NMR-based metabolomics to identify bioactive compounds in herbs and fruits

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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

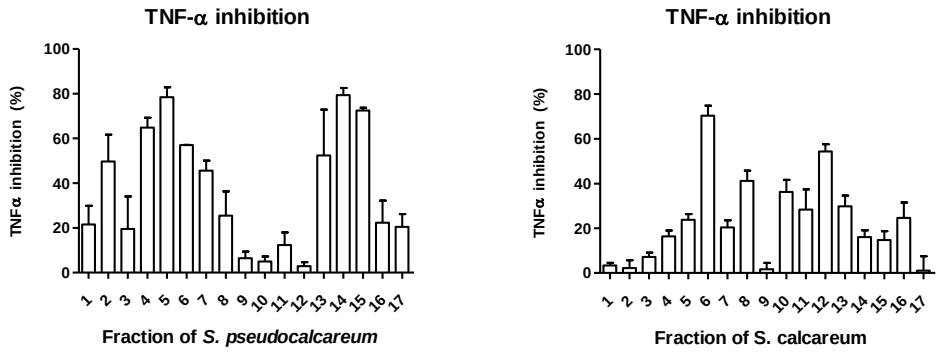

Figure 1: TNF-α (%) inhibition exhibited by different fractions (1-17) of *S. pseudocalcareum* and *S. calcareum* eluted with comprehensive extraction. Each bar represents ±SEM of three replicates.

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

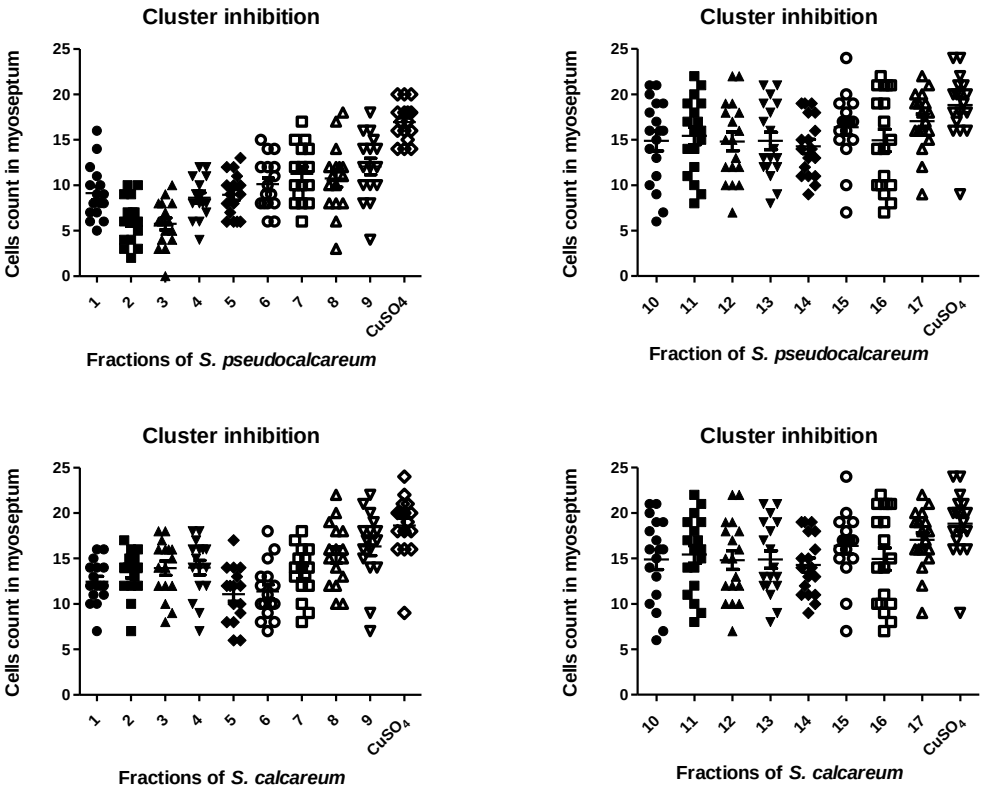

Figure 2: Inhibition of leukocytes infiltration or clusring by fractions (1-17) from *S. pseudocalcareum* and *S. calcareum* towards wounded area (myoseptum) of zebrafish. Less cells means high activity. Each error bar represents ±SEM n= 16.

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

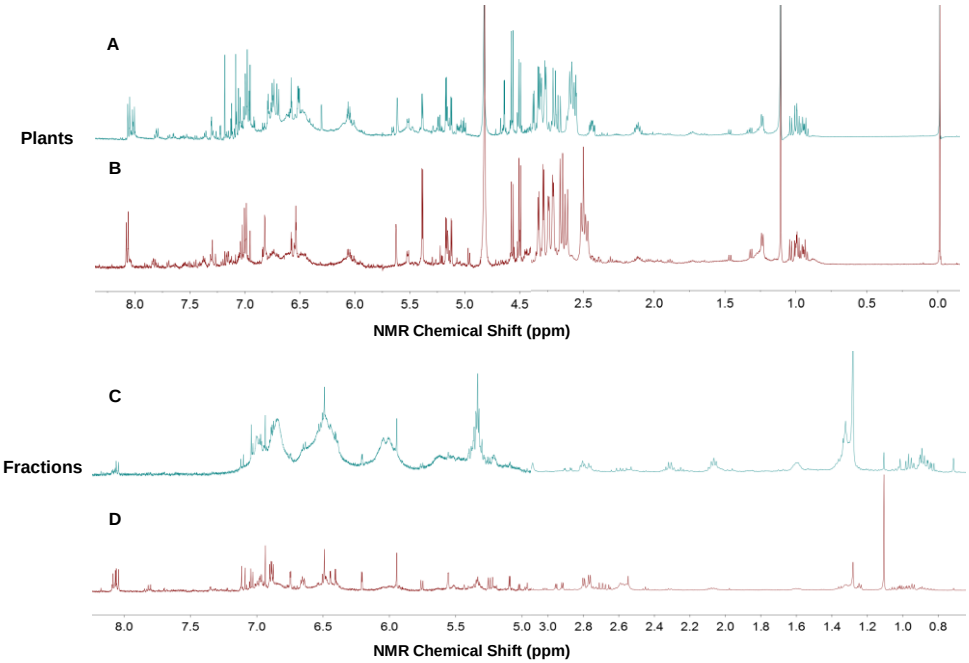

Figure 3: Comparison of ^{1}HNMR spectra of cultivar *Sempervivum pseudocalcareum* (A) and *Sampervivum calcareum* (B). Spectra of fractions which showed strongest activity *in-vitro* (C, 5) and *in-vivo* (D, 3)are presented in (C) and (D) respectively.

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.

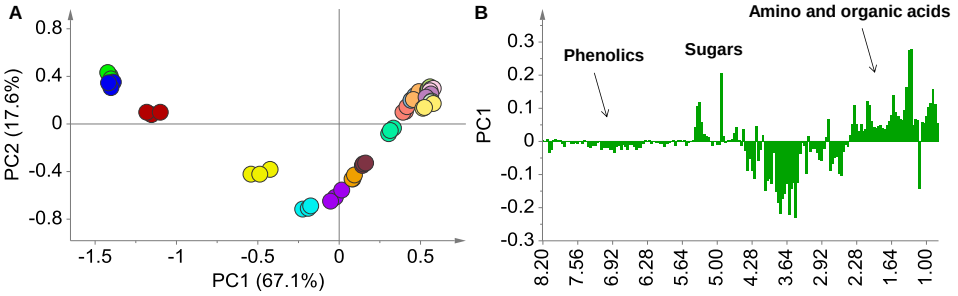

Figure 4. Score plot (A, PC1+PC2) and loading plot (B, PC1) of principal component analysis (PCA) of fractions from *Sempervivum pseudocalcareum*. All fractions are separated from each other. Samples are colored according to different fractions (1-17).

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.

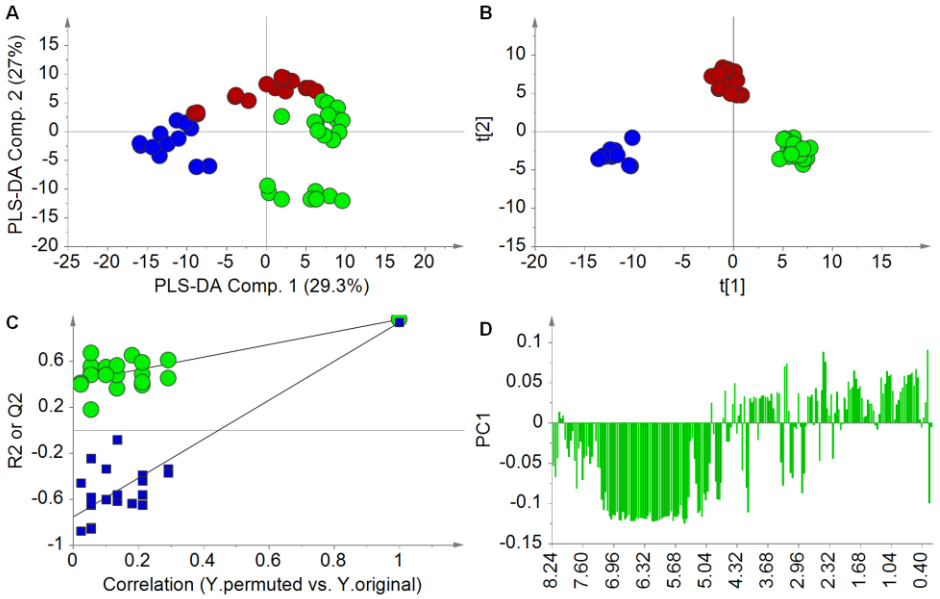

Figure 5. Score plot of PLS-DA (A); OPLS-DA (B); validation plot of permutation test for PLS-DA (C). The samples in blue represent high anti-TNF-α activity while samples in green and red represent medium and low activity. Loading plot of PLS-DA (D).

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

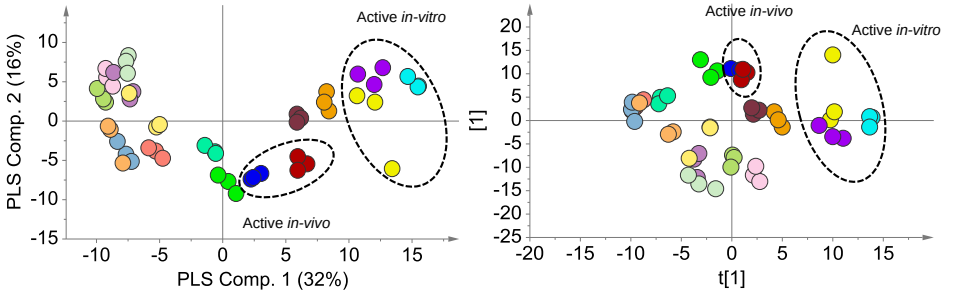

Figure 6: Score plot of PLS (A), and OPLS (B).

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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