. Administration of luteolin significantly reduced the levels of lactoperoxidase (LPO) and OH- in plasma and colonic mucosa as well as increases the antioxidant enzymes which might be due to the strong antioxidant property of luteolin[297].

VIII.1.3-Cytoprotective activity

Inflammation is a complex process, which is frequently associated with pain and involves events such as increased vascular permeability, protein denaturation and membrane alterations, leading to important pathological conditions in humans. Harmful stimuli, including ionizing radiation, toxic substances or damaged cells, can generate cellular alterations through the deterioration of key biomolecules with consequent loss of functionality and initiate an inflammatory response in vascular tissue.

Different mechanisms may be involved in this process, such as oxidative stress, cell membrane permeability, enzyme activity, cell adherence, ATP production, and nucleotide uptake activity, that could lead to diseases associated with oxidative and inflammatory processes[219, 221]. Therefore, under such extreme conditions, if the protective mechanisms of the cells are compromised, cell injury and/or cell death can occur.

In this study, extracts with high content of flavonoids obtained from the aerial parts of *A. ricinella* L. exhibited a cytoprotective action on red blood cells, leucocytes and vascular endothelium cells. These last mentioned cells play a key role as a selective barrier between blood and tissues, being pivotal in the control of the homeostasis and inflammation. Those results were demonstrated using different methods based on different cellular functions: the integrity of the cell cytoskeleton, the effects on cellular respiration and DNA damage[298].

The results declared in this manuscript shown how the extracts are able to stabilise the red blood cells membranes when exposed to hypotonic and oxidative medium. The AR2 extract was the one that presented the most favourable effect, which may be correlated to its highest concentration in polyphenols and flavonoids. This makes it highly susceptible for

interaction with lipids and membrane proteins and modification of their properties, especially based on their antioxidant capacity derived from it planar chemical backbone. In this way, these flavonoids can interact with the inner and outer membrane of the erythrocyte, stabilizing it, exerting cytoprotective activity and therefore preventing its lysis as shown in the microphotographs presented in figure V.5.

Studies show that orientin (a luteolin-C-glycoside) and luteolin decreased erythrocyte haemolysis in H_2O_2-induced oxidative stress in a dose-dependent manner. In addition, both compounds decreased oxidative products, protect the activities of antioxidant enzymes, and preserve the integrity of the morphology and structure of erythrocytes[299]. Other compounds such as vitexin, quercetin and *Panax japonicus* extract demonstrated their protective effects against oxidative damage induced by H_2O_2 by attenuating the haemolysis of erythrocytes by inhibiting lipid peroxidation[300-302]. At the same time, similar results were obtained when cell viability was evaluated in endothelial cells incubated with the extracts of *A. ricinella* using the crystal violet method. No negative effect on cell viability at the doses evaluated was found, suggesting that the extracts do not affect cell integrity and functionality.

Likewise, using the resarzurin oxidation/reduction method to evaluate the metabolic activity (cellular respiration) of RAW 264.7, VERO, HUVECs, and THP-1 cells, the extracts tested were not cytotoxic at the concentrations evaluated, estimating an IC50≥256 (see figure). Miao Xin *et al*., performed a proteomic study focused in the protective effect of apigenin on palmitate-induced lipotoxicity in human aortic endothelial cells (HAEC). They obtained that HAEC pre-incubated with 10 μM apigenin for 6 h, significantly increase the cell viability from 71.55 ± 3.62 to 91.06 ± 4.30 percent; modifiying the mitochondrial membrane potential to the normal level (101.62 ± 11.72% of control)[303].

On the other hand, *Adelia ricinella* L. extracts were evaluated on endothelial cells by flow cytometry using trypan blue, and only the AR1 extract at the higher concentration evaluated (300 μg/ml) showed an apoptotic effect. Thangaiyan R *et al*., in 2018 investigated the effect of apigenin on apoptotic signalling induced by isoproterenol hydrochloride (ISO) in cardiomyoblast H9C2 cells. The results showed that apigenin treatment (10 μM) prevented ISO (31.25 μM)-induced lipid peroxidative levels and antioxidant status in H9C2 cells. Apigenin prevented ISO-induced DNA damage and apoptotic signalling through modulating the expression of Bax, caspase-3, -8, and -9, cytochrome c, and Fas proteins in H9C2 cells. It also inhibited the expression of inflammatory markers such as: TNF-α, IL-6,

NF-κB, COX-2, and iNOS in H9C2. All those facts demonstrating the cytoprotective effect of this flavone on cardiomyoblast cells, suggesting a similar mechanism for the *A, ricinella* L. components [304]. In the same way, this flavonoid prevents neuronal apoptosis by protecting the neurons against inflammatory stresses[295].

VIII.1.4- Anti-inflammatory activity

The leukocytes and vascular endothelium play a crucial regulator and effector of immune response, actively contributing to both initiation and resolution of inflammation. These immune cells constitute a key element in the development of some diseases that are associated with inflammatory processes such as: cancer, metabolic diseases, neurodegenerative diseases, among others. In this work, the *in vitro* anti-inflammatory activity was evaluated on macrophages RAW264.7 after stimulation with LPS and IFN-γ. These cells, once stimulated, exert classical M1 activation through activation of Toll-like receptor 4 (TLR4)[305]. TLR4 modulation by LPS promotes activation of effector functions, production of NO and ROS, PGE_2, cytokines (IFN-β, IL-1β, IL-6, IL-12, and TNF-α) and chemokines, as well as induction of the expression of key enzymes such as iNOS, COX-2, and of transcription factors such as NF-κB. For this reason, macrophages constituted a useful model to evaluate potential substances as regulators of the inflammatory response.

In this research, we found that treatment with ethanol and aqueous extracts of the *A. ricinella* L. were able to inhibit the release of important mediators of inflammation in murine macrophages. This includes the production of nitrites, ROS and pro-inflammatory cytokines (such as IL-1β, IL-6 and TNF-α). IL-1β functions as a master cytokine that can further induce the expression of other pro-inflammatory cytokines, such as IL-6 and TNF-a, chemokines, adhesion molecules and other inflammation-associated compounds to amplify the inflammatory response. This cytokine (IL-1β), was inhibited by all three extracts tested at the light of the results presented in this study. Similarly, the extracts inhibit the release of lipid mediators such as eicosanoids, especially PGE2, by inhibiting the enzyme cyclooxygenase (COX-1 and COX-2). These are key enzymes that mediate the inflammatory process activated by the release of various membrane components, including phospholipids that are then converted to arachidonic acid by the enzyme phospholipase A2. The arachidonic acid generated is converted to different eicosanoids such as prostaglandins, leukotrienes and thromboxanes. These lipid mediators mediate pain and oedema associated with inflammation[37]. The ethanol extracts (AR1 and AR2) strongly inhibited the activity of these enzymes in a concentration-dependent manner, being significantly more

active on COX-2 than on COX-1(Figure VI.3). This effect could be due to the decrease of the expression of COX-2, where the aqueous extract (AR3) also showed activity. These results can be correlated with the high content of phenolic compounds in these extracts, especially flavonoids such as luteolin and apigenin, as well as the antioxidant capacity that they present, as we discussed in previous chapters.

Flavonoids, especially luteolin, act by inhibiting iNOS expression, iNOS activity and NO production. It has been reported to act as a ROS scavenger, an inhibitor of ROS production and an activator of antioxidant enzymes. In addition, this flavone inhibits the production and release of eicosanoids and suppresses the expression of pro-inflammatory cytokines[284]. Other researchers demonstrated that luteolin and apigenin inhibit the production of NO, IL-6, IL-1β and TNF-α in RAW 264.7 cells in a dose-dependent manner and in turn regulate the expression of proteins that code for TNF- α, IL-1β, iNOS and COX-2[306-309].

In addition, Huang et al. and Kim et al., found that apigenin *C*-glycosides decreased the expression of COX-2 in RAW 264.7 cells stimulated with LPS[310, 311]. Those results disagreed with the report of Choi et al., who found that isovitexin did not inhibit the expression of this enzyme in the same experimental model[312]. On the other hand, and taking into consideration the observation that luteolin has stronger inhibitory effects than its derivatives, Park and Song demonstrated that luteolin inhibited NO and PGE_2 production in LPS-stimulated RAW 264.7 cells, as well as the corresponding enzyme activities of iNOS and COX-2, more strongly than luteolin-7-*O*-glucoside[313].

Similarly, Pena et al. also found that luteolin and apigenin were present in several Brazilian plant species and that can inhibit pro-inflammatory cytokines (IL-1β, IL-2, IL-6, IL-8, IL-12, IL-17, TNF-α, IFN-β, and granulocyte-macrophage colony-stimulating factor). Furthermore, in the case of luteolin, it can increase the level of IL-10 (an anti-inflammatory cytokine)[86].

The anti-inflammatory activity of luteolin glycosides has also been reported. Luteolin-5-*O*-glucoside, isolated from the active ethyl acetate fraction from Korean Thistle *Cirsium maackii*, inhibited NO production, iNOS expression, COX-2 and ROS generation in macrophages; but to a lesser extent than its aglycone[314].

Cellular adhesion molecules, selectins, integrins, and others vascular cells; mediates the adhesion of blood leukocytes to the endothelial cell surface and their transmigration from the subendothelial space to the inflammatory focus, favouring oedema, inflammation, and oxidative stress. Therefore; inhibiting the expression of these molecules in vascular

endothelial cells can be considered as a promising therapeutic approach for treating vascular inflammatory diseases.

In the present research, the plant extracts did not show any effects in the leukocyte adhesion on endothelial cells-stimulated TNFα. However, it was described that the EtOH 50% extract (AR2) and the aqueous extract (AR3) were able to reduce the adhesive interactions between LPS-stimulated THP-1 monocytes and endothelial cells-stimulated TNF-α. They probably acting by decreasing the expression of LFA-1 and L- integrins, selectin. This is a very important step in the inflammatory process as it prevents subsequent leukocyte extravasation and inhibits the formation of oedema.

Previous investigations, showed that apigenin acts by blocking the endothelial adherence of human monocytes by reducing VCAM-1, ICAM-1 and E-selectin-mRNA to the basal levels[315]. This flavone also realized its protective function on palmitate-induced lipotoxicity in HAEC cells mainly via regulating pathways such as IL-17, TNF, and endoplasmic reticulum protein processing. Furthermore, apigenin upregulated of gene ICAM-1, VCAM-1, and endothelin-1 transcription in different degrees inhibited the cell adhesion[303]. At the same time, orientin inhibited expression of cell adhesion molecules (CAMs), and adhesion of monocytes to human endothelial cells[316]

Lolito and Frei in 2006, as well as Chen et al. in 2004 demonstrated the ability of several flavonoids to attenuate TNFα-induced adhesion molecules expression in aortic endothelial and respiratory epithelial cells. Both studies found that the 5,7-dihydroxyl substitution on the A-ring of the flavonoids was essential for this activity. In addition, in the first work also discuss the importance of the 2,3-double bond and the 4-keto group in the C-ring for the inhibition of adhesion molecule expression, where luteolin and apigegenin show good activity similar to that observed in this research[317, 318].

Turner et al. suggest that the regulation of pro-inflammatory cytokines is crucial because they are key modulators of both acute and chronic inflammation. TNF-α is a pro-inflammatory cytokine that has different functions such as the activation of the production of other mediators such as IL-1β, IL-6, local activation of the vascular endothelium, release of NO with vasodilation and increased vascular permeability, which leads to the recruitment of inflammatory cells[319]. *Adelia ricinella* L. extracts are able of inhibiting this cytokines and in turn inhibiting the activation of the vascular endothelium, preventing diapedesis. Studies suggest that orientin protects vascular barrier integrity by inhibiting

hyperpermeability, expression of CAMs, and adhesion and migration of leukocytes, thereby endorsing its usefulness as a therapy for vascular inflammatory diseases[316].

Similarly, other researchers showed that luteolin is capable of inhibiting a set of chemotactic cytokines (CCL2, CXCL2, CXCL8, CXCL9), which help to control the migration and positioning of immune cells, together with prostaglandins and leukotrienes. However, these effects could be dependent on the specific stimulus (inducer), the type of cell and the experimental model used[284].

This anti-inflammatory activity was corroborated through an *in vivo* model of acute inflammation of the paw oedema induced by 1% formalin as a phlogistic agent. It is well known that this model is one of the most suitable test procedures to select arthritic and anti-inflammatory agents[55, 320]. The plant extracts under study demonstrated anti-inflammatory activity, mainly AR2 at a dose of 50 mg/ml. *In vivo* studies using genetically modified mice deficient in LFA-1 or Mac-1 clearly delineate fundamental different mechanisms for each of these β2 integrins. While firm adhesion is mediated by LFA-1, locomotion depends from Mac-1. Therefore, an inhibition of these integrins could cause poor migration and in turn inhibit oedema[321].

Sayuri Suyenagan E *et al*., investigated the role of different flavonoids including luteolin and apigenin on leukocyte migration in an *in vivo* model consisting in the carrageenan-induced pleurisy model in rats. Both flavones were able to reduce cell migration to the pleural cavity and inhibited rolling, adhesion, and transmigration. Furthermore, flow cytometry assays showed that *in vitro* treatment with all compounds (15–60 μm) did not cause cell death and 1 inhibited cleavage of L-selectin and β2 integrin expression. These authors clearly show the ability of these compounds to inhibit *in vivo* neutrophil influx into inflamed tissue, by acting on different neutrophil migration mechanisms. Result that could be very useful for the treatment of different diseases associated with inflammatory processes and can be associated to the results obtained for *A. ricinella* L. extracts[322].

At the same time, Funakoshi *et al*., performed the evaluation of the anti-inflammatory activity in structurally related flavonoids, and they included luteolin, apigenin and fisetin. They clearly demonstrated that luteolin and apigenin effectively inhibited inflammation in the paw oedema model where inflammation was induced by carrageenan in Jcl-ICR mice[323]. Sridhar at al. demonstrated the ability of a vitexin-rich methanolic extract obtained from *Teramnus labialis* leaves to inhibit the formation of carrageenan-induced rat paw oedema, which also showed inhibitory action of the 5-LOX enzyme[324].

The effect of *A. ricinella* L. extracts on the selectivity of cyclooxygenase enzymes was compared to the effect shown by the most marketed NSADs drugs such as aspirin, indomethacin, ibuprofen, naproxen and piroxicam (see figure VIII.2). Most of them are more selective to COX1 than to COX2 or non-selective, and in consequence; they show adverse effects related to gastrointestinal and cardiovascular disorders[325, 326]. Considering that the COX1/COX2 inhibition ratio of AR2 *A. ricinella* L. extract classifies as 10-fold more selective for COX2 than for COX1 this places it in the same desirable group that paracetamol, diclofenac and celecoxib (from 5-50 fold more selective). Ratio values above 50 times like those of rocecoxif, increase cardiovascular risks and, consequently, are also not desired (see table VIII.1). These results obtained for the extracts of *A. ricinella* L. places it in a very good position and could have a high impact on clinical practice, since it would be very beneficial to disposes of a medicinal plant able to be used for the treatment of different pathologies associated with inflammatory processes, reason that constitute one of the main reasons for consultation in the primary health care services [327-330].

The ability of the extracts to inhibit the activity and expression of the cyclooxygenase enzyme, the inhibition of the production of PGE_2, NO, proinflammatory cytokines and the adhesive interactions between THP-1 monocytes stimulated with LPS and endothelial cells, leads us to the hypothesis that the regulation of these mediators could be correlated with the inhibition of the signalling pathway of the transcription factor NF-κB. This is one of the most important nuclear factors and plays a key role in the expression of pro-inflammatory genes, including cytokines, chemokines, and adhesion molecules.

Reports indicate that luteolin and apigenin regulate in a coordinated way several signalling pathways of NF-κB, MAPK/AP-1 and JAK (Janus tyrosine kinase) / STAT that regulate the expression of both pro-inflammatory and anti-inflammatory mediators (see figure VIII.3)[313, 331-335]. Luteolin decreased the degradation of IκBα and nuclear translocation of the p65 fraction of NF-κB[336]. Similarly, Lee et al. demonstrated that this flavone inhibits IFN-β-dependent signalling, induces the TRIF adapter (which contains the TIR domain), and observed an inhibition of the expression of pro-inflammatory cytokines, IL-10, chemokines, among others, in macrophages derived from bone marrow[319].

Nicholas C et al. demonstrated in 2007 that apigenin inhibited LPS-induced inflammation through inhibition of NF-kB activation by hypophosphorylation of Ser536 in the p65 subunit in an *in vivo* mouse model[334]. In agreement with these results, Zhang et al. showed

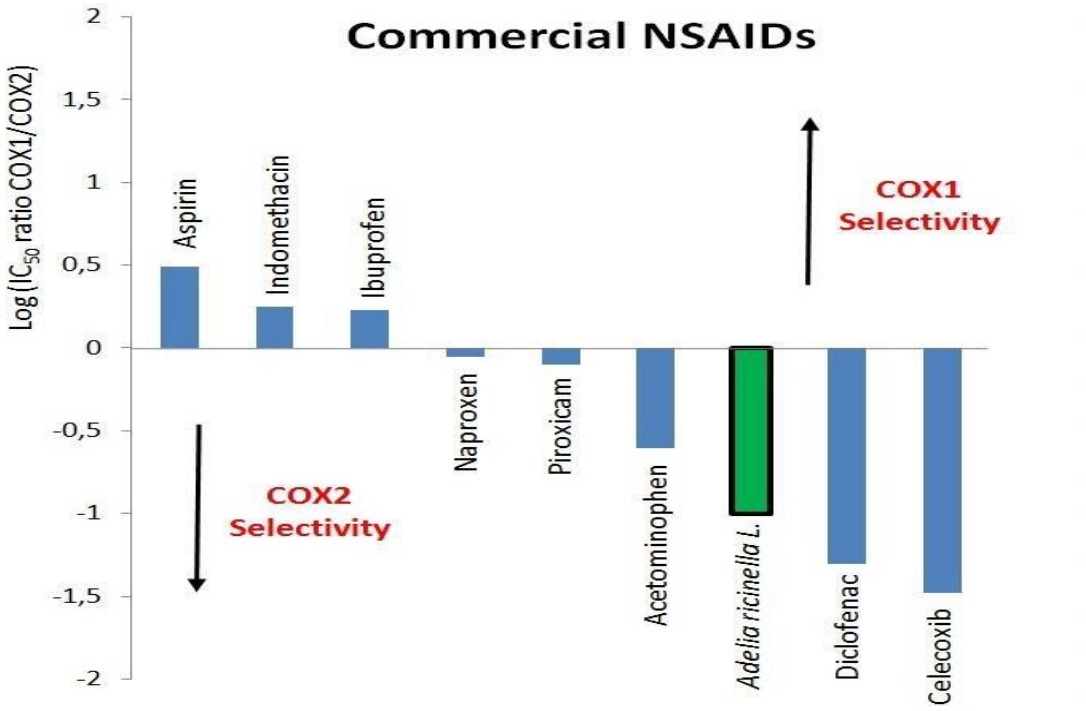


Figure VIII.2 Relative COX-1/COX-2 selectivity on main used commercial NSAIDs. Adapted from Bacchi S et al., 2012[325].

Table VIII.1 COX-1/COX-2 ratio selectivity of commons NSAIDs. Adapted from Laight D., 2018[325].

More COX-1 Selective	Nonselective	5-50 Fold COX-2 selective	>50 Fold COX-2 selective
Ketorolac (Acular) Flubiprofen (Ocufen) Ketoprofen (Generic) Indomethacin (Indocin) Aspirin (Generic) Ibuprofen (Advil, Motrin) Tolmetin (Generic) Meclofenamate (Generic)	Naproxen (Aleve) Piroxicam (Feldene) Fenoprofen (Nalfon) Sodium salicylate (Generic) Diflunisal (Generic)	Sulindac (Clinoril) Diclofenac (Cambia) Celecoxib (Celebrex) Meloxicam (Mobic) Etodolac (Generic)	Etoricoxib (Arcoxia) Lumiracoxib (Prexige)
Increased gastrointestinal effects ⟷ Increased cardiovascular effects			

COX: Cyclooxygenase, NSAIDs Non-steroidal anti-inflammatory drugs.

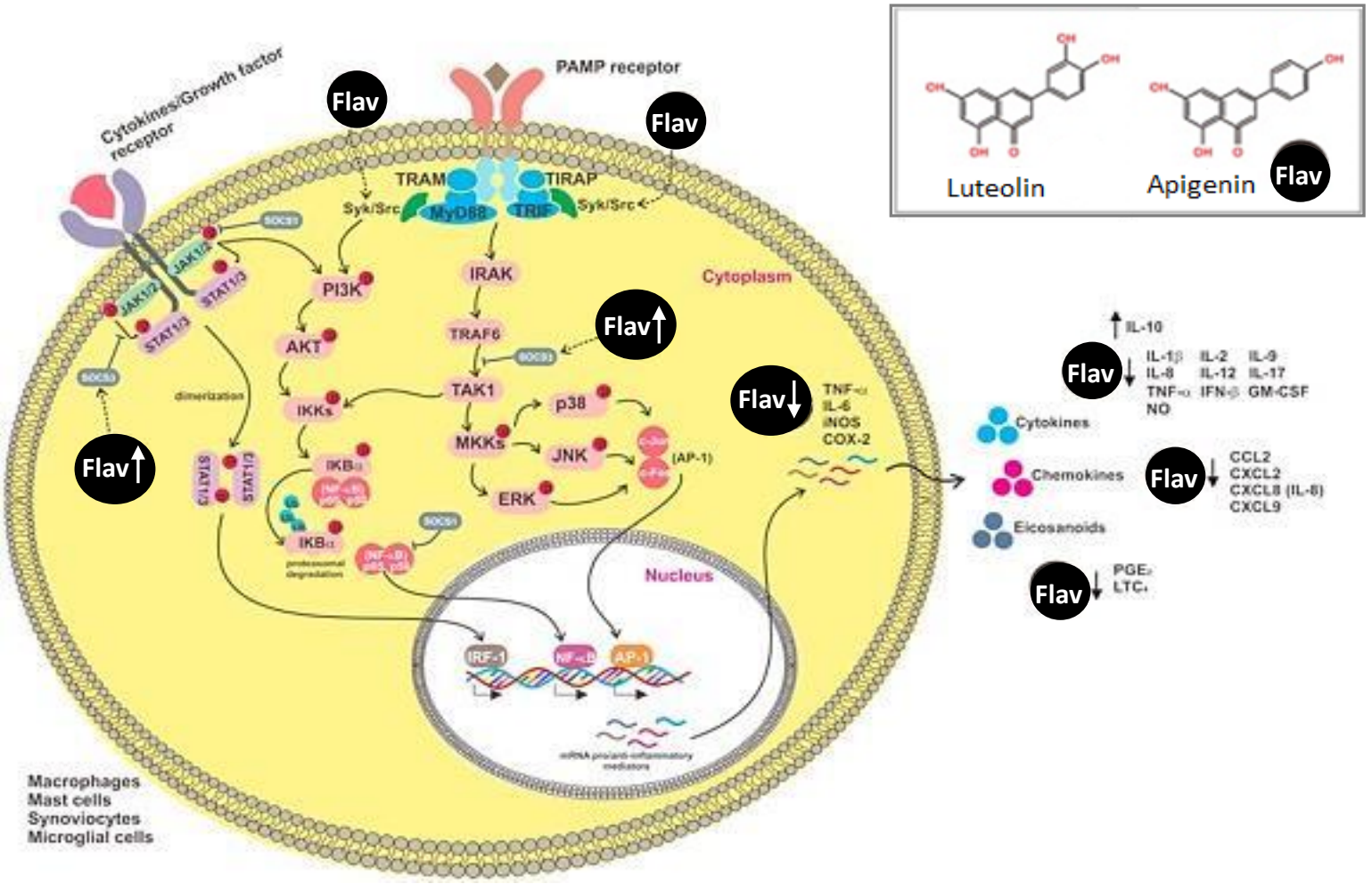


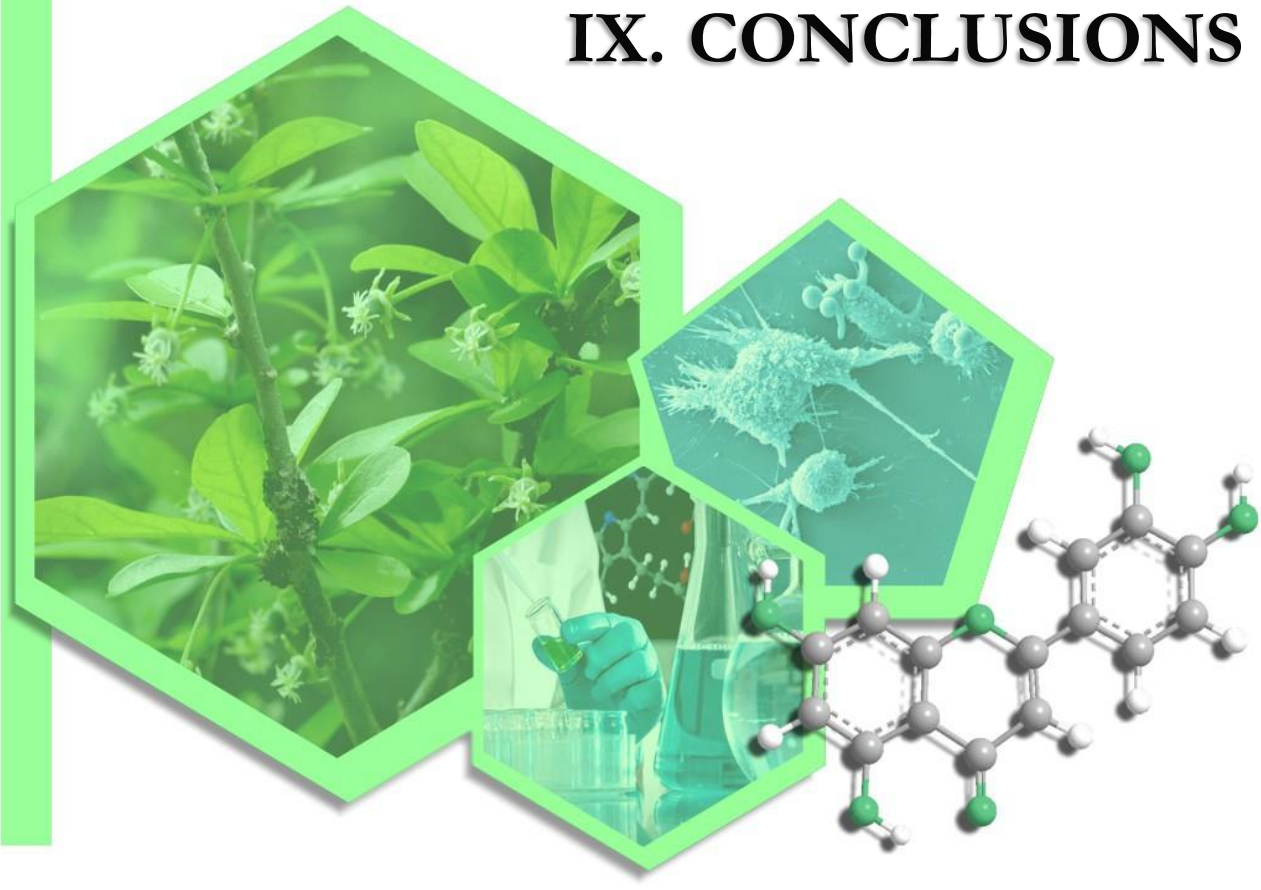
Figure VIII. 3. Schematic illustration of various flavone-targeted inflammatory signalling pathways. Flav: Flavone (Luteolin and Apigenin). Adapted from Aziz N et al., 2018 [(338)] .

in 2014 that this flavone significantly inhibited the activation of ERK1/2 and NF-kB induced by LPS in human THP-1 macrophages[333].

Zhou et al. reported that luteolin protects against zinc-induced apoptosis in human neuroblastoma cells (SH-SY5Y). This observed effect is due, at least in part, to the suppression of the signalling pathways of PI3K/AKT, NF-κB, ERK 1/2 (extracellular signal-regulated protein kinases 1 and 2) and subsequently the decrease in ROS[337]. In turn, this flavone is capable of suppressing NF-κB activation more strongly than luteolin-7-*O*-glucoside[338].

Altogether, the results presented in this thesis, show that ethanol and aqueous extracts (AR1, AR2, and AR3) of *Adelia ricinella* L. aerial parts can act on different molecular targets related to inflammation. These evidences emerged as the first reports that could support the ethnomedicinal use made by the inhabitants of the Caribbean and could contribute to the development of a future natural medicine to treat diseases associated with inflammatory processes. However, further studies should be performed in order to fully elucidate the molecular mechanism by which they are able to exert the anti-inflammatory effect described.

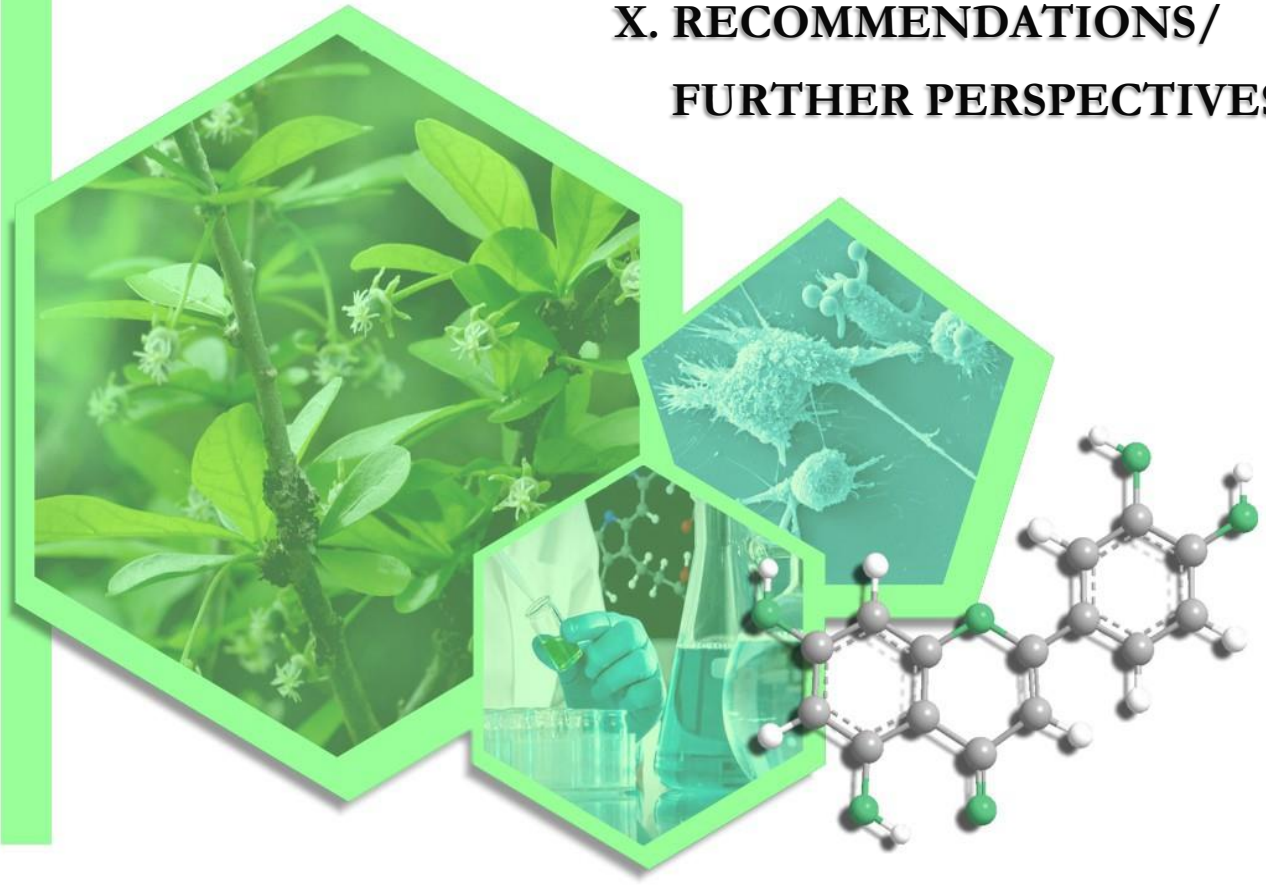
IX. CONCLUSIONS



IX. CONCLUSIONS

- The determination of the pharmacognostic, physico-chemical, and chemical features of the plant material allowed to define a first approach of the Quality control parameters for *A. ricinella* L. and their extracts which prove to be rich in flavonoids derived from luteolin and apigenin as basic structures.
- Antioxidant and cytoprotective effect of the *Adelia ricinella* L. extracts favours its anti-inflammatory activity and supports it relative safeness documented by the Caribbean inhabitants.
- *Adelia ricinella* L. extracts interferes the inflammatory process due to an inhibitory effects on: the release inflammatory mediators, reactive oxygen and nitrogen species, and enzymes expression which allowed for a preliminary approach to a mechanism of action
- *Adelia ricinella* L. extracts inhibit the adhesion of leucocyte on endothelial cells and extravasation to the site of inflammation, leading to a reduction the oedema in mice using paw oedema model, justifying the traditional use by the Caribbean inhabitants.

X. RECOMMENDATIONS/ FURTHER PERSPECTIVES



X. RECOMMENDATIONS/FURTHER PERSPECTIVES

- To continue the phytochemical studies in order to identified other components of the extracts, especially in AR2.
- To delve into preclinical pharmacology using other *in vivo* and *in vitro* models, in order to offer more evidence about the mechanism by which these extracts achieve their biological activities.
- To perform studies related to the acute and subchronic toxicity according to the Organization for Economic Co-Operation and Development (OECD) test guidelines to assess the extracts safety.

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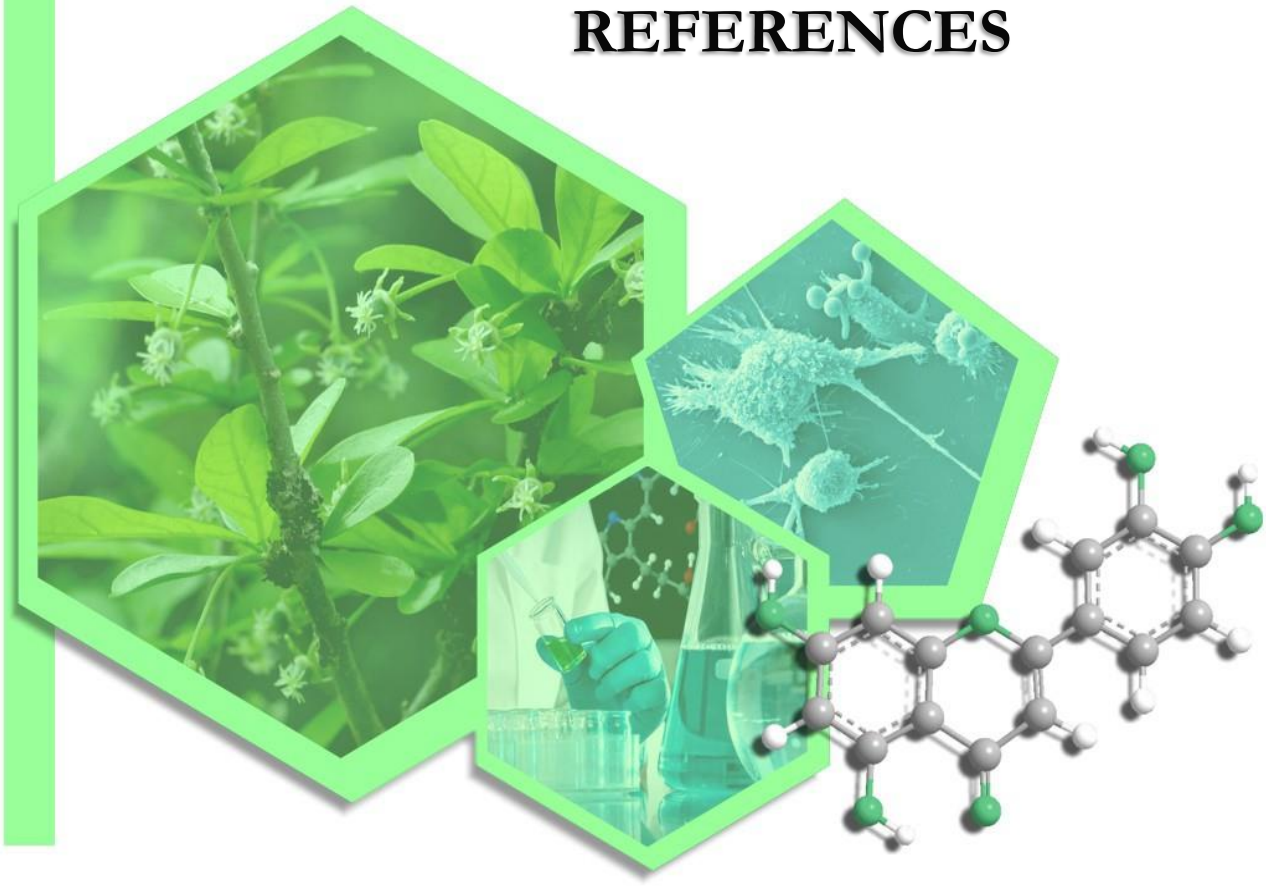

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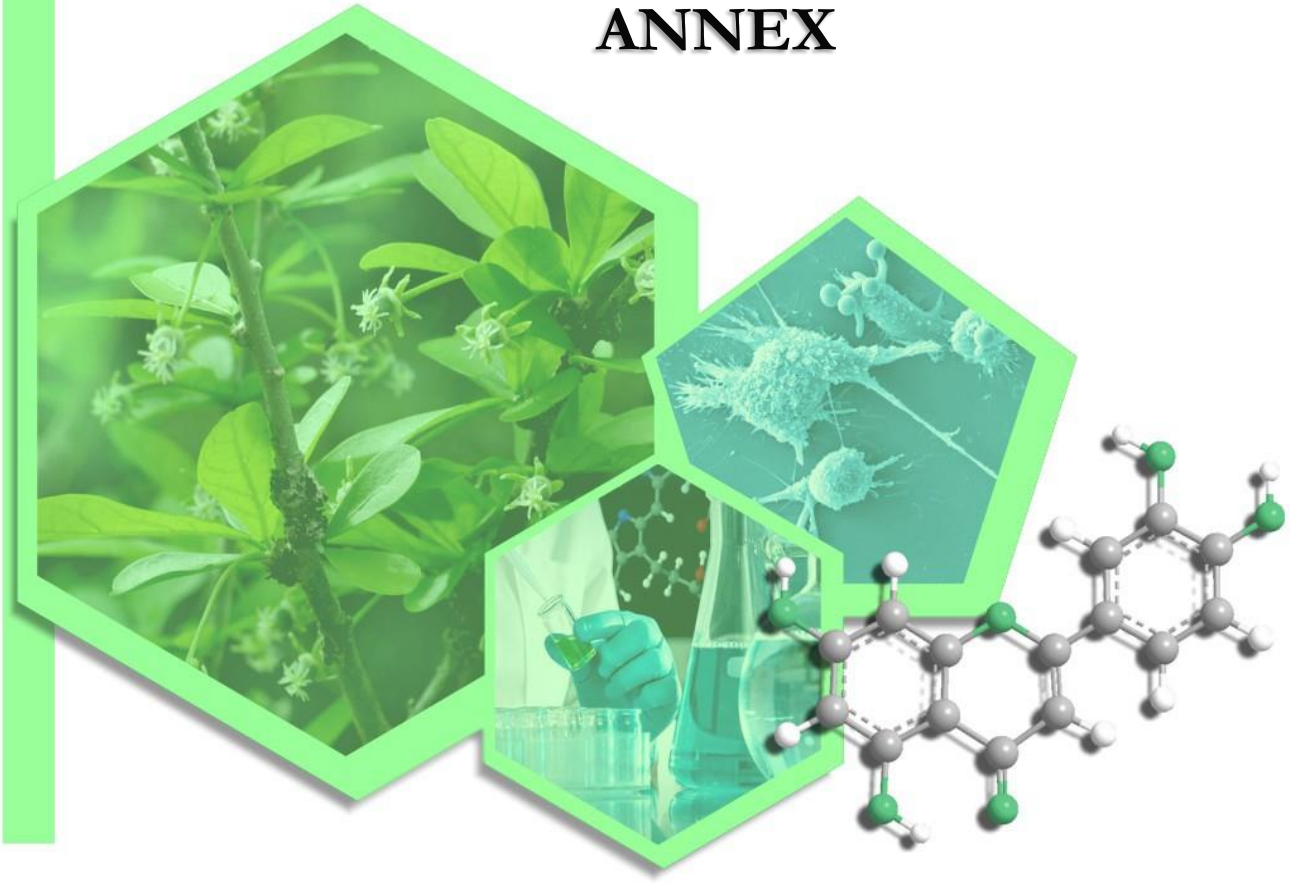
ANNEX



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HERBARIO BSC "DR. JORGE SIERRA CALZADO"

Nombre y apellidos: Clara Azalia Beunguer Rivas

Institución a que pertenece: Dpto. de Farmacia. Universidad de Oriente

Servicio que solicita:
[X] Identificación taxonómica Adelia vicinella L.
[ ] Utilidad medicinal
[ ] Montaje de muestra
[ ] Asesoría
[ ] Otros

Finalidad del estudio:
[ ] Docente
[ ] Estudio fitoquímico
[ ] Estudio genético
[X] Investigativo
[ ] Estudio molecular
[ ] Otros

Depósito de la muestra en la colección
[X] Si [ ] No

Estado de la muestra:
[X] Buena [ ] Regular [ ] Mala

Número de voucher de la colección: 14780

Identificador: Lic. Jaimer Costa Acosta

Fecha: 16/03/2017

Certifica: Lic. Josefina Blando Ojeda
Investigador Agregado
Herbario BSC

M.Sc. Gustavo Solano Durán
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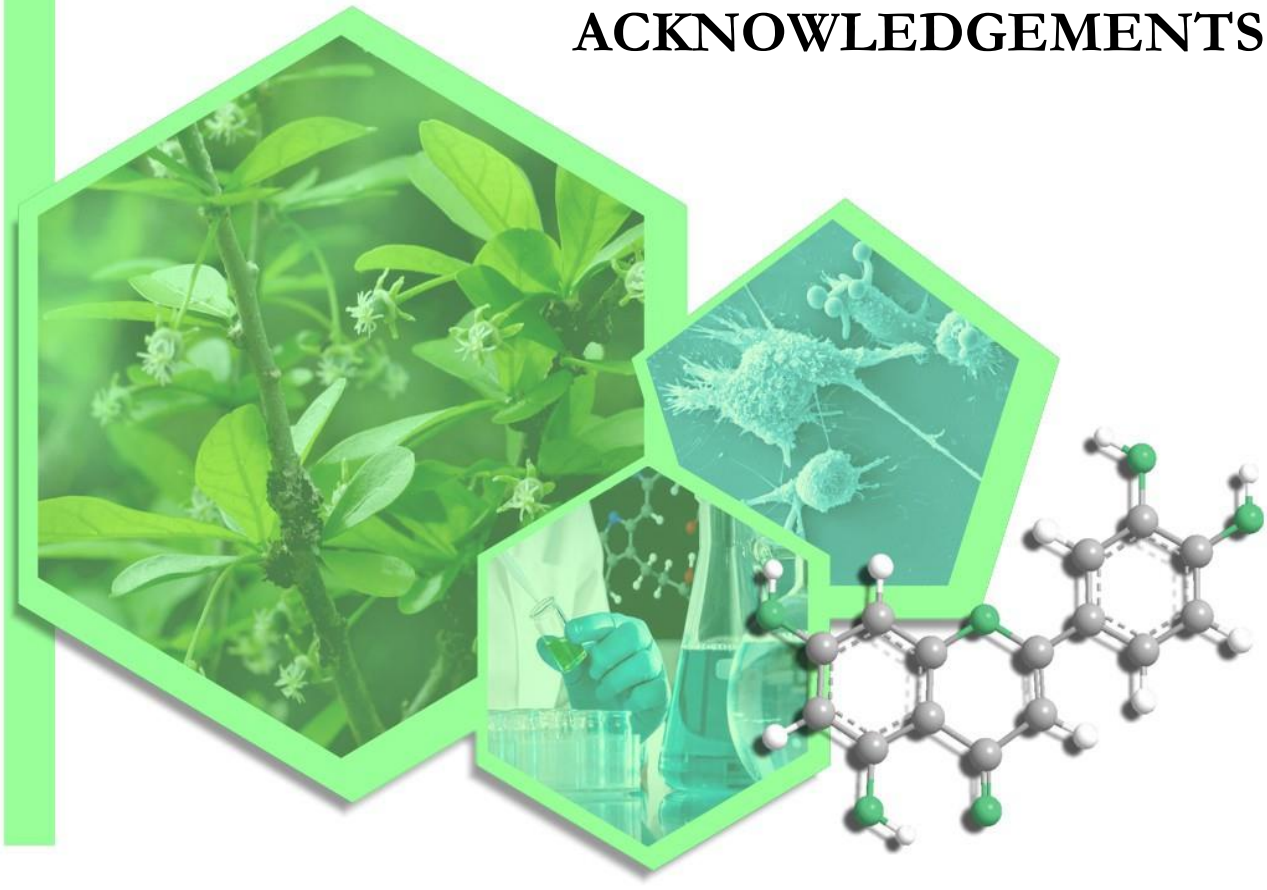
ACKNOWLEDGEMENTS



ACKNOWLEDGEMENTS

It is a great pleasure to thank the many people who provided me valuable support, assistance and make then this research project possible.

- *First and foremost I wish to express my heartfelt gratitude to my family in special for to my father (rest in peace), my mother, my husband, my children and my sister, for their love, understanding, inspiration and moral support during this long way.*

- *I would like to express my deepest gratitude to my Belgian promotors Prof. Dr. Paul Coss and Prof. Dr. Luc Pieters for giving me the opportunity to conduct my research in the University of Antwerp and for their invaluable guidance throughout the course of this investigation. Thanks also to give me the possibility to increase my experimental skills in their research group.*

- *I would also like to express my unwavering gratitude to my supervisor Prof. Dr. Julio Cesar Escalona Arranz for many years working together and constant source of motivation and guidance in the completion of this research study. I am also grateful to him for his understanding and support in overcoming numerous obstacles I have been facing. It was a great privilege and honor to work under his supervision.*

- *The work would not be possible without the financial assistance from the Belgian Development Cooperation through VLIR-UOS (Flemish Interuniversity Council-University Cooperation for Development) in the context of the TEAM Project "Installing a center of excellence in the Central- Eastern region of Cuba to enhance production and research on bioactive plants" with the University of Camagüey and the Institutional University Cooperation Program with University of Oriente, especially by means of the P-3 project "Biopharmaceutical Products from Natural Sources in the Development of Biotechnology.*

- *I am also most grateful to all members of staff in the NatuRA Department and the Laboratory for Microbiology, Parasitology and Hygiene (LMPH) for their unconditional support, excellent cooperation and contributing their generous time in helping me to carry out the research, very specials thanks to Anastasia, Stephano, Andrés, Stefania, Yunita, Mart, Tania, Kenn, Deborah, Maíra, Freya, Eveline, Natascha, An ……*

- *My profound special thanks go to Prof. Dr. Humberto Morris Quevedo and Prof. Dr, Gabriel LLauradó Maury for their copious assistance and moral support throughout my PhD work. I really appreciated their knowledge and invaluable experience in this field.*
- *I would also like to take this opportunity to dedicate my sincere appreciation to my colleagues of the Pharmacy Department at the University of Orient for sharing together the countless hours and good ideas to carry out completing this research successfully. Thanks to all my friends.*
- *I also wish to extend my sincere appreciation to all members of the Center of Toxicology and Biomedicine (TOXIMED) and Centre of Studies for Industrial Biotechnology (CEBI) in special to Onel, Mereidis, Jorgito De la Vega, Ernesto, Betty, Niola, Diana, Elizabeth, Edgar, Serrat, Miladis, Yaima,….*

Last, I would like to say many thanks to my friends and others colleagues for their moral support, friendship and timely help to complete this project successfully.