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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 anti-inflammatory 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 bowel 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 anti-inflammatory, 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 monocyctic 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 monocyctic 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/ synergism 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 bioactivity 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.

References


