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VALIDATION OF PLANTS TRADITIONALLY USED FOR SKIN INFLAMMATION

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ARC	Acetone extract of *Rhus coriaria* L.
CE	Cyanidin-3-O-glucoside equivalent
DCMRC	Dichloromethane extract of *Rhus coriaria* L.
DMEM F12	Dulbecco's modified eagle medium F12;
DMSO	Dimethyl sulfoxide
DWAL	Decoction water extract *Arctium lappa* L.
EARC	Ethyl acetate extract of *Rhus coriaria* L.
EGCG	Epigallocatechin-3-gallate
ELISA	Enzyme-linked immunosorbent assay
EEA	*Echium amoenum* Fisch. ethanol extract
ERC	*Rhus coriaria* L. ethanol extract
EWAL	*Arctium lappa* L. ethanol-water extract
EWEA	*Echium amoenum* Fisch. ethanol-water extract
EWRC	*Rhus coriaria* L. ethanol-water extract
GAE	Gallic acid equivalent
HaCaT	HaCaT, spontaneously immortalized human keratinocyte line1
IC_{50}	Half maximal inhibitory concentration
IFN-γ	Interferon γ; IL-1, Interleukin 1
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-8	Interleukin 8
ICAM-1	Intercellular adhesion molecule 1
HPLC-UV-DAD	High-performance liquid chromatography-ultra violet-diode array detection
LUC	Luciferase
mEEA	*Echium amoenum* Fisch. macerated ethanol extract
mERC	*Rhus coriaria* L. macerated ethanol extract
MMP-9	MMP-9, matrix metalloproteinase-9
MTT	MTT, 3,4,5-dimethylthiazol-2-yl-2–5-diphenyltetrazolium bromide
Oil-ARC	Oil derived from acetone extract of *Rhus coriaria* L.
Oil-EARC	Oil derived from ethyl acetate extract of *Rhus coriaria* L.
Oil-mERC	Oil derived from macerated ethanolextract of *Rhus coriaria* L.
QE	Quercetin-O-glucoside
NF-κB	Nuclear factor κB
TNF-α	Tumor necrosis factor alpha
TMB	3,31,5,51-tetramethylbenzidine
VEGF	Vascular endothelial growth factor

WAL	*Arctium lappa* L. water extract
WEA	*Echium amoenum* Fisch. water extract
WRC	*Rhus coriaria* L. water extract
s.d.	Standard deviation

LIST OF SYMBOLS

α	Alpha
κ	Kappa
ºC	Degree Celsius
%	Percent

RIASSUNTO

Rhus coriaria L. (*R. coriaria*), *Arctium lappa* L. (*A. lappa*) ed *Echium amoenum* Fisch. (*E. amoenum*) sono piante medicinali con lunga tradizione d'uso, per le quali si annovera anche l'attività anti-infiammatoria a livello cutaneo. La presente ricerca ha lo scopo di studiare gli effetti antinfiammatori di diversi estratti di frutti di *R. coriaria*, radici di *A. lappa* e fiori di *E. amoenum* in cheratinociti umani (cellule HaCaT), valutando diverse tecniche estrattive.

In primo luogo, gli estratti delle piante sono stati preparati utilizzando vari metodi tra cui estrazione a freddo, macerazione e decozione. I solventi di estrazione utilizzati sono stati acqua, etanolo, etanolo-acqua (50:50), acetone, acetato di etile e diclorometano. Nella prima fase di questo studio sono stati valutati gli estratti di *E. amoenum*, *A. lappa* e gli estratti polari di *R. coriaria*: acquoso (WRC), idroalcolico (EWRC), etanolico (ERC) e macerato etanolico (mERC). La citotossicità e il rilascio di IL-8 sono stati valutati utilizzando, rispettivamente, saggi MTT ed ELISA, eseguiti dopo 6 ore di trattamento con TNF-α (10 ng/mL). Sulla base di questi risultati, mERC e EWRC sono stati selezionati come estratti attivi e valutati per la trascrizione guidata da NF-κB, seguendo le medesime condizioni di trattamento. Un estratto non citotossico di *A. lappa* (EWAL) è stato inoltre saggiato sulla via di segnalazione di NF-κB. Secondo i dati preliminari ottenuti, *R. coriaria* ha mostrato gli effetti piu coerenti e promettenti nell'inibizione della segnalazione di IL-8 e NF-κB. Pertanto, a partire da questa fase della ricerca, il focus principale dello studio è stato valutare le attività biologiche associate a questa pianta e l'effetto degli estratti mERC e il EWRC. In cellule stimolate da TNF-α e trattate con mERC ed EWRC, sono stati valutati il rilascio di ICAM-1, VEGF e MMP-9, nonché la traslocazione di NF-κB, mediante saggi ELISA. Inoltre è stato studiato il ruolo di entrambi gli estratti attivi nell'infiammazione indotta da UVB (40 mJ / cm^2) ed è stata ottenuta una caratterizzazione degli stessi attraverso analisi HPLC-UV-DAD.

Nella seconda fase di questa ricerca, gli estratti lipofili di *R. coriaria* (ARC, EARC e DCMRC) sono stati valutati usando i test di citotossicità e rilascio di IL-8 in cellule trattate con TNF-α o PMA. Sulla base di questi risultati, sono stati valutati gli effetti sulla trascrizione guidata da NF-κB.

Tra le piante valutate, solo gli estratti di *R. coriaria* hanno mostrato attività inibitoria sulla secrezione di IL-8. Sebbene tutti gli estratti polari di questa pianta abbiano inibito il rilascio di IL-8 indotto da TNF-α, solo mERC ed EWRC hanno soppresso l'attivazione di NF-κB, ICAM-1 e la secrezione di MMP-9. EWRC ha mostrato un maggiore effetto inibitorio su ICAM-1 e MMP-9 con IC_{50} di 1.76 ± 0.24 e 1.24 ± 0.33 μg/mL, rispettivamente (media ± s.d.). Al contrario, mERC ha ridotto significativamente i livelli di VEGF mentre EWRC non ha mostrato alcun effetto. La caratterizzazione HPLC-UV degli estratti ha rivelato una maggiore quantità di antociani in EWRC rispetto a mERC, il quale, a dispetto di ciò, ha inibito sia il rilascio di IL-8 indotto da UVB (con IC_{50} di 7.61 μg/mL) sia la traslocazione di NF-κB (a 10 μg/mL), misurata mediante saggio ELISA.

Gli estratti in acetone (ARC) ed etilacetato (EARC) di *R. coriaria*, hanno significativamente inibito il rilascio di IL-8 (a 25 μg/mL), nonché la trascrizione guidata da NF-κB (a 50 μg/mL), quando indotta da TNF-α. L'estratto in diclorometano (DCMRC), invece, non ha mostrato attività inibitoria della secrezione di IL-8 indotta da TNF-α. ARC (con IC_{50} di 25.77 μg/mL) e mERC (a 50 μg/mL) hanno mostrato un effetto significativo nell'inibire il rilascio di IL-8 indotto da PMA, inoltre ARC ha ridotto la trascrizione guidata da NF-κB indotta da PMA, con IC_{50} di 27.82 μg/mL.

I nostri risultati suggeriscono il potenziale effetto positivo degli estratti di frutto di *R. coriaria,* in particolare mERC, come agenti utili nella prevenzione dell'infiammazione dei cheratinociti attraverso il loro effetto inibitorio sulla produzione di mediatori pro-infiammatori della pelle. Le nostre promettenti scoperte sugli effetti inibitori di *R. coriaria* nell'induzione di NF-κB

sembrano confermare l'uso tradizionale di queste piante come rimedi per trattare gli stati infiammatori della pelle. L’impiego come antinfiammatorio cutaneo di queste piante merita ulteriori approfondimenti.

ABSTRACT

Rhus coriaria L. (*R. coriaria*), *Arctium lappa* L. (*A. lappa*) and *Echium amoenum* Fisch. (*E. amoenum*) are medicinal herbs with extensive traditional uses, covering anti-inflammatory skin therapy. The present research aims to investigate the anti-inflammatory effects of different extracts of *R. coriaria* fruit, *A. lappa* roots and the *E. amoenum* flowers in human keratinocytes (HaCaT cells), evaluating extracts prepared using different techniques.

Firstly, the herbal extracts were prepared using different methods including cold extraction, maceration, and decoction. The extraction solvents used were water, ethanol, ethanol-water (50:50), acetone, ethyl acetate, and dichloromethane. In the first phase of this study the extracts of *E. amoenum*, *A. lappa* and the polar (water (WRC), ethanol-water (EWRC), ethanol (ERC) and macerated ethanol (mERC)) extracts of *R. coriaria* were tested. The cytotoxicity and IL-8 release using, respectively, MTT and ELISA assays, were performed after 6 hours of TNF-α (10 ng/mL) treatment. Based on these results, the mERC and the EWRC were selected as the active extracts and assessed for NF-κB driven transcription, following identical treatment conditions. A non-toxic extract of *A. lappa* (EWAL) was also tested for its effect on NF-κB signaling. According to the preliminary data obtained, *R. coriaria* showed the most consistent and promising effects in inhibition of lL-8 and NF-κB signaling. Therefore, starting from this phase of the research the main focus of the study was on this herb and selectively mERC and the EWRC as the active extracts.

The challenged cells by TNF-α, under treatment conditions with mERC and EWRC, were analysed for ICAM-1, VEGF, and MMP-9 releases, as well as NF-κB translocation, by ELISA assays. In addition, both active extracts were investigated for the inflammation induced by UVB (40 mJ/cm^2) and were chemically profiled through HPLC-UV-DAD analysis.

In the second phase of this research, the lipophilic extracts of *R. coriaria* (ARC, EARC and DCMRC) were evaluated using the cytotoxicity and IL-8 release assays in TNF-α or PMA-treated cells. Based on these results, active extracts were measured for their effects on NF-κB driven transcription.

Among the evaluated herbs, only *R. coriaria* extracts achieved anti-IL-8 activity. Although all the polar extracts of this plant inhibited the TNF-α-induced IL-8 release, just mERC and EWRC suppressed NF-κB activation, ICAM-1, and MMP-9 secretion. EWRC showed higher inhibition on ICAM-1 and MMP-9 with IC_{50}s of 1.76 ± 0.24 and 1.24 ± 0.33 μg/mL, respectively (mean ± s.d.). On the contrary, mERC significantly decreased VEGF levels whereas EWRC did not show any effect. The HPLC-UV profile of the extracts revealed higher amount of anthocyanins in EWRC in comparison with mERC. mERC, which showed lower amount of anthocyanins than EWRC, blocked both UVB-induced IL-8 release (with IC_{50} of 7.61 μg/mL) and translocation of NF-κB (at 10 μg/mL), measured by ELISA assay.

The *R. coriaria* acetone (ARC) and ethyl acetate (EARC) extracts significantly inhibited the IL-8 release (at 25 μg/mL), as well as NF-κB driven transcription (at 50 μg/mL), when induced by TNF-α. The dichloromethane extract of this herb failed to show IL-8-inhibitory activity against TNF-α. ARC (with IC_{50s} of 25.77 μg/mL) and mERC (at 50 μg/mL) showed a significant effect in inhibiting the PMA induced IL-8 release. ARC modulated PMA-induced NF-κB signaling with IC_{50} of 27.82 μg/mL.

Our results suggest the potential positive effect of *R. coriaria* fruit extracts, mostly mERC, as preventive agent in the treatment of keratinocyte inflammation through their inhibitory effect on the production of skin pro-inflammatory mediators. Our findings on the promising inhibitory effects of *R. coriria* on NF-κB signaling seem to confirm the traditional use of this

herb as a remedy to treat skin inflammatory conditions. Its use as skin anti-inflammatory agent deserves further investigation.

Keywords: HaCaT; TNF-α; PMA, mERC, *R. coriaria*; anthocyanins; skin inflammation; polyphenols

CHAPTER ONE

1. Introduction

1.1 Basic structure and functions of the skin

The skin is the outside layer and the largest organ of the body with approximately 16% of body weight (Aghmiuni and Khiavi, 2017; Gawkrodger and Ardern-Jones, 2016). It is a continuously self-renewing organ that covers the surface area around 1.8 m^2 and holds other organs and tissues (Baroni et al., 2012). The skin isolates the body from the exterior world with which it interfaces in a dynamic way and controls crucial processes in the human body (Aghmiuni and Khiavi, 2017). It is responsible for numerous fundamental body functions such as sense, regulation of body temperature and elimination of waste products by sweating, producing vitamin D and mainly prevention of the passage of hurtful substances within in the body. Keeping the interior systems intact, it acts as a barrier and provides defense against noxious external factors such as mechanical and chemical insults, heat, infections, water, and microorganisms, ultraviolet radiation (Terui, 2000). The skin is composed of three basic fundamental compartments which are themselves multilayered (Fig. 1-1):

1. **Epidermal layer,** the surface coating the epithelial component which is principally composed of keratinocytes (Varani, 1998).
2. **Dermal layer,** which could be a connective constituent of nourishment, contains the capillary network to provide nutrient and to remove toxic waste.
3. **Hypodermis,** a subcutaneous fat compartment which anatomically provides stabilization, dynamic interface and division between the other two layers (Baroni et al., 2012).

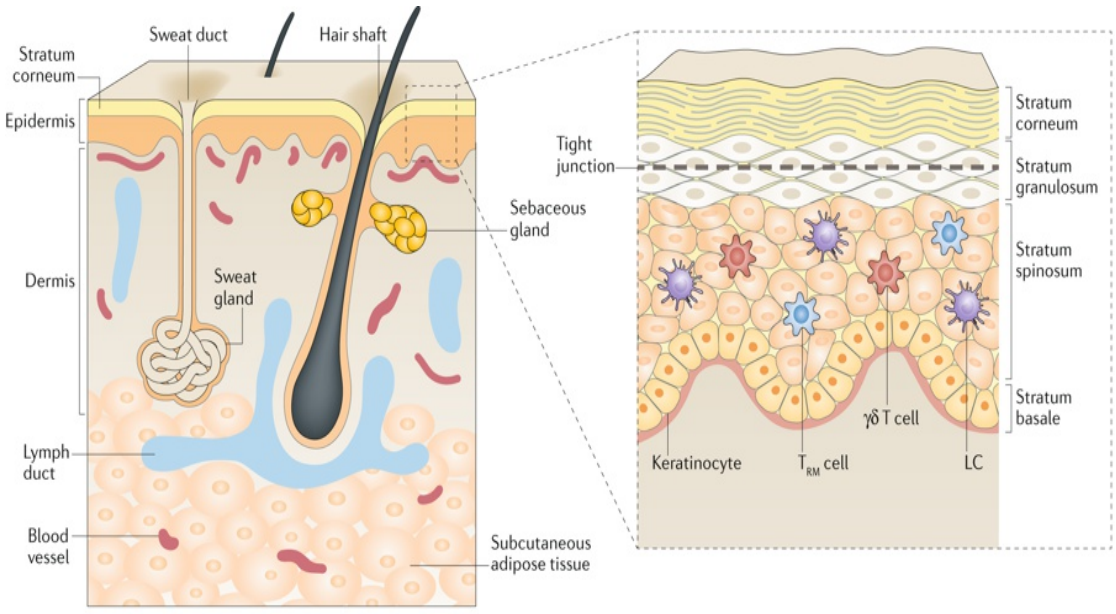

Fig. 1-1 Anatomy of the skin
Adapted from: (Kabashima et al., 2019).

Skin is the dynamic organ in a consistent state of altering, as cells of the external layers are continually shed and supplanted by internal cells moving up to the surface. Moreover, hair, nails, sebaceous, sweat, apocrine glands are respected as derivatives of skin (Gallo and Nizet, 2003; Gawkrodger and Ardern-Jones, 2016; Terui et al., 2000). Both epidermal and dermal layers cooperate in the formation of a specific matrix structure, the basement membrane (Mikesh et al., 2013).

The human skin represents the first line of protection against potentially hazardous environmental threats, such as infection by microbes (viruses, bacteria and fungi). This complex organ has the capability to instantly recognize between self and not-self, functioning as an interface between the human host and the environment, initiating an adequate innate particular and non-specific immune response.

1.1.1 Epidermis

The epidermis is the external layer of skin with around 0.1–1.4 mm thick (on areas of the body). The foremost abundant cells of the epidermis are keratinocytes which are nucleated

and viable from the basal layer to the granular layer and generate the protein keratin (Swindell et al., 2016; Wu et al., 2012). This layer is additionally containing other cells as inhabitant populaces or in reaction to temporal conditions: Langerhans, Melanocyte and Merkel cells. The organized layers of the epidermis from the outermost to the innermost layers are as follows:

1. **Stratum corneum:** The foremost distinctive anatomical feature of the epidermis is stratum corneum containing heaps of corneocytes which are dead keratinocytes and intercellular lipids (Petkova et al., 2014; Shenefelt, 2010). This fundamental structure blocks the entrance or exit of aqueous and aqueous-soluble constituents. It contains numerous holes for dermal appendages, such as hair follicles and sweat ducts, to protect against environmental insults such as mechanical damage, ultraviolet light, temperature changes and dryness (Akay et al., 2002; Kabashima et al., 2019).
2. **Stratum lucidum:** this layer contains nucleated cells occurring between the stratum granulosum and stratum corneum. It can be found whithin the thicker skins whithin the palms and soles.
3. **Stratum granulosum:** this layer contains flattened cells which are losing their nuclei, keratohyalin granules within the cell and the lipid-filled membrane coating vesicles. The lysosomal activity in this stratum is responsible for digestion of the cell content and cells disintegration. Fatty acids, cholesterol and cholesterol and ceramides are the main type of lipids in Stratum granulosum.
4. **Stratum spinosum:** this layer of polyhedral cells mostly contains Langerhans cells. It is formed by upwards migration of daughter basal cells, which are interconnected by desmosomes.
5. **Stratum basale:** the basal cell layer of the epidermis is composed of keratinocytes. Keratin tonofibrils, melanocytes and Merkel cells are found in the stratum basale.

Although the total skin structure is effectively involved in the defense mechanisms, epidermis plays vital role as both “inside–outside” and “outside– inside” barrier. This function basically includes inhibition of unregulated loss of water and other constituents protecting the body from potentially hazardous environmental threats. The epidermal barrier function can be better described as follows:

1. **The physical barrier:** Main component for this function is the stratum corneum. Additionally, cell–cell junctions and associated cytoskeletal proteins also participate in this role (Baroni et al., 2012).
2. **The chemical and biochemical barrier:** Lipids, acids, hydrolytic enzymes, antimicrobial peptides, and macrophages are the main components involved in this function.
3. **The immunologic barrier:** Humoral and cellular constituents of the immune system serve immunologic barrier function.

When the skin is challenged with exogenous insults, the relatively inactive skin immune system is changed to activated state and immunological cascades are set in motion. In some conditions such as psoriasis disorder, these immunological changes are not within a normal range and cause severe inflammation and hyper-proliferation of skin cells (Terui et al., 2000).

1.1.1.1 Keratinocytes

The structure of the epidermis is tightly connected to the life cycle of its predominant cell type, keratinocyte (Proksch et al., 2008; Wickert and Visscher, 2006). These cells stem from epidermal stem cells located in the stratum basale and synthesize and express several structural proteins and lipids during their maturation (Baroni et al., 2012). Keratinocytes represent the first barrier against pathogens in human skin (Kim et al., 2011). In the skin immune system, these cells are connected with resident immune cells such as lymphocytes, Langerhans cells and macrophages, to contribute to innate immunity and inflammatory

processes. Exposure to agents causing inflammation as well as environmental aggressors can induce the secretion of proinflammatory cytokines and chemokines (IL-1, IL-6, IL-8, IL-10, IL-18) by keratinocytes. These soluble mediators, in turn, carry out their functions in paracrine and autocrine manners (Huber and Petersen, 2015; Loschke et al., 2016; Nestle et al., 2009; Suter et al., 2009). Keratinocytes are major structural components of epidermis and participate in the initiation and/or regulation of cutaneous inflammatory and immune responses as a result of their ability to produce a variety of cytokines and chemokines. In response to pro-inflammatory cytokines (e.g. IL-1β, IFN-γ and TNF-α), they synthesize and release IL-6 and IL-8 (Raingeaud and Pierre, 2005).

1.1.1.2 Other cell types

Other cell types in the epidermis include the pigment producing melanocytes, Langerhans' cells and Merkel cells. Melanocytes are located in the stratum basale (Tsatmali et al., 2002) and make up approximately 5 % of the cells of the epidermis (Thingnes et al., 2012). An epidermal-melanin unit consists of a dendritic melanocyte and an average of 36 keratinocytes (Seiberg, 2001). Melanosomes are the melanin containing granules which are translocated to the dendritic tips of the melanocytes and transferred to keratinocytes (Kippenberger et al., 1998). They are trapped by microvilli on keratinocytes and incorporated into the cytosol where they form a perinuclear cap in the keratinocytes able to protect DNA from UV-light induced damage (Ando et al., 2012). The Langerhans cells are immature dendritic cells and the only immune cells resident in the epidermis. In the case of an injury and infection they migrate to the peripheral lymph nodes, loose the antigen processing capabilities but upregulate major histocompatibility complex and present antigens at a high level (Clausen and Stoitzner, 2015). Merkel cells are mechanosensory cells located in the basal layer and form synapse-like connections to somatosensory system in the dermis (Maksimovic et al., 2014).

1.1.2 Dermis

The dermis is defined as a connective tissue matrix, providing nutrients and physical support to the epidermis. It is located below the epidermis and is mainly composed of the two papillary and reticular layers.

The papillary dermis is the most superficial part containing tactile receptors, the Meissner corpuscles (Vega et al., 1996), and free nerve endings are present in the papillae that are responsible for the conduction of sensations, including pain. It is characterized by areolar connective tissue organized in dermal papillae forming projections into the epidermal side and the Merkel cell-neurite complexes in conjunction with the basement membrane making up a direct contact between the two tissue compartments (Maksimovic et al., 2014; Moll et al., 2005).

The reticular dermis is a dense network of collagens and coarse elastic fibers making up the bulk of the skin and providing for its integrity and extensibility. Interspersed in the spaces between the connective tissue bundles are adipocytes, glands, nerves and the hair follicles. Epidermal cells are present in two of the structures of the hair follicle: in the matrix surrounding the connective tissue papilla and in the bulge of the hair follicle. Keratinocytes and melanocytes are resident in the matrix of the hair follicle and the origin of the epithelial cells is the epidermal stem cells of the bulge of the hair follicle (Cotsarelis, 2006). The bulge cells only contribute to the structure of the epidermis in the case of re-epithelialization of a wound where the cells respond quickly to damage but give rise to a transit amplifying population that later is replaced by strictly epidermal keratinocytes (Cotsarelis, 2006).

The different types of cells in the reticular layer of dermis are as follows:

1. **Fibroblasts:** they are mainly involved in repairing damaged tissues;
2. **Mast cells:** these cells are playing a major anti-infection role;
3. **Lymphatic vessels:** as a defense system they are mainly fighting against infection;

4. **Epidermal appendages:** they are active in connecting epidermis and dermis for preventing skin damages;
5. **Ground substance:** as a gel-like component it has supporting effect on the structure of dermis.

The connective tissue that constitutes the dermis is produced by dermal fibroblasts. Additional cell types present are macrophages and adipocytes. The dermis harbors nerves, glands, hair follicles and blood vessels that supply the skin and participate in thermal regulation. The structural division of the dermis in two layers is based on the packing of the connective tissue. Connective tissue consists of a ground substance with protein fibers containing water and a mixture of large organic molecules including the combination of polysaccharides as complex carbohydrates and proteins (Zaidi and Lanigan, 2010). This tissue matrix is called the extracellular matrix (ECM) and contains collagens as the most abundant proteins and fibronectin, a protein with high importance for cell adhesion and migration (Bachman et al., 2015; Gelse et al., 2003; Pickford et al., 1997; Zhu and Marchant, 2011).

1.1.3 Hypodermis

Subcutaneous supportive fat compartment or the hypodermis layer lays below the dermis as an innermost region of the human skin. It is composed up largely of fat cells and connective tissue for protection of internal structures against traumatic and thermal insults (Zaidi and Lanigan, 2010).

1.2 Skin inflammation and skin inflammatory disorders

Inflammatory skin diseases are the most common dermatologic conditions which are influenced by genetic and environmental factors. Chronic inflammation results from the dysregulation and abnormal expression of inflammatory mediators or their receptors in keratinocytes (Chandel et al., 2000). Although the inflammation is an essential innate immunity

response that is crucial to fight pathogens, dysregulated and untimely inflammation, contributes to several chronic inflammatory skin diseases such as psoriasis, atopic dermatitis and cancer (Fukumura et al., 2016; Griffiths et al., 2007; Hotamisligil, 2006). There are many types of skin diseases such as eczema, fungal/yeast, bacterial, viral, and parasitic infections, autoimmune disease and miscellaneous skin disease. The inflammatory skin disorders can be classified by means of their responsibilities in the specific layers of the host defense system of the skin, as follows (Dainichi et al., 2014):

1) Disorders of acquired immunity
2) Disorders of innate immunity
3) Disorders of the skin barrier

1.2.1 Disorders of acquired immunity

These disorders can induce an inflammatory skin disease, which partly simulates the actual protective response against infections (bacteria, viruses, fungus and parasites) and dangers (venoms, poisons, toxic haptens and allergens). These conditions might be related to genetic lack of the specific molecules that are essential in acquired immunity. They can be classified into three groups as follows:

1) immunodeficiency: severe combined immunodeficiency (SCID); acquired immunodeficiency syndrome (AIDS) and chronic mucocutaneous candidiasis (CMCC)
2) immunohyperactivity (allergy): allergic contact dermatitis; graft-versus-host disease (GVHD); chronic eczema and papullo-erythroderma.
3) qualitative disorder (autoimmunity): pemphigus, pemphigoid alopecia areata.

1.2.2 Disorders of innate immunity:

These disorders can occur following the activation of transcription factors including NF-κB, in response to dangers (physical damages, foreign body, dead cells cellular and oxidative stress) or infectious agents (bacteria, viruses, fungus and parasites).

The skin disorders of innate immunity can be classified into three groups:

1) innate immunodeficiency: myeloid differentiation primary response 88 (MyD88), IL-1 receptor-associated kinase 4 (IRAK4) and phagocyte dysfunction, etc.

2) innate immunohyperactivity, which can be divided into two groups:

- Systemic innate immunohyperactivity and autoinflammatory diseases,
- Organ-specific innate immunohyperactivity which includes autoinflammatory folliculitis (acne, pyoderma chronica), and Autoinflammatory perifolliculisitis (rosacea).

3) qualitative disorder (general or local innate autoimmunity) which can be classified as follows:

- Systemic innate autoimmunity: systemic lupus erythematosus (SLE), scleroderma.
- Organ-specific innate autoimmunity: psoriasis, pityriasis rubra pilaris (PRP).

1.2.3 Disorders of the barrier of the skin;

The barrier disorders cannot yet be clearly determined quantitatively or qualitatively. These skin conditions can be considered as defects in the physical barrier (e.g. trauma, burns atopic dermatitis) or the chemical barrier (atopic dermatitis). The physical barrier diseases are usually accompanied by the chemical barrier disorders.

In general, skin conditions have been overlooked through-out the world, not only as beauty threatening but life-threatening disorders. For example, psoriasis has been classified as a rare inflammatory chronic recurrent dermatologic condition due to great impact on patient's quality of life (Akay et al., 2002; Petkova et al., 2014; Shenefelt, 2010). Among the above-mentioned types of disorders, psoriasis can be considered among the severe autoimmune

diseases (Swindell et al., 2016; Wu et al., 2012). This immune-mediated skin disease affects approximately 2–3% of the general population (Fig.1-2).

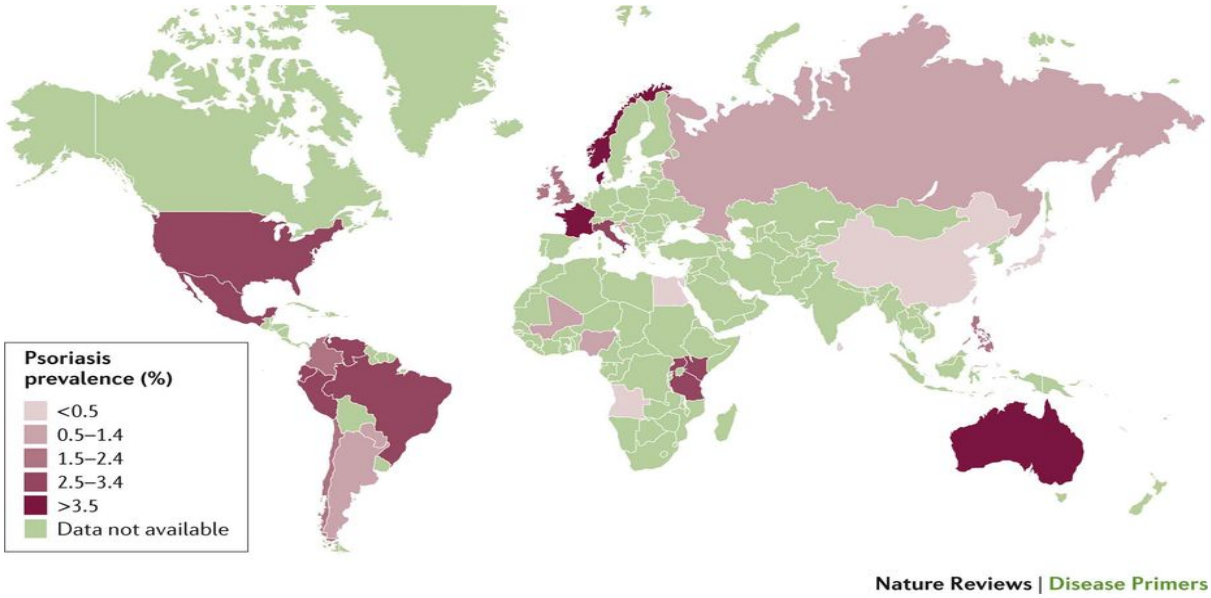

Fig. 1-2 The prevalence of psoriasis as a type of skin inflammatory disorder.

Adapted from: (Greb et al., 2016)

1.3 Role of epidermal keratinocytes in the pathogenesis of skin inflammation

Keratinocytes are the most abundant cells of the outer epidermis layer of the skin which play a key role in the induction and maintenance of inflammation in this organ (Colombo et al., 2017). Epidermal keratinocytes play a critical role in skin inflammation through their production of inflammatory chemokines (Albanesi, 2010; Khalilpour et al., 2019). Therefore, chemokines are considered as pivotal mediators in the progress of inflammatory skin diseases. Numerous skin disorders, such as psoriasis, atopic dermatitis and contact dermatitis are associated with dysregulation of immune responses in the skin. Although its pathogenesis is not fully understood, there is an underlying interaction between numerous immune effector cells and aberrant hyperproliferation and differentiation of pro-inflammatory role of epidermal keratinocytes. Figure 1-3 shows the involvement of keratinocytes and other cell types in the

mechanism of skin inflammation. As the most abundant cells in epidermis, keratinocytes play a key role in immune response and act as initiators of inflammation. Pathophysiological conditions of psoriasis involve hyperproliferation of keratinocytes in combination with abnormal differentiation of the epidermis layer and infiltration of inflammatory cytokines secreted from activated T cells or antigen presenting cells from the dermis and epidermis cell layers.

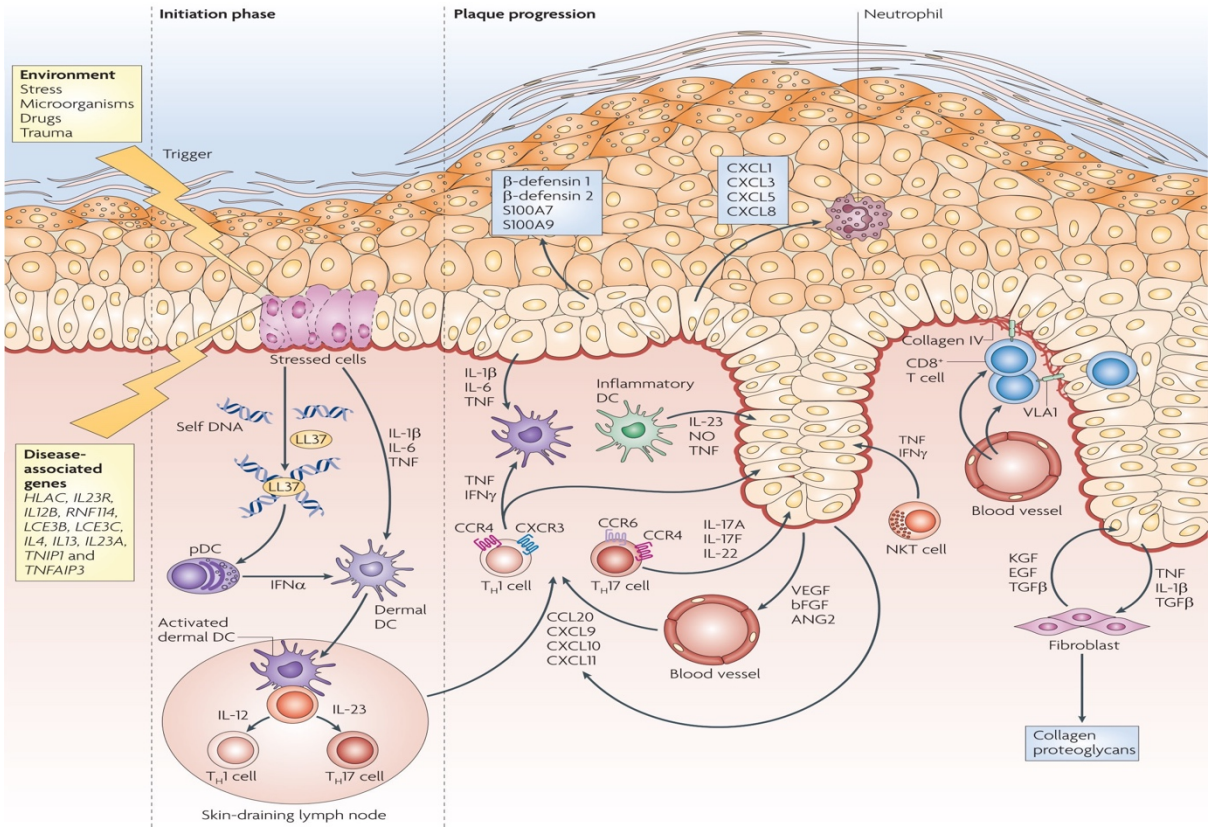

Fig. 1-3 Key Cells and Mediators in skin inflammation and keratinocytes as targets for cytokines in skin inflammation
Adapted from: (Nestle et al., 2009)

As a protective interface between internal organs and the environment, the skin encounters the first line of defense against the physical, chemical and immune system-specific aggressions that may rapidly develop into an epidermal response. To defend against such challenges to the cutaneous micro-environment, such as pathogenic organisms as well as

physical and environmental stressors, such as toxic chemicals and UV rays, the skin functions are more than just a physical barrier; it is also an active immune organ (Bito et al., 2000). Previous studies have demonstrated the production of proinflammatory cytokines by T cells, monocytes and keratinocytes in response to stimulation by skin-related bacteria (Chen et al., 2002; Grange et al., 2009; Nagy et al., 2005; Wang et al., 1997). Inflammatory skin diseases, such as psoriasis and atopic dermatitis, are characterized by activation of keratinocytes and infiltration of activated T cells into the dermis and epidermis, where they modulate the local environment through the release of cytokines (Matjeka, 2012). Activated keratinocytes release potent cytokines acting on cells in the local environment to boost the inflammatory response (Pasparakis et al., 2002). An increase in the production of soluble factors, such as cytokines, chemokines and antimicrobial peptides, induce the oriented migration of different subpopulations of leukocytes (Grange et al., 2009).

Keratinocytes can produce numerous cytokines, including IL-1, IL-3, IL-6, IL-8, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, transforming growth factor (TGF)-α, TGF-β, tumor necrosis factor (TNF)-α and platelet-derived growth factor, upon activation with proinflammatory mediators, such as IFN-γ and TNF-α (Ansel et al., 1990; Khalilpour et al., 2019; Sangiovanni et al., 2019b).

Furthermore, they produce the IL-8 (CXCL8), a chemokine induced by IFN-γ, IFN-inducible protein-10 and IFN-inducible T cell α-chemoattractant; and the macrophage inflammatory protein-1, regulated on activation, normal T expressed and secreted, macrophage inflammatory protein (MIP)-3α, macrophage-derived chemokine and cutaneous T-cell-attracting chemokines (Albanesi et al., 2001; Lebre et al., 2003) after stimulation, which regulate migration of diverse types of leukocyte in the skin lesion.

1.3.1 NF-κB signaling

Nuclear factor κB (NF-κB) is a protein transcription factor that initiates inflammation and other complex biological processes. It is a key regulatory element in a variety of immune and inflammatory pathways, in cellular proliferation and differentiation and in apoptosis. Therefore, NF-κB is a crucial mediator involved in the pathogenesis of psoriasis and this inflammatory dermatosis is marked by elevated levels of active, phosphorylated NF-κB. Genomic studies have also linked psoriasis with mediators in the NF-κB pathway. NF-κB has been hypothesized to connect the altered keratinocyte and immune cell behavior that characterizes the psoriatic milieu in human skin.

There are numerous inducers of NF-κB, including pro-inflammatory cytokines, such as tumor necrosis factor (TNF) and interleukin IL-1, foreign antigens, such as carbohydrates and peptides, as well as factors that induce oxidative stress and DNA damage, such as ultraviolet radiation and reactive oxygen species. Stimulation of keratinocytes with TNF-α leads to activation of the NF-κB pathway, and subsequently increases the expression of adhesion molecules and proinflammatory genes (Bahar-Shany et al., 2010; Seo et al., 2015).

NF-κB is composed from a family of proteins, including RelA (p65), RelB and c-Rel. Two additional subunits are formed from the precursor proteins NF-κB 1 (p105) and NF-κB 2 (p100), which are processed into p50 and p52 (Perkins et al., 1992). All the NF-κB proteins have a Rel homology domain responsible for DNA binding and dimerization. NF-κB transcription factors form dimers from two of the five protein building blocks, bind κB sites and enhance genes responsible for induction or repression of transcriptional activities. Selection of specific NF-κB dimers occurs at control regions within DNA sequences. The selective gene expression may also occur by interactions of specific subunits.

NF-κB activation by TNF-α is due to the activation of the inhibitor of κB (IκB)-kinases (IKKs) complex, an upstream kinase complex of IκBα, which phosphorylates IκBα at Ser32/36, resulting in its ubiquitination and subsequent proteasomal degradation (Kim et al., 2016; Seo et al., 2015). Degradation of IκBα leads to translocation of cytosolic NF-κB complexes into the nucleus, where they activate the transcription of proinflammatory target genes (Cho et al., 2017; Kim et al., 2016; Seo et al., 2015). Stimulation of keratinocytes with TNF-α leads to activation of nuclear factor-κB (NF-κB) and subsequently increases the expression of adhesion molecules and proinflammatory genes (Fig. 1-4) (Ghosh and Baltimore, 1990; Kock et al., 1990).

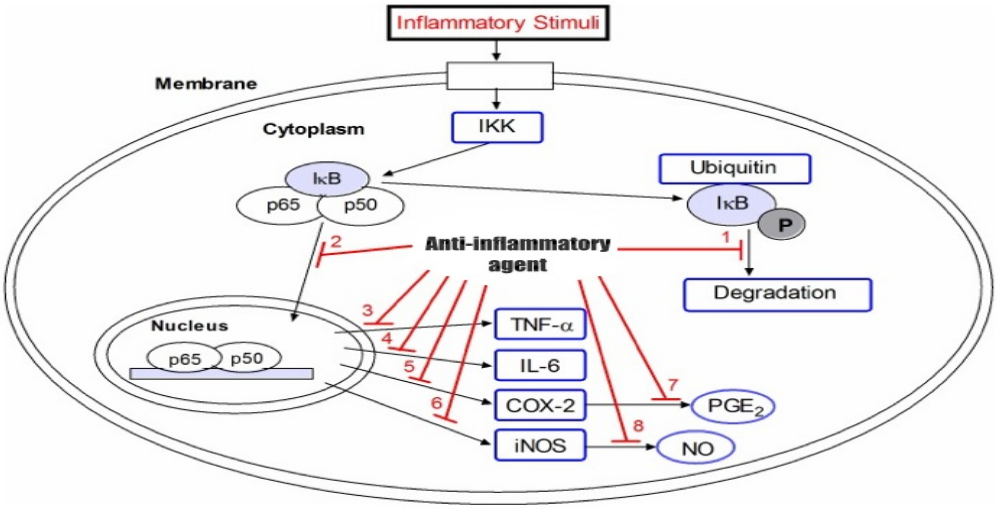

Fig. 1-4 NF-κB: an essential transcription factor involved in keratinocytes inflammation

Adapted from: (Hussein et al., 2013)

1.3.2 TNF-α

Tumor necrosis factor (TNF-α) is one of the key pro-inflammatory mediators involved in the pathophysiology of the chronic skin inflammatory disorders such as psoriasis, atopic dermatitis and rheumatoid arthritis and is often found in high amounts around psoriasis skin

lesions (Kristensen et al., 1992). This pro-inflammatory cytokine plays a key role in cell survival and apoptosis of normal and malignant cells (Brenner et al., 2015; Pennica et al., 1984). A critical step in the development of inflammatory skin diseases is the infiltration of various immune cells, such as monocytes, neutrophils, and activated T cells, from the blood into the skin (Grone, 2002). TNF-α is expressed in several cell types including activated macrophages, T cells, natural killer cells, dendritic cells, mast cells and keratinocytes (Kock et al., 1990). This cytokine induces inflammation in keratinocytes which causes the release of different proinflammatory mediators. It serves as a master regulator, playing a role in the regulation of cell survival, apoptosis, and necrosis for various cells and tissues. Upon stimulation with proinflammatory agents, including TNF-α and interferon (IFN)-γ, keratinocytes in the skin express proinflammatory cytokines, chemokines, and adhesion molecules, like inter-cellular adhesion molecule-1 (ICAM-1), which contribute to the entry of immune cells from the blood into the site of inflammation in the skin (Dustin et al., 1988; Trefzer et al., 1991).

1.3.3 IL-8

Interleukin-8 (IL-8) is a cytokine belonging to the CXC chemokine family involved in recruitment of leukocytes to the site of inflammation (Baggiolini, 1998). IL-8, also known as neutrophil activating peptide, CXCL8, is a small protein that is encoded by the *IL-8* gene, which is located on chromosome 4q12-q21 (Modi and Chen, 1998; Vandamme et al., 1989). IL-8. It is an important factor in the pathogenesis of psoriasis vulgaris, which is characterized by proliferation of keratinocytes, neutrophil infiltration, and angiogenesis. This chemokine is widely accepted as an important pro-inflammatory factor involved in the proliferation of keratinocytes, neutrophil infiltration and angiogenesis in psoriasis (Arican et al., 2005; Biro et al., 1997; Pietrzak et al., 2008). In normal skin, many types of cells can produce IL-8 to maintain the homeostasis of the skin barrier (Roebuck, 1999). In psoriasis vulgaris, activated

keratinocytes can produce higher levels of IL-8 (Gillitzer et al., 1991). Thus, local level of IL-8 positively correlates to disease severity (Sticherling et al., 1999). Many cytokines derived from immunocytes involved in the pathogenesis of psoriasis vulgaris can upregulate IL-8 production and act as therapeutic targets in psoriasis vulgaris treatment (Homey, 2004; Krueger, 2002). In healthy tissues, IL-8 is barely detectable, but is rapidly induced by 10- to 100-fold in response to pro-inflammatory cytokines, such as tumor necrosis factor- (TNF-) or IL-1, bacterial or viral products, and cellular stress (Hoffmann et al., 2002). A high level of IL-8 in serum has been found to be significantly associated with the presence of oral ulcer and IL-8 activated neutrophils is believed to be a major source of enzymes involved in tissue destruction (Srosiri et al., 2010). Therefore, the elevation of IL-8 secreted by oral keratinocytes may be the initial sign of the acute inflammatory response following lead exposure to oral mucosal surfaces and may reflect the pathogenesis of lead stomatitis in the oral cavity. This chemokine with mitogenic activity in keratinocytes is also a chemoattractant involved in the recruitment of neutrophils, the predominant cell type in acne-related lesions (Grange et al., 2009).

1.3.4 MMP-9

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that contain a zinc ion in their active site. MMPs take part in many physiological processes such as cell migration and angiogenesis (Manicone and McGuire, 2008; Sternlicht and Werb, 2001). MMPs are a family of Ca-Zn-dependent proteases with common functional and structural properties (Goetzl and Banda, 1996).

The epidermis is routinely exposed to numerous stresses, such as mechanical injury, UV radiation, infectious organisms, and xenobiotics. These challenges invoke a multifaceted interactive response including inflammation and tissue remodeling. The maintenance of tissue architecture requires coordinated degradation and synthesis de novo of ECM constituents.

During normal tissue repair MMPs carry out controlled degradation of ECM constituents, and increase the bioavailability of growth factors and cytokines (Stamenkovic, 2003). On the other hand, excessive activity of MMPs leads to degradation of growth factors and of necessary ECM constituents and interferes with cell migration and tissue repair (Trengove et al., 1999; Turchi et al., 2003)

MMP-9 (gelatinase B) belongs to the gelatinase subgroup of the MMP family, and its expression and activity have been correlated with different stages of carcinoma progression (Zeng and Guillem, 1996). In the skin compartment, eosinophils, mast cells, Langerhans cells, and keratinocytes express MMP-9 and MMP-3 (Kobayashi, 2014; Kobayashi and Shinkai, 2005; Kobayashi, 1997; Onoue et al., 2003; Sawicki et al., 2005; Schwingshackl et al., 1999). MMP-9, an inducible gelatinase secreted by several cell types including stressed keratinocytes, can degrade collagen IV the main constituent of the basement membrane and plays a major role in tissue remodeling following cutaneous injury (Crowe et al., 2001; Kim et al., 2008). It was found that MMP-9 is highly but transiently expressed in newly wounded tissue (Soo, 2000). On the other hand, in chronic wounds and in persistent inflammation, where the balance between tissue degradation and formation is disturbed, levels of MMP-9 remain high (Ekekezie et al., 2004; Ladwig et al., 2002). MMP-9 also exerts a pro-inflammatory activity in the epidermis by increasing migration of immune cells through the basement membrane (Purwar et al., 2008) and by increasing the local availability of a variety of chemokines and cytokines (Van Lint and Libert, 2007). The inflammatory response to cutaneous stress, which accompanies tissue remodeling, is characterized by elevated levels of inflammatory cytokines among which TNF-α plays a major role. Indeed, dysregulation of TNF-α has been implicated in a wide variety of cutaneous inflammatory diseases (Trent and Kerdel, 2005). TNF-α is also a well-known regulator of MMPs' gene expression. Chronic exposure to TNF-α might bring about unbalanced MMPs activity that might result in irreversible damage to the inflamed

epidermis. MMP-9 gene expression is modulated by various signals, among them growth factors and cytokines. Two transcription factors are implicated in the induction of MMP-9 expression in many cellular systems, namely AP-1 and NF-κB (Chakraborti et al., 2003). Activation of the NF-κB pathway is a well-characterized outcome of TNF receptor activation, involving activation of IκB kinase (IKK), phosphorylation and subsequent degradation of the endogenous NF-κB inhibitors, members of the IκB family (please refer to 1.3.1 paragraph) (Boersma and Meffert, 2008)(Bahar-Shany et al., 2010; Boersma and Meffert, 2008; Purwar et al., 2008; Wittmann et al., 2007).

1.3.5 ICAM-1

The Inter-cellular adhesion molecule-1 (ICAM-1 or CD54) is a 76–114- kDa cell surface glycoprotein and a member of the immunoglobulin superfamily of proteins, contributing to the local infiltration of leukocytes in immune responses by mediating the adhesion and activation of these cells (Roebuck and Finnegan, 1999). A critical step in the development of inflammatory skin diseases is the infiltration of various immune cells, such as monocytes, neutrophils, and activated T cells, from the blood into the skin (Grone, 2002). Upon stimulation with proinflammatory agents, including TNF-α, keratinocytes in the skin express proinflammatory cytokines, chemokines, and adhesion molecules, like ICAM-1, which contribute to the entry of immune cells from the blood into the site of inflammation in the skin (Dustin et al., 1988; Trefzer et al., 1991). The contribution of ICAM-1 has been demonstrated in several inflammatory diseases (Sampath et al., 1999; Stanciu and Djukanovic, 1998; Watanabe and Fan, 1998). An up-regulation in the expression of ICAM- 1 in keratinocytes was demonstrated to be an important feature in cutaneous inflammation (Wu et al., 2009). Inflammatory keratinocytes express ICAM-1 to promote adsorption of leukocytes into skin tissue (Youn, Gi Soo et al., 2013). This adhesion molecule plays a critical role to cause the inflammatory response in the interaction between leukocytes and keratinocytes. The release of

inflammatory mediators such as TNF-α can induce surface expression of ICAM-1 in activated keratinocytes (Youn, G. S. et al., 2013).

These cells subsequently proliferate, ultimately resulting in epidermal hyperplasia. In addition, over-expression of ICAM-1 and chemokine release are induced, leads in immune cell infiltration into the inflamed tissues (Han et al., 2015; Kalish, 1991; Kim et al., 2016).

Exposure of keratinocytes to inflammatory cytokines derived from infiltrated cells, particularly T cells, which are recruited by chemokines and ICAM-1 expression of keratinocytes leads to high expression of soluble ICAM-1 (Andrea Cavani and Girolomoni, 1998; Han et al., 2015).

In case of the suppression of ICAM-1 expression, the infiltration of immune cells into the skin may decrease, which could ameliorate inflammatory skin conditions. ICAM-1 is involved in recruitment to the inflammatory site through its binding to β_2 integrin lymphocyte function-associated antigen 1 (LFA-1, CD11a/CD18) which is located on macrophages, neutrophils, T cells and B cells (Han et al., 2015; Youn, Gi Soo et al., 2013).

1.3.6 VEGF

Vascular endothelial growth factor (VEGF) is one of the major mediators involved in inflammatory processes associated with aberrant angiogenesis (Baggiolini et al., 1994; Ballaun et al., 1995; Ferrara and Davis-Smyth, 1997; Neufeld and Gospodarowicz, 1988). In skin inflammation, epidermal cells are important VEGF-producing cells which probably contribute to the pathogenesis of a variety of epidermal diseases through their chemotactic activity on inflammatory cells and through their angiogenic effect. Cultured human keratinocytes constitutively secrete VEGF (Ballaun et al., 1995; Frank et al., 1995; Viac et al., 1997), and multiple factors including proinflammatory cytokines increase VEGF expression *in vitro* (Frank et al., 1995; Larsen et al., 1988; Trompezinski et al., 2003). Angiogenesis has an important role in tumor growth and metastasis. However, vascular remodeling also occurs in many inflammatory and autoimmune disorders (Carmeliet, 2003; Pober and Sessa, 2007),

including rheumatoid arthritis, inflammatory bowel disease, and the chronic inflammatory skin disease, psoriasis (Bainbridge et al., 2006; Danese et al., 2006; Leong et al., 2005). A crucial role of VEGF in the pathogenesis of psoriasis has been demonstrated in several studies (Canavese et al., 2010; Lei and Yang, 2012; Yan et al., 2018). It has been reported that epidermis-derived VEGF is strongly up-regulated in psoriatic skin lesions and VEGF serum levels are correlated with the severity of this disorder (Detmar et al., 1997). There are also evidences of involvement of a genetic predisposition caused by single-nucleotide polymorphisms of the VEGF gene in the pathogenesis of psoriasis (Magnani et al.; Young et al., 2004). In an animal study on K14-VEGF transgenic mice expressing mouse, VEGF164 in the epidermis spontaneously develop a chronic psoriasis form skin inflammation (Xia et al., 2003). Moreover, VEGF transgenic mice show the characteristic Koebner phenomenon, because induction of skin inflammation in transgenic mice results in the development of chronic, psoriasis-like skin inflammation (Kunstfeld et al., 2004). Treatment of K14-VEGF transgenic mice with a VEGF receptor tyrosine-kinase inhibitor, NVP-BAW288, reduced the number of blood and lymphatic vessels and the infiltrating leukocytes in the skin and normalized the epidermal structure (Halin et al., 2008).

1.4 In vitro models of skin inflammation

The skin is a useful organ for the basic biological and pharmacological studies. For instance, it is a suitable tissue for investigation of basis of wound repair, mechanisms of tissue injury, ageing process, and hyper-proliferative disorders. Due to its structure and amenability to topical treatments, it is a proper model to evaluate the effects of several therapeutic agents which could not be assessed systematically. *In vitro* studies with cells in monolayer culture have been successfully used for understanding the events which contribute to maintenance of structure and function of normal skin and also evaluating the skin inflammation (Varani, 1998).

1.4.1 HaCaT cells

HaCaT (spontaneously immortalized human keratinocytes) is a nontumorigenic monoclonal cell line which has been widely used as a model for epidermal keratinocytes in various studies related to their functions and also irritancy and drug development in the skin (Magcwebeba et al., 2012; Micallef et al., 2009). The comparative studies have been demonstrated the normal phenotype, similar differentiation and expression of the surface markers and functional activities of these cells with respect to normal keratinocytes in normal and stimulated conditions (Boukamp et al., 1988; Deyrieux and Wilson, 2007; Fusenig and Boukamp, 1998; Micallef et al., 2009; Schurer et al., 1993).

This in vitro system has been successfully used as a skin carcinogenesis model and in the *in vitro* models of infectious diseases (Colombo et al., 2017; Khalilpour et al., 2019). It has been reported by Colombo et al. that this cell line is a reliable model for the screening of anti-inflammatory agents against skin diseases involving keratinocytes inflammation. Although in general, the availability, convenience, cost and time effectiveness limit the use of cell lines, utilization of this reliable and reproducible cells, results in lower variability compared to keratinocytes from skin biopsies (Colombo et al., 2017).

1.4.2 Triggering of keratinocytes inflammation by TNF-α

Triggering of HaCaT cells with TNF-α is a current *in vitro* cell-based model used for anti-TNF-α activity screening in keratinocytes (HaCaT cells). This model involves treating cells with recombinant purified TNF-α before or after treatment with chemical compounds or herbal extracts and TNF-α antagonists (Cho et al., 2007; Coll et al., 2009; Reinartz et al., 1996; Saelee et al., 2011). Anti-TNF-α antibodies targeting specific signaling pathways, have been used efficiently for the management of immune-mediated inflammatory diseases including psoriasis (Jung et al., 2014; Kriegler et al., 1988; Udommethaporn et al., 2016).

1.4.3 UVB-induced inflammation

Chronic exposure of human skin to solar ultraviolet (UV) radiations may lead to several skin damages including sunburn, skin cancer, and oxidative stress as well as photoaging (Ichihashi et al., 2003; Magnani et al.). The UV region of the electromagnetic spectrum can be divided into three regions as follows (Fig. 1-5):

1) UVA, from 320 to 400 nm; reaches the deeper layers of the epidermis and dermis and provokes the premature aging of the skin (Ebrahimzadeh et al., 2014; Gallagher and Jones).
2) UVB, from 290 to 320 nm, reaches the epidermis and cause sunburn and erythema.
3) UVC, from 200 to 290 nm is altered by the atmosphere before reaching the earth (Dutra et al., 2002; Dutra et al., 2006; Mensah et al., 2001).

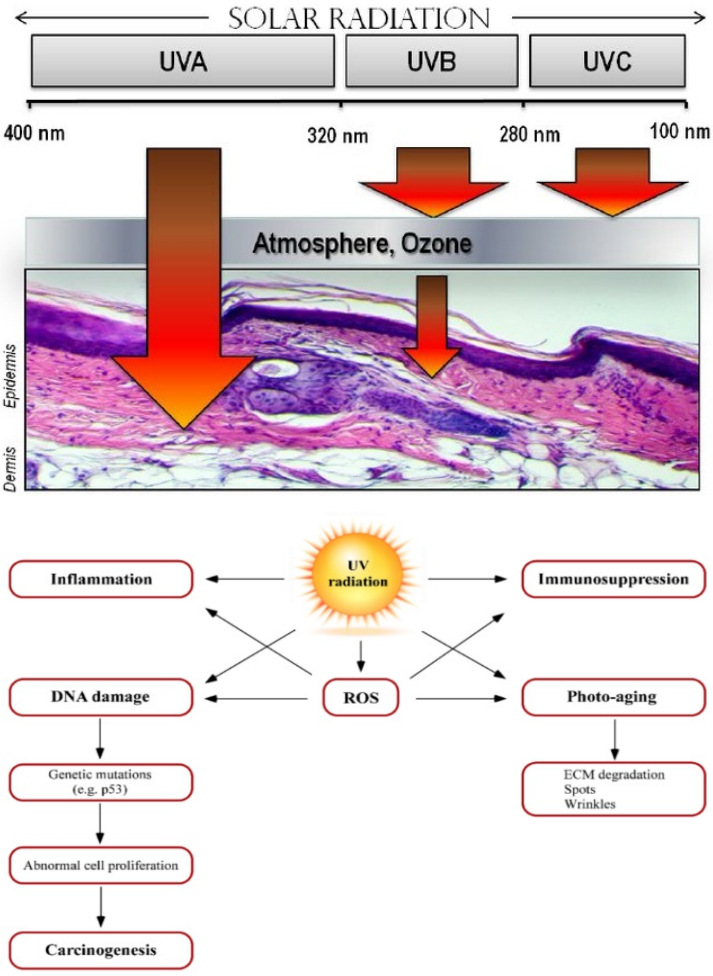

Fig. 1-5 UV penetration into the layers of the skin

Adapted from: (D'Orazio et al., 2013)

Human keratinocytes are natural target cells of UVB radiations. *In vitro*, exposure of these cells to high amounts of UVB is a model system which results in the synthesis of a large variety of cytokines such as TNF-α, IL-1α, IL-6, IL-8 and IL-10. Induction of the NF-κB activity has been demonstrated as one of the main factors involved in the process of inflammation, in human keratinocytes after UVB-irradiation (Fig. 1-6) (Grandjean-Laquerriere et al., 2005).

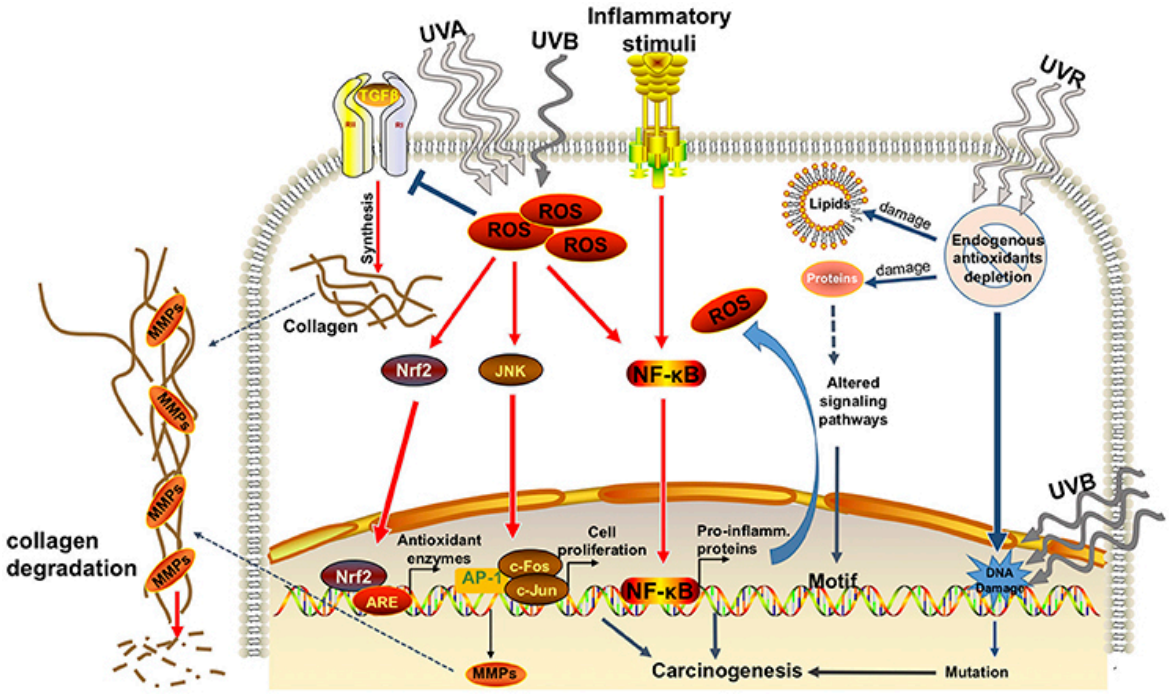

Fig. 1-6 UVB-induced NF-κB activation in keratinocytes
Adapted from: (Dunaway et al., 2018)

1.4.4 PMA-induced inflammation

PMA or TPA (phorbol-12-myristate 13-acetate) is used as stimulating factor in an *in vitro* model of skin inflammation and is known to increase the production of intracellular IL-1α and IL-8 in keratinocytes at noncytotoxic concentrations (Magcwebeba et al., 2012; Wilmer et al., 1994). In response to this exogenous stimulus, human keratinocytes produce soluble mediators that are important in primary contact irritancy including cytokines that are associated

with proinflammatory properties (e.g. TNF- *a*) and chemotaxis (IL-8) through the activation of NF-κB signaling (Wilmer et al., 1994; Xu et al., 2017). The qualitative and quantitative changes in selected intracellular and secreted cytokines have been evaluated by Wilmer et al, in human keratinocyte cultures in response to non-sensitizing contact irritants containing PMA (Wilmer et al., 1994).

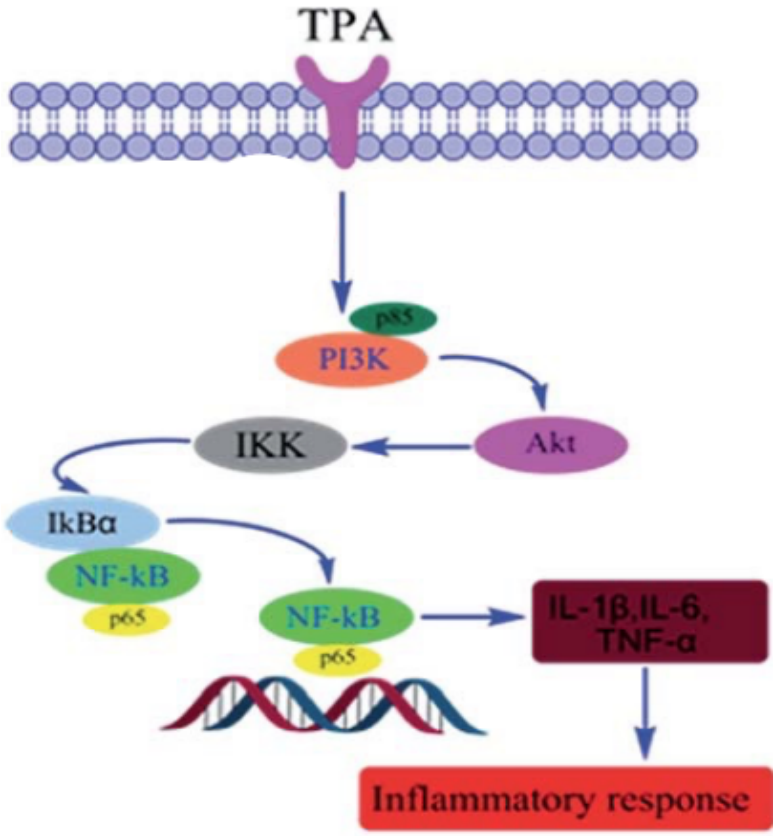

Fig. 1-7 TPA-induced signal transduction pathways

Adapted from: (Xu et al., 2017)

1.5 Inhibitors of NF-κB signaling as therapeutic agents against skin inflammation

Given the significance of inflammation in a variety of common skin diseases, anti-inflammatory drugs are currently being used in therapeutic regimens, especially in psoriasis and dermatitis (Brown and DuBois, 2005; Cargill et al., 2007; Rosbotham et al., 1994). With an established role in inflammation and apoptosis, NF-κB provides a novel target for chronic inflammatory disorders. Numerous compounds have already been shown to inhibit the NF-κB pathway, acting on different molecular steps in the complex machinery of the NF-κB signaling (Gilmore and Herscovitch, 2006). Development of more targeted therapies could potentially limit side effects seen with the current treatments. These include an upstream blockade by specifically targeting the IKK complex or IκB degradation, or interrupting translocation and transcription of NF-κB, a process further downstream at the nuclear level (Wong and Tergaonkar, 2009). A more complete understanding of the interaction between NF-κB and the psoriasis phenotype may lead to effective therapies, possibly directed toward specific cell types or steps within the NF-κB pathway. Several studies have demonstrated baseline elevations of NF-κB activity in lesional and non-lesional psoriatic skin with reduction in NF-κB levels following systemic and localized treatments e.g. etanercept and bortezomib (Heenen and Simonart, 2006; Tung et al., 2002; Zollner et al., 2002).

1.6 Natural products for skin inflammation

According to world health organization (WHO), 70-80% of the world population relies on traditional medicine, mostly plant-based drugs, for their primary healthcare needs (Valery, 2007). Since ancient times, natural products have been used successfully in Europe and Asia for the treatment of skin diseases (Bedi and Shenefelt, 2002). The worldwide use of approximately one-third of all traditional herbal remedies in management of dermatologic

conditions has been reported (Mantle et al., 2001). The traditional crude form of remedy has emerged as standardized herbal extracts and their formulations. Some available scientific literature has revealed the skin anti-inflammatory effects of plants such as *Silybum marianum*, *Arctium lappa, Rhus coriaria, Harpagophytum procumbens*, *Santalum album, Avena sativa, Cydonia oblonga, Pinus eldarica, Aloe barbadensis, Matricaria chamomilla* and *Foeniculum vulgare* (Behnammanesh et al., 2015; Chan et al., 2011; Khalilpour et al., 2019; Lin et al., 1996). The ethnopharmacological approaches are aimed at identifying potential new drugs from plant sources, and it is apparent that a variety of plants show or have the potential to show activities relevant for the usage against skin inflammatory conditions.

Plant-derived phytochemicals are an important and promising group of potential anti-inflammatory agents because of their low toxicity and apparent benefit in acute and chronic diseases (Chung et al., 2007; Mengoni et al., 2011). Natural substances have been also recently considered as potential sunscreen resources because of their absorption in the UV region and their antioxidant activity (Bonina et al., 1996; Liu et al., 1996). There is now an increasing body of evidence that the use of sunscreen is not entirely safe for photoprotection (Vainio and Bianchini, 2000).

Natural products are therefore important sources to search for new active compounds. This offers the possibility of discovering new biological mechanisms, to obtain new active molecules, and to study their structure-function relationships in order to develop more active drugs and to avoid side effects. Furthermore, if the natural sources are common and widely occurring, it might be possible to produce a high quantity at a low price (Farasat et al., 2014; Mirshafa et al., 2013). Green tea polyphenols, *Aloe barbadensis* extract, aromatic compounds isolated from *lichens*, glycosides of *aesculin* and *Murraya koenigii* leaf essential oil are examples of natural substances evaluated for their sunscreen specifications (Patil et al., 2010). Anti-psoriatic or anti-dermatitis properties of several herbs including *Aloe vera*, *Spinacia*

oleracea, Plecranthus amboinicus, *Mahonia aquifolium*, *Vitis vinifera* L., *Cannabis sativa* L., *Lavandula multifida*, *Hypericum perforatum* L. *Leontopodium alpinum* have been reported (Dobner et al., 2004; Sangiovanni et al., 2019a; Sangiovanni et al., 2019b; Siddiqui et al., 2019; Syed et al., 1996; Vijayalakshmi et al., 2019).

1.6.1 Rhus coriaria L.

1.6.1.1 Taxonomic classification

Kingdom:	Plantae (Plants)
Subkingdom:	Tracheobionta (Vascular plants)
Superdivision:	Spermatophyta (Seed plants)
Division:	Magnoliophyta (Flowering plants)
Class:	Magnoliophyta (Dicotyledons)
Subclass:	Rosidae
Order:	Sapindales
Family:	Anacardiaceae
Genus:	*Rhus*
Species:	*coriaria*

1.6.1.2 Botanical description

Rhus coriaria L. (*R. coriaria*) is a traditional medicinal herb belonging to the family of Anacardiaceae, growing to 1-3 m high as a small tree, with pinnately compound leaves and greenish-white flowers (Mozaffarian, 2013; Peter, 2006; Shabbir, 2012). Well-known as

sumac, *R. coriaria* belongs to the *Anacardiaceae* family and genus *Rhus* which covers over 200 species throughout the world (Morshedloo et al., 2018; Peter, 2006).

R. coriaria is also known as Sicilian Sumac, Elm-Leaved Sumach, Tanner's Sumach (Beltsville, 2007; sumac, 2014). The term *sumac* is derived from the Syriac word “sumâqâ”, meaning red. It is a common name belonging to the *Rhus* genus. The genus *Rhus* has been introduced in tropical and temperate non-agriculturally viable regions. *Rhus* is considered a medicinal herb, with several applications in different cultures and geographical locations (Jalal Pourahmad, 2010).

Tanner's Sumach is a wild herb native to the Mediterranean region and is widespread from the Canary Islands, southern Europe, Turkey and Middle Eastern countries to Afghanistan (Beretta, 2009; Candan and Sokmen, 2004b; Jalal Pourahmad, 2010). *R. coriaria* is an annual plant which can attain small tree or a shrub height from 1 to 3 meters. It can grow 1000 meters above sea level and is usually found in stony places. It grows on dry rocky slopes in forests and in mountainous areas, with high tolerance of temperatures as low as -20 °C.

Sumac has alternate and odd-pinnate leaves. The surface of the leaf is dark green above and gray below, with 4-8 pairs of opposite toothed leaflets (Mills, 2010). The leaf is 15-20 cm in length and 1.5-3 cm in width (Behnammanesh et al., 2015; Pharmacy, 2014). The flowers are small, inconspicuous greenish-white and unisexual. They are clustered in large male and female panicles. Male panicles are 25 cm long and female panicles are 15 cm long with conical, apical and axillary buds. The petals are white, ovate and oblong and sepals are green rounded-ovate. The plant flowers in northern hemisphere in June and July, and the fruits are ripe in September and October. Sometimes a secondary bloom occurs in the fall (Khalilpour, 2015). The fruit is small and brownish-purple or drupe, with red seeds that are kidney-shaped or spherical (Behnammanesh et al., 2015; Mills, 2010).

Fig. 1-8 *Rhus coriaria* L. fruits

Fig. 1-9 Dried fruits of *Rhus coriaria* L.

1.6.1.3 Chemical constituents

Recent phytochemical studies of *R. coriaria* have proved its richness in strong antioxidants called tannins (Pourahmad et al., 2010). The leaves contain up to 25-33% tannins. Hydrolysable tannins, condensed tannins and gallic acid derivatives have been found. The hydrolysable gallotannins have not been structurally characterized by Nuclear magnetic

resonance or physicochemical mass spectrometry. They have been extensively used in tanning leather (Mestres, 2004; Tang et al., 2005). Gallic, protocatechuic, linolenic, p-OH-benzoic, and vanillic are the most abundant phenolic acids found in the leaves of this herb. *In vitro* assays indicated that gallic acid may be considered among the active principles of sumac (Franziska Ferk, 2007). Anthocyanins like cyanidin, peonidin, pelargonidin and petunidin have also been reported in the leaves of *R. coriaria.* Other flavonoids detected in this herb are quercetin and kaempferol glycosides (Panico et al., 2009b; Zalacain A, 2003).

1.6.1.4 Ethnopharmacological approach

The brown to red fruits of *R. coriaria* are used as a very popular spice in food production with a sour lemon taste. The red fruits have an extensive range of applications in Persian traditional medicine such as treatment of diarrhea, hemorrhoids, gout and decreasing effect on cholesterol, uric acid and blood sugar levels (Abu-Reidah et al., 2014; Candan, Ferda, 2003; El Hasasna et al., 2016; Kosar, 2007; Mohammad Moazeni, 2012; Mozaffarian, 2013; Pourahmad et al., 2010; Rayne and Mazza, 2007; Shabbir, 2012; Sierra Rayne 2007).

The traditional use of *R. coriaria* fruits to heal skin injuries and disorders including burns, wounds and eczema has been also recorded (Altundag and Ozturk, 2011). However, the mechanism of action of *R. coriaria* is poorly understood.

Historically, *R. coriaria* has been found to possess remarkable medicinal value (Khalilpour, S. et al., 2018; Khalilpour et al., 2019; Mohammad Moazeni, 2012). The leaves and berries of this herb have been used extensively as remedies in folk medicine (Khalilpour, 2015; Khalilpour et al., 2017; Panico et al., 2009a). The red fruits of *R. coriaria* that contain one seed are commonly consumed in Middle Eastern countries, especially in Turkey, as spice (Beretta, 2009; Jalal Pourahmad, 2010; Khalilpour et al., 2019; Kosar, 2007). The ground dried drupes of this herb with salt have a sour, lemony taste and are usually used in salad and as a

condiment sprinkled over the traditional Turkish cuisine, kebab. The citric acid and malic acids in *R. coriaria* are responsible for the sour taste (pH=2.5) of the spice (Mohammad Moazeni, 2012).

Several studies have been focused on *R. coriaria* fruits and leaves. The leaves with their high content of tannins have been used as a tanning agent; berries have a diuretic and antimicrobial effect and have been used for wound healing, fever reduction, and intestinal discomforts (Behnammanesh et al., 2015; S and G, 2007). Reports from the Iranian traditional medicine have shown an athero-protective effect of this herb (Jalal Pourahmad, 2010).

Studies of the biological activity of *R. coriaria* leaves have demonstrated that they have significant antimicrobial, antifungal, and antiviral activities (Sierra Rayne 2007). Studies on the methanolic extract of the leaves and the ethanolic and water extract of the fruits have shown antibacterial effects against twelve different species of bacteria and nine tested fungal strains (Iauk et al., 1998; Nasar-Abbas and Halkman, 2004; Zhaleh et al., 2018).

The neuroprotective effect of the ethanol extract of fruits prepared using maceration has been successfully *in vitro* and *in vivo* tested by Khalilpour et al.(Behnammanesh et al., 2015; Khalilpour, 2015; Khalilpour et al., 2017). The fruit ethanol extract of *R. coriaria* inhibited the ischemia in an optic nerve degeneration model in mice (Khalilpour, 2015; Khalilpour, S. et al., 2018). This extract also protected the retinal ganglion cells in an *in vitro* model of retinal degenerations (Khalilpour, 2015; Khalilpour et al., 2017).

The antioxidant properties of *R. coriaria* have been investigated by different experiments. Food products such as stored sunflower and peanut oil were tested for stabilizing with methanolic extracts of the seeds of *R. coriaria* and the antioxidant properties were reported (Candan and Sokmen, 2004a; Ozcan, 2003a, b). Ozcan *et al*. (2003) reported that the extract of *Rhus coriaria* L. fruits showed antioxidant activity (Ozcan, 2003a). The radical

scavenger properties of this extract have been shown in cell-free systems. For instance, in the xanthine oxidase system the extract has exhibited an inhibitory effect on superoxide anion formation and lipid peroxidation (Candan, F, 2003; Candan and Sokmen, 2004b). The water extract of fruits of *Rhus coriaria* L. has demonstrated a hepatoprotective effect against oxidative stress in isolated hepatocytes from Sprague–Dawley rats.

Beretta *et al*. (2009) investigated the cardiovascular protective activity of hydro-alcoholic extract of *R. coriaria* leaves and the gallotannin fraction in isolated rabbit heart. Limited post-ischemic myocardial injury was demonstrated (Beretta, 2009). The vasorelaxant ability of the extract was also showed in isolated thoracic aorta of rabbit (Beretta, 2009). The antioxidant potency of gallic acid as an active compound of the extract of *R. coriaria* fruit was compared with vitamins C and E and was found to be 50 times more protective (Franziska Ferk, 2007).

The anticarcinogenic and tumor formation and growth inhibitory effect of tannins of *R. coriaria* leaves has also been exhibited in animal models. Studies on sumac extracts have also demonstrated other bioactivities of this herb, such as antifibrogenic (Lee, 2003), anti-inflammatory, antimalarial (Ahmed et al., 2001), antithrombin, antiatherosclerosis (Zargham, 2008) properties, and astringent potency (Behnammanesh et al., 2015). Sumac can be used externally against burns, weeping ulcers, wounds, eczema, and internally against bleeding of the gastrointestinal tract, diarrhea, enteritis, and colitis, because of its high tannin content. Despite this information, the potential modulatory effect of *R. coriaria* fruit extracts on epidermal keratinocytes inflammation has not been reported so far.

1.6.2 *Echium amoenum* Fisch.

1.6.2.1 Taxonomic classification

Kingdom:	Plantae (Plants)
Clade:	Angiosperms
Clade:	Eudicots
Clade:	Asterids
Order:	Boraginales
Family:	Boraginaceae
Genus:	*Echium*
Species:	*amoenum*

1.6.2.2 Botanical description

Echium amoenum Fisch. (*E. amoenum*, Iranian Borage) is an annual herb belonging to the family of Boraginaceae which is native to Europe and Mediterranean region and also grows in the northern mountains of Iran at an altitude ranging from 60-2200m (Abolhassani, 2010; Behnammanesh et al., 2015; Mehrabani et al., 2004; Ranjbar et al., 2006). Flowers, stems, roots and leaves from this plant are used for medicinal purposes (Behnammanesh et al., 2015). *Echium* genus has four species, however only *Echium vulgare* and *Echium amoenum* have been used in traditional medicine of Iran for a long time (Mehrabani et al., 2004).

Fig. 1-10 *Echium amoenum* Fisch**.** Plant

Fig. 1-11 Dried flowers of *Echium amoenum* Fisch**.** Plant

1.6.2.3 Chemical constituents

E. amoenum is considered as a promising source of bioactive compounds with various beneficial biological activities. It has been reported that the petals of *E. amoenum* is rich in rosmarinic acid (RA), a potent antioxidant (Abolhassani, 2010), anthocyanins including delphinidin and cyanidin glicosides, gamma-linolenic acid (GLA), alpha-linolenic acid (ALA), and flavonoids (Ranjbar et al., 2006). Cyanidin 3-glucoside, which is present in petals of this herb, is considered the most common anthocyanin (Behnammanesh et al., 2015).

1.6.2.4 Ethnopharmacology, experimental pharmacology and clinical data

The petals of *E. amoenum* are traditionally either brewed or boiled in water before drinking. The advantages of this herb have initially been discovered by the Romans, 300 B.C. (Behnammanesh et al., 2015). The flowers and the leaves has been used as an anti-inflammatory, antioxidant, antibacterial, analgesic, antiviral, anxiolytic, antidepressant and recently possible protective factor against cancer (Ranjbar et al., 2006; Sayyah et al., 2012). The antioxidant activity of *E. amoenum* petals aqueous extract has been investigated in humans (Ranjbar et al., 2006). The results showed a significant reduction in blood lipid peroxidation after 14 days of treatment with the extract (7 mg/kg). It is suggested that the flavonoids in this plant play an important role in its potential antioxidant activity (Sayyah et al., 2012). In a human study, effects of borage oil rich in gamma-linolenic acid on atopic dermatitis was evaluated. Treatment for two weeks with this oil showed significant effect against erythema and itch and reduced trans-epidermal water loss (Kanehara et al., 2007).

This herb also showed neuroprotective effect and attenuated the brain superoxide levels resulted from blocking apoptosis-inducing factor release in mitochondria (Behnammanesh et al., 2015). Flavonoids with anti-inflammatory, antioxidant, and gastroprotective effects are widely distributed in the plant kingdom (Talhouk et al., 2015). The influence of usage of the *E. amoenum* petal extract on the oxidative stress of healthy subjects has been studied. It has been indicated that the concentration of reactive oxygen species (ROS) markedly decreased after consumption of *E. amoenum* (Ranjbar et al., 2006). Its antioxidant property acts as a free radical scavenger which protects cells from free radical exposure and cellular damage (Ranjbar et al., 2006)(Behnammanesh et al., 2015).

Various substances have been suggested to act as antioxidant in this plant. Numerous phenolic antioxidants such as flavonoids, rosmarinic acid, tannins, coumarins, xanthenes, and

procyanidins have been shown to scavenge radicals in a dose-dependent manner (Uysal et al., 2015; Yao et al., 2004). Rosmarinic acid, an important constituent of *E. amoenum*, is an ester of caffeic acid and 3,4-dihydroxyphenylacetic acid. It is commonly found in species of the Boraginaceae and the subfamily Nepetoideae of the Lamiaceae (Mehrabani et al., 2004). There are number of reports on the antioxidant activities of rosmarinic acid which all confirm that rosmarinic acid has strong antioxidant activity even higher than vitamin E (Englberger et al., 1988; Sanbongi et al., 2004).

1.6.3 *Arctium lappa* L.

1.6.3.1 Taxonomic classification

Kingdom:	Plantae
Clade:	Angiosperms
Clade:	Eudicots
Clade:	Asterids
Order:	Asterales
Family:	Asteraceae
Genus:	*Arctium*
Species:	*lappa*

1.6.3.2 Botanical description

Arctium lappa L. (*A. lappa,* Burdock) is a medicinal herb which has been used therapeutically in China, Europe, North America and Asia for hundreds of years (Lin et al., 1996; Lou et al., 2010). Burdock's stem has multiple branches, each of which is topped by many crimson-violet flowerheads that produce the famous "burrs" that give burdock its name (Fig. 1-12). The biennial grows to three to nine feet in height. The root (the medicinal part of

the plant) has a very hard, horny, brown, longitudinally wrinkled bark and a white interior. The plant is readily grown from seed in moist, rich soil and full sun. Roots can be harvested the fall of the first year of growth or the spring of the second year (Fig. 1-13)(Kemper, 2010).

Fig. 1-12 *Arctium lappa* L. Plant

Fig. 1-13 Dried root sample of *Arctium lappa* L.

1.6.3.3 Chemical constituents

The useful properties of burdock are mostly due to the lignans arctigenin, arctiin, diarctigenin, but burdock roots contain a variety of constituents which include tannins, inulin, sulfur-containing polyacetylenes, caffeic, acetic, butyric, chlorogenic, gamma-guanidino-n-butyric, isovaleric, linoleic, linolenic, myristic, oleic, palmitic, proprionic, stearic acids (Chan et al., 2011; Ito et al., 1986; Kemper, 2010; Wang and Yang, 1993). Characterization of burdock lignans has been performed by Liu et al. using single quadrupole mass spectrometry. Liu et al. reported the isolation and identification of arctiin in burdock leaves (Liu et al., 2005). Ferracane et al, identified some phenolic components for the first time from this plant. Presence of caffeic acid, chlorogenic acid and cynarin in the seeds of this herb has been reported by Ferracane et al (Ferracane, 2010). Arctiin, luteolin and quercetin rhamnoside have been detected in burdock roots. Phenolic acids, quercetin, quercitrin and luteolin were identified in burdock leaves (Ferracane et al., 2010).

1.6.3.4 Ethnopharmacology, experimental pharmacology and clinical data

Burdock has been also used in several countries throughout history to treat problems arthritis and skin disorders. This plant is one of the key herbal ingredients in the twentieth-century cancer remedies (Lin et al., 1996). It is used topically for furunculosis, sciatica, radiculitis and a variety of dermatologic conditions such as eczema, ulcers, skin eruptions, scrapes, burns and wounds (Chan et al., 2011; Lou et al., 2010). According to traditional Chinese medicine, these pathological conditions are mainly due to the increase of toxin in the body. Burdock root is effective in eliminating heavy metals from the body, draining toxins, detoxifying blood and stimulating blood circulation to the skin surface, improving the skin texture and curing dermatologic disorders (Chan et al., 2011; Kemper, 2010). The Chinese used

burdock to treat upper respiratory infections. In fourteenth-century Europe, a combination of burdock and wine was used to treat leprosy. In traditional medicine, the dried root of burdock is the major part used, as a tincture, decoction, extract, and oil (Kemper, 2010). Burdock root has a use as a diuretic and pathogenic agent for treatment of gout, rheumatism, and several dermatologic conditions. The consumption of burdock root tea in the improvement of lipid profile and blood pressure status in patients with knee osteoarthritis, has been suggested by Maghsoumi-Norouzabada et al.(Maghsoumi-Norouzabad et al., 2016). The pharmacological properties recorded for the roots of this herb are as follows: antioxidant and free radical scavenging activity, neuroprotective activity; anti-anaphylaxis, antiviral activity; antitumor; immuno-modulator; antihypertension; antidiabetic and anti-obesity (Chan et al., 2011). A clinical study indicated the hepatoprotective, anti-inflammatory and free radical scavenging activities associated to presence of caffeoylquinic acid derivatives in the roots of this herb (Lin et al., 1996). It has been reported that the chemopreventive effects of burdock seeds are attributed to lignans such as arctiin and arctigenin (Hirose et al., 2000). Awale et al reported the antiproliferative and apoptotic effects of lignans from this herb on leukemic cells (Awale et al., 2006). Mastumoto et al described the antitumoral properties of arctigenin on pancreatic cancer cell lines (Matsumoto et al., 2006).

CHAPTER TWO

2. Aims of the study

The anti-inflammatory potential of selected plant extracts at the skin level has been investigated by *in vitro* studies using human HaCaT keratinocytes. Different extracts from *R. coriaria* fruits, *E. amoenum* flowers, *A. lappa* roots were prepared using different extraction methods. The goal of the present work was to deduce whether any of the investigated herbal extract might be candidate agents for use as anti-inflammatory agents in attenuating different triggers for keratinocytes as might occur in skin inflammatory condition such as Psoriasis or dermatitis. The influence of the extracts on IL-8 release were assessed following cytotoxicity assays in TNF-α stimulated HaCaT cells. The aim of this screening was to select the most active extracts. *R. coriaria* showed the most promising effects and selectively two types of preparations from the fruits of this herb (macerated ethanol (mERC) and ethanol water (EWRC) extracts) were selected for further studies.

The aim of second phase of this study was to determine the mechanisms by which the *R. coriaria* extracts can act as IL-8 inhibitor, in TNF-α stimulated keratinocytes. The effect of the selected mERC and EWRC on VEGF, MMP-9, ICAM-1 secretion and NF-κB signaling, in TNF-α-stimulated HaCaT cells were measured. Phytochemical studies have been carried out to characterize the active extracts and to quantify their main constituents. This research is concerned with evaluating the properties of mERC and EWRC, particularly in relation to UVB-induced skin inflammation in human keratinocytes. This was investigated by exposing HaCaT cells in culture to an insult of UVB 40 mJ/cm^2 and determining the influences of mERC and EWRC.

This research work also aimed to develop and evaluate other preparations of *R. coriaria* which could be suitable for topical application. The goal of this section was to evaluate the

efficacy of lipophilic extracts of *R. coriaria* which have higher skin permeability. In order to determine the most suitable active extracts of *R. coriaria* fruits, for topical anti-inflammatory activity test, the lipophilic extracts were prepared and tested in PMA-induced inflammation in HaCaT. The general conclusion from the studies was that *R. coriaria* is a very effective anti-inflammatory agent because of its ability to regulate the release of inflammatory mediators in human keratinocytes inflammation. This study is worthy enough to be expanded in further investigation level *in vivo*.

CHAPTER THREE

3. Materials and methods

3.1 Materials

Acetone	Carlo Erba, Milan, Italy
Bradford (Bio-Rad),	Bio-Rad laboratories, USA
Britelite Plus reagent	PerkinElmer Inc. Massachusetts, USA
CE, Cyanidin-3-O-glucoside equivalent	Sigma-Aldrich, Milan, Italy
DCM, Dichloromethane	Carlo Erba, Milan, Italy
DMEM F12, Dulbecco's modified eagle medium F12	Gibco, Life Technologies, Monza, Italy
DMSO, Dimethyl sulfoxide	Sigma-Aldrich, Milan, Italy
EGCG, Epigallocatechin-3-gallate	Sigma-Aldrich, Milan, Italy
EDTA	Gibco, Life Technologies, Monza, Italy
Ethanol	Carlo Erba, Milan, Italy
Ethyl acetate	Carlo Erba, Milan, Italy
FBS, Fetal bovine serum	Euroclone S.P.A., Milan, Italy
GAE, Gallic acid equivalent	Sigma-Aldrich, Milan, Italy
HaCaT, Spontaneously immortalized human keratinocyte line	Cell Line Service GmbH, Eppelheim, Germany
HRP-conjugated streptavidin	RayBio®, Norcross, GA

Human ICAM-1 ELISA kit	Peprotech, Rocky Hill, NJ, USA
Human MMP-9 ELISA kit	RayBio®, Norcross, GA
Human VEGF-165 ELISA Kit	Peprotech, Rocky Hill, NJ, USA
IL-8 enzyme-linked immunosorbent assay Kit	Peprotech, Rocky Hill, NJ, USA
LDH	Takara, Italy
L-glutamine	Gibco, Life Technologies, Monza, Italy
MTT, 3,4,5-dimethylthiazol-2-yl-2–5-diphenyltetrazolium bromide	Sigma-Aldrich, Milan, Italy
NF-κB (p65) transcription factor assay kit	Cayman
Nuclear Extraction Kit	Cayman Chemical Company Michigan, USA
Penicillin	Gibco, Life Technologies, Monza, Italy
Plasmid NF-κB-luc	Department of Internal Medicine-Cardiology, University of Ulm, Germany
Quercetin-O-glucoside	Sigma-Aldrich, Milan, Italy
Quercetin	Sigma-Aldrich, Milan, Italy
Resveratrol	Sigma-Aldrich, Milan, Italy
Streptomycin	Gibco, Life Technologies, Monza, Italy
TNFα, Tumor necrosis factor alpha	Peprotech, Rocky Hill, NJ, USA

TMB, 3,31,5,51-tetramethylbenzidine	Gibco, Life Technologies, Monza, Italy
Trypsin	Gibco, Life Technologies, Monza, Italy
Tween 20 emulsifier	Sigma-Aldrich, Milan, Italy
VEGF human ELISA kit	Peprotech, Rocky Hill, NJ, USA

3.2 Equipment and Apparatus

Autoclave	Fedegari, Italy
Balance	Fisher Scientific, Germany
Biosafety cabinet	Gelaire flow laboratories, Milan, Italy
Cell culture flasks (75 cm2)	Euroclone S.P.A., Milan, Italy
Centrifue	Eppendorf S.R.L Milan, Italy
Corning 96-well EIA/RIA plates	Sigma-Aldrich, Milan, Italy
Envision	PerkinElmer, United States
Eppendorf Tips	Euroclone S.P.A., Milan, Italy
Eppendorf tube 1.5 mL	Euroclone S.P.A., Milan, Italy
Filter paper	Whatman International Ltd., UK
-20 ˚C freezer	Samsung, Korea
-80 ˚C freezer	Angelantoni Industrie S.R.L., Italy

Freeze drier	Edwards Modulyo, UK
Grinder	Moulinex, Italy
Hemocytometer	BRAND GMBH, Germany
Incubator	Euroclone S.p.A., Milan, Italy
Inverted light microscope	Leitz labovert, Germany
LC-940 analytical/ semipreparative HPLC system	Agilent, Ex Varian, Leinì, Torino, Italy
Magnetic stirrers	VELP Scientifica S.R.L, Italy
Microcentrifuge tubes	Euroclone S.p.A., Milan, Italy
Microplate reader, Victor X3	Perkin Elmer, Walthman MA, USA
PIPETMAN G Starter Kit, P20G, P200G, P1000G	Gilson, Milano, Italy
Refrigerator	Samsung, Korea
Round bottom flasks	Pyrex, USA
Rotary evaporator	Heidolph WBECO, Germany
Shaker	Stuart Scientific, UK
UV light source (Triwood 31/36, W36, V230)	Helios Italquartz, Milan, Italy
Volumetric flasks	Pyrex, USA
Vortex mixer	Fisher Scientific, Italy
Water bath	Julabo Italia SRL, Milan, Italy

24-well cell culture cluster	DB FalconTM
96-well plates	DB FalconTM

3.3 Collection and authentication of the herbs

Our studies did not involve any endangered or protected species. *R. coriaria* fruit*, A. lappa* root *and E. amoenum* flower samples were purchased from a local market in Alborz region located 120 km northwest of Tehran, Iran. The *R. coriaria* sample was authenticated by the Herbarium Unit, School of Biological Sciences, University of Science Malaysia, where the voucher specimen was deposited (reference number 11525). *A. lappa* and *E. amoenum* samples were authenticated by the laboratory of Pharmacognosy at Università degli Studi di Milano.

3.4 Preparation of extracts

To prepare the extracts, three different methods were used: cold extraction, maceration or decoction. In all cases the dried fruits were ground with an electric grinder and 5 g of dried fruit powder were used. Cold extraction was performed with three different solvent or mixtures (50 mL each) using a double extraction method (6 h plus overnight incubation on a shaker at room temperature). After cooling, the insoluble material was removed by filtration (Whatman filter paper), the extraction solvent evaporated under reduced pressure (Heidolph WBECO, Germany) and the dry residues freeze-dried (Edwards Modulyo, UK). Maceration process was performed by 48 h incubation with ethanol (Khalilpour, 2015; Khalilpour et al., 2017; Khalilpour, Saba et al., 2018; Mercati et al.). In brief, the mixture of herbal material (5 g) and solvent (50 mL) was placed in a shaker for 48 h at room temperature and mixed gently using a magnetic stirrer. Then, the mixture was filtered, the extract was taken to dryness under reduced pressure. Using decoction method, the herb material was soaked in water for 2h and boiled at 85˚C for 1h and filtered for further use. The extracts were kept in sterile glasses at 2˚C until use.

R. coriaria extracts were prepared using cold extraction and maceration methods. Using cold extraction, the extracts were prepared in three different solvent or mixtures (ethanol-water 50:50, EWRC; ethanol, ERC; water, WRC). Maceration process was performed by 48h incubation in ethanol. At the end of this procedure an oily residue was obtained and further separated using centrifuge, obtaining the oil phase (oil-mERC) and the residue mERC. Lipophilic extracts of *R. coriaria* were prepared using cold extraction method. The solvents used for this extraction were acetone, dichloromethane and ethyl acetate, and the extracts were named as follows: ARC, DCMRC and EARC, respectively. The oil phases of ARC and EARC were separated and named as oil-ARC and oil-EARC respectively (Fig. 2-1).

E. amoenum extracts were prepared using the same methods. The extracts obtained using cold extraction method were as follows: ethanol-water 50:50, EWEA; ethanol, EEA; water, WEA. The product of maceration method was also an oily residue which was similarly separated in two phases of oil (oil-mEEA) and the residue (mEEA) (Fig. 1-2).

A. lappa root extracts were prepared using cold extraction in water and ethanol-water 50:50 and named respectively WEA and EWEA. Using decoction method, the extract named as DWEA (Fig. 2-3).

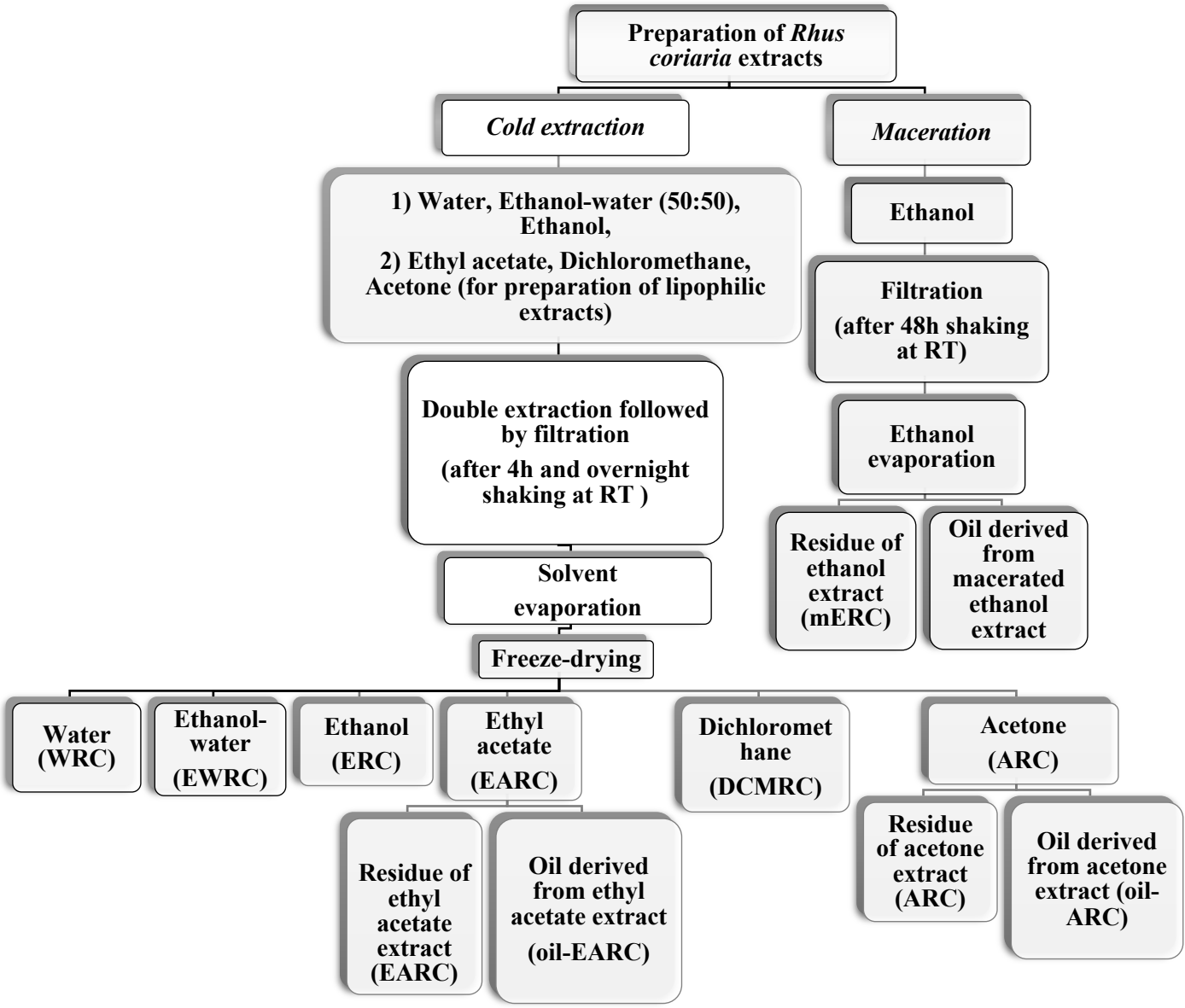

Fig. 3-1 Extraction of *R. coriaria* fruits

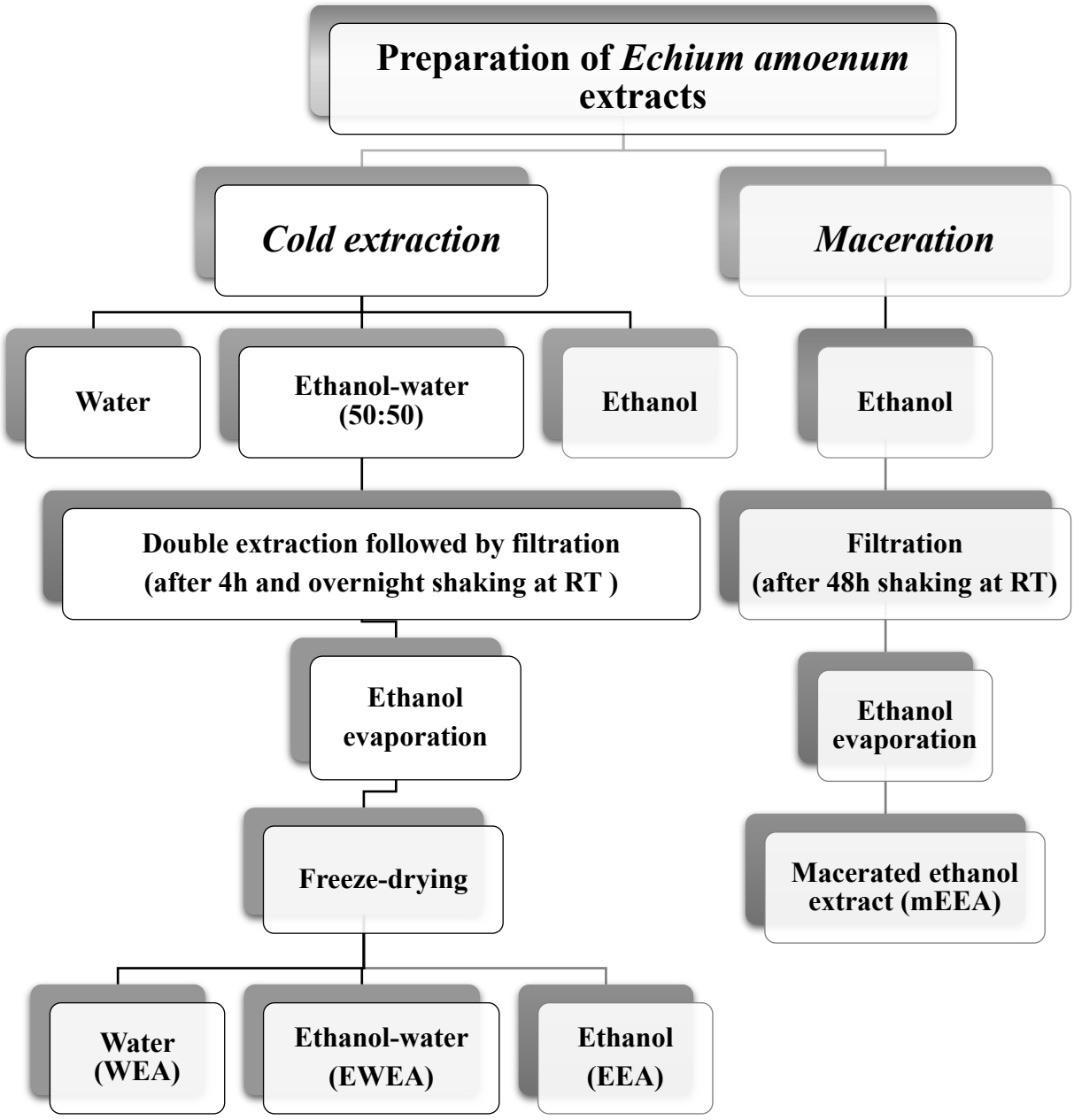

Fig. 3-2 Extraction of *E. amoenum* fruits

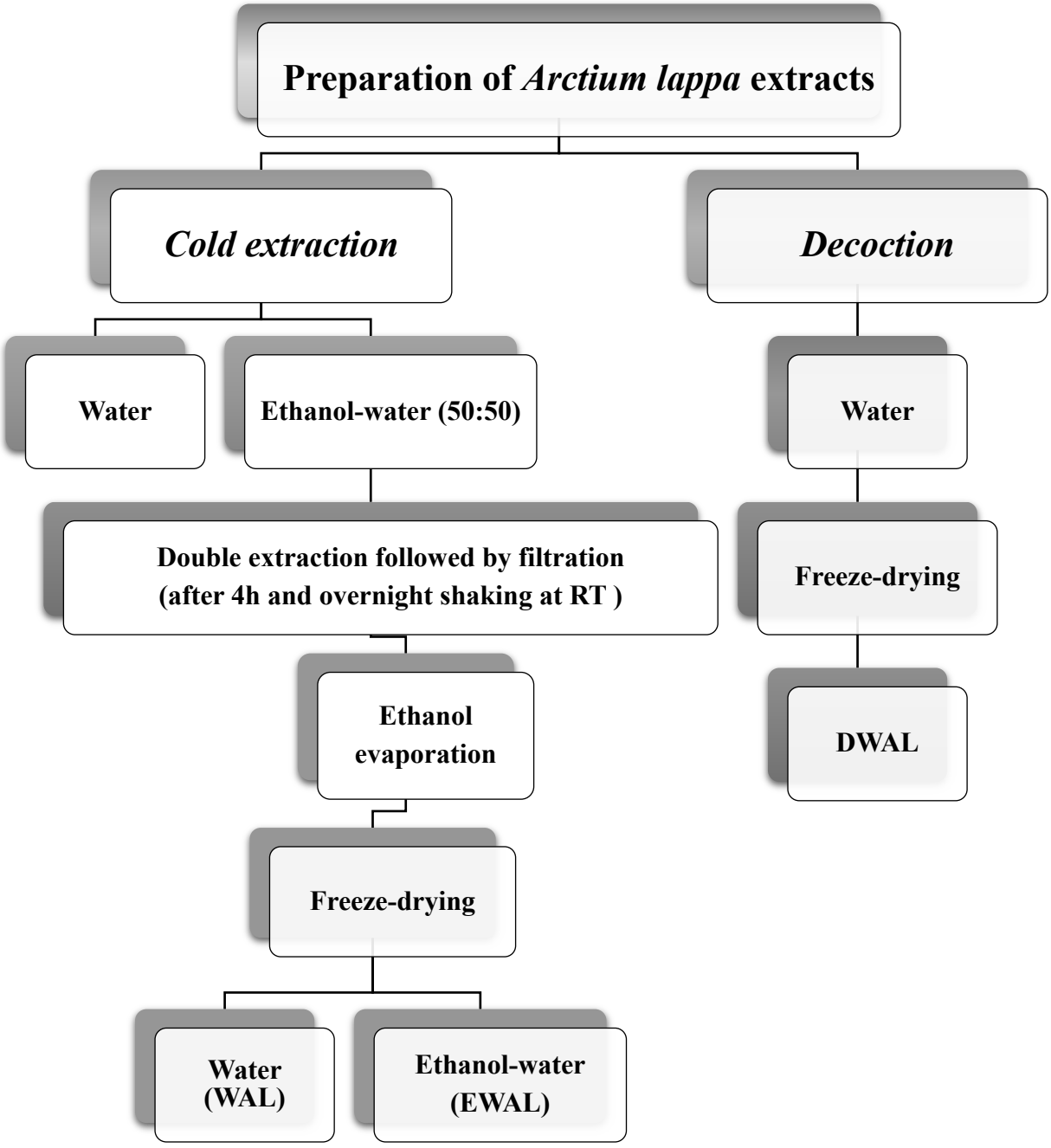

Fig. 3-3 Extraction of *A. lappa* roots

3.5. Cell culture

HaCaT cells, spontaneously immortalized human keratinocyte line (Cell Line Service GmbH, Eppelheim, Germany) were cultured in 5% CO_2 at 37°C in DMEM F12 (Gibco, Life Technologies, Monza, Italy (Boukamp, 1988 #5043). The media was supplemented with 2 mM

L-glutamine (Gibco, Life Technologies, Monza, Italy), 10% heat-inactivated fetal calf serum (FCS) (Euroclone S.P.A., Milan, Italy), penicillin (100 U/mL) and streptomycin (100 mg /mL) (Gibco, Life Technologies, Monza, Italy). The cells were seeded in 75 cm^2 flask (Euroclone S.p.A., Milan, Italy) at the density of 1.5×10^6 cells/flask, to allow the cell line growth. Then they were sub-cultured every four days, at 80-90% of confluence. The cells were detached from the flask using tripsin-EDTA 0.25% (Gibco, Life Technologies, Monza, Italy), counted and replaced in a new flask. The remaining cells were seeded in 24-well plates (DB FalconTM) at a density of 5.7×10^3 cells⁄cm^2 for the biological tests.

3.6. Cell treatment

After 72 hours of growth, HaCaT cells (CLS Cell Lines Service, GmbH, Eppelheim, Germany) were subjected to treatment with the extracts (*R. coriaria*, *E. amoenum* or *A. lappa* extracts) or individual compound and the pro-inflammatory stimulus (TNFα 10 ng/mL or PMA 10 or 100 nM) using DMEM medium supplemented with L-glutamine (2 mM), penicillin (100 U⁄mL) and streptomycin (100 mg⁄mL). Treatment times were varied depending on the parameters evaluated, from 6 (IL-8 release, NF-κB driven transcription) to 24 hours (VEGF, ICAM-1 and MMP-9 release). UVB assays were performed following 1-hour pre-treatment and 9 or 24 hours post-treatments with *R. coriaria* extracts. Prior to UV irradiation, cells were properly washed with PBS and covered with a thin layer of PBS. Dishes with keratinocytes were UVB irradiated (40 mJ/cm2) on ice-cold plates to eliminate UV-induced thermal stimulation. In parallel, non- irradiated cells were treated similarly and kept in the dark in a cell incubator. The viability of cells following 1h pre-treatment or 24 h post-treatment with mERC or EWRC were measured at 24 h after the UVB exposure using MTT assay. Then the viability of HaCaT cells were tested following 9 h post-treatment with different concentrations of mERC and EWRC, and the supernatants were collected and analyzed using IL-8 ELISA assay. At the

end of the treatment, medium or cell lysates were collected and stored at -20°C till the biological assay.

3.7. Cytotoxicity

HaCaT cells morphology was evaluated by light microscopy before and 6 or 24 h after their exposure to the tested substances. The influence of *A. lappa*, *R. coriaria* and *E. amoenum* extracts on cell viability was tested after 6 or 24-hours treatment in HaCaT cell lines. The extracts cytotoxicity was assessed measuring the mitochondrial succinate dehydrogenase activity by the 3,4,5-dimethylthiazol-2-yl-2-5-diphenyltetrazolium bromide (MTT) method (Sigma-Aldrich, Milan, Italy) as well as evaluating cells membrane damage by the lactate dehydrogenase release assay (Mishra et al., 2017). Briefly, 200 µL of MTT solution were added to each well containing the culture medium followed by incubation for 30-40 minutes up to the development of a violet color due to the formazan formation. 200 µL of a solution made by isopropanol:DMSO 90:10 was added in each well to extract formazan from the cells. The absorbance was read through spectroscopy at 570 nm (Envision, PerkinElmer, United States). The LDH assay was performed spectrophotometrically by measuring LDH content of the cell supernatant by the disappearance of NADH during the LDH-catalyzed conversion of pyruvate to lactate(Morliere et al., 1991; Tebbe et al., 1997). The effect of *R. coriaria* or *E. amoenum* extracts on TNF-stimulated cells was assessed at concentrations ranging from 1 to100 µg/mL, whereas that of *A. lappa* extracts was evaluated at 50 µg/mL. The effect of *R. coriaria* extracts on UV exposed cells was evaluated at 1-50 µg/mL, whereas that on cells stimulated by 10 or 100 nM PMA was evaluated at 50 or 25 µg/mL, respectively.

3.8. Measurement of IL-8 levels

For measurement of IL-8 secretion, HaCaT cells were grown in 24-well plates (6×10^5 cells/well) for 48 h; then, cells were treated with the pro-inflammatory stimulus (TNF-α at 10

ng/ml or PMA at 10 or 100 nM) and extracts/compound under study or vehicle alone (<0.2% DMSO). The cytotoxicity of extracts and reference compounds were evaluated by MTT assay, as previously described. After 6 h treatment, the supernatants were removed and stored at −80°C until the assay. Quercetin (10 μM) was used as a reference compound. In UV experiments, the samples were collected for IL-8 assays after 9 hours of treatment with *R. coriaria* extracts after the UVB exposure. IL-8 was quantified by an enzyme-linked immunosorbent assay Kit (Peprotech, Rocky Hill, NJ, USA) following the manufacturer's instructions.

3.9. NF-κB driven transcription

To evaluate NF-κB driven transcription, cells were plated in 24-well plates (6×10^5 cells/well). After 48 h cells were transiently transfected by lipofectamine with a reporter plasmid (NF-κB-luc, 250 ng/well); the plasmid contained the luciferase gene under control of a NF-κB responsive promoter (E-selectin promoter): NF-κB-luc. The plasmid NF-κB-luc was a gift of Dr. N. Marx (Department of Internal Medicine-Cardiology, University of Ulm, Germany). After 16 h, cells were placed in a medium deprived of FCS, and stimulated with TNF-α at 10 ng/ml or PMA 100 nM. mERC and EWRC were tested at 1, 5, 10, 25 and 50 μg/mL whereas the reference compound at 20μM. After 6 h the luciferase assay was performed using Britelite Plus reagent (PerkinElmer Inc. Massachusetts, USA) according to manufacturer's instructions; signal was read with Victor X3 (Perkin Elmer, Walthman MA, USA). Data were expressed considering 100% the luciferase activity related to the cytokine-induced NF-κB driven transcription. EGCG (20 μM) was used as a reference compound.

3.10. NF-κB nuclear translocation assay

To assess the effect of the extracts and individual compounds on the NF-κB (p65) nuclear translocation, HaCaT cells were plated at the density of 1.5×10^5 cells/mL in 100 mm

plates. After 48 h, cells were treated for 1 h with the pro-inflammatory mediators and the extracts/reference compound under study. Nuclear extracts were prepared using Nuclear Extraction Kit (Cayman Chemical Company, Michigan, USA) and stored at −20 °C until assayed. The same amount of total nuclear proteins (10 μg/well), measured by the method of Bradford (Bio-Rad), was used to assess the NF-κB nuclear translocation using the NF-κB (p65) transcription factor assay kit (Cayman) followed by spectroscopy at 450 nm, 0.1s (VictorX3, Perkin Elmer, Walthman MA, USA). Data were expressed considering 100% the absorbance related to the TNF-α-induced NF-κB nuclear translocation. EGCG (20 μM) was used as a reference compound. Similarly, UV-induced NF-κB nuclear translocation was performed after exposure of UVB in HaCaT cells.

3.11. Assessment of ICAM-1 and VEGF release

For measurement of ICAM-1 and VEGF release, HaCaT cells were grown in 24-well plates (6×10^5 cells/well) for 48 h; then, cells were treated with pro-inflammatory stimuli (TNF-α at 10 ng/ml) and extracts/reference compound under study. After 24 h treatment the supernatants were removed and stored at −80°C until the assay. Then the cytotoxicity of extracts and reference compounds were assessed by MTT test as previously described (Khalilpour et al., 2017; Khalilpour et al., 2019). The release of ICAM-1 and VEGF from HaCaT cells was quantified using two different high sensitivity human ELISA sets (Peprotech, Rocky Hill, NJ, USA) following the method described below. Briefly, Corning 96-well EIA/RIA plates from Sigma-Aldrich (Milan, Italy) were coated with the antibodies provided, overnight at 4°C. In all cases, 300 μl of samples were transferred in duplicate into wells at room temperature for 2 h. The results were detected by spectroscopy (signal read 450 nm, 0.1 s, by VictorTM X3) using biotinylated and streptavidin-HRP conjugate antibodies, evaluating 3,5,3,59-tetramethylbenzidine (TMB) substrate reaction. The quantification of analytes was

done using an optimized standard curve supplied with the ELISA sets. Results were expressed as pg/10^6 cells. Curcumin (20 μM) and EGCG (20 μM) were used as reference inhibitors of ICAM-1 and VEGF release, respectively.

3.12 Evaluation of MMP-9 secretion

Firstly, the HaCaT cells were grown in 24-well plates (6×10^5 cells/well) for 48 h. Then the cells were treated with pro-inflammatory stimuli (TNF-α at 10 ng/ml) and extracts under study or the reference compound. After 24 h treatment the supernatants were removed and stored at −80 °C until the assay. MMP-9 secretion from HaCaT cells was evaluated by ELISA set (RayBio® Human MMP-9 ELISA kit, Norcross, GA) using a precoated 96-well plate. 300 μl of samples were transferred in duplicate into wells at room temperature for 2.5 h. MMP-9 was detected by spectroscopy (signal read 450 nm, 0.1 s, by VictorTM X3) using biotinylated and streptavidin-HRP conjugate antibodies, evaluating TMB substrate reaction. The quantification of analytes was done using an optimized standard curve supplied with the ELISA set. Resveratrol (50 μM) was used as a reference compound. The data are expressed as pg/10^6 cells.

3.13. UVB irradiation system

The effect of UVB on viability of the HaCaT cells was tested following 1h pre-treatment or 24 h post-treatment with different concentrations of mERC and EWRC. Prior to UV irradiation, cells were properly washed with PBS and covered with a thin layer of PBS. Dishes with keratinocytes were UVB irradiated (40 mJ/cm^2) light source (Triwood 31/36, W36, V230, Helios Italquartz, Milano, Italy) on ice-cold plates to eliminate UV-induced thermal stimulation. Radiation time (about 50 seconds) was adjusted for each experimental day, measuring energy emission by LP 471 UVB probe (Delta OHM, Padova, Italy). After

irradiation, fresh serum-free medium was immediately added. In parallel, non-irradiated cells were treated similarly and kept in the dark in a cell incubator.

The viability of cells following 1h pre-treatment or 24 h post-treatment with mERC or EWRC were measured at 24 h after the UVB exposure using MTT assay. Then the viability of HaCaT cells were tested following 9 h post-treatment with different concentrations of mERC and EWRC, and the supernatants were collected and analyzed using IL-8 ELISA assay. UVB-induced NF-κB nuclear translocation was measured following exposure of HaCaT cells to UVB (40 mJ/cm^2) as above described and treated for 1 hour with increasing concentrations of mERC and EWRC (10-50 μg/ml).

3.14. HPLC-UV-DAD analysis

For analysis, mERC and EWRC extracts (50 mg) were solubilized in ethanol/water (10 mL) under sonication in a sonication bath at maximal power, until complete dissolution (30 min). The corresponding solutions were analyzed using an LC-940 analytical/semipreparative HPLC system (Agilent, Ex Varian, Leinì, Torino, Italy) equipped with binary pumps, autosampler, fraction collector, with a UV-DAD detector operating in the 200-400 nm range. Separations were done using a Kinetex™ Biphenyl chromatographic column (particle size 2.6 μm, pore size 100 Å, 100 × 4.6 mm, Phenomenex, Castel Maggiore, Bologna, Italy). Gradient solvents: 0.05% aqueous formic acid (A) and 0.1% formic acid in acetonitrile (B); gradient program: 5% B for 0-3 min, from 5% to 40% B in 47 min, 40% B for 10 min. Injection volume: 10 μL. Total flow rate: 1.6 mL/min. Column temperature: 25°C.

Total polyphenols, flavonoids and anthocyanidins were measured by comparison with calibration curves built using ethanol hydroalcoholic (30:70 v/v water/ethanol) solutions standard gallic acid (observation λ=270 nm), quercetin-O-glucoside (observation λ=350 nm) and cyanidin-3-O-glucoside (observation λ=510 nm) in the concentration ranges 0.050-1.0

mg/mL, 5-50 µg/mL and 0.35-5 µg/mL respectively. Results are expressed as percentage (% w/w) of gallic acid equivalent (GAE), quercetin-O-glucoside (QE) or cyanidin-3-O-glucoside equivalent (CE) in the extracts.

3.15. Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0 via one-way ANOVA followed by Bonferroni as post-hoc test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. stimulus alone. Results are the mean ± s.d of at least three individual experiments performed in duplicate or triplicate. The half maximal inhibitory concentration (IC_{50}) was calculated using GraphPad Prism 7.0.

CHAPTER FOUR

4. Results

4.1. Preparation of extracts and percent recovery

The extraction yields of *R. coriaria* fruit are reported in Tab.1. The WRC extract showed the highest yield of extraction (23.2%), while EWRC, ERC and mERC extracts had lower or comparable extraction yields (14.8%, 16.2%, and 15.2%, respectively).

Table 1 %Yield of different extracts of *R. coriaria* fruits

Extract	Yield of extracts (%)
Water extract (WRC)	23.2
Ethanol-water extract (EWRC)	14.8
Ethanol extract (ERC)	16.2
Macerated ethanol extract (mERC)	12.8

The % extraction yields of *E. amoenum* flowers are shown in Tab. 2. EWEA showed the highest yield of extraction (21.4%). The yields of extraction of WEA, mEEA and EEA were 9.8, 7.6, 6.2 % respectively.

Table 2 %Yield of different extracts of *E. amoenum* flowers

Extract	Yield of extracts (%)
Water extract (WEA)	9.8
Ethanol-water extract (EWEA)	21.4
Ethanol extract (EEA)	6.2
Macerated ethanol extract (mEEA)	7.6

Among the three types of *A. lappa* extracts, DWAL showed the highest (6.4%) yield of extraction (Tab. 3). WAL and EWAL showed lower yield of extractions, 2.78 and 1.4 % respectively.

Table 3 %Yield of different extracts of *A. lappa* roots

Extract	Yield of extracts (%)
Water extract (WAL)	2.78
Ethanol-water extract (EWAL)	1.4
Decoction water extract (DWAL)	6.4

4.2. Effect of R. coriaria extracts on IL-8 release by HaCaT cells stimulated with TNFα

The effect of the extracts on the viability of HaCaT cells was analyzed by MTT assay. Quercetin (10 μM), a known IL-8 inhibitor, was used as reference compound. None of the extracts was found to be toxic in HaCaT cells, but slight toxicity occurred after treatment with 100 μg/mL EWRC. Therefore, this concentration was excluded from further analysis on the biological effects of this extract (Fig. 4-1).

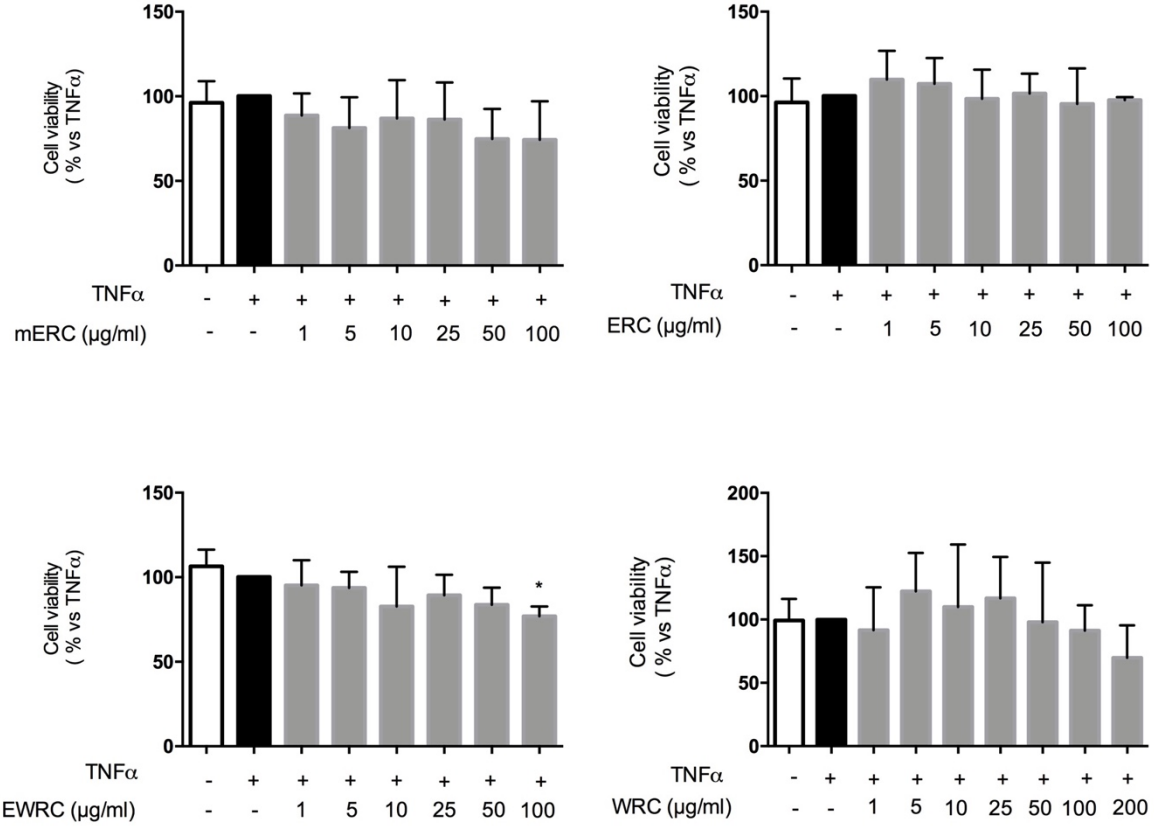

Fig. 4-1 Effect of 6 h treatment of *R. coriaria* fruit extracts on viability of TNF-α treated HaCaT cells. Results are the mean ± s.d. of at least three experiments performed in duplicate; * $p< 0.05$ vs. control (n=3).

The preliminary screening of the extracts on the TNF-α-induced IL-8 release in human keratinocyte HaCaT cells was performed. All the extracts differently inhibited IL-8 release, as depicted in Fig. 4-2. mERC was the most active extract, with significant inhibition ranging between 5 and 25 μg/mL (IC_{50}=3.15±1.14 μg/mL; mean ± s.d.); EWRC was active as well, which a doubled IC_{50} value (IC_{50}=6.61±0.55 μg/mL; mean ± s.d.).

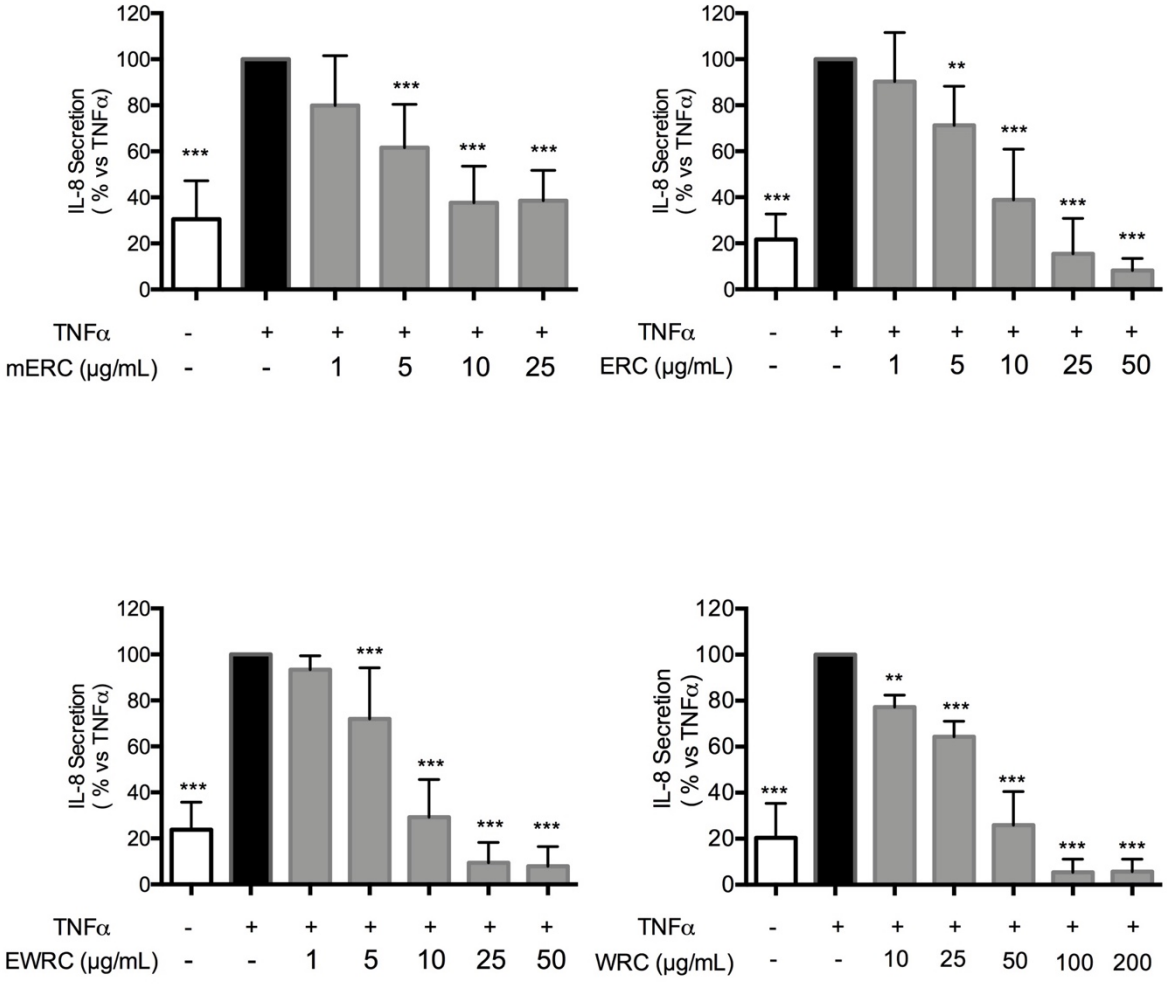

Fig. 4-2 *R. coriaria* fruit extracts inhibit IL-8 secretion in TNF-α treated HaCaT cells. Results are the mean ± s.d., of at least three experiments performed in duplicate. * $p< 0.05$, ** $p< 0.01$, and *** $p< 0.001$ vs. control (n=3).

The ERC effect was similar to EWRC, while WRC showed the lowest inhibitory activity (Tab. 2). IC_{50s} of the extracts under study were as follows: WRC > ERC > EWRC > mERC. Quercetin 10 µM was used as reference compound (~25% inhibition). Based on these results, mERC and EWRC were selected for further studies aimed to assess the inhibitory effect on release of pro-inflammatory mediators involved in skin diseases (Tab. 4)

Table 4 Summary of IC_{50s} (µg/mL) of different extracts of *R. coriaria* on IL-8 secretion

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Extract	IC_{50} (mean± s.d. ; µg/mL)
Macerated ethanol extract (mERC)	3.15±1.14
Ethanol-water extract (EWRC)	6.61±0.55
Ethanol extract (ERC)	7.15 ±1.13
Water extract (WRC)	26.87±2.96

4.3. Effect of E. amoenum extracts on IL-8 release by HaCaT cells stimulated with TNFα

The cytotoxic effects of *E. amoenum* extracts were evaluated in HaCaT cells. WEA, EEA, mEEA didn't show cytotoxicity, while EWEA was cytotoxic at concentrations 5-100 µg/mL (Fig 4-3).

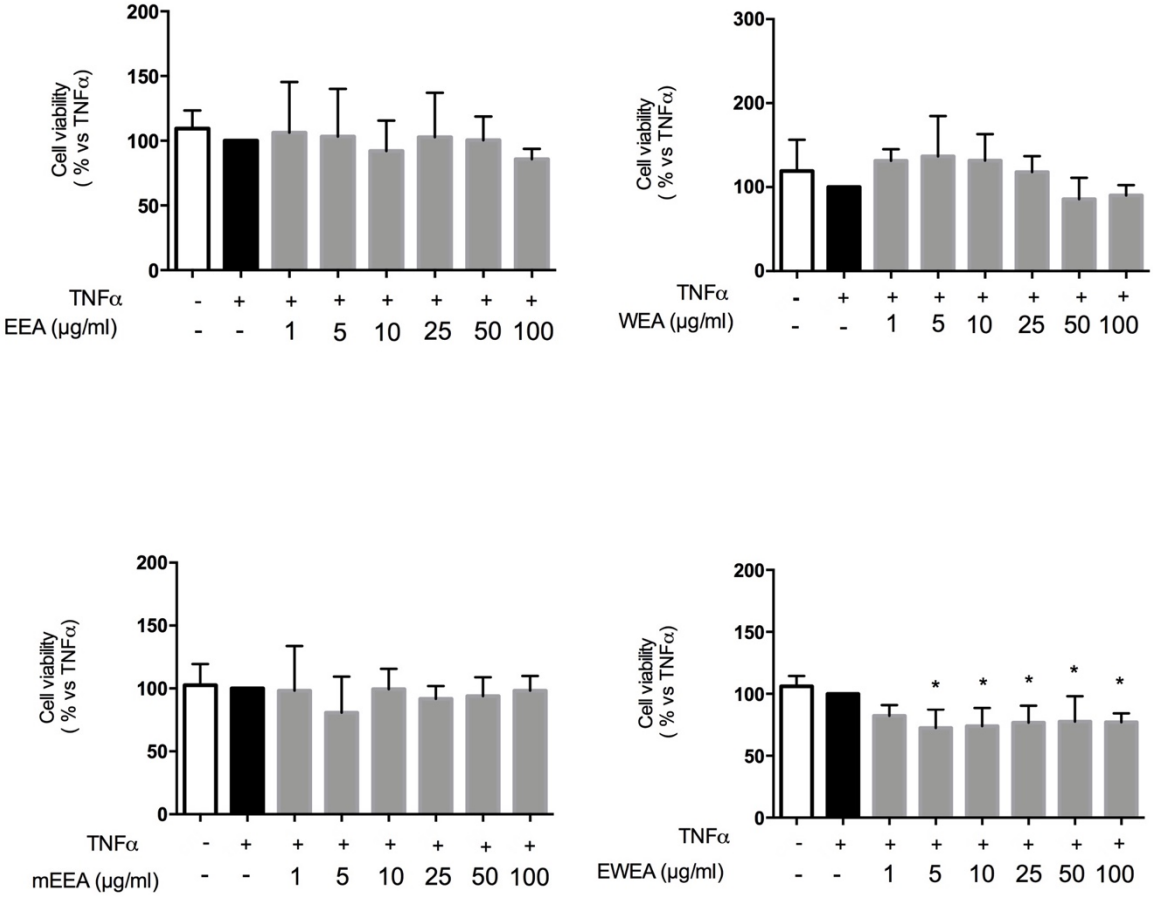

Fig. 4-3 Effect of 6 h treatment of *E. amoenum* fruit extracts on viability of TNF-α treated HaCaT cells. Results are the mean ± s.d. of at least three experiments performed in duplicate; * $p< 0.05$ vs. control (n=3).

All the extracts, except EWEA, were evaluated using TNFα induced-IL-8 release assay. The extracts at tested concentrations (1-100 μg/mL) failed to inhibit release of IL-8 in HaCaT cells (Fig. 4-4). Quercetin 10 μM was used as reference compound (~25% inhibition).

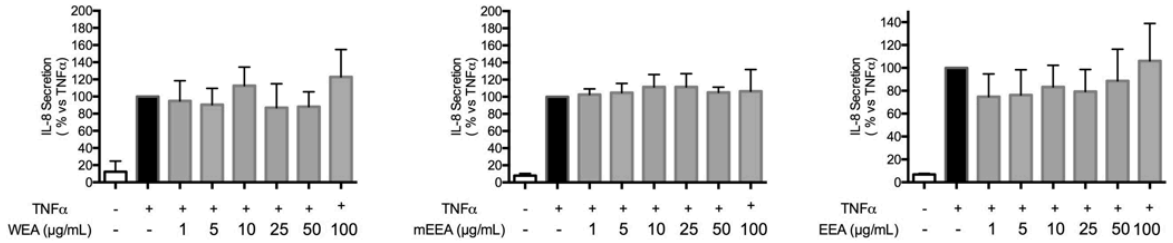

Fig. 4-4 *E. amoenum* flower extracts failed to inhibit IL-8 secretion in TNF-α treated HaCaT cells. Results are means ± s.d., of three experiments performed in duplicate.

4.4. Effect of A. lappa extracts on IL-8 release by HaCaT cells stimulated with TNFα

The cytotoxicity of *A. lappa* extracts at concentration 50 µg/mL were tested following TNFα treatment. WAL showed cytotoxic effect and excluded from further analysis. Effect of EWAL and DWAL were assessed on IL-8 release. Both extracts didn't show inhibitory effect on IL-8 production in HaCaT (Fig. 4-5). Quercetin 10 µM was used as reference compound (~25% inhibition).

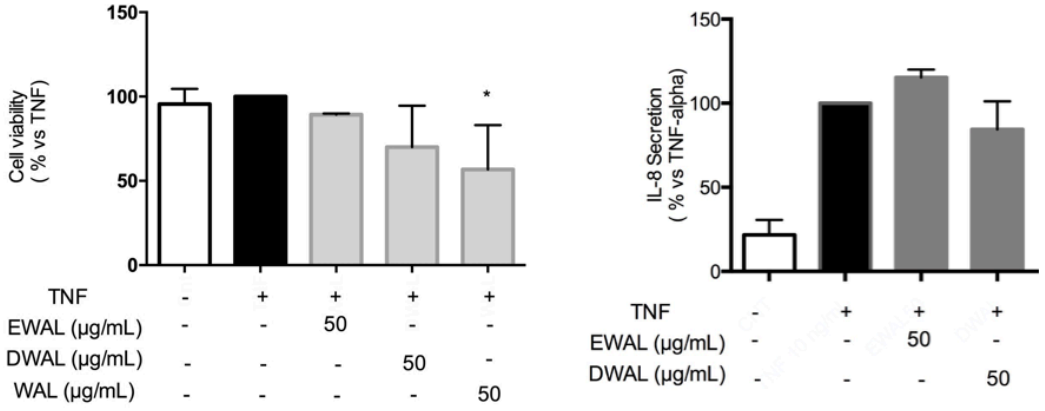

Fig. 4-5 *A. lappa* root extracts didn't show any inhibitory effects on IL-8 secretion and DWAL showed cytotoxicity in TNF-α treated HaCaT cells. Results are means ± s.d., of three experiments performed in duplicate * $p < 0.05$ vs. control (n=3).

4.5. Macerated ethanol and ethanol-water extracts of R. coriaria inhibit the TNF-α induced IL-8 secretion through suppression of the NF-κB signaling

To evaluate the mechanism of action by which mERC and EWRC are able to inhibit IL-8 release, and to find out the involvement of NF-κB pathways, HaCaT cells were transiently transfected with the NF-κB-luc plasmid and treated for 6 h with different concentrations of the extracts (10-50 µg/mL) or the reference compound in the presence of TNF-α (10 ng/mL). As shown in Fig. 4-6, both the extracts inhibited TNF-α-induced NF-κB driven transcription in a concentration dependent fashion, with comparable activity. IC_{50}s for mERC and EWRC were 11.48 ± 0.21 and 18.51 ± 0.08 µg/mL (mean ± s.d.), respectively (Tab. 5). EGCG 20 µM was used as reference compound (~65.80 % inhibition).

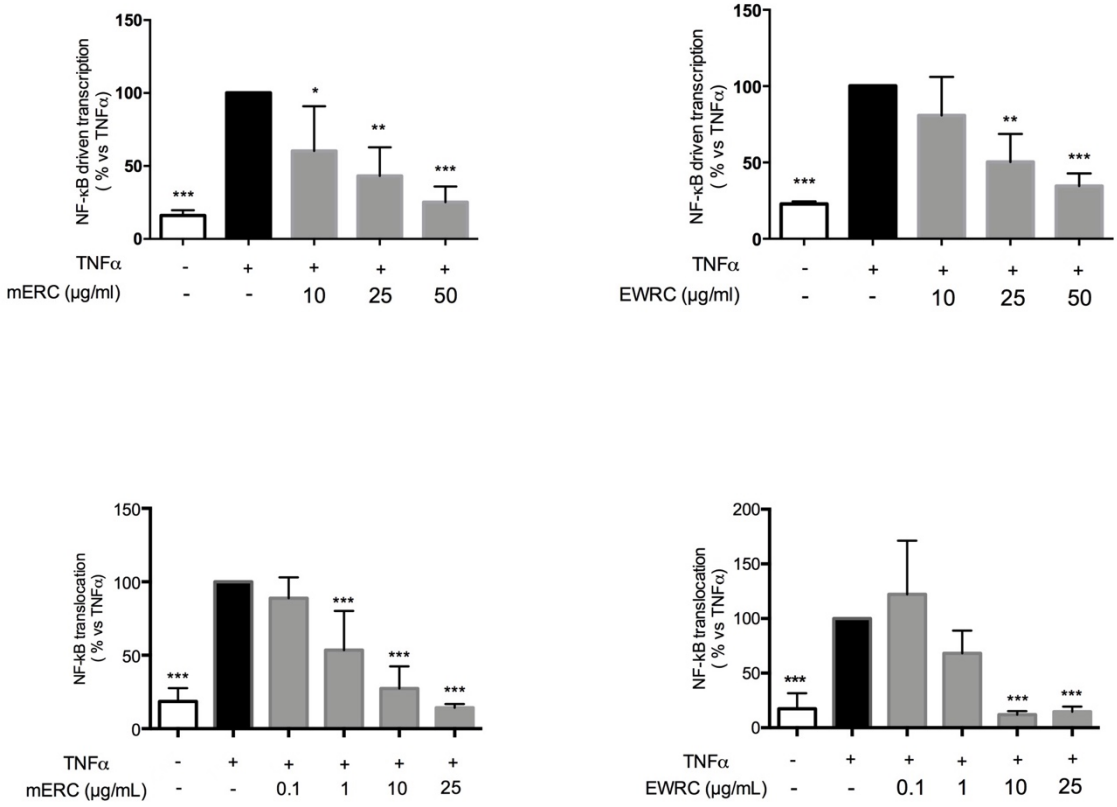

Fig. 4-6 Effect of *R. coriaria.* macerated ethanol and ethanol-water extracts on NF-κB pathway. Macerated ethanol and ethanol-water extracts of *R. coriaria* fruit inhibit NF-κB-luc transcription and NF-κB translocation in TNF-α treated HaCaT cells. Results are the mean ± s.d. of at least three experiments performed in duplicate. * $p< 0.05$, ** $p< 0.01$, and *** $p< 0.001$ vs. control (n=3)

To gain further insights into the molecular mechanisms by which the mERC and EWRC exert anti-inflammatory activity in keratinocytes, we tested the extracts on the NF-κB translocation. The HaCaT cells were treated for 1 h with different concentrations (0.1, 1, 10, and 25 µg/mL) of the extracts in serum free medium containing TNF-α (10 ng/mL). The supernatants were used for nuclear extraction and assessment of p65 translocation by ELISA. mERC and EWRC extracts inhibited the NF-κB translocation in a concentration dependent fashion (Fig. 4-6), with similar IC_{50}s (0.84 ± 0.45 and 1.03 ± 0.31 µg/mL, respectively) (Tab. 5). The reference compound EGCG 20 µM showed about 50.60% inhibitory activity.

4.6. Ethanol-water extracts of A. lappa inhibit the TNF-α-induced NF-κB signaling

The effect of non-toxic extracts of *A. lappa* (DWAL and EWAL) at concentration 50 µg/mL were tested on NF-κB signaling. EWAL at this concentration significantly inhibited the NF-κB driven transcription whereas DWAL did not show any activity at 50 µg/mL (Fig. 4-7). EGCG 20 µM was used as reference compound (~65.80 % inhibition).

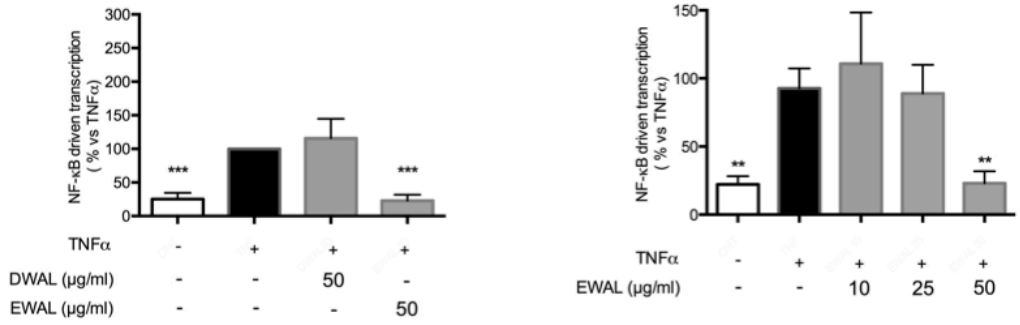

Fig. 4-7 Effect of *A. lappa.* extracts on NF-κB pathway. Ethanol-water extract of *A. lappa* root inhibit NF-κB-luc transcription in TNF-α treated HaCaT cells. Results are the mean ± s.d. of at least three experiments performed in duplicate. ** $p< 0.01$, and *** $p< 0.001$ vs. control (n=3)

4.7. Two different extracts of R. coriaria fruits inhibit TNF-α-induced ICAM-1 release in HaCaT cells

According to the preliminary data obtained on the screening of the three herbs traditionally used as skin inflammatory agent, *R. coriaria* showed the most consistent and promising effects in inhibition of IL-8 and NF-κB signaling. Therefore, starting from this phase of the research the main focus of the study was on *R. coriaria* and selectively the two different extracts of this herb, macerated ethanol (mERC) and ethanol-water (EWRC) extracts. As shown in Fig. 4-8, TNF-α induced the highest ICAM-1 release at 24 h in HaCaT cells, and this time was selected for the following experiments aimed to test the effect of the extracts (1–50 μg/mL).

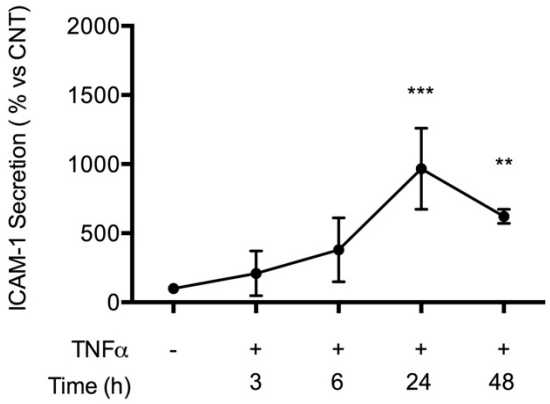

Fig. 4-8 Time course study result of release of ICAM-1 in TNF-α treated HaCaT cells. Result is the mean ± s.d. of at least three experiments performed in duplicate; ** $p< 0.01$, and *** $p< 0.001$ vs. control (n=3).

Viability of both the extracts was tested by MTT at 24 h and none of them was found to be toxic up to the highest concentration tested (50 μg/ml, Fig. 4-9).

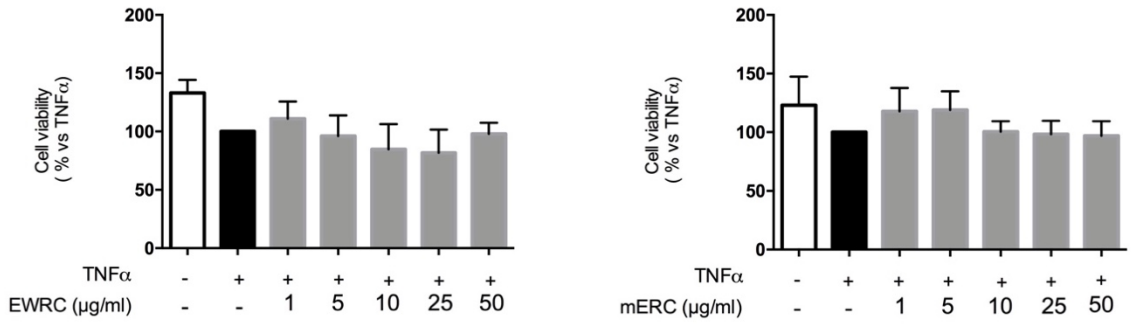

Fig. 4-9 Effect of 24 h treatment of *R. coriaria* macerated ethanol extract (mERC) and ethanol-water extract (EWRC) on viability of TNF-α treated HaCaT cells. Results are the mean ± s.d. of at least three experiments performed in duplicate; (n=3).

mERC and EWRC inhibited ICAM-1 release in a concentration dependent fashion; IC_{50s} were 2.59 ± 0.46 and 1.76 ± 0.24 µg/mL for mERC and EWRC, respectively (Fig. 4-10). The reference compound curcumin 20 µM significantly inhibited ICAM-1 release (53.55%), as expected.

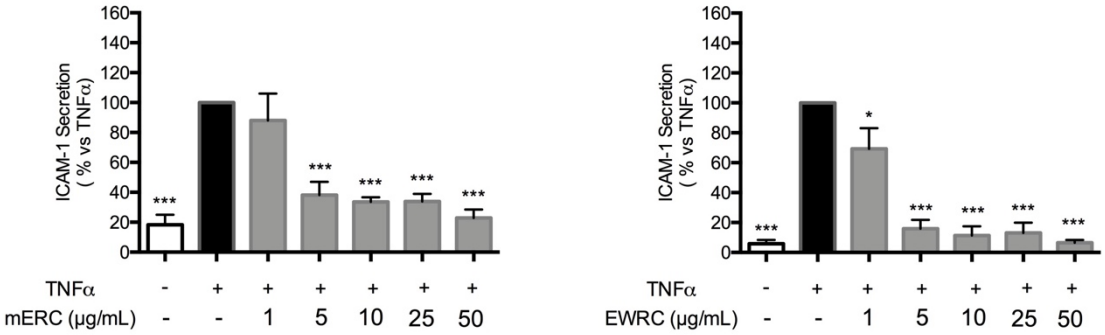

Fig. 4-10 Effect of *R. coriaria* macerated ethanol and ethanol-water extracts on ICAM-1 secretion in TNF-α treated HaCaT cells. Results are the mean ± s.d. of at least three experiments performed in duplicate. * $p< 0.05$, and *** $p< 0.001$ vs. control (n=3).

4.8. Macerated ethanol and ethanol-water extracts of R. coriaria inhibited TNF-α-induced VEGF release in HaCaT cells

Only mERC inhibited the TNF-α-induced VEGF release at 5-50 μg/mL (Fig. 4-11). The IC_{50} was 1.48 ± 0.47 μg/mL; the reference compound EGCG 20 μM showed about 60% inhibitory activity. EWRC did not show any inhibitory effect on the secretion of VEGF, one of the key factors in the process of angiogenesis, thus suggesting that the extracts may have different composition.

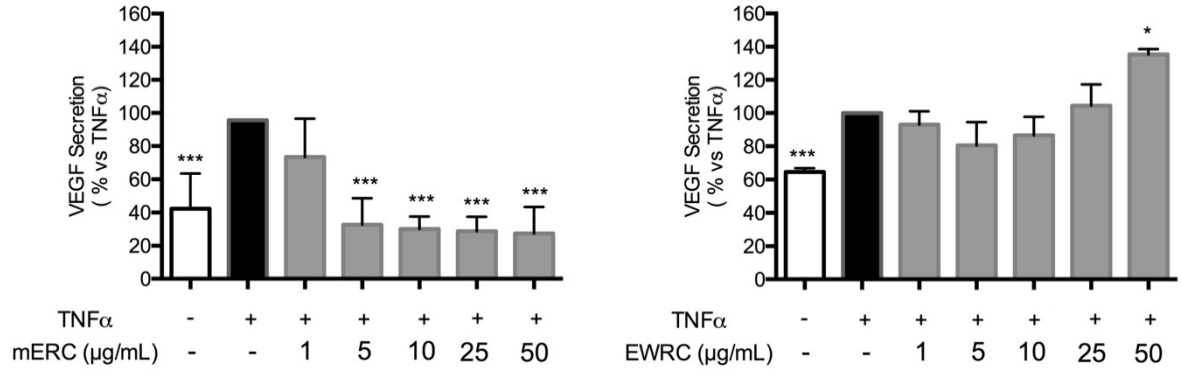

Fig. 4-11 Effects of macerated ethanol (left) and ethanol-water (right) extracts of *R. coriaria* fruit on VEGF secretion in TNF-α treated HaCaT cells. Results are the mean ± s.d. of at least three experiments performed in duplicate. * $p< 0.05$, and *** $p< 0.001$ vs. control (n=3).

4.9. Macerated ethanol extract of R. coriaria inhibits TNF-α-induced MMP-9 release in HaCaT cells

HaCaT cells were treated with different concentration of the extracts described above, then MMP-9 production, a protease which plays a key role in matrix remodeling and degradation, was investigated. The MMP-9 secretion reached the maximum at 24 h and this time was selected for the experiments (Fig. 4-12).

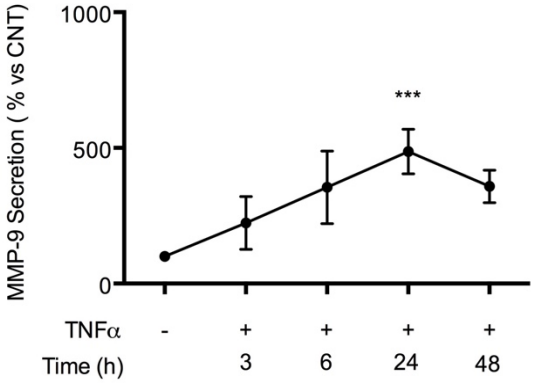

Fig. 4-12 Time course study result of release of MMP-9 in TNF-α treated HaCaT cells. Result is the mean ± s.d. of at least three experiments performed in duplicate; *** $p< 0.001$ vs. control (n=3).

mERC showed an inhibitory effect at the concentrations of 5-25 μg/mL (Fig. 4-13) with the IC_{50} of 3.37 ± 0.77 μg/mL (Tab. 5), thus reflecting results obtained on IL-8 release. EWRC was also active on MMP-9 secretion with the IC_{50} of 1.24 ± 0.33 μg/mL, in comparison with the significant inhibitory activity (67.21 %). Resveratrol 50 μM inhibited the MMP-9 release (66.19 %).

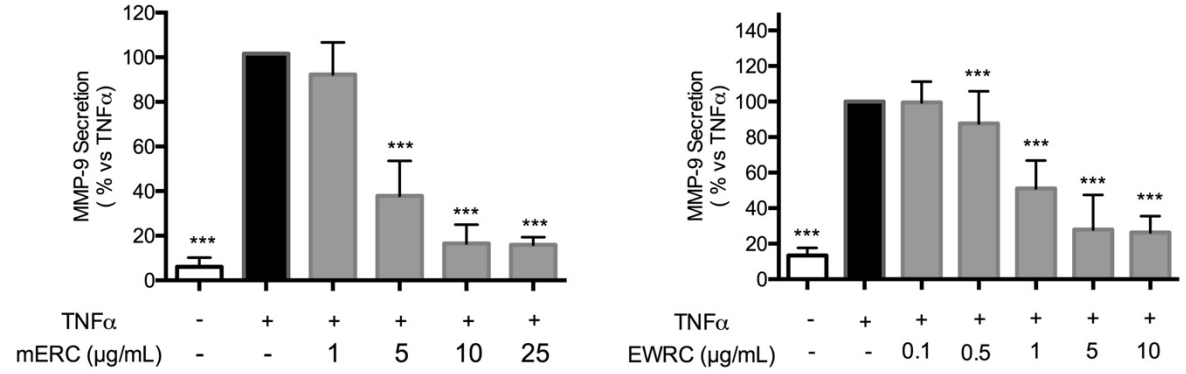

Fig. 4-13 Macerated ethanol (left) and ethanol-water (right) extracts of *R. coriaria* fruit inhibit MMP-9 secretion in TNF-α treated HaCaT cells. Results are the mean ± s.d. of at least three experiments performed in duplicate. *** p< 0.001 vs. control (n=3).

Table 5 Summary of IC_{50} (mean ± s.d.; µg/mL) of macerated ethanol (mERC) and ethanol-water extract (EWRC) on the different parameters related to skin inflammation

Parameter	Macerated ethanol extract (mERC)	Ethanol-water extract (EWRC)
IL-8	3.15±1.14	6.61±0.55
VEGF	1.48 ± 0.47	-
ICAM-1	2.59±0.46	1.76±0.24
MMP-9	3.37 ± 0.77	1.24 ± 0.33
NF-κB transcription	11.48± 0.21	18.51± 0.08
NF-κB translocation	0.85 ± 0.45	1.03 ± 0.31

4.10. Phytochemical characterization of macerated ethanol and ethanol-water extracts of R. coriaria

The phytochemical profile of *R. coriaria,* which includes hydrolyzable gallotannins, gallic acid derivatives and anthocyanins, have been previously reported in the literature (Abu-Reidah et al., 2015; Abu-Reidah et al., 2014; Beretta et al., 2009; Kossah et al., 2010; Shabbir, 2012).

However, to get deeper insights into the composition of macerated and ethanol-water extracts, their composition was investigated through HPLC-UV/DAD analysis. The corresponding chromatographic profiles (observation λ=270 nm) are reported in Fig. 4-14A and Fig. 4-14B respectively. The chromatograms were almost superimposable and, in both cases, dominated by the presence of several derivatives with UV spectra typical of the gallotannin derivatives, indicating that the employed extraction procedures and solvent compositions did not have significant impact on extraction yield and phytochemical profile of this class of compounds in the extracts.

Similarly, also the quantitative amount of the substances with UV spectral absorptions characteristic of quercetin derivatives (λ_{max}=254, 350 nm, spectra not shown) generating the

peaks at RT~12.5 min and RT~20.5 min, did not change significantly. By contrast, the EWRC extract showed a comparatively higher concentration of at least three anthocyanin derivatives at RT=6.5 min (I, λ_{max}=216, 273, 350, 511 nm), RT=11.55 min (II, λ_{max}=217, 273, 350, 511 nm) and at RT= 16.5 min (III, λ_{max}=218, 275, 350, 517 nm).

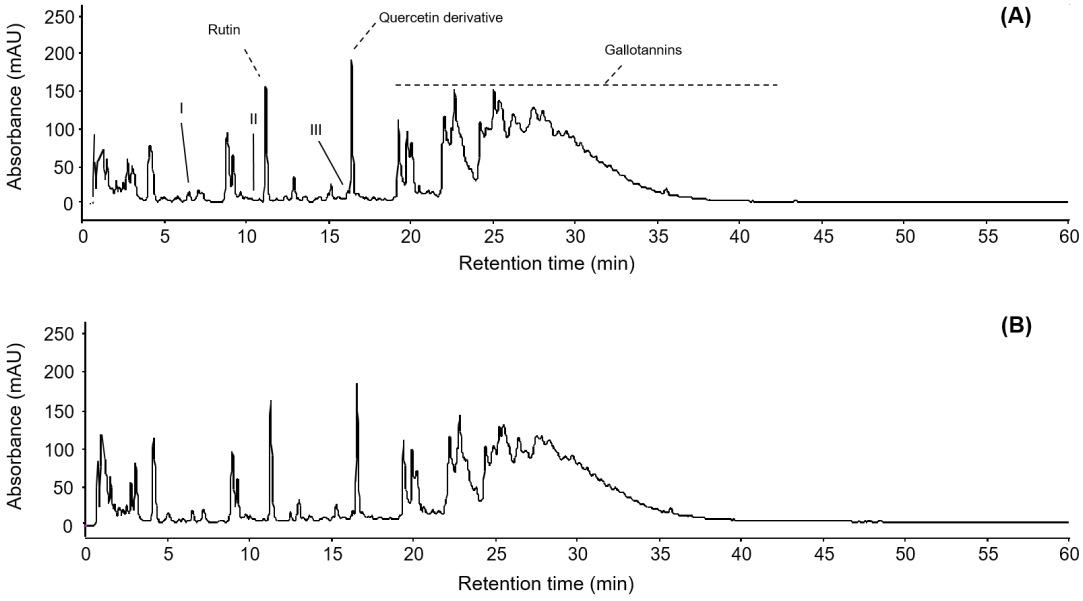

Fig. 4-14 HPLC-UV chromatographic profile of (A) EWRC (observation λ=270 nm) and of (B) mERC extracts. Peaks generated by the main phytochemical substances of interest are indicated (see text for spectroscopic data details).

Cyanidin derivatives, consistent with those previously identified in *R. coriaria* fruits (cyanidin-3-O-(2”galloyl)-galactoside, 7-O-methyl-delphinidin-3-O-(2”galloyl)-galactoside, methyl delphinidin aglycone, 7-O-methyl-cyanidin-3-O-(2”galloyl)-galactoside), were responsible for the characteristic pink color of the hydroalcoholic extract compared to the pale brownish ethanol extract (see Fig. 4-15 for the spectrophometric comparison of the visible light absorptions of the two extracts analyzed by HPLC).

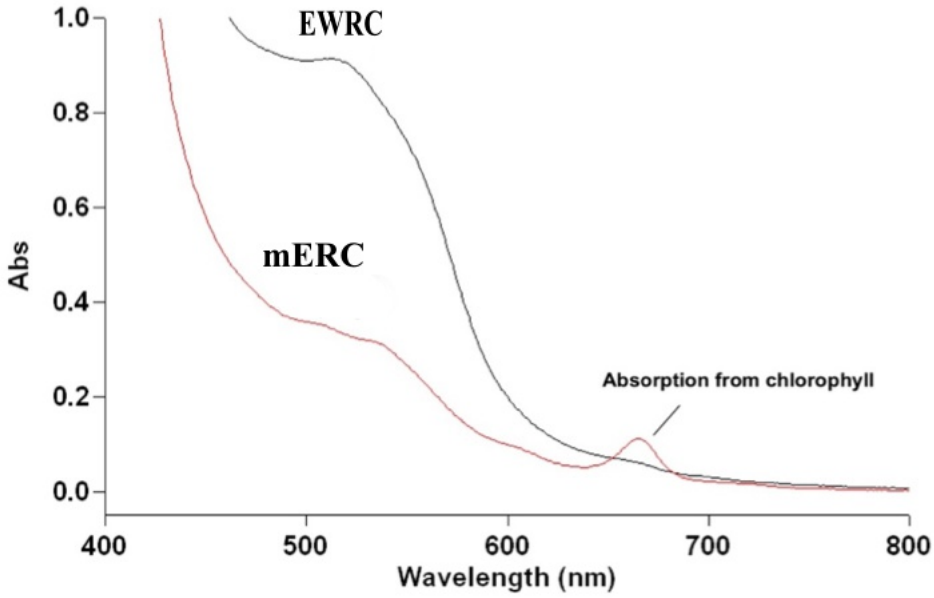

Fig. 4-15 Spectrophotometric profile of the extracts (400-800 nm). EWRC showed a significantly higher absorbance at λ~510 nm, the typical visible light absorption maximum of anthocyanins.

The quantitative analysis of these extracts is reported in Tab. 4. Both extracts show similar amounts of flavonoids (0.23±0.02% QE and 0.23± 0.03% Q; mERC vs. EWRC, P>0.05) and tannins (4.33± 0.32% GAE and 4.54±0.23% GAE; mERC vs. EWRC, P>0.05). By contrast, EWRC showed a significantly higher anthocyanins content 0.207± 0.023% CE compared to mERC 0.031±0.005 % CE (P<0.05) (Tab.6).

Table 6 Quantitative analysis of macerated ethanol (mERC) and ethanol-water extract (EWRC)

Extract	Flavonoids Expressed as quercetin-3-O-glucoside (%)	Tannins Expressed as gallic acid (%)	Anthocyanins Expressed as cyanidin-3-O-glucoside (%)
EWRC	0.23±0.02	4.54±0.23	0.207±0.023
mERC	0.23 ± 0.03	4.33±0.32	0.031±0.005

4.11. Macerated ethanol and ethanol-water extracts of R. coriaria do not show cytoprotective effect against UVB radiation

Effects of 1h pre-treatment or 24h post-treatment with mERC or EWRC on viability of cells were measured at 24h after the UVB exposure using MTT assay. The 1h and 24h treatment with mERC and EWRC failed to attenuate the negative effect of UVB on HaCaT cells (Fig. 4-16).

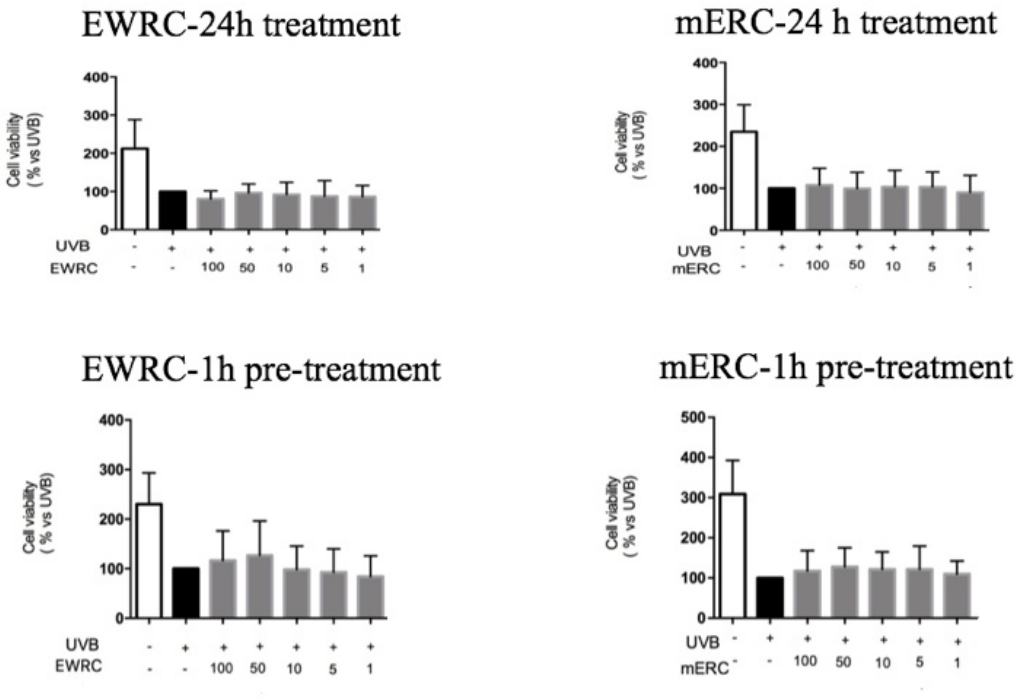

Fig. 4-16 The pre-treatment (1 h) and post-treatment (24 h) with macerated ethanol and ethanol-water extracts of *R. coriaria* fruit didn't show protective effect on HaCaT cells against UVB exposure. Results are the mean ± s.d. of at least three experiments performed in duplicate (n=3).

4.12. Macerated ethanol of R. coriaria extract inhibits UVB-induced IL-8 levels

The viability of HaCaT cells were tested following 9h post-treatment with different concentrations of mERC and EWRC (1, 5, 10, 25, 50 and 100 μg/mL) and the supernatants were collected and analyzed using IL-8 Elisa assay. The 9h post treatment with mERC and EWRC had not any cytotoxicity on HaCaT cells following the UVB exposure (Fig. 4-17).

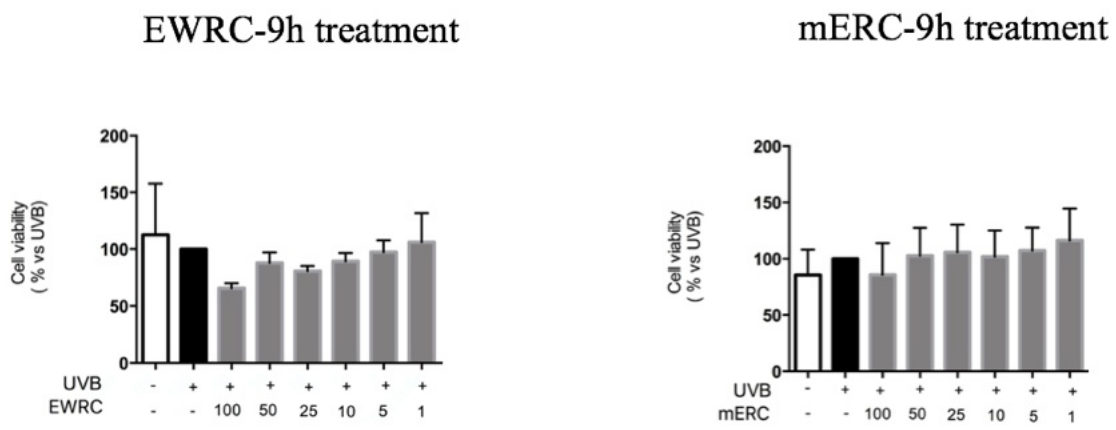

Fig. 4-17 Effect of 9 h treatment of *R. coriaria* macerated ethanol (right) and ethanol-water extract (left) on viability of HaCaT cells after exposure to UVB. Results are the mean ± s.d. of at least three experiments performed in duplicate; (n=3).

The IL-8 secretion was suppressed by 9h post-treatment with mERC. EWRC had no effect against the secretion of IL-8 (Fig. 4-18).

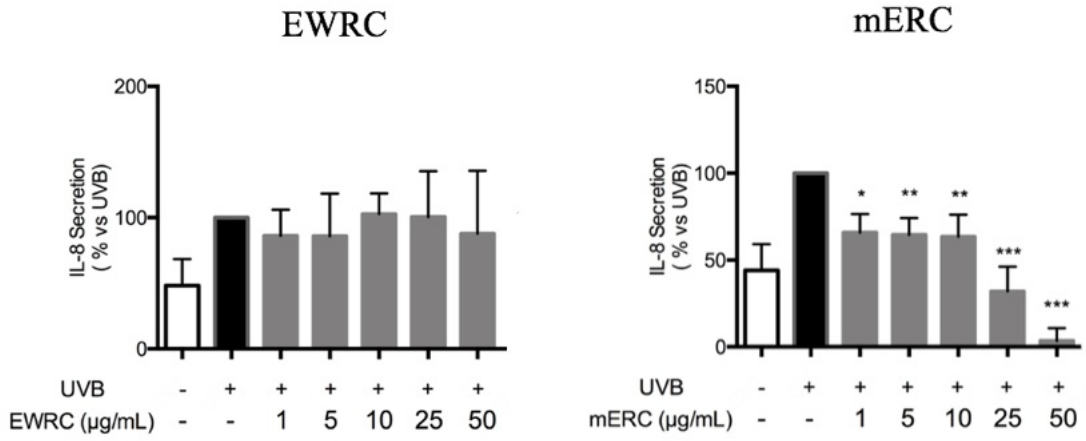

Fig. 4-18 Macerated ethanol (right) extracts of *R. coriaria* fruit inhibit IL-8 secretion in UVB-induced inflammation in HaCaT cells. The ethanol-water extract of this herb (left) didn't show inhibitory effect on UVB-induced IL-8 release in these cells. Results are the mean ± s.d. of at least three experiments performed in duplicate. * $p< 0.05$, ** $p< 0.01$, and *** $p< 0.001$ vs. control (n=3).

4.13. Macerated ethanol extract of R. coriaria inhibits NF-κB translocation after UVB exposure

The HaCaT cells were treated for 1 h with different concentrations of mERC and EWRC (1, 10, and 25 µg/mL) following UVB exposure (40 mJ/cm^2). The supernatants were used for nuclear extraction and assessment of p65 translocation by ELISA. mERC inhibited the NF-κB translocation at 10 and 25 µg/mL while EWRC was not active in inhibiting of NF-κB translocation (Fig. 4-19).

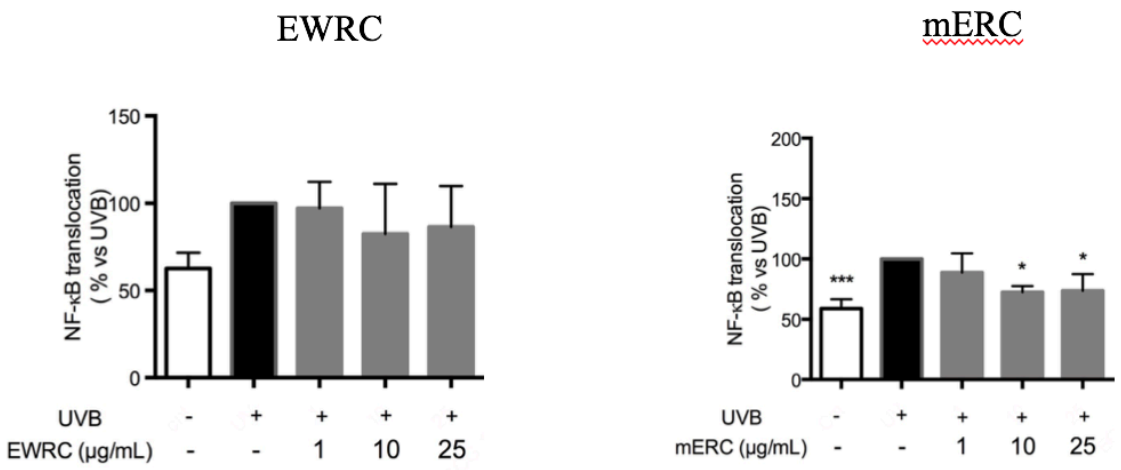

Fig. 4-19 Effect of *R. coriaria.* macerated ethanol (right) and ethanol-water extracts (left) on NF-κB pathway after UVB exposure. Macerated ethanol extract of *R. coriaria* fruit inhibits NF-κB translocation in UVB-induced inflammation in HaCaT cells. Results are the mean ± s.d. of at least three experiments performed in duplicate. * $p< 0.05$ vs. control (n=3)

4.14. Preparation of lipophilic extracts of R. coriaria and percent recovery

The aim of this phase of the study is to develop and evaluate the new preparations of *R. coriaria* which are more suitable for topical administration. The lipophilic extracts of *R. coriaria* fruits were prepared using cold extraction method (Fig. 3-1). To measure the yield of each extract, mass of the extract was divided by the initial mass of the dry matter. The % yield of lipophilic extracts of *R. coriaria* is reported in Tab. 7 The dichloromethane extract showed the lowest %yield of extraction 8.5%. The ethyl-acetate and acetone extracts showed the similar

yield of extraction around 27%. The % yield of extraction for the oil phase of acetone extract was 9.7% and for ethyl acetate was about 7.9%.

Table 7 %Yield of different lipophilic extracts of *R. coriaria* fruit

Extract	Yield of extract (%)
Acetone extract (ARC)	27.78
Oil derived from acetone extract (oil-ARC)	9.7
Ethyl acetate extract (EARC)	26.8
Oil derived from ethyl acetate extract (oil-EARC)	7.9
Dichloromethane extract (DCMRC)	8.5

4.15. Effects of lipophilic extracts of R. coriaria on TNF-induced IL-8 secretion

The lipophilic extracts were tested for both cytotoxicity and the effect on IL-8 secretion in TNF-treated HaCaT cells. Fig. 4-20 shows the effect of these extracts on viability of HaCaT cells after 6h treatment with TNF (10 ng/mL). The extracts did not show any cytotoxicity at the concentrations of 25 µg/mL in TNF-treated cells.

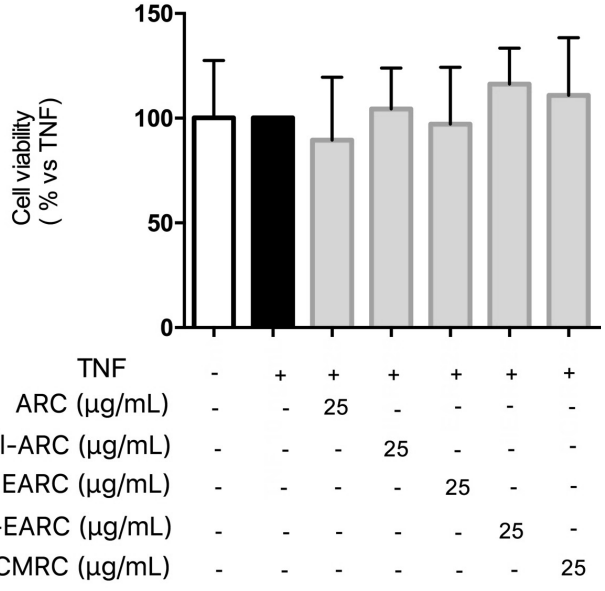

Fig. 4-20 Effect of lipophilic extracts of *R. coriaria in TNF-treated HaCaT cells.* Results are the mean ± s.d. of at least three experiments performed in duplicate. (n=3)

ARC and EARC inhibited the release of IL-8 significantly at concentrations 25 µg/mL in TNF-treated cells (Fig. 4-21). DCMRC, oil-ARC and oil-EARC similarly were not active in inhibition of TNF-induced IL-8 release. Quercetin 10 µM was used as reference compound (~25% inhibition).

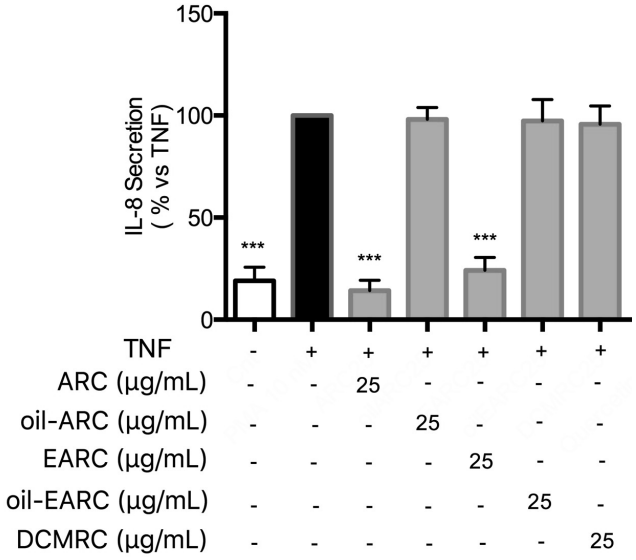

Fig. 4-21 Effects of *R. coriaria* lipophilic extracts on IL-8 secretion in TNF-α treated HaCaT cells. ARC and EARC inhibit the release of IL-8 in these cells. Results are the mean ± s.d. of at least three experiments performed in duplicate. *** $p < 0.001$ vs. control (n=3).

4.16. Acetone and ethyl acetate extracts of R. coriaria inhibit TNF-induced IL-8 secretion

The cytotoxicity of acetone and ethyl acetate extracts of *R. coriaria* at concentration 1-50 µg/mL were evaluated in TNF-induced inflamed HaCaT cells. Both extracts at the tested concentrations didn't show any cytotoxicity in presence of TNF (Fig. 4-22).

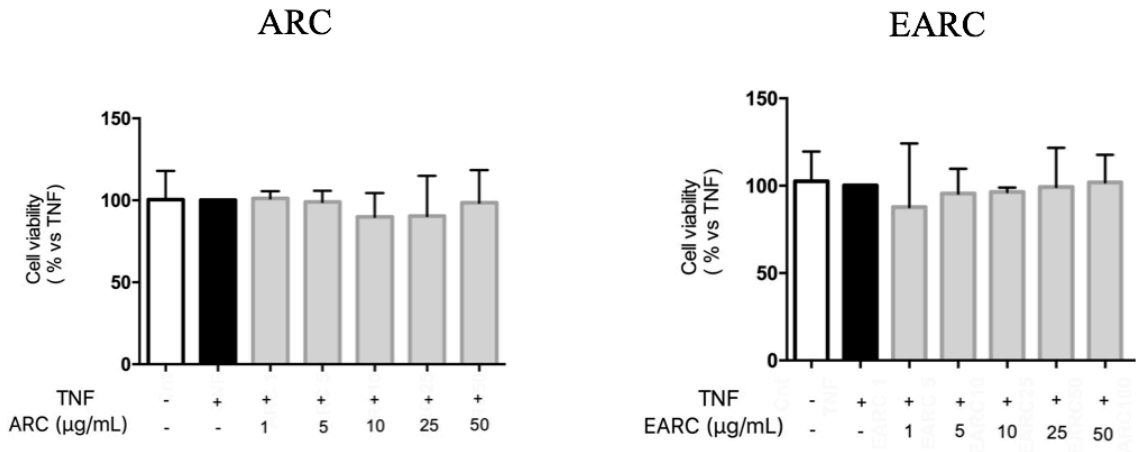

Fig. 4-22 Effects of acetone (left) and ethyl acetate (right)extracts of *R. coriaria* on viability of TNF-treated HaCaT cells. Results are the mean ± s.d., of at least three experiments performed in duplicate. (n=3).

ARC and EARC significantly suppressed the IL-8 production with IC_{50s} 2.708 ± 0.37 and 5.247 ± 1.54 µg/mL, respectively (Fig. 4-23). Quercetin 10 µM was used as reference compound (~25% inhibition).

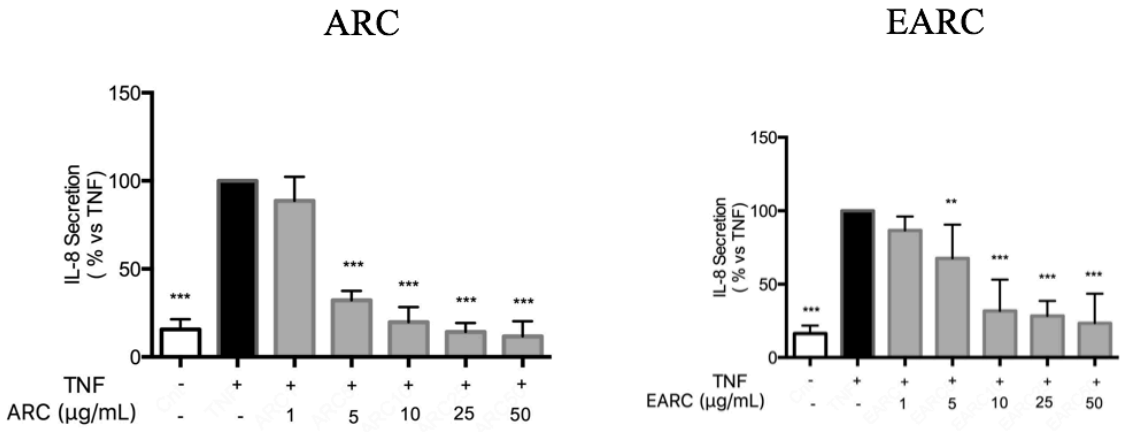

Fig. 4-23 Effects of acetone (left) and ethyl acetate (right) extracts of *R. coriaria* on IL-8 secretion in HaCaT cells. Both extracts at concentration 5-50 µg/mL significantly suppressed the IL-8 production. Results are the mean ± s.d., of at least three experiments performed in duplicate. ** $p< 0.01$, and *** $p< 0.001$ vs. control (n=3).

4.17. Acetone and ethyl acetate extracts of R. coriaria suppress TNF-induced NF-κB driven transcription

In TNF treated cells, ARC and EARC, significantly inhibited NF-κB-luc transcription with IC_{50s} 17.18±3.45 and 21.92 ± 3.07 µg/mL respectively (Fig. 4-24 and Tab. 8).

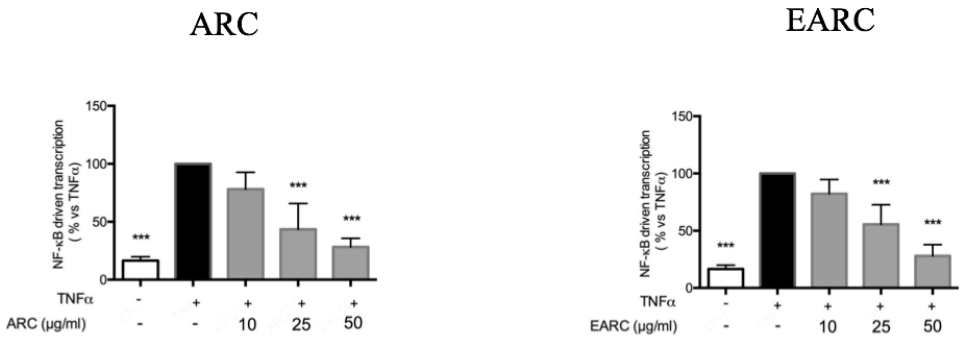

Fig. 4-24 Effects of acetone (left) and ethyl acetate (right) extracts of *R. coriaria* on NF-κB-luc transcription in TNF treated HaCaT cells. Both extracts at concentration 25 and 50 µg/mL significantly suppressed the NF-κB-luc transcription. Results are the mean ± s.d., of at least three experiments performed in duplicate. *** $p< 0.001$ vs. control (n=3).

Table 8 shows summary of IC_{50s} of selected extracts of *R. coriaria* extracts. This table reveals that ARC and mERC show the lowest IC_{50s} for both IL-8 release and NF-κB driven transcription induced by TNF.

Table 8 Summary of IC_{50s} (µg/mL) of macerated ethanol and ethanol-water, acetone, and ethyl acetate extracts on the different parameters related to TNF-induced skin inflammation

Extracts	IL-8	NF-κB-LUC transcription
Macerated ethanol extract (mERC)	3.15±1.14	11.48±0.21
Ethanol-water extract (EWRC)	6.61 ± 0.55	18.51±0.08
Acetone extract (ARC)	2.708±0.37	17.18±3.45
Ethyl acetate extract (EARC)	5.247 ± 1.54	21.92 ± 3.07

4.18. R. coriaria fruit extract at 25 µg/ml did not show inhibitory effect against IL-8 secretion in PMA-treated HaCaT cells

The cells were subjected to PMA (10 nM) and all the *R. coriaria* extracts at 25 µg/mL treatment for 6 hours. The cytotoxicity effects of the extracts were measured by MTT the results are reported in Fig. A. 9. EARC showed cytotoxicity which verified by LDH release assay (Fig. 4-25).

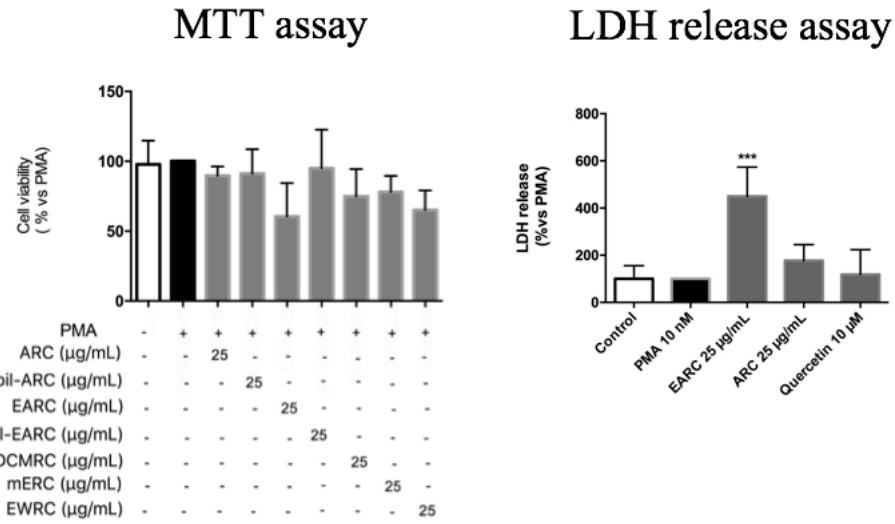

Fig. 4-25 Effects of extracts of *R. coriaria* on viability of PMA (10nM) treated HaCaT cells. Ethyl acetate extract at concentration of 25 µg/mL showed cytotoxicity by MTT assay (left) which verified by LDH release assay (right). Results are the mean ± s.d., of at least three experiments performed in duplicate. *** $p< 0.001$ vs. control (n=3).

Therefore, this extract has been excluded for further studies. Rest of the extracts were not toxic in 10 nM PMA-treated HaCaT cells and were assessed on their effects on IL-8 releases. As shown in Fig. 4.26 the extracts were not effective in inhibiting IL-8 secretion at the tested concentration (25 µg/mL).

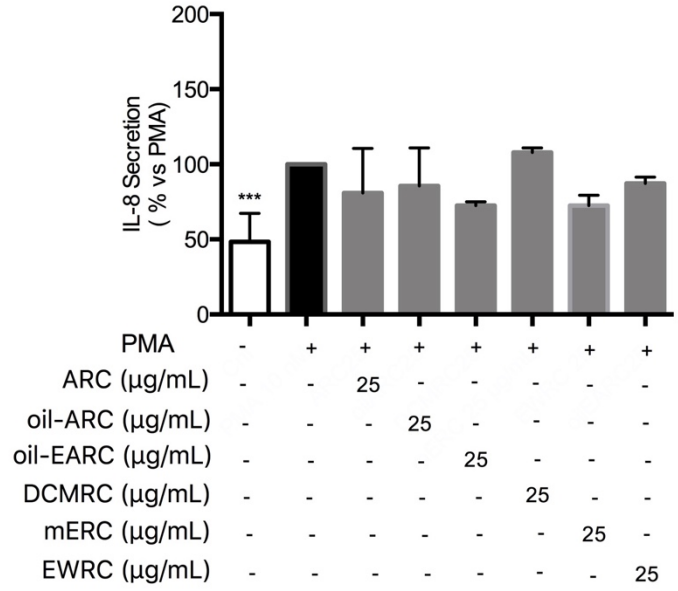

Fig. 4-26 Effects of extracts of *R. coriaria* on IL-8 secretion in PMA (10 nM) treated HaCaT cells. Results are the mean ± s.d., of at least three experiments performed in duplicate. (n=3).

4.19. Macerated ethanol and acetone extracts of R. coriaria inhibited PMA-induced IL-8 secretion

The extracts were tested at 50 μg/mL in HaCaT cells treated with 100 nM PMA, and EWRC showed significantly toxic effect (Fig. 4-27).

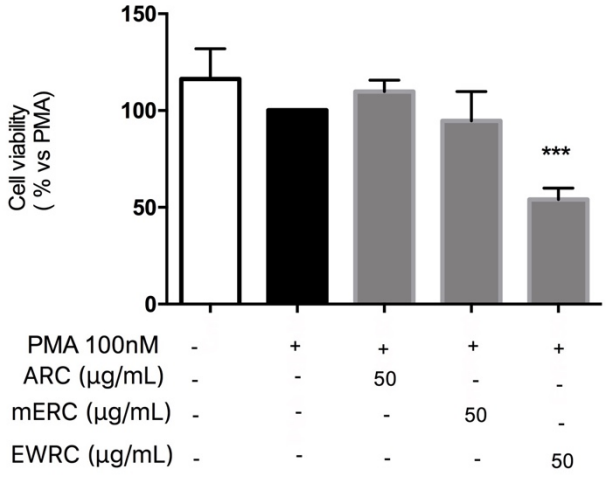

Fig. 4-27 Effects of extracts of *R. coriaria* on viability of PMA (100 nM) treated HaCaT cells. Ethanol-water extract at concentration of 50 μg/mL showed significant cytotoxicity by MTT assay. Results are the mean ± s.d., of at least three experiments performed in duplicate. *** $p<$ 0.001 vs. control (n=3).

ARC and mERC showed significant inhibitory effect on IL-8 release in PMA induced inflammatory conditions (Fig. 4-28).

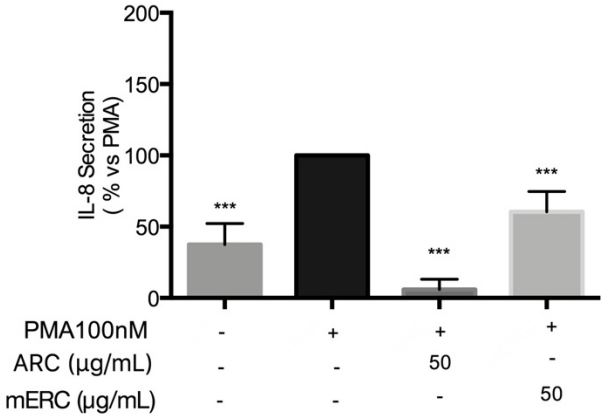

Fig. 4-28 Effects of acetone and macerated ethanol extracts of *R. coriaria* on IL-8 secretion in PMA (100nM) treated HaCaT cells. Both extracts inhibit the IL-8 secretion significantly at 50 µg/mL. Results are the mean ± s.d., of at least three experiments performed in duplicate. *** $p< 0.001$ vs. control (n=3).

Both extracts were tested also at different concentrations ranging from 1 to 50 µg/mL using IL-8 assay; both the extracts showed a concentration dependent inhibitory activity. The IC_{50s} obtained for ARC were 25.77 µg/mL and mERC just at 50 µg/mL showed a significant inhibitory effect against IL-8 (Fig. 4-29).

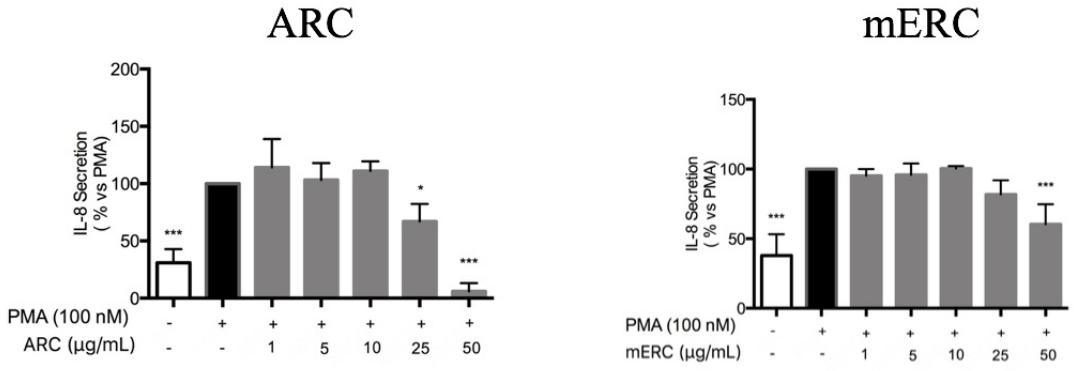

Fig. 4-29 Effects of different concentrations of acetone and macerated ethanol extracts of *R. coriaria* on IL-8 secretion in PMA (100 nM) treated HaCaT cells. Results are the mean ± s.d., of at least three experiments performed in duplicate. * $p< 0.05$, and *** $p< 0.001$ vs. control (n=3).

4.20. Acetone extract of R. coriaria inhibited PMA-induced NF-κB driven transcription

Both active anti-IL-8 *R. coriaria* active extracts in PMA induced inflammatory conditions (mERC and ARC) were analyzed for their effect on NF-κB signaling at concentration of 50 µg/mL. ARC inhibited NF-κB-LUC transcription, with IC_{50} 27.82 µg/mL in comparison with the significant inhibitory effect of EGCG 20 µM (~35.5 % inhibition) (Fig. 4-30).

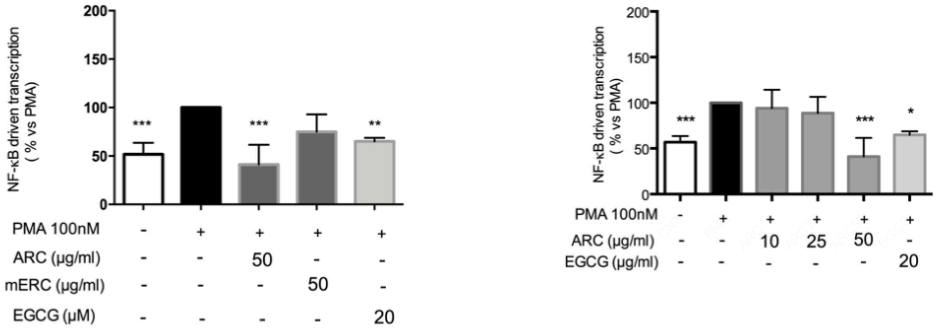

Fig. 4-30 Effects of macerated ethanol and acetone extracts of *R. coriaria* on NF-κB-LUC transcription in PMA (100 nM) treated HaCaT cells. Results are the mean ± s.d., of at least three experiments performed in duplicate. ** $p< 0.01$, and *** $p< 0.001$ vs. control (n=3).

CHAPTER FIVE

5. Discussion and conclusion

5.1. Discussion

The skin is the largest organ of the body and a protective barrier against any environmental stress (Hänel et al., 2013). Keratinocytes are the most abundant cells of the outer epidermis layer of skin which play a key role in the induction and maintenance of inflammation in this organ (Colombo et al., 2017). Although inflammation is an essential innate immunity response, dysregulated and untimely inflammation plays a central role in many disorders including psoriasis, atopic dermatitis, rheumatoid arthritis, coronary heart disorders and cancer (Chandel et al., 2000; Khalilpour et al., 2019). The process of inflammation in the progression of these disorders has been poorly understood. Inflammatory skin diseases are the most common dermatologic conditions which are influenced by genetic and environmental factors including UV solar radiations. Chronic skin inflammation results from dysregulation and abnormal expression of pro-inflammatory mediators in keratinocytes, including TNF-α and IL-8. Stimulation of keratinocytes with TNF-α leads to activation of NF-κB and subsequently increases the expression of adhesion molecules and proinflammatory genes (Bahar-Shany et al., 2010; Seo et al., 2015). Although blockage of TNF-α, by anti-TNF-α agents, such as Infliximab, Etanercept and Adalimumab, which bind TNF-α with high affinity, has been shown to successfully treat psoriasis, they can cause serious side effects (Chan et al., 2008; Ganguly, 2009; Makol and Grover, 2008; Silva et al., 2010). Thus, the development of novel anti-TNF-α and NF-κB inhibitor with low side effects is the main aim of research in the field of skin inflammations including psoriasis.

Accumulating evidence shows that many traditional herbal medicines have successfully exhibited anti-inflammatory activities in the skin (Choi et al., 2009; Choo et al., 2009; Hon et

al., 2011; Hughes et al., 2007). *Rhus coriaria* L. (*R. coriaria*), *Arctium lappa* L. (*A. lappa*) and *Echium amoenum* Fisch. (*E. amoenum*) are medicinal herbs with wide-ranging traditional applications covering anti-inflammatory effects also in the skin. The present research firstly aims to validate the traditional use of these herbs and find out the most active herb with most promising extracts for skin inflammatory conditions.

In the first part of this study, *R. coriaria*, *E. amoenum* and *A. lappa* extraction with water, ethanol water 50:50 or ethanol (EtOH), were carried out via maceration and cold extraction technique (see Materials and Methods, section 2.4). Extraction is the process used to treat plant tissue with specific solvents whereby the medicinally active constituents are dissolved out.

Then, the anti-inflammatory properties of the extracts prepared using different extraction methods were evaluated in human keratinocytes (HaCaT cells). Epidermal keratinocytes play critical role in skin inflammation through their production of inflammatory chemokines (Albanesi 2010). Keratinocytes are active players in epidermal repair and in the skin's immune defense through the secretion of different growth factors, cytokines, and chemokines. The *in vitro* model used for this screening involves the treatment of cells with TNF-α prior to incubation with reference compounds or extracts. TNF-α is one of the most critical chemokines involved in the initiation and development of chronic skin inflammation, such as psoriasis. This cytokine stimulates production of pro-inflammatory molecules such as IL-6, IL -8, VEGF, MMP-9, and ICAM-1. Moreover, TNF-α activates the NF-κB pathway leading to the recruitment of inflammatory cells in the dermal and epidermal layers of psoriatic skin (Nickoloff, Xin et al. 2007, Udommethaporn, Tencomnao et al. 2016).

All the extracts were tested for cytotoxicity, after 6 hours of TNF-α treatment. WAL at 50 µg/mL, EWEA at concentrations 5-100 µg/mL, and 100 µg/mL of EWRC showed cytotoxic effects. Since IL-8 expression is a sign of initiation of keratinocyte inflammation, we

investigated this chemokine and the inhibitory effect of all non-toxic extracts on TNF-α activated HaCaT cells. Our results showed that the extracts of *A. lappa* and *E. amoenum* were not active in inhibiting IL-8 release. All the extracts prepared from *R. coriaria* showed inhibitory effect on IL-8 secretion, and mERC and EWRC revealed the highest activity. Following these studies on screening of different extracts of *R. coriaria* on the TNFα-induced IL-8 release in HaCaT cells, EWRC and mERC were selected as the most active extracts.

R. coriaria is a medicinal herb native to middle east and Mediterranean region and well-known as "sumac" or "Sicilian sumac". The traditional use of *R. coriaria* fruits to heal skin injuries and disorders including burns, wounds and eczema has been also reported in the literature (Altundag and Ozturk, 2011; Behnammanesh et al., 2015; Mehrabani Natanzi et al., 2017). However, the mechanism of action of *R. coriaria* is poorly understood. Recent phytochemical studies on *R. coriaria* have proved its richness in hydrolysable tannins, condensed tannins, gallic acid derivatives, anthocyanin and various organic acids such as malic and citric acids, fatty acids, vitamins, flavonoids and terpenoid derivates (Kossah, Nsabimana et al. 2010, Shabbir 2012, Abu-Reidah, Jamous et al. 2014). Protocatechuic acid, linolenic acid, p-OH-benzoic acid, and vanillic acid were the phenolic acids found in the leaves of this herb which contain up to 25-33% tannins. Several researches on biological activities of *R. coriaria* has indicated its antimicrobial, antifungal, antiviral, antioxidant, hypoglycemic and anti-inflammatory, neuroprotective, hepatoprotective, and cardiovascular protective properties (Rayne and Mazza 2007, Beretta, Rossoni et al. 2009, Panico, Cardile et al. 2009, Pourahmad, Eskandari et al. 2010, Behnammanesh, Khalilpour et al. 2015, Khalilpour 2015, Khalilpour, Behnammanesh et al. 2017). Despite this information, an inhibitory effect of fruit extracts of *R. coriaria* on epidermal keratinocytes inflammation has not been reported so far.

In the present study, following the screening of different extracts of *R. coriaria* on the TNFα-induced IL-8 release in HaCaT cells, EWRC and mERC were selected as the most active extracts. Since IL-8 expression is dependent on the NF-κB activation, contributing to exacerbate inflammation, the inhibitory effect of mERC and EWRC on TNF-α- activated keratinocytes was investigated. The NF-κB signaling is involved in the regulation of several pro-inflammatory chemokine in HaCaT cells (Choi et al., 2009). In keratinocytes, TNF-α stimulation activates several intracellular signaling pathways, including those regulated by NF-κB (Sung et al., 2012). TNF-α, among other cytokines, induces the phosphorylation and degradation of IκB-α which is found in combination of NF-κB/p65 in the cytoplasm. This action of TNF-α leads to the phosphorylation of NF-κB/p65 and its nuclear translocation (Kang et al., 2013). In the nucleus, NF-κB induces the expression of genes that promote the inflammatory reaction.

The extracts selected based on their efficacy in inhibiting IL-8 release have been investigated on the NF-κB signaling. The mERC and EWRC inhibited the NF-κB-luc transcription with the IC_{50} 11.48 ± 0.21 and 18.51 ± 0.08 μg/mL, respectively. The translocation of NF-κB was also suppressed by both the extracts. The obtained IC_{50}s for mERC and EWRC were 1.06 ± 0.61 and 1.82 ± 0.67 (mean ± s.d.), respectively. Our results showed that the suppressing activation of the NF-κB pathways by mERC and EWRC was the main reason of their inhibitory activity on the expression of IL-8. Two types of non-toxic extract of *A. lappa* also were tested for NF-κB signaling. EWAL suppressed the NF-κB-luc transcription at 50 μg/mL.

Then, the activity of the selected extracts on other pro-inflammatory mediators, including ICAM-1, VEGF, and MMP-9, in response to TNF-α as pro-inflammatory stimuli were assessed in HaCaT cells. These factors were selected as strictly involved in the pathogenesis of inflammatory skin conditions and overexpressed during psoriasis (Chandel et

al., 2000; Jeong et al., 2010; Kang et al., 2013). Several studies have reported evidences that link the TNF-α in keratinocytes with regulation of inflammation-related genes, including IL-1, ICAM-1, VEGF, TGF-β, chemokines and the CXCL8 family (Jeong et al., 2010; Sung et al., 2012). The levels of pro-inflammatory mediators, including VEGF, MMP-9 and ICAM-1 were analyzed following 24 h treatment of HaCaT cells with mERC and EWRC at 1-50 μg/mL in serum free medium in the presence of this stimulus.

Our results show that the release of VEGF, and MMP-9 was significantly lower in HaCaT cell following the treatments. The presence of mERC reduced the TNF-α-induced release of MMP-9 at the concentrations 5-25 μg/mL and VEGF secretion at the concentrations 5-50 μg/mL. The mERC significantly decreased the VEGF level (IC_{50} 1.46 ± 0.47 μg/mL) whereas EWRC did not show any activity. The prominent involvement of angiogenesis in the pathogenesis of psoriasis and the validated use of anti-angiogenic therapy in human cancers using a monoclonal antibody directed against VEGF (bevacizumab, AvastinR)(Folkman, 2006; Kerr, 2004) suggests anti-VEGF treatment as a possible therapy for patients suffering from psoriasis. Recently, a patient was reported to experience complete remission of psoriasis during bevacizumab treatment of metastatic colon cancer (Akman et al., 2009; Schonthaler et al., 2009; Trompezinski et al., 2003).The level of MMP-9 release was also decreased significantly by mERC and EWRC, with IC_{50s} 3.37 ± 0.77 and 1.24 ± 0.33 μg/mL, respectively. The ICAM-1 level was also significantly decreased after treatment with mERC at concentrations of 5-50 μg/mL.

We evaluated the effect of EWRC on the secretion of the same factors in HaCaT cell line and it showed inhibitory effect on all the analyzed parameters which were upregulated significantly by TNF-α, except VEGF. The mERC (5-50 μg/mL) showed significant suppression of ICAM-1 secretion with IC_{50} 2.59 ± 0.46 μg/mL, while the EWRC had the highest inhibitory effect (IC_{50} 1.76 ± 0.24 μg/mL).

Both the extracts were chemically profiled through HPLC-UV-DAD analysis. The HPLC-UV profile of the extracts revealed higher amounts of anthocyanins in EWRC in comparison with mERC. The two extracts were characterized by similar composition, both in terms of gallotannins and flavonol derivatives contents, with the presence of a significantly higher proportion of anthocyanins as the only evident difference between them. Anthocyanins are polyphenols that are responsible for the pink, red, violet, and blue coloration of many fruits and flowers (Osmani et al., 2009). These polyphenols are present as glycosides linked to 2-phenylbenzopyrylium or flavylium moiety. They have potent antioxidant and anti-inflammatory properties, and it has been reported that they stimulate the wound-induced VEGF production in keratinocytes (Nizamutdinova et al., 2009). Therefore, higher amount of anthocyanins in EWRC may be the main reason of its stimulating effect on VEGF levels in HaCaT cells. The HPLC-UV analysis of both extracts revealed higher concentration of anthocyanins in EWRC. In this extract gallotannins, flavonol (quercetin- 3-O-glucoside) and rutin were also identified as the most abundant compounds. Then, anti-inflammatory action of active components in comparison with the extracts will be measured on HaCaT cells as well as keratinocytes derived from psoriatic patients.

This research work also aims to find out the most interesting extracts from *R. coriaria* fruits and their effects in rescuing keratinocytes from the detrimental effect of inflammation induced by UVB exposure. Exposure to UV radiations, which comprise approximately 90–99% UVA and 1–10% UVB, is a critical risk factor in the initiation of dermatologic conditions. The genotoxicity and sun burning effect of UVB (280–315 nm) is 1000 times more than UVA (315–400 nm) (Svobodová, A et al. 2012). The UVB is involved in skin inflammatory processes including a cascade of events leading to release of TNF-α, IL-8 and activation of the NF-κB pathways. Consequently, the downregulation of keratinocytes inflammatory chemokine production and the inhibition of their interaction with immune cells may be an effective target

in the treatment of inflammatory skin diseases (Kwon, Bae et al. 2012, Yang, Hwang et al. 2015).

The viability of cells following 1h pre-treatment or 24 h post-treatment with mERC or EWRC were measured at 24 h after the UVB exposure using MTT assay. The 1 h and 24 h treatments with mERC and EWRC failed to attenuate the negative effect of UVB on HaCaT cells. Then the viability of HaCaT cells were tested following 9 h post-treatment with different concentrations of mERC and EWRC, and the supernatants were collected and analyzed using IL-8 ELISA assay. The 9 h post treatment with mERC and EWRC did not show any cytotoxicity in HaCaT cells following the UVB exposure. The IL-8 secretion was suppressed by 9 h post-treatment with mERC whereas EWRC did not show any effect. The macerated ethanol extract inhibited the release of IL-8 in a concentration-dependent manner in an *in vitro* model of UVB induced skin inflammation.

Among the pro-inflammatory cytokines synthesized in response to UVB radiation, IL-8 is a potent pro-inflammatory CXC chemokine known to play a prominent part both in inflammatory and hyperproliferative skin disorders. IL-8 production by epidermal cells contributes to the development of inflammatory skin diseases through induction of the migration of inflammatory and immunocompetent cells. Furthermore, biologically active IL-8 produced in large amounts in psoriatic skin acts as an autocrine growth factor and promotes epidermal cell proliferation (Tuschil et al., 1992).

This study also determined the most suitable active extracts of *R. coriaria* fruits, for topical anti-inflammatory activity test. To prepare the apolar and lipophilic extracts with higher skin permeability, the process of extraction was performed with three different solvents including acetone, dichloromethane and ethyl acetate using a double extraction method. Firstly, the extracts were evaluated for cytotoxicity and effect on the production of IL-8 in TNF-treated HaCaT cells. An important class of immunobiologic therapies that has been introduced over

the past decade is TNF-inhibitors, which offer additional treatment options for patients with moderate-to-severe psoriasis. As a deeper understanding of disease pathogenesis is reached, more specific therapeutic targets for psoriasis can be found with potentially greater efficacy and fewer side effects. The cytotoxicity results revealed that, at 25μg/mL, none of the extracts showed toxic effect in TNF-treated cells. ARC and EARC inhibited the release of IL-8 significantly at concentrations 25 μg/mL in TNF-treated cells. At concentrations 1-50 μg/mL ARC and EARC did not show any cytotoxicity in this condition. ARC and EARC at concentrations 5-50 μg/mL significantly suppressed the IL-8 production with IC_{50s} 2.7 and 5.2 μg/mL respectively. In these cells ARC and EARC, significantly blocked NF-κB -luc transcription with IC_{50s} 17.18 and 21.92 μg/mL, respectively. As shown in Tab. 8 ARC and mERC are the extracts with the lowest IC_{50s} for IL-8 release and NF-κB-LUC transcription.

Then the extracts were analyzed in HaCaT cells stimulated with a chemical insult, PMA/TPA a phorbol ester, which activate the NF-κB transcription and release of proinflammatory mediators like TNF-α, IL-6 and 8. This inflammatory agent is usually used in an *in vivo* model of skin inflammation (TPA-induced ear edema). The aim of this part of our research is an *in vitro* assessment of the extracts against TPA and the results will be valuable for further validation of this herbs in *in vivo* studies. Firstly, EARC has been excluded for further studies due to its cytotoxic effect in presence of PMA. In this condition also the extracts were not active against IL-8 release. The extracts were tested at 50 μg/mL in HaCaT cells treated with 100 nM PMA, and EWRC showed significantly toxic effect. EARC and ARC showed significant inhibitory effect for IL-8 release. ARC at 25 and 50 and mERC at 50 μg/mL significantly inhibited the release of IL-8. At concentration 50 μg/mL, ARC also modulated NF-κB -LUC transcription, with IC_{50} 27.82 μg/mL.

To summarize, both mERC and EWRC affect the ability of TNF-α-stimulated HaCaT cells to produce pro-inflammatory mediators including ICAM-1, MMP-9, and IL-8 acting, at

least in part, on the NF-κB pathway whereas only mERC impaired VEGF release. On the other hand, the activities of mERC and also ARC also demonstrate suitability of use of these extracts for *in vivo* model of TPA induced inflammation. In fact, it appears that in comparison also with the other evaluated herbal extracts (*E. amoenum* and *A. lappa*), mERC can be considered as the most active extract of this research. Our findings on the inhibitory effects of the *R. coriaria* on NF-κB signaling seem to confirm the traditional use of this herb as a remedy to treat skin inflammatory conditions. Other mechanisms may be involved in the response of the other extracts to skin inflammation; however, this point requires further investigations.

5.2. Conclusion

Keratinocytes produce cytokines and chemokines involved in the development of inflammatory skin disorders, such as psoriasis and atopic dermatitis (Han et al., 2011). The ability of TNF-α and UVB, and chemical insults including TPA or PMA, to stimulate the production of cytokines by keratinocytes has been previously demonstrated in skin inflammation (Jung et al., 2012). In the present study, both selected extracts (*Rhus coriaria* L. macerated ethanol extract (mERC) and *Rhus coriaria* L.ethanol-water extract (EWRC)) were active as anti-TNF agents to impair the NF-κB pathway, which plays a key role in the potentiation of the processes leading to keratinocytes inflammation. mERC and EWRC reduced MMP-9 and ICAM-1 release from HaCaT cells in a concentration dependent manner, but only mERC was active against VEGF secretion. The mERC also inhibited the IL-8 release following UVB exposure, and similar to apolar extract of this herb (ARC) showed a significant effect in inhibiting the PMA–induced IL-8 releases.

In conclusion, inhibition of the production of pro inflammatory mediators, which are involved in recruitment of neutrophils, infiltration of leukocytes, degradation of matrix and formation of new blood vessels, reveals the potency of *R. coriaria* as novel opportunity against

skin inflammation. The concentration-dependent decrease of pro-inflammatory mediators like ICAM-1, VEGF, MMP-9, inhibition of NF-κB signaling and anti-IL-8 activity in UVB, TNF and PMA induced conditions were achieved in cells treated with mERC, suggesting it as the most potential active extract in this study. These findings particularly on the effects of mERC, confirms for the first time the traditional use of *R. coriaria* in the treatment of inflammatory skin diseases. Its use for the treatment of skin inflammation is worthy of further *in vivo* investigation. In addition, the effect of *A. lappa* on inhibition of TNF-induced NF-κB activation, could be also an evidence, providing a theoretical basis for the traditional use of this herb in treating skin inflammation.

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