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

Iqbal, M.

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.

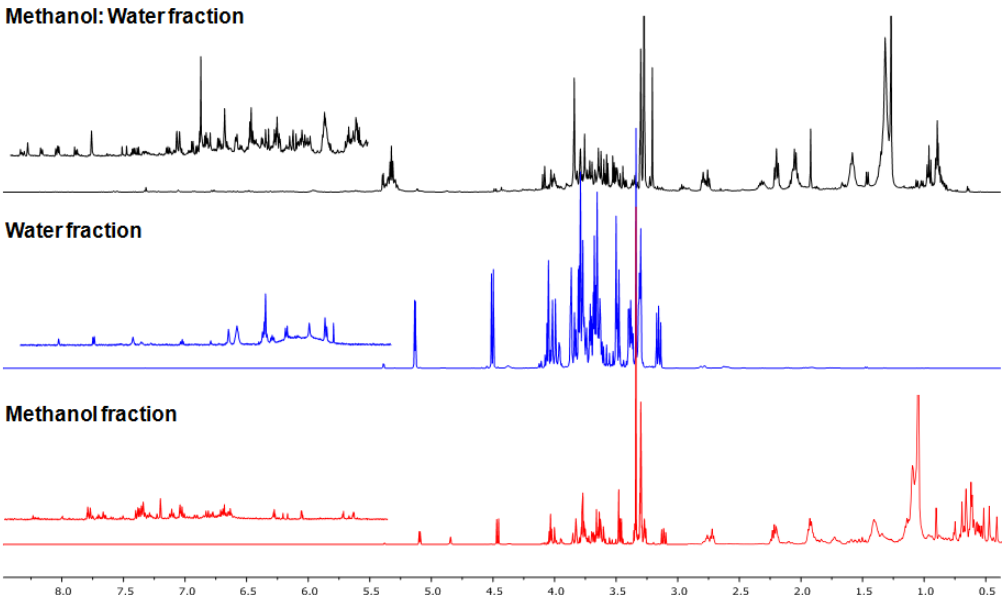

Fig. 1: Comparison of ^{1}H NMR spectra of SPE fractions of black grapes extract.

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.

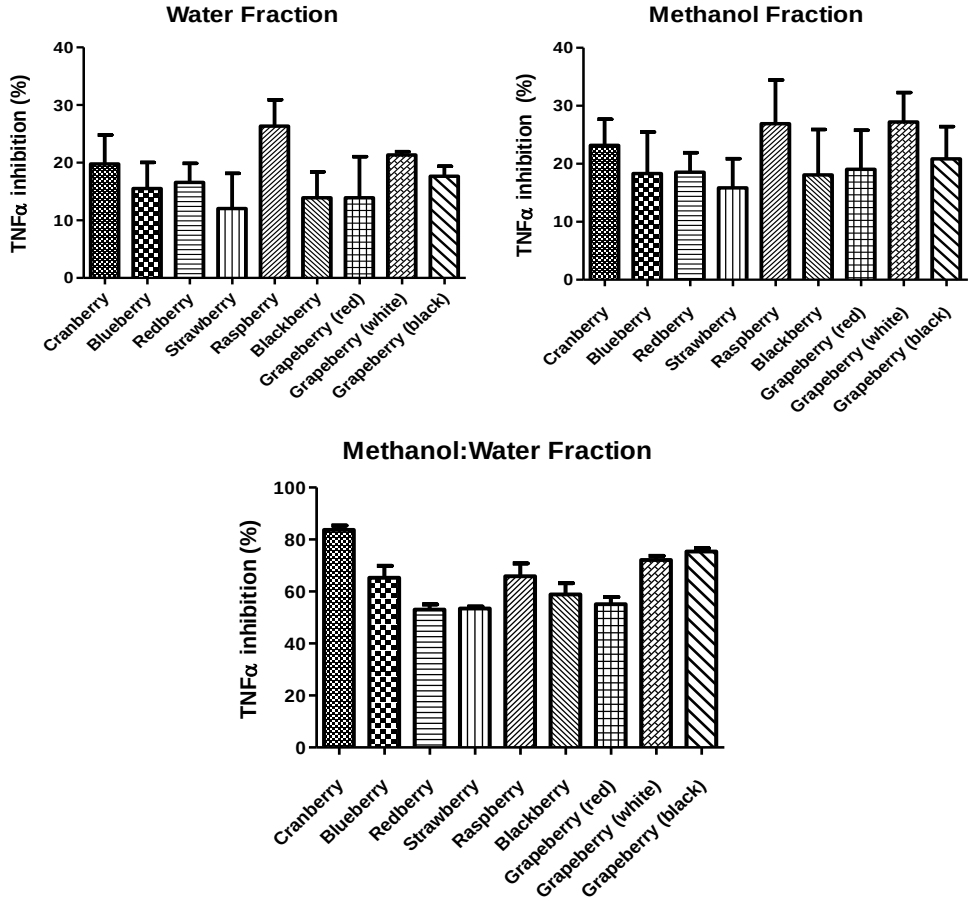

Fig. 2: TNF-α inhibition ratio (%) exhibit by different berries. Bars represent standard error of Mean (*n*=3).

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

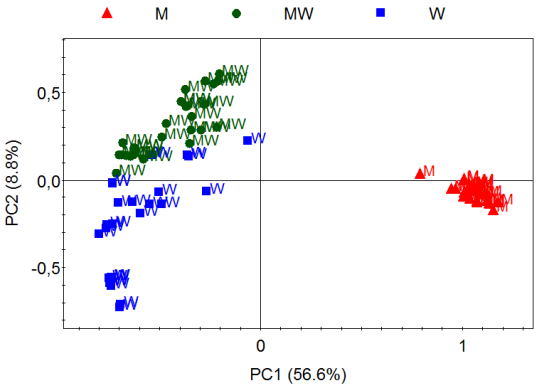

Fig. 3: Principal component analysis score plot of SPE fractions of different kind of berries. Red represents methanol, green represents methanol:water (1:1), and blue represents water fractions.

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.

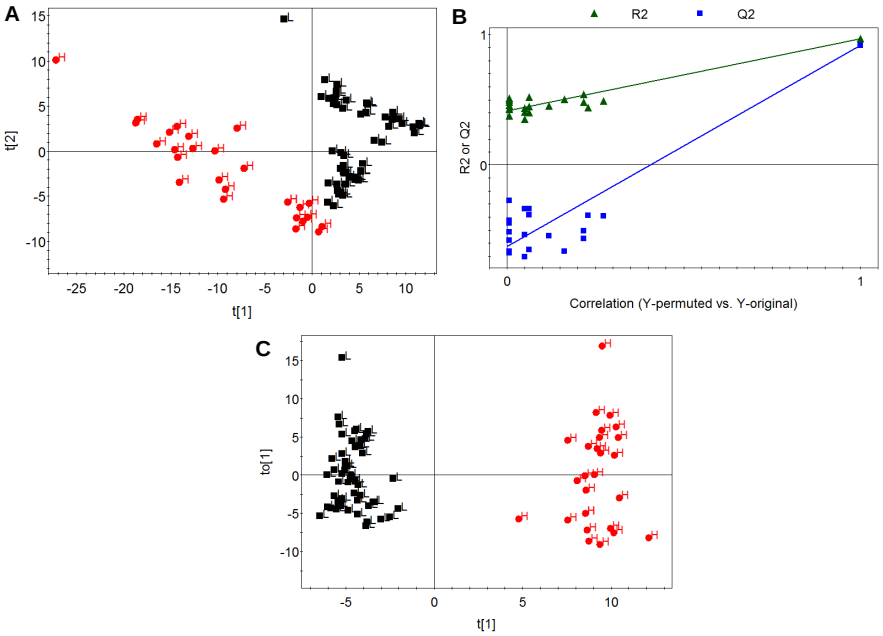

Fig. 4: The PLS-DA score plot (A), PLS-DA permutation test (B), and O2PLS-DA score plot (C) of anti-TNF-α activity of SPE fractions of different kind of berries. Black squares represent samples with low activity, whilst red circles represent samples with high activity.

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

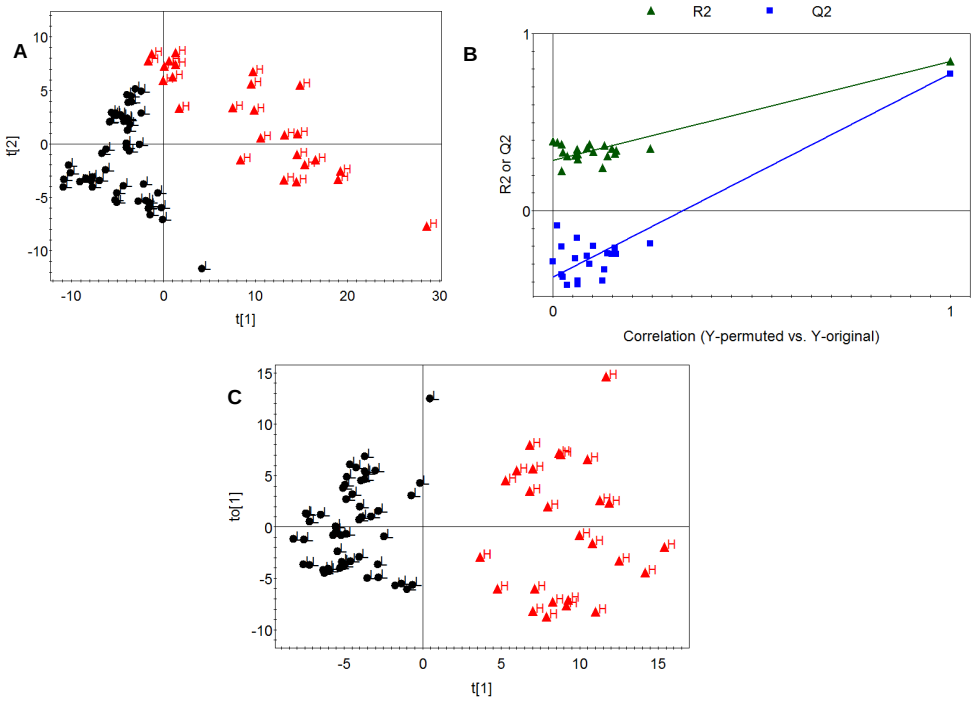

Fig. 5: The PLS score plot (A), PLS permutation test (B), and score plot of O2PLS (C) of anti-TNF-α activity of SPE fractions of different kind of berries. Black circles represent samples with low activity, red triangles represent samples with high activity.

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

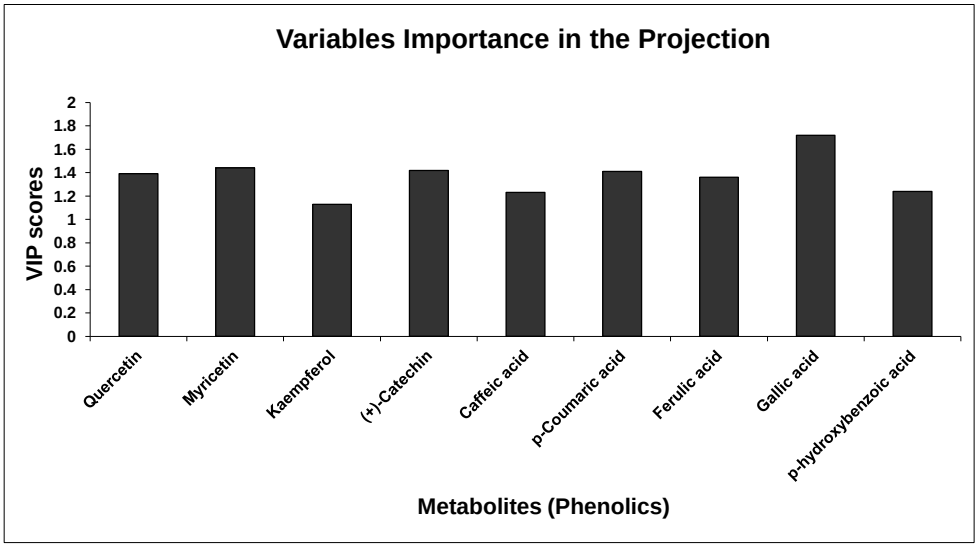

Fig. 6: The VIP scores of different phenolics identified in berries.

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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