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To my family…!!!

Contents

Chapter 1

General Introduction

Inflammation is an immune reaction of the body to the external stimuli such as toxins or pathogens, and is characterized by redness, swelling, pain, and heat, which are localized at the site of infection (Ruslan, 2010). The process of inflammation is regulated by several pro-inflammatory and antiinflammatory cytokines. Tumor necrosis factor-α (TNF-α) is a major pro-inflammatory cytokine involved in the inflammatory response. Besides inflammatory diseases like rheumatoid arthritis and inflammatory bowl disease, elevated TNF-α expression has been found to be associated with the development of diabetes, atherosclerosis, septic shock, and tumorigenesis. Thus inhibition of TNF-α at any step of inflammatory pathways provides an attractive treatment for inflammatory diseases as well as for series of other common diseases. TNF-α is secreted by macrophages, monocytes, neutrophils, T-cells, and NK-cells after their stimulation by lipopolysaccharides (LPS) (Paul et al., 2006).

LPS, the major component of bacterial cell walls, is known as potent inducer of TNF-α production. It normally consists of three parts: Lipid A, a core oligosaccharide, and an O side chain (Raetz and Whitfield, 2002). The Lipid A portion is responsible for the biological activity of LPS, whilst recent evidence suggests that the polysaccharide tail determines the antigenic properties (Lien et al., 2000; Poltorak et al., 1998). Lipopolysaccharides are very potent molecules known to activate macrophages at concentration as low as 1 nM (Aderem, 2000), and have been used as a stimulus for promoting inflammation in many studies (Old, 1985; Yuliana et al., 2011a).

Currently several types of clinically approved drugs are available for inhibition of TNF-α production in different disease conditions. These include Etnercept, Infliximab, and Adalimumab. Although these drugs are potentially beneficial to human health, unfortunately they also exert some devastating effects such as an increased chance of infections, heart failure, neurological changes and several problems related to autoimmunity (Palladino et al., 2003; Scheinfeld, 2004). Thus, it is essential to develop safer, less toxic, and beneficial TNF-α inhibitor drugs.

Plants provide an alternative sources of medicines used traditionally by people worldwide since thousands of years ago. Around 80% of the World's population relies upon plants for primary health care. Currently, about 25–30%

of all drugs available as therapeutics are derived from natural products (plants, microbes and animals) or are natural product derivatives (Calixto, 2005). About 50,000 flowering plants are used as medicinal plants out of the total 422,000 flowering plants reported from this world (Schippmann et al., 2002). It is very well documented that plants produce a vast and diverse nature of compounds, known as primary and secondary metabolites. Plants utilize secondary metabolites in its interaction with its environment as defense against attack by pathogens or herbivores, or to attract pollinators (Verpoorte, 1998). According to a rough estimate, around 30,000 metabolites are present in a single plant (Verpoorte et al., 2008). These metabolites have been classified into different classes such as flavonoids, phenolics, glucosinolates, terpenoids, and alkaloids. Plants create species specific compounds by sharing core biosynthetic pathways and then utilizing unique modification enzymes at the end of the pathway to generate novel chemical structures with significantly changed specific biological activities (Kliebenstein, 2011). Secondary metabolites display diverse pharmacological activities which include antiinflammatory, antiviral, antibacterial, antitumor, antihypertension, antidepressive, sedative and many more (Erlund, 2004; Lovkova et al., 2001; Shaheen et al., 2005).

Finding a lead with particular activity as TNF-α inhibitors requires a reliable *in-vitro* assay as the preliminary step. Several human monocytic cell lines (U937, HL-60, THP-1, and Mono Mac 6) are available that are widely used as *in-vitro* model systems for monocytes and macrophages (Verhoeckx et al., 2004). Among them, U937 cell lines have been used extensively as an *in-vitro* model in biomedical research (Lee et al., 2007; Yuliana et al., 2011a). U-937 is a tumor cell line derived from the pleural effusions of a patient with histiocytic lymphoma. Phorbol 12-myristate 13-acetate (PMA), one of the most potent tumor promoting agents has been shown to induce monocytic differentiation. The PMA-stimulated cells acquired morphological, ultrastructural, and functional characteristics typical of cells of the monocyte/macrophage lineage. The PMA-treated U-937 cells became adherent, and are no longer able to proliferate. Furthermore, the cells become functionally similar to monocyte/macrophage-like cells that can perform phagocytosis, antibody dependent cellular cytotoxicity, antigen presentation and chemotaxis (Minta and Pambrun, 1985; Verhoeckx et al., 2004).

In-vivo assay using animal model is the next step after a compound or an extract is determined "active" by an *in-vitro* assay. The zebrafish embryo (*Danio rerio*) has become an important vertebrate model for assessing drug effects. Zebrafish embryos exhibit unique characteristics, including ease of maintenance and drug administration, short reproductive cycle, and

transparency that permits visual assessment of developing cells and organs. Because of these advantages, zebrafish bioassays are cheaper and faster than mouse assays, and are suitable for large-scale drug screening (Parng et al., 2002). There is strong conservation between zebrafish and humans when compared with other model organisms, such as the fruit fly *Drosophila melanogaster* and the worm *Caenorhabditis elegans*, which makes zebrafish an excellent model organism for studying complex biological processes, such as generation of the nervous system, kidney, heart, hematopoietic system, and notochord, as well as assessing angiogenesis, apoptosis, and toxicity response (Kari et al., 2007).

An active plant extract (mixture of compounds) must contain individual active compounds or perhaps interaction/ synergisim among the compounds that contribute to the activity if compared to the non active plant extract. Further step is studying the mixture of compounds present in the extract / biological matrix by metabolomics. Metabolomics is an approach aimed for the monitoring of primary and secondary metabolism and can be defined as a metabolic snapshot of a living system. Several analytical techniques (GC, HPLC or UPLC combined with UV and/ or MS, and MR) have been used in metabolomics e.g., for metabolic fingerprinting of different plants. NMR spectroscopy is an effective technique for both metabolite fingerprinting and metabolite profiling applications in samples of plant origin. It has some unique advantages over chromatography and MS-based methods. Despite of its low sensitivity, the ease of NMR spectroscopy in identification of compounds make it a popular tool in the area of metabolome analysis. The most prominent features of NMR are its non destructive nature, simple sample preparation, and the relative short measuring time. Moreover, NMR-metabolomics data stand for ever, as long as the same extraction procedures and the same NMR-solvents are used. An NMR spectrum represents the physical characteristic of a compound and thus highly reproducible. NMR can be used to identify metabolites of biological origin of which no a-prior knowledge is available. Furthermore, structure elucidation of unknown compounds in a complex mixture can be performed using 2D NMR methods like *J* resolved, COSY, NOESY, HMBC and HSQC. In addition, one can easily get information regarding quantity and quality of metabolites as signal intensity of NMR spectrum is directly proportional to the molar concentration of the metabolites.

The development of methods and algorithms for the multivariate statistical modeling have contributed much to metabolomics as they opened the way for handling the huge datasets of large-scale metabolic analyses. NMR spectroscopy together with multivariate data analysis has been widely practiced

for metabolic profiling of various samples (Ali et al., 2011a; Choi et al., 2004a). Several studies have been published regarding correlation of metabolic profiles of plant extracts with its bioactivity profile (Ali et al., 2012). This approach has allowed the identification of the active compounds from crude extracts without extensive chromatographic steps and techniques. Application of this approach requires the consideration of important factors like extraction, identification and statistical methods. Thus, an untargeted metabolomic approach is important to find correlation between NMR data and the bioactivity profile of fractionated different types of extracts or extracts of different individual plant accessions. For application of this method, one requires an extraction method which can cover a broad range of metabolites present in the plants (polar to non polar). Solid phase extraction and comprehensive extraction methods have been used recently for such studies (Ali et al., 2012; Yuliana et al., 2011c).

Multivariate data analysis algorithms are an essential component of any metabolomics study. These methods are used to reduce the dimensionality of a multivariate dataset and thus enable to recognize possible differences or similarities among the samples. Principal component analysis (PCA) is considered as a primary tool in metabolomics, helping to better understand possible differences between samples. It is an unsupervised method; hence the separation of samples is purely due to differences among the samples. In order to identify the metabolites responsible for activity, a supervised methods are applied e.g. partial least squares-discriminant analysis (PLS-DA), is used. In this case samples are, for example, classified in high and low active classes by creating dummy *Y*-variables. Projection to latent structures (PLS) is another supervised method in which instead of creating dummy *Y*-variables, the actual data from anti-TNF-α assay can be used as a *Y*-data set. The application of bidirectional orthogonal-PLS (O2PLS) resulted in much better distinction of the samples with different activities than the PLS model. One of the key aspects of a supervised regression algorithm is model validation. A permutation test is often used for validation of methods like PLS and PLS-DA. A permutation test is the calculation of goodness of fit and the predictive ability of the model, R2 and Q2, respectively.

Application of NMR spectroscopy together with multivariate data analysis makes identification of compounds responsible for activity easy and thus these compounds can be further identified and elucidated by means of 1D and 2D NMR techniques.

Aim of the thesis

The aim of this study was to develop methods for the rapid identification of active compounds in plant extracts by correlating NMR metabolomics and bioassay results by means of multivariate data analysis. Various food plants were thus studied for antiinflammatory activity. Following objectives were addressed in this general aim.

- Development of high throughput antiinflammatory bioassays for screening plant extracts
- Development of rapid, fast and reliable extraction and fractionation methods for bioacticity based screening of plants
- Development and validation of chemometrics methods for identification of compounds related to bioactivity

Different extraction methods like comprehensive extraction method and solid phase extraction together with multivariate data analysis were used to detect the active compounds in different plant extracts. TNF-α bioassay and zebrafish bioassay were used to measure the bioactivity of plant extracts *in-vitro* and *in-vivo*. NMR spectroscopy was used to characterize the metabolic profile of different plant extracts. Several multivariate data analysis methods were used to determine the correlation between metabolic profile and bioactivity.

Outline of the thesis

The thesis begins with a comprehensive review discussing the phytochemicals as a potential source for TNF-α inhibitors. This review briefly summarizes the role of TNF-α in the, its receptors in the signaling cascades of the cellular immune response, and assess briefly various natural compounds which are known to inhibit TNF-α release **(Chapter 2).** Sixty six different plant extracts were screened out for their ability to inhibit TNF-α release in LPS stimulated U937 cell lines. The active extracts were further tested for their antiinflammatory activity *in-vivo* using transgenic (MPO) Zebrafish embryo as a model system **(Chapter 3).** Anti TNF-α inhibition of major cannabinoids isolated from *Cannabis sativa* using U937 cell lines was presented in **Chapter 4.** Screening of different fruit berries against TNF-α production, NMR spectroscopy and multivariate data analysis-based study was described in **Chapter 5.** The use of solid phase extraction along with multivariate data analysis to predict anti-TNF-α activity in different grape cultivars, at different developmental stages is presented in **Chapter 6.** The inhibition potential of

different red wines from different vintages against TNF-α production is also assessed and presented in **Chapter 7.** NMR spectroscopy coupled with multivariate data analysis to measure antiinflammatory activities (*in-vitro, in-vivo*) of *Eugenia uniflora* is presented in **Chapter 8.** Comprehensive extraction integrated with multivariate data analysis to identify set of compounds form *Sempervivum pseudocalcareum* responsible of antiinflammatory activities is presented in **Chapter 9.** Finally general discussion, conclusions and future prospects related to metabolic profiling and bioactivity screening of different plants are presented in **Chapter 10.**

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Chapter 2

Phytochemicals as a potential source for TNF-α inhibitors

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Abstract

Cytokines play an important role in the immune system. Any disorder in the regulation of cytokines can lead to the development of inflammatory diseases. Tumor necrosis factor- α (TNF-α) is one of the most important inflammatory cytokines that controls different types of cell functions. The overproduction of TNF-α is linked with the development of various diseases such as asthma, rheumatoid arthritis, psoriatic arthritis, inflammatory bowel disease, septic shock, diabetes and atherosclerosis. Plants are considered as excellent sources of pharmacologically active compounds. Currently, scientists are searching for natural products with anti-TNF-α properties for the treatment of various inflammatory disorders. At present, protein-based drugs are available for the inhibition of TNF-α, however these have some limitations. Plant might provide an alternative and cost-effective source of drugs that can regulate TNF-α level. This review briefly highlights the physiological and pathological roles of TNF-α along with a description of plant-derived compounds capable of interfering with TNF-α activity and production.

Key words: Inflammatory disorders, medicines, natural products, plants, TNF-α inhibitors

Definitions

NF-κB: A protein complex which is found in all animal cells and is actively involved in regulating immune response to infection.

T cells: They belong to a group of white blood cells known as lymphocytes, and play a central role in cell mediated immunity.

NK cells: (Natural killer cells), these are lymphocytes and they are considered as a major component of the immune system.

B cells: These are lymphocytes that play a large role in the humoral immune response.

IL-1α and β: These are pro-inflammatory cytokines involved in immune defense against infection.

IC50: Half maximal inhibitory concentration: is a measure of effectiveness of a compound in inhibiting a biological function.

IFN-γ: Interferon-gamma; a soluble cytokine that is important for innate and adaptive immunity against viral and intracellular bacterial infections.

TNF-α: (Tumor necrosis factor alpha) is a pro inflammatory cytokine involved in the process of inflammation. It is produced by several types of cells but especially macrophages.

Abbreviations:

AKT	A serine/threonine kinase
AP-1	Activator protein-1
COX-2	Cycloxygenase 2
DD	Death domain
EGB-761	Extract of *Ginkgo biloba*
ERK	Extracellular signal-regulated kinases
ICAM-1	Intercellular adhesion molecule-1
IGF	Insuline- like growth factor
IKB	Inhibitor of nuclear factor-Kappa B
INOS	Inducible nitric oxide
IRA	Insulin receptor activation
LOX-2	Lipoxygenase-2
LPS	Lipopolysaccharides
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MEKK1	Mitogen-activated protein kinase kinase 1

NF-κB	Nuclear factor kappa B
NIK	Nuclear factor-kB-inducing kinase
NO	Nitric oxide
PDE4	Phosphodiesterase 4
PGE2	Prostaglandin E2
PK	Protein kinase
RAW-264.7	Rat leukemia monocyte macrophage cell line
RBL-2H3	Rat basophilic leukemia mast cell line
RIP	Receptor activating protein
TACE	TNF-α converting enzyme
TNF-α	Tumor necrosis factor alpha
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
ZZ-CWE	Crude Water extract of *Zingeber zerumbet*

2.1. Introduction

Tumor necrosis factor-α (TNF-α) is one of the most important regulatory proteins in the immune system of animal cells. It was discovered in 1975 by Lloyd Old and his colleagues because of its anti-tumor activity (Bradley 2008; Tracey and Cerami 1994), although previous observations in the 19th century had already shown that heat-killed bacteria could be used to induce tumor regression in cancer patients (Carswell et al. 1975). Presently TNF-α is also known to mediate tumor initiation, metastasis and inflammation.

Extensive studies of cell signaling cascades have shown that dysregulation of TNF-α can result in a wide variety of inflammatory diseases including asthma, dermatitis, cystic fibrosis, rheumatoid arthritis, inflammatory bowel/Crohn's disease, multiple sclerosis, psoriasis, systemic lupus erythematosus, diabetes type II, atherosclerosis, Alzheimer's disease, osteoporosis and autoimmune deficiency disease (Bjo¨rnsdottir and Cypcar 1999; Cohen et al. 1996; Medana et al. 1997; Murphy et al. 1998; Sekut and Connolly 1996). Those diseases can be initiated by several genetic factors, pathogenic bacterial or viral infections, as well as by lifestyle such as diet, smoking and alcohol consumption, which can promote metabolic or oxidative stress (Hu 2011), and aging (Hunt et al. 2010; Sarkar and Fisher 2006). The molecular mechanisms underlying these diseases are complex and many are still unknown, although it has been shown that proper regulation of TNF-α is necessary to keep the immune system in balance (homeostasis).

It is evident that the suppression of TNF-α could be beneficial in different inflammatory diseases. Significant efforts have been made in developing drugs that interfere with TNF-α production (Cohan et al. 1996; Moreira et al. 1993). Currently, several types of clinically approved drugs are available for the inhibition of TNF-α production in different disease conditions. These include, Etnercept, Infliximab, and Adalimumab (Scheinfeld 2004). Although these drugs are potentially beneficial to human health, they can also exert some devastating effects such as an increased chance of infection, heart failure, neurological changes, and problems related to autoimmunity (Palladino et al. 2003; Scheinfeld 2004). Thus, it is essential to develop safer, less toxic, and more beneficial anti- TNF-α drugs.

Plants are sources of medicines which are used since thousands of years in China or India, and the core of global medicine has its roots in plant products. Some 150,000 plant compounds are already known, and many more are expected to be discovered since only a small percentage of plants have been studied phytochemically and/or pharmacology. Together with the known compounds, plant compounds form a great source for screening for TNF-α inhibitors. By screening plant extracts one may find single active compounds or

mixtures that work in synergy. Hits from screening can be isolated and be further developed into new leads with improved activity and low toxicity.

This review briefly summarizes the role of TNF-α and its receptors in the signaling cascades of the cellular immune response, and assess briefly various natural compounds which are known to inhibit TNF-α release.

2.2. TNF-α, cell signaling pathways and pathology

Tumor necrosis factor-α (a transmembrane protein of 26-kDa) is released in its active form (a 17-kDa protein) by macrophages and a wide variety of cells, particularly immune cells (e.g. mast cells, T cells, neutrophyls, NK cells and synovial cells) upon stress stimuli. This process is tightly regulated and involves an enzyme called TNF-α ctivating converting enzyme (TACE), a membrane bound disintegrin metalloprotease, which belongs to the family of mammalian adama-lysins (ADAMs), and can be a target for inhibition of TNF-α release (Black et al. 1997; Moss et al. 1997).

There are two types of receptors that bind TNF-α with comparable binding activity and through which its effects are mediated; TNF receptor I (TNF-R1, also known as p60, p55 or CD120a), which is expressed in all cell types in the body, and TNF receptor II (TNFR2, also called p80, p75 or CD120b). The latter is only expressed on cells of the immune system and on endothelial cells (Aggarwal et al. 2006).

Several (downstream) pathways are activated whenTNF-α binds to TNF-R1. For example, it activates the pathway leading to apoptosis when the cytoplasmic ‘‘death domain’’ of TNF-R1 recruits proteins including ‘‘TNF-receptor-associated death domain’’ (TRADD), ‘‘Fas-associated death domain’’ (FADD) and ‘‘FADD-like ICE’’ (FLICE, also called caspase-8) leading to activation of caspase-3, which is responsible for degradation of multiple proteins (Nagata and Golstein 1995).

The signaling cascade leading to inflammation occurs when the ‘‘death domain’’ of TNF-R1 recruits TRADD, which subsequently recruits a protein called ‘‘TNF receptor-associated factor’’ (TRAF2). Mediated by ‘‘receptor-interacting protein’’ (RIP), the complex activates IκBα kinase (IKK), which is needed for the activation of IκBα. Activated/phosphorylated IκBα is necessary to interact with and inhibit ‘‘nuclear factor kappa-light-chain-enhancer of activated B cells’’ (NFκB). Subsequently, ubiquitination and degradation of IκBα complex releases the free NF-κB. The latter translocates to the nucleus, binds to the promoter or enhancer regions of target genes to enhance transcription (Bonizzi and Karin 2004; Campbell et al. 2004; Luqman and Pezzuto 2010). There are many genes known to be regulated by NF-κB

including TNF-α itself and those that play roles in inflammation, such as cyclooxygenase-2 (COX-2), lipoxygenase-2 (LOX-2), cell-adhesion molecules (CAMs), inflammatory cytokines and inducible nitric oxide synthase (iNOS). Thus, the proinflammatory effects of TNF-α are mainly due of its ability to activate NF-κB (Aggarwal et al. 2006). This pathway is a particular therapeutic target for arthritis and other inflammatory diseases. Any agent that can block activation of this pathway that results in down regulation of NF-κB, TNF-α and other inflammatory enzymes and cytokines can be a drug candidate (Aggarwal et al. 2006; Khanna et al. 2007). Similar to that of IκBα, degradation of IκBβ releases NF-κB dimers and upregulates TNF-α, however, recently, Rao et al. (2010) reported that in vivo IκBβ served both to inhibit and facilitate the inflammatory response. The absence of IκBβ in IκBβ-/- mice resulted in a dramatic reduction in TNF-α levels in response to lipopolysaccharide (LPS) challenge even though activation of NF-κB was normal. The inhibition of the mRNA for TNF-α correlated with the absence of nuclear hyposphorylated-IκBβ bound to p65:c-Rel heterodimers at specific κB site on the TNF-α promoter. Thus, blocking IκBβ might be a promising strategy for selective TNF-α inhibition at the chronic phase of TNF-α production during the inflammatory response.

Aberrant regulation of NF-κB is found to occur in many types of cancer cells. Some studies showed that NF-κB could modulate the transcriptional activation of genes associated with cell proliferation, angiogenesis, metastasis, tumor promotion, suppression of apoptosis and drug resistance (Luqman and Pezzuto 2010), meaning that downregulation of NF-κB can also be a target for anticancer therapies (Karin et al. 2006; Luqman and Pezzuto 2010).

Several other pathways can also be induced by TNF-α through the recruitment of TRAF2 leading to the activation of activator protein-1 (AP-1), c-Jun amino terminal kinase (JNK), p38 mitogen activated protein kinase (p38MAPK), p44/p42MAPK (also called extracellular signal-regulated kinases 1/2, ERK1/2), and AKT, which in turn can upregulate TNF-α activation. The AP-1, JNK and p38MAPK pathways can contribute to stress responses and inflammation. For example, a reversible ATP-competitive inhibitor ‘‘SP600125’’ inhibited the phosphorylation of c-Jun and the expression of the inflammatory genes COX-2, IL-2, IFN-c and TNF (Bennett et al. 2001). It also prevented the activation and differentiation of primary human CD4 cell cultures. Campbell et al. (2004) showed that p38MAPK regulated the TNF-α production relevant to inflammation suggesting that this kinase could be a therapeutic target in rheumatoid diseases. Some other pathways seem to provide negative feed-back in TNF-α- induced apoptosis. For example, TNF-induced ERK1/2 activation is necessary for cell proliferation and has a dominant protective effect over apoptotic signaling from the death receptors (Aggarwal et

al. 2006; Tran et al. 2001). In addition, the TNF-α induced AKT pathway is involved in cell growth, as it was shown that inhibition of AKT kinase resulted in suppression of cell growth and induction of apoptosis in human cancer cells (Yang et al. 2004). Thus, both the ERK1/2 and AKT pathways can be targets for developing anticancer drugs.

The present day knowledge about the cell signaling cascade shows the importance of TNF-α as a regulatory factor in many cellular processes in the immune system and the complexity of the signaling network.

It is clear that in pro-inflammatory state, TNF-α is upregulated in the inflamed organs/tissues together with some proinflammatory cytokines which are involved in the initiation of inflammation (e.g. IL1-β, IL-6 and IL-18), and also some anti-inflammatory cytokines (e.g. IL-10, IGF-β and IRA) which inhibit the production of inflammation (Choy and Panayi 2001). TNF-α is not the only cytokine involved in inflammation, at least 100 different types of cytokines and their receptors have been identified (Kim et al. 2004b). Upregulation of TNF-α (and some other cytokines) is part of the complex signaling mechanism that results in the production, activation, and recruitment of immune cells (leucocytes) to sites of infection in order to combat pathogens. For example, infection by pathogenic gram-negative bacteria can generate inflammation because the major component of bacteria cell wall, LPS, is potent inducer of TNF-α production.

Lipopolysaccharide normally consists of three parts: Lipid A, a core oligosaccharide, and an O side chain (Raetz and Whitfield 2002). The Lipid A portion is responsible for the biological activity of LPS, whilst recent evidence suggests that the polysaccharide tail determines the antigenic properties (Lien et al. 2000; Poltorak et al. 2000). LPS are very potent molecules known to activate macrophages at concentration as low as 1 nM (Aderem 2000), and have been used as a stimulus for promoting inflammation in many studies (Old 1985; Yuliana et al. 2011a).

Lipopolysaccharides-stimulation of mammalian cells occurs through a series of interactions involving several proteins including the LPS-binding protein (LBP) in serum, followed by the binding of the LBP/ LPS complex to CD14. This subsequently activates the signal transduction pathways including the MAPK family, ERK1/2, JNK, p38MAPK, and the transcription factor NF-κB, and induces gene expression of various pro-inflammatory cytokines (Fig. 1) (Guha et al. 2001; Kishore et al. 2004). Macrophages exposed to LPS produce the transmembrane 27 KDa TNF-α, that binds directly to TNF-R1 and TNF-R2 receptors through cell-to-cell contact or the secreted 17 KDa TNF-α that can bind these receptors in its soluble form (Jones et al. 1989). Production of TNF-α by LPS stimulated macrophages is highly accelerated in the presence of interferon-γ (IFNγ), resulting in differentiation and activation of nitric oxide

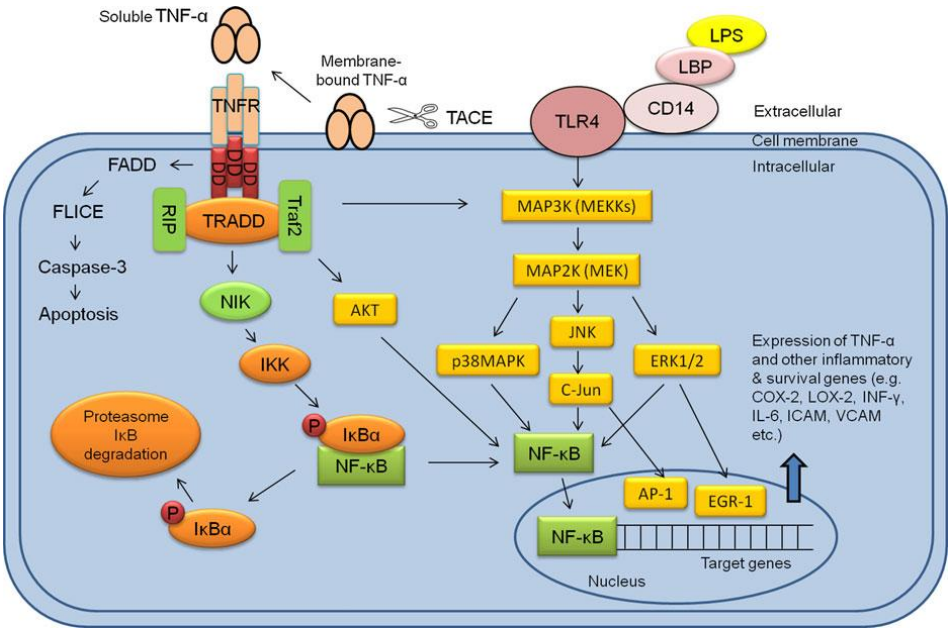


Figure 1: TNF-α and signaling cascade. Binding of TNF-α to its receptor TNFR results in the recruitment of TRADD, TRAF2 and RIP. This is followed by the activation of IKK, binding of IκBα to NFκB, phosphorylation of IκBα, and releasing and transport of NFκB into nucleus; the latter binds to several target (inflammatory) genes. Binding of LPS to LBP and subsequently to CD14 and TLR4 activates the signaling pathways of JNK, p38MAPK and ERK1/2 for upregulation of TNF-α through activation of NFκB or some other pathways. Recruitment of TRAF2 can also activate JNK, p-38MAPK, ERK1/2 and AKT pathways for regulation of TNF-α.

synthase, which in turn induces the production of nitric oxide in large amounts. The latter ultimately enhances the killing of microorganisms. The absence of IFNγ causes the macrophages to produce an insulin like growth factor (Heller et al. 1990).

Infection by pathogens is not the only method to cause inflammation. Although the mechanisms are not clear, some oxidative and metabolic stress associated with an ‘‘unhealthy’’ life style e.g. excessive consumption of palatable food, alcohol, and cigarettes/ smoking (Hu 2011), as well as aging (Hunt et al. 2010) may also generate an inflammatory state and trigger autoregulation/dysregulation of the immune system. Recent study has even shown that a life-style associated with sleep deprivation can lower insulin sensitivity, whilst disruption of the circadian rhythm (e.g. for persons working night-shift or frequently experiencing jetlag) decreases the production of insulin (Buxton et al. 2012).

This suggests a coupling between how the biological clock orchestrates the metabolism and the light-cycle, where perturbation can result in

dysregulation and even destruction of the whole system/body. Thus, stress can cause the immune system response by upregulation of some inflammatory cytokines. A chronic dysregulation of TNF-α, due to improper activation of one or more TNF-induced pathway(s), can cause a wide variety of diseases. For example, obesity can promote inflammation. Once an inflammatory state is established and becomes uncontrolled, it can develop into several diseases such as metabolic syndrome, diabetes type II, atherosclerosis and cardio-vascular diseases. Adipose tissue can synthesize TNF-α and IL-6 (Yudkin et al. 1999). Increased TNF-α levels in the adipose tissues of obese people is probably also aimed to decrease insulin sensitivity for lowering the sugar influx to the cells. It is known that the ligand-receptor complex of TNF-α and soluble TNFR1 is linked to the secretion of insulin (Ferna´ndez-Real and Ricart 1999; Ferna´ndez-Real et al. 1999), whereas TNF-α/TNFR2 is linked to the action of insulin (insulin sensitivity for glucose transport into the cell) (Ferna´ndez-Real et al. 1998). Thus, obesity promotes inflammation and can lead to the emergence of diabetes type II (high production but low sensitivity to insulin), which can also develop further to diabetes type I due to the damage of the Islet cells of the pancreas. Ferna´ndez-Real et al. (2006) identified a biologically active form of plasma/soluble TNFR2 which is produced by differential splicing (called DS-TNFR2) in ‘‘healthy’’ people, which can antagonize TNF-α biological activity. Higher plasma concentrations of DS-TNFR2 seem to have an anti-inflammatory role, which may be a target for the treatment of obesity and related metabolic disorders.

Since obesity can promote inflammation, it can also increase the risk for atherosclerosis and further cardiovascular complications. In obese people, the atherogenic diet can cause disturbed vascular blood flow and abnormal shear stress near the arterial wall. This can reduce local production of NO (which acts as a vasodilator and antiinflammatory molecule) and initiate inflammation of the endothelial monolayer. The cells start to express vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), which can mediate binding precisely to various classes of leucocytes (e.g. monocytes and T-lymphocyte). Proteins such as selectins and integrins mediate leukocyte interactions and firmer attachments with the inflamed luminal endothelium, and together with monocyte chemoattractant protein-1 (MCP-1) and other pro-inflammatory cytokines cause penetration/ migration of the adhered macrophages into the intima. As the inflammation continues, many inflammatory leukocytes (macrophages) extravasate and since they express scavenger receptors for modified lipoproteins (which may derive from low density lipoprotein, LDL), ingest lipids and become foam cells. This can attract T cells and enhance the production of cytokines (e.g. γ-interferon, lymphotoxin/ TNF-β). The activated macrophages and vascular cells can subsequently release

fibrogenic mediators and growth factors, which promote replication of smooth muscle cells and the formation of a dense extracellular matrix to form a plague. The large number of activated macrophages in the atheroma release proteolytic enzymes that can degrade the collagen making up the plaque's fibrous cap, which makes the latter susceptible to rupture. In addition, the macrophages also produce a procoagulant that can trigger thrombosis in the plaques. A physical disruption of the atherosclerotic plaque in the coronary artery is the most common cause of a fatal acute myocardial infarction. This shows the link between inflammation, atherosclerosis and cardiovascular complications (Libby et al. 2002). TNF-α induces the production of VCAM-1, ICAM-1, E-selectin, IFN-γ and some other cytokines in atherosclerotic lesion (Joyce et al. 2001).

The dysregulation of TNF-α (inflammation) in patients with rheumatoid arthritis and autoimmune diseases results in an increased chance to develop diabetes, atherosclerosis and cardiovascular diseases.Some studies have reported the possible effectiveness of statins not only for treatment of atherosclerosis and cardiovascular diseases, but also for treatment of other inflammatory diseases like metabolic syndrome and diabetes type II, which are associated with increased levels of C reactive protein (CRP), a sensitive inflammatory biomarker in blood plasma (Libby et al. 2002). Statins may also be considered for treatment of selected cases of rheumatic and autoimmune disorders (Kotyla 2010). This is because statins also have a potent anti-inflammatory activity apart from their LDL-lowering effect. However, even though statins are labeled as safe, there are groups of people for whom statins cannot be prescribed because of adverse side effects such as muscle pain.

Obviously, TNF-α is an important pleotropic cytokine which is central to the development of various inflammatory diseases. Any agent that can suppress the overproduction of TNF-α through inhibition at any step of the various inflammatory pathways, can be considered a potential drug candidate for inflammatory diseases. This review will discuss about natural products as novel TNF-α inhibitors.

2.3. Natural compounds as a source of TNF-α inhibitors

Plants produce a huge diversity of compounds belonging to different classes from which drugs can be developed. Many compounds are known to reduce TNF-α levels or disrupt the various pro-inflammatory mediators that are actively involved in TNF-α expression (Kuhnau 1976). These compounds may provide an alternative for treatment of inflammatory diseases. Here we will review these compounds according to their biosynthetic-chemical classification. For reasons of comparison in Tables 1, 2 and 3 we have summarized some

activity data of well known anti-inflammatory medicines that effect TNF-α.

2.3.1. Flavonoids

Flavonoids (Fig. 2a, b) are among the most widespread secondary metabolites in the plant kingdom. The capacity of flavonoids to act as anti-inflammatory agents has long been utilized in Chinese medicine and in the cosmetic industry in the form of crude plant extracts (Ratty and Das 1988). The Western diet is rich in flavonoids; the daily intake is about 1 g of flavonoids per day (Kuhnau 1976). Many *in vitro* and *in vivo* studies have proven the efficacy of phenolic compounds as anti-inflammatory agents and their ability to modulate pro-inflammatory cytokines.

In studies, it has been observed that flavonoids such as flavones, flavonols, and chalcones can inhibit the production of TNF-α. For example, a study with LPSstimulated J774.1 cells (Herath et al. 2003) showed that various dietary flavonoids including luteolin (1), apigenin (2), kaempferol (3), quercetin (4), myricetin (5), naringenin (6), catechin (7), phloretin (8), butein (9), pelargonidin (10) and cyanidin (11) were potent inhibitors of TNF-α with IC_{50} values ranging from 3 to 37 μM. (Xagorari et al. 2001) reported that pretreatment of RAW264.7 cells with luteolin, genistein (12) luteolin- 7-glucoside (13), and quercetin, inhibited both LPSinduced TNF-α and -IL-6, whereas eriodictyol and hesperetin only inhibited TNF-α release. Among the tested compounds, luteolin and quercetin were the most potent inhibitors of TNF-α with an IC_{50} of 1 and 5 μM, respectively.

Scutellaria baicalensis has been used traditionally in China for the treatment of various inflammatory diseases (Chung et al., 1995). This plant contains wogonin (5,7-dihydroxy-8-methoxyflavone) (14) which has been shown to inhibit NO production (by reducing iNOS) and PGE2 production (by down-regulating COX II expression) in LPS-induced RAW 264.7 macrophages. The IC_{50} values of wogonin were 31 and 0.3 μM for the inhibition of NO and PGE2 production, respectively (Kim et al., 2001). Wogonin has also been found to strongly inhibit TNF-α secretion by LPS-stimulated RAW264.7 cells (Dien et al., 2001). Morin (15), a flavonoid present in various fruits and Chinese herbs, has been reported to have many beneficial biological activities. The effect of morin and its sulphated/glucuronated derivatives on the production of NO and cytokines by LPS-stimulated macrophages were investigated; it was found that these compounds inhibited the release of TNF-α with IC_{50} values of 2.0 and 2.5 μM by morin and morin sulphates/glucuronides, respectively (Fang et al., 2003). It has also been observed that amoradicin (16), a flavanone isolated from *Amorpha fruticosa* extracts, significantly inhibits TNF-α production by LPS-stimulated RAW264.7 cells, with an IC_{50} value of 28.5 μM (Cho et al., 2000b).

Four kaempferol glycosides were isolated from the leaves of *Cinnamomum osmophloeum* Kaneh, a Taiwan endemic tree; kaempferitrin (17), kaempferol-3-*O*-β-D-apiofuranosyl-(1→2)-α-L-arabinofuranosyl-7-*O*-α-L-rhamnopyranoside (18), and kaempferol-3-*O*-β-D-apiofuranosy-(1→4)-α-L-rhamnopyranosyl-7-*O*-α-L-rhamnopyranoside (19). These compounds inhibited LPS and IFN-γ-induced NO, TNF-α and IL-12 with IC_{50} values of 40, 15 and 20 μM, respectively (Fang et al., 2005). Several polymethoxylated flavones were isolated and identified from *Citrus depressa*, which decreased the release of TNF-α with IC_{50} values ranging from 5 to 120 μM (Manthey et al., 1999). These include 5,6,7,4′-tetramethoxyflavone (tetra-*O*-methylscutellarein) (20), tangeretin (21), 3,5,6,7,8,3′,4′-heptamethoxyflavone (22), nobiletin (23), sinensetin (24), 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone (25), 5,7,8,3′,4′-pentamethoxyflavone (26), 7-hydroxy-3,5,6,7,3′,4′ hexamethoxyflavone (27), 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (28), 5,6,7,3′,4′,5′-hexamethoxyflavone (29), 5,6,3′,4′-tetramethoxyflavone hydroxyflavones, 3-*O*-methylquercetin (30), quercetin, apigenin, kaemferol, limocitrin (31), chrysoeriol (32), rhamnetin (33) and acacetin (34). Of all of these flavonoids, 3,5,6,7,8,3′,4′-heptamethoxyflavon (HMF) shows inhibition of LPS-induced TNF-α with an IC_{50} of 5 μM (Manthey et al., 1999). In another study, velutin (35), isolated from the pulp of *Euterpe oleracea* commonly known as açaí fruit, was evaluated for its effects on reducing LPS-induced TNF-α in RAW 264.7 peripheral macrophages and mice peritoneal macrophages. Velutin showed effective inhibition of TNF-α through the suppression of NF-κB activation and inhibited protein kinase p38 and JNK phosphorylation (Xie et al., 2012).

Although various *in-vitro* studies have confirmed the effects of some flavonoids on gene expression of pro-inflammatory cytokines, there have been only a few investigations to prove the same effects of flavonoids *in-vivo*. Intraperitoneal administration of flavonoids such as quercetin to mice suppressed the lethal endotoxic shock induced by LPS, or LPS plus D-galactosamine, whereas rutin inhibited the production of TNF-α (Takahashi et al., 2001). Wogonin was found to inhibit COX-II induction when it was applied topically to TPA-treated mouse skin. Intraperitoneal administration of wogonin also inhibited TNF-α production and lethal shock in mice induced by LPS and D-galactosamine (Dien et al., 2001; Park et al., 2001). Woganin also showed an antiinflammatory activity as did its analogues, baicalein and baicalin, when administered orally in several animal models of inflammation (Kubo et al., 1984). Oral administration of luteolin in LPS-treated mice showed an inhibition of TNF-α production, whilst intraperitoneally administration of luteolin increased the survival rate and inhibited the expression of TNF-α and ICAM-1 (Hiroshi Ueda et al., 2004; Kotanidou et al., 2002). Morikawa et al. (2003)

showed that the administration of quercetin in carrageenan-induced rats inhibited the release of TNF-α, RANTES (Regulated upon activation, normal T-cell expressed, and secreted), MIP-2 (macrophage inflammatory protein 2) and the mRNA of COX-2. All of these investigations prove that several flavonoids including wogonin, luteolin, and quercetin inhibit the expression of pro-inflammatory molecules in experimental animals suggesting that the modulation of pro-inflammatory gene expression is most probably the major mechanism of action of flavonoids which explains their antiinflammatory activity. (For a mechanism of action see Table 1).

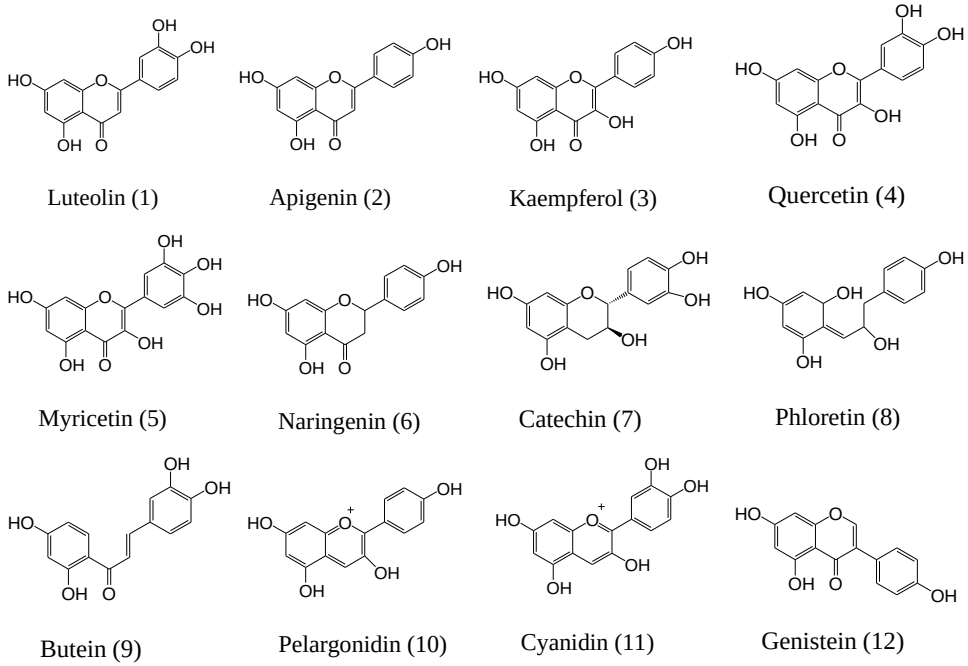


Figure 2a: Structure of natural TNF-α inhibitors: Flavonoids (1-12)

The structure-activity relationship of flavonoids have been discussed in some studies (Chen et al., 2006b; Comalada et al., 2006; Herath et al., 2003; Shanmugam et al., 2008; Xagorari et al., 2001). There are some key rules regarding the structure of a flavonoid that is regard to have an antiinflammatory activity (Herath et al., 2003); (a) flavonoid aglycone shows a stronger activity than its glycoside, (b) a double bond between carbon 3 and 4 with a ketone group at position 4 is necessary, and (c) four hydroxyl groups at positions 5, 7, 3' and 4' are necessary for an optimal antiinflammatory effect. Many *in-vitro* and *in-vivo* studies have shown the efficacy of flavonoids as potential

candidates for treatment of different diseases, however, the bioavailability of flavonoids, is still a matter of debate. There is a large bioavailability of flavonoids, is still a matter of debate. There is a large difference between the amounts of flavonoids consumed in our food or administered as a herbal medicine with the actual absorption of flavonoids in the gastro-intestinal tract. The poor uptake and the rather high activity levels (> 1 μM range) of flavonoids is hampering their development as drug candidates (Scalbert and Williamson, 2000). Biological activities of flavonoids depend on their bioavailability; which includes their GI-tract metabolism, degree of absorption (which takes place in small intestine), metabolism in the liver and body, and excretion. These factors vary considerably among the individual flavonoid subclasses. Bioavailabilty of flavanones seem to be more than other related flavonoids such as flavonols or flavan-3-ols. This can be associated with the fact that these compounds are found to be less degraded than other flavonoids by colonic microbiota and, therefore, are more available for absorption (Selma et al., 2009; Williamson and Clifford, 2010). It has also been observed that flavonoids undergo extensive metabolism and transformation in the colon by microbiota before absorption by the human body. This conversation is often essential and modulates the biological activity of these dietary agents (Crozier et al., 2010; Selma et al., 2009). Flavonoid glycosides are first deglycosylated prior to intestinal uptake, subsequently the aglycones are transported to the liver and metabolized to form conjugates with glucoronide or sulphate, and may undergo various chemical conversions such as methylation and oxidation. It has been proposed that conjucated forms of flavonoids might be involved in the health promoting effects of flavonoids (Crozier et al., 2010; Miyake et al., 2000). Table 2.1 shows the possible mechanism underlying TNF-α inhibition by flavonoids.

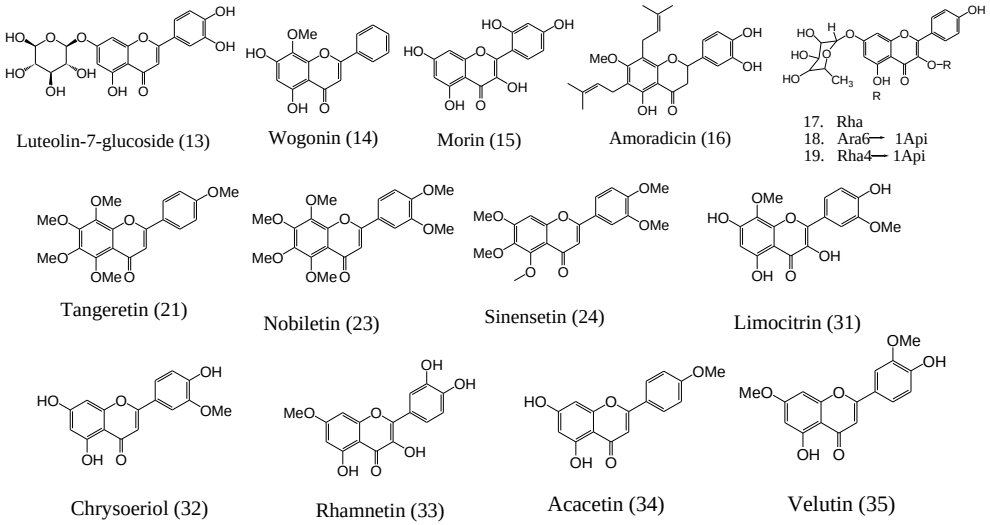


Figure 2b: Structure of natural TNF-α inhibitors: Flavonoids (13-35)

Compounds	Source	IC50/Dose	Possible inhibition mechanism	References
Luteolin	*Perilla frutescens*	14.44 µM	Inhibition of LPS-stimulated TNF-α release by blocking the activation of MAPK, ERK, P38 and CK2 pathways.	(Herath et al., 2003; Ueda et al., 2002; Xagorari et al., 2002)
Genistein	*Glycine max*	65.08 µM	Inhibition of TNF-α release via inactivation of NF-κB.	(Castro et al., 2012; Chen et al., 2012; Herath et al., 2003; Sarkar and Li, 2002)
Apigenin	Apium graveolens	3.27 µM	Inhibition of LPS-induced TNF-α by down regulating NF-κB pathway through the suppression of P65 phosphorylation.	(Herath et al., 2003; Kang et al., 2011; Ko et al., 1991; Nicholas et al., 2007)
Kaempferol	*Capsicum annum*	3.99 µM	Down regulation of TNF-α expression through inhibition of NF-κB, NIK, IKK and MAPK activation.	(Herath et al., 2003; Kim et al., 2007; Miean and Mohamed, 2001)
Quercetin	*Brassica oleracea*	3.11 µM	Suppression the production of TNF-α by inhibiting the phosphorylation and activation of JNK/SAPK and also by inhibition of NF-κB pathway.	(Herath et al., 2003; Nair et al., 2006; Wadsworth et al., 2001)
Myricetin	*Brassica oleracea*	3.84 µM	Inhibition of TNF-α release by suppressing IκB activity.	(Herath et al., 2003; Miean et al., 2001; Rathee et al., 2009)
Naringenin	*Citrus paradisi*	14.41 µM	Inhibition of LPS-induced TNF-α by suppressing phosphorylation of P38, serines 63 and 73. It also increases ERK5 phosphorylation which inhibits inflammatory response.	(Bodet et al., 2008; Herath et al., 2003; Vafeiadou et al., 2009; Wilcox et al., 1999)
Catechin	*Camellia sinensis*	3.11 µM	Inhibition of TNF-α release by blocking NF-κB activation.	(Baumann et al., 2001; Herath et al., 2003; Yang et al., 1998)

(Herath et al., 2003; Lommen et al., 2000; Lu et al., 2009)	Inhibition of LPS-induced TNF-α via inactivation of PKC pathways.	3.00 µM	*Malus domestica*	Phloretin
(Cheng et al., 1998; Herath et al., 2003; Jung et al., 2007)	Suppression of TNF-α release by inactivation of NF-κB and JNK1/2.	6.29 µM	*Dalbergia odorifera*	Butein
(Chandra et al., 2001; Herath et al., 2003; Min et al., 2010)	Inhibition of TNF-α production through NF-κB and MAPK inactivation.	36.91 µM	*Prunus cerasus*	Cyanidin
(Lee, 2011; Wang et al., 1998)	Inhibition of LPS-induced NO by blocking NF-κB, phosphorylation of P38 of MAPK, ERK1/2 and JNK.	50 µM	*Thymus vulgaris*	Eriodictyol
(Comalada et al., 2006; Swatsitang et al., 2000; Vafeiadou et al., 2009)	Inhibition of LPS-stimulated TNF-α by blocking ERK1/2, JNK and P38 activation	50 µM	*Citrus sinensis*	Hesperetin
(Chiu et al., 2002; Lee et al., 2003)	Inhibition of TNF-α production through inactivation of NF-κB.	1-50 µM	*Scutellaria baicalensis*	Wogonin
(Arima and Danno, 2002; Fang et al., 2003)	Suppression of TNF-α release through inhibition of NF-κB activation.	1.5 mM	*Psidium guajava*	Morin
(Cho et al., 2000b)	Inhibition of TNF-α production by suppression of protein tyrosine kinase.	28.5 µM	*Amorpha fruticosa*	Amoradicin
(Xie et al., 2011)	Inhibition of LPS-induced TNF-α release via suppression of NF-κB activation, protein kinase p38 and JNK phosphorylation.	2.5 µM	*Euterpe oleracea*	Velutin

2.3.2. Lignans

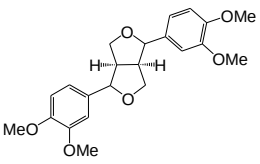
Lignans (Figure 3) form a class of plant secondary metabolites which is widely distributed throughout the plant kingdom. They are most commonly present as free form in roots, rhizomes, stems, leaves, seeds and fruits. Some are found as glycosides. Lignans have been reported to have interesting pharmacological activities including antitumor, anti-inflammatory, immunosuppressive, cardiovascular, antioxidant and antiviral activity (Saleem et al., 2005).

Three different types of lignans were isolated from the flower buds of *Magnolia fargesii*. They were identified as eudesmin (36), magnolin (37), and lirioresinol-B dimethylether (38). These lignans were found to have inhibitory effects on TNF-α production by the LPS-stimulated murine macrophage cell line, RAW264.7 (Chae et al., 1998) when used at 12.5μg/ml. Eudesmin had strongest activity with an IC_{50} value of 51 μM. Two neolignans, magnosalin [1β, 2α, 3β, 4α-1,2-dimethyl-3,4-bis-(2,4,5-trimethoxyphenyl)-cyclobutane] (39) and andamanicin [1α, 2β, 3β, 4α-1,2-dimethyl-3,4-bis-(2,4,5-trimethoxyphenyl)-cyclobutane] (40) were isolated and purified from the leaves of *Perilla frutescens*. These compounds were found to inhibit the induction of NO synthesis (IC_{50} of 5.9 μM and 53.5 μM, respectively), and TNF-α by LPS-activated RAW 264.7 cells (Ryu et al., 2002). Savinin (41) and calocedrin (42), isolated from the heartwood of *Pterocarpus santalinus*, were able to inhibit the release of TNF-α by LPS stimulated RAW264.7 cells without any toxicity (IC_{50} values of 31.9 μM and 150 μM respectively) (Cho et al., 2001b). The presence of a butyrolactone ring and its polar function at the C-9 position of dibenzylbutyrolactone–type lignans plays an important role in the inhibition of TNF-α. It was also reported that arctigenin (43), a dibenzyl butyrolactone lignan, inhibited TNF-α production by LPS-stimulated murine RAW264.7 macrophages and by differentiated human U937 macrophages with IC_{50} values of 5.0 and 3.9 μM, respectively, without displaying any cytotoxicity (Cho, 1999).

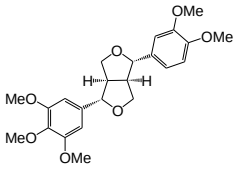
Coptis japonica (Ranunculaceae**)** has a long history of use in traditional medicine for the treatment of various diseases. Alkaloids, phenolic compounds and lignans are the major constituents of this plant (Yahara et al., 1985). Five different woorenosides (I, II, III, IV, V) (44-48) were isolated and identified from *C. japonica*, and showed inhibition of LPS-induced TNF-α and -NO release from RAW264.7 cells in a dose-dependent manner, with IC_{50} values ranging from 15 to 65 μM (Cho et al., 2000a). In another study, isolariciresinol (49), lariciresinol glycoside (50), pinoresinol (51), pinoresinol glycoside (52) and syringaresinol glycoside (53) were isolated from the rhizomes of *C.*

japonica and tested for *in-vitro* antiinflammatory effects. All of the compounds significantly inhibited TNF-α production with IC50 values of 39.3 μM (pinoresinol), 54.1 μM (lariciresinol glycoside) and 123.8 μM (isolariciresinol) by LPS-stimulated RAW264.7 macrophages (Cho et al., 2001a).

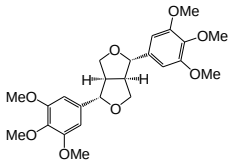
Magnolia officinalis (called "Houpu" in Chinese) is one of the most popular traditional Chinese medicinal herbs and is widely used for the treatment of various diseases which are classified in the oriental medicine as: stroke, cold damage, headache, cold and heat, and blood impediment. Honokiol (53) is considered one of the main bioactive components. The bark of this plant is a particularly rich source for honokiol (Wang et al., 2004). Honokiol is a dimer of two phenylpropanoids with a single C-C coupling, different from other lignans that usually are coupled via the C-3 side chains (Bai et al., 2003). Honokiol was found to inhibit the LPS- induced TNF-α release in activated macrophages (Son et al., 2000). Moreover, it has been shown to suppress TNF-induced NF-κB activation through inhibition of IKKs (Tse et al., 2005). In conclusion the lignans have some similarity to the flavonoids with activities in the μM range. Table 2.2 shows the possible mechanism underlying TNF-α inhibition by lignans and terpenoids.



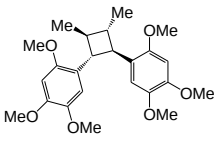
Eudesmin (36)



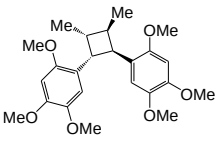
Magnolin (37)



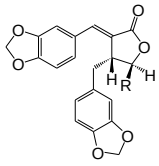
Lirioresinol-B-dimethylether (38)



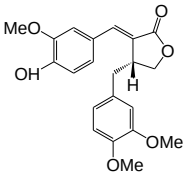
Magnosalin (39)



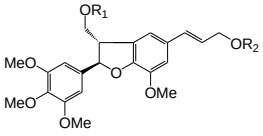
Andamanicin (40)



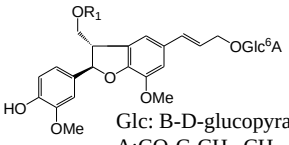
Savinin R = H (41)
Calocedrin R = OH (42)



Arctigenin (43)

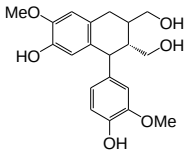


R1 R2
(44): -H Glc Woorenoside 1
(45): -Ac Glc Woorenoside II
(46): -H Glc^6A Woorenoside III
(47): -Ac Glc^6A WoorenosideIV

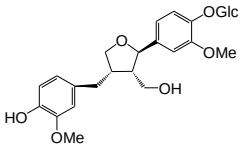


Glc: B-D-glucopyranosyl
A:CO-C-CH_2-CH_2-OH
‖
CH_2

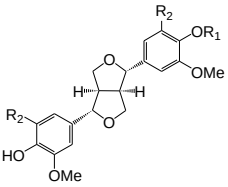
Woorenoside V(48)



+ Isolariciresinol (49)



Laricisesinol glycoside (50)



RI = R2 = H ; Pinoresinol (51)
R1 = GIc, R2 = H ; Pinoresinol glycoside (52)
RI = Glc, R2 = OMe= ; Stringaresinol glycoside (53)

Figure 3: Structure of natural TNF-α inhibitors: Lignans (36-53)

References	Possible inhibition mechanism	IC50/Dos	Source	Compounds
(Chae et al., 1998; Kim et al., 2009)	Inhibition of LPS-induced TNF-α through suppression of NF-κB.	51.3 µM	*Magnolia fargesii*	Eudesmin
(Chae et al., 1998; Kim et al., 2009)	Inhibition of LPS-induced TNF-α through suppression of NF-κB.	12.5µg/ml	*Magnolia fargesii*	Magnolin
(Chae et al., 1998; Kim et al., 2009)	Inhibition of LPS-induced TNF-α through suppression of NF-κB.	12.5µg/ml	*Magnolia fargesii*	lirioresinol-B dimethylether
(Ryu et al., 2002)	Inhibition of TNF-α by blocking of LPS-induced NF-κB.	5.9 µM	*Perilla frutescens*	Magnosalin
(Ryu et al., 2002)	Inhibition of TNF-α by inactivation of of LPS-induced NF-κB.	53.5 µM	*Perilla frutescens*	Andamanicin
(Cho et al., 2001b)	Supression of cyclic AMP phosphodiesteras, which in turn inhibits TNF-α transcriptional level.	31.9 µM	*Pterocarpus santalinus*	Savinin
(Cho et al., 2001b)	Supression of cyclic AMP phosphodiesteras, which in turn inhibits TNF-α transcriptional level.	150 µM	*Pterocarpus santalinus*	Calocedrin
(Cho et al., 2004; Zhao et al., 2009)	Inhibition of LPS-stimulated TNF-α release through the inhibition of MAP kinases including ERK1/2, p38 kinase and JNK (inhibition of MKK activities). It also inhibits LPS-induced NF-κB activation and I-κB-α phosphorylation.	3.88 µM	Arctium lappa	Arctigenin
(Kim and Cho, 2008; Tse et al., 2005; Wu et al., 2011)	Inhibition of LPS-induced TNF-α by suppressing the phosphorylation of Akt, P42/P44 MAPK, NF-κB and P65 at Ser536.	15 µM	*Magnolia officinalis*	Honokiol

(Jin et al., 2000; Koch et al., 2001; Lee et al., 1999)	Inhibition of LPS-induced TNF-α through inactivation of NF-κB.	0.6-10 mM	*Saussurea lappa*	Dehydrocostus lactone
(Takahashi et al., 2003)	Activation of peroxisome proliferator-activated receptor-γ (PPARγ) ligand, which inhibits TNF-α by blocking the activity of NF-κB, STAT-1 and AP1.	1-100 µM	*Abies grandis*	Abietic acid
(Cho et al., 2000a)	Inhibition of TNF-α release through selective inhibition of NF-κB.	8.24 µM	*Saussurea lappa*	Cynaropicrin
(Pae et al., 2007)	Inhibition of TNF-α via inactivation of NF-κB.	0.1-1 µM	*Saussurea lappa*	Costunolide
(Kang et al., 1996; Kang et al., 2010; Kim et al., 2004c)	Inhibition of TNF-α release through MAPKs and NF-κB pathways.	5.46 µg/ml	*Acanthopanax koreanum*	Acanthoic acid
(Jang et al., 2003; Jang et al., 2006)	Inhibition of TNF-α through suppression of NF-κB and MAPKs (p38, ERK1/2, and JNK) pathways.	0.34-34 µM	*Salvia miltiorrhiza*	Tanshinone IIA
(Jeong et al., 2002)	Inhibition of TNF-α through suppression of NF-κB.	0.101 µg/ml	Aucuba japonica	Aucubin
(Wu et al., 2007)	Inhibition of TNF-α through suppression of NF-κB activation.	51.3 µM	*Panax notoginseng*	Ginsenoside Rb1
(Zhang et al., 1997)	Inhibition of TNF-α by interfering NF-κB and IκB-α.	100 µg/ml	*Panax notoginseng*	Notoginsenoside R1
(Wu et al., 2007)	Inhibition of TNF-α through suppression of NF-κB activation.	26.8 µM	*Panax notoginseng*	Ginsenoside Rb2
(Kobori et al., 2007)	Inhibition of TNF-α through suppression of NF-κB activation and MAPKs phosphorylation.	15-60 µM	*Sarcodon aspratus*	Ergosterol peroxide
(Manjula et al., 2006)	Inhibition of TNF-α through interfering with MAPK pathways.	n.d	*Commiphora mukul*	Guggulsterol

2.3.3. Terpenoids

The terpenoids (Figure 4a-c), also known as terpenes, constitute the largest family of natural compounds and have been associated with all kinds of biological activities including antibiotic, antiparasitic, antitumor and antiinflammatory activities (Heras et al., 2003). Steroids play a particularly important role as endogenous signal compounds (hormones). The worldwide market for terpene-based pharmaceuticals was approximately around US $ 12 billion (Wang et al., 2005a).

Roots of *Saussurea lappa* (Compositae), a Chinese medicinal herb, have been traditionally used to treat asthma, inflammations and rheumatism (Gokhale et al., 2002). Dehydrocostus lactone (54) and costunolide (55), the active sesquiterpene lactones in the roots of *Saussurea lappa*, have been shown to inhibit TNF-α release by LPS-stimulated RAW264.7 macrophages with a dose range of 0.6-10 mM and 0.1-1 μM, respectively (Lee et al., 1999; Pae et al., 2007). Cynaropicrin (56), reynosin (57), and santamarine (58) were isolated from this plant and were shown to inhibit the production of TNF-α, IL-6 and NO production by LPS-activated RAW264.7 and U937 cells by suppressing the expression of inducible NO synthase (See Table 2). Cynaropicrin, reynosin, and santamarine show the highest activities with IC50 value of 8.24, 87.4, and 105 μM, respectively (Cho et al., 1998). *Tripterygium wilfordii* (Celastraceae) is a Chinese medicinal plant (Duan et al., 2001); its alcoholic extract has been reported to be effective in the treatment of a variety of inflammatory and autoimmune diseases, like rheumatoid arthritis (Gu and Brandwein, 1998; Wenyan et al., 1985). Triptolide (59), known as PG490, is an oxygenated diterpene, which has been identified as the major component responsible for the immunosuppressive and antiinflammatory effects of *T. wilfordii*. This compound was evaluated for its ability to suppress various inflammatory cytokines and was reported to inhibit strongly LPS-induced NO, TNF-α and IL-1β production by microglia cells in a dose-dependent manner ranging from (10^{-10}-10^{-8}M) (Zhou et al., 2003).

Abietic acid, a diterpene (60), is a major component of the resin fraction of the oleoresin produced by conifer species, such as the grand fir (*Abies grandis*) and the lodgepole pine (*Pinus contorta*) (Aranda and Villalaín, 1997). It has been shown to suppress the expression of genes involved in inflammation

such as TNF-α and COX-II by activated macrophages at a concentration of 50 μM (Takahashi et al., 2003). *Zingiber zerumbet*, a form of ginger commonly found in Southeast Asia, has been used as a traditional herbal medicine for a variety of diseases (Jang et al., 2004). The main bioactive

component in this ginger species is Zerumbone (61), a sesquiterpene which reduces NO synthase, COX-II activity, and the release of TNF-α by mouse macrophages (Table 2) (Murakami et al., 2002). Furthermore, the water extracts of *Z. zerumbet* (50 and 500 mg/ml) was reported to inhibit the release of TNF-α in a dose dependent manner *in-vitro* as well as *in-vivo* in response to LPS stimulation (Chaung et al., 2008).

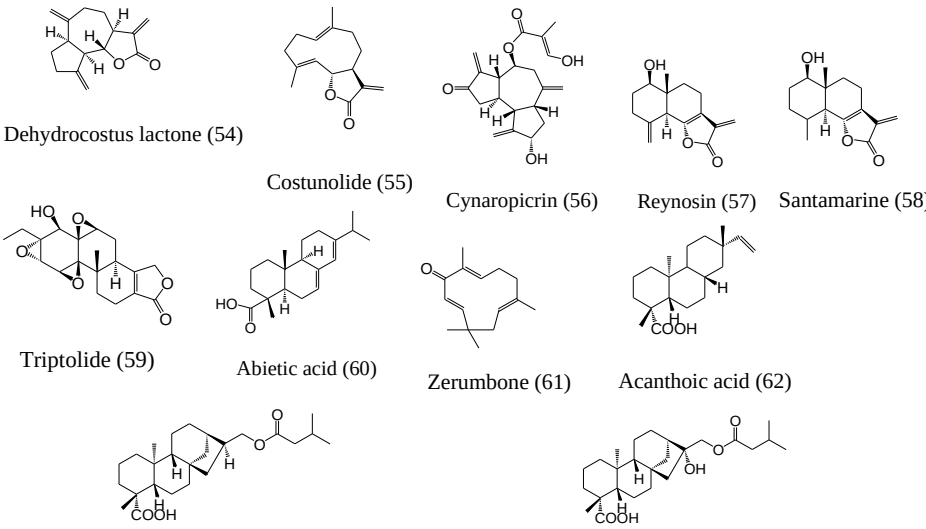
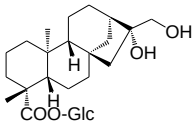
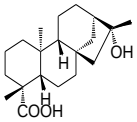


Figure 4a: Structure of natural TNF-α inhibitors: Terpenoids (54-64)

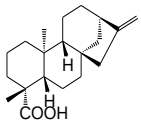
Acanthopanax koreanum is a Korean medicinal plant, used traditionally in the treatment of rheumatoid arthritis and diabetes (Kim et al., 2004d). Acanthoic acid, (-)-pimara-9(11),15-di-en-19-oic acid (62), is a diterpene isolated from the root bark of this plant. This compound has been reported to suppress the production of IL-1β and TNF-α up to 90% by human monocytes and macrophages stimulated with silica at concentrations of 19.88 μM (Kang et al., 1996). Five kaurane type diterpenoids, 16αH,17-isovaleryloxy-ent-kauran-19-oic acid (63), 16α-hydroxy-17-isovaleryloxy-ent-kauran-19-oic acid (64), paniculoside (65), 16α-hydroxy-ent-kauran-19-oic acid (66), and ent-kaur-16-en-19-oic acid (67) were isolated and tested for their ability to inhibit TNF-α secretion. All of them were able to inhibit TNF-α secretion, with 16αH, 17-isovaleryloxy-ent-kauran-19-oic acid showing the highest inhibitory activity with an IC_{50} value of 16.2 μM (Cai et al., 2003).



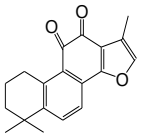
Paniculoside-IV(65)



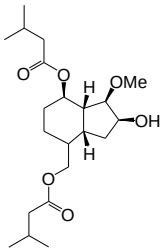
16α - hydroxy-ent-kauran-19-oic acid (66)



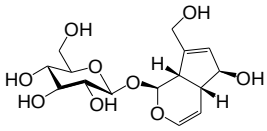
Ent-kaur-16-en-19-oic acid (67)



Tanshinone II A (68)



Nardostachin (69)

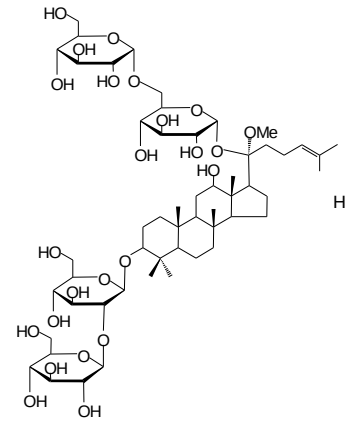


Aucubin (70)

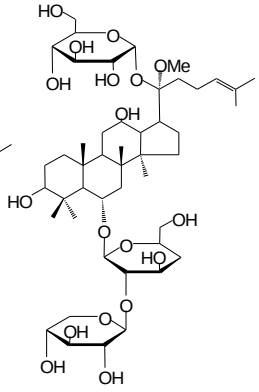
Figure 4b: Structure of natural TNF-α inhibitors: Terpenoids (65-70)

The roots of *Salvia miltiorrhiza,* have been widely used in the treatment of various diseases including coronary artery diseases, angina pectoris, myocardial infarction, cerebrovascular diseases, various types of hepatitis, chronic renal failure, dysmenorrhea, and also to improve microcirculation in the human body (Jiang et al., 2005). Tanshinone II A (68), a diterpene isolated from the *Salvia miltiorrhiza* root, has been shown to inhibit the production of TNF-α, IL-1β and IL-6 by activated RAW264.7 cells in a dose-dependent manner (0.34 – 34.0 μM) (Jang et al., 2003).

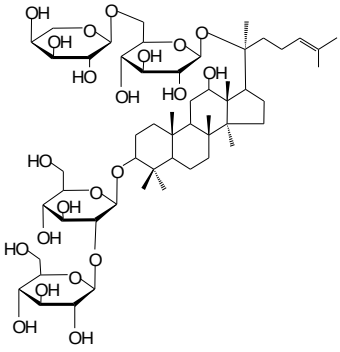
Iridoids represent a large group of highly oxygenated monoterpenoids, structurally based on a cyclopentan-[C]-pyran skeleton, which usually consist of ten carbons, but forms containing nine-, or rarely eight carbon iridoids occur in nature (Sampaio-Santos and Kaplan, 2001). *Patrinia saniculaefolia* (Valerianaceae) is a Korean native plant. The roots of the genus *Patrinia* have been used in Korean and Chinese traditional medicine for treating inflammation, edema, appendicitis, and abscesses (Lee, 1980). Nardostachin (69), an important constituent of *Patrinia saniculaefolia,* was reported to suppress the production of LPS-induced NO and TNF-α with IC_{50} values of 12.3 μM and 16.2 μM, respectively. Moreover, it has been reported to reduce the COX-2 expression level and PGE_2 production in LPS-stimulated macrophages (Ju et al., 2003). Aucubin (70), another iridoid glycoside, is a common constituent of many traditional oriental medicinal plants. The effect of



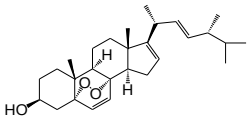
Ginsinoside Rb_1 (71)



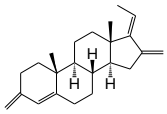
Notoginsinoside R_1 (72)



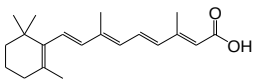
Ginsinoside Rb_2 (73)



Ergosterol peroxide (74)



Guggulsterol (75)



Retinoic acid (76)

Figure 4c: Structure of natural TNF-α inhibitors: Terpenoids (71-76)

aucubin on TNF-α and IL-6 expression was studied in antigen-stimulated rat basophilic leukemia (RBL)-2H3 mast cells. This compound was found to inhibit antigen-induced TNF-α and IL-6 production with IC_{50s} of 0.101 and 0.19 µg/ml, respectively. Maximum inhibition of TNF-α and IL-6 production was 73% ± 4.3 and 88.8% ± 5, respectively. Aucubin also inhibited antigen-induced nuclear translocation of the p65 sub-unit of NF-κB and the degradation of IκB, which might explain its beneficial effect in the treatment of chronic allergic inflammatory diseases (Jeong et al., 2002; Salminen et al., 2008).

Panax notoginseng, known as "sanchi" in the Chinese language, has a long history of use as medicine in China. It is mainly used for the treatment of cardiovascular diseases, inflammation, different body pains, trauma, and internal and external bleeding due to injury (Cicero et al., 2003; Dong et al., 2003; Ma et al., 1999). The important components of *Panax notoginseng* are ginsenosides (Rb1, Re, Rg1, and Rh1) and notoginsenosides (R1) (Zhou et al., 1991), see Fig. 4c. BT-201, n-Butanol fraction of *P. notoginseng*, was found to inhibit the release of TNF-α through inactivation of the NF-κB and MAPK pathways. BT-201 contains the ginsenosides Rb1, Re, and Rg1, and

notoginsenosides R1, R2, Fa, and Fc. Among these, ginsenoside Rb1 (71) is a major component (24.1%) and the major active constituent of BT-201 mediating the antiinflammatory effects of the extract (Chang et al., 2007). Furthermore, notoginsenoside R1 (72) from *P. notoginseng* has been reported to inhibit LPS-induced TNF-α production *in-vitro* as well as *in-vivo* in human whole blood cells (Zhang et al., 1997). The inhibitory activity of protopanaxadiol ginsenosides (PPDGs), such as Rb_1, Rb_2 and Rc (and their combined effect with a known (TNF)-α antagonists), against LPS-induced TNF-α production has been studied in murine (RAW264.7) or human (U937) macrophages (Cho et al., 2001c). Rb_1 and Rb_2 (73) suppressed TNF-α production in RAW264.7 cells with an IC_{50} of 56.5 μM and 27.5 μM, respectively, and in differentiated U937 cells with an IC_{50} of 51.3 μM and 26.8 μM, respectively (see also Table 3).

Phytosterols which are also derived from terpenes, are structurally similar to cholesterol, but differ in their side chain configurations (Ling and Jones, 1995). Ergosterol peroxide (74) (Fig. 4c) is a major anti-tumor sterol present in edible and medicinal mushrooms; it has been shown to reduce LPS-induced TNF-α secretion and IL-1α/β expression by RAW264.7 cells through inhibition of NF-κB and C/EBPb transcriptional activity and phosphorylation of MAPKs (Kobori et al., 2007). (See table 3).

Commiphora mukul (guggul) is one of the most commonly used drugs in Ayurveda medicine for the treatment of several disorders such as gout, arthritis, rheumatism, obesity, hypercholesterol, and inflammation (Giridharan et al., 2002). The crude ethyl acetate extract and pure compound (guggulsterol) (75) isolated from *C. mukul* were investigated for antiinflammatory properties by evaluating the inhibitory effects on a variety of key mediators that regulate immune responses *in-vitro*. Both crude extracts (5-50 μg/ml) and the pure compound (< 30 μg/ml) were shown to downregulate the level of inflammatory mediators such as IFN-γ, IL-12, TNF-α, IL-1β and NO (Manjula et al., 2006).

Retinoic acid (76) was evaluated for its effects on the expression of TNF-α and inducible nitric oxide synthase (iNOS) in microglia activated by β-amyloid peptide (Aβ) and LPS. Retinoic acid inhibited TNF-α (29–97%) and iNOS (61–96%) mRNA expression in microglia exposed to either (Aβ) or LPS, in a dose-dependent manner (0.1–10.0 μM) (Dheen et al., 2005).

2.3.4. Alkaloids

Plant alkaloids (Fig. 5) comprise next to terpenoids the second largest classes of plant secondary metabolites with 16,000 representatives (Verpoorte et al., 2000). The term alkaloid generally refers to basic substances containing one or more nitrogens, usually as part of a cyclic system (Sato et al., 2001). Because of their basic character they are water soluble at low pH in the protonated from, but at high pH they are in the lipohilic neutral form. That makes them ideal drugs as they are water soluble compounds that can pass through membranes. In fact most medicines, both natural and synthetic do contain tertiary nitrogen (Barbosa-Filho et al., 2006; Cordell et al., 2001; Herraiz and Galisteo, 2003).

Cigarette smoking not only plays an important role in the development of cancer but surprisingly can also exert some beneficial effects against chronic diseases such as Alzheimer disease and ulcerative colitis (Ernster, 1988; Harries et al., 1982; van Duijn and Hofman, 1991). It was observed that cigarette smoke contains a variety of compounds with different pharmacological activities. Nicotine (77) is a toxic substance with a LD50 value for rats of 50 mg/kg, for mice of 3 mg/kg and for adult humans of 40-50 mg/kg (Okamoto et al., 1994; Pullan et al., 1994). Nicotine was found to strongly inhibit TNF-α production in a dose dependent manner ranging from 10^{-9}-10^{-5}M (Madretsma et al., 1996) using a mechanism involving inhibition of NF-κB activation (Sugano et al., 1998). This could be linked with smoker's immunity for autoimmune diseases.

The Amaryllidaceae alkaloids, lycorine (78) and lycoricidinol (79), were found to inhibit TNF-α production in murine macrophages stimulated with LPS at ID_{50} values of 696.11 nM and 6.50 nM, respectively (Yui et al., 2001).

Furthermore, fangchinoline (80) and isotetrandrine (81) were evaluated for suppression of the pro-inflammatory cytokines IL-1 and TNF-α in human peripheral blood mononuclear cells infected with *Staphylococcus aureus*. It was observed that these two alkaloids inhibited cytokine production at concentrations of 4.6 and 4.3 μM respectively (Onai et al., 1995). Tetrandrine (82) at a concentration of 8.029 μM has been reported to inhibit the release of TNF-α and may be useful in the treatment of inflammatory diseases (Ferrante et al., 1990).

Berberine (83), a common quarternary isoquinoline alkaloid produced in *Coptis japonica,* was found to suppress the expression and secretion of TNFα, monocyte chemoattractant protein-1 (MCP-1), and IL-6 (*in-vitro*) in macrophages stimulated by acetylated low-density lipoprotein (AcLDL) in a

dose-dependent manner, ranging from 5-10 µM (Chen et al., 2008). For the mechanism, see Table 3.

Piperine (84), a well known alkaloid produced by *Piper nigrum*, was shown to inhibit the production of LPS-induced TNF-α (*in-vitro*) in L929 cells at the concentration of 5 µg/ml. Balb/C mice were used to examine the *in-vivo* effects of piperine. The level of TNF-α was significantly reduced in the piperine-treated animals (105.8 pg/mL) compared to control animals (625.8 pg/mL) (Pradeep and Kuttan, 2003).

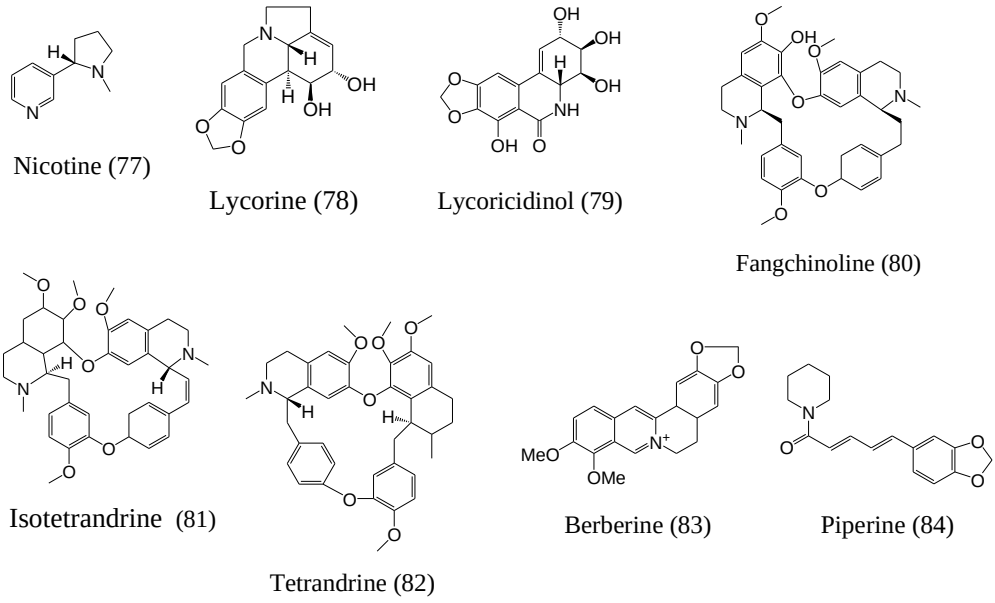
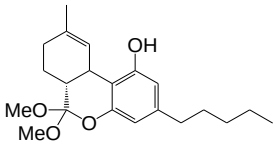


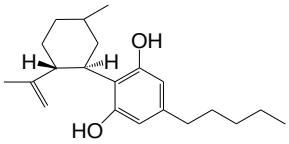
Figure 5: Structure of natural TNF-α inhibitors: Alkalods (77-84)

2.3.5. Cannabinoids

Cannabinoids (Fig. 6) are terpenophenolic compounds chemically related to terpenoids since part of the structure is derived from geranyl diphosphate, a C10 terpenoid unit which is attached to a polyketide-derived aromatic ring. The cannabinoids are the active constituents in *Cannabis sativa* and are utilized in a number of cannabis-based medicines (Hazekamp and Grotenhermen 2010). Cannabinoids are present in the stalks, leaves, flowers and particularly in the resin secreted by female flowers (Ashton 2001).



△^{9} Tetrahydrocannabinol (85)



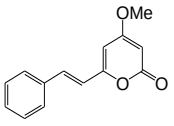
Cannabidiol (86)

Figure 6: Structure of natural TNF-α inhibitors: Cannabinoids (85-86)

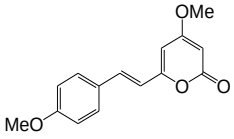
Tetrahydrocannabinol is the psychoactive constituent formed from the main component in the resin, the nonpsychoactive constituent D9-tetrahydrocannabinoid acid (THCa). The cannabinoids have been utilized in a number of cannabis-based medicines (Mechoulam and Gaoni 1967). The immunomodulating activity of unheated *C. sativa* extracts were investigated; it was found that unheated cannabis extracts and THCa (85) could inhibit LPS-induced TNF-α release in the supernatant of U937 macrophages at a dose of 4–58 µg/ml (Verhoeckx et al. 2006). This activity is due to its main non-psychoactive constituent D9- tetrahydrocannabinoid acid, the precursor of THC which is formed by decarboxylation upon heating. Another in vivo study revealed that a low dose of cannabidiol CBD (86), another non psychoactive constituent of *C. sativa*, decreases the TNF-α production in LPS-treated mice (Malfait et al. 2000).

2.3.6. Kawapyrones

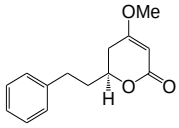
Piper methysticum (Piperaceae), popularly known as kawa, is an oceanic pepper plant widely used in the Pacific islands (Schmidt and Molnar, 2002). A drink prepared from the root of this plant is very popular on the island of Fiji, and is thought to be responsible for the low incidence of cancer in the population of this island as compared to other Pacific islands. Different kawapyrones (Figure 7) were isolated from *Piper methysticum* and evaluated for their antiinflammatory activities. It was found that 5,6-dehydrokawain (87) and yangonin (88) suppress TNF-α release from BALB/3T3 cells treated with okadaic acid. The compounds have IC_{50} values of 17 µM and 40 µM, respectively (see Table 3). Dihydrokawain (89) was found to be the strongest inhibitor of TNF-α release in mice (Hashimoto et al., 2003).



5,6 Dehydrokawain (87)



Yangonin (88)



Dihydrokawain (89)

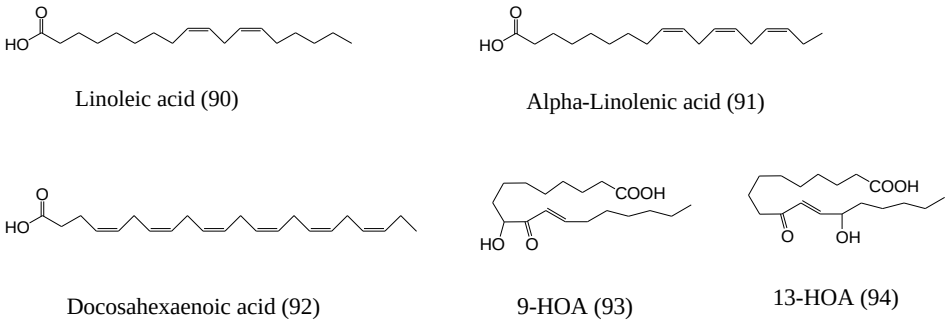
Figure 7: Structure of natural TNF-α inhibitors: Kawapyrones (87-89)

2.3.7. Fatty acids

Fatty acids (Fig. 8) are also known to play an important role in immunomodulation (Calder 2007). The effects of linoleic acid (LA) (90), a-linolenic acid (ALA) (91), and docosahexaenoic acid (DHA) (92) on inflammatory responses in human monocytic THP-1 cells were studied (Zhao et al. 2005); it was found that THP-1 cells treated with LA, ALA, and DHA inhibited the LPS-stimulated production of IL-6, IL-1β, and TNF-α in a dose–response manner ranging from 0 to 100 µM. Furthermore, the inhibitory effects of these polyunsaturated fatty acids were associated with the inhibition of NF-κB activation via activation of peroxisome proliferator-activated receptor-γ (PPARγ).

Corn germ and rice bran are known to contain fatty acids, which include (±)-9-hydroxy-trans, -cis-10, 12-octadecadienoic acid (9-HOA from rice) (93), and (±)-13-hydroxy-10-oxo-trans-11-octadecenoic acid (13-HOA from corn) (94) (Hayashi et al. 1996, 1998). They have been reported to reduce the expression of pro-inflammatory genes in LPS-stimulated macrophages via a

blockage of the NF-κB and AP-1 pathways. At higher concentrations, 10-ODO, 13-HOA and 9-HOA exhibited profound suppressive effects on the expression of iNOS, COX-II, IL-6, and TNF-α. Murakami et al. (2005) also observed that 13-HOA strongly inhibits the expression of proinflammatory genes such as COX-II, iNOS, TNF-α, and IL-6



Linoleic acid (90) Alpha-Linolenic acid (91)

Docosahexaenoic acid (92) 9-HOA (93) 13-HOA (94)

Figure 8: Structure of natural TNF-α inhibitors: Fatty acids (90-94)

2.4. Miscellaneous compounds from nature as source of TNF-α inhibitors

Some other natural products known to inhibit TNF-α release are shown in Figure 9 (See table 3).

2.4.1. Curcumin

Curcuma longa has been used widely in India and Indonesia for the treatment of inflammation since ancient times. Curcumin (95) (Fig. 9) is a polyphenol which is present in the rhizome of the plant *C. longa*. It inhibits LPS-induced production of TNF-α and IL-1β in a dose-dependent manner (ranging from 0.5 to 5 μM) in the human monocytic macrophage cell line, MonoMac 6. Curcumin was also reported to inhibit LPS-induced activation of NF-κB and reduced the biological activity of TNF-α in a fibroblast lytic assay in L929 cells (see Table 3) (Chan 1995).

2.4.2. Capsaicin

Capsaicin (96), the most important flavor component of hot peppers, has been reported to contain many anti-inflammatory properties (Caterina et al. 1997; Han et al. 2001). It has been observed that capsaicin effectively inhibits the production of proinflammatory mediators through NF-κB inactivation in LPS-stimulated macrophages (see also Table 3). Capsaicin significantly inhibits the production of TNF-α following LPS treated of RAW264.7 cells in a dose-dependent manner (ranging from 10 to 50 μM). The inhibitory action of capsaicin against TNF-α expression is mediated by peroxisome proliferator-activated receptors (PPARγ) (Park et al. 2004).

2.4.3. Coumarines

Scoparone (6,7-dimethoxycoumarin) (97) is the major component of the shoots of Artemisia capillaries (Compositae), which has been used as antipyretic, anti-inflammatory, diuretic, and for the treatment of hepatitis and bilious disorder (Jamwal et al. 1972). Scoparone inhibits the production of TNF-α, NO, PGE2 and interleukins (IL-1β, IL-6) in either IFN-γ/ LPS- or LPS-stimulated RAW264.7 cells in a dose dependent manner (ranging from 0 to 50 μg/ml) (Jang et al. 2005). Another species of *Artemisia* (*Artemisia feddei*), has been used as a folk medicine in different countries for treatment of various inflammatory and digestive disorders (Kang et al. 1999). Scopoletin (6-hydrooxy-7-methoxycoumarin) (98) was isolated as an active compound from an *A. feddei* aqueous extract. It has been reported that scopoletin strongly inhibits the release of LPS-stimulated TNF-α, IL-1β, IL-6, and PGE2 over a dose range of 5–260 μM in murine RAW264.7 macrophage (Kim et al. 2004a).

2.4.4. Resveratrol

Resveratrol (99), an antioxidant phytoalexin isolated from multiple sources including grapes, was investigated for its effect on the inhibition of TNF-α production by LPS-activated microglia. This compound inhibited the production of TNF-α in a dosedependent manner (ranging from 0-10 μg/ml). In addition to TNF-α inhibition, resveratrol has been found to suppress LPS-induced degradation of IκBα, expression of iNOS, and phosphorylation of p38 MAPKs in N9 microglial cells which could be the possible mechanism of its anti-inflammatory effects (Bi et al. 2005; Boscolo et al. 2003).

2.4.5. Thymoquinone

Nigella sativa, a Middle East plant commonly known as black cumin, belongs to the family Ranunculaceae. Besides the use in food, seeds of this plant are of great importance because of their use in traditional medicine (Salomi et al. 1992). Thymoquinone (100), an important constituent of this plant, has been reported to lower the levels of TNF-α, IL-1β and COX II produced in a dose- and time-dependent manner (ranging from 25 to 75 μM) (Chehl et al. 2009; Haq et al. 1999). Furthermore, oral administration of thymoquinone resulted in a significant decrease in the level of several pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IFN-γ and PGE2 at a concentration of 5 mg/kg (Umar et al. 2012).

2.4.6. Anthralin

Anthralin (102) is a medically interesting compound found in rhubarb roots. Varieties of rhubarb (*Rheum palmatum*) have a long history as medicinal plants in traditional Chinese medicine (TCM) (Ashnagar et al. 2007). Anthralin has been reported to be a safe and efficient drug for treatment of various diseases like psoriasis although its mode of action is still unclear. The effect of anthralin on the secretion of cytokines from stimulated monocytes was investigated. The results showed a dose-dependent inhibition of IL-6, IL-8, and TNF-α release from with an IC_{50} value of 1–2.7 μM of anthralin (Mrowietz et al. 1997).

2.4.7. Paeoniflorin, Paeonol and Pentagalloylglucose

Moutan cortex, a root bark of *Paeonia suffruticosa* (Paeoniaceae), is commonly used as a TCM for treating various diseases. It has been reported to possess different types of biological activities (Chen et al. 2006a). Compounds purified from Moutan cortex were evaluated by measuring the expression of TNF- α in rat synoviocytes. It was found that paeoniflorin (102), paeonol (103) and pentagalloylglucose (104) inhibit the release of TNF-α and IL-6 production in a dose-dependent manner (ranging from 6.1 to 200 μM) in rat synoviocytes (Wu and Gu 2009).

2.4.8. EGb 761 (*Ginkgo biloba* extract)

An extract of *Ginkgo biloba*, called EGb 761, is commonly used in France and Germany for the treatment of a variety of diseases. This extract is

known to improve peripheral vascular, cardiovascular, and cerebrovascular blood flow, and for the treatment of acute mountain sickness. All of these diseases are known to be associated with oxidative stress (Le Bars et al. 1997; Philippe et al. 1996). Flavonoids, like rutin, the glycoside of quercetin, and terpenes (bilobalide and ginkgolides A, B and C) are the main components of EGb 761 (Satoh and Nishida 2004). It has been reported that EGB 761 (400 µg/ml) inhibits the release of LPS-induced TNF-α in vitro (RAW264.7 cells) and in vivo (C56BL/6 mice) in a dose-dependent manner ranging from 20 to 100 mg/kg. Furthermore, it was found that EGB 761 also inhibits (ERK1/2) phosphorylation and P38MAPK activity, which are very important in the post-transcriptional regulation of TNF-α mRNA (Wadsworth et al. 2001). Table 2.3 shows the possible mechanism underlying TNF-α inhibition by alakaloids, kawapyrones and other miscellaneous compounds.

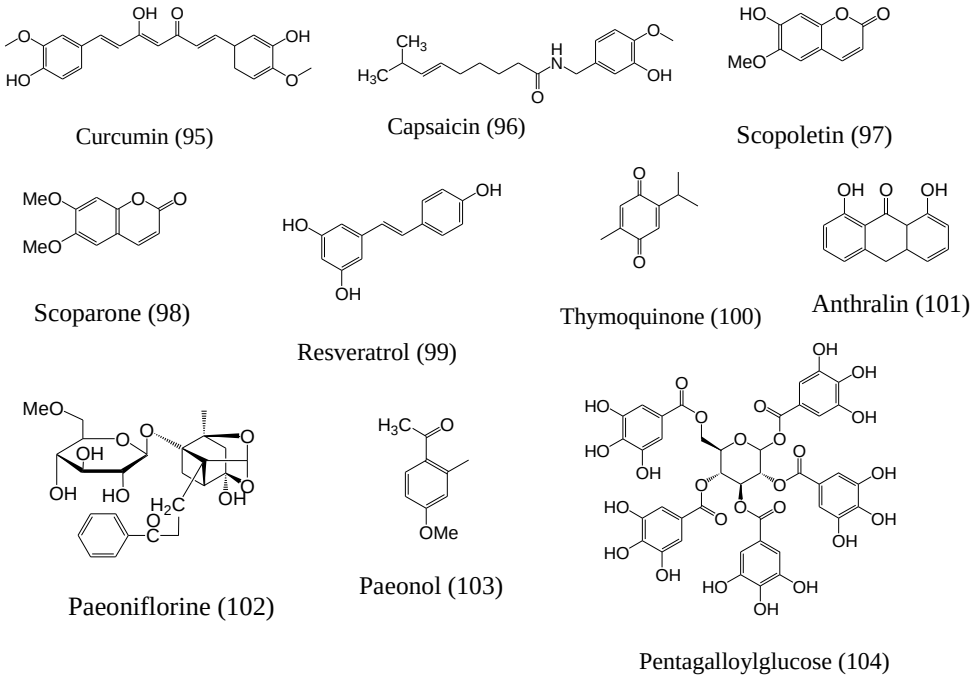


Figure 9: Structure of natural TNF-α inhibitors: Miscellaneous compounds (95-104)

References	Possible inhibition mechanism	IC50/Dose	Source	Compounds
(Li et al., 2011; Madretsma et al., 1996)	Inhibition of TNF-α expression through an α7 nAChR/MyD88/ NF-κB pathway.	10^{-8} M	*Nicotiana tabacum*	Nicotine
(Chen et al., 2008)	Inhibition of TNF-α through a PPAR-γ inhibitor blockage and also through inactivation of NF-κB activity.	5 µM/L	*Berberis heterophylla*	Berberine
(Hashimoto et al., 2003; Pollastri et al., 2009)	Inhibition of TNF-α through suppression of NF-κB activation.	17 µM	*Piper methysticum*	5, 6-Dehydrokawain
(et al., 2003b; Pollastri et al., 2009)	Inhibition of TNF-α through suppression of NF-κB activation.	40 µM	*Piper methysticum*	Yangonin
(Hashimoto et al., 2003; Pollastri et al., 2009)	Inhibition of TNF-α through suppression of NF-κB activation.	>100 µM	*Piper methysticum*	Dihydrokawain
(Jagetia and Aggarwal, 2007)	Inhibition of LPS-induced TNF-α through inhibition of MAPK phosphorylation and NF-κB nuclear translocation.	2.5 µM	*Curcuma longa*	Curcumin

(Kim et al., 2003; Park et al., 2004)	Inhibition of TNF-α through NF-κB inactivation.	50 µM	*Capsicum annuum*	Capsaicin
(Moon et al., 2007)	Inhibition of TNF-α via IκBα phosphorylation and degradation (in NF-κB pathway).	10 µg/ml	*Artemisia feddei*	Scopoletin
(Zhong et al., 2012)	Inhibition of LPS-induced TNF-α via down-regulation (and phosphorylation) of NF-κB and MAPKs pathways.	10 µg/ml	*Vitis vinifera*	Reserveratrol
(Sethi et al., 2008)	Inhibition of TNF-α through suppression of the NF-κB activation pathway.	25-75 µM	*Nigella sativa*	Thymoquinone
(Jiang et al., 2009; Wu et al., 2009)	Inhibition of TNF-α release through suppression of NF-κB pathway by inhibiting IκB kinase activity.	6.1-200 µM	Moutan Cortex	Paeoniflorin
(Tsai et al., 2008; Wu et al., 2009)	Inhibition of TNF-α through NF-κB inactivation.	6.1-200 µM	Moutan Cortex	Paeonol

2.5. Conclusions

Studies have shown that dysregulation of a proinflammatory cytokine, like TNF-α, can lead to inflammation and the emergence of a wide variety of inflammatory diseases and cancer. Several clinically approved protein-based TNF-α inhibitors are capable of modulating TNF-α activity, but these are associated with unwanted side effects. Moreover, patients with rheumatoid arthritis also have an increased risk for diabetes, atherosclerosis and cardiovascular complications. The use of the microbial natural products derived statins have been proposed to treat several inflammatory diseases like metabolic syndrome, diabetes, rheumatic and autoimmune disorders. Even though statins are labeled as safe, these drugs cannot be applied to some patients because of adverse effects such as muscle pain.

Low molecular weight compounds provide many advantages over protein-based drugs, particularly concerning production, stability and route of administration. Plants are considered a good source for the development of novel drugs and already many natural compounds, belonging to various chemical classes like flavonoids, terpenoids, alkaloids, cannabinoids, ginsenosides, and phytosterols, have been found to inhibit the upstream signaling molecules that are involved in TNF-α expression. The dose at which most of these compounds are active in the various in vitro tests is about 1–50 μM, which is quite high in comparison with the presently used medicines like Etanercept, Infliximab, and Adalimumab. The compounds discussed in this review have been found either by random screening of plant or microorganism extracts or by studying traditionally used herbal medicines. So far no real leads have been reported from all the screening efforts of plants. The complexity of the inflammation might be a reason for not finding a novel drug on the basis of ‘‘single target— single compound’’ paradigm for the present day approach to drug development. The use of a mixture of natural products or plant extracts for prevention or stopping the development of such diseases should be considered as an alternative approach. Particularly various traditional medicines may contain interesting leads for synergy in mixtures of compounds and also pro-drugs, such as salix bark, which contains a salicylalcohol glucoside that is converted to salicylic acid in the G.I tract. However, using the classical bioassay guided fractionation, synergy will not be detected. Therefore, a novel systems biology approach is required in which a direct link is made between all compounds in an extract that correlate with activity (Verpoorte 2012; Verpoorte et al. 2005; Wang et al.2005b). Yuliana et al. (2011b) reported such an approach combining a comprehensive extraction, bioassays of the fractions and metabolomics to identify the compounds in the mixtures that are correlated to activity.

Pharmacologists are increasingly realizing that the concept of ‘‘one disease—one target—one drug’’ does not always provide the best cure, particularly for the treatment of chronic inflammatory diseases (Georgiou et al. 2011). Thus, both finding novel potent drugs from nature and the development of mixtures of compounds/ medicinal plant extracts or health food for treatment and/or prevention of diseases is becoming a hot topic. One example is the development of an anti-inflammatory dietary mixture that modulates inflammation and oxidative and metabolic stress using a nutrigenomics approach (Bakker et al. 2010). In the case of TCM, standardization of raw plant materials, good manufacturing practise (GMP), good laboratory practise (GLP) and other requirements are amongst the most important issues before a TCM can be launched as a scientifically- based (legal) medicine in European- or other developed countries (Verpoorte 2012). A ‘‘systems biology’’ approach which involves metabolomics (or several ‘‘omics’’) as the tool(s) is now applied in order to gain more insight into the molecular mechanisms of some inflammatory diseases (Buriani et al. 2012; Pelkonen et al. 2012; van der Greef et al. 2006; van der Greef and McBurney 2005; Verpoorte et al. 2005; Wang et al. 2005b), including the possible effect of TCM in the treatment of these diseases (van der Greef 2011). The compounds in a plant extract, a herbal medicine/TCM or a nutraceutical may work based on several different mechanisms synergistically, resulting to moderate the immune system.

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Chapter 3

Anti-TNF-α activity of extracts from herbs and spices

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Abstract

Inflammation plays a vital role in the pathogenesis of many diseases. Process of inflammation is modulated by several cytokines**.** Tumor necrosis factor-alpha (TNF-α) is a cytokine, which plays an important role in inflammation. In this study, 66 different plant extracts were screened for their ability to inhibit LPS-stimulated TNF-α release in U937 cell lines. Tested plants were extracted with a mixture of methanol-water (80:20, v/v) and dried under vacuum. The dried extract was redissolved in DMSO for TNF-α assay. Out of 66 plants, extracts of *Urtica dioica*, *Houttuynia cordata*, *Salvia officinalis*, *Adhatoda vasica*, *Sempervivum smaragd*, *Syzygium aromaticum*, *Pimenta officinalis*, *Myristica fragrans*, *Capsicum annuum*, *Alpinia galanga*, *Zingiber officinale*, *Kaempferia galanga*, *Bixa orellana*, *Pistacia lentiscus* show significant inhibition without any toxicity while *Origanum vulgare*, *Rosmarinus officinalis*, *Curcuma xanthorrhiza*, *Bosenbergia rotunda*, *Orthosiphon stamineus*, *Cannabis sativa*, *Psoralea corylifolia*, *Curcuma longa*, and bark of *Pistacia lentiscus* show highly significant inhibition of TNF-α but also cytotoxicity toward the tested U937 cell lines. Plants which showed activity *in-vitro* were further tested using zebrafish larvae. We have found that extracts which were active *in-vitro* also shows activity *in-vivo*. In this study, we have successfully employed zebrafish as a model system for plant extracts screening. Furthermore it also shows that a diet rich in fruits, herbs and spices may contribute to the reduction of the inflammatory response and related diseases.

Keywords: Herbs, inflammation, screening, spices, TNF-α, zebrafish

3.1. Introduction

Inflammation is an immune reaction of body to external stimuli such as toxins and pathogens, aiming at the production, activation, and recruitment of immune cells (leucocytes) to sites of infection in order to combat the stimuli/pathogens. Inflammation is commonly characterized by redness, swelling, pain, and heat, localized at the site of infection (Ruslan, 2010). The process of inflammation is regulated by several pro-inflammatory and antiinflammatory cytokines. Tumor necrosis factor alpha (TNF-α) is one of the proinflammory cytokines and also a regulatory protein in the cell signaling cascade. TNF-α is produced by immune cells including macrophages, neutrophils, monocytes and fibroblasts in response to lipopolysaccharide (LPS).

Overproduction of TNF-α and inflammation were found to occur not only due to a pathogenic infection, but also due to dysregulation of proteins in the immune system like in several chronic inflammatory diseases such as rheumatoid arthritis, crohn's disease (CD), inflammatory bowel disease, and other autoimmune diseases. Suppression of TNF-α could be a method for preventing and treating diseases associated with inflammation (Paul et al., 2006). Currently several proteins based TNF-α inhibitors are available for treatment of rheumatoid arthritis, but these drugs are associated with risk, high cost and potential side effects (Scheinfeld, 2004). To find a safe, cheap source for drug development, plants are of interest.

Many currently known natural products were originally identified using *in-vitro* assays such as cell lines (Lee et al., 2007; Wang and Mazza, 2002). However, for *in-vivo* studies zebrafish embryo has become an important vertebrate model for assessing pharmacological and toxic effects. It is well suited for studies in genetics, embryology, development, and cell biology. Zebrafish embryos exhibit unique characteristics, including ease of maintenance and drug administration, short reproductive cycle, and transparency that permits visual assessment of developing cells and organs. Because of these advantages, zebrafish bioassays are cheaper and faster than mouse assays, and are suitable for large-scale drug screening. Transgenic lines of zebrafish which express the green fluorescent protein in neutrophils were used for *in-vivo* activity test. Due to the transparency of the embryo, movement of neutrophils toward effected site can be seen (d'Alencon et al., 2010; Parng et al., 2004). By screening plant extracts one may find not only single active compounds but also mixtures including synergy. Hits from the screening can be isolated and be further developed into new leads with good activity and low toxicity.

Plants have been and continue to be the greatest sources of medicines being used traditionally by people in different parts of the world since thousands of years. Until now, around 150,000 plants compounds are already known, and many more will be discovered as only a small percentage of all plants have been studied phytochemically and/or pharmacologically. As each plant contains probably as many compounds as genes, there is a huge potential for finding new compounds. Together with the already known compounds, they form a great source for screening for TNF-α inhibitors.

Natural dietary agents including fruits, vegetables, and spices have drawn a great deal of attention from the scientific community regarding the presence of bioactive compounds. These dietary agents are excellent sources of active ingredients like flavonoids, phenolics, terpenoids, and alkaloids which have been shown to be protective against different diseases. Several plant derived agents like curcumin, quercetin, resveratrol, kaempferol, capsaicin, and eugenol have been found to be potent inhibitors of TNF-α. Recently several reports have been published regarding TNF-α inhibitors from plant sources (Ali et al., 2012; Khanna et al., 2007; Paul et al., 2006; Yuliana et al., 2011).

We used *in-vitro* and *in-vivo* models to confirm the anti-inflammatory activities of different plant extracts. Lipopolysaccharide (LPS)-induced U937 human cell lines were used in the *in-vitro* study, and zebrafish larvae served as the *in-vivo* study. Inflammation is considered as major characteristic of several diseases related to autoimmunity. Thus, this study was designed to investigate the antiinflammatory activities of spices, herbs and fruits *in-vitro* and *in-vivo* model system.

3.2. Materials and Methods

3.2.1. Spices and medicinal plants

Kandol (*Benincasa hispida* Thunb.), chameleon (*Houttuynia cordata* Thunb.), white mustard (*Sinapis alba* L.), black mustard (*Brassica nigra* L.), purslane (*Portulaca oleracea* L.), radish (*Raphanus sativus* L.), oregano (*Oreganum vulgare* L.), rosemary (*Rosmarinus officinalis* L.), celery (*Apium graveolens* L.), cress (*Barbarea verna* P. Mill. Aschers.), ajwain (*Trachyspermum ammi* [L.] Sprague.), wolfberry (*Lycium chinense* Mill.), Asian ginseng (*Panax ginseng L.*), marijuana (*Cannabis sativa* L.), babchi

(*Psoralea corylifolia* L.), houseleeks *(Sempervivum smagard L.)*, and tora *(Cassia tora* [L.] Roxb.), were provided by Dr. Henrie Korthout (Fytagoras BV Plant Science, Leiden, The Netherlands). Leaves of vasaka (*Adhatoda vasica L.)* were kindly provided by a lab colleague Muhammad Jahangir.

The following spices were obtained from TRS Co. Ltd, London, UK, in a dried form and identified by Mr. Anil Shah from TRS Co. Ltd; Anis (seed of *Foeniculum vulgare* P. Mill.), coriander (seed of *Coriandrum sativum* L.), cumin (seed of *Cuminum cyminum* L.), dill (seed of *Anethum graveolens* L.), lovage (seed of *Levisticum officinale* Koch.), annato (seed of *Bixa orellana* L.), brown mustard (seed of *Brassica juncea* (L.) Czern.), candle nuts (seed of *Aleurites moluccana* (L.) Willd.), fenugreek (seed of *Trigonella foenum-graecum* L.), star anise (fruit of *Illicium floridanum* Ellis), cinnamon (stem bark of *Cinnamomum verum*), cloves (flower bud of *Syzygium aromaticum* (L.) Merr. et Perry), piment (fruit of *Pimenta officinalis* Lindl.), mace (arillus seed of *Myristica fragrans* Houtt.), nutmeg (seed of *Myristica fragrans* Houtt.), poppy seeds (seed of *Papaver somniferum* L.), sesame seeds (seed of *Sesamum indicum* L.), black pepper (seed of *Piper nigrum* L.), pomegranate seeds (seed of *Punica granatum* L.), black onion (seed of *Nigella sativa* L.), red chili (fruit of *Capsicum annuum* L.), and black cardamom (seed of *Amomum subulatum* Roxb.). Lemon grass, (stalk of *Cymbopogon citratus* (DC) Stapf), greater galangal (rhizome of *Alpinia galanga* (L.) Willd.), ginger (rhizome of *Zingiber officinale* Rosc.), sand ginger (rhizome of *Kaempferia galanga* L.), onion (bulb of *Allium cepa* L.), garlic (bulb of *Allium sativum* L.), and kluwek nut (seed of *Pangium edule* Reinw. ex Blume) were purchased fresh from local supermarket in Leiden, The Netherlands. Temulawak (rhizome of *Curcuma xanthorrhiza* Roxb.) and temukunci (rhizome of *Boesenbergia rotunda* (L.) Mansf.) were purchased from traditional market in Bandung, Indonesia.

Orthosiphon stamineus Benth. leaves were purchased from drugstore van der Pigge, Haarlem, The Netherlands, and identified by Nancy Dewi Yuliana (Leiden University, Leiden, The Netherlands). *Astragalus membranaceus* (Fisch.) Bunge roots were purchased from Shanxi Hunyuan Hengshan Huangqi Company of Limited Liability (Hunyuan County, Shanxi Province, China). *Codonopsis pilosula* Franch roots were purchased from Beijintonrentang Lingchuandanshen Youxianzerendongsi (Linchuan County, Shanxi Province, China). Both were identified by Dr. Young Hae Choi (Leiden University, Leiden, The Netherlands) and Prof. Xue-Mei Qin (Shanxi University, Taiyuan, Shanxi Province, China). *Morus alba* L. stem bark and leaves were purchased from Korean Export and Import Federation of Drugs, Seoul, Korea, and identified by Dr. Young Hae Choi. *Curcuma kwangsiensis* S.

G. Lee and C. F. Liang rhizome. *Plantago major* L. leaves, *Morus alba* L. fruit were purchased from TongRengTang TCM Pharmacy, Chengdu City, Sichuan province and identified by Dr. Henrie Korthout (Fytagoras BV Plant Science, Leiden, The Netherlands). *Urtica dioica* L. leaves was purchased from drug store van der Pigge, Haarlem, The Netherlands and identified by Dr. Henrie Korthout. *Hoodia gordonii* (Masson) Sweet ex Decne was provided by BZH exporters and Importers CC, Hermanus, South Africa, and identified by Mr. Adolf Joubert (BZH exporters and Importers CC, Hermanus, South Africa). All voucher specimens are stored in Pharmacognosy Department, Section Metabolomics, Leiden University, Leiden, The Netherlands.

3.2.2. Chemicals and reagents

Methanol and DMSO were purchased from Biosolve BV (Valkenswaard, The Netherlands). Fetal bovine serum (FBS), penicillin, streptomycin and RPMI1640 were purchased from GIBCO (Grand Island, NY) and U937 cell lines were purchased from ATCC (CRL-1593.2). Lipopolysaccharide (*Escherichia coli* O111:B4) and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Human TNF-α ELISA kit was purchased from BioSource International Inc. (Camarillo, CA, USA). All solvents and reagents were of analytical grade.

3.2.3. Extraction Method

Lemon grass, (*Cymbopogon citratus*), galangal (*Alpinia galanga*), ginger, (*Zingiber officinale*), sand ginger (*Kaempferia galanga*), onion (*Allium cepa*), garlic (*Allium sativum* L.), and kluwek nut *(Pangium edule* Reinw. ex Blume) were powdered and subsequently dried in a freezedryer, while other dry spices were just powdered and directly extracted.

One gram of each dried powdered spice was placed in a reaction tube, to which 2 mL of MeOH 80% is added, vortexed, and sonicated for 15 minutes. The filtrate was collected by filtration and the extraction on the solid phase was repeated two times. The solvent was evaporated by a vacuum rotavapor. The dried extracts were subsequently dissolved in DMSO at 10 mg/mL concentration and ready for the assays.

3.2.4. Growth of cells, LPS stimulation and treatment with extract

Human monocyte-like histiocytic lymphoma cells U937 obtained from the ATCC (CRL-1593.2) were grown in RPMI-1640 medium, supplemented with 10% (v/v) fetal calf serum and 2 mM l-glutamine (Life technologies, Breda, The Netherlands) at 37 °C, 5 % CO2 in a humidified atmosphere. U937 monocytic cells ($5x10^5$ cells per well) were plated in 96-well culture plates and then differentiated into macrophages using phorbol 12- myristate 13-acetate (PMA, 10 ng/ml, overnight, Omnilabo, Breda, The Netherlands). The PMA-differentiated macrophages were allowed to recover from PMA treatment for 48 h, during which the culture medium was replaced daily. Cells were stimulated with Lipopolysaccharide (LPS) as described by (Sajjadi et al., 1996). Treatment of cells were performed immediately after LPS stimulation at different concentrations ranging from (1-100µg mL^{-1}) and then incubated at 37 °C for 4 hours. Supernatants were then collected and measured for TNF-α content using the Human TNF-α enzyme linked immunosorbent assay (ELISA) kit (R and D systems, Europe Ltd).

3.2.5. TNF-α assay

TNF-α in culture supernatants were determined by quantitative ‘‘sandwich’’ enzyme-linked immunosorbent assay using paired antibodies purchased from (Biosource Etten-Leur, The Netherlands) (Verhoeckx et al., 2006). In brief, wells of high-binding Immulon plates (Millipore, Bedford, MA, USA) were coated with 100 µl of the capture antibody (anti-Human TNF-α) (0.250 mg/0.125 mL). After overnight incubation at 4 °C, the plates were washed with the washing buffer and blocked for 1 hour with 1% bovine serum albumin in phosphate-buffered saline. After washing one time, 100 µl of culture supernatants, various concentrations of standard (recombinant Human TNF-α protein) along with 50 µl of detection antibody, was incubated for 2 hours at room temperature with continuous shaking (at 700 rpm). The mixture was discarded and wells were washed again 5 times with washing buffer before addition of 100 µl of streptavidin-HRP to the wells and incubated at room temperature further for 30 minutes with continuous shaking (at 700rpm). The wells were aspirated and washed again 5 times before addition of 100 µl of TMB substrate. The plates were incubated for 30 minutes at room temperature with continuous shaking (at 700 rpm). After 30 minutes the reactions were terminated by addition of 100 µl of 2 M H_2SO_4, and absorbance was determined using a microtiter plate reader (Bio-Tek Instruments Inc., Winooski, VT, USA) at 450 nm. The concentration of TNF- α in the unknown samples was calculated

by comparison of the absorbance of the unknown samples to the standard curve. The ratio (%) of TNF-α inhibition release was calculated by the equation; Inhibition (%) = (1 – T/C) x 100%, of which 'T' represents the concentration of TNF-α released from the cells treated with plant extracts while 'C' is the concentration of TNF-α produced by control cells (treated only with LPS+DMSO).

3.2.6. Cell viability assay

Cell viability (after treatment with different plant extracts) was determined by using MTT assay (Lee et al., 2007). After removal of supernatant for TNF-α measurement, cells were treated with MTT at the rate of 2mg/ml. After 2.5 hours of incubation at 37 °C, the medium was discarded and formazan blue, which is formed from MTT by the mitochondrial dehydrogenase in the living cells, was dissolved in 100 µL DMSO. The optical density (OD) was measured at 540 nm. The background signal (inherent to the wells when no cell was present) was subtracted from the absorbance obtained from each sample.

3.2.7. Zebrafish

Standard procedures (in agreement with local animal welfare regulations) were adopted to raise and maintain Zebrafish (*Danio rerio*) embryos. The GFP Transgenic lines (MPO,s) of zebrafish were used in this study (Lawson and Weinstein, 2002). Embryos were obtained by natural crosses. Fertilized eggs were collected and staged as previously described by (Kimmel *et al*., 1995).

3.2.8. Chemical Induced Inflammation Assay (ChIn assay)

Assay was performed as described by (d'Alencon *et al*., 2010). Briefly, E3 medium was used to grow zebrafish larvae of the GFP strain. They were kept in petri dish until 56 hours post fertilization. Spontaneously hatched larvae were transferred to 48- well plates at the rate of 1 larva/well in a volume of 500 µL of E3 solution. Fruit extracts and controls were pipette to the wells containing embryos 1 hour before the addition of $CuSO_4$. Plates were incubated for 40 minutes at 28 °C. E3 medium was replaced with 4% paraformaldehyde in PBS buffer which was used to fix the embryo and further incubation was carried out for 1 hour at room temperature. Fixing and subsequent steps normally

carried out in dark to evade fading of the fluorescent protein signal. Larvae were washed with PBS-Tween20. Fluorescent cells were examined and counted within the next 48 hours after fixation using a Leica (Wetzlar, Germany) MZ-12 fluorescent stereoscope. Labeled cells were within a specific area known as myoseptum which consist of between the first somite and the end of the tail on one side of each larva. Sixteen embryos were used for each concentration and cells were counted by two independent observers.

3.2.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. One way ANOVA followed by posthoc Tucky's test was performed for TNF-α assay n=3 while for zebrafish assay one way ANOVA followed by posthoc comparison between means and controls were made by Dunnett's test. Value of $P \leq 0.05$ was considered statistically significant for both assays.

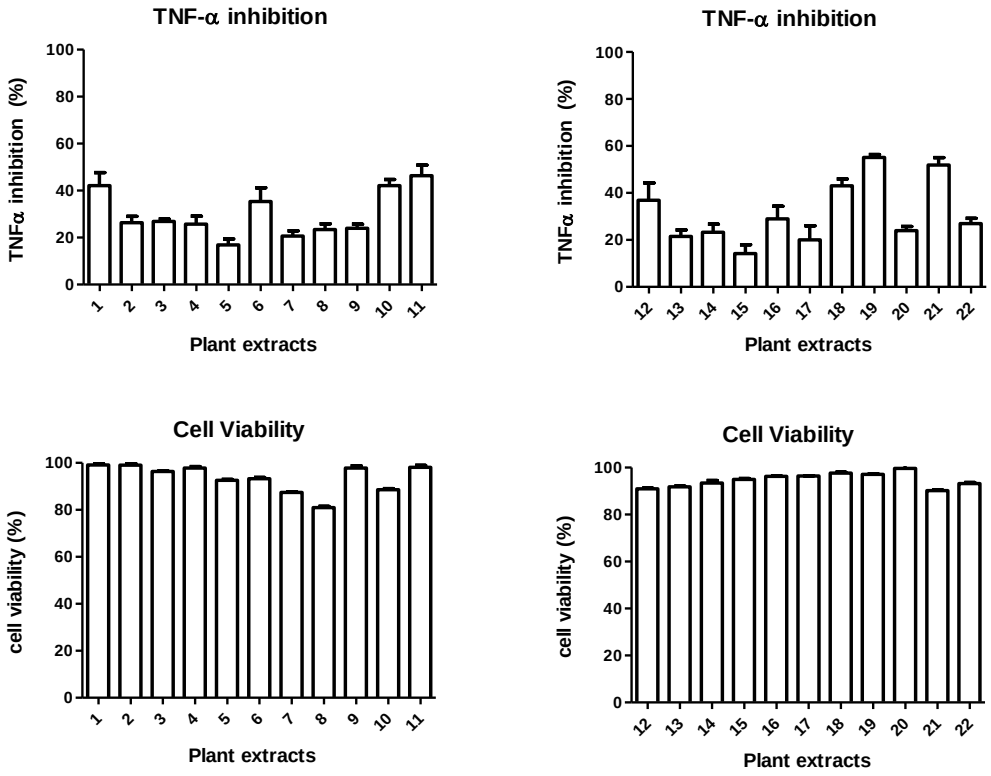

Figure 1: TNF-α inhibition and cell viability % exhibited by various plant extracts (for plants names 1-66, see table 2.1) at a concentration of 10µgmL^{-1}. Each error bar represents ±SEM of three replicates.

The most significant inhibition was observed from the extracts of *Rosmarinus officinalis*, *Curcuma xanthorrhiza*, *Bosenbergia rotunda* and the bark of *Pistacia lentiscus* without exerting cytotoxicity. In addition, the extracts of *Orthosiphon stamineus*, *Cannabis sativa*, *Psoralea corylifolia* and *Curcuma longa* also show significant inhibition of TNF-α, however, they were cytotoxic at the highest concentration applied. It is noteworthy that extracts from *Cannabis sativa* and *Psoralea corylifolia* inhibit TNF-α release even at a concentration of 1 µg/ml. Furthermore, extracts from *Urtica dioica*, *Houttuynia*

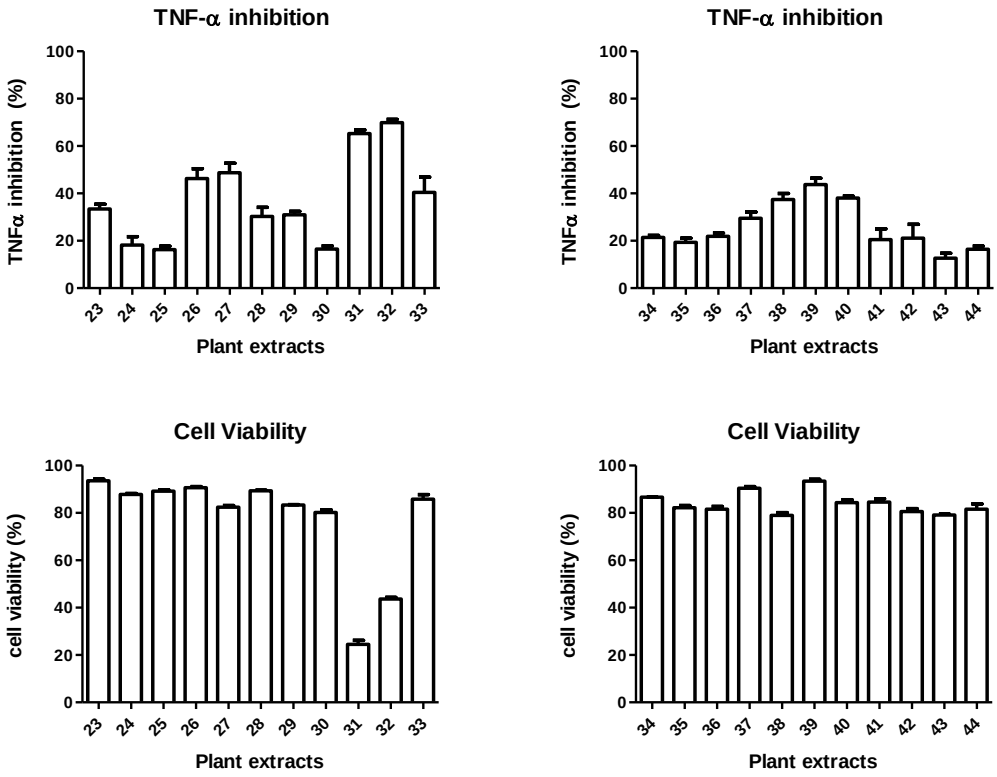

Figure 2: TNF-α inhibition and cell viability % exhibited by various plant extracts (for plants names 1-66, see table 2.1) at a concentration of 10µgmL^{-1}. Each error bar represents ±SEM of three replicates.

cordata, Salvia officinalis, Acacia alata, Origanum vulgare, Sempervivum smagard, Syzygium aromaticum, Pimenta officinalis, Myristica fragrans, Capsicum annuum, Alpinia galanga, Zingiber officinale, Kaempferia galanga, Bixa orellana and *Pistacia lentiscus* leaves also show significant inhibition of TNF-α (Fig1-3).

Extracts which showed activity *in-vitro* were subjected to zebrafish for *in-vivo* studies to confirm their antiinflammatory activity. Copper sulphate was used to induce injury in 56hpf embryo followed by treatment with plant extracts. From this study, we have found that extracts from *Salvia officinalis, Rosmarinus officinalis, Curcuma xanthorrhiza, Myristica fragrans, Curcuma*

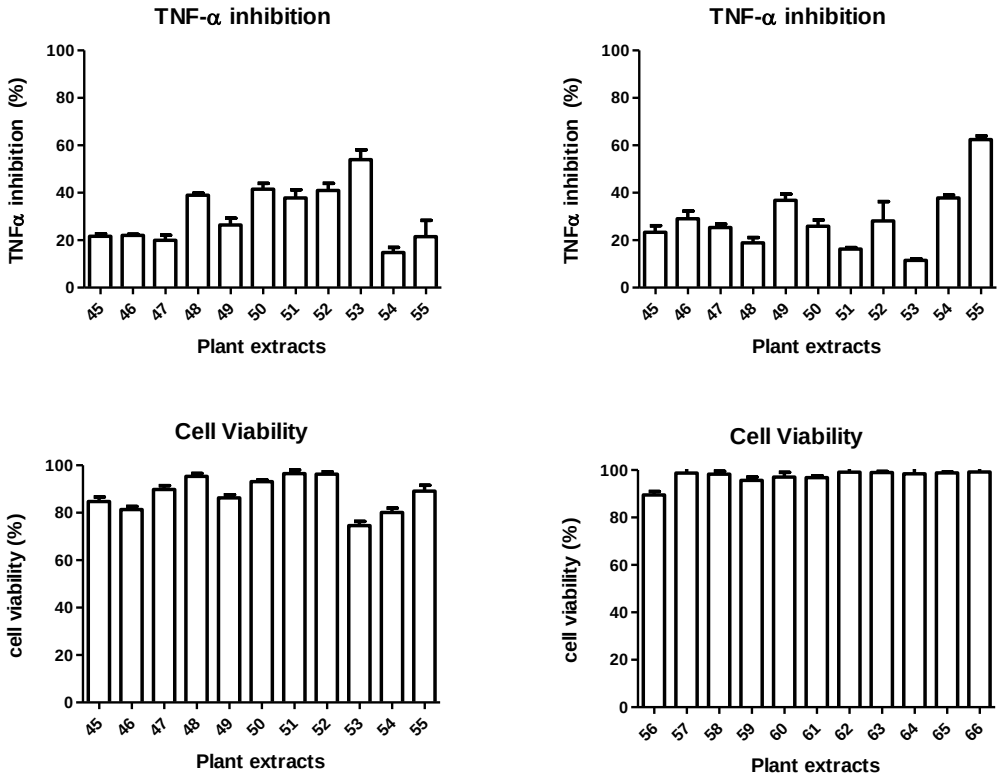

Figure 3: TNF-α inhibition and cell viability % exhibited by various plant extracts (for plants names 1-66, see table 2.1) at a concentration of 10µgmL^{-1}. Each error bar represents ±SEM of three replicates.

longa and bark of *Pistacia lentiscus* showed highly significant inhibition of neutrophils migration towards the wounded areas in zebrafish embryos (Fig.4).

Nettle (*Urtica dioica L.*) extract was found to be active in lowering TNF-α level. The plant has been used as drug, food, dye, cosmetic and for fibres for centuries. Stalk and leaves of the nettle have been used in treatment of diabetes, hypertension, antirheumatic, diuretic, antidiuretic, and cholagogue. Several phenolic compounds have been identified including rutin, quercetin, quercetin glycoside, kaempferol and kaempferol glycosides (Otles and Yalcin, 2012). Extract of *Urtica dioica* folium (IDS 23, Rheuma-Hek) have been reported to inhibit significantly LPS-stimulated TNF-α level in human whole blood of healthy volunteers (Obertreis et al., 1996; Teucher et al., 1996).

Vasaka *(Adhatoda vasica* (L.) Nees) is a well-known plant drug in Ayurvedic and Unani medicine. It has been used for the treatment of various diseases and disorders, particularly for the respiratory tract ailments. The major alkaloid of the plant, vasicine, has been found to be biologically active and is the subject of many chemical and pharmacological studies (Claeson et al., 2000). Tea prepared from the stems and leaves of *H. cordata*, has been shown to possess a variety of pharmacological activities like antiviral, antibacterial, antileukemic, and antiallergic activities. It is reported that *H. cordata* contains a wide range of polyphenols such as rutin, quercetin, hyperoside, quercitrin, and chlorogenic acid, which have been considered to be responsible for the antioxidant activity (Meng et al., 2009; Nuengchamnong et al., 2009).

Salvia officinalis L. has been used as medicinal plant since centuries. This plant is very rich in biologically active compounds like carnosol, carnosic acids, and ursolic acids, which are used for the treatments of soar throat, dyspepsia and diverse inflammatory diseases in the Western world (Bauer et al., 2012).

Fresh juice of leaves of *Sempervivum* has been used as traditional medicine since ancient times to treat wounds, skin burns, insect bites and inflammation of the ears. Drinking tea prepared from the leaves of this plant is used to treat ulcer. All of these activities have been attributed to the phenolic compounds (quercetin, myricetin, herbacetin, kaempferol) present in *Sempervivum* (Abram and Donko, 1999; Sentjurc et al., 2003).

Clove (*Syzygium aromaticum* L.) and nutmeg has been used by the traditional Ayurvedic healers of India since ancient times to treat respiratory problems, fever, skin diseases and digestive ailments (Banerjee et al., 2006; Chung et al., 2006). Many different essential oils have been identified from clove and nutmeg, in which the most abundant one is eugenol, which is reported to inhibit LPS-stimulated TNF-α release in U937 cell lines (Chung et al., 2006; Lee et al., 2007). It has also been reported that myristicin is an important component of essential oil of the nutmeg, significantly inhibiting TNF-α release at 50 μM (Lee and Park, 2011).

Pimenta dioica has been used in the treatment of digestive ailments and abdominal pain, high blood pressure, hyperglycemia, obesity, menstrual cramps and inflammatory conditions in traditional medicine of South American, Middle

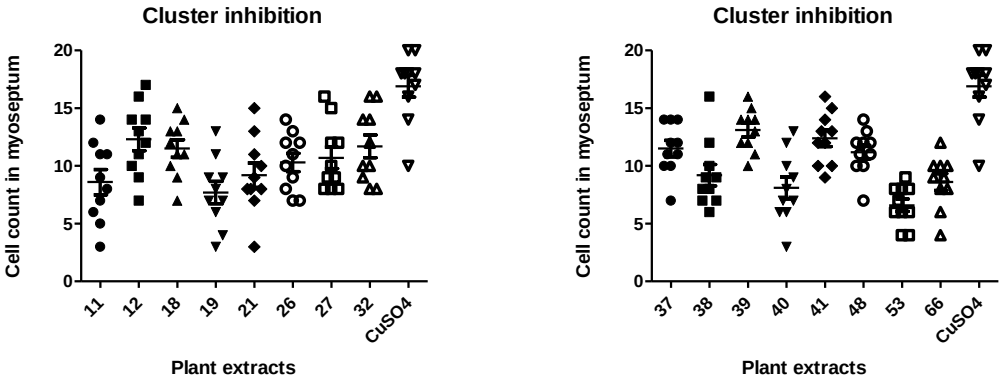

Figure 4: Leukocyte cluster inhibition in zebrafish embry exhibited by various plant extracts (for plants names 1-66, see table 2.1) at a concentration of 10µgmL^{-1}. Each error bar represents ±SEM of N= 10.

Eastern and Asian countries (Al-Rehaily et al., 2002). Allspice has been reported to contain flavonoids and an essential oil containing eugenol, which is considered to be the active principal (Kikuzaki et al., 1999; Kikuzaki et al., 2008). Several flavonoids and alkaloids have been isolated from *Capsicum annuum* with a wide range of biological activities (Lee et al., 1995). Capsaicin, a major ingredient of hot pepper has been reported to suppress TNF-α release (Park et al., 2004). *Alpinia galanga* has been used for the treatment of bronchitis, heart disease, renal calculus, diabetes, and rheumatism. Phytochemical investigation has led to the isolation of flavonoids, phenolic acids and several other compounds (Kaushik et al., 2011; Mayachiew and Devahastin, 2008). The rhizome of *Kaempferia galanga* is used by people in many regions for relieving toothache, abdominal pain, muscular swelling and rheumatism. The extract of (dried) rhizome contains volatile oils as the major components, which includes ethyl-*p*-methoxycinnamate (31.77%), methylcinnamate (23.23%), carvone (11.13%), eucalyptol (9.59%) and pentadecane (6.41%); these oils were found to possess anti-bacterial properties (Ridtitid et al., 2008; Tewtrakul et al., 2005).

Table 3.1 shows all the plants which we have tested in our studies. It also shows their botanical names, respective families and their ethanobotanical/trdaitional use in different parts of the world.

Sr.no	Species	Family	Common name	Part of plant used	Biological activity and/or traditional medicinal use	References
1	*Urtica dioica*	Urticaceae	Stinging nettle	Leaves	Treatment of diabetes, rheumatism, eczema, anaemia and hair loss.	(Akbay et al., 2003)
2	*Morus alba*	Moraceae	Mulberry	Leaves	Treatment of throat infections, irritations and inflammations; for reducing stress and preventing diabetes mellitus.	(Isabelle et al., 2008;
3	*Curcuma kwangsiensis*	Zingiberaceae	Ezhu	Rhizome	Antitumor and antioxidant activities.	Kumar and Chauhan, 2008)
4	*Astragalus membranaceus*	Fabaceae	Membranous milk-vetch root	Roots	Immunostimulant	(Xu et al., 2009)
5	*Codonopsis pilosula*	Campanulaceae	Poor man,s ginseng	Roots	Antitumor, antimicrobial, antioxidant activities.	(Cho and Leung, 2007)
6	*Morus alba*	Moraceae	Mulberry	Fruit	Antioxidative, antiinflammatory, and antidiabetic activities.	(Yongxu and Jicheng, 2008)
7	*Plantago major*	Plantaginaceae	Common plantain	Leaves	To treat skin diseases, infectious diseases, and problems concerning the digestive organs, respiratory organs, reproduction, blood circulation, tumours, for pain relief and reducing fever.	(Isabelle et al., 2008)

(Kumar et al., 2008)	Purgative and vermifuge.	Stem bark	Mulberry	Moraceae	*Morus alba*	8
(Sabale et al., 2011)	Treatment of dyspepsia, burning sensation, diabetes and used also as vermifuge. Reported to have antiinflammatory, anti-diuretic and anti-cancer activities	Fruit	Winter melon	Cucurbitaceae	*Benincasa hispida*	9
(Lu et al., 2006)	To treat cough, leucorrhea and ureteritis	Leaves	chameleon plant	Saururaceae	*Houttuynia cordata*	10
(Baricevic et al., 2001)	Anti-bacterial, fungistatic, virustatic, astringent, eupeptic and anti-hydrotic effects	Leaves	Sage	Lamiaceae	*Salvia officinalis*	11
(Chakraborty and Brantner, 2001)	Antiallergic, antiinflammatory and antiasthmatic	Leaves	vasaka	Acanthaceae	*Adhatoda vasica*	12
(Ma et al., 2005)	To treat anorexia, chronic diarrhea, diabetes, problems with seminal emission, and excessive leukorrhea and to promote immune functions	Rhizome	Chinese yam	Dioscoreaceae	*Dioscorea opposita*	13
Eskin et al., 2007)	Antineoplastic and anticancer	seedling	White mustard	Brassicaceae	*Sinapis alba*	14

(Rahmatullah et al., 2010)	Anti-hyperglycemic	Seeds	Black mastard	Brassicacea e	*Brassica nigra*	15
(Chan et al., 2000)	Anti-scorbutic, antiseptic, antispasmodic, diuretic, vermifuge, refrigerant, and used as a vulnerary herb against sore nipples, ulcers of the mouth and urinary disorders.	Leaves	Pusley	Portulacace ae	*Portulaca oleracea*	16
(Moon and Kim, 2012)	To treat inflammation.	Seedlin g	Radish	Brassicacea e	*Raphanus sativus*	17
(Ocaña-Fuentes et al., 2010)	Antioxidant, antimicrobial, antimutagenic and anticarcinogenic	Leaves	Oregano	Lamiaceae	*Origanum vulgare*	18
(Peng et al., 2007)	Antioxidant, antitumor, antiinflammatory and anti HIV	Leaves	Rosemar y	Lamiaceae	*Rosmarinu s officinalis*	19
(Mencherini et al., 2007)	Antiinflammatory	Leaves	Celery	Apiaceae	*Apium graveolens*	20
(Devaraj et al., 2010)	To treat hepatitis, liver complaints, diabetes, rheumatism, cancer, hypertension and heart disorders.	Rhizom e	Temula wak	Zingiberace ae	*Curcuma xanthorrhi za*	21

(Dey et al., 2006)	Antiinflammatory	Seed	American cress	Brassicaceae	*Barbarea verna*	22
(Bairwa et al., 2012; Thangam and Dhananjayan, 2003)	Antiinflammatory, antihypertensive, hepatoprotective, antispasmodic, broncho-dilating and treatment of stomach disorder.	Seeds	Ajwain	Apiaceae	*Trachyspermum ammi*	23
(Park et al., 2011)	Anti-aging and neuroprotective effects.	Fruits	Wolfberry	Solanacea e	*Lycium chinense*	24
(Madgula et al., 2010)	Appetite suppressant; treatment of severe abdominal cramps, hemorrhoids, tuberculosis, indigestion, hypertension and diabetes.	Stem	Cactus	Apocynaceae	*Hoodia gordonii*	25
(Ching et al., 2007)	Treatment of aphthous ulcer, dry mouth, stomach discomfort, leucorrhea and dysentery, a tonic for women after childbirth; to treat rheumatism and muscular pains (added into lotions), application to the body after confinement (added into pastes).	Root	Temukunci	Zingiberaceae	*Bosenbergia rotunda*	26
(Yam et al., 2007)	Catarrh of the bladder, nephritis, nephrolithiasis, hydronephrosis, vesical calculi, atherosclerosis, goat, jaundice, and rheumatism. As beverage to improve health, kidney, bladder inflammation and diabetes.	Leaves	Cat whiskers	Lamiacea e	*Orthosiphon stamineus*	27
(Eguale et al., 2007)	Antifertility, antidiabetic, antihyperlipidemic, antioxidant and hypotensive activities.	Seeds	Coriander	Apiaceae	*Coriandrum sativum*	28

(McKay and Blumberg, 2006)	Antiinflammatory, antiviral, antiallergenic, antibacterial, antitumor and antioxidant.	Leaves	Peppermint	Labiatae	*Mentha piperita*	29
(Calixto et al., 2001)	Alleviating certain type of pains such as toothache, abdominal pain, chest pain, and neuralgia.	Root	Ginseng	Araliaceae	*Panax ginseng*	30
(Choi et al., 2004)	To treat menstrual cramps and convulsions, inflamed tonsils, migraine and headaches, glaucoma, asthma and pain relief.	Flowers	Marijuana	Cannabaceae	*Cannabis sativa*	31
(Matsuda et al., 2009)	Used as a tonic, treatment of uterine hemorrhage, and a coronary vasodilatory in traditional Chinese medicine.	Seeds	Babchi	Fabaceae	*Psoralea corylifolia*	32
(Abram et al., 1999)	To treat ulcers, wounds and pain.	Leaves	Houseleeks	Crassulaceae	*Sempervivum smaragd*	33
(Benjamin and Lamikanra, 1981; Maity et al., 1998)	Antifungal, antiinflammatory, antirheumatic; and treatment of skin diseases.	Leaves	Sickle senna	Caesalpinaceae	*Cassia tora*	34
(Khanum et al., 2004)	Antibacterial, antiviral, and antifungal activity.	Bulb	Garlic	Lilliceae	*Allium sativum*	35

(Wang et al., 2011)	Antimicrobial, antioxidant, insecticidal, analgesic, sedative and convulsive activities.	Nuts	Star anise	Illiaceae	*Illicium verum*	36
(Jayapraka sha and Rao, 2011)	Antiemetic, antidiarrheal, antiflatulent, and general stimulant.	Bark	Cinnamo n	Lauraceae	*Cinnamom um verum*	37
(Miyazaw a and Hisama,	Antibacterial, vermifuge and to treat toothaches.	Flower s	Cloves	Myrtacea e	*Syzygium aromaticu m*	38
(Al-Rehaily et al., 2002)	Treatment of digestive ailments and abdominal pain, high blood pressure, hyperglycemia, obesity, menstrual cramps and inflammatory conditions.	Fruit	Piment	Myrtacea e	*Pimenta officinalis*	39
(Barceloux , 2009)	To treat rheumatism, cholera, psychosis, stomach cramps, nausea, diarrhea, flatulence, anxiety, in addition as an aphrodisiac and an	Seeds	Nutmeg	Myristica ceae	*Myristica fragrans*	40
(Sindhu et al., 2012)	Treatment of diabetes, high cholesterol, wounds, inflammation, and gastrointestinal ailments.	Seeds	Fenugre ek	Fabaceae	*Trigonella foenum-graecum*	41
(Calixto et al., 2001)	Analgesic.	Seeds	Poppy	Papaverac eae	*Papaver somniferu m*	42

(Shenoy et al., 2011)	Used to treat burns and wounds; anti-aging.	Seeds	Sesame	Pedaliaceae	*Sesamum indicum*	43
(Gülçin, 2005)	To improve appetite and digestion, and to treat cold, cough, dyspnoea, diseases of the throat, intermittent fever, cholic, dysentery, worms and	Seeds	Black pepper	Piperaceae	*Piper nigrum*	44
(Lee et al., 2010)	Antioxidant, antitumour, antihepatotoxicity, antilipoperoxidation and antibacterial.	Seeds	Pomegranate	Punicaceae	*Punica granatum*	45
(Figueirinha et al., 2010)	Treatment of digestive disorders, inflammation, diabetes, neuro disorders, and fever.	Stem	Lemon grass	Poaceae	*Cymbopogon citratus*	46
(Ilaiyaraja and Khanum,	To improve respiratory, stomach, intestinal, kidney, liver, circulatory and immune system function.	Seeds	Black onion	Ranunculaceae	*Nigella sativa*	47
(Conforti et al., 2007)	Radical scavenging and anti-oxidant.	Fruit	Red chilli	Solanaceae	*Capsicum annuum*	48
(Verma et al., 2010)	Treatment of dyspepsia, cough, nausea, vomiting, itching, for throat troubles, congestion of lungs, inflammation of eyelids, digestive	Seed pod	Black cardamom	Zingiberaceae	*Amomum subulatum*	49
(Rao et al., 2010)	To treat rheumatism, bronchial catarrh, breath disorder, ulcers, whooping colds in children, throat infections and fever.	Rhizome	Great galangal	Zingiberaceae	*Alpinia galanga*	50

(Ghasemzadeh et al., 2010)	Treatment of diabetes, high blood pressure, cancer, and for body fitness.	Rhizome	Ginger	Zingibera ceae	Zingiber officinale	51
(Vittalrao et al., 2011)	Stimulant, expectorant, diuretic and carminative; treatment of skin infections, diabetes mellitus and various inflammatory diseases.	Rhizome	Sand ginger	Zingibera ceae	Kaempfer ia galanga	52
(Abbas et al., 2010)	To treat liver obstruction, jaundice, ulcers, inflammation, dysentery, stomach disorders, fresh wounds, insect stings and viral infections including	Rhizome	Turmeric	Zingibera ceae	Curcuma longa	53
(Corea et al., 2005)	Treatment of cancer, coronary heart disease, obesity, hypercholesterolemia, type-2 diabetes, hypertension, cataract, and disturbances of the gastrointestinal tract.	bulbs	Onion	Alliaceae	Allium cepa	54
(Chye and Sim, 2009)	Antioxidant and antibacterial.	Seeds	Kluwek nut	Flacourtia ceae	Pangium edule	55
(Santos et al., 2005)	Diaphoretic, emmenagogue, expectorant, stomachic and stimulant.	Seeds	Lovage	Apiaceae	Levisticu m	56
(Barros et al., 2010)	To treat digestive system disorders, diabetes, bronchitis and chronic coughs, kidney stones; having diuretic, stomachic and galactogogue properties.	Seeds	Anis	Apiaceae	Foeniculu m vulgare	57
(Jana and Shekhawat, 2010)	To improve digestion and stomach functions; to treat ulcers and abdominal pain.	Seeds	dil	Apiaceae	Anethum graveolen s	58

(Dhandapani et al., 2002)	Stomachic, diuretic, carminative, emmanogogic and antispasmodic; used to treat dyspepsia, diarrhea, jaundice	Seeds	Cumin	Apiaceae	Cuminum cyminum	59
(Ferreira et al., 2012)	To treat fevers, inflammatory conditions and parasitic diseases.	Seeds	Annatto	Bixaceae	Bixa orellana	60
(Rahmatullah et al., 2010)	Antidiabetic.	Seeds	Brown mustard	Brassicaceae	Brassica juncea	61
(Pedrosa et al., 2002)	To treat headache, fever, inflammation and gonorrhoea, and also for lowering of cholesterol level in blood.	Leaves	Candle nut	Euphorbiaceae	Aleurites moluccana	62
(Çalış et al., 1993)	Used as diuretic, and to treat constipation, dropsy, arthritis, rheumatic pain, cystitis and itching scalp.	Leaves	Ash	Oleaceae	Fraxinus angustifolia	63
(Atmani et al., 2009)	Antioxidant.	Bark	Ash	Oleaceae	Fraxinus angustifolia	64
(Duru et al., 2003)	To treat eczema, paralysis, throat infections, renal stones, jaundice, asthma and stomach-ache; used as astringent, pectoral and stimulant; and leaves to have antiinflammatory	Leaves	Mastic	Anacardiaceae	Pistacia lentiscus	65
(Kordali et al., 2003)	A traditional folk medicine in some regions of Spain for treatment of hypertension.	Bark	Mastic	Anacardiaceae	Pistacia lentiscus	66

Previous studies reported the presence of leucocyanidin, ellagic acid, apogenin, luteolin, and the flavonoid bisulphates, ishwarane and bixaghanen in *B. orellana* leaf oil (Harborne, 1975; Lawrence and Hogg, 1973). The pharmacological action of its leaves' extract includes anticonvulsant, analgesic, antidiarrheal, antimicrobial, antileishmanial and antifungal activity (Fleischer et al., 2003; Shilpi et al., 2006).

Gum of Chios mastic (*Pistacia lentiscus*) is a natural antimicrobial agent that has found extensive use in pharmaceutical products and as a nutritional supplement. Antiinflammatory activities have been well described (Mahmoudi et al., 2010) and can be attributed to a variety of compounds such as triterpenes of the oleanane, euphane, and lupine type; alpha-tocopherol and polyphenols (Assimopoulou and Papageorgiou, 2005; Sanz et al., 1992). It has also been reported that the essential oil of *Pistacia lentiscus* reduces leukocyte migration to the damaged tissue and exhibits antiinflammatory activity (Maxia et al., 2011).

Ginger extract and its pungent constituents gingerol, shogaol, and zingerone have been found to possess many interesting pharmacological and physiological activities, such as antiinflammatory, analgesic, antipyretic, antihepatotoxic, and cardiotonic effects (Mascolo et al., 1989; Mustafa et al., 1993).

Several sesquiterpenoids have been isolated from *C. xanthorrhiza*. The most popular one is xanthorrizol which has anti-bacterial activity (Hwang et al., 2000). The hexane-soluble fractions from *C. xanthorriza* were found to decrease the level of serum and liver triglycerides in rats. The major compound of *C. xanthorriza* essensial oil, α-curcumene, was thought to be one of the active principles (Yasni et al., 1994).

From the methanolic extract of *B. rotunda*, several prenylchalcones and prenylflavanones have been isolated. Among them are krachaizin B, 4-hydroxypanduratin A, 4-hydroxypanduratin A, isopanduratin A, alpinetin, cardamonin, and 2, 6-dihydroxy-4-methoxy dihydrochalcone (Morikawa et al., 2008). Most of the compounds isolated from *C. xanthorriza* and *B. rotunda* have a prenyl substituent, which may be involved in activity, as it is thought to be important for protein-binding (Magee and Seabra, 2003).

Curcumin, the major constituent isolated from the rhizomes of *C. longa* L., is responsible for the antiinflammatory effects (Araújo and Leon, 2001). Several compounds namely rosmarinic acid (RA), caffeic acid (CA), chlorogenic acid, carnosolic acid, rosmanol, carnosol and different diterpenes

were identified in the aqueous extract of rosemary and these compounds were reported for different biological activities (Hoefler et al., 1987; Wu et al., 1982). It is also worth to mention that the rosemary essential oil has been reported to have antiinflammatory activities (de Melo et al., 2011).

3.4. Conclusion

In conclusion, our results demonstrate that extracts of *Urtica dioica, Houttuynia cordata, Salvia officinalis, Acacia alata, Origanum vulgare, Sempervivum smaragd, Syzygium aromaticum, Pimenta officinalis, Myristica fragrans, Capsicum annuum, Alpinia galangal, Zingiber officinale, Kaempferia galangal, Bixa orellana* and *Pistacia lentiscus* show significant inhibition of TNF-α in LPS stimulated U937 cells lines. In addition, there are some other plants including *Origanum vulgare, Rosmarinus officinalis, Curcuma xanthorrhiza, Bosenbergia rotunda, Orthosiphon stamineus, Cannabis sativa, Psoralea corylifolia, Curcuma longa*, and bark of *Pistacia lentiscus* which show highly significant inhibition of TNF-α, but they also exhibited toxicity at the highest concentration applied in this study. We have also found that extracts which were active in *in-vitro* also showed activity *in-vivo* with few wxceptions which suggest that *in-vivo* system behaves totally different. Extracts from Salvia *officinalis, Rosemarinus Officinalis, Curcuma Xanthorrhiza, Myristica fragrans, Curcuma longa* and bark of *Pistacia lentiscus* showed highly significant inhibition of neutrophil migration towards wounded area in zebrafish embryo. This study demonstrates the potential applications of the medicinal constituents of these plants in the prevention and treatment of inflammatory diseases.

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Chapter 4

Evaluation of anti-TNF-α activity of eight major cannabinoids isolated from *Cannabis sativa*

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Abstract

We investigated the antiinflammatory effect of a series of cannabinoids in an *in-vitro* systems, human U937 cells stimulated with LPS to secrete immunoregulatory cytokine tumor necrosis factor alpha (TNF-α). Phytocannabinoids like the psychoactive delta-9- tetrahydrocannabinol (THC) and nonpsychoactive cannabidiol (CBD), cannabigerol (CBG), cannabinol (CBN), cannabichromene (CBC), cannabidiolic acid (CBDA), Δ9-tetrahydrocannabinolic acid (THCA), cannabigerolic acid (CBGA) showed activity which suggest that cannabinoids can potentially alter cytokine secretion of human U937 cell lines.

Keywords: Cannabinoids, inflammation, TNF-α, U937 cell lines

4.1. Introduction

Inflammation is a response of a tissue to injury, which can be caused (or followed by) pathogen invasion. It is characterized by redness, heat, swelling, pain and dysfunction of the organs involved. The process of inflammation is mediated by several pro-inflammatory and antiinflammatory cytokines. Tumor necrosis factor alpha (TNF-α) is one of the most important pro-inflammatory cytokines which promotes inflammation. TNF-α is mainly produced by macrophages upon stimulation by a bacterial cell wall component lipopolysaccharide (LPS) at nanogram per milliliter concentration. Wounds can never heal without inflammation, so release of TNF-α plays an essential role in host defense against pathogens and tissue recovery. However, excessive production of TNF-α can lead to endotoxic shock, rheumatoid arthritis, and cachectic states associated with malignancies, chronic parasitic infections and several diseases related to autoimmunity. In these cases anti-TNF-α therapies are recommended for the treatment of several inflammatory diseases. Several protein based drugs are available for the inhibition of TNF-α but these are associated with high costs and side effects. Thus, it is important and even essential to develop safer and perhaps more-cost-effective TNF-α inhibitors. Many natural compounds belonging to various classes have been found to reduce TNF-α level (Paul et al., 2006).

Nature is a main source of compounds for pharmaceutical purposes. Because of the great structural diversity, natural products or natural product-derived compounds offer great opportunities for the development of antiinflammatory drugs. Their origin extends to plants, fungi, bacteria, and marine organisms. Plants have been and continue to be the greatest source of natural compounds from which drugs can be synthesized. Of the 1184 new chemical entities registered as medicine in the period of 01/1981 to 06/2006, 60% are derived from or based on natural products. Natural products clearly play a dominant role in the discovery of leads for drug development (Gautam and Jachak, 2009).

Cannabis is considered one of the oldest psychotropic drugs known to humanity. It is difficult to trace the beginnings of its use by humans because it was cultivated and consumed long before the appearance of writing (McKim, 2000). There are several species of cannabis. The most relevant are *Cannabis sativa*, *Cannabis indica* and *Cannabis ruderalis*. *Cannabis sativa*, the largest variety, grows in both tropical and temperate climates. The two main preparations derived from cannabis are marijuana and hashish. Marijuana is a Mexican term initially attributed to cheap tobacco but referring today to the dried leaves and flowers of the hemp plant. Hashish, the Arabic name for Indian hemp, is the viscous resin of the plant (Ben Amar, 2006).

More than 460 different compounds have been identified from cannabis plants, around 60 of which are grouped under the name cannabinoids. The major psychoactive ingredient of cannabis is delta-9-tetrahydrocannabinol, commonly known as THC. Other cannabinoids present in Indian hemp include delta-8-tetrahydrocannabinol (Δ^8 THC), cannabinol (CBN), cannabidiol (CBD), cannabicyclol (CBL), cannabichromene (CBC) and cannabigerol (CBG), but they are present in small quantities and have no significant psychotropic effects compared to THC. However, they may have an impact on the product's overall effect.

The therapeutic effects of cannabis and its derivatives have been extensively investigated, and they have been shown to exhibit a wide variety of beneficial properties, inhibiting cancer, neuropathic pain, multiple sclerosis, Alzheimer's disease, atherosclerosis, rheumatoid arthritis, asthma and many inflammatory diseases (Alexander et al., 2009; Ligresti et al., 2009; Nolin et al., 2002; Pacher et al., 2006). Inflammation plays a crucial role in most of the mentioned health issues, and cannabinoids have been proven to influence these processes. Their biological activity is connected to the activation of specific receptors: CB1, expressed mostly in the central nervous system; and CB2, found mainly in peripheral tissues. CBD, a non-psychoactive cannabinoid, is responsible for the antiinflammatory activity of marihuana, acting mostly on the CB2 receptor in peripheral tissues (Rajesh et al., 2007; Zoratti et al., 2003).

Antinflammatory studies are performed using the U937 cell line derived from a human histiocytic lymphoma (Sundström and Nilsson, 1976). This cell line is maintained as replicative non-adherent cells having many of the biochemical and morphological characteristics of blood monocytes (Harris and Ralph, 1985). When treated with phorbol myristate acetate (PMA), U937 cells differentiate to become adherent, non-replicative cells with characteristics of tissue macrophages, including isoenzyme patterns, 17 CR3 expressions, 18 and other phenotypic markers (Pearlman et al., 1988). The purpose of this study was to investigate antiinflammatory activities of cannabinoids using U937 cell lines (*in-vitro*).

4.2. Materials and Methods

4.2.1. Chemicals and reagents

Fetal bovine serum (FBS), penicillin, streptomycin and RPMI1640 were purchased from GIBCO (Grand Island, NY) and U937 cell lines from ATCC (CRL-1593.2). Lipopolysaccharide (Escherichia coli O111:B4) and phorbol 12-myristate 13-acetate (PMA) were from Sigma–Aldrich (St. Louis, MO, USA). The Human TNF-α ELISA kit was purchased from BioSource International

Inc.(Camarillo, CA, USA). DMEM, fetal bovine serum (FBS), penicillin and streptomycin solution, phosphate buffered saline, were supplied by GIBCO Netherlands BV (Breda, The Netherlands). DMSO was purchased from Biosolve BV (Valkenswaard, The Netherlands).

4.2.2. Plant Cannabinoids

The cannabinoids used in this study were kindly provided by Dr Arno hazekamp.

4.2.2. Cell culture

Human monocyte-like histiocytic lymphoma cells U937 were cultured as described in chapter 3.

4.2.4. TNF-α ELISA

TNF-α in culture supernatants were performed as described in chapter 3.

4.2.5. Cell viability assay

Cell viability assay was performed as described in chapter 3.

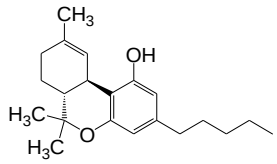
4.2.6. Data analysis

Statistical analyses were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. One way ANOVA was performed. Posthoc comparison between means and controls were made using Dunnett's test. Value of $P \leq 0.05$ was considered statistically significant.

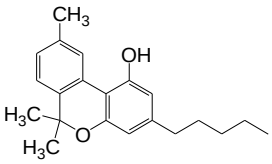
4.3. Results and Discussions

Cannabis has a long history as a medicinal preparation, mainly for properties such as analgesia, antiemesis, ocular hypotension, and anticonvulsion

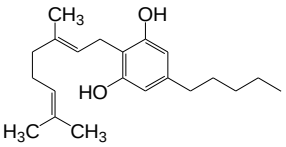
(Mechoulam et al., 1998). Recent research *in-vitro* and in animal models has led to increasing evidence that cannabinoids are also important modulators of the immune system (Klein et al., 1998) and thus could cannabinoids have a role in the treatment of chronic inflammatory diseases. It is therefore important to find out whether nonpsychoactive cannabinoids are suitable for treating chronic inflammatory diseases.



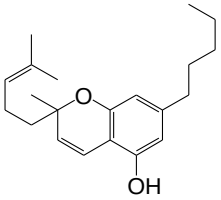
Δ^9- Tetrahydrocannabinol (Δ^9- THC)



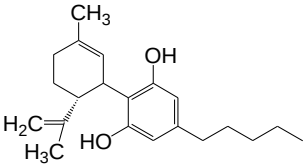
Cannabinol (CBN)



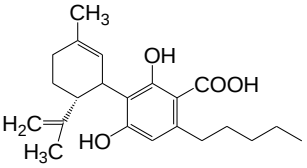
Cannabigerol (CBG)



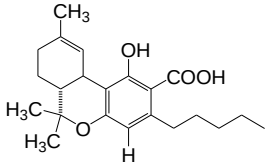
Cannabichromene (CBC)



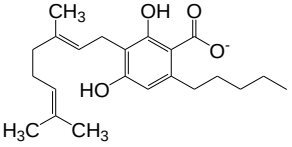
Cannabidiol (CBD)



Cannabidiolic acid (CBDA)



Δ^9- Tetrahydrocannabinolic acid (Δ^9- THCA)



Cannabigerolic acid (CBGA)

Figure 1: Structures of cannabinoids studied in this chapter.

The inhibitory effects of eight major cannabinoids and *Cannabis sativa* extract on TNF-α inhibition was evaluated in U937 cell line stimulated by LPS. Crude extract, Δ^9-THC, Δ^9-THCA, CBD, CBDA, CBG, CBGA, CBC and CBN were evaluated for their ability to inhibit TNF-α at a concentration of 10 μgmL^{-1}, 1 μgmL^{-1} and 0.01 μgmL^{-1} respectively. In this study, it was found that phytocannabinoids show highly significant inhibition of TNF-α at a concentration of 1µg/ml. Maximum inhibition was observed in Δ^9-THCA followed by CBDA > CBGA > CBD >Δ^9-THC > CBG > CBN and CBC. They all show activity in a concentration-dependent manner. It was also found that all these compounds show toxicity towards cell lines at concentration of 10µg/ml. TNF-α inhibition and cell viability of cannabinoids after stimulated with LPS has shown in Figure 2-4.

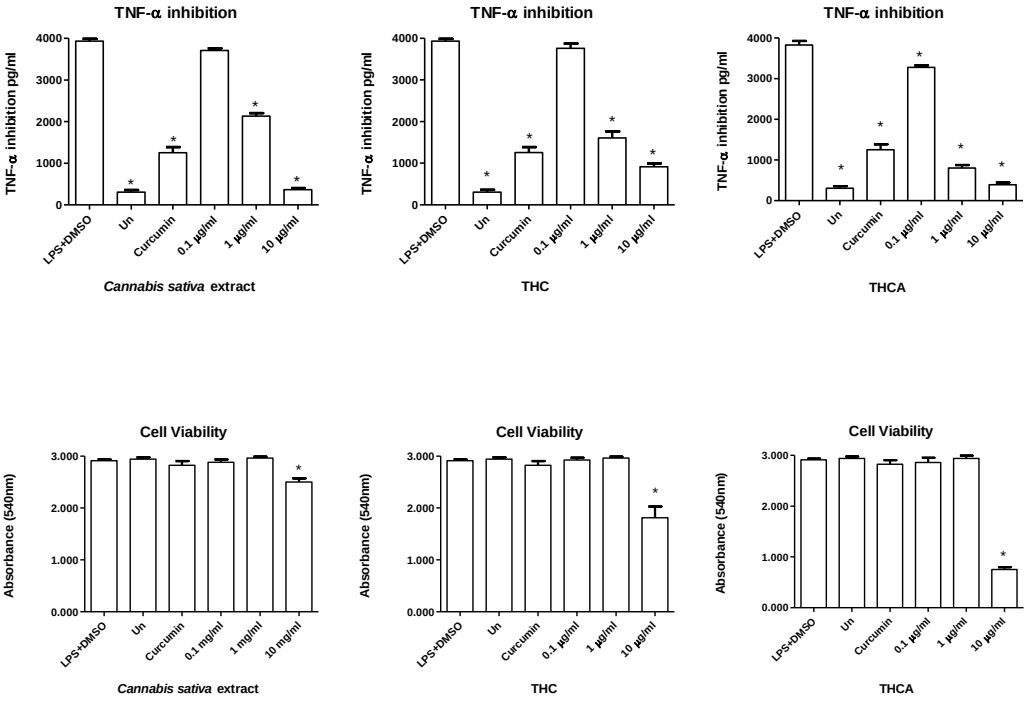


Figure 2: TNF-α inhibition and cell viability of LPS stimulated U937 cell lines treated with different cannabinoids. One way ANOVA was performed. Posthoc comparison between means and controls (LPS+DMSO) were made using Dunnett's test. Each error bar represents ±SEM of three replicates. * = the value shows a significant difference ($P \leq 0.05$).

Delta-9-tetrahydrocannabinol (THC) is one of more than 80-100 cannabinoids in the marijuana plant and has been recognized as the major psychoactive component of this plant. THC being psychoactive and most studied component of marijuana is widely acknowledged because of its therapeutic effects including relief of nausea and vomiting associated with cancer and its treatments; stimulation of appetite in AIDS patients and patients with anorexia and wasting syndrome; analgesia; and muscle relaxation. In this study, we show that Δ^9-THC inhibits TNF-α release in LPS stimulated U937 cell line. THC shows toxicity towards cells at highest concentration while at low concentration (1µg/ml), it shows significant inhibition of TNF-α. Various *in-vitro* studies have shown that THC exhibits a variety of inhibitory effects on immune functions. THC has been used with success in controlling severe cachexia seen in patients with cancer or AIDS (Kusher et al., 1994; Razdan, 1986). THC has been shown to inhibit TNF-α production in various models of cell lines (Fischer-Stenger et al., 1993; Zheng and Specter, 1996; Zhi-Ming et al., 1992). The mechanism by which THC inhibits TNF-α production is not yet clear but there are several possibilities proposed (Specter et al., 1990). There are reports saying THC as a lipophilic compound, can be incorporated into the cell membranes and cell membrane altercation could be the reason of inhibitory action. There is also a report regarding THC acid and its potential to inhibit TNF-α release in LPS stimulated U937 cell lines. Moreover, it was also found that the inhibitory effect on TNF-α production by THC and THCA are not mediated via CB1 and CB2 receptors instead via TLR4 and IFN receptors (Figure 3) (Kozela et al., 2010; Verhoeckx et al., 2004).

Cannabidiol (CBD; Fig. 3), the most abundant nonpsychoactive cannabinoid in the plant has been studied more extensively in recent years. CBD is well-known for its immunosuppressive, antiinflammatory and antioxidant properties both *in-vitro* and in various preclinical models (Fernández-Ruiz et al., 2005; Mechoulam et al., 2007). We here report that CBD significantly suppresses the level of TNF-α associated with LPS in U937 cell lines. At higher dose (10µg/ml) CBD shows significant toxicity towards cell lines but at lower dose (1µg/ml), it strongly inhibits the release of TNF-α in LPS stimulated cell lines.

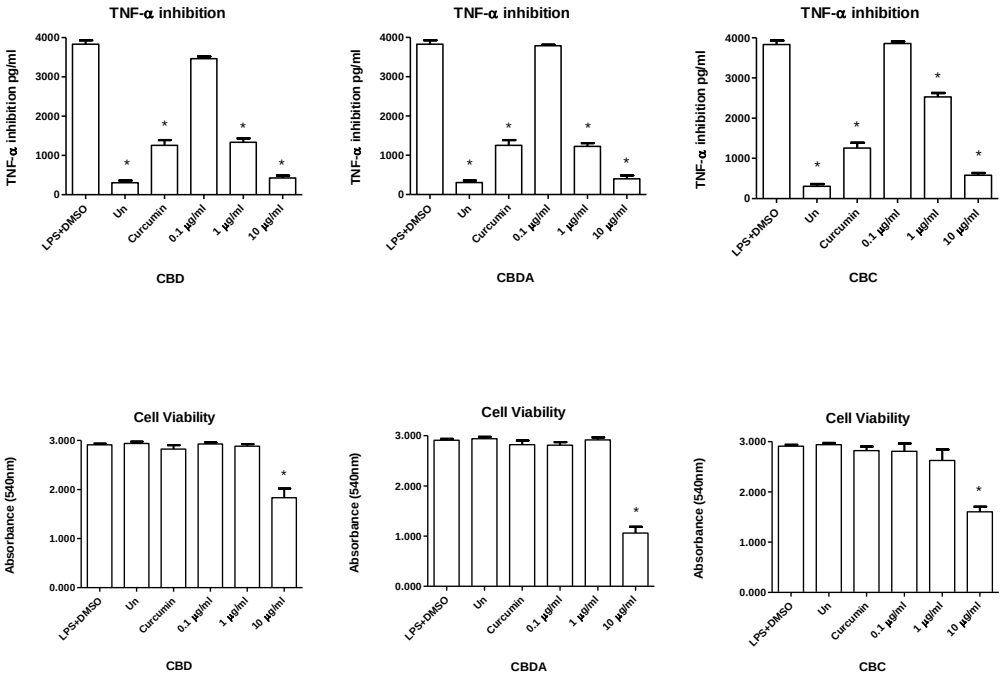


Figure 3: TNF-α inhibition and cell viability of LPS stimulated U937 cell lines treated with different cannabinoids. One way ANOVA was performed. Posthoc comparison between means and controls (LPS+DMSO) were made using Dunnett's test. Each error bar represents ±SEM of three replicates. * = the value shows a significant difference ($P \leq 0.05$).

There are several reports which support our results regarding the inhibition of TNF-α. Costa et al., (2007) reported that in synovial cells isolated from mice, CBD treatment inhibits the release of TNF-α. In addition, oral administration of CBD (2.5–20 mg/kg) reduces neuropathic and inflammatory pain in rats. In another study it has been shown that a low dose of CBD suppresses TNF-α production induced by lipopolysaccharide (LPS) in mice (Carrier et al., 2006). CBD has been shown to reduce joint inflammation in collagen-induced arthritis (CIA) in mice (Sumariwalla et al., 2004) and carrageenan paw edema in rats (Costa et al., 2004). Though CBD did not reduce inducible nitric oxide synthase (iNOS) in these studies, others (Esposito et al., 2006; Esposito et al., 2007) have reported that CBD does inhibit iNOS in a beta-amyloid induced murine model of neuroinflammation. CBD also reduces intestinal inflammation in mice (Capasso et al., 2008). In addition to its ability to suppress production of the inflammatory cytokine TNF-α, CBD appears to

exert antiinflammatory activity by suppressing fatty acid amidohydrolase (FAAH) activity, thereby increasing concentrations of the antiinflammatory endocannabinoid anandamide (Ben-Shabat et al., 2006).

The complex mechanisms whereby these compounds exert their effects is illustrated by the fact that hydrogenation at different double bonds has different effects on bioactivities, none of which appear dependent on CB1 activation. Further, insight into mechanisms whereby CBD exerts therapeutic effects is provided by experiments which indicate that CBD attenuates inflammation induced by high glucose in diabetic mice (Rajesh et al., 2007). Specifically, CBD treatment reduces mitochondrial superoxide, iNOS, nuclear factor kappa B (NF-κB) activation, and transendothelial migration of monocytes. Another potential therapeutic use of CBD may lie in its ability to counter some undesirable effects of THC (sedation, psychotropic effects, tachycardia), thus suggesting that if given together with THC, it may allow higher doses of THC (Russo and Guy, 2006). Several studies pointed out that cannabinoids could have CB1/CB2 receptor-independent mechanisms of action. CBD exhibits very low affinity towards CB1 and CB2 and thus shows immunosuppressive effects through non CB1 and non-CB2 mechanisms (Kaplan et al., 2003). There was reported that CBD inhibits production of pro-inflammatory cytokines by decreased activity of NF-κB (Kozela et al., 2010).

Cannabichromene (CBC) is, together with Δ^9-tetrahydrocannabinol, cannabidiol and cannabinol, the most abundant naturally occurring cannabinoid (Brown and Harvey, 1990; Holley et al., 1975). It is particularly abundant in freshly harvested dry-type cannabis material and it is the second most abundant cannabinoid in some strains of marijuana growing in the USA (Brown et al., 1990). It is reported that in USA during period 1993–2008, CBC represented 0.7 and 0.9% of the constituents from hashish or hash oil, respectively (Mehmedic et al., 2010). Despite the relative abundance of this compound in cannabis preparations, very little is known about its pharmacology. Cannabichromene was reported to have anti-inflammatory activity in the carageenan paw edema assay (DeLong et al., 2010; Wirth et al., 1980) and has analgesic effects (Davis and Hatoum, 1983). CBC inhibits prostaglandin synthesis *in-vitro*, but less potently than CBD or THC (Burstein et al., 1973). CBC exhibits strong antibacterial activity and mild antifungal activity, superior to THC and CBD in most instances (Eisohly et al., 1982). The mechanism by which CBC exerts its antiinflammatory effects is not known but it is confirmed

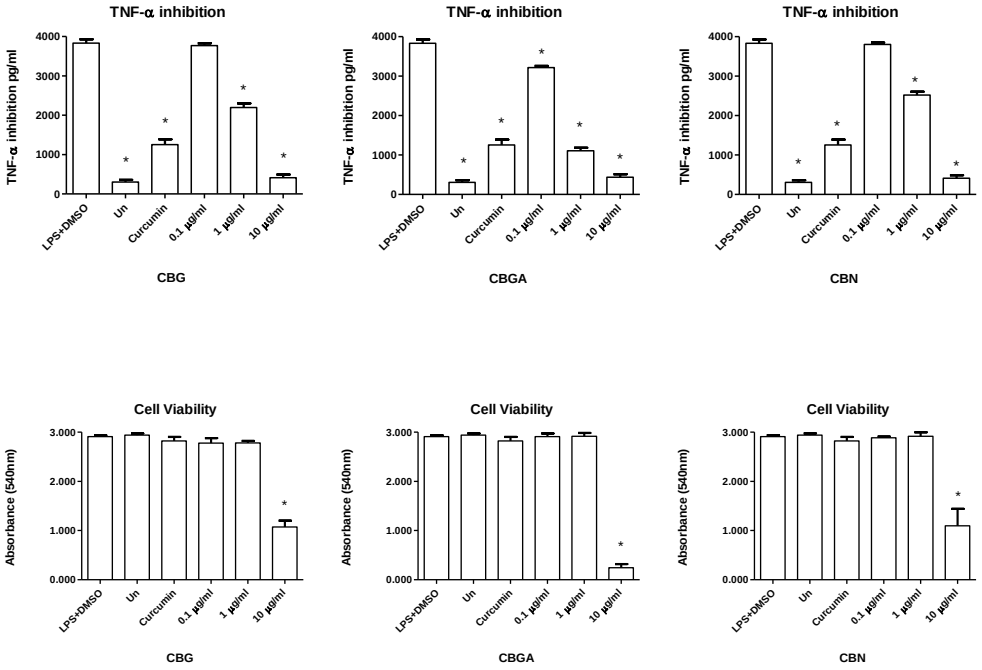


Figure 4: TNF-α inhibition and cell viability of LPS stimulated U937 cell lines treated with different cannabinoids. One way ANOVA was performed. Posthoc comparison between means and controls (LPS+DMSO) were made using Dunnett's test. Each error bar represents ±SEM of three replicates. * = the value shows a significant difference ($P \leq 0.05$).

that these effects are mediated through a non cannabinoid receptor mechanism of action (DeLong et al., 2010).

Cannabigerol (CBG; Fig. 4) is the biosynthetic precursor of CBC, CBD, and THC, and is present only in minor amounts. CBG has being shown less affinity towards CB1 receptors as compared to THC, approximately the same as CBD (Devane et al., 1988). CBG is also reported to inhibit the uptake of serotonin and norepinephrine in rat brains, less effectively than CBD and THC, but CBG inhibits GABA uptake more effectively than CBD and THC (Banerjee et al., 1975). CBG acts as an analgesic (more potently than THC), it inhibits erythema (much more than THC), and it blocks lipoxygenase, again more

effectively than THC (Evans, 1991). CBG has antibacterial (Appendino et al., 2008; Mechoulam and Gaoni, 1965) and antitumoural activities (Baek et al., 1998b).

Its activity against gram-positive bacteria, mycobacteria, and fungi is superior to that of THC, CBD, and CBC (Eisohly et al., 1982). CBG inhibits the growth of human oral epitheloid carcinoma cells (Baek et al., 1998a). CBG has been found to activate alpha (2)-adrenoceptors, to block 5-HT1A and CB1

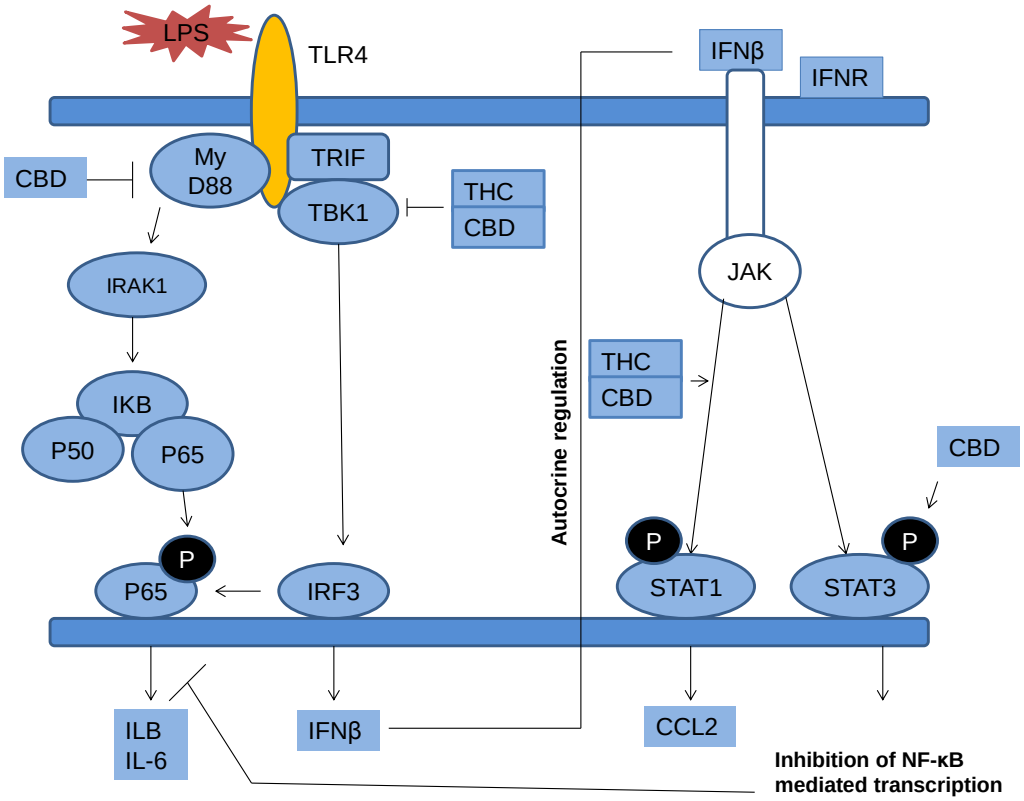


Figure 5: Possible antiinflammatory mechanism exhibited by THC & CBD. Pointed arrows represent activation while blunt arrows show suppression.

receptors and bind to CB2 receptors (Cascio et al., 2010) and may serve as a treatment for glaucoma (Colastani, 1990). It has been recently reported that CBG strongly inhibits the synthesis of IL-1β, IL-6, PGE2 and TNF-α in a dose-

dependent manner. PPARγ receptors have been shown to be involved in the modulation of inflammation, as PPARγ agonists downregulate the expression of several proinflammatory cytokines. Activation of PPARγ receptors might explain the TNF-α inhibitory action of CBG (Granja et al., 2012; Jiang et al., 1998).

Cannabinol (CBN; Fig. 4) is the degradation product of THC, and is found most often in aged cannabis products. CBN has been reported for its anti-convulsant and antiinflammatory activities (Evans, 1991; Turner et al., 1980). CBN shows greater affinity for CB2 receptors thus it may affect cells of the immune system more than the central nervous system. Furthermore, it is also reported that CBN modulates thymocytes by attenuating the activity of the c-AMP response element-binding protein (CREB), nuclear factor κB (NF-κB), and interleukin-2 (IL-2). CBN inhibits the expression of these proteins in splenocytes, via decreased activation of ERK MAP kinases (Faubert and Kaminski, 2000).

4.4. Conclusion

Cannabis sativa has been used throughout the history not only for its fiber, but also as a medicinal plant. Here, we have demonstrated that acidic forms of different cannabinoids are more active and strongly inhibit the release of TNF-α. Maximum activity was found in Δ^9-THCA followed by CBDA and CBGA. These acidic forms also showed strong toxicity towards U937 cells at highest concentration of 10μg/ml. Our studies support earlier findings that cannabinoids are potent antiinflammatory agents and they exert their effects through suppression of cytokine production.

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Chapter 5

Screening of fruit berries against TNF-α production: NMR spectroscopy and multivariate data analysis-based study

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Abstract

Nuclear magnetic resonance (NMR) spectroscopy and multivariate data analysis methods were applied to the metabolite profiling of different types of berries. The water, methanol-water (1:1), and methanol fractions from solid phase extraction (C18) were subjected to *in-vitro* TNF-α activity assay. All the SPE fractions were clearly separated on a score plot of principal component analysis (PCA). In order to find correlations between metabolites and activities, partial least squares-discriminant analysis (PLS-DA) and partial least squares-discriminant analysis (OPLS-DA) were used. Signals related to the TNF-α inhibition observed in the SPE fractions of berries were identified as a wide range of phenolics. By calculating variable importance in the projection (VIP), the active ingredients in the high activity samples have been identified as gallic acid, caftaric acid, quercetin, myricetin, and (+)-catechin. The present study shows the usefulness of NMR spectroscopy in combination with chemometrics to identify the possible bioactive metabolites in the crude extracts.

Keywords: Berries, chemometrics, NMR spectroscopy, TNF-α inhibition, phenolic identification

5.1. Introduction

Inflammation plays a crucial role in diseases like asthma, atherosclerosis, and rheumatoid arthritis. The imbalance between pro-oxidants and antioxidants in an organism lead to a condition known as oxidative stress which can be a trigger in the autoregulation of cytokines in the inflammatory diseases. Pro-inflammatory cytokines including interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) are known to be released during inflammation and. Tumor necrosis factor-α is a regulatory pro-inflammatory cytokine produced mainly by macrophages, but various other cells like T-cell, neutrophils, NK cells, and synovial cells are also known to release TNF-α (Vilcek and Lee, 1991). TNF-α is secreted during the early phase of inflammatory diseases and responsible to initiate the secretion of other cytokines like IL-1, IL-6, and IL-8 (Cho et al., 2001; Cho et al., 1998). Low production of TNF-α is advantageous for the host but overproduction of TNF-α during infection plays a pivotal role in the development of several diseases (Björnsdottir and Cypcar, 1999; Medana et al., 1997; Murphy et al., 1998). The suppression of TNF-α or anti TNF-α therapy could be beneficial for the treatment of these acute and chronic diseases.

It is reported that a negative correlation exists between the consumption of diet rich in fruits, and vegetables and the risks for chronic angiogenic diseases, such as cardiovascular diseases, arthritis, chronic inflammation and cancers (Chen et al., 2006; Prior, 2003; Saleem et al., 2002; Zhang et al., 2005). Fruits and vegetables, especially some deep-coloured varieties, are good sources of phenolics (Cieślik et al., 2006), including flavonoids (Qian et al., 2004) and anthocyanins, as well as carotenoids (Sass-Kiss et al., 2005). Among fruits, berries such as bilberries, blackberries, blueberries, cranberries, elderberries, raspberries and strawberries contain diverse anthocyanins and exert differential inhibition effects on COXs activity (Seeram et al., 2001). Among these berries, strawberries contain abundant amounts of phenolic compounds (Häkkinen and Törrönen, 2000) and have demonstrated anticancer activity in several diferent experimental systems (Hannum, 2004). Phenolic compounds have been reported to have a strong antioxidant activity (Johnson, 2005) and exhibit a wide range of biological and pharmacological activities both *in-vitro* and *in-vivo*, such as the inhibition of cyclooxygenase (COX), induction of CD95 signalling dependent apoptosis, effects on cell division cycle and the modulation of NF-Kb activation (Falchetti et al., 2001). Therefore, a diet rich in fruits and vegetables is suggested to have immuno-modulatory effects such as antiinflammation (Devereux and Seaton, 2005; Sanchez-Moreno et al., 2006).

Considering the great chemical diversity, for finding active compounds in plants it is unlikely that a single analytical method could provide information about all the metabolites, and at the same time be unbiased, rapid, reproducible, and stable over time, while requiring only simple sample preparation. Metabolomics is an approach aimed for the better understanding of primary and secondary metabolism and can be defined as a metabolic snapshot of a living system (Andrew Clayton et al., 2006). Other than the use of NMR spectroscopy in structure elucidation of novel compounds, NMR based metabolomics is now a popular tool in the area of metabolome analysis (Son et al., 2009). Often criticized for its low sensitivity as compared to other platforms for metabolomics analysis, the most promising features of NMR are its non-destructive nature, simple sample preparation, and spectra are obtained in relative short time.

The development of methods and algorithms for the multivariate statistical modeling have contributed much to metabolomics as they opened the way for handling the huge datasets of large-scale metabolic analyses (Crockford et al., 2005). In combination with different multivariate data analyses methods, NMR has been widely used for metabolic profiling of various samples (Brescia et al., 2002; Charlton et al., 2002). Several studies showed the analysis of the extracts (Bailey et al., 2004; Cardoso-Taketa et al., 2008; Cho et al., 2009; Roos et al., 2004) for the prediction of different pharmacological activities using NMR spectroscopy in combination of chemometrics methods.

The present study is aimed to measure the *in-vitro* anti-TNF-α activity of different berry types. Several phenolics were also identified using 1D and 2D NMR techniques. The correlation of activity data and NMR data using different multivariate data analyses methods in order to identify the active ingredients is also presented.

5.2. Materials and Methods

5.2.1. Sampling

All fruit berries [Cranberry, blueberry, redberry, strawberry, raspberry, blackberry, grapeberry (green), grapeberry (red), and grapeberry (black)] were purchased from local market in The Netherlands. The berries were milled using mortar and pestle and susequently dried in a freeze drier.

5.2.2. Solid phase extraction (SPE)

A sample of 1 g dry weight powder of each berry was extracted with MeOH-H_2O (8:2). Subsequently the extract was dried using a vacuum evaporator at room temperature and redissolved in 1 mL of deionized water before application to solid phase extraction (SPE) of a SPE-C18 cartridge (Waters, Milford, MA, USA). Prior to its use, the SPE cartridge was preconditioned by elution with 10 mL of methanol followed by 10 mL of water. The extract was eluted successively with 5 mL of water, 5 mL of methanol-water (1:1) and finally with 5 mL of methanol. All three fractions were collected separately in a round-bottom flask, evaporated under vacuum and used for further NMR analysis. All the solvents were purchased from Biosolve B.V. (Valkenswaard, The Netherlands).

5.2.3. Preparation of berry's extracts for bioassay

One gram DW of (each) berry powder was extracted with 30 mL of methanol-water (8:2). The crude extract was subsequently dried using a rotary evaporator at room temperature, weighed, redissolved in DMSO and diluted several times to achieve a concentration of 100 µg/mL. The dried extracts achieved from SPE experiment were also redissolved and diluted in DMSO to provide the same concentration (100µg/ml).

5.2.4. Growth of cells and treatment with berry's extract

Human monocyte-like histiocytic lymphoma U937 cells were cultured as dscribed in chapter 3.

5.2.5. ELISA for TNF-α

TNF-α in culture supernatants was determined by quantitative 'sandwich' enzyme-linked immunosorbent assay as described in chapter 3.

5.2.6. Cell viability assay

Cell viability was determined by MTT assay as described in chapter 3.

5.2.7. ^{1}H NMR Spectroscopy

The dried extracts of the three fractions eluted from SPE were redissolved in 1 mL of methanol-d_4. An aliquot of 800 µL of sample was transferred to the 5-mm NMR tube and used for the NMR analysis. The deuterated methanol was purchased from Cambridge Isotope Laboratories, Inc., Andover, MA, USA. ^{1}H NMR spectra were recorded at 25 °C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz. Methanol-d_4 was used as the internal lock. Each ^{1}H NMR spectrum consisted of 128 scans requiring 10 min and 26 sec acquisition time with the following parameters: 0.16 Hz/point, pulse width (PW) = 30° (11.3 µsec), and relaxation delay (RD) = 1.5 sec. A pre-saturation sequence was used to suppress the residual H_2O signal with low power selective irradiation at the H_2O frequency during the recycle delay. FIDs were Fourier transformed with LB = 0.3 Hz. The resulting spectra were manually phased and baseline corrected, and calibrated to MeOH- d_4 at 3.3 ppm, using XWIN NMR (version 3.5, Bruker). 2D NMR techniques were performed on a 600 MHz Bruker DMX-600 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 600.13 MHz. *J*-resolved NMR spectra were acquired using 8 scans per 128 increments for F1 and 8 k for F2 using spectral widths of 5000 Hz in F2 (chemical shift axis) and 66 Hz in F1 (spin–spin coupling constant axis). A 1.5 s relaxation delay was employed, giving a total acquisition time of 56 min. Datasets were zero-filled to 512 points in F1 and both dimensions were multiplied by sine-bell functions (SSB = 0) prior to double complex FT. *J*-resolved spectra tilted by 45°, were symmetrized about F1, and then calibrated, using XWIN NMR (version 3.5, Bruker). ^{1}H–^{1}H correlated spectroscopy (COSY) and heteronuclear multiple bonds coherence (HMBC) spectra were recorded on a 600 MHz Bruker DMX-600 spectrometer (Bruker). The COSY spectra were acquired with 1.0 s relaxation delay, 6361 Hz spectral width in both dimensions. Window function for COSY spectra was sine-bell (SSB = 0). The HSQC spectra were obtained with 1.0 sec relaxation delay, 6361 Hz spectral width in F2 and 27,164 Hz in F1. Qsine (SSB = 2.0) was used for the window function of the HSQC. The HMBC spectra were recorded with the same parameters as the HSQC spectra except for 30,183 Hz of spectral width in F2. The optimized coupling constants for HSQC and HMBC were 145 Hz and 8 Hz, respectively.

5.2.8. Data analysis

The ^{1}H NMR spectra (from all SPE fractions) were automatically reduced to ASCII files. Spectral intensities were scaled to methanol signal (δ

3.30) and reduced to integrated regions of equal width (δ 0.04) corresponding to the region of δ 0.0–10.0. The regions of δ 4.85–4.95 and δ 3.28–3.4 were excluded from the analysis because of the residual signal of D_2O and CD_3OD, respectively. Bucketing was performed by AMIX software (Bruker) with scaling on total intensity. The SIMCA-P software (v. 12.0, Umetrics, Umeå, Sweden) was used for application of principal component analysis (PCA) with scaling based on Pareto, and also projection to latent structures (PLS) such as PLS-discriminant analysis (PLS-DA), bidirectional orthogonal PLS (O2PLS), and O2PLS-discriminant analysis (O2PLS-DA) with scaling based on Unit Variance. The TNF-α content was arbitrarily set as 100 in the negative control (LPS+DMSO) and all the other values are normalized to this (% activity) as shown in results. Means and standard deviations were calculated and means comparisons were made with one way ANOVA followed by posthoc Tucky's test at a significance level <0.01.

5.3. Results and Discussions

The ^{1}H NMR spectra of three SPE fractions resulted from grape berry extract are shown in Fig. 1.

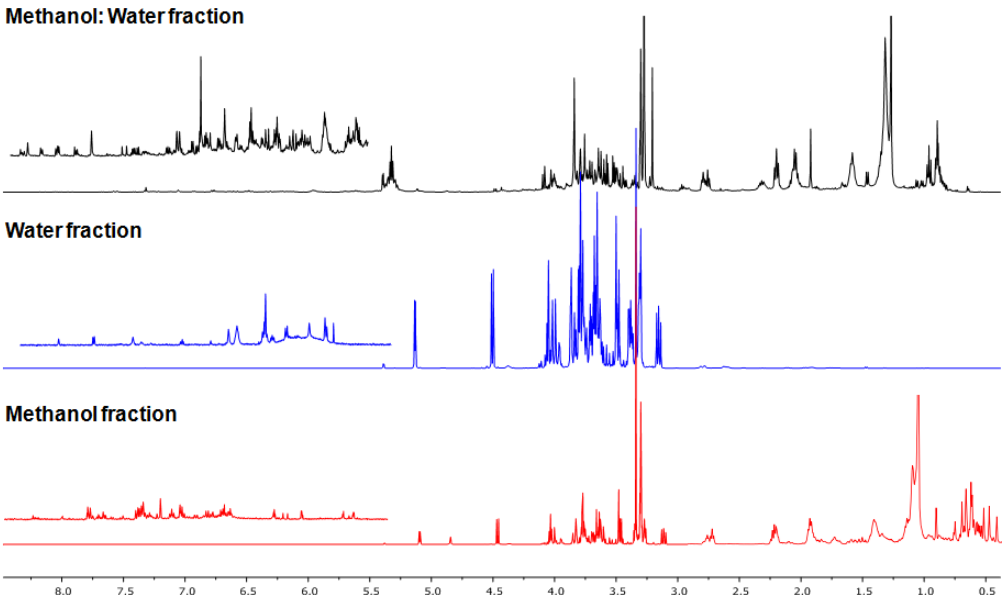

Fig. 1: Comparison of ^{1}H NMR spectra of SPE fractions of black grapes extract.

All the three SPE fractions are quite different from each other with respect to the contained metabolites. Sugars and amino acids are more concentrated in water fraction while the high signals intensity of amino acids and fatty acids are observed higher in the methanol fraction with some resonances in the phenolics region. The 50% methanol fraction shows maximum amount of phenolics with higher signals related to amino acids and fatty acids.

In NMR-based metabolomics studies, signals congestion is the biggest obstacle in metabolite identification. Several 2D NMR techniques, like *J*-resolved, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HMBC, and ^{1}H-^{13}C HSQC, provide additional information which facilitates the identification of metabolites. *J*-resolved and ^{1}H-^{1}H COSY are proved effective and widely used in metabolomics due to short measuring time with good quantitative features (Viant et al. 2003; Liang et al. 2006). Recently the potential of ^{1}H-^{13}C-related NMR techniques, like ^{1}H-^{13}C HMBC and ^{1}H-^{13}C HSQC for application in metabolomics has also been discussed (Hyberts et al., 2007; Lewis et al., 2007).

In this study, several flavonoids and phenolic acids are identified using our in-house library of NMR data of common metabolites. Flavonols like quercetin, kaempferol, and myricetin were identified in the aromatic region along with (+)-catechin of the flavan-3-ols group. The signals at δ 6.46 and δ 6.77 correlated with each other in the COSY spectrum with a coupling constant of 2.0 Hz were assigned to be H-6 and H-8 of kaempferol. The correlation between the signals at δ 7.00 (H-2′, H-6′, d, *J*=8.8 Hz) and δ 8.07 (H-3′, H-5′, d, *J*=8.8 Hz) led to the elucidation of the B-ring protons of kaempferol. The flavonoids quercetin and myricetin were also identified in the aromatic region. The quercetin signal at *δ* 6.49 of H-8 was correlated in the ^{1}H-^{1}H COSY spectrum with the signal at *δ* 6.27 of H-6 and a signal at *δ* 6.95 of H-5′ with one at *δ* 7.56 of H-6′. Similar correlations for the signals of myricetin at δ 6.51 of H-8 with δ 6.29 of H-6 also showed ^{1}H-^{1}H COSY correlations.

The aromatic part of the ^{1}H NMR spectra shows some signals of *p*-hydroxybenzoic acid and gallic acid. The singlet at δ 7.03 was assign to gallic acid while resonances at δ 7.94 and δ 6.83 were assign to *p*-hydroxybenzoic acid which are also found correlated in ^{1}H-^{1}H COSY spectrum. Resonances of H-8′ and H-7′ (olefinic protons) of *trans*-hydroxycinnamic acids are clearly observed as doublets of 16.0 Hz in the range of δ 6.39-6.50 and δ 7.59-7.70, respectively, in *J*-resolved spectrum. These protons are also found correlated in the ^{1}H-^{1}H COSY spectra, with the coupling with carbonyl carbon at δ 168.3 in the HMBC spectra. These signals are assigned to cinnamic acid derivatives

including caffeic acid, *p*-coumaric acid, and ferulic acid. In the ^{1}H NMR spectra of grape berry samples, these resonances were assigned to three different hydroxycinnamic acids moieties which include *trans*-caffeoyl, *trans*-coumaroyl, and *trans*-feruloyl derivatives. The ^{1}H-^{1}H COSY spectra showed correlations among signals like δ 6.41 with δ 7.62; and δ 7.02 with δ 6.88 of caffeoyl; δ 7.51 with δ 6.87; and δ 6.45 with δ 7.65 of coumaroyl; δ 6.46 with δ 7.56 of feruloyl derivative.

These hydroxycinnamic acids were also found to be conjugated with tartaric acid via an ester linkage. The signal for tartaric acid was observed in the region of δ 5.32-5.44 in ^{1}H NMR spectrum, being shifted downfield from the typical tartaric acid signal at δ 4.30 due to the bonding to the carboxylic function of cinnamic acids which was confirmed by their correlation with the signal at the region of δ 167.5-168.5 in the HMBC spectra. Based on these assignments, these compounds were identified as *trans*-caftaric acid (caffeic acid conjugated with tartaric acid), *trans*-fertaric acid (ferulic acid conjugated with tartaric acid), and *trans*-coutaric acid (coumaric acid conjugated with tartaric acid).

Along with the *trans*- forms, the *cis*- forms of these hydroxycinnamic acids, i.e. *cis*-caffeic and *cis*-*p*-coumaric acid, were also detected. When compared to their *trans*-configuration, the *cis*-forms showed an upfield shift of the signals for H-8′ and H-7′ along with the reduction in the coupling constant from 16.0 Hz to 13.0 Hz. Two clear doublets of 13.0 Hz at δ 5.92 and δ 5.94 were detected for the H-8′ in the *cis*-configuration. The ^{1}H-^{1}H COSY spectra also confirmed this by showing the correlation of these signals with the respective H-7′ protons at δ 6.81 and δ 6.86. It was also confirmed by the correlation of this signal with the carbonyl resonance at δ 167.2 in the HMBC spectra. All the phenolics are identified by comparing the spectra of the reference compounds analyzed under same condition from our in-house library and our previous reports (Abdel-Farid et al., 2007; Liang et al., 2006).

In this study all three fractions of SPE from different berries extracts were tested for their potential inhibition against TNF-α production (Fig. 2). The 100% water and 100% methanol fractions provided the least anti-TNF-α activity with no significance difference among different berry types. The methanol-water fraction showed maximum TNF-α inhibition, with cranberry showing significantly higher activity than all of the other berry types except black grape berry. Among the grape berries, it is interesting to note that black and white grapes provided significantly higher activities than red grapes.

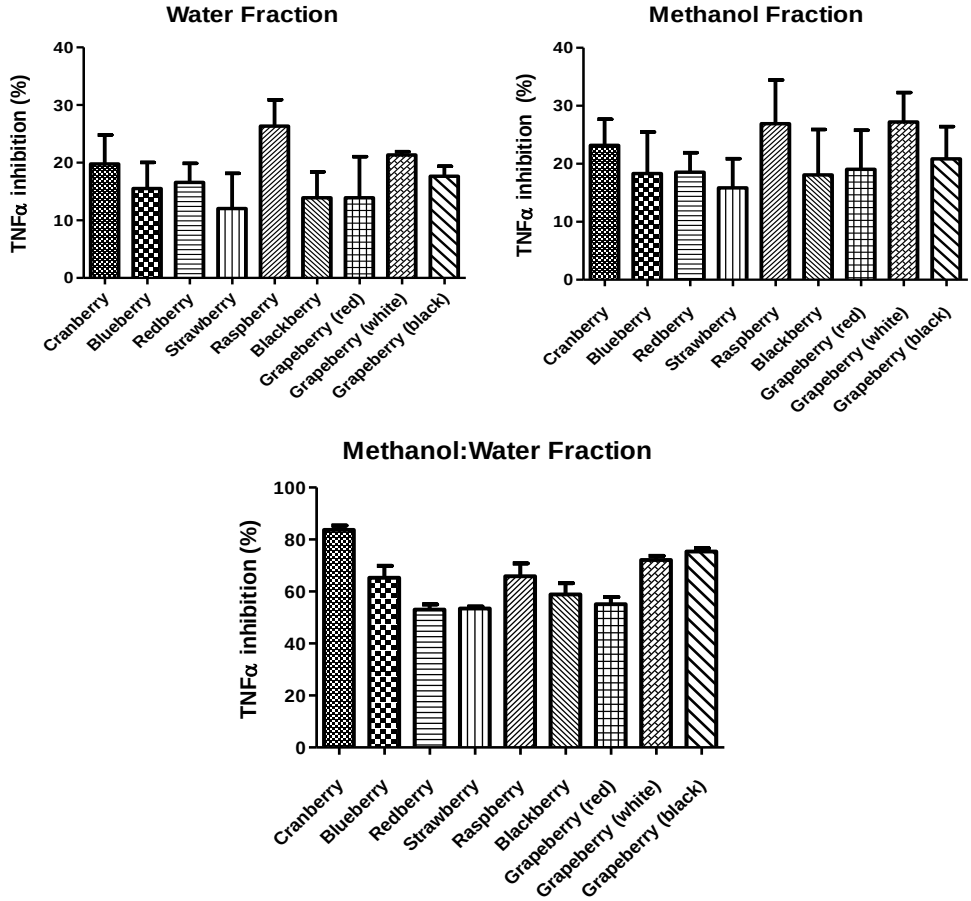

Fig. 2: TNF-α inhibition ratio (%) exhibit by different berries. Bars represent standard error of Mean (*n*=3).

Among the other berry type, raspberry and blueberry showed significantly higher activities than the redberry and strawberry.

Multivariate data analysis algorithms are an essential component of any metabolomics study. These methods are used to reduce the dimensionality of a multivariate dataset and thus enable to recognize possible differences or similarities among the samples. Principal component analysis (PCA) is considered as a primary tool in metabolomics and helps to better understand possible differences between samples. It is an unsupervised method; hence the separation of samples is purely due to differences among the samples. The NMR data from the SPE fractions of all samples were subjected to PCA in

order to highlight the differences existed among the SPE fractions and to identify the metabolites responsible for that distinction. The PCA score plot shows good separation among the SPE fractions (Fig. 3).

The methanol fractions are totally separated from the other two fractions by PC1 (56.6%) while the water and methanol-water fractions are separated by PC2 (8.8%). By examining the loadings plot and the respective NMR spectra, it is clear that all the three fractions are quite different in their

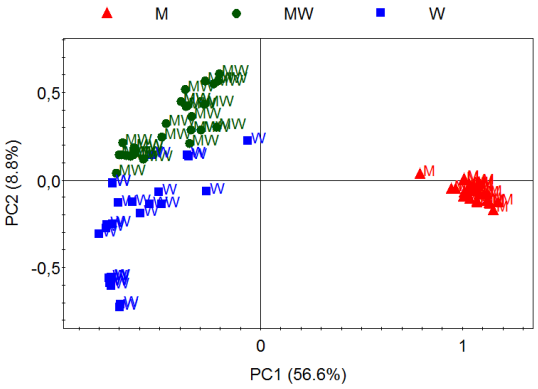

Fig. 3: Principal component analysis score plot of SPE fractions of different kind of berries. Red represents methanol, green represents methanol:water (1:1), and blue represents water fractions.

metabolic contents. The methanol fraction was also found higher in fatty acids with very small quantities of phenolics and amino acids. The water fraction was relatively higher in sugars and some amino acids and organic acids. Most of the phenolics were found to be eluted in the methanol:water fraction. In order to identify the metabolites responsible for anti-TNF-α activity, a supervised method, i.e. partial least squares-discriminant analysis (PLS-DA), was used. For PLS-DA, samples were classified in high and low active classes by creating dummy *Y*-variables. The score plot shows nice separation among the high and low activity samples (Fig. 4A). To achieve better clustering, bidirectional orthogonal-PLS-DA (O2PLS-DA) was used which was resulted in a clear distinction among the samples from different classes (Fig. 4C). The corresponding loadings plot indicated the accumulation of phenolics like quercetin, caffeic acid, gallic acid, and (+)-catechin in the samples with high activity.

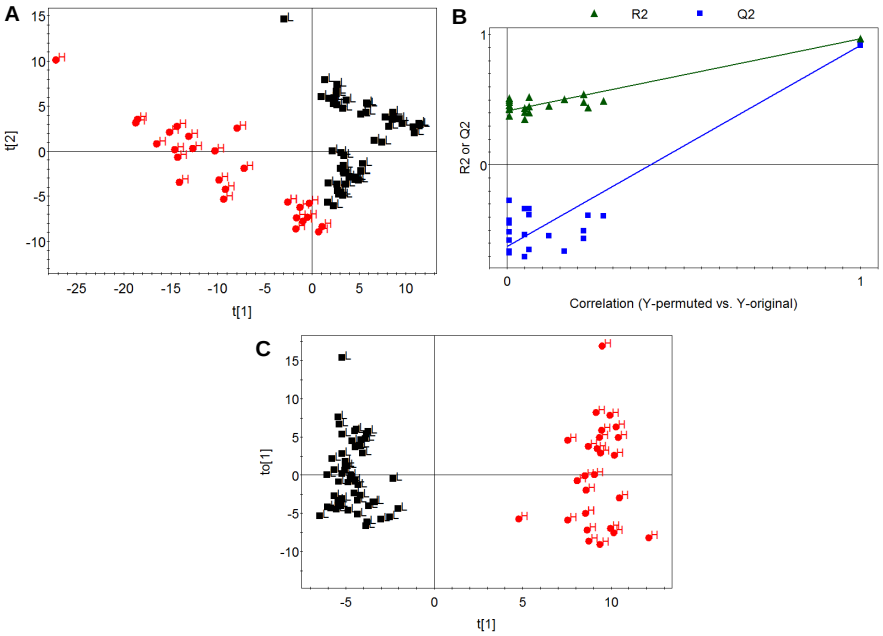

Fig. 4: The PLS-DA score plot (A), PLS-DA permutation test (B), and O2PLS-DA score plot (C) of anti-TNF-α activity of SPE fractions of different kind of berries. Black squares represent samples with low activity, whilst red circles represent samples with high activity.

Projection to latent structures (PLS) is another supervised method in which instead of creating dummy *Y*-variables, the actual data from anti-TNF-α assay can be used as a *Y*-data set. The PLS analysis was found effective in separating the high and low activity samples similar to PLS-DA method (Fig. 5A). The application of bidirectional orthogonal-PLS (O2PLS) resulted in much better distinction of the samples with different activities than PLS model (Fig. 5C). By examining the loadings plot, the findings from the O2PLS-DA model were endorsed as similar phenolics were found responsible for higher activity in the samples.

One of the key aspects of a supervised regression algorithm is model validation. A permutation test is often used for validation of methods like PLS and PLS-DA. A permutation test is the calculation of goodness of fit and the predictive ability of the model, R2 and Q2, respectively. The R2 and Q2 values of PLS and PLS-DA were calculated using four and six components for PLS and PLS-DA, respectively. For anti-TNF-α activity the R2 and Q2 values for

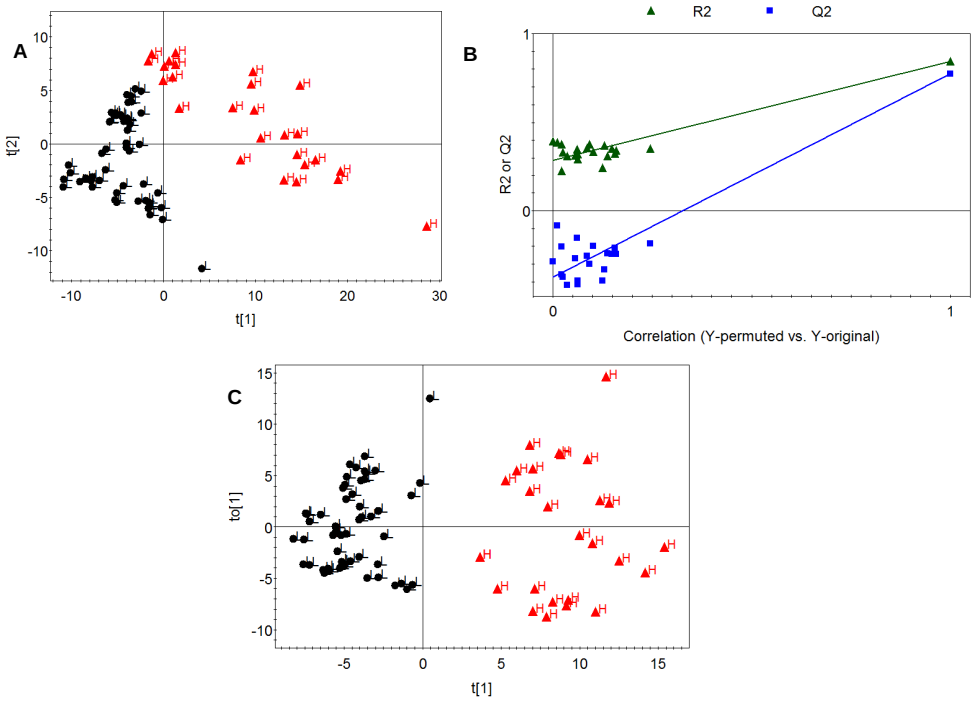

Fig. 5: The PLS score plot (A), PLS permutation test (B), and score plot of O2PLS (C) of anti-TNF-α activity of SPE fractions of different kind of berries. Black circles represent samples with low activity, red triangles represent samples with high activity.

PLS analysis were 0.84 and 0.77, respectively, while for PLS-DA these figures were 0.96 and 0.91. These PLS and PLS-DA models were validated by the permutation method through 20 applications in which all Q2 values of permuted Y vectors were lower than original ones and the regression of Q2 lines intersect at below zero (Fig. 4B and 5B).

It has been indicated that VIP (variable importance in the projection) score is directly proportional with the influence of a factor on the separation on score plot, meaning, factors have higher VIP values are more important in the separation of samples. For O2PLS-DA analysis, VIP values for identified phenolic compounds are calculated. It has been reported that factors with VIP values more than 0.7 could be regarded influential for the separation of samples (Eriksson et al., 2006). The VIP values of the major contributing metabolites are

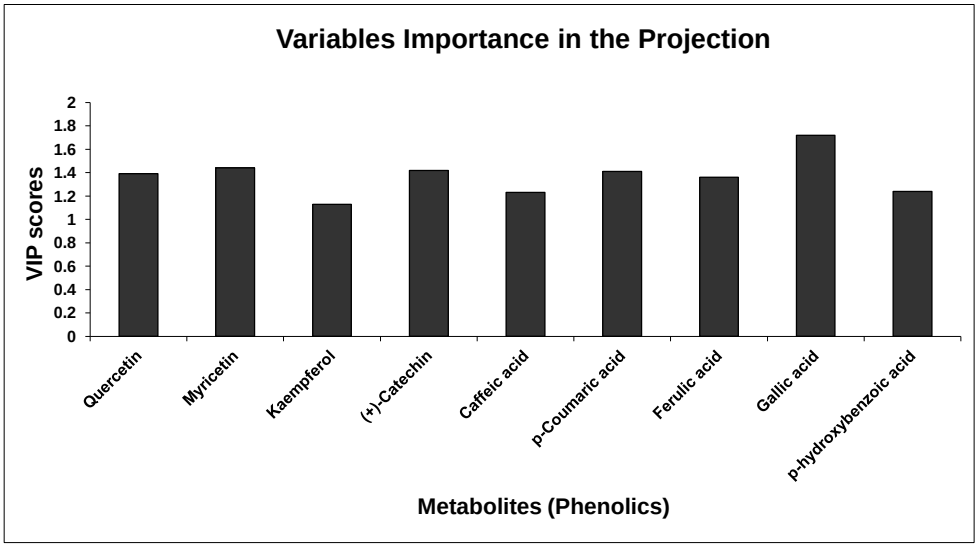

Fig. 6: The VIP scores of different phenolics identified in berries.

shown in Fig. 6. Among the phenolics, gallic acids was found with the highest VIP score followed by myricetin and (+)-catechin. The *p*-coumaric acid score is the highest among the cinnamic acids. Kaempferol shows the least VIP score among all the identified phenolics. These VIP scores for the identified phenolics authenticate their involvement in the separation of high activity samples and suggest a role of these compounds in inhibiting TNF-α production.

Chemometrics methods in combination with NMR spectroscopy are gaining popularity among the researchers. In this study, various multivariate data analysis methods were used in combination with NMR spectroscopy in order to correlate the activity data with the spectroscopy data of the same extracts. Such analyses of extracts from *Hypericum perforatum*, *Artemisia annua*, *Citrus grandis*, and *Galphimia glauca*, were successful in linking pharmacological activities with certain compounds (Bailey et al., 2004; Cardoso-Taketa et al., 2008; Cho et al., 2009; Roos et al., 2004). This approach is very effective in the screening of plant extracts in order to identify active compounds without laborious fractionation and chromatographic separation of the crude extract. Fractions from SPE of various berries were analyzed for anti-TNF-α activity and the combination of NMR spectroscopy and chemometrics was successfully applied to identify metabolites like quercetin, myricetin, gallic

acid, caffeic acid, and ferulic acid, which are responsible for the high anti-TNF-α activity.

Phenolics is a class of metabolites which are well known for their anti-oxidant and antiinflammatory activities, both *in-vivo* and *in-vitro* (Miles et al., 2005; Pietta, 2000). Many reports have been published with regard to activities of flavonoids like quercetin, myricetin, and kaempferol against TNF-α production (Chuang et al., 2010; Park et al., 2008). Similarly, phenolics acids including caffeic, ferulic, and *p*-coumaric acid are well known in inhibiting TNF-α production (Sakai et al., 1997; Shin et al., 2004). Benzoates like gallic acid and *p*-hydroxybenzoic acid are also known for their free radical scavenging potentials and antiinflammatory activities (Giftson et al., 2010; Kroes et al., 1992; Yeh et al., 2004). In the present study, all these metabolites were found to be correlated with the TNF-α inhibition in LPS stimulated cell line. Gallic acid was found to have the highest correlation whereas the kaempferol is the least correlated among the identified phenolics.

5.4. Conclusion

In conclusion, combination of chemometric methods and nuclear magnetic resonance spectroscopy (1D and 2D) has been applied for the phenotyping of different type of berries. In order to separate phenolics from the other metabolites, solid phase extraction was used which resulted in water, methanol:water (1:1), and methanol fractions and have been tested for TNF-α inhibition. The assay showed that the methanol:water fraction from SPE strongly inhibit TNF-α production. Various multivariate data analysis methods showed good correlation between the NMR resonances for phenolics and anti-TNF-α activity. Algorithms like PLS and PLS-DA showed good separation among the samples classified as high and low activity with high model validity. The application of bidirectional orthogonality, i.e. O2PLS-DA and O2PLS, showed even better distinction among the classes. The VIP plot showed that NMR signals related to metabolites like quercetin, myricetin, (+)-catechin, caftaric acid, coutaric acid, and gallic acid, were statistically significantly correlated with high activity. Using the presented approach, the analysis of NMR shifts in relation to pharmacological activity can provide information about what part of the NMR spectrum (aromatic or aliphatic regions) correlates with the activity which gives information about the active ingredients in crude extracts of medicinal plants.

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Chapter 6

Identification of active metabolites in grape cultivars with different ripening stage using NMR and multivariate data analysis

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Abstract

Nuclear magnetic resonance combined with multivariate data analysis were applied to the evaluation of TNF-α inhibition from three grapes, 'Trincadeira', 'Touriga Nacional', and 'Aragoneˆs', at four different developmental stages. The initial stages of grape development, green and veraison, were found more active against TNF-α production as compared to the later ripe and harvest stages. Among the cultivars, 'Touriga Nacional' was found to be the most potent inhibitor. Different multivariate data analyses algorithms based on projections to latent structures were applied to correlate the NMR and TNF-α inhibition data. The variable importance in the projections plot showed that phenolics like quercetin, myricetin, (+) - catechin, (-)-epicatechin, caftarate, and coutarate, were positively correlated with high activity. This work demonstrates the great potential of NMR spectroscopy in combination with chemometrics for the screening of large set of crude extracts, to study the effects of different variables on the activity, and identifying sets of active compounds in complex mixtures like plant extracts.

Keywords: Grapes, developmental stages, NMR spectroscopy, multivariate data analysis, anti-TNF-α activity, PLS modeling

6.1. Introduction

Inflammation is a key characteristic of many diseases related to autoimmunity. It is a complex process and regulated by various pro-inflammatory and antiinflammatory cytokines (Choy and Panayi, 2001). TNF-α is one of the most important pro-inflammatory cytokines which promotes inflammation. It was discovered in 1975 as having an anti-tumor activity, but is now recognized as a host defense factor in immunological and inflammatory responses (Tracey and Cerami, 1994). TNF-α is known to be secreted during early stages of acute and chronic inflammatory diseases such as rheumatoid arthritis, asthma, septic shock and other allergic diseases (Cho et al., 2001; Herath et al., 2003). Low production of TNF-α is advantageous for the host but overproduction of TNF-α during infection plays a pivotal role in the development of diseases like disseminated intravascular coagulation, death in septic shock, cerebral malaria, along with a wide range of other inflammatory diseases including asthma, dermatitis, multiple sclerosis, inflammatory bowel disease, cystic fibrosis, rheumatoid arthritis, and immunological disorders (Björnsdottir and Cypcar, 1999; Cohen et al., 1996; Medana et al., 1997; Murphy et al., 1998; Sekut and Connolly, 1996). Therefore it is evident that the suppression of TNF-α or anti TNF-α therapy could be beneficial for the treatment of these acute and chronic diseases.

Recently, several studies have been published regarding natural compounds inhibiting TNF-α release (Paul et al., 2006; Yuliana et al., 2011). Chemical phenotyping has become the focal point in recent years, as the analysis of the low molecular weight compounds reflect the physiological activities of an organism or tissue under certain conditions. The observable chemical profile or fingerprint, referred as 'metabolome', is highly complex consisting of a variety of compounds of very different nature. Considering the great chemical diversity it is unlikely that a single analytical method could provide information about all the metabolites, and at the same time be unbiased, rapid, reproducible, and stable over time, while requiring only simple sample preparation.

An accurate snap shot of the metabolome is highly important in metabolomics which requires a reliable metabolite extraction (Colquhoun, 2007; Kopka et al., 2004). Many platforms are now available for the high throughput analysis of metabolites, varying in their sensitivity (Beckonert et al., 2007). In case of a pure organic compound, two of the most widely used parameters for solvent selection are total solubility and constituent partial solubility, but in metabolomics extraction is a totally different state of affairs.

Based on sample chemistry and aim of the research, many extraction protocols for metabolomics studies have been published, offering different advantages but also having some limitations (De Vos et al., 2007; Kim et al., 2010; Kruger et al., 2008; Lisec et al., 2006). In grapes, the high sugar concentration may hamper metabolite identification especially if they are in low quantities, like secondary metabolites. Solid Phase Extraction (SPE) has been an effective sample handling technique with advantages like high recovery, high pre-concentration factors, low organic solvent consumption, simplicity, and ease of operation (Zhao et al., 2007) and has been successfully used in many studies (Cai et al., 2003; Fraccaroli et al., 2008; Zou et al., 2007).

NMR has a unique place not only in structure elucidation and characterization of molecules, but is now also considered as a major tool in metabolomics studies. Though criticized because of its low sensitivity, NMR is known for advantages like non-destructive nature, easy sample preparation, and a relatively short analysis time. These striking features of NMR, its non-selectivity and the use of NMR data directly for quantification, makes NMR an optimum choice for a broad range of metabolite analyses and quantification (Dixon et al., 2006; Son et al., 2009). NMR is now widely used in combination with different multivariate data analyses methods to do metabolic profiling of various samples (Brescia et al., 2002; Charlton et al., 2002). Characterization of different plant species (Hye Kyong Kim et al., 2005), and cultivars (Ali et al., 2009), monitoring grape berry growth (Ali et al., 2011), and the effects of growing regions, vintage, soil, and microclimate have been reported using NMR based metabolomics (Pereira et al., 2005; Pereira et al., 2006b). Many reports have been published on correlating the NMR and bioactivity data using various multivariate data analysis methods (Bailey et al., 2004; Cardoso-Taketa et al., 2008; Cho et al., 2009; Roos et al., 2004).

Food items like spices, herbs and fruits are well known for their anti-inflammatory properties (Mueller et al., 2010). Among the fruits, the use of grapes for multiple purposes like juice, fresh and dried fruit, and most importantly in wine production, make them one of the most economically important and widely cultivated fruit crops across the world. In addition to their economic importance, an increasing number of medicinal advantages have been attributed to grapes. Grapes phytochemistry is known to have relatively high concentrations of phenolics which in turn resulted in many health effecting properties, for instance, cardioprotective, anti-oxidant, antiinflammatory, and anti-cancer activities (Ali et al., 2010). Studies using human (Zern et al., 2005), and animal (Cui et al., 2002; Fuhrman et al., 2005; Seymour et al., 2008) models have shown that due to abundance of polyphenols possessing anti-

oxidative and antiinflammatory properties, dried grape powder has cardioprotective effects.

The present study was designed to measure TNF-α inhibition of Portuguese grape varieties at different development stages of berries in combination with SPE. Two different vintages of the 'Trincadeira' cultivar are also compared. Several primary and secondary metabolites (especially, phenolics) using 1D and 2D NMR techniques are identified. The correlation of activity and NMR data using different multivariate data analyses methods in order to identify the active ingredients in grape berries are also presented.

6.2. Materials and Methods

6.2.1. Sampling

Three elite Portuguese grapes cultivars i.e. 'Trincadeira', 'Touriga Nacional', and 'Aragoneˆs', were used in this study. Five biological replicates of each cultivar of 80–100 berries from 8 to 10 plants were collected in 2008 and 2007 (for 'Trincadeira' only) corresponding to the developmental stages of EL 32 (green), 35 (veraison), 36 (ripe), 38 (harvest) (EL refers to the modified Eichhorn and Lorenz developmental scale as described by Coombe, 1995 (Viant, 2003). Each biological replicate contained berries from a single row of plants. Four rows distant 3 to 10 m from each other were used for each variety. Plants from the three varieties were growing in the vineyard 15 to 30 m apart. Seeds were removed from all the berries prior to extraction.

6.2.2. Solid Phase Extraction (SPE)

A sample of 100 mg of lyophilized grape berries was extracted with 2 ml of the mixture of water and methanol (2:8), with ultrasonication for 20 minutes at 25 ^{o}C. The suspension was then centrifuged at 3500 rpm and the supernatant was transferred to a round bottomed flask. The same procedure was repeated two more times and the supernatants were pooled together in the flask and taken to dryness with a rotary evaporator. This grape berries extract was subjected to solid phase extraction (SPE) on SPE-C18 cartridges (Waters, Milford, MA, USA). Prior to its use, the SPE cartridge was prepared by elution

of 6 mL of methanol followed by 6 mL of water. Then, the redissolved grape berry extract (1 mL of deionized water) was applied to the cartridge and eluted successively with 5 mL of water and then 5 mL of methanol:water (1:1) and finally with 5 mL of methanol. All three fractions were collected in round bottomed flasks and evaporated under vacuum and were used for further NMR analysis and TNF-α bioassay. All the solvents were purchased from Biosolve B.V. (Valkenswaard, the Netherlands).

6.2.3. Cell culture and treatment with plant extract

Human monocyte-like histiocytic lymphoma U937 cells were cultured as described in chapter 3.

6.2.4. TNF-α ELISA assay

TNF-α in culture supernatants were determined by quantitative ‘‘sandwich’’ enzyme-linked immunosorbent assay as described in chapter 3.

6.2.5. Cell viability assay

Cell viability was determined by using MTT assay (Lee et al., 2007) as described in chapter 3.

6.2.6. ^{1}H NMR Spectroscopy

Fractions eluted from SPE were redissolved in 1 mL of methanol-d_4. An aliquot of 800 µL of sample was transferred to the 5-mm NMR tube and used for the NMR analysis. The deuterated methanol was purchased from Cambridge Isotope Laboratories, Inc., Andover, MA, USA. ^{1}H NMR spectra were recorded at 25 °C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz. MeOH- d_4 was used as the internal lock. Each ^{1}H NMR spectrum consisted of 128 scans requiring 10 min and 26 sec acquisition time with the following parameters: 0.16 Hz/point, pulse width (PW) = 30° (11.3 µsec), and relaxation delay (RD) = 1.5 sec. A pre-saturation sequence was used to suppress the residual H_2O signal

with low power selective irradiation at the H_2O frequency during the recycle delay. FIDs were Fourier transformed with LB = 0.3 Hz. The resulting spectra were manually phased and baseline corrected, and calibrated to MeOH- d_4 at 3.3 ppm, using XWIN NMR (version 3.5, Bruker). 2D NMR techniques were performed on a 600 MHz Bruker DMX-600 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 600.13 MHz. *J*-resolved NMR spectra were acquired using 8 scans per 128 increments for F1 and 8 k for F2 using spectral widths of 5000 Hz in F2 (chemical shift axis) and 66 Hz in F1 (spin–spin coupling constant axis). A 1.5 s relaxation delay was employed, giving a total acquisition time of 56 min. Datasets were zero-filled to 512 points in F1 and both dimensions were multiplied by sine-bell functions (SSB = 0) prior to double complex FT. *J*-resolved spectra tilted by 45°, were symmetrized about F1, and then calibrated, using XWIN NMR (version 3.5, Bruker). ^1H–^1H correlated spectroscopy (COSY) and heteronuclear multiple bonds coherence (HMBC) spectra were recorded on a 600 MHz Bruker DMX-600 spectrometer (Bruker). The COSY spectra were acquired with 1.0 s relaxation delay, 6361 Hz spectral width in both dimensions. Window function for COSY spectra was sine-bell (SSB = 0). The HSQC spectra were obtained with 1.0 sec relaxation delay, 6361 Hz spectral width in F2 and 27,164 Hz in F1. Qsine (SSB = 2.0) was used for the window function of the HSQC. The HMBC spectra were recorded with the same parameters as the HSQC spectra except for 30,183 Hz of spectral width in F2. The optimized coupling constants for HSQC and HMBC were 145 Hz and 8 Hz, respectively.

6.2.7. Data analysis

The ^1H NMR spectra (from all SPE fractions) were automatically reduced to ASCII files. Spectral intensities were scaled to methanol signal (δ 3.30) and reduced to integrated regions of equal width (0.04) corresponding to the region of δ 0.0–10.0. The regions of δ 4.85–4.95 and δ 3.2–3.4 were excluded from the analysis because of the residual signal of D_2O and CD_3OD, respectively. Bucketing was performed by AMIX software (Bruker) with scaling on total intensity. Principal component analysis (PCA) with scaling based on Pareto while projection to latent structures (PLS), PLS-discriminant analysis (PLS-DA), bidirectional orthogonal PLS (O2PLS), and O2PLS-discriminant analysis (O2PLS-DA), with scaling based on Unit Variance were performed with the SIMCA-P software (v. 12.0, Umetrics, Umeå, Sweden). TNF-α level was arbitrarily set as 100 in the negative control (LPS+DMSO) and all the other values are normalized to this (% activity). Means and standard

deviations were calculated and means comparisons were made with ANOVA followed by posthoc Tucky's test at a significance level <0.01.

6.3. Results and Discussion

By looking at NMR spectra, it is clear that all three SPE fractions are quite different from each other in terms of contained metabolites Fig. 1A. Mostly sugars and organic acids can be seen in the water fraction while the methanol fraction shows mostly amino acids and fatty acids with some resonances in the phenolic region Fig. 1B. The methanol: water fraction shows the presence of maximum amount of phenolics with relatively few sugars and amino acids. The phenolic regions of ^{1}H NMR spectra from all three cultivars are shown in Fig. 1C. Among the cultivars, 'Touriga Nacional' is found to have highest phenolic content. It is also observed from NMR spectra that high levels of phenolics with fewer sugars and organic acids characterize the initial stage in berry growth. As the berry grows, the level of sugars and organic acids seems to increase with a decrease in phenolics content.

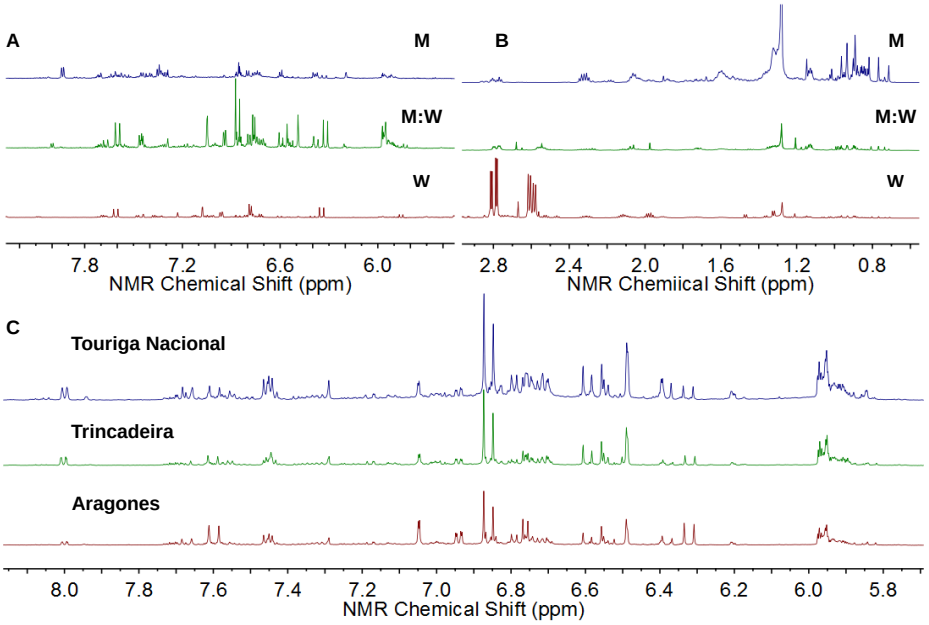

Figure 1a: Comparison of ^{1}H NMR spectra of phenolic (A) and aliphatic (B) regions of SPE fractions, and phenolic region of three cultivars at green stage (C).

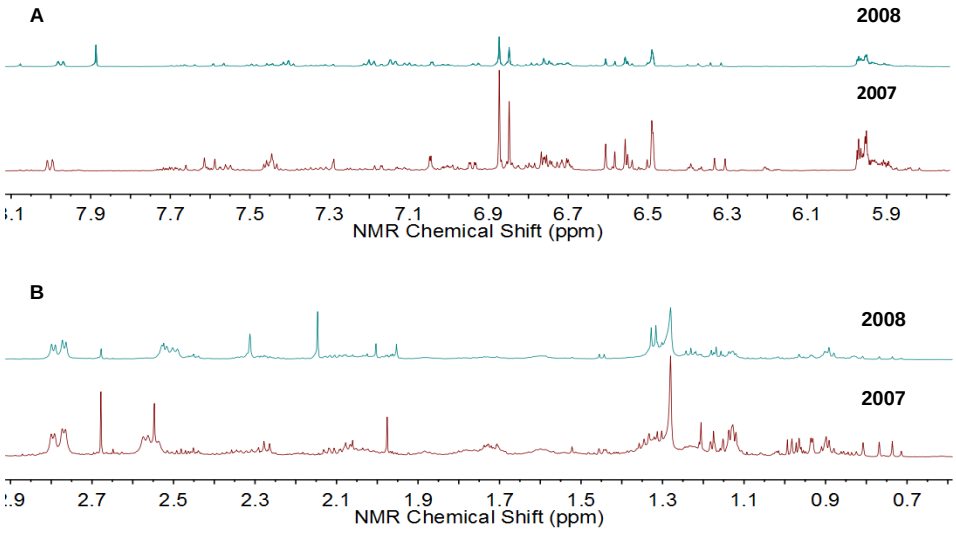

Figure 1b: Comparison of ^{1}H NMR spectra of phenolic 'A' and aliphatic 'B' regions of 'Trincadeira' 2007 and 'Trincadeira' 2008 vintages.

As a powerful analytical tool, ^{1}H NMR offers many advantages in metabolomics studies but signals congestion in NMR spectra hampers the metabolite identification. Several 2D NMR techniques, like *J*-resolved, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HMBC, and ^{1}H-^{13}C HSQC, provide additional information which facilitates the identification of metabolites. Among the above mentioned techniques *J*-resolved and ^{1}H-^{1}H COSY are widely used due to short measuring time, for signal purity and to sort out signals from same molecule respectively. They showed to be quite effective in the confirmation of metabolites like phenylpropanoids and flavonoids (Ali et al., 2009; Lewis et al., 2007; Mark R, 2003). Several compounds (Flavonoids, amino acids and organic acids) were identified using our in-house library of NMR data of common metabolites. The ^{1}H NMR chemical shifts for all these metabolites are shown in Table 1. All these assignment of metabolites are based on previous studies (Ali et al., 2011; Ali et al., 2009).

Table 1: ^{1}H NMR chemical shifts (δ) and coupling constants (Hz) of grape metabolites identified by references and using 1D and 2D NMR spectra (CD3OD-KH2PO4 in D2O, pH 6.0)

Compounds	**Chemical Shifts (δ)**
Gallic acid	7.03 (s)
Syringic acid	3.89(s), 7.31(s)
Vanillic acid	3.90 (s), 6.77 (d, *J*=8.2), 7.22 (m)
(+) - Catechin	2.49 (dd, *J*=16.1, 8.2), 2.83 (dd, *J*=16.0, 5.4), 4.04 (m), 4.55 (d, *J*=7.5), 5.91 (d, *J*=2.2), 6.75 (d, *J*=8.0)
(-) - Epicatechin	2.72 (dd, *J*=16.8, 2.8), 2.85 (dd, *J*=16.7, 4.6), 5.91 (dd, *J*=10.0, 2.3), 6.96 (d, *J*=2.2)
Quercetin-3-*O*-glucoside	5.30 (d, *J*=7.6), 6.27 (d, *J*= 2.0), 6.49 (d, *J*=2.0), 6.95 (d, *J*=8.6), 7.56 (dd, *J*=8.5, 2.0), 7.81 (d, *J*=2.0)
Myricetin	6.28 (d, *J*= 2.0), 6.51 (d, *J*=2.0), 7.30 (s)
trans-Caftaric acid	5.34 (s), 6.41 (d, *J*=16.0), 6.88 (d, *J*=8.4), 7.02 (dd, *J*=8.4, 2.0), 7.12 (d, *J*=2.0), 7.62 (d, *J*=16.0)
trans-Fertaric acid	3.89 (s), 5.38 (s), 6.32 (d, *J*=16.0), 6.89 (d, *J*=8.4), 7.01

	(dd, *J*=8.4, 2.0), 7.19 (d, *J*=2.0), 7.56 (d, *J*=16.0)
trans-p-Coutaric acid	5.42 (s), 6.45 (d, *J*=16.0), 6.87 (d, *J*=8.8), 7.51 (d, *J*= 8.8), 7.65 (d, *J*=16.0)
cis-Caftaric acid	5.34 (s), 5.92 (d, *J*=13.0), 6.71 (d, *J*=8.4), 6.81 (d, *J*=13.0), 7.03 (dd, *J*=8.4, 2.0), 7.44 (d, *J*=2.0)
cis-p-Coutaric acid	5.41(s), 5.94 (d, *J*=13.0), 6.73 (d, *J*=9.2), 6.86 (d, *J*=13.0), 6.93 (d, *J*=9.2), 7.61 (d, *J*=9.2)
Valine	1.01 (d, *J*=7.0), 1.06 (d, *J*=7.0), 2.28 (m)
Leucine	0.96 (d, *J*=7.5), 0.98 (d, *J*=7.5)
Alanine	1.48 (d, *J*=7.4), 3.73 (q, *J*=7.4)
GABA	1.90 (m), 2.31(t, *J*=7.5), 3.01 (t, *J*=7.5)
Proline	2.35 (m), 3.37 (m)
Methionine	2.15 (m), 2.65 (t, *J*=8.0)
Threonine	1.32 (d, *J*=6.5), 3.51 (d, *J*=5.0), 4.27 (m)
Glutamic acid	2.13 (m), 2.42 (m), 3.71 (dd, *J*=7.0, 1.9)
α-Glucose	5.17 (d, *J*=3.78)
β-Glucose	4.58 (d, *J*=7.89)
Fructose	4.08 (d, *J*=7.80)
Sucrose	5.39 (d, *J*=3.94)
2,3-butanediol	1.14 (d, *J*=6.47)
Acetic acid	1.94 (s)
Choline	3.20 (s)
Succinic acid	2.53 (s)
Citric acid	2.56 (d, *J*=17.6), 2.74 (d, *J*=17.6)

Tartaric acid (free)	4.30 (s)
α-Linolenic acid	0.95 (t, *J*=7.5)
Ascorbic acid	4.52 (d, *J*=2.0)
Malic acid	2.68 (dd, *J*= 16.6, 6.6), 2.78 (dd, *J*=16.6, 4.7), 4.34 (dd, *J*=6.6, 4.7)
Formic acid	8.45 (s)
Fumaric acid	6.52(s)

All three fractions from SPE of grape extracts were tested for TNF-α inhibition at 100 µg mL^{-1}. The methanol: water fractions show significantly higher activity than the water and methanol fractions (Fig. 2). It has been shown in the previous section that the metabolic compositions of these fractions are quite different from each other and the methanol: water fraction contained most of the grape phenolics. The water and methanol, fractions also showed some activity, though mostly not significantly different from each other. Methanol:water extracts of three cultivars show different activity at different developmental stages.

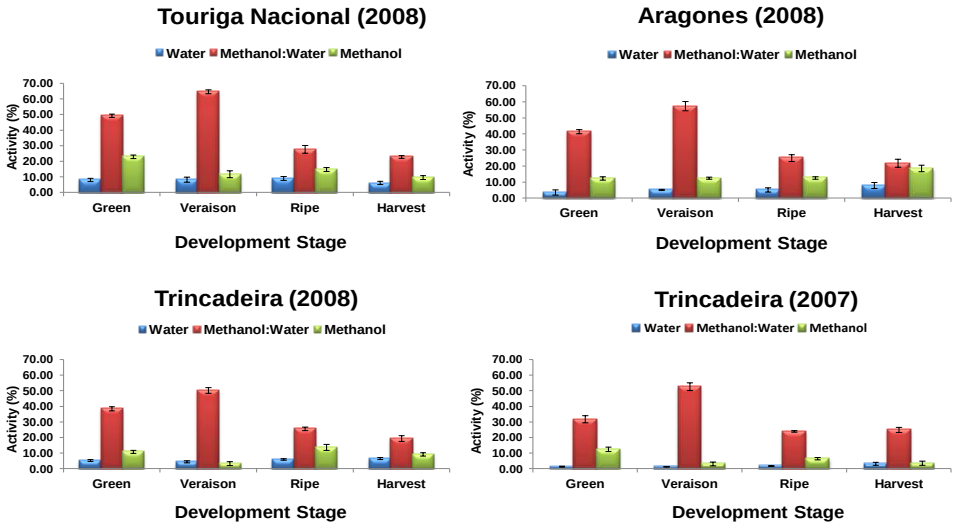

Figure 2: **TNF-α inhibition ratio (% of positive control) exhibited by grape cultivars and vintages at different developmental stages at the concentration of 100µg/ml. Bars represent the Mean ± S.D. (*n*=3) *p*<0.01.**

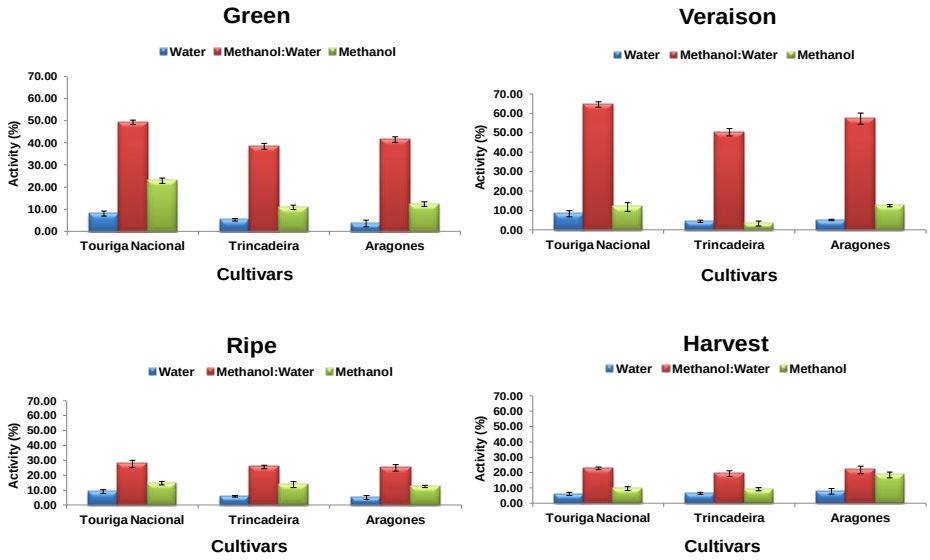

Figure 3: **TNFα inhibition ratio (% of positive control) exhibited by different grape cultivars at same developmental stages at the concentration of 100µg/ml. Bars represent the Mean ± S.D. (*n*=3) at *p*<0.01.**

The veraison stage is found to have maximum TNF-α inhibition in every cultivar followed by the green stage. For two cultivars, 'Touriga Nacional' and 'Aragonês', the ripe and harvest stages are not significantly different in TNF-α inhibition. The ripe stage of 'Trincadeira' is significantly higher than the harvest stage in inhibiting the TNF-α production. Among the green stages of all three cultivars, the green 'Touriga Nacional' grapes are found more active than the green grapes of 'Trincadeira' and 'Aragonês' (Fig. 3). At veraison, the 'Aragonês' and 'Touriga Nacional' grapes are not different but both are significantly more active than 'Trincadeira'. All the three cultivars show similar potency of inhibiting TNF-α production at the ripe and harvest stages.

In order to highlight the vintage effect on anti-TNF-α activity, the 2007 and 2008 vintage of 'Trincadeira' cultivar are compared. 'Trincadeira' 2007 also shows highest TNF-α inhibition at veraison stage followed by green stage but unlike 'Trincadeira' 2008 (see above), the 2007 vintage shows no significant difference in TNF-α inhibition at later stages of development i.e. ripe and harvest (Fig. 2). Comparing every developmental stage of these two vintages, only green and harvest are different. Green 'Trincadeira' 2008 grapes show higher activity while at harvest 'Trincadeira' 2007 grapes show significant inhibition of TNF-α production (Fig. 4). The ^{1}H NMR spectra (Fig. 1b) analysis shows that 'Trincadeira' 2007 has more phenolics as compared to 'Trincadeira' 2008, this suggesting a relationship between phenolics and activity.designed to prevent further pathogen spread or plant damage.

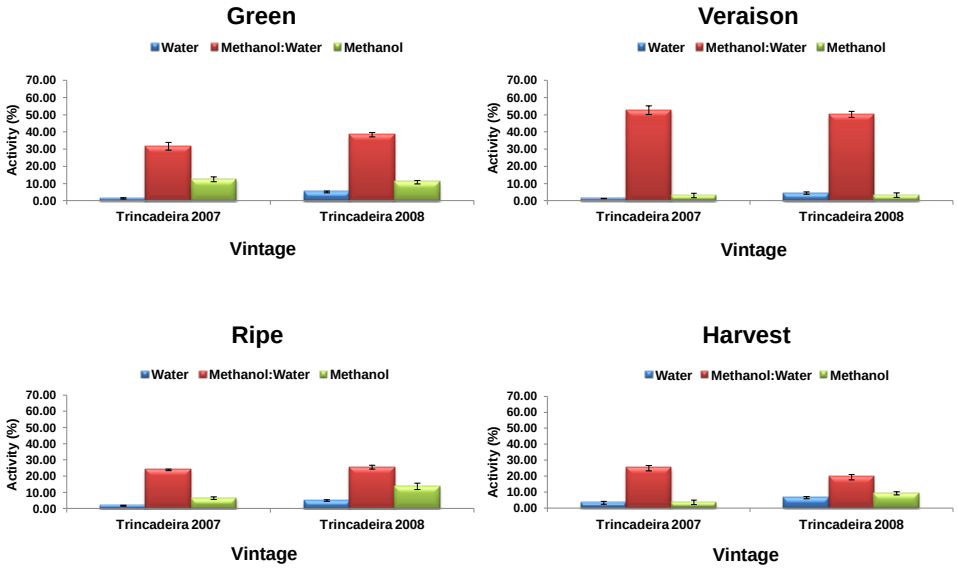

Figure 4: TNFα inhibition ratio (% of positive control) exhibited by 'Trincadeira' 2007 and 'Trincadeira' 2008 at similar developmental stages at the concentration of 100μg/ml. Bars represent the Mean ± S.D. (*n*=3) at *p*<0.01.

Principal component analysis (PCA) is considered as a primary tool in metabolomics used to reduce the dimensionality of a multivariate dataset, and thus helping to better understand possible differences between classes. It is an unsupervised method hence the clustering or separation of samples is purely due to similarities or differences, respectively, among all the samples. The NMR data from the SPE fractions of all the samples have been subjected to PCA in order to identify possible markers for the different cultivars, developmental stages, and SPE fractions. Fig. 5 shows the score plots of PCA where samples are colored according to SPE fractions, cultivars, and developmental stages.

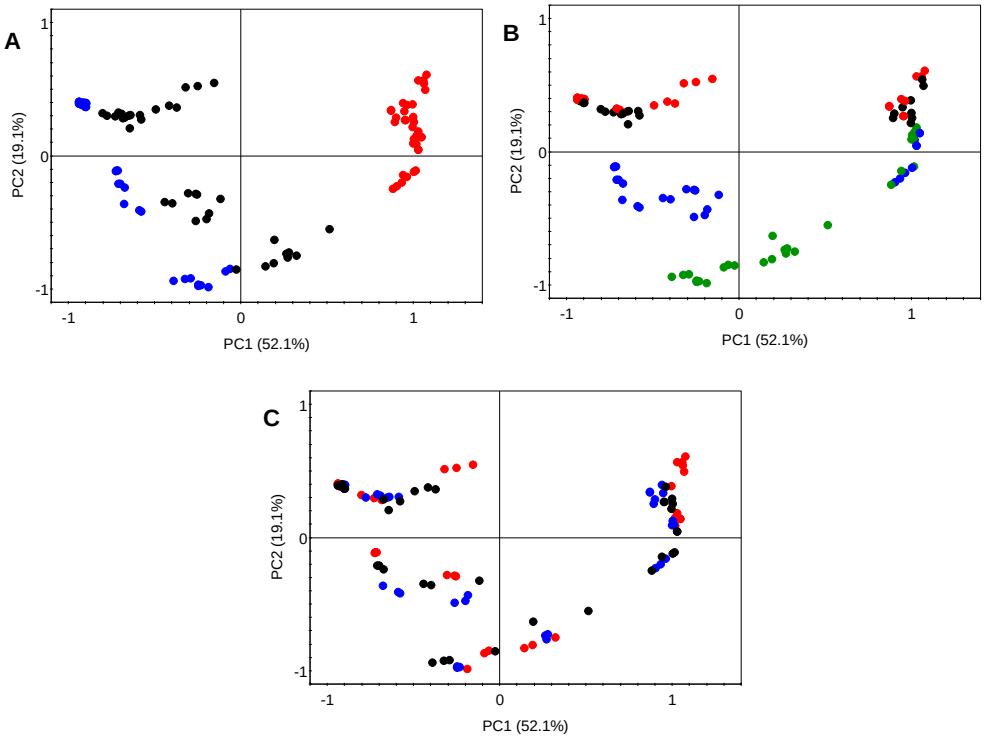

Figure 5: **Principal component analysis score plots. In 'A', samples are colored according to SPE fractions where red represents methanol, black represents methanol:water (1:1), and blue represents water fraction. In 'B', samples are colored according to developmental stages where green represents green stage, blue represents veraison, black represent ripe, and red represents harvest stage. In 'C', samples are colored according to grape cultivars where red represents samples from Aragone, blue represents samples from 'Touriga Nacional', and black represents samples from 'Trincadeira'.**

Fig. 5 represents the PCA score plot where samples are colored according to SPE fractions. It is clear from the figure that PC1 (51%) is responsible for the separation of all three SPE fractions. The water fractions are clustered on the negative side of PC1 while the methanol fractions are grouped on the positive side of PC1. The methanol:water fractions are located in between the methanol and water fractions, mostly having negative PC1 values. To highlight the differences based on developmental stages, samples from the same PCA are colored according to developmental stages in Fig. 5. It is obvious from the score plot that while PC1 is responsible for the separation of SPE

fractions, PC2 (19%) is quite effective in discriminating the developmental stages of grapes. The initial stages, like green and veraison, are on the negative side of PC2 whereas the remaining stages, like ripe and harvest, mostly have positive PC2 scores. The same PCA score plot is shown in Fig. 5C but this time the samples are colored according to grape cultivar. It is evident from this figure that PCA is not very effective as the samples are not clustered based on the grape cultivars.

The corresponding loading plots with the respective NMR spectra reveal the information regarding the metabolites responsible for the differentiation of samples on the score plots. As also shown by Fig. 1A, the SPE fractions are very much distinct in their metabolic contents. The water fraction is relatively higher in sugars and some amino acids and organic acids. Most of the phenolics are found to elute in the methanol:water fraction while the remaining amino acid and phenolics come out with the last methanol fraction. The methanol fraction is also found higher in fatty acids. The PCA is also found very effective in discriminating the developmental stages and by examining the loading plot the metabolites involved in differentiation could be identified. The grapes in green and veraison stages have higher levels of phenolics with relatively less sugar and organic acid contents. As the berries grow, the level of phenolics starts to decrease whereas sugars and organic acids concentrations increase (Ali et al., 2011). SPE fractions of grapes from 2007 and 2008 vintages of the 'Trincadeira' cultivar at four developmental stages were analyzed and compared for metabolic differences. Principal component analysis, also in this case, is found effective in highlighting the metabolic differentiation among the samples based on developmental stages and SPE fractions and responsible metabolites are identified. As discussed above, phenolics are the main discrimination factor in SPE fractions while a similar metabolic behavior of developmental stages was observed in all grape cultivars. However, to analyze specifically the vintage effects on the grape metabolic profile, supervised multivariate data analysis was applied.

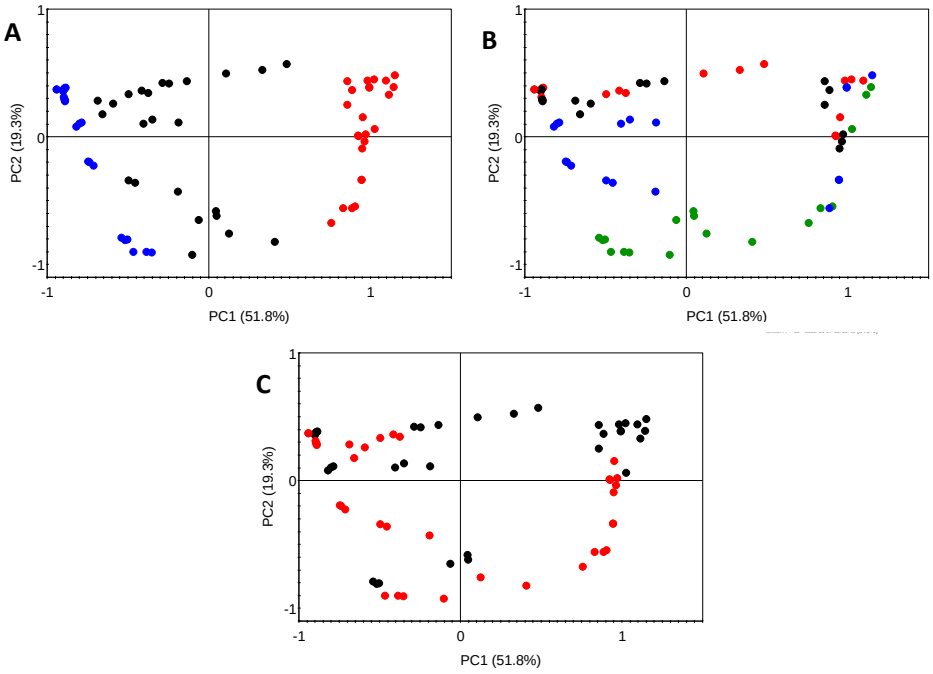

Figure 6: **Principal component analysis score plots. In 'A', samples are colored according to SPE fractions where red represents methanol, black represents methanol:water (1:1), and blue represents water fraction. In 'B', samples are colored according to developmental stages where green represents green stage, blue represents veraison, black represent ripe, and red represents harvest stage. In 'C', samples are colored according to 'Trincadeira' vintages where red represents samples from 'Trincadeira' 2008, and black represents samples from 'Trincadeira' 2007.**

First, projection to latent structures-discriminant analysis (PLS-DA) was used in which samples are classified in to two classes based on samples from 2007 and 2008 vintages. The score plot (Fig. 7A) shows good separation among the samples belonging to the two different classes but none of the components is found totally effective. The PLS-DA model was validated using permutation test with 20 applications (Fig. 7B). To draw clear conclusions, bidirectional orthogonal projection to latent structures-discriminant analysis (O2PLS-DA) was applied. The score plot (Fig. 7C) shows very clear distinction among the different vintages. Component 1 is responsible for the separation as samples from 2007 and 2008 vintages are on the positive and negative side, respectively. The loading plot shows that the 2007 vintage has higher levels of

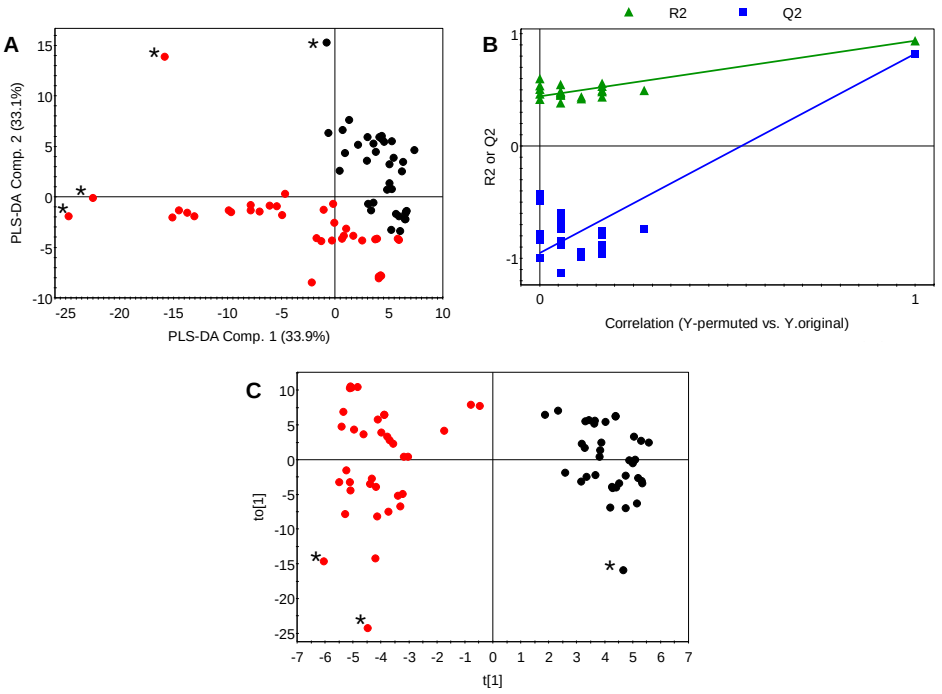

Figure 7: **Multivariate data analysis of 'Trincadeira' 2007 and 2008. The PLS-DA score plot (A), permutation test for PLS-DA (B), and score plot of O2PLS-DA (C) are shown. Samples with black color are from 'Trincadeira' 2007 while samples with red color are from 'Trincadeira' 2008. Samples with '*' represents an outlier.**

phenolics than the 2008 vintage. The 2008 vintage shows elevated levels of organic acids like malate and citrate with some sugars like glucose and fructose. Projection to latent structures-discriminant analysis (PLS-DA) is considered as the second step of metabolomics studies. It is a supervised method in which samples are classified into different groups on the basis of creating a set of dummy *Y*-variables. In this study, based on activity data, we classify our samples into low (<10%), medium (≥10% and <25%), and high (≥25%) activity classes as *Y*-variables, and used these in a PLS-DA. Fig. 8A shows that this gives a clear separation especially the samples with the lowest and medium activity are grouped separately. Samples with high activity are scattered and some are mixed with the samples with medium activity.

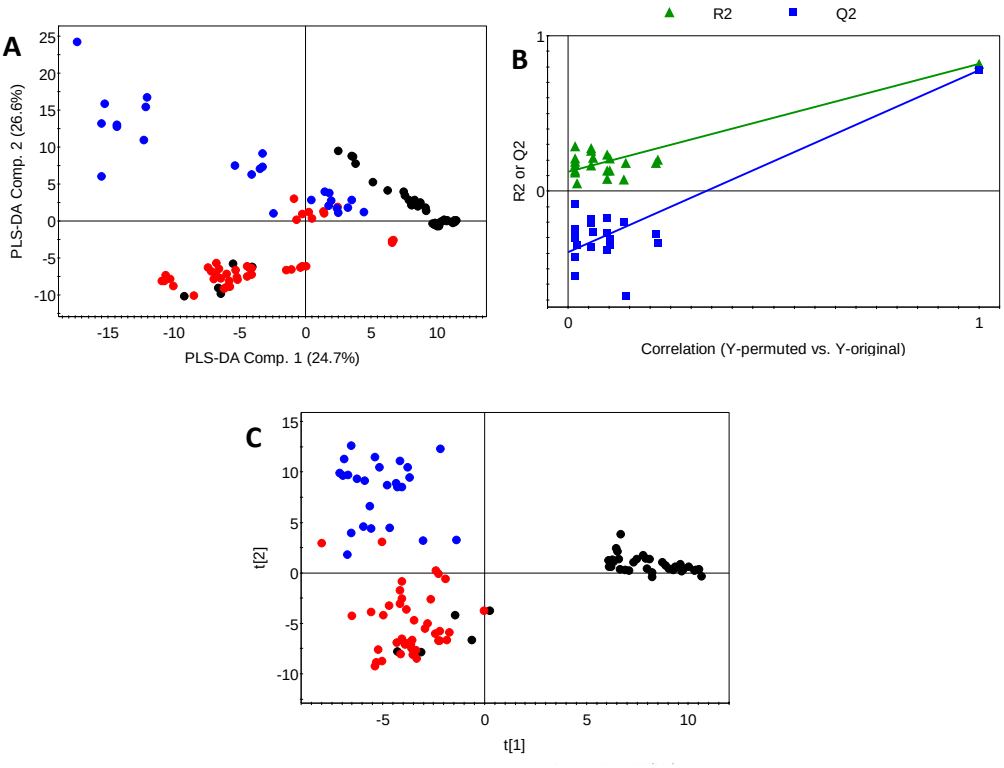

Figure 8: **Score plots of PLS-DA (A) and O2PLS-DA (C) are represented. Samples with black color show lowTNF-α inhibition while samples with red and blue colors show medium and high TNF-α inhibition, respectively. The permutation test for PLS-DA (C) is also presented.**

Validation of the supervised regression model is one of the key aspects in such analysis. For supervised algorithms, like PLS and PLS-DA, permutation test is used for validation which is a calculation of goodness of fit and the predictive ability of the model, represented by R2 and Q2, respectively. Generally R2 describes how well the data in the training set are mathematically reproducible. The R2 value can vary from 0 to 1, where 1 means a model with a perfect fit. If the Q2 value is more than 0.5, the model is considered to have good predictability and if it is higher than 0.9 and less than 1.0, then the model is considered to have an excellent predictability. It is suggested that if more than five PLS components are included in the model the training set data generally reproduce excellently. The R2 and Q2 values of PLS-DA using six components

were calculated. For anti-TNF-α activity the figures were 0.82 and 0.78, respectively. This PLS-DA model was validated by the permutation method through 20 applications in which all Q2 values of permuted Y vectors were lower than original ones and the regression of Q2 lines intersect at below zero (Fig. 8B).

In order to get better separation, especially for the samples with high activity, bidirectional orthogonal projection to latent structures-discriminant analysis (O2PLS-DA) is applied. The score plot of O2PLS-DA (Fig. 8C) shows much better separation among the samples based on anti-TNF-α activity. Samples with the low activity are grouped separately on the positive side of component 1 while the samples with medium and high activity are having negative component 1 scores. Component 2 was found effective in separating the high and medium activity samples as they clustered on positive and negative side of component 2, respectively. Few samples from low activity and medium activity classes are mixed with the medium activity and high activity classes, respectively, as their anti-TNF-α activity values are on the border line of their classes. The O2PLS-DA model is validated by cross validation-analysis of variance (CV-ANOVA) with a *p*-value of 8.35×10^{-38}. By examining the corresponding loadings plot, the metabolites responsible for separation are identified. Samples with different activity levels mainly differ in their phenolic contents. The high anti-TNF-α activity samples have higher levels of phenolics like cinnamic acids, flavonols, and flavan-3-ols while the medium and low activity samples have less or no phenolic contents.

The next step is to perform the direct correlation between the activity and NMR data using original anti-TNF-α assay values. Instead of classifying samples as high, medium, and low activity groups, the activity data from TNF-α assay for each sample are directly used as such. In such approaches PLS and/or PLS-DA are used and two different data sets, independent variable (like NMR spectral data) and dependent variable (like anti-TNF-α activity), are correlated using regression. For this purpose projection to latent structures (PLS) analysis was performed using the NMR and activity data. The PLS score plot (Fig. 9A) shows relatively good separation among the samples but many are overlapping with the other groups. Component 1 is mainly responsible for the separation as the samples are arranged from low to high activity along the negative to positive side of component 1, respectively. For PLS modeling again the permutation method through 20 applications was used for validation. The regression of Q2 lines intersect at below zero with all Q2 values of permuted Y vectors were lower than original. Variance (R2) and cross-validated variance (predictive ability of the model, Q2) values of PLS using seven components were

calculated and for anti-TNF-α activity the figures were 0.95 and 0.89, respectively (Fig. 9B).

Finally for the identification of metabolites responsible for high activity in grapes, we used another multivariate data analysis method known as bidirectional orthogonal projection to latent structures (O2PLS). Analyses like PLS regression can cause systematic variation of any data block due to

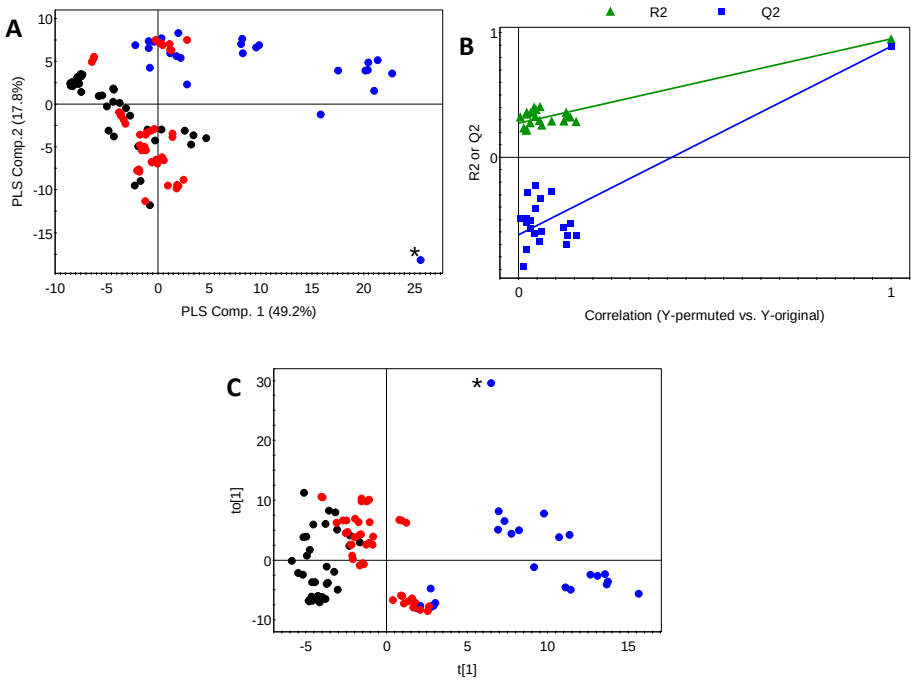

Figure 9: **Score plots of PLS (A) and O2PLS (C) are represented. Samples with black color show low TNF-α inhibition while samples with red and blue colors show medium and high TNF-α inhibition, respectively. The permutation test for PLS (C) is also presented.**

structured noise present in the data blocks. Other algorithms, like O2PLS-DA and O2PLS, are multivariate projection methods which remove the structured noise by extracting linear relationships from independent and dependent data blocks, in a bidirectional way, and results in the decomposition of systematic variation into two model parts: the predictive or parallel part and the orthogonal part (Baur et al., 2006; Chuang et al., 2010). The score plot, Fig. 9C, shows very nice separation among low, medium and high activity samples based on

component 1. This O2PLS model was validated by CV-ANOVA with *p*-value of $1.4x10^{-37}$. Like PLS-DA and O2PLS-DA the corresponding loadings plot show that the samples with high anti-TNF-α activity contained more phenolics, such as cinnamates and flavonoids, when compared to samples with low and medium activity.

In PLS based regression, VIP can be defined as a weighted sum of squares of the PLS weights. It has been indicated that it is directly proportional with the influence of factor on the separation on score plot, meaning, factors have higher VIP values are more important for the samples separation. For O2PLS-DA and O2PLS analyses, VIP (variable importance in the projection) values for several phenolic compounds, responsible for separation on the score plot, are presented in Table 2. As indicated, in O2PLS-DA analysis, caftaric acid, and (+)-catechin are the metabolites with top two VIP scores while quercetin and myricetin have relatively less VIP values. In O2PLS model, again (+)-catechin is one of the top two followed by coutaric acid while caftaric acid showned a much lower VIP score. This high VIP scores for the identified phenolics legitimate their involvement in the separation of high activity samples and suggest a role of these compounds in inhibiting TNF-α production.

Table 2: The VIP (variable importance in the projection) values of the major contributing compounds for the separation in the score plots derived from O2PLS-DA and O2PLS models.

Compounds	Chemical shift (ppm)	VIP values	
		O2PLS-DA	O2PLS
Quercetin-3-*O*-glucoside	6.27	1.38	2.06
Myricetin	6.51	1.17	1.62
(+)- Catechin	6.75	1.44*	2.16*
(-)- Epicatechin	6.96	1.40	2.09
Caftaric acid	7.62	1.55*	1.80
Fertaric acid	6.32	1.43	2.08
p-Coutaric acid	7.65	1.41	2.10*

The ^{1}H NMR spectra clearly shows distinction among different cultivars and vintages, their developmental stages, and the SPE fractions. This clearly advocates the enormous analytical potential of NMR spectroscopy as compared to other platforms for metabolomics studies (Verpoorte et al., 2008). Multivariate data analysis in combination with NMR is very popular in metabolic phenotyping studies of plants. Many reports have been published regarding grape berries using the same approach (Pereira et al., 2006a; Pereira et al., 2005; Pereira et al., 2006b; Son et al., 2009). In this study, metabolic profiling of different grapes at different stages of ripening has been successfully performed. The initial stages, green and veraison, have been characterized with high phenolics, whereas high sugar and organic acids content is observed in the later stages i.e. ripe and harvest, as also reported previously (Ali et al., 2011). This metabolic distinction among the developmental stages is reflected in the associated anti-TNF-α activity as green and veraison are found more active than ripe and harvest.

The vintage effect on the grape metabolome is quite obvious now as it is widely accepted that the several climatic factors are involved in the biosynthesis of several key metabolites in grapes (Pereira et al., 2006a). The green and harvest stages of these two vintages present significant metabolic differences, characterized by higher and lower phenolic contents in 2008 vintage, respectively, as compared to 2007 vintage. Since vintage has shown its effects on the phenolic contents of 'Trincadeira', the anti-TNF-α activity shown by these vintages is also affected. The green and harvest stages from 2008 and 2007 vintages, respectively, showed significantly different anti-TNF-α activity. As shown by the NMR spectra, this is due to difference in phenolic contents. It has been reported that different factors like hot and dry climate can result in higher phenolic contents in grapes (Pereira et al., 2006a). For instance, the insolation totals were higher in July and August of 2007 and differences in rain totals and average temperature were also observed in between seasons and may influence the fine tuning of phenolics´ biosynthesis (unpublished data). It is interesting to note that transcriptomic analysis using Affymetrix GrapeGen® genome array showed that a gene coding for anthocyanidin reductase which is involved in proanthocyanidins biosynthesis such as catechin was more expressed in 2007 samples. Since catechin seems to present high anti-TNF-α activity as suggested by the results hereby presented this may constitute a good example of positive integration of transcriptomic and metabolomic data, and medicinal properties that deserves further attention.

Data correlation using different multivariate data analysis tools is now increasingly popular and found efficient in predicting the unknown NMR

signals (metabolites) by using the resulting training model (Eriksson et al., 2006). Many reports have been published on developing predictive models for certain pharmacological activities in plants. Plants like St. John Wort (*Hypericum perforatum*) (Roos et al., 2004), *Artemesia annua* (Bailey et al., 2004), *Citrus grandis* (Cho et al., 2009), and *Galphimia glauca* (Cardoso-Taketa et al., 2008), have been efficiently studied for the prediction of different medicinal properties, using this approach. Such chemometrics based approach can provide firsthand knowledge regarding the plant extracts and any related bioactivity without tedious chromatographic separations. Since grapes are one of the richest sources of polyphenolics, many studies (Chuang et al., 2010; Seymour et al., 2008; Zern et al., 2005) have shown their potency against TNF-α production and grape polyphenolics are widely acclaimed and accepted to have anti-oxidative and antiinflammatory properties (Baur et al., 2006; Breksa Iii et al., 2010). Phenolics in grapes, like resveratrol (Stewart et al., 2008) and quercetin (Rivera et al., 2008) are known to reduce inflammation, while others like cinnamates, benzoates, flavonols, flavan-3-ols, and anthocyanins, are well known antioxidants (Lee et al., 2009). The present study is the only known attempt to analyze different grape cultivars, their developmental stages, and vintages for TNF-α inhibition. The identified NMR signals, responsible for the activity, are related to quercetin, myricetin, (+)- catechin, (-)- epicatechin, coutaric acid, fertaric acid, and caftaric acid, which are found relatively higher in the samples with high activity using different chemometrics methods.

6.4. CONCLUSION

Nuclear magnetic resonance spectroscopy (1D and 2D) has been applied for the phenotyping of three grape cultivars from Portugal at different stages of their development. Solid phase extraction was used in order to separate the phenolics from the other components of grape metabolome which resulted in water, methanol: water (1:1), and methanol fractions which were tested for TNF-α inhibition. The assay showed that grapes from all three cultivars at veraison and green stages strongly inhibit TNF-α production. Various multivariate data analysis methods showed good correlation between the NMR resonances for phenolics and TNF-α inhibition. Algorithms like PLS and PLS-DA showed good separation among the samples classified as high, medium, and low activity with high model validity. The application of bidirectional orthogonality, i.e. O2PLS-DA and O2PLS, showed even better distinction among the classes. The VIP plot showed that NMR signals related to metabolites like quercetin, myricetin, (+)-catechin, (-)-epicatechin, caftaric acid,

and coutaric acid, were statistically significantly correlated with high activity. Using the presented approach, the analysis of NMR shifts in relation to pharmacological activity can provide information about what part of the NMR spectrum (aromatic or aliphatic regions) correlates with the activity which gives information about the active ingredients in crude extracts of medicinal plants. In the approach shown here the compounds related to activity can be identified without extensive and elaborate chromatographic separation, and thus allows rapid identification of active compounds in extracts with biological activity.

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Chapter 7

Application of NMR spectroscopy and chemometrics based approach to examine the effect of red wine polyphenols on TNF-α production

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Abstract

It is very well documented that bioactive compounds from plant derived food may have beneficial effects on human health and can reduce the risk of various chronic inflammatory diseases. In this study, eleven different types of red wine from Portugal were screened for their potential TNF-α inhibition. Nuclear magnetic resonance (NMR) spectroscopy together with multivariate data analysis methods are applied for the metabolite profiling of different red wines. All the fractions from solid phase extraction were subjected to *in-vitro* TNF-α bioassay. Principal component analysis (PCA) was used to see separation among the different SPE fractions. Supervised multivariate data analysis, i.e. Partial least square (PLS), Partial least squares-discriminant analysis (PLS-DA), orthogonal bidirectional OPLS-DA and orthogonal partial least square (OPLS) were used as statistical methods to find correlation between metabolite profile of the extracts and their respective bioactivities. OPLS-DA was found most effective in discriminating the high activity samples from the low and medium activity samples. According to variable importance in the projection (VIP), different phenolic compounds were found to correlate with high activity samples and identified as caftaric acid, quercetin, and (+)-catechin. Among the

different vintages, maximum TNF-α inhibition was found in samples from the 2010 vintage. The results obtained in this study advocate the efficacy of NMR spectroscopy in combination with chemometrics to identify the possible bioactivity in the several crude extracts of red wines.

Keywords: Chemometrics, NMR, red wines, TNF-α,

7.1. Introduction

Inflammation is the response of the body (immune system) against an external stimuli e.g. a pathogenic invasion aiming at the production, activation, and recruitment of immune cells (leucocytes) to sites of infection in order to combat the pathogens. The process of inflammation involves a complex mechanism of cell signaling cascades and is regulated by different pro-inflammatory cytokines and antiinflammatory cytokines. However, besides pathogenic infection, dysregulation of one or more regulatory proteins in the cell signaling pathways can also lead to inflammation and the emergence of wide varieties of chronic inflammatory diseases such as metabolic disorders, diabetes, atherosclerosis and cardiovascular complications, rheumatoid arthritis, Alzheimer's disease and cancer (Neuman, 2007).

Tumor necrosis factor-α is one of the regulatory proteins of the immune system in which dysregulation of this protein can lead to the emergence of the diseases. The proper regulation of TNF-α is necessary to keep the immune system in balance (homeostasis). TNF-α is secreted by immune cells; (mainly) by macrophages and also by T-cells, neutrophils, NK cells, and synovial cells (Vilcek and Lee, 1991). Increased level of TNF-α occurs during the early phase of inflammatory diseases and it contributes significantly to the secretion of other cytokines like IL-1, IL-6, and IL-8 (Cho et al., 2001; Cho et al., 1998). Overproduction of TNF-α can lead to systemic toxicity, thus, suppression of the secretion of TNF-α in this case can be a method for prevention and/or therapy.

The imbalance between prooxidants and antioxidants in an organism leads to a condition known as oxidative stress, which can be a trigger of a chronic inflammatory disease (Rahman et al., 2006). Healthy life style includes a rich antioxidant diet, which moderates the immune system and prevents diseases. Plants are known to be a rich source of antioxidants. Food and beverage from plants contain compounds that may work as TNF-α inhibitor. Wine for example, is a beverage of long tradition and high value and known to contain a complex mixture of compounds at a wide range of concentrations. The compounds in wine cover a diverse range of metabolites including primary (e.g. sugars, organic acids, amino acids) and secondary metabolites (e.g. flavonoids, hydroxycinnamates, hydroxybenzoates, anthocyanins). Wine phenolics have been proved to posses several health promoting activities (Ali et al., 2010a; Halpern, 2008) and nearly all of these beneficial effects associated to wine are due to anti-oxidant and radical scavenging properties of wine phenolics (German and Walzem, 2000). Since grape skin, seeds, and stem are the main source of phenolics in wine, red wines contain much higher concentrations of

these compounds as compared to white wines as skin, seeds, and stem are left in contact with must in red wine making, but is rapidly separated from the must in the case of white wine.

Metabolomics is a powerful tool to be applied on food or beverages such as wines in order to gain more insight in the compounds that are responsible for an interesting pharmacological activity. Metabolomics is an approach aimed for the better understanding of primary and secondary metabolism and can be defined as a metabolic snap shot of a living system (Andrew Clayton et al., 2006). More powerful and sophisticated tools (like mass spectrometry and NMR) for such chemical analyses have been introduced with the advancement in the field of analytical chemistry. Other than the use of NMR spectroscopy in structure elucidation of novel compounds, it is now a popular tool in the area of metabolome analysis (Son et al., 2009). Often criticized for its low sensitivity as compared to other platforms for metabolomics analysis, the most promising features of NMR are its non destructive nature, simple and fast sample preparation, and quantification is easy as for all compounds it is only dependent on the molar concentration. Nuclear magnetic resonance now has an important place in the chemical analyses of food. Some recent publications reported the use of NMR for the studies like quality control, authenticity or geographical characterization of different food products like coffee, olive oil, tomato and orange juices, wine and beer (Charlton et al., 2002; D'Imperio et al., 2007; Le Gall et al., 2001; Nord et al., 2004; Sobolev et al., 2003).

The development of methods and algorithms for the multivariate statistical modeling have contributed much to metabolomics as they opened the way for handling the huge datasets of large-scale metabolic analyses (Crockford et al., 2005). In combination with different multivariate data analyses methods, NMR has been widely used to do metabolic profiling of various samples (Brescia et al., 2002; Charlton et al., 2002). Several other studies have been published using the same combination focusing on the characterization of different plant species (Hye Kyong Kim et al., 2005) and cultivars (Ali et al., 2009), monitoring grape berry growth (Ali et al., 2011), and studying the effects of growing areas, vintage, soil, and microclimate (Pereira et al., 2005; Pereira et al., 2006). NMR based metabolomics was used for the analysis of the extracts of *Hypericum perforatum* (Roos et al., 2004), *Artemisia annua* (Bailey et al., 2004), *Citrus grandis* (Cho et al., 2009), and *Galphimia glauca* (Cardoso-Taketa et al., 2008), to correlate metabolites with the aid of pharmacological activities.

As wine in many countries is considered to be beneficial for health, with antioxidant thought to be responsible for this, we were interested to learn if also other effects could be present. With inflammation being a major disease, the present study was aimed at measuring the effect on an important mediator of inflammation. We therefore measured *in-vitro* TNF-α inhibition of some Portuguese red wines from different vintages. Several wine phenolics and other primary metabolites were identified using 1D and 2D NMR techniques. The correlation of activity data and NMR data using different multivariate data analyses methods in order to identify the active ingredients in red wines is also presented.

7.2. Materials and Methods

7.2.1 Wine samples

Wine samples analyzed in this study were kindly provided by Eng. Inês Aranha and Esporão (http://www.esporao.com).

7.2.2 Solid Phase Extraction (SPE)

A sample of 10 mL of each wine was completely dried under vacuum and redissolved in 1 mL of deionized water before subjected to solid phase extraction (SPE) on SPE-C18 cartridges (Waters, Milford, MA, USA). Prior to its use, the SPE cartridge was preconditioned by elution using 10 mL of methanol followed by 10 mL of water. The sample was subsequently applied to the cartridge and eluted successively with 5 mL of water, 5 mL of methanol:water (1:1) and 5 mL of methanol. 1mL of each fraction was taken into separate 2ml vial, dried under Nitrogen gas and dissolved in DMSO with concentration of 10mg/ml. The remaining fractions were collected, each in a round bottom flask and evaporated using a rotary evaporator except water fraction which was dried using freeze drier. The dried samples were in MeOD for NMR analysis. All the solvents were purchased from Biosolve B.V. (Valkenswaard, the Netherlands).

7.2.3. Growth of cells, Lipopolysaccharides stimulation, and treatments with wine

Human monocyte-like histiocytic lymphoma U937 cells were cultured and treated with extracts as described in chapter 3

7.2.4 ELISA for TNF-α

TNF-α in culture supernatants were determined by quantitative 'sandwich' enzyme-linked immunosorbent assay as described in chapter 3.

7.2.5. Cell viability assay

Cell viability after treatment with different wine samples was determined by using MTT assay (Lee et al., 2007) as described in chapter 3.

7.2.6. ^{1}H NMR Spectroscopy

The three fractions eluted from SPE were redissolved in 1 mL of methanol-d_4. An aliquot of 800 µL of sample was transferred to the 5-mm NMR tube and used for the NMR analysis. The deuterated methanol was purchased from Cambridge Isotope Laboratories, Inc., Andover, MA, USA. ^{1}H NMR spectra were recorded at 25 °C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz. MeOH- d_4 was used as the internal lock. Each ^{1}H NMR spectrum consisted of 128 scans requiring 10 min and 26 sec acquisition time with the following parameters: 0.16 Hz/point, pulse width (PW) = 30° (11.3 µsec), and relaxation delay (RD) = 1.5 sec. A pre-saturation sequence was used to suppress the residual H_2O signal with low power selective irradiation at the H_2O frequency during the recycle delay. FIDs were Fourier transformed with LB = 0.3 Hz. The resulting spectra were manually phased and baseline corrected, and calibrated to MeOH- d_4 at 3.3 ppm, using XWIN NMR (version 3.5, Bruker). 2D NMR techniques were performed on a 600 MHz Bruker DMX-600 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 600.13 MHz. *J*-resolved NMR spectra were acquired using 8 scans per 128 increments for F1 and 8 k for F2 using spectral widths of 5000 Hz in F2

(chemical shift axis) and 66 Hz in F1 (spin–spin coupling constant axis). A 1.5 s relaxation delay was employed, giving a total acquisition time of 56 min. Datasets were zero-filled to 512 points in F1 and both dimensions were multiplied by sine-bell functions (SSB = 0) prior to double complex FT. *J*-resolved spectra tilted by 45°, were symmetrized about F1, and then calibrated, using XWIN NMR (version 3.5, Bruker). ^{1}H–^{1}H correlated spectroscopy (COSY) and heteronuclear multiple bonds coherence (HMBC) spectra were recorded on a 600 MHz Bruker DMX-600 spectrometer (Bruker). The COSY spectra were acquired with 1.0 s relaxation delay, 6361 Hz spectral width in both dimensions. Window function for COSY spectra was sine-bell (SSB = 0). The HSQC spectra were obtained with 1.0 sec relaxation delay, 6361 Hz spectral width in F2 and 27,164 Hz in F1. Qsine (SSB = 2.0) was used for the window function of the HSQC. The HMBC spectra were recorded with the same parameters as the HSQC spectra except for 30,183 Hz of spectral width in F2. The optimized coupling constants for HSQC and HMBC were 145 Hz and 8 Hz, respectively.

7.2.7 Data analysis

The ^{1}H NMR spectra (from all SPE fractions) were automatically reduced to ASCII files. Spectral intensities were scaled to methanol signal (δ 3.30) and reduced to integrated regions of equal width (0.04) corresponding to the region of δ 0.0–10.0. The regions of δ 4.85–4.95 and δ 3.2–3.4 were excluded from the analysis because of the residual signal of D_2O and CD_3OD, respectively. Bucketing was performed by AMIX software (Bruker) with scaling on total intensity. Principal component analysis (PCA) with scaling is based on Pareto scaling, while projection to latent structures (PLS), PLS-discriminant analysis (PLS-DA), bidirectional orthogonal PLS (O2PLS), and O2PLS-discriminant analysis (O2PLS-DA), is based on Unit Variance scaling, all were performed with the SIMCA-P software (v. 12.0, Umetrics, Umeå, Sweden). The TNF-α level was arbitrarily set as 100 in the positive control and all the other values are normalized to this (% activity) and shown in results. Means and standard deviations were calculated and means comparisons were made with ANOVA at a significance level <0.01.

7.3. Results and Discussion

TNF-α inhibition results from the SPE fractions of the different wine samples are shown in Table 1. Water fraction of Petit Verdot 2008 shows least inhibition while the most active water fraction is of Aragones 2010 wine. The most active methanol fraction of SPE is from the Aragones (2009) while the least active fraction is from Alicante 2008. By comparison among the three SPE fractions maximum TNF-α inhibition was found in the methanol:water fraction. In the fractions, the vintage effect is obvious as Petit Verdot, Touriga Nacional, Aragones, and Alicante from 2010 vintage are significantly more active than the vintages of 2008 (Petit Verdot, Touriga Nacional, and Alicante) and 2007 (Aragones). Among all SPE fractions of different wine types, the Touriga Nacional (2010) showed the maximum inhibition of TNF-α production.

Wine type	Vintage	TNF-α inhibition (%)*		
		Water Fraction	Methanol:Water Fraction (1:1)	Methanol Fraction
Petit Verdot	2008	**7.51±2.72**	56.53±3.14	34.10±6.10
	2010	39.77±0.58	83.82±2.41	37.40±6.96
Touriga Nacional	2008	34.39±2.00	64.84±5.64	28.11±2.04
	2009	20.03±4.16	79.51±4.59	26.39±2.25
	2010	35.93±0.09	**88.81±2.56**	35.23±2.90
Aragones	2007	36.22±2.90	57.86±2.11	34.14±2.28
	2009	**48.93±0.94**	63.71±1,29	**43.08±5.38**
	2010	54.47±3.68	77.83±7.49	24.68±2.65
Alicante	2008	36.77±5.52	**55.14±1.97**	**17.71±2.87**
	2009	25.45±3.76	61.26±3.62	25.93±3.12

2010	38.11±4.92	73.47±1.35	27.69±3.35

* Values are expressed as mean ± standard deviation of n=3 experimental replicates. Means in a column differ significantly (P<0.01).

NMR spectroscopy of the SPE fractions was used for the metabolic profiling of the different red wines. Figure 1 shows the ^{1}H NMR spectra of water, methanol:water (1:1), and methanol fractions. From the NMR spectra it is clear that the SPE fractions are different from each other in terms of metabolites contained. Sugars and organic acids are mostly found in the water fraction. The methanol fraction shows high signal intensity in amino acids and fatty acids regions with relatively less sugars and no phenolics. The methanol:water fraction shows more signals in the phenolic region (δ 5.50 to

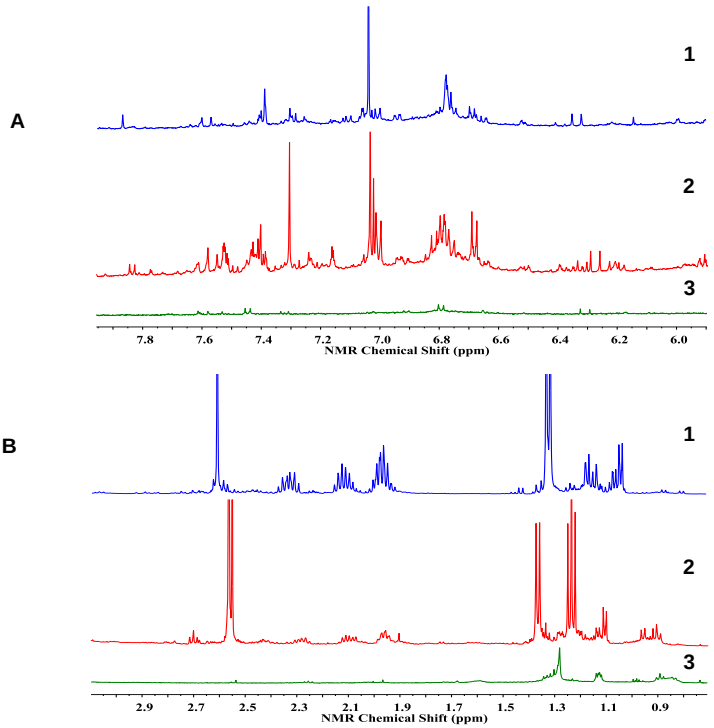

Figure 1: Comparison of 1H NMR spectra of phenolics (A) and amino acids (B) regions of SPE fractions. Both (A) and (B) show water (1), methanol:water (1:1) (2), and methanol (3) fractions from top to bottom.

8.50) with relatively less sugars and amino acids.

1D and 2D NMR techniques like *J*-resolved, ^{1}H-^{1}H COSY, HMBC and HSQC were used to identify the metabolites responsible of activity. Among the flavonoids, quercetin, myricetin, and kaempferol, are identified in the aromatic region. Signal correlation is observed between δ 6.49 of H-6 and δ 6.27 of H-8, and also between 6.99 of H-5′ and δ 7.66 of H-6′ of quercetin in the ^{1}H-^{1}H COSY spectrum. Likewise myricetin signals, δ 6.47 of H-8 with δ 6.25 of H-6, and kaempferol signals at δ 8.04 of H-2′ and H-6′ with δ 6.74 of H-3′ and H-5′, and at δ 6.52 of H-8 with δ 6.28 of H-6 also showed ^{1}H-^{1}H COSY correlations. Compounds like (+)-catechin and (-)-epicatechin were also identified. For both the (+)-catechin and (-)-epicatechin, signals of H-6′ and H-5′ along with signals of H-6 and H-8 showed correlations in ^{1}H-^{1}H COSY spectra. Resonances like δ 6.21 (t, J = 2.1 Hz), δ 6.31 (d, J = 2.1 Hz), δ 6.68 (d, J = 13.3 Hz), δ 6.71 (d, J = 8.5 Hz), δ 6.76 (d, J = 13.3 Hz), and δ 7.18 (d, J = 8.5 Hz) are assigned to resveratrol. This compound is identified as *cis*- isomer of resveratrol as the olefinic protons signals are shifted, i.e. H-8: from δ 6.79 to δ 6.68, and H-7: from δ 6.89 to δ 6.76, with reduced coupling constants (from 16.1 Hz to 13.2 Hz). These olefinic protons are also found to be correlated in the ^{1}H-^{1}H COSY spectrum along with other signal correlations like H-4 (δ 6.21) with H-2 and H-6 (δ 6.30), and H-6′ (δ 7.18) with H-3′ (δ 6.71).

The aromatic part of the ^{1}H NMR spectra also showed some signals of benzoic acid derivatives such as gallic acid, syringic acid, *p*-benzoic acid, and vanillic acid. The presence of characteristic doublets of 16.0 Hz in the range of δ 6.39-6.50 and δ 7.59-7.70 represent the H-8′ and H-7′ (olefinic protons) of *trans*-cinnamic acids, respectively, which are also found correlated in the ^{1}H-^{1}H COSY spectra and also coupled with the carbonyl carbon at δ 168.3 in the HMBC spectra. These metabolites are identified as caffeic acid, and *p*-coumaric acid. These two cinnamic acids derivatives, along with *trans*-feruloyl derivative, were also identified conjugated with tartaric acid through an ester linkage. Based on these assignments, these compounds were identified as *trans*-caftaric acid (caffeic acid conjugated with tartaric acid), *trans*-fertaric acid (ferulic acid conjugated with tartaric acid), and *trans*-coutaric acid (coumaric acid conjugated with tartaric acid). Along with the *trans*- forms, the *cis*- forms of these conjugated cinnamic acids, i.e. *cis*-caftaric acid and *cis*-coutaric acid, were also detected as an upfield shift of the signals for H-8′ and H-7′ along with the reduction in the coupling constant from 16.0 Hz to 13.0 Hz was observed in the J-resolved spectrum. Different amino acids like alanine, threonine, valine, proline, methionine, tyrosine, phenylalanine, glutamic acid, glutamine, arginine, and aspartic acid were identified by comparison with the reference spectra of

these compounds. All the compounds were identified (Table 2) by comparing the spectra with previous reports (Ali et al., 2011; Ali et al., 2009; Choi et al., 2004; Choi et al., 2005; Liang et al., 2006) and 1D and 2D NMR spectra of common plant metabolites in our in-house library.

Table 2: ^{1}H NMR chemical shifts (δ) and coupling constants (Hz) of wine metabolites identified by using 1D and 2D NMR Spectra of the reference compounds (CD3OD-KH2PO4 in D2O, pH 6.0)

Compounds	Chemical Shifts (δ)
Alanine	1.48 (d, *J*=7.4)
Threonine	1.32 (d, *J*=6.5), 3.51 (d, *J*=5.0), 4.27 (m)
Valine	1.01 (d, *J*=7.0), 1.06 (d, *J*=7.0), 2.28 (m)
Proline	2.35 (m), 3.37 (m)
Methionine	2.15 (m), 2.65 (t, *J*=8.0)
Tyrosine	6.85 (d, *J*=8.5), 7.19 (d, *J*=8.5)
Phenylalanine	3.15 (dd, *J*=8.2, 14.5), 3.91 (t, *J*=9.6)
Glutamine	2.14 (m), 2.41 (td, *J*=16.2, 7.5)
Glutamate	2.13 (m), 2.42 (m), 3.71 (dd, *J*=7.0, 1.9)
Arginine	1.75 (m), 3.75 (t, *J*=5.5)
Aspartate	2.80 (m), 3.80 (m)
β-glucose	4.58 (d, *J* = 7.8)
α-glucose	5.17 (d, *J* = 3.7)
Sucrose	5.39 (d, *J* = 3.9)
GABA	1.90 (m), 2.31(t, *J*=7.5), 3.01 (t, *J*=7.5)
Choline	3.20 (s)

Glycerol	3.56 (m), 3.64 (m)
2,3-butanediol	1.14 (d, *J*=6.5)
Acetic acid	1.94 (s)
Succinic acid	2.53 (s)
Fumaric acid	6.52 (s)
Formic acid	8.45 (s)
Citric acid	2.56 (d, *J* = 17.6), 2.74 (d, *J* = 17.6)
Malic acid	2.68 (dd, *J* = 16.6, 6.6), 2.78 (dd, *J* = 16.6, 4.7), 4.34 (dd, *J* = 6.6, 4.7)
Lactic acid	1.40 (d, *J*=7.0)
Tartaric acid	4.35 (s)
cis-Resveratrol	6.21 (t, *J*=2.1), 6.31 (d, *J*=2.1), 6.68 (d, *J*=13.3), 6.71 (d, *J*=8.5), 6.76 (d, *J*=13.3), 7.18 (d, *J*=8.5)
Gallic acid	7.03 (s)
Syringic acid	3.89(s), 7.31(s)
Vanillic acid	3.90 (s), 6.77 (d, *J*=8.2), 7.22 (m)
p-Benzoic acid	6.83 (d, *J*=8.7), 7.94 (d, *J*=8.6)
p-Coumaric acid	6.38 (d, *J*=16.0), 6.84 (d, *J*= 8.8), 7.50 (d, *J*=8.8), 7.59 (d, *J*=16.0)
Caffeic acid	6.24 (d, *J*=16.0), 6.87 (d, *J*=8.4), 7.02 (dd, *J*=8.4, 2.0), 7.12 (d, *J*=2.0), 7.52 (d, *J*=16.0)
(+)- Catechin	2.52 (dd, *J*=16.1, 8.2), 2.83 (dd, *J*=16.0, 5.4), 4.04 (m), 4.55 (d, *J*=7.5), 5.89 (d, *J*=2.2), 6.75 (d, *J*=8.0), 6.80 (dd, *J*=8.5, 2.0), 6.88 (d, *J*=8.5), 6.9 (d, *J*=2.0)
(-)- Epicatechin	2.72 (dd, *J*=16.8, 2.6), 2.89 (dd, *J*=16.9, 4.6), 4.26 (m),

	6.03 (d, *J*=2.0), 6.06 (d, *J*=2.0), 6.88 (brs), 6.96 (d, *J*=2.2)
Quercetin	6.27 (d, *J*= 2.0), 6.49 (d, *J*=2.0), 6.99 (d, *J*=8.6), 7.66 (dd, *J*=8.5, 2.0), 7.71 (d, *J*=2.0)
Myricetin	6.28 (d, *J*= 2.0), 6.51 (d, *J*=2.0), 7.30 (s)
Kaempferol	6.28 (d, *J*= 2.0), 6.52 (d, *J*=2.0), 6.74 (d, *J*=8.6), 8.04 (d, *J*=8.6),
trans-Caftaric acid	5.77 (s), 6.29 (d, *J*=16.0), 6.88 (d, *J*=8.4), 7.02 (dd, *J*=8.4, 2.0), 7.12 (d, *J*=2.0), 7.52 (d, *J*=16.0)
trans-p-Coutaric acid	5.84 (s), 6.36 (d, *J*=16.0), 6.87 (d, *J*=8.8), 7.51 (d, *J*= 8.8), 7.59 (d, *J*=16.0)
Fertaric acid	3.89 (s), 5.38 (s), 6.32 (d, *J*=16.0), 6.89 (d, *J*=8.4), 7.01 (dd, *J*=8.4, 2.0), 7.19 (d, *J*=2.0), 7.56 (d, *J*=16.0)
cis-Caftaric acid	5.34 (s), 5.92 (d, *J*=13.0), 6.71 (d, *J*=8.4), 6.81 (d, *J*=13.0), 7.03 (dd, *J*=8.4, 2.0), 7.44 (d, *J*=2.0)
cis-p-Coutaric acid	5.41(s), 5.94 (d, *J*=13.0), 6.73 (d, *J*=9.2), 6.86 (d, *J*=13.0), 6.93 (d, *J*=9.2), 7.61 (d, *J*=9.2),

Multivariate data analysis algorithms (supervised or unsupervised) were used to reduce the dimensionality of multivariate dataset and thus enable us to discriminate among the samples. The NMR data from the SPE fractions of all the samples were subjected to PCA to see the differences among the SPE fractions and to identify the metabolites responsible for that distinction. Figure 2 shows the score plots of PCA where samples are colored according to SPE fractions. By examining the corresponding loadings plot, metabolites responsible for this separation are revealed (Table 1).

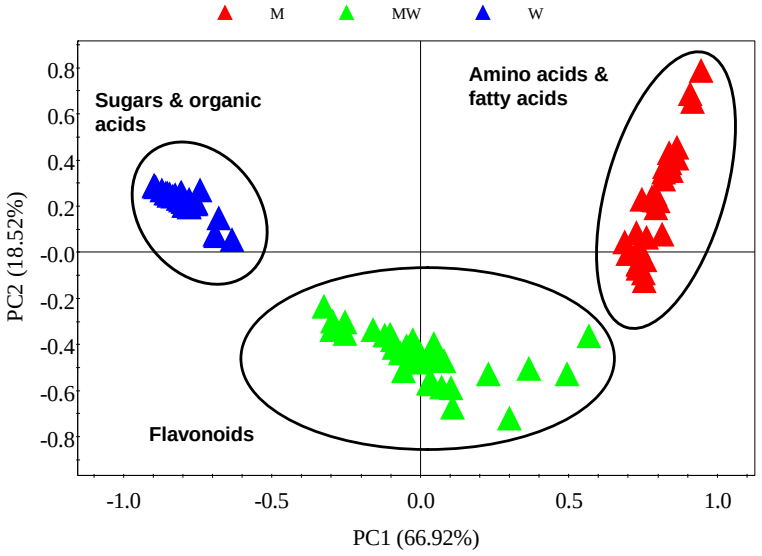

Figure 2: **Principal component analysis (PCA) score plot of SPE fractions of all the wine samples. Three fractions are clearly separated from each other. Samples in blue, green, and red indicates water, methanol:water (1:1), and methanol fractions, respectively.**

PLS-DA is a supervised multivariate data analysis method in which samples are classified into various classes on the basis of activity by creating dummy *Y*-variables. The score plot of PLS-DA (Figure 3A) shows good separation of samples with high activity from the others but no clear distinction was found among samples with medium and low activity. To see the difference among low and medium activity samples, another supervised algorithm, bidirectional orthogonal PLS-DA (O2PLS-DA), was used. Figure 3B shows very nice separation among all the three classes of samples. Metabolites responsible for the separation are identified by examining the corresponding loadings plot. Samples with high activity are found with higher levels of phenolics like quercetin, myricetin, (+)-catechin, caftaric acid, and coutaric acid while metabolites like glucose, sucrose, valine, proline, methionine, and alanine are found more concentrated in low and high activity samples.

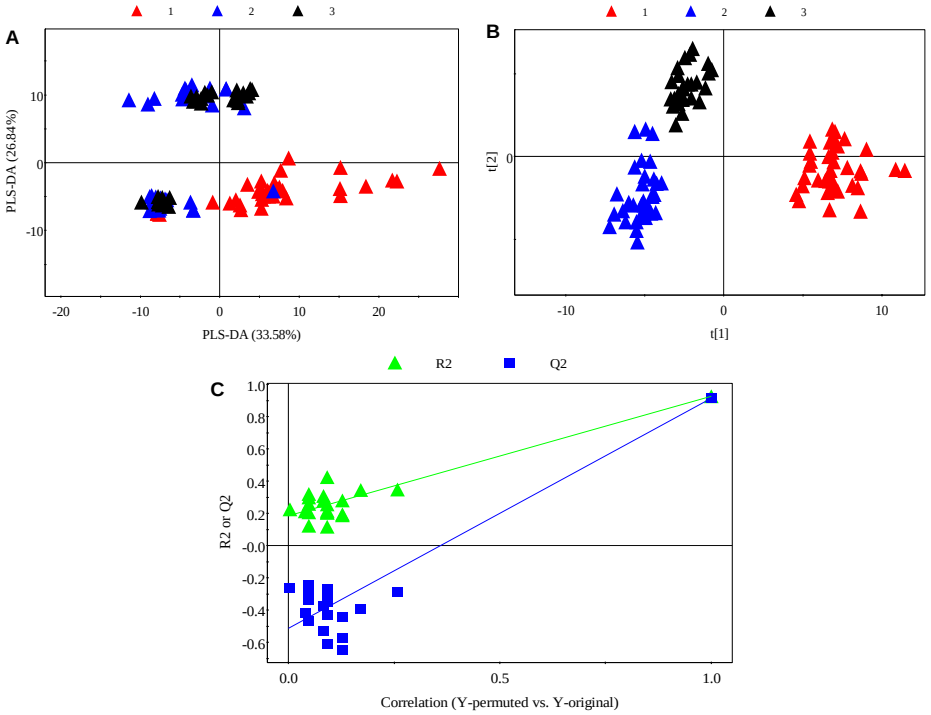

Figure 3: Score plots of PLS-DA (A) and O2PLS-DA (B) and validation plot of permutation test for PLS-DA (C). Samples with high, medium, and low activity are presented red, blue and black colors.

Projection to latent structures (PLS) is a supervised data reduction algorithm in which actual activity values were used instead of dummy *Y*-variables. The score plot of PLS analysis (Figure 4A) shows that samples with high activity are well separated, but no distinction was found among low and medium activity samples. Bidirectional orthogonal PLS was also used in order to separate all the three classes of samples but as shown by the score plot (Figure 4B), only high activity samples are clearly separated from the others. Permutation test is often used for validation of PLS and PLS-DA. The R2 and Q2 values for PLS and PLS-DA were calculated using six components for both analyses.

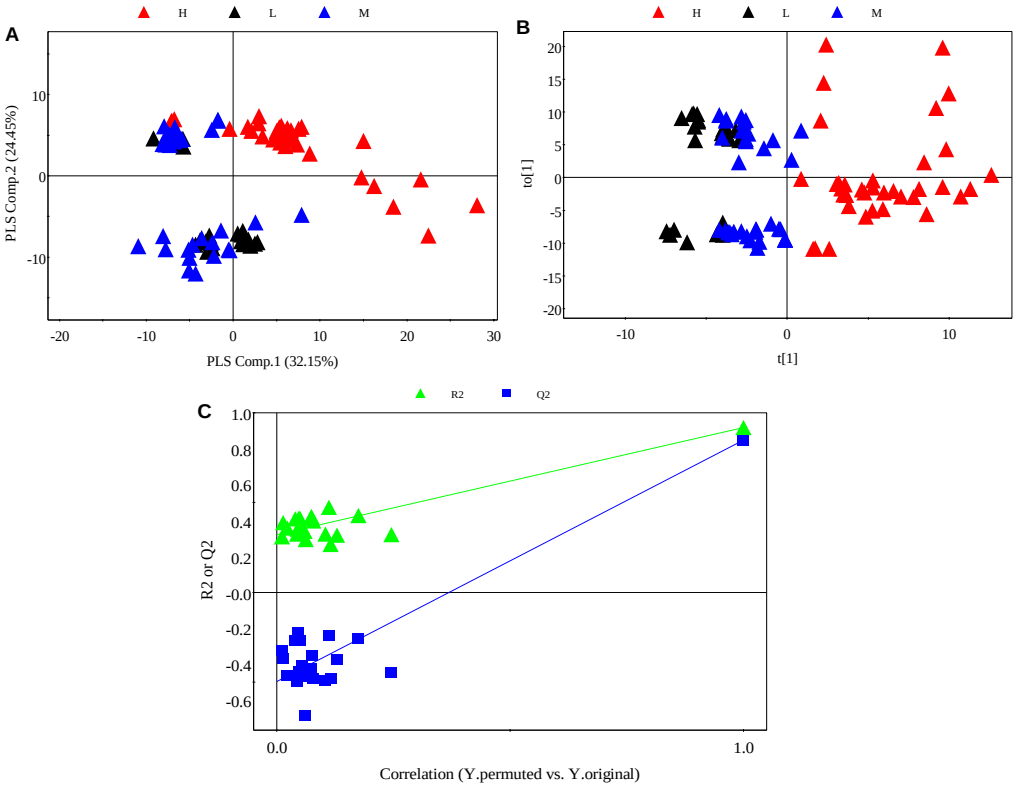

Figure 4: Score plots of PLS (A), O2PLS (B) and validation plot of permutation test for PLS (C). Samples with high, medium, and low activity are presented in red, blue and black colors.

For anti-TNF-α activity the R2 and Q2 values for PLS analysis were 0.91 and 0.84, respectively, while for PLS-DA these figures were 0.92 and 0.91 (Figure 4C and 3C). For O2PLS-DA and O2PLS analyses, VIP values for several phenolic compounds, responsible for separation on the score plot, are calculated. It has been reported that factors with VIP values more than 0.7 could be regarded influential for the separation of samples (L. Eriksson et al., 2006). VIP values of the major contributing metabolites are as follows; caftaric acid at δ 7.02: 1.91, quercetin at δ 7.81: 1.74, coutaric acid at δ 7.68: 1.42, and (+)-catechin at δ 5.92: 1.18. It is thus assumed that these compounds with high VIP scores are directly related to the inhibition of TNF-α production.

Various multivariate data analysis methods are used in combination with NMR spectroscopy in order to correlate the activity data of the extract with the spectroscopy data of the same. Several studies showed the analysis of the

extracts (Bailey et al., 2004; Cho et al., 2009; Roos et al., 2004) with NMR spectroscopy using chemometrics methods to identify the active compounds. This approach is very effective in the screening of various plant extracts in order to identify the plant compounds with an activity without laborious fractionation and chromatographic separation of the crude extract. Fractions from SPE of various red wines from Portugal were analyzed for anti-TNF-α activity and the combination of NMR spectroscopy and chemometrics was successfully applied to identify the active ingredients.

The vintage effect on the metabolic profile of grapes and ultimately on wine has been extensively studied (Lee et al., 2009; Pereira et al., 2006) and the importance of the vintage is widely accepted. The amino acids and polyphenols contents are highly affected by the climatic conditions of a grape production area. It has been shown that a hot and dry climate results in a higher proline and phenolic contents in wine (Lee et al., 2009). This study is also capable to highlight the effects of vintage on the TNF-α inhibition potential of different wines. It is evident from the results that samples from 2010 are more active than samples from the other vintages and based on this observation it can be postulated that in the 2010 vintage the berries experienced a hot and dry climate which ultimately resulted in higher phenolic contents and more potency towards TNF-α inhibition.

Our diet has beneficial effects on health and the consumption of antioxidant rich food (fruits, vegetables, tea, and wine) may have health promoting effects. The medicinal importance of moderate wine consumption has been proven by many studies. Wine metabolites, especially phenolics, are now well known to act against cardiovascular diseases (Cordova and Sumpi, 2009), renal disorders (Bertelli et al., 2005), Alzheimer's disease (Marambaud et al., 2005), cancer (Barstad et al., 2005), and also against bacteria (Murray et al., 2002) and viruses (Takkouche et al., 2002). Several health promoting activities associated to wine polyphenols were comprehensively reviewed recently (Ali et al., 2010b; Cordova et al., 2009; Halpern, 2008; Opie and Lecour, 2007). Phenolics are well known for their potency to inhibit TNF-α production as they are widely accepted to have anti-oxidative and antiinflammatory properties (Baur et al., 2006; Chuang et al., 2010). Phenolics like resveratrol (Stewart et al., 2008) and quercetin (Rivera et al., 2008) are known to reduce inflammation, while others like cinnamates, benzoates, flavonols, flavan-3-ols, and anthocyanins, are well known antioxidants (Lee et al., 2009; Meyer et al., 1997).

7.4. Conclusion

In this study, solid phase extraction integrated with NMR spectroscopy and multivariate data analysis methods were used to identify the correlation of metabolites with a TNF-α inhibition data set. Active ingredients in an extract could be identified using PLS-based regression models with ^{1}H NMR and activity data set. Phenolics like quercetin, caftaric acid, and (+)-catechin are identified as most influential in inhibiting TNF-α production among the other wine metabolites. The approach presented here, can be applied for the identification of TNF-α inhibiting compounds in crude plant extracts using NMR and multivariate data analysis. The methodology can also be applied to infer the various bioactivities associated to wine without any laborious chromatographic separation of metabolites.

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Chapter 8

NMR spectroscopy coupled with multivariate data analysis to assess antiinflammatory activities of *Eugenia uniflora* fruits in different developmental stages

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Abstract

Eugenia uniflora is widely used in Argentina, Brazilian and Paraguayan folk medicine. In this study, crude extracts of berries in different developmental stages were examined by assessing their effect on the production of TNF-α in lipopolysaccharide (LPS) stimulated U937 cell lines. Zebrafish embryos expressing fluorescent protein were used for *in-vivo* studies. Berries were staged into green, yellow, red and purple according to the period towards maturity. The fruits at the green stages presented significant antiinflammatory activity in both the assays followed by yellow, purple and red stages. NMR spectroscopy together with multivariate data analysis was applied to identify the compounds responsible for activity. Projections to latent structures (PLS) were found effective in discriminating high activity samples from low activity samples. By analysing the coefficient plot, the active constituents in the high activity samples have been identified as quercetin, myricetin, kaempferol, cinnamic acid

and chlorogenic acid. NMR spectroscopy proved to be a valuable tool for identifying compounds responsible for activity.

Keyword: *Eugenia uniflora*, NMR, TNF-α, inflammation, zebrafish, multivariate data analysis

8.1. Introduction

Inflammation is a response of the innate immune system to external stimuli and an essential part of the healing process without which the affected area could not be cured. Several factors such as infections, ultraviolet exposure and injuries play an important role in inducing inflammation. In an inflammatory process, macrophages, mast cells or other monocytes release many types of mediators including chemokines and cytokines, one of which is the tumor necrosis factor-alpha (TNF-α). Primarily, it plays an important role in regulation of immune cells but also takes part in the initiation of several inflammatory diseases (Habtemariam, 2000).

The healing process involves not only the release of mediators such as TNF-α but also the recruitment of leukocytes, which in turn release other mediators (Comalada *et al*., 2006). Any injury or infection accelerates the release of pro-inflammatory cytokines, chemokines or prostaglandins, which in turn produce the adhesion of leukocytes or white blood cells to the infected area. Among these, the first in situ responders are neutrophils, type of white blood cells present in large amount in blood. Neutrophil-induced inflammation is important in the wound-healing process however failure to regulate the recruiting can cause irreparable damage to the infected site (Renshaw *et al*., 2006).

Once the infected site is healed, the process is interrupted. In case of the overproduction of mediators, such as TNF-α, however, chronic conditions including inflammatory bowel disease, rheumatoid arthritis or even septic shock can occur (De Rycke *et al*., 2005; Singh *et al*., 2001). Furthermore, people prone to chronic inflammation are diagnosed with various types of cancer in several studies (Karin and Greten, 2005; Mantovani *et al*., 2008).

Due to an increasingly unhealthy life style, inflammatory diseases are becoming ever more common and the synthetic drugs used to treat them are not entirely satisfactory, among other reasons, for their negative side effects (Hu, 2011). An alternative to these drugs is the use of natural products, a potential source of new bioactive compounds (Iqbal *et al*., 2012).

Eugenia uniflora, also known as "arrayán" in Argentina,"Surinam cherry", "cerezo Brazileño", "cereza de Cayena", "pitanga" in Brazil, "pendanga" in Venezuela, "guinda" in El Salvador, ñanga-piré and "cereza cuadrada" in Colombia, belongs to the family Myrtaceae, indigenous to

Argentina, Southern Brazil, Surinam, Guyana, Uruguay and also commonly dispersed in other South American countries. People consume this fruit as fresh fruit, juice, frozen pulp, or jam. It is also used to make typical liquor in Northeastern Brazil (Porcu and Rodriguez-Amaya, 2008). It is a 7- to 10-ribbed berry that ranges between 1.5 and 5.0 cm long and is known for its exotic flavor. While ripening, its taste varies from very acid to sweet (Malaman *et al*., 2011). Epicarp of the fruit changes from green to yellow, orange, dark red and finally almost black in order from (Celli *et al*., 2011).

Polyphenols like flavonoids and leucoanthocyanidins are characteristic secondary metabolites of this species, apart from steroids and/or triterpenoids in leaves (Bandoni *et al*., 1972). High amounts of catechins, flavonols, and proanthocyanidins are found in the ripe fruits collected from Brazil and Argentina, all of which are known for their antioxidant activity (Einbond *et al*., 2004). The fruits are also rich in carotenoids which are described as vitamin A precursors: the carotenoids present in the Brazilian and Argentine berries are *trans*-lycopene, *trans*-rubixanthin, *trans*-β-cryptoxanthin, 13-*cis*-lycopene and lower amounts of zeaxanthin, *cis*-rubixanthin, lutein and γ-, α- and β-carotene (Azevedo-Meleiro *et al*., 2004; Porcu and Rodriguez-Amaya, 2008). Lycopene is the most important carotenoid, comprising of 46% of the total carotenoid content (Filho *et al*., 2008). The characteristic flavour of the Brazilian cherry was credited to sesquiterpenes and ketones identified by (Malaman *et al*. 2011).

Several disease and disorder like bronchitis, chest cold, cough, gout, sore throat, hypertension, headaches, influenza, hepatic diseases, painful urination, rheumatism, diarrhea, fever stomach diseases and other gastro-intestinal disorders are treated from the extracts of *E.uniflora*. There are also reports of its diuretic and insect repelling properties and of its ingestion as a tea to ease the process of child-birth (Begossi *et al*., 2002; Consolini *et al*., 1999; Schapoval *et al*., 1994). It is used to treat obesity, diabetes and to stimulate menstrual flow. Volatile oil of this plant has been reported to contain digestive, eupeptic and carminative properties. Hot water extract of the fresh leaf and unripe fruit is used as remedy to treat malaria and fever in Nigeria. Due to its high content of carotenoids and phenolic compounds the *E. uniflora* fruit can be considered to be a strong candidate for cancer prevention (Bagetti *et al*., 2011; Celli *et al*., 2011).

Transgenic line of zebrafish expressing green fluorescent protein in neutrophils was used to study in-vivo. Due to the transparency of zebrafish embryo, it is possible to visualize the movement of neutrophils towards the

affected site. Other advantages of zebrafish embryo are their limitless availability, low cost and ease of handling. Apart from this they require very little medium for growth, so that they are cost effective if compared with other mammalian models (Kari *et al.*, 2007).

The metabolomic study and identification of active compounds in natural products requires the use of different platforms, e.g., gas or liquid chromatography in combination with mass spectrometry (Kobayashi *et al*., 2012; Staszkow *et al*., 2011) or nuclear magnetic resonance (NMR) spectroscopy. In this case, we chose the latter as the main tool to characterize the compounds responsible for the pharmacological effects. Objections to NMR for its low sensitivity are outweighed by its numerous advantages since it involves simple sample preparation, short analysis time, it is non-destructive, non-selective and highly reproducible. Additionally it allows the direct quantification of all compounds without the need of calibration curves or reference substances. Altogether it is thus the ideal tool for a broad metabolomic analysis (Son *et al*., 2009). The low sensitivity of NMR that hinders the detection of secondary metabolites present at low concentrations can be counteracted with different extraction techniques such as liquid-liquid fractionation, removal of sugars by solid phase extraction or 2D NMR methods (Ali *et al*., 2012; Kim and Verpoorte, 2010). Based on previous report that showed that TNF-α activity differs according to their developmental stage (Ali *et al*., 2012), our main objective here was to characterize the metabolic profile of *E. uniflora* fruits at different stages of their development and explore their potential as anti-inflammatory agents.

8.2. Materials and Methods

8.2.1. Sampling

Fruits of *Eugenia uniflora* L from 4 different ripening stages were used. Fruits were classified as followed: green (inmature), orange, red and purple (mature). The berries were collected around 10 a.m. in November of 2009 at Nogalito's wood located in El Siambón, Tucumán (Northwestern Argentina). Samples were immediately transported to the laboratory in dry ice. Five biological replicates (each including 80-100 berries) were realized. Each replicate contained berries from a single plant, and from the sunny and shady

sides of the plants. Berries were grinded in liquid nitrogen, seeds removed and kept at -20 ºC until use.

8.2.2. Extraction

A sample of 50 mg of lyophilized fruit was extracted according to (Kim *et al*., 2010). Briefly, powdered dry plant material 50 mg was taken into 2mL eppendorf tube and extracted with 50% aqueous methanol followed by ultrasonication at room temperature for 20 minutes. The procedure was repeated three times. The supernatant was pooled together and dried using rotary evaporator.

8.2.3. Cell Culture

Human monocyte-like histiocytic lymphoma U937 cells were cultured and treated with plant extracts as described in chapter 3.

8.2.4. TNF-α assay

TNF-α in culture supernatants were determined by quantitative ‘‘sandwich’’ enzyme-linked immunosorbent assay as described in chapter 3.

8.2.5. MTT assay

Cell viability after treatment with fruit extracts in different ripening stages was determined by using MTT assay (Lee et al., 2007) as described in chapter 3.

8.2.6. Zebrafish culture

Standard procedures (in agreement with local animal welfare regulations) were adopted to raise and maintain Zebrafish (*Danio rerio*) embryos. The GFP Transgenic lines (MPO,s) of zebrafish were used in this study (Lawson & Weinstein, 2002). Embryos were obtained by natural crosses.

Fertilized eggs were collected and staged as previously described by (Kimmel *et al*., 1995).

8.2.7. Chemical induced inflammation assay (ChIn)

Assay was performed as described by (d'Alencon *et al*., 2010). Briefly, E3 medium was used to grow zebrafish larvae of the GFP strain. They were kept in Petri dish until 56 hours post fertilization. Spontaneously hatched larvae were transferred to 48- well plates at the rate of 1 larva/well in a volume of 500 µL of E3 solution. Fruit extracts and controls were pipette to the wells containing embryos 1 hour before the addition of $CuSO_4$. Plates were incubated for 40 minutes at 28 °C. E3 medium was replaced with 4% paraformaldehyde in PBS buffer which was used to fix the embryo and further incubation was carried out for 1 hour at room temperature. Fixing and subsequent steps normally carried out in dark to evade fading of the fluorescent protein signal. Larvae were washed with PBS-Tween20. Fluorescent cells were examined and counted within the next 48 hours after fixation using a Leica (Wetzlar, Germany) MZ-12 fluorescent stereoscope. Labeled cells were within a specific area known as myoseptum which consist of between the first somite and the end of the tail on one side of each larva. Sixteen embryos were used for each concentration and cells were counted by two independent observers.

8.2.8. ^{1H} NMR spectroscopy

50 mg lyophilized sample of berries was extracted according to (Kim *et al*., 2010). Briefly samples were transferred to 2 ml eppendorf tubes, 1ml of MeOD and D_2O buffer with 0.01% TSP (1:1) was added. Sample was vortexed for 30 second and then sonicated for 15 minutes. After sonication sample was centrifuged and clear supernatant (800 ul) was transferred to the 5-mm NMR tube and used for NMR analysis. Deuterated methanol was purchased from Cambridge Isotope Laboratories, Inc., Andover, MA, USA. ^{1}H. NMR spectra were recorded at 25 °C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz. MeOH- d_4 was used as the internal lock. Each ^{1}H NMR spectrum consisted of 128 scans requiring 10 min and 26 sec acquisition time with the following parameters: 0.16 Hz/point, pulse width (PW) = 30° (11.3 µsec), and relaxation delay (RD) = 1.5 sec. A pre-saturation sequence was used to suppress the

residual H_2O signal with low power selective irradiation at the H_2O frequency during the recycle delay. FIDs were Fourier transformed with LB = 0.3 Hz. The resulting spectra were manually phased and baseline corrected, and calibrated to TSP at 0 ppm, using XWIN NMR (version 3.5, Bruker). 2D NMR techniques were performed by using parameters described by (Ali *et al*., 2012)

8.2.9. Data analysis

The ^{1}H NMR spectra were reduced to ASCII files. Bucketing was performed by AMIX software (Bruker) with scaling to total intensity. Spectral intensities were scaled to the TSP signal (δ 0.0) and reduced to integrated regions of equal width (0.04) corresponding to the region of δ 0.3–10.0. During analysis, regions between δ 4.75–4.9 and δ 3.28–3.40 were excluded because of the residual signal of water and methanol-d_4, respectively. SIMCA-P software (v. 12.0, Umetrics, Umeå, Sweden) was used to perform principal component analysis (PCA) with scaling based on Pareto while partial least square (PLS) with scaling based on Unit Variance. Means and standard deviations were calculated. ANOVA was performed for comparison with means and significance level was set at <0.05.

8.3. Results and discussion

Metabolites variations of four different developmental stages of *Eugenia uniflora* have been evaluated. All these four stages differ both quantitatively and qualitatively from each other. The differences between these stages can be sorted out by using a simple metabolomic approach with the help of ^{1}H NMR. First NMR spectra were phased; base line corrected and compared visually to see any visible change during the development of the fruit (Fig.1). Here the developmental stages of *Eugenia uniflora* has been divided into four phases namely green, yellow or yellow orange, red and purple. Green is the earliest and purple to be the most ripened. Representative ^{1}H NMR spectra's of these four stages are shown in Figure 1. From this figure we can see that the developmental stages follow a pattern. Green stage contain a high amount of phenolics like quercetin, myricetin, kaempferol, shikimic acid, chlorogenic acid and amino acids like glutamate and glutamine which decreases gradually as the fruit ripens. While we also see the decrease in the chlorogenic acid. On the other

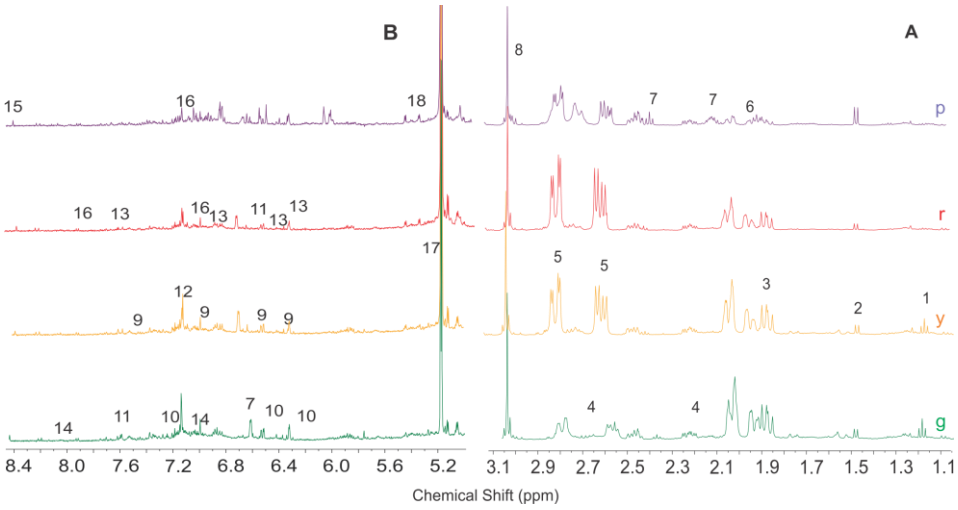

Figure 1: Comparison of ^{1}H NMR spectra of four developmental stages from *Eugenia uniflora.* g (green), y (yellow), r (red), p (purple). 1. Ethanol. 2. Alanine, 3. Quinic acid, 4. Glutamine/Glutamate, 5. Malic acid, 6. GABA, 7. Shikimic acid, 8. Creatine, 9. Quercetin, 10. Myricetin, 11. Cinnamic acid, 12. Gallic acid, 13. Chlorogenic acid, 14. Kaempferol, 15. Formic acid, 16. Histidine, 17. Fumaric acid, 18. Glucose, 19. Sucrose.

hand malic acid seems to increase up to red stage and to decrease in the purple stage, whereas alanine increases along with the ripening stages.

Multivariate data analysis is a technique to filter out the most important variables affecting the results. The main aim of using this statistical technique is to reduce the dimensionality of the data (Eriksson et al., 2006). Among the different tools available the most common used are Principal component analysis (PCA), partial least square analysis (PLS) and Orthogonal partial least square analysis (OPLS). Where PCA is an unbiased analysis the other two mentioned methods depend upon the input information given. The most important information obtained by the supervised biased analyses is the correlation of the certain variables with the given information, e.g. identification of markers.

To analyze and identify the most important metabolites characteristic for the developmental process of *Eugenia uniflora*, we subjected NMR data to PCA. The obtained results are displayed in Fig. 2. The PCA score plot reveals a pattern in which green stages tend to cluster on the negative side of PC1 along with the yellow stages. Few replicates of the yellow stages lie closer to the red stage in the positive side of PC1. Both Red and purple ripened stages are on the positive side of PC1. This result indicates good separation of the four developmental stages on the basis of the time of development. The loading

column plot which is the projection of the variables in our case metabolites of the cherries revealed that green and yellow stages are rich in phenolics, organic acids and amino acids while the more ripened stage obviously has high content of sugars like sucrose, fructose and glucose.

Among the four stages, low TNF-α inhibition was exhibited by mature stages, while highest activity was obtained by the green stage followed by the yellow stage as shown in Fig 3A. From the figure it is clear that initial developmental stage (green) has highest inhibition activity as compared to the others, similar results were observed in grapes (Ali et al., 2012).

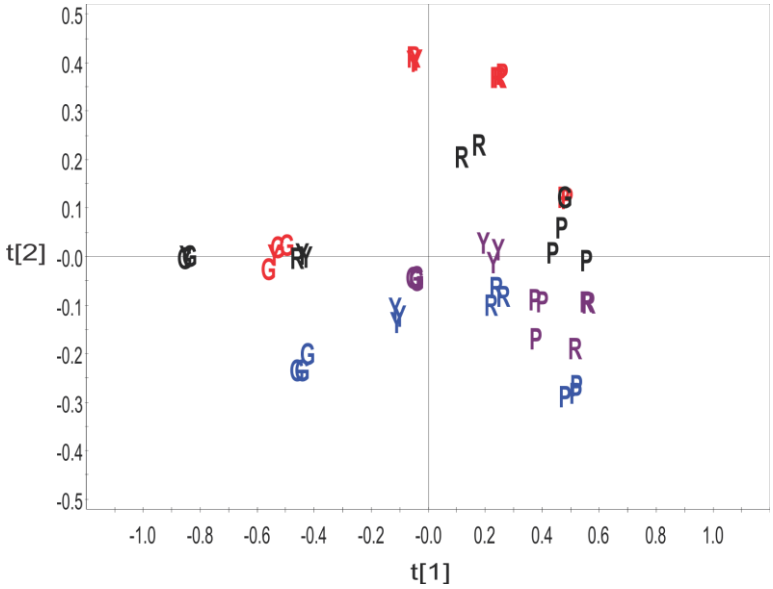

Figure 2: Scatter plot of stage development. G stands for green, P stands for purple, Y stands for yellow and R stands for red.

In-vivo analysis was done by using mutant zebrafish embryos as a model. A representative figure of control and zebrafish embryo treated with $CuSO_4$ with and without fruit extract is shown in Fig 3. B. The fluorescents cells represent the sites of injury after treating the embryo with 10 µM $CuSO_4$. The zebrafish embryo treated with $CuSO_4$ in presence of fruit extract exhibit less number of leukocytes movement as compare to embryo treated with $CuSO_4$ without fruit extract. Fig 3.C shows results similar to the *in-vitro* TNF-α inhibition. The green stage of fruits would seem to be dominantly significant from other stages in term of controlling or inhibiting the injury. To identify

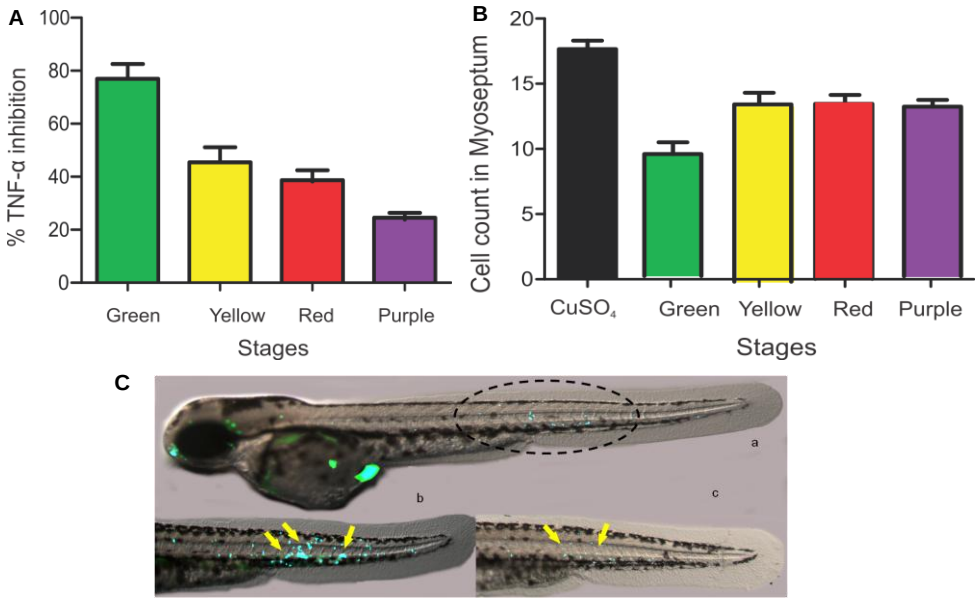

Figure 3 A: TNF-α inhibition (%) exhibited by four different developmental stages of *Eugenia uniflora* at the dose rate of 100 µg/ml. Bars represent standard error of Means (*n*=3). 3B (a): 56hpf zebrafish embryo with fluorescent leukocytes. (b) myoseptum area of 10 µM $CuSO_4$ treated embryo (yellow arrows shows the area of injury with fluorescent clusters of leukocytes). (c) myoseptum area of extract treated embryo exhibit less number of leukocytes movement as compare to $CuSO_4$ treated embryo. 3 C. Quantification of migrating leukocytes after treatment with extract at the dose rate of 100 µg/ml. The higher the number of cells the lower the activity of extracts.

compounds related to activity activity for TNF-α inhibition, a supervised method (Partial least square model, PLS) was applied. TNF-α inhibition value was added as *Y* -input to the model. Activity values were divided into to two classes: active (>60%) and non active (<60%). The score plot for the resulting model is shown in Fig 4 A. The red dots show the stage responsible for high TNF-α inhibition activity, while the black triangles show samples with less activity for TNF-α inhibition. One replicate from the study has been excluded due to some experimental error. As the PLS score plot shows, a nice separation between active and non active groups have been achieved with PLS1 (65%) and

and PLS2 (13%). The stage related to high activity is on the positive side of vector t1 of the PLS scatter plot while stages correlating with less activity cluster on the negative side of t1. By examining the corresponding Y-coefficient loadings plot Fig 4.b, we find high content of phenolics like quercetin, myricetin, kaempferol, cinnamic acid, chlorogenic acid, and amino acids like glutamate and glutamine which are corresponding with the inhibition of TNF-α.

For *in-vivo* analysis cell counts were used as *Y*-input value. Fruit extracts able to reduce the cell count to less than10 were assigned as active while extracts unable to reduce the cell count less than 10 were assigned as non active. The resulting model does not show a clear distinction among the active and non-active samples (data not shown) which can be due to noise in the spectra. To improve the correlation of activity with metabolites, a built-in filter was used called orthogonal signal correction (OSC). This filter removes the

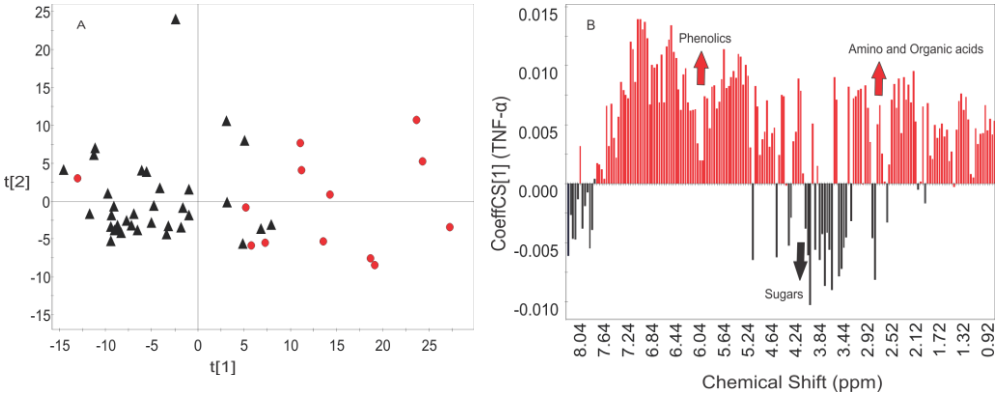

Figure 4A: PLS score plot for *in vitro* activity based on whole range of ^{1}H NMR spectra. Triangle shows non active extracts, while red dots represent active extracts for *in vitro* anti inflammatory activity. 5B. Coefficient plot for *in vitro* PLS score plot. Black bars on negative side of coefficient plot relate with the non active extracts while red bars on the positive side of coefficient plot relate with active extract for *in vitro* activity.

unwanted and uncorrelated signals by using orthogonal logarithm (Ali et al., 2012; Eriksson et al., 2006). After applying their filter the remaining data were again subjected to PLS modeling, the resulting model was highly improved with PLS1 (37%) and PLS2 (32%) values. From the score scatter plot (Fig.5 A), we can see that the active stages are on the negative side of the score plot or PLS1 while the non active stages are on the positive side of the PLS1 plot. The relevant coefficient plot (Fig.5.B) showed a high content of phenolics compounds like quercetin, myricetin, kaempferol, organic acids like cinnamic

acid, chlorogenic acid, malic acid and amino acid like glutamate and glutamine for the active stages the PLS1 while high sugar content is on the non active side of PLS1. These results confirm the findings that high phenolic contents are responsible for antiinflammatory activities both *in-vivo* as *in-vitro*.

It is a well known fact that synthetic drugs are costly and associated with risks to human health; hence efforts to develop safer and more effective medicines are essential. Natural products provide an alternative source for developing antiinflammatory drugs. Agents derived from plants can modulate the expression of pro-inflammatory signals. These include flavonoids, quinones, catechins, anthocyanins and anthoxanthins, terpenes and alkaloids, all of which are known to have anti-inflammatory effects (Paul et al., 2006).

Flavonoids present in the plants either simple or complex glycosides. These polyphenolic compounds and their sugar derivatives display a remarkable spectrum of biological activities including anti-inflammation (Miles et al., 2005; Pietta, 2000). Several reports have been published related to activities of the mentioned flavonoids against TNF-α production (Chuang et al., 2010; Park et al., 2008). There are reports that quercetin inhibits TNF-α secretion selectively in different cell studies (Wadsworth and Koop, 1999; Wadsworth et al., 2001). Myricetin, another flavonoids have been reported to inhibit TNF-α production

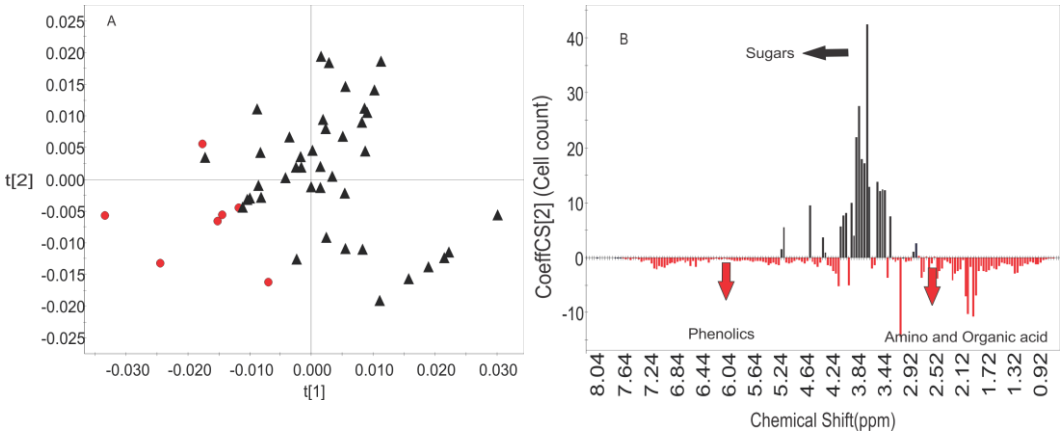

Figure 5A: OSC-PLS scatter plot for *in-vivo* activity. Triangle shows non active extract, while red dots represent active extract for *in-vivo* anti inflammatory activity. 6B. Coefficient plot for *in-vivo* OSC- PLS score plot. Black bars on positive side of coefficient plot relate with the non active extracts while red bars on the negative side of coefficient plot relate with active extract for in vitro activity.

in LPS stimulated J774.1 cell lines (Herath et al., 2003). Similarly, phenolics

acids including caffeic acid, gallic acid, ferulic acid, *p*-coumaric acid and chlorogenic acids are well known inhibiting TNF-α production (Chauhan et al., 2011; Kim et al., 2006; Sakai et al., 1997; Shin et al., 2004).

The application of combinations of chemometric methods with NMR spectroscopy is gaining popularity among researchers due to the wealth of information they provide. This approach is very effective in the screening of plant extracts allowing the identification of active compounds without laborious fractionation and chromatographic separation of the crude extract. When applied to extracts of *Hypericum perforatum*, *Artemisia annua*, *Citrus grandis*, and *Galphimia glauca* it proved to be very successful in linking pharmacological activities with certain compounds (Bailey et al., 2004; Cardoso-Taketa et al., 2008; Cho et al., 2009; Roos et al., 2004). In this study, diverse multivariate data analysis methods were used in combination with NMR spectroscopy in order to correlate the activity data of the extracts with their spectroscopic data. Crude extracts from *E.uniflora* fruit were studied for anti-TNF-α activity and the combination of NMR spectroscopy and chemometrics was successfully applied to identify the metabolites quercetin, myricetin, gallic acid, cinnamic acid, and chlorogenic acid as those responsible for their high anti-TNF-α activity.

8.4. Conclusion

The present study is the first to analyze *Eugenia uniflora* at different developmental stages for TNF-α inhibition and neutrophils migration towards wounded area in Zebrafish using an NMR based metabolomic approach.NMR spectroscopy (1D and 2D) was applied for the metabolic profiling of *Eugenia uniflora* berries. The crude extracts (8:2) methanol:water of berries were tested for TNF-α inhibition and antiinflammatory activity. Green stage of berries was found active in both assays. Various multivariate data analysis methods showed good correlation between the NMR resonances for phenolics and anti-TNF-α activity. Algorithms like PLS and PLS-DA showed good separation among the samples classified as high and low activity with high model validity. Metabolites like quercetin, myricetin, gallic acid, cinnamic acid, and chlorogenic acid, were statistically significantly correlated with high activity. Using the presented approach, the analysis of NMR shifts in relation to pharmacological activity can provide information about what part of the NMR spectrum (aromatic or aliphatic regions) correlates with the activity which in

turn gives information about the active ingredients in crude extracts of functional food. Our study suggests a potential use of edible fruit as a source of anti-inflammatory agents.

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Chapter 9

Application of NMR spectroscopy integrated with comprehensive extraction method to find antiinflammatory active compounds in the plant of *Sempervivum pseudocalcareum*

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Abstract

A novel approach was applied to investigate the potential antiinflammatory activities of genus *Sempervivum in-vitro* by using U937 cell lines assay and zebrafish embryos for *in-vivo* studies. Twenty four different species/cultivar of *Sempervivum* were screened for their ability to inhibit TNF-α release. *Sempervivum Smaragd, Sempervivum Pilatus, Sempervivum Noir, Sempervivum Pseudo-calcareum, Sempervinum microcephalum, Sempervivum tectorum Glaucum* showed maximum TNF-α inhibition respectively. Two different *Sempervivum pseudocalcareum* (active) and *Sempervivum calcareum* (non active) (cultivar of *Sempervivum tectorum* L.) were selected for further studies on the basis of our screening results. Comprehensive extraction was applied to cover a broad range of metabolites by using a gradient from water to methanol in combination. Seventeen fractions were eluted and subjected to NMR analysis and activity related studies. NMR based metabolomics coupled with multivariate data analysis was used to identify bioactivity related metabolites in fractionated plant extracts. Fractions (4-6) of *S. pseudocalcareum* show strong inhibition of TNF-α *in-vitro* and for *in-vivo* studies fractions (2, 3) were found most promising whereas in case of *Sempervivum calcareum* only fraction 6 was found active *in-vitro* but none of the fraction showed activity *in-vivo* .

Multivariate data analysis likes PCA, PLS, PLS-DA, O2PLS-DA and O2PLS were used to see discrimination among samples and to correlate NMR signals with activity data. The VIP plot showed that kaempferol analogues were positively correlated with high activity. The bioassays presented here allow us to screen thousands of plant extracts and pure compounds which show this is a rapid way of finding new lead compounds for drug development.

Key words: *Sempervivum pseudocalcareum*, *Sempervivum calcareum*, inflammation, zebrafish, bioactivity, metabolomics, multivariate data analysis

9.1. Introduction

Inflammation is the reaction of the body's defense system (immune system) which protects the body from harmful stimuli such as pathogens, damaged tissues and irritant. The process of inflammation is regulated by several cytokines. Some cytokines induce inflammation, make the diseases worse and are called pro-inflammatory cytokines such as TNF-α, IL-1 and IL-18 while others take part to reduce inflammation, accelerate the process of healing and are called antiinflammatory cytokines like IL-4, IL-6, IL-10, IL-11, and IL-13 (Dinarello, 2000). Tumor necrosis factor alpha (TNF-α) is one of the most important pro-inflammatory cytokines which promotes inflammation in different disease conditions like septic shock, AIDS, cancer, rheumatoid arthritis, diabetes and several other disease. An optimum level of TNF-α is beneficial for the human body but increased production can lead to the development of chronic diseases related to autoimmunity (Paul et al., 2006). Thus anti-TNF-α therapies are considered for treatment of inflammatory diseases. Although protein based drugs are available for TNF-α inhibition, they have limitations such as high costs and health affecting side effects. Plants are considered as an alternate source for drugs for treatment of diseases mediated through TNF-α. Several investigations have been published regarding inhibition of TNF-α by plant crude extracts and pure natural compounds (Yuliana et al., 2011a).

The plant kingdom is able to produce an enormous amount of different chemicals as a response to variable and harsh environmental conditions. Among these chemicals, a vast number expose pharmacological interesting properties. Since ancient times plants with medicinal activities were used by mankind. Knowledge on the specific medicinal activity of a particular plant or herbal mixture was based on long historical clinical use. In modern science however knowledge of the active constituents (metabolites) in an herbal- or plant derived preparation is required in order to meet the demands of quality control, safety and efficacy. The complexity of the (multi) metabolic content, the variability in the metabolic content due to the plants responses to changes in environmental conditions and synergistic effects are serious bottlenecks for the identification of bioactive metabolites in herbal extracts (Li et al., 2011). In recent years it became more and more clear that a holistic approach based on metabolomics technology is very promising to overcome these bottlenecks (Jiang et al., 2010; van der Kooy et al., 2009; Yuliana et al., 2011b; Zhang et al., 2010). Such a technology enables to link metabolites in multi-constituent plant extracts,

obtained by variations in cultivation or extraction procedures, to bioactivity data by using statistical data analysis (Hou et al., 2010; Yuliana et al., 2011c).

Nuclear magnetic resonance (NMR) spectroscopy has been commonly used to study metabolic profiles of plants, as well as for identification, characterization and structure elucidation of molecules (Ali et al., 2010; Son et al., 2009). NMR spectroscopy is a prime tool for metabolomic studies. NMR is often criticized because of its low sensitivity but NMR also provides advantages of being non destructive in nature, simple sample preparation, short analysis time and ease in quantitation (Dixon et al., 2006). NMR based metabolomics has been applied successfully in combination with different multivariate data analysis methods in different types of biological or medical studies (Ali et al., 2011b; Charlton et al., 2002). For example several reports have been published on NMR in combination with multivariate data analyses to identify pharmacologically active substances in plants extracts (Bailey et al., 2004; Cardoso-Taketa et al., 2008; Cho et al., 2009; Roos et al., 2004).

A large amount of variable extracts have to be tested *in-vitro* cell based assays and molecular based assays in order to discover novel bioactive compounds. However, using *in-vitro* assays of herbal or plant extracts have some major drawbacks: (i) the multi-constituents of a herbal or plant extract may probably act on multiple targets in an organism (Lan and Jia, 2010) and (ii) the effected composition of the herbal/plant extract in *in-vitro* assays may significantly differ from the *in-vivo* effect due to miscellaneous and uncontrolled (enzymatic) biotransformations and/or differential absorption characteristic during intake in the body of an organism (Lan et al., 2010; Unger, 2010). Therefore the availability of high-throughput *in-vivo* models is highly desirable for active compound identification in herbal extracts. In the last decade, Zebrafish has emerged as a model organism for different scientific studies. Zebrafish as a model organism offers lot of advantages over other animals in term of cost, availability, and handling. Recently the Zebrafish as an *in-vivo* model has been explored for metabolomic studies and discovery of bioactive molecules from nature (Deo and MacRae, 2011; Mandrekar and Thakur, 2009). By using transparent larvae it was successfully demonstrated that zebrafish could be used as a high-throughput screening assay for antiinflammatory activity after chemically induced inflammation (d'Alencon et al., 2010).

Sempervivum or Housleek (Crasssulaceae) genus comprises 30 species and over 1200 varieties. This plant is mostly found 200-2800 m above sea level

and is widely distributed in the Europe and Asia. It normally grows on chimneys, old walls, rocks and roofs. Although it is an ornamental plant, it has also been used as medicine since ancient times. Several reports have been published regarding its utilization as folk medicine. Fresh juice prepared from the leaves has been used to treat wounds, skin burns, insect bites and inflammation of the ears. A tea is also prepared from the leaves of this plant to treat ulcers. All of these activities have been attributed to the presence of phenolic compounds (quercetin, myricetin, herbacetin, kaempferol) (Abram and Donko, 1999; Šentjurc et al., 2003; Swart, 1991).

The present study first describes the screening of different cultivar of Genus *Sempervivum* for *in-vitro* TNF-α inhibition. In the second step, two cultivars *Sempervivum pseudocalcareum* (active) and *Sempervivum calcareum* (non active) were selected on the basis of the screening using an *in-vivo* Zebrafish assay. Several primary and secondary metabolites (especially phenolics) using 1D and 2D NMR techniques were identified. The correlation of activity data and NMR data using different multivariate data analyses methods for identification of the the active ingredients in *Sempervivum pseducalcareum* is also presented.

9.2. Materials and Methods

9.2.1. Plant Material

All species, subspecies and cultivars of the genus *Sempervivum* were obtained from Radder (Margraten, the Netherlands). Plants were cultivated under organic conditions. After harvesting, the aerial parts of the plants were lyophilized and subsequently milled to obtain a fine homogenized powder.

9.2.2. Extraction and Fractionation

All organic solvents were purchased from Sigma. For "crude" extraction, 150 mg powdered plant material was dissolved in 2 mL *n*-hexane, chloroform or methanol-water (80:20 v/v). The mixtures were placed in an ultrasonic bath for 30 minutes followed by intensive shaking for 30 minutes. Thereafter the mixture was centrifuged for 10 minutes at 2000 rpm, the

supernatant removed and evaporated under N_2. The dried supernatants were solved in 100 µL DMSO for *in-vitro* and *in-vivo* studies.

For "comprehensive" extraction, 750 mg of the powdered plant material was used and simultaneously extracted and fractionated as described by (Yuliana et al., 2011c). Briefly the plant material was extracted/fractionated in a linear gradient of 100% solvent A (H_2O) to 100% solvent B (methanol). During extraction/fractionation 17 fractions of 10 mL were collected; 1 mL was dried and dissolved in DMSO for determination of *in-vitro* and *in-vivo* bioactivity, the remaining 9 mL was dried and dissolved in MeOD for ^{1}H-NMR analysis.

9.2.3. In -vitro Bioactivity Assay

Human monocyte-like histiocytic lymphoma U937 cells were cultured and treated with the extracts as described in chapter 3.

9.2.4. TNF-α Assay

TNF-α in culture supernatants were determined by quantitative ''sandwich'' enzyme-linked immunosorbent assay as described in chapter 3.

9.2.5. Zebrafish

Standard procedures in agreement with local animal welfare regulations were adopted to raise and maintain Zebrafish (*Danio rerio*) embryos. The GFP Transgenic lines (MPO,s) of zebrafish were used in this study (Lawson & Weinstein, 2002). Embryos were obtained by natural crosses. Fertilized eggs were collected and staged as previously described by (Kimmel *et al*., 1995).

9.2.6. ChIn Assay

ChIn assay (Chemical induced inflammation) was performed as described by (d'Alencon et al., 2010). Briefly Zebrafish larvae of the GFP strain were grown in E3 medium in groups of 20-25 larvae per 10-cm Petri dish until

56 hours post fertilization. Spontaneously hatched larvae were used for the assay. Selected larvae were transferred to forty eight well plates at the rate of 1 larva/well in a volume of 500 µl of E3 solution. Furthermore wells were pasted with 1 drop of 1% Agarose gel to avoid sticking of embryos to the bottom before addition of larvae to the wells. Plant extracts and controls were added to the wells containing embryos 1 hour before the addition of $CuSO_4$, and incubation was carried out for 40 minutes at 28 °C. Larvae were then fixed by transferring them to 1.5-ml microfuge tubes and replacing the E3 medium with 4% paraformaldehyde prepared in phosphate-buffered saline (PBS) and incubating for 1 hour at room temperature. During fixation and subsequent handling, the tubes were kept in the dark to avoid bleaching or fading of the fluorescent protein signal. After fixation, larvae were washed three times for 5 minutes each in PBS-Tween20 with gentle agitation. Examination of fluorescent cells and counting was carried out within the next 48 hours after fixation using a Leica (Wetzlar, Germany) MZ-12 fluorescent stereoscope. Labeled cells were counted under fluorescent illumination within 10 cell diameters of the horizontal myoseptum between the first somite and the end of the tail on one side of each larva. All experiments were carried out with a minimum of 16 larvae for each condition, and counts were carried out by two observers.

9.2.7. ^{1}HNMR Spectroscopy

All the fractions eluted from comprehensive extraction were dissolved in 1mL of methanol D4. Samples were transferred to 2 ml eppendorf tubes, 1ml of MeOD and D_2O buffer with 0.01% TSP (1:1) was added. Sample was vortexed for 30 second and then sonicated for 15 minutes. After sonication sample was centrifuged and clear supernatant (800 ul) was transferred to the 5-mm NMR tube and used for NMR analysis. Deuterated methanol was purchased from Cambridge Isotope Laboratories, Inc., Andover, MA, USA. ^{1}H. NMR spectra were recorded at 25 °C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz. MeOH- d_4 was used as the internal lock. Each ^{1}H NMR spectrum consisted of 128 scans requiring 10 min and 26 sec acquisition time with the following parameters: 0.16 Hz/point, pulse width (PW) = 30° (11.3 µsec), and relaxation delay (RD) = 1.5 sec. A pre-saturation sequence was used to suppress the residual H_2O signal with low power selective irradiation at the H_2O frequency during the recycle delay. FIDs were Fourier transformed with LB = 0.3 Hz. The resulting spectra were manually phased and baseline corrected, and calibrated to TSP at 0 ppm, using XWIN NMR (version 3.5, Bruker). 2D NMR techniques were performed by using parameters described by (Ali *et al*., 2012).

9.2.8. Data Analysis

The ^{1}HNMR spectra were automatically reduced to ASCII files. Bucketing was performed by AMIX software (Bruker). Spectral intensities were scaled to total intensity and reduced to integrated regions of equal width (0.04) corresponding to the region of δ 0.3-10.0. The regions of δ 4.85-4.95 and δ 3.28-3.34 were excluded from the analysis because of the residual signal of H2O and methanol-*D4*, respectively. Principal component analysis (PCA) with scaling based on Pareto while projections to latent structures (PLS), PLS-discriminant analysis (PLS-DA), bidirectional orthogonal PLS (O2PLS), and O2PLS- discriminant analysis (O2PLS-DA) with scaling based on Unit variance were performed with the SIMCA-P+ software (v. 12.0, Umetrics, Umeå Sweden).

9.2.9. Statistical analyses

Statistical analyses were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. One way ANOVA was performed for both assays. Tukey's multiple comparison test was performed for in-vitro studies while Posthoc comparison between means and controls were made using Dunnett' s multiple comparison test. Value of $P \leq 0.05$ was considered statistically significant for both assays.

9.3. Results and discussion

The Genus *Sempervivum* has not been studied much regarding its metabolic profile and medicinal properties. *Sempervivum tectorum* L., common houseleek is a wild-growing succulent belonging to the family of Crassulaceae. In traditional folk medicine grounded leaves and leaf juice are used to relieve pain and sore ailments, particularly earache and ear inflammations. Leaves have been used traditionally for ear inflammations, warts, ulcers, skin rash and corns in Bosnia and Herzegovina. In Serbia houseleek has been used for earache as a compress made from leaf juice and applied to the sore area. In Italy fresh leaves of *S. tectorum* have been used for insect bites, while beaten aerial parts have been placed on the brow with handkerchief for headache Recently few reports have been published describing antinociceptive, liver protecting and membrane

stabilizing effect related to *Sempervivum tectorum* and flavonoids like kaempferol, quercetin, myricetin, and herbacetin have been credited for these activities (Alberti et al., 2012). This is the first study which was designed to measure antiinflammatory properties of *Sempervivum pseudocalcareum* and *Sempervivum calcareum in-vitro* and *in-vivo*. A preliminary study was performed to screen 4 species and 20 cultivars of the genus *Sempervivum* against TNF-α inhibition. Only a few cultivars showed strong TNF-α inhibition. TNF-α release caused by all these cultivars are presented in Table 1.

Species of Genus *Sempervivum*	**% TNF-α inhibition**
Sempervivum tectorum L.	59
Sempervivum pittonii NYM. et Kotschy	25
Sempervivum calcareum Jord.	0
Sempervivum arachnoideum L.	0
Cultivar of *Sempervivum tectorum* L.	
Sempervivum smaragd	96
Sempervivum pilatus	93
Sempervivum noir	90
Sempervivum pseudo-calcareum	85
Sempervivum microcephalum	83
Sempervivum tectorum glaucum	76
Sempervivum crimson piratey	70
Sempervivum feldmaier	67
Sempervivum silbergroenarneol	67
Sempervivum packardian	64
Sempervivum seerosenstern	63
Sempervivum lipari	55
Sempervivum cordeurs	53
Sempervivum brons	52
Sempervivum borisii	45
Sempervivum rubin	25
Sempervivum Van der Steen	21
Sempervivum Monique	10

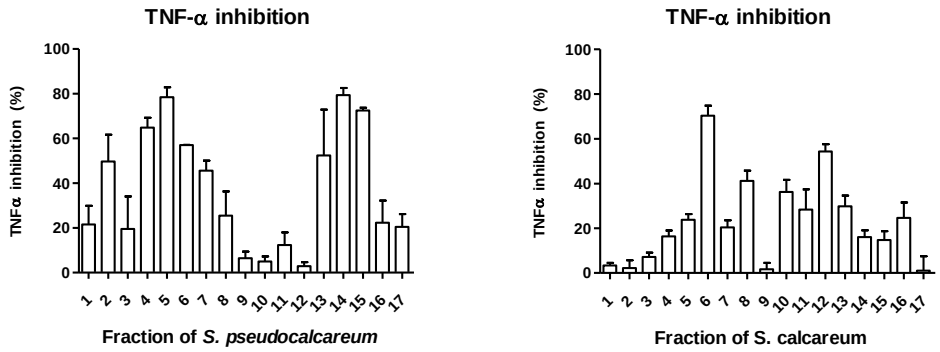

Figure 1: TNF-α (%) inhibition exhibited by different fractions (1-17) of *S. pseudocalcareum* and *S. calcareum* eluted with comprehensive extraction. Each bar represents ±SEM of three replicates.

Based on the screening, further experiments were designed to identify the metabolite responsible for this activity by comparing an active (*Sempervivum pseudocalcareum*) and non active cultivar (*Sempervivum calcareum*). The comprehensive extraction method was used to evaluate the potential antiinflammatory activity of *Sempervivum pseudocalcareum* and *Sempervivum calcareum*. Seventeen fractions were obtained and subjected to TNF-α bioassay *in-vitro*. To determine the TNF-α inhibition by plant extracts, U937 cells were treated with LPS and plant extracts simultaneously. The activity of all the fractions is presented in (Figure 1). It is clear from the figure that fractions (4-6) and (14,15) of *Sempervivum pseudocalcarem* show significant high activity as compared to other fractions while in case of *Sempervivum calcareum* only fraction 6 inhibits TNF-α production.

As *in-vivo* bioassay, zebrafish larvae were used in this study. It is well documented that zebrafish larvae establish a primary lateral line system by 3 days of post fertilization (dpf). For chemical inflammation, exposure of zebrafish to copper sulphate rapidly destroys hair cells of the lateral line system by inducing oxidative stress followed by cell death. Immediately after the treatment with copper sulphate, fluorescent leukocytes start making clusters at the damaged neuromast (d'Alencon et al., 2010). Fluorescent neutrophils were counted manually within a specific area known as myoseptum which runs from

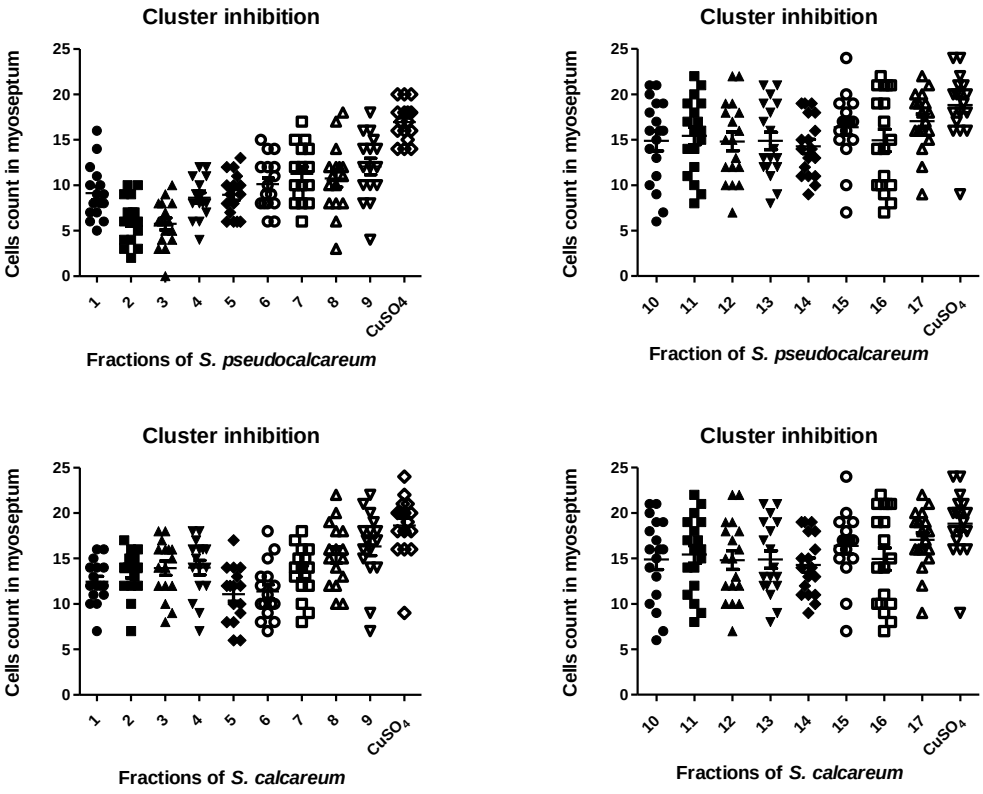

Figure 2: Inhibition of leukocytes infiltration or clusring by fractions (1-17) from *S. pseudocalcareum* and *S. calcareum* towards wounded area (myoseptum) of zebrafish. Less cells means high activity. Each error bar represents ±SEM n= 16.

the first somite to the end of the tail. It is interesting to observe that fractions (2) and (3) of *Sempervivum pseudocalcareum* show strong activity as compared to other fraction which is unlike the *in-vitro* bioassay, while in case of *Sempervivum calcareum* none of the fraction show any significant activity.

The ^{1}HNMR spectras of both cultivars have been shown in Figure 3 (A, B) and it is evident from visual inspection of the spectra that both cultivars are different from each other in terms of metabolic profile specially in the phenolic region. The ^{1}HNMR spectra of active fractions (3) and (5) of *S. pseudocalcareum* are presented in the figure 3 (C, D) respectively. By looking at the spectra, it can be

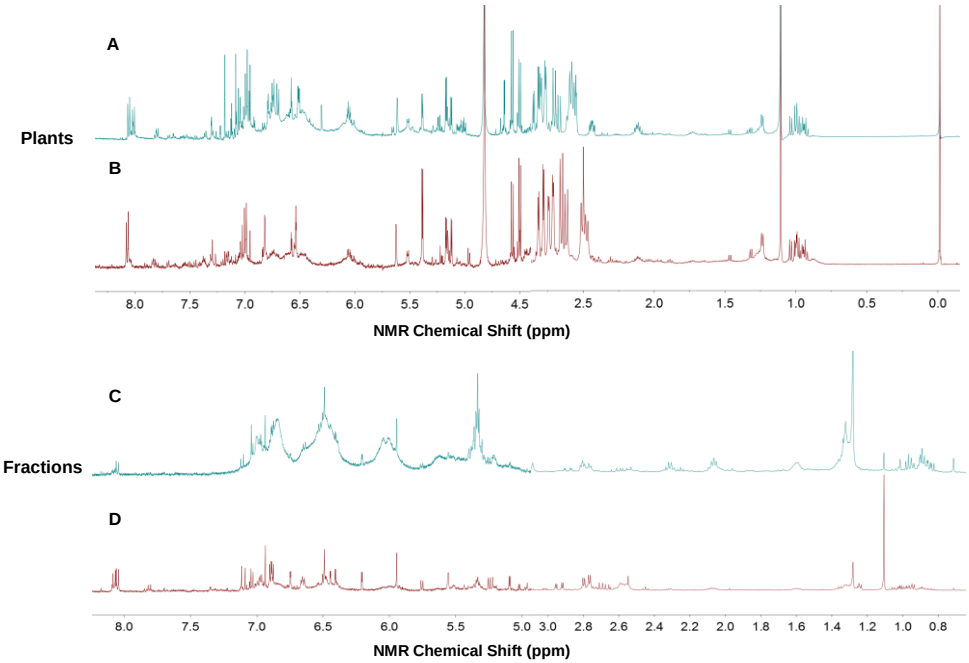

Figure 3: Comparison of ^{1}HNMR spectra of cultivar *Sempervivum pseudocalcareum* (A) and *Sampervivum calcareum* (B). Spectra of fractions which showed strongest activity *in-vitro* (C, 5) and *in-vivo* (D, 3)are presented in (C) and (D) respectively.

observed that there are not so many compounds present in the phenolic region of fraction (5) as in case of fraction (3). Several compounds (Flavonoids, amino acids and organic acids) were identified using our in-house library of NMR data of common metabolites. The ^{1}H NMR chemical shifts for all these metabolites are shown in Table 1. All these assignment of metabolites are based on previous studies (Abdel-Farid et al., 2007; Ali et al., 2011a; Ali et al., 2009). A number of amino acids and organic acids were identified due to high signal intensities in the amino acid region. Amino acids like alanine, glutamine, isoleucine, threonine, and valine were identified by comparing spectra with our inhouse NMR spectral library of reference compounds. The signals in the carbohydrate region are highly clustered and overlapping. Signals of sucrose, α-glucose, β-glucose, mannose and arabonise could be identified.

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Table 2: ^{1}H NMR chemical Shifts (δ) and coupling constants (Hz) of *Sempervivum pseudocalcareum* metabolites identified by references and using 1D and 2D NMR spectra (CD3OD-KH2PO4 in D2O, pH 6.0).

Compounds	**Chemical Shifts (δ)**
Gallic acid	7.03 (s)
Syringic acid	3.89(s), 7.31(s)
Kaempferol analogue 1	6.23 (d, J=2.0 Hz), 6.43 (d, J=2.0 Hz), 6.90 (d, J=9 Hz), 8.07 d, J=8.8 Hz)
Kaempferol analogue 2	6.46 (d, *J* = 2.1 Hz), 6.77 (d, *J* = 2.1 Hz), 7.00 (d, *J* = 8.8 Hz), 8.09 (d, *J* = 8.8 Hz)
Myricetin	6.28 (d, *J*= 2.0), 6.51 (d, *J*=2.0), 7.30 (s)
Valine	1.01 (d, *J*=7.0), 1.06 (d, *J*=7.0), 2.28 (m)
Leucine	0.96 (d, *J*=7.5), 0.98 (d, *J*=7.5)
Alanine	1.48 (d, *J*=7.4), 3.73 (q, *J*=7.4)
GABA	1.90 (m), 2.31(t, *J*=7.5), 3.01 (t, *J*=7.5)
Methionine	2.15 (m), 2.65 (t, *J*=8.0)
Threonine	1.32 (d, *J*=6.5), 3.51 (d, *J*=5.0), 4.27 (m)
Glutamine	2.46 (t of d), 2.16-2.10 (m)
Glutamic acid	2.13 (m), 2.42 (m), 3.71 (dd, *J*=7.0, 1.9)
α-Glucose	5.17 (d, *J*=3.78)
β-Glucose	4.58 (d, *J*=7.89)
Fructose	4.08 (d, *J*=7.80)
Sucrose	5.39 (d, *J*=3.94)
Choline	3.20 (s)

Citric acid	2.56 (d, *J*=17.6), 2.74 (d, *J*=17.6)
α-Linolenic acid	0.95 (t, *J*=7.5)
Ascorbic acid	4.52 (d, *J*=2.0)
Malic acid	2.68 (dd, *J*= 16.6, 6.6), 2.78 (dd, *J*=16.6, 4.7), 4.34 (dd, *J*=6.6, 4.7)
Formic acid	8.45 (s)
Aspartic acid	2.82 (dd) *J* = 17.0, 8.5, 2.64 (dd) *J*=17.0, 10.0
Shikimic acid	2.18 (dt, *J*=18.1, 1.7), 2.69 (dt, *J*=18.0, 5), 3.70 (dd *J*=10,4), 4.00 (m), 4.32 (t, *J*=4.5), 6.56 (dt, *J*=4.0, 1.7 Hz)

Multivariate data analysis methods are generally used to see possible similarities and differences for the metabolomic studies. Principal component analysis (PCA) is considered as primary tool among all these multivariate data analysis methods. It is an unsupervised method in which samples are clustered or separated purely on the basis of metabolic similarities or differences respectively. The NMR data from all the fractions of *Sempervivum pseudocalcareum* have been subjected to PCA to find out the differences among the fraction and to identify possible candidate for the separation. The score plot of PCA (Figure 4A) shows a clear separation among all the fractions.

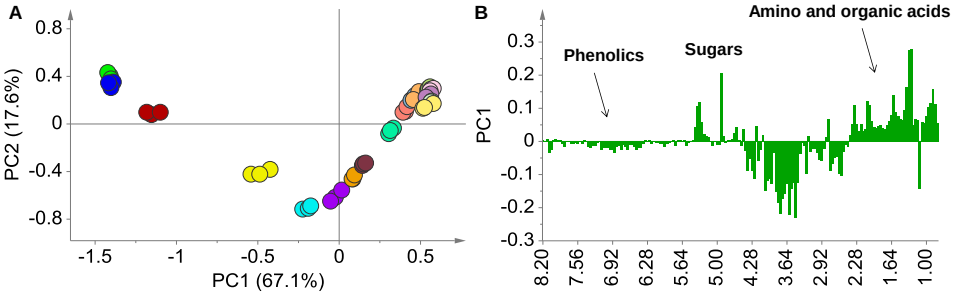

Figure 4. Score plot (A, PC1+PC2) and loading plot (B, PC1) of principal component analysis (PCA) of fractions from *Sempervivum pseudocalcareum*. All fractions are separated from each other. Samples are colored according to different fractions (1-17).

In order to find compounds responsible of activity, supervised methods are used. Projection to latent structures-discrimination analysis (PLS-DA) was employed to separate active from less active samples, a supervised method in which samples were classified into three different classes by creating dummy Y-variables. The samples were classified into high (>50%), medium (<50% and >30%), and low (≤30%) activity classes. The score plot of PLS-DA (Figure 5A) shows the clear separation among the fractions based of the different classes. By looking at the corresponding loading plot of PLS-DA (Figure 5D), it is quite clear that mostly phenolic compounds are present in the active fractions. The PLS-DA method was validated using permutation test with 20 applications (Figure 5C). For activity data R2 and Q2 values for PLS-DA analysis were 0.94 and 0.88 respectively. Although, a separation was observed in PLS-DA, bidirectional orthogonal projection to latent structures –discriminant analysis (O2PLS-DA) was also employed. The score plot of O2PLS-DA (Figure 5B) shows much better separation among different activity classes. Samples with medium activity are grouped on the positive side of PC1 while samples with low and high activity are grouped on the positive sides of component 2 and negative sides of component 1 respectively. The O2PLS-DA method was validated by cross validation-analysis of variance (CV-ANOVA) with a *p*-value of 7.42×10^{-24}. By examining the loading plot, the metabolites responsible for separation are identified. The high TNF-α activity samples have higher levels of phenolic contents while the medium or low activity samples have less or no phenolic contents.

The next step was to perform the direct correlation between the activity and NMR data using original anti-TNF-α assay values. Instead of classifying samples as high, medium, and low activity groups, the activity data from TNF-α

assay for each sample are directly used as such. In such approaches PLS and/or PLS-DA are used and two different data sets, independent variable (like NMR spectral data) and dependent variable (like anti-TNF-α activity), are correlated using regression.

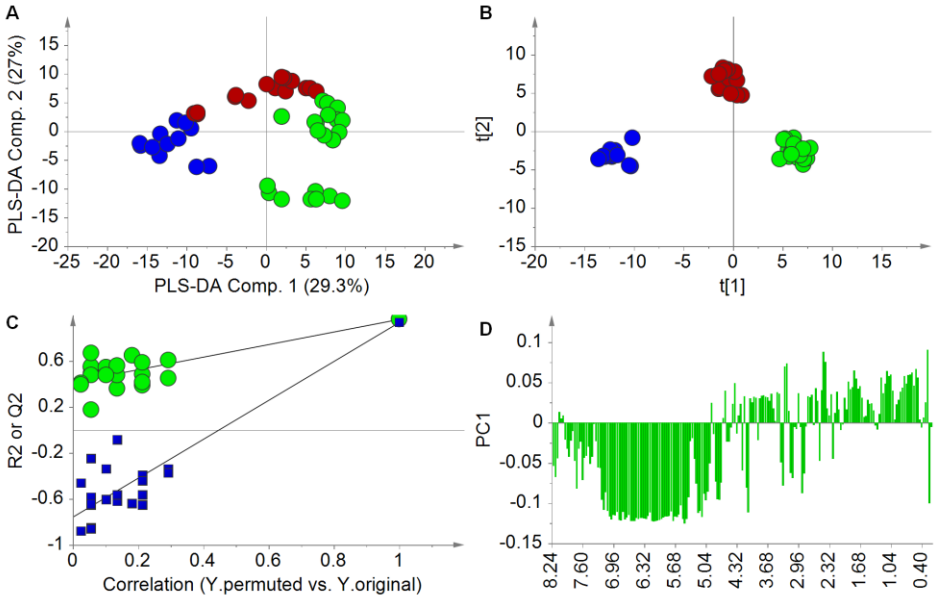

Figure 5. Score plot of PLS-DA (A); OPLS-DA (B); validation plot of permutation test for PLS-DA (C). The samples in blue represent high anti-TNF-α activity while samples in green and red represent medium and low activity. Loading plot of PLS-DA (D).

For this purpose projection to latent structures (PLS) analysis was performed using the NMR and activity data. The PLS score plot (Fig. 6A) shows relatively good separation among the samples but many are overlapping with the other groups. Component 1 is mainly responsible for the separation as the samples are arranged from low to high activity along the negative to positive side of component 1, respectively. For PLS modeling again the permutation method through 20 applications was used for validation.

Finally for the identification of metabolites responsible for high activity in *Sempervivum pseudocalcareum*, we used another multivariate data analysis method known as bidirectional orthogonal projection to latent structures (O2PLS). Analyses like PLS regression can cause systematic variation of any data block due to structured noise present in the data blocks. Other algorithms, like O2PLS-DA and O2PLS, are multivariate projection methods which remove

the structured noise by extracting linear relationships from independent and dependent data blocks, in a bidirectional way, and results in the decomposition of systematic variation into two model parts: the predictive or parallel part and the orthogonal part (Baur et al., 2006; Chuang et al., 2010). The score plot, (Figure 6B) shows very nice separation among low, medium and high activity samples based on component 1. This O2PLS model was validated by CV-ANOVA with *p*-value of 1.10×10^{-17}. Like PLS-DA and O2PLS-DA the corresponding loadings plot show that the samples with high anti-TNF-α activity contained more Kaempferol derivatives, when compared to samples with low and medium activity.

Variable importance in the projection can be defined as a weighted sum of the squares of the PLS weights. It has been indicated that it is directly proportional with the influence of a factor of separation on the score plot which means factors having high VIP values are more important for the separation. It is also worth to mention that factors having VIP values more than 0.7 could be

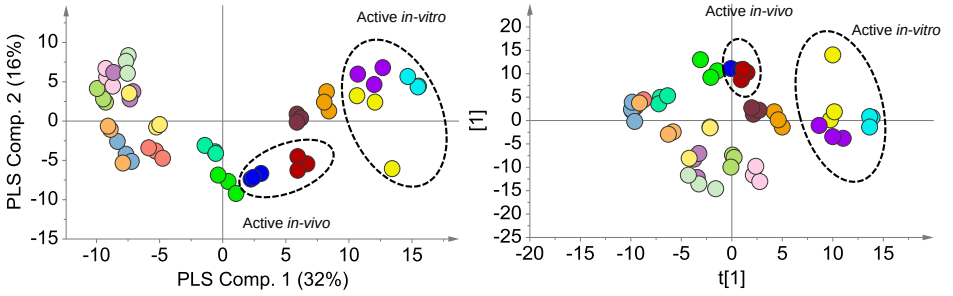

Figure 6: Score plot of PLS (A), and OPLS (B).

considered as most significant for the separation of samples (Eriksson et al., 2006). VIP values for O2PL-DA and O2PLS are calculated for different compounds responsible for activity. Here it was found that for *in-vitro* study, signals from the kaempferol molecule (6.90) are strongly correlating with activity data and have high VIP values 2.52, while in case of *in-vivo* study sugar molecules with signal at (5.10) have the highest VIP value (2.0), whereas kaempferol has 1.91 and myrecetin 1.70. The high VIP scores for the identified phenolics legitimate their involvement in the separation of high activity samples and suggest a role of these compounds in inhibiting TNF-α production and neutrophil migration in the *in-vivo* study.

TNF-α has been implicated in the pathogenesis of many vascular diseases such as atherosclerosis, heart attack, shock and sepsis (Brånén et al.,

2004; Li et al., 2005). Therefore, inhibition of cytokine production or function is a key mechanism in the control of inflammation. In this study, we have found that fractions (4-6) from *Sempervivum pseudocalcareum* significantly inhibits the production of TNF-α in LPS stimulated U937 cell lines. Furthermore, we have also shown that fractions (1-3) significantly inhibited neutrophils migration towards wounded area in Zebrafish. The NMR profile of these active fractions has led to the identification of flavonoids like kaempferol and myricetin as related to the activity.

Flavonoids were reported to possess various biological/pharmacological activities including anticancer, antimicrobial, antiviral, antiinflammatory, immunomodulatory, and antithrombotic activities (Havsteen, 1983). Of these biological activities, the antiinflammatory capacity of flavonoids has long been utilized in Chinese medicine and the cosmetic industry in a form of crude plant extracts. Many investigations have proven that various flavonoid molecules possess antiinflammatory activity in various animal models of inflammation. Especially, some flavonoids (quercetin and rutin) were found to inhibit chronic inflammation in several experimental animal models (Takahashi et al., 2001). It has been reported that flavones, flavonols, and chalcones are the potent inhibitors of production of TNF-α in LPS stimulated J774.1 cell lines (Herath et al., 2003). Recently, it has been reported that NMR spectroscopy integrated with multivariate data analysis was applied successfully for the identification of flavonoids responsible of activity (Ali et al., 2012).

Kaempferol is a common flavonoid in the human diet and has various biological activities including antioxidant, anticancer, and antiinflammatory effects (Kang et al., 2008; Mahat et al., 2010). A number of reports have shown the immunomodulatory effect of kaempferol on T lymphocytes (Okamoto et al., 2002), B cells (Zunino and Storms, 2009), macrophages (Comalada et al., 2006; Hamalainen et al., 2007; Harasstani et al., 2010; Kim et al., 2005; Liang et al., 1999), neutrophils (Moreira et al., 2007; Selloum et al., 2001; Wang et al., 2006), basophils (Shim et al., 2009), and mast cells (Kempuraj et al., 2005; Lee et al., 2010). A similar inhibition of inflammatory cytokines such as TNF-α, IL-12, and IL-1β by kaempferol is also observed in LPS-stimulated macrophages (Fang et al., 2005; Harasstani et al., 2010; Kowalski et al., 2005). Myricetin, another flavonoid commonly found in tea, wines, berries, fruits and medicinal plants, have been reported to possess antiproliferative and antiinflammatory effects (Yanez et al., 2004).

The suppression of TNF-α production by flavonoids may occur by several pathways: by inhibition of a key enzyme activity involved in production of a group of powerful pro-inflammatory signaling molecules; by inhibition of enzyme activity of protein kinases involved in cell activation processes; by inhibition of biosynthesis of protein cytokines that mediate various inflammatory processes or any combination of these. Recently, it has been reported that kaempferol down-regulates inflammatory iNOS and TNF-α production in aged rat gingival tissues via the inhibition of NF-kB activation, by interfering with the activation of NIK/IKK and MAPK (Kim et al., 2007).

9.4. Conclusion

Nuclear magnetic resonance (NMR) spectroscopy integrated with comprehensive extraction and multivariate data analysis was applied for the identification of active metabolites in *Sempervivum pseudocalcareum*. Various multivariate data analysis methods show good correlation between NMR and activity data. Algorithms like PLS and PLS-DA show good separation among samples classified as high, medium and low activity. Application of bidirectional orthogonality like O2PLS-DA and O2PLS show even more discrimination among the classes. The VIP plot showed the NMR signals that are correlated to activity. The signals were identified as belonging to kaempferol-derivatives, myricetin and sugars. This novel approach allows us to screen thousands of plant extracts, and fractions, without an immediate need of chromatographic techniques.

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Chapter 10

Concluding remarks and prospective

Since long ago, plants have been used as the primary source of food, timber, fuel, and medicine, among other purposes. The ability of plants to produce different kinds of complex compounds makes them a very special, and one of the most important sources for developing new drugs. Currently plants, plant extracts and plant-derived components are being used as herbal remedies against different diseases in different parts of the world. Chronic inflammation is linked to numerous human diseases and a serious health problem around the world so scientists are trying to find cures for inflammatory diseases. Increasing number of evidences suggest that modulation of inflammatory response by natural phytochemicals plays an important role in the prevention, mitigation, and treatment of many chronic inflammatory diseases.

The work described in this thesis shows the application of NMR spectroscopy integrated with multivariate data analysis for metabolic profiling and to find active ingredients from the crude extracts of different plants. The supervised multivariate data analysis methods like partial least square (PLS) and orthogonal partial least square (OPLS) analysis to study the correlations of metabolic profile of the extracts with their bioactivities. Mostly phenolic compounds were identified by using this approach. Recently lot of attention has been given to a class of secondary metabolites which are extensively present in a wide range of food plants: the flavonoids, for which many different biological activities have been reported. Epidemiological studies suggest that a high intake of vegetables and fruits are associated with lower risk of chronic diseases. However, the mechanism of action and the components involved in this effect have not been identified clearly. The antiinflammatory actions of flavonoids *in-vitro* or in cellular models involve the inhibition of the synthesis and activities of different pro-inflammatory mediators such as eicosanoids, cytokines, adhesion molecules and C-reactive protein. Molecular activities of flavonoids include inhibition of transcription factors such as NF-kB and activating protein-1 (AP-). However, the impact of *in-vitro* studies on flavonoids are limited due to the non-physiological concentrations utilized (5-100µM). In addition, it has been observed in the human studies that flavonoid absorption in the gastrointestinal tract is only between 1 and 5% of the ingested food. Moreover, *in-vivo* flavonoids are extensively metabolized to molecules with different chemical structures and activities compared with the ones originally present in the food. Human studies investigating the effect of flavonoids on markers of inflammation are insufficient, and are mainly focused on flavonoid-rich food

but not on pure molecules. Most of the studies lack assessment of flavonoid absorption or fail to associate an effect on inflammation with a change in circulating levels of flavonoids. Human cinical trials on pure flavonoid molecules are needed to clarify if flavonoids represent key molecules involved in the antiinflammatory properties of food plants. In this study, we tested several reference compounds from active extracts. Compounds like curcumin, quercetin, kaempferol, myricetin and eugenol were highly active in *in-vitro* studies but none of them showed activity in *in-vivo* except curcumine. The activity might be due to the combination of certain compounds (synergism) or unidentified compounds present in the extracts. Further studies are needed to confirm this. With the emergence of new analytical tools and techniques with lot more precision, accuracy and sensitivity we can deeply look inside plant metabolome and find active ingredient from them. Furthermore, proteomics, transcriptmics and genomics data is necessary to determine their mechanism of action.

Summary

Cytokines play an important role in the immune system. Any disorder in the regulation of cytokines can lead to the development of inflammatory diseases. Tumour necrosis factor- α (TNF-α) is one of the most important inflammatory cytokines that controls different types of cell functions. The overproduction of TNF-α is linked with the development of various diseases such as asthma, rheumatoid arthritis, psoriatic arthritis, inflammatory bowel disease, septic shock, diabetes and atherosclerosis. Currently, several types of clinically approved drugs are available for the inhibition of TNF-α production in different disease conditions. These include, Etnercept, Infliximab, and Adalimumab. Although these drugs are potentially beneficial to human health, they can also exert some devastating effects such as an increased chance of infection, heart failure, neurological changes, and problems related to autoimmunity. Thus, it is essential to develop safer, less toxic, and more beneficial anti-TNF-α drugs. Low molecular weight compounds provide many advantages over protein-based drugs, particularly concerning production, stability and route of administration. Plants are considered a good source for the development of novel drugs and already many natural compounds, belonging to various chemical classes like flavonoids, terpenoids, alkaloids, cannabinoids, ginsenosides, and phytosterols, have been found to inhibit the upstream signalmolecules that are involved in TNF-α expression. The dose at which most of these compounds are active in the various *in-vitro* tests is in the range of about 1-50 µM **(Chapter 2)**.

Since ancient times, in various cultures worldwide, inflammatory disorders and related diseases have been treated with plants or plant-derived formulations. The anti-inflammatory activity of several plant extracts and isolated compounds has already been scientifically demonstrated. In this study, we examined the antiinflammatory activities of approximately 66 plant extracts in LPS-stimulated macrophages, an *in-vitro* model for studying antiinflammatory drugs or herbs. However, for *in-vivo* studies the zebrafish embryo has become an important vertebrate model for assessing pharmacological and toxic effects. It is well suited for studies in genetics, embryology, development, and cell biology. Zebrafish embryos exhibit unique characteristics, including ease of maintenance and drug administration, short reproductive cycle, and transparency that permits visual assessment of developing cells and organs. Because of these advantages, zebrafish bioassays are cheaper and faster than mouse assays, and are suitable for large-scale drug

screening. The results from our study demonstrate that extracts of *Urtica dioica, Houttuynia cordata, Salvia officinalis, Adhatoda vasica, Origanum vulgare, Sempervivum smaragd, Syzygium aromaticum, Pimenta officinalis, Myristica fragrans, Capsicum annuum, Alpinia galanga, Zingiber officinale, Kaempferia galanga, Bixa orellana* and *Pistacia lentiscus* show significant inhibition of TNF-α in LPS stimulated U937 cells lines. In addition, there are some other plants including *Origanum vulgare, Rosmarinus officinalis, Curcuma xanthorrhiza, Boesenbergia rotunda, Orthosiphon stamineus, Cannabis sativa, Psoralea corylifolia, Curcuma longa*, and bark of *Pistacia lentiscus* which show highly significant inhibition of TNF-α, but they also exhibited toxicity at the highest concentration applied in this study **(Chapter 3).**

Recent studies have suggested that psychotropic and non-psychotropic phytocannabinoids exert a wide range of pharmacological effects. Based on our screening results, we designed an experiment to explore the antinflammatory activities of different cannabinoids isolated from *Cannabis sativa.* These include delta9-tetrahydrocannabinol (delta9-THC), cannabidiol (CBD), cannabigerol (CBG), cannabinol (CBN) cannabichromene (CBC), as well as cannabinoid acids such as delta9-tetrahydrocannabinolic acid (delta9-THCA), cannabidiolic acid (CBDA) and cannabigerolic acid (CBGA). We found that the acid form of cannabinoids show more significant inhibition of TNF-α as compared to the decarboxylated form. These compounds show toxicity towards cell lines at concentration of 10 µg/ml, maximum inhibition was observed in delta9-THCA followed by CBDA > CBGA > CBD >Δ^9-THC > CBG > CBN and CBC at a concentration of 1 µg/ml **(Chapter 4)**.

Eating diet rich in fruits and vegetables is thought to be the best and safest means of preventing cancer and many chronic inflammatory diseases. An experiment was designed to detect the anti-TNF-α activity of different fruit berries [Cranberry, blueberry, redberry, strawberry, raspberry, blackberry, grapeberry (green), grapeberry (red), grapeberry (black)] and find out the responsible compounds by using Solid phase extraction (SPE), NMR spectroscopy and multivariate data analysis. All the SPE fractions were clearly separated on a score plot of principal component analysis (PCA). In order to find correlations between metabolites and activities, partial least squares-discriminant analysis (PLS-DA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) were used. Signals related to the TNF-α inhibition observed in the SPE fractions of berries were identified as a wide range of phenolics. By calculating variable importance in the projection (VIP), the active ingredients in the high activity samples have been identified as gallic acid, caftaric acid, quercetin, myricetin, and (+)-catechin. This study shows the

usefulness of NMR spectroscopy in combination with chemometrics to identify the possible bioactive metabolites in crude extracts. Furthermore, this study also supports the thought that a diet rich in fruit could contribute to the prevention of inflammatory diseases **(Chapter 5)**.

Based on the results of chapter 5, three Portuguese grape varieties at different development stages were analyzed for the same activity. Two different vintages of 'Trincadeira' cultivar were also compared. The initial stages of grape development, green and veraison, were found more active against TNF-α production as compared to the later ripe and harvest stages. Among the cultivars, 'Touriga Nacional' was found to be the most potent inhibitor. Different multivariate data analyses algorithms based on projections to latent structures were applied to correlate the NMR and TNF-α inhibition data. The variable importance in the projections plot showed that phenolics like quercetin, myricetin, (+)- catechin, (-)-epicatechin, caftarate, and coutarate, were positively correlated with high activity. Using this approach, compounds related to activity can be identified without extensive and elaborate chromatographic separation, and thus allows rapid identification of extracts with biological activity **(Chapter 6)**.

Based on the results in chapter 6, we further tested samples of wine which were prepared from those grapes. There are several reports regarding cancer preventive properties of polyphenolic compounds present in wines. In this study, eleven different types of red wine from Portugal were analyzed for their potential TNF-α inhibition. NMR spectroscopy together with multivariate data analysis was applied to find active ingredients of wine. OPLS-DA was found most effective in discriminating the high activity samples from the low and medium activity samples. According to variable importance in the projection (VIP), different phenolic compounds were found to correlate with high activity samples and identified as caftaric acid, quercetin, and (+)-catechin. Among the different vintages, maximum TNF-α inhibition was found in samples from the 2010 vintage. This study shows again the usefulness of NMR spectroscopy in combination with chemometrics to identify the possible active compounds in crude extracts **(Chapter 7)**.

Eugenia uniflora is widely used in Argentina, Brazilian and Paraguayan folk medicine. The study was designed with the aim of the metabolic characterization of fruits of *Eugenia uniflora* and to determine antiinflammatory activities of crude extracts using NMR spectroscopy and chemometrics methods. Berries were staged into green, yellow, red and purple according to the period towards maturity. The fruits at the green stages presented significant antiinflammatory activity in both the assays followed by yellow, purple and red

stages. NMR spectroscopy together with multivariate data analysis was applied to identify the compounds responsible for activity. Projections to latent structures (PLS) were found effective in discriminating high activity samples from low activity samples. By analyzing the coefficient plot, the active constituents in the high activity samples have been identified as quercetin, myricetin, kaempferol, cinnamic acid and chlorogenic acid. NMR spectroscopy proved to be a valuable tool for identifying compounds responsible for activity **(Chapter 8).**

The potential antiinflammatory activities of Genus *Sempervivum* were evaluated by using NMR spectroscopy and multivariate data analysis. Twenty four different species of *Sempervivum* were screened for their ability to inhibit TNF-α release. *Sempervivum smaragd, Sempervivum pilatus, Sempervivum noir, Sempervivum pseudo-calcareum, Sempervinum microcephalum, Sempervivum tectorum glaucum* showed maximum TNF-α inhibition respectively. In the second stage of the experiment, a comparison was made between *Sempervivum pseudocalcareum* (active) and *Sempervivum calcareum* (non active) to find out the responsible compounds for activity. Comprehensive extraction integrated with NMR spectroscopy was successfully applied for metabolic characterization and identification of compounds responsible of the antiinflammatory activity. The approach presented here allows us to screen thousands of plant extracts and pure compounds without using laborious chromatographic techniques **(Chapter 9)**.

This bioassay mentioned above was an important goal of this thesis: the development and description of a relatively simple, robust and easily adaptable *in vitro* model for screening anti-inflammatory activity. This was achieved using the human monocytic cell line, U937, for screening of anti-TNF-α activity of different plant extracts and isolated pure compounds.

The zebrafish embryos proved to be a good model for evaluation of antiinflammatory activity of crude extracts and pure compounds, confirming the utility of zebrafish embryos as an *in-vivo* model for assessing drug effects by offering unique characteristics, including ease of maintenance and drug administration, short reproductive cycle, and transparency that permits visual assessment of developing cells and organs. Because of these advantages, zebrafish bioassays are cheaper and faster than mouse assays, and are suitable for large-scale drug screening. Because of the complexity of the mixtures and compounds present in minor quantities, it is impossible to extract all from a plant using one single solvent system. Thus, solid phase extraction (SPE) was used to enrich minor compounds and comprehensive extraction methods to extract the total metabolome of the plant material. SPE can be used to extract

specific class of compounds. The major advantage of this method is removal of sugars and fatty acids which may interfere in activity based assays. Thus using this approach, we were able to extract three different types of fractions from one extract based on differences in polarity. Moreover, comprehensive extraction was used as an alternative method to obtain the total metabolome.

Multivariate data analysis of the NMR spectra of active extracts or fractions was used to identify the signals correlating with biological activity. By further 2D NMR spectroscopy the active compounds could be identified. Application of comprehensive extraction together with multivariate data analysis makes the identification of active compounds (dereplication) fast and easy without any need for chromatographic steps. Using this approach, a wide range of compounds can be extracted from non-polar to polar. Comprehensive extraction is a simple, efficient, robust and reproducible method for identification of biologically active compounds from crude extracts.

In conclusion, NMR metabolomics in combination with bioassays is an effective method for rapid dereplication, i.e. the identification of the active compounds in active extracts. Interestingly in most cases several active compounds were found in active extracts. Using NMR spectroscopy integrated with chemometric based methods like (PLS, PLS-DA and OPLS), thousands of unexplored plant extracts can be screened in a short time for lead finding without laborious and costly chromatographic techniques.

Samenvatting

Cytokines spelen een belangrijke rol in het immuun systeem. Ontregeling van de regulatie van cytokines kan leiden tot de ontwikkeling van ontstekingsziektes. De tumor necrosis factor- α (TNF-α) is een van de belangrijkste ontsteking gerelateerde cytokines die verschillende typen celfuncties controleert. De overproductie van TNF-α is verbonden met de ontwikkeling van verschillende ziektes zoals astma, reumatoïde arthritis, psoriasis, artritis, inflammatoire darmziekten, septic shock, diabetes en atherosclerose.

Momenteel zijn er verschillende klinisch goedgekeurde medicijnen beschikbaar voor de inhibitie van TNF-α productie bij verschillende ziektes. Tot deze medicijnen behoren o.a. Etnercept, Infliximab, en Adalimumab. Hoewel deze medicijnen potentiële voordelen hebben voor de menselijke gezondheid, hebben ze ook negatieve effecten zoals een toegenomen kans op infecties, hart falen, neurologische veranderingen en problemen gerelateerd aan auto-immuniteit. Zodoende is het essentieel om veiligere, minder schadelijke en beter werkzame anti-TNF-α medicijnen te ontwikkelen.

Natuurstoffen van laag moleculair gewicht hebben vele voordelen ten opzichte van eiwit gebaseerde medicatie, vooral wat betreft de productie, stabiliteit en manier van toediening. Planten worden beschouwd als een goede bron voor de ontwikkeling van nieuwe geneesmiddelen. Van veel natuurstoffen, behorende tot verschillende chemische klasses, zoals flavonoïden, terpenoïden, alkaloïden, cannabinoïden, ginsenosiden, en fytosterolen, is ontdekt dat zij een remming geven op het niveau van signaal moleculen upstream van de TNF-α expressie. De dosis waarbij de meeste van deze stoffen actief zijn in de verschillende *in-vitro* testen is in de orde van ongeveer 1-50 µM **(Hoofdstuk 2)**.

Sinds lang vervlogen tijden, werden en worden wereldwijd in verschillende culturen ontsteking gerelateerde ziektes behandeld met planten of daarvan afgeleide producten. De ontstekingsremmende activiteit van sommige plantenextracten en geïsoleerde stoffen zijn al wetenschappelijk aangetoond. In deze studie onderzochten we de ontstekingsremmende werking van 66 plantenextracten in LPS-gestimuleerde macrofagen, een *in-vitro* model voor het bestuderen van ontstekingsremmende medicijnen in plantenextracten.

Voor *in-vivo* studies is het zebravis embryo een belangrijk model geworden voor het bepalen van farmacologische en toxicologische effecten. Het

is ook zeer geschikt voor studies in genetica, embryologie, ontwikkelingsbiologie en celbiologie. Zebravis embryos bezitten unieke eigenschappen, zoals het gemak van het kweken en onderhouden van de vissen, de eenvoudige toediening van de te testen monsters, een korte reproductie cyclus en hun transparantie, wat de visuele beoordeling van ontwikkelende cellen en organen mogelijk maakt. Vanwege deze voordelen zijn zebravis bioassays goedkoper en sneller dan testen met muizen en zijn ze geschikt voor het op grote schaal screenen van nieuwe leads voor geneesmiddelen.

Uit de resultaten van onze studie blijkt dat extracten van *Urtica dioica, Houttuynia cordata, Salvia officinalis, Adhatoda vasica, Origanum vulgare, Sempervivum smaragd, Syzygium aromaticum, Pimenta officinalis, Myristica fragrans, Capsicum annuum, Alpinia galanga, Zingiber officinale, Kaempferia galanga, Bixa orellana* en *Pistacia lentiscus* een significante remming geven van TNF-α productie in LPS gestimuleerde U937 cellijnen. De extracten van, *Origanum vulgare, Rosmarinus officinalis, Curcuma xanthorrhiza, Boesenbergia rotunda, Orthosiphon stamineus, Cannabis sativa, Psoralea corylifolia, Curcuma longa*, en de schors van *Pistacia lentiscus*, geven een significante remming van de TNF-α productie, maar waren toxisch bij de hoogste concentraties die in de studie werden gebruikt **(Hoofdstuk 3).**

Recente studies hebben aangetoond dat psychotropische en niet-psychotropische fytocannabinoïden een breed scala aan farmacologische effecten teweeg kunnen brengen. Gebaseerd op de resultaten van de screening, hebben we een experiment opgezet om de ontstekingsremmende activiteiten van verschillende cannabinoïden te bepalen. De volgende, uit *Cannabis sativa* geïsoleerde, cannabinoïden werden onderzocht: delta9-tetrahydrocannabinol (delta9-THC), cannabidiol (CBD), cannabigerol (CBG), cannabinol (CBN) en cannabichromene (CBC), en ook de cannabinoïde zuren delta9-tetrahydrocannabinolzuur (delta9-THCA), cannabidiolzuur (CBDA) en cannabigerolzuur (CBGA).

De zure vormen van de cannabinoïden bleken een significante hogere inhibitie van TNF-α te vertonen dan hun gedecarboxyleerde vorm. Deze stoffen zijn toxisch voor cellijnen bij concentraties van 10 µg/ml. Maximale inhibitie werd gevonden voor delta9-THCA gevolgd door CBDA > CBGA > CBD >Δ^9-THC > CBG > CBN en CBC bij een concentratie van 1 µg/ml **(Hoofdstuk 4)**.

Een dieet rijk aan fruit en groenten is, zo wordt algemeen gedacht, de beste en veiligste methode voor het voorkomen van kanker en vele chronische ontstekingsziektes. Daarom werd een experiment opgezet om een mogelijke vermindering van TNF-α activiteit te detecteren door extracten van

verschillende besvruchten (cranberry, blauwe bosbes, rode bes, aarbei, framboos, braam, groene/rode/zwarte druif) en in geval van activiteit de actieve stoffen te vinden, door middel van een combinatie van 'solid phase extraction (SPE)', NMR spectroscopie en multivariaat data analyse. Alle SPE fracties waren duidelijk van elkaar gescheiden in een score plot van een 'principal component analysis (PCA)' van de NMR spectra. Om correlaties te vinden tussen de signalen en activiteit, werden 'partial least squares-discriminant analysis (PLS-DA)' en 'orthogonal partial least squares-discriminant analysis (OPLS-DA)' gebruikt. De NMR signalen gerelateerd aan de remming van TNF-α productie, werden geïdentificeerd als behorend tot een brede groep van fenolen. Door het berekenen van de 'variable importance in the projection (VIP)' konden de actieve stoffen in de extracten met hoge activiteit worden geïdentificeerd als: galzuur, caftaarzuur, quercetin, myricetin, en (+)-catechin.

Deze studie toont de bruikbaarheid aan van NMR spectroscopie, in combinatie met chemometrie om potentiële biologisch actieve natuurstoffen te identificeren in ruwe extracten. Verder ondersteunt deze studie ook de gedachte dat een dieet rijk aan fruit, zou kunnen bijdragen aan het voorkomen of beheersen van ontstekingsreacties (**Hoofdstuk 5**).

Gebaseerd op de resultaten van hoofdstuk 5, werden drie Portugese druiven variëteiten in verschillende ontwikkelingsstadia geanalyseerd op remming van TNF-α activiteit. Twee verschillende oogstjaren van de druiven van de cultivar *Trincadeira* werden ook vergeleken. De initiële ontwikkelingsstadia van druiven, groen en veraison (net kleurend), bleken een sterkere remming te geven van de TNF-α productie, dan de extracten van de meer rijpere stadia. Van de cultivars bleek *Touriga Nacional* de meest potente inhibitor. Verschillende multivariaat data analyse algoritmes, gebaseerd op projecties naar latente structuren, werden toegepast om de NMR data te correleren aan TNF-α productieremmings data. De 'variable importance in the projections plot' liet zien dat fenolen zoals quercetin, myricetin, (+)- catechin, (-)-epicatechin, caftaarzuur, en coutaarzuur, positief correleren met hoge activiteit. Gebruik makend van deze aanpak kunnen stoffen worden geïdentificeerd die gerelateerd zijn aan activiteit, zonder uitgebreide en ingewikkelde chromatografische scheidingen en staat daardoor snelle identificatie toe van de actieve stoffen in extracten met biologische activiteit **(Hoofdstuk 6)**.

Gebaseerd op de resultaten van hoofdstuk 6, hebben we wijn monsters getest, die bereid waren van die druiven. Er zijn verschillende publicaties over de preventieve werking op kanker van in wijn voorkomende fenolische componenten. In deze studie werden elf verschillende soorten Portugese rode

wijn getoetst op hun potentiële TNF-α inhibitie. NMR spectroscopie, samen met multivariaat analyse werden toegepast om actieve componenten te vinden in wijn. Het bleek dat OPLS-DA het meest effectief is in het discrimineren tussen de hoog actieve en de laag en medium actieve monsters. Met de 'variable importance in the projection (VIP)', werden verschillende fenolische componenten gevonden die correleren met monsters met hoge activiteit. Deze werden geïdentificeerd als caftaarzuur, quercetin, en (+)-catechin. Van de verschillende oogstjaren, werd maximale TNF-α inhibitie gevonden in monsters van de oogst uit 2010. Deze studie toont wederom de bruikbaarheid aan van NMR spectroscopie in combinatie met chemometrie, voor het identificeren van mogelijk actieve stoffen in ruwe extracten **(Hoofdstuk 7)**.

Eugenia uniflora wordt veel gebruikt in traditionele Argentijnse, Braziliaanse en Paraguayaanse medicijnen. De studie was opgezet met als doel de metabolieten in de vruchten van de *Eugenia uniflora* te karakteriseren en om een eventuele ontstekingsremmende activiteit te meten van ruwe extracten, om vervolgens gebruik makend van NMR spectroscopie en chemometrics methoden de actieve stoffen te identificeren. Aan de hand van hun rijpheid werden de bessen ingedeeld in de groepen: groen, geel, rood en paars. De vruchten in het groene stadium vertoonden significante ontstekingsremmende activiteit in beide meet methoden, gevolgd door geel, paars en rode stadia. NMR spectroscopie samen met multivariaat data analyse werden toegepast om stoffen te identificeren die verantwoordelijk waren voor de activiteit. De methode 'projections to latent structures (PLS)' gaf een duidelijk onderscheid van monsters met hoge en van lage activiteit. Door het analyseren van de coëfficiënt plot, konden de actieve bestandsdelen van de hoge activiteit monsters worden geïdentificeerd als quercetin, myricetin, kaempferol, kaneelzuur en chlorogeenzuur. Met NMR spectroscopie kon de structuur van deze stoffen bevestigd worden. **(Hoofdstuk 8)**.

De potentiële ontstekingsremmende activiteit van extracten van planten van het genus *Sempervivum* werd geëvalueerd door gebruik te maken van NMR spectroscopie en multivariaat analyse. Vierentwintig verschillende soorten van *Sempervivum* werden gescreend op hun vermogen om TNF-α productie te remmen. *Sempervivum smaragd, Sempervivum pilatus, Sempervivum noir, Sempervivum pseudo-calcareum, Sempervinum microcephalum* en *Sempervivum tectorum glaucum* toonden de sterkste remming. In de tweede fase van het experiment werden *Sempervivum pseudocalcareum* (actief) en *Sempervivum calcareum* (niet actief) vergeleken om de stoffen te vinden die verantwoordelijk zijn voor de activiteit. 'Comprehensive extraction', geïntegreerd met NMR spectroscopie, werd succesvol toegepast voor

karakterisatie van de stoffen die verantwoordelijk zijn voor de ontstekingsremmende activiteit. De aanpak zoals hier gepresenteerd, stelt ons in staat om duizenden plantextracten en zuivere stoffen te screenen, zonder gebruik te hoeven maken van bewerkelijke chromatografische technieken **(Hoofdstuk 9)**.

Met de bioassay voor de TNF-α activiteit werd een belangrijk doel van deze thesis bereikt: het ontwikkelen en beschrijven van een relatief simpel, robuust en eenvoudig adapteerbaar *in-vitro* model voor het vinden van onstekingsremmende natuurstoffen. Dit werd gerealizeerd met de menselijke monocytische cellijn U937, waarin de remming van TNF-α activiteit door plantenextracten en plantenstoffen gescreend kan worden.

Zebravis embryos bleken een goed model voor de evaluatie van anti-ontstekings activiteit van ruwe extracten en zuivere stoffen, waardoor het zebravis model gebruikt kan worden als een *in-vivo* model voor het bepalen van farmacologische effecten. Vanwege de complexiteit van de mengsels en omdat veel stoffen slechts in kleine hoeveelheden aanwezig zijn, is het onmogelijk om alle stoffen uit een plant te extraheren met slechts een enkel oplosmiddel. Daarom werd ‘solid phase extraction (SPE)’ gebruikt om specifiek bepaalde groepen van stoffen, die in kleinere hoeveelheden aanwezig zijn, te verrijken. SPE kan worden gebruikt om specifieke klasses van stoffen te extraheren. Het grote voordeel van deze methode is dat suikers en vetzuren worden verwijderd, die mogelijk interfereren in activiteit gebaseerde toetsen. Met deze aanpak konden we drie verschillende typen fracties extraheren van een extract, gebaseerd op verschillen in polariteit. Tevens werd de ‘comprehensive extraction’ methode toegepast om het totale metaboloom uit de planten te extraheren.

Multivariaat data analyse van de NMR spectra van actieve extracten of fracties, werd gebruikt om de signalen te identificeren die correleren met biologische activiteit. Met verdere 2D NMR spectroscopie konden de actieve componenten worden geïdentificeerd. Het toepassen van ‘comprehensive extraction’, samen met multivariaat data analyse, maakt het identificeren van actieve componenten (‘dereplication’) snel en eenvoudig zonder dat chromatografische stappen nodig zijn. Met deze methode kan een breed scala aan stoffen worden geëxtraheerd van niet-polair tot polair. ‘Comprehensive extraction’ is een simpele, efficiënte, robuuste en reproduceerbare methode voor het identificeren van biologisch actieve stoffen in ruwe extracten.

NMR metabolomics is, in combinatie met biotoetsen, een effectieve methode voor snelle identificatie van actieve stoffen in actieve extracten. In veel

gevallen bleken er meerdere actieve stoffen aanwezig te zijn in de actieve extracten. Door gebruik te maken van NMR spectroscopie, geïntegreerd met chemometrics methoden, zoals PLS, PLS-DA and OPLS, kunnen duizenden nog niet onderzochte plantenextracten in korte tijd worden gescreend voor het vinden van leads, zonder de noodzaak voor bewerkelijke en dure scheidings methodes.

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Curriculum Vitae

Muzamal Iqbal was born on 30th March 1983 in Narowal (Punjab) Pakistan. He obtained his high school education from the Government High School Maingri, Narowal in 1998 and completed higher secondary education from Government Forman Christian (F.C) College Lahore in 2000. For further studies, he joined University of Agriculture Faisalabad (UAF) Pakistan. From there, he obtained his bachelor (B.Sc Hons) in 2005 and his master (M.Sc Hons) in 2007 with major subjects Plant Breeding and Genetics. His master thesis was titled Genetic Studies of Some Quantitative Traits in Cotton (*Gossypium hirsutum* L.). In 2007, he won the Overseas Scholarship offered by Higher Education Commission (HEC) of Pakistan for PhD studies. He perused his PhD research at Natural Products Laboratory (NPL), Institute of Biology (IBL), Leiden University, The Netherlands under the supervision of Prof. Dr. Robert Verpoorte. He started his PhD project named NMR-based metabolomics to identify bioactive compounds in herbs and fruits. The results of this research are presented in this thesis.

List of Publications

- **Iqbal M**, Verpoorte R, Korthout HAAJ, Mustafa NR. Phytochemicals as a potential source of TNF-α inhibitors. Phytochem Rev DOI 10.1007/s11101-012-9251-7
- Yuliana, N. D., **Iqbal, M**., Jahangir, M., Wijaya, C. H., Korthout, H., Kottenhage, M., Kim, H. K., and Verpoorte, R. 2011. Screening of selected Asian spices for anti obesity-related bioactivities. Food Chem 126, 1724-1729.
- Ali, K., **Iqbal, M**., Korthout, H., Maltese, F., Fortes, A., Pais, M., Verpoorte, R., and Choi, Y. 2012. NMR spectroscopy and chemometrics as a tool for anti-TNFα activity screening in crude extracts of grapes and other berries. Metabolomics, 1-14.
- Ali, K., **Iqbal, M**., Yuliana, N., Maltese, Verpoorte, R., and Choi, Y. 2013. Identification of bioactive metabolite against adenosine A1 receptor using NMR based metabolomics
- Ali, K., **Iqbal, M**., Yuliana, N., Maltese, Verpoorte, R., and Choi, Y. Red wines attenuate TNF-α production in U937 cell lines: NMR spectroscopy and chemometrics-based study. (Accepted in journal of food chemistry).
- **Iqbal, M**., Mushtaq, MY., Ali, K., Korthout HAAJ., Verpoorte, R., Cardozo, ML., Ordoñez, RM., Isla, MI., Choi, YH. Anti-inflammatory activities of *Eugenia uniflora* fruits in different developmental stages using NMR spectroscopy and multivariate data analysis. (Submitted in journal of phytochemical analysis)
- **Iqbal M**, Verpoorte R, Korthout HAAJ, Mustafa NR.Comprehensive extraction integrated with NMR metabolomics as a new way of bioacticvity guided identification of biologically active compounds in *Sempervivum* species. (Submitted in Planta medica)