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Analytical Methods

Red wines attenuate TNFα production in human histiocytic lymphoma cell line: An NMR spectroscopy and chemometrics based study

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ABSTRACT

Nuclear magnetic resonance (NMR) spectroscopy and multivariate data analyses methods are applied to the metabolic profiling of different red wines from Portugal. The water, methanol–water (1:1), and methanol fractions from solid phase extraction (SPE) with C18 resin were subjected to in vitro TNFα activity assay. Principal component analysis allowed the clear separation among the different SPE fractions according to the activity. Various supervised data reduction algorithms were tested and compared to highlight the TNFα inhibition by SPE fractions of wines. Partial least squares-discriminant analysis and orthogonal bidirectional PLS-DA were found most effective in discriminating the samples with different activities. By calculating variable importance in the projection, the active ingredients in the high activity samples have been identified as caftaric acid, quercetin, and catechin. Among the different vintages, samples from 2010 vintage were found with maximum anti-TNFα activity. The effectiveness of NMR spectroscopy in combination with chemometrics to identify the possible bioactivity in the several crude extracts is highlighted.

1. Introduction

The advancement in the statistical methods for the analysis of multivariate dataset aids largely to metabolomics by providing a platform to handle large dataset of metabolomics studies (Crockford et al., 2006). In combination with different chemometrics methods, NMR has been used to do metabolic profiling of different types of samples (Brescia et al., 2002; Charlton, Farrington, & Brereton, 2002). Several other studies have been published using the same combination focusing characterisation of plant species (Kim, Choi, Erkelens, Lefeber, & Verpoorte, 2005) and cultivars (Ali et al., 2009), monitoring and studying different physical effects on grape berry growth (Ali et al., 2011; Pereira et al., 2005, 2006).

Many studies showed analysis of the extracts from *Hypericum perforatum* (Roos, Roeseler, Bueter, & Simmen, 2004), *Artemisia annua* (Bailey et al., 2004), *Citrus grandis* (Cho et al., 2009), and *Galphimia glauca* (Cardoso-Taketa, Pereda-Miranda, Choi, Verpoorte, & Villarreal, 2008), for the prediction of different pharmacological activities using NMR spectroscopy with the combination of chemometrics methods. Recently our group has published two successful attempts on using chemometrics methods to highlight the active components in the crude extracts from plants (Ali et al., 2012; Yuliana, Khatib, Verpoorte, & Choi, 2011).

Inflammation plays a vital role in various high occurring diseases in the world like asthma, atherosclerosis, and rheumatoid arthritis. Mediators such as pro-inflammatory cytokines including interleukin-1 (IL-1), tumour necrosis factor-α (TNFα), and interferon-γ (IF-γ) are known to be released during an inflammatory response. The imbalance between pro-oxidants and antioxidants leads oxidative stress, which is known to play critical role in various degenerative diseases like diabetes, cancer, cardio vascular diseases, and artherosclerosis. Tumour necrosis factor-α is produce mainly by macrophages but can also be formed by various cells like T-cell, neutrophilis, NK cells, and synovial cells (Vilcek & Lee, 1991). TNFα is secreted during the early phase of inflammatory diseases and responsible to initiates the secretion of other cytokines like IL-1, IL-6, and IL-8 (Cho et al., 1999, 2001). Hence the local effect of TNFα can be considered as beneficial but it's over production can leads to systemic toxicity.

Wine is one of the most important beverages with long tradition and high value and known to contain a complex mixture of compounds at a wide range of concentrations. Wine phenolics

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have been proved to posses several health promoting activities (Ali, Maltese, Choi, & Verpoorte, 2010; 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 & Walzem, 2000). Since grape skin, seeds, and stem are the main source of phenolics in wine, red wines contains much higher concentrations of these compounds as compared to white wine as skin, seeds, and stem are left in contact with must in red wine making but rapidly separated from the must in the case of white wine.

The present study is aimed to perform the in vitro anti-TNFα assay to measure the inhibitory activity in different red wines from different vintages. Several wine phenolics and other primary metabolites are also identified using different NMR techniques. The activity data and NMR data is also correlated using different multivariate data analyses methods to identify the active components in red wines.

2. Materials and methods

2.1. Wine samples

All the wine samples analysed in this study are kindly provided by Eng. Inês Aranha and Esporão (http://www.esporao.com).

2.2. SPE and ELIZA for TNFα

A sample of 10 mL of each wine was completely dried under vacuum and then subjected to solid phase extraction (SPE) on SPE-C18 cartridges (Waters, Milford, MA, USA) according to Ali et al. (2012). The ELIZA for TNFα, which include the growth of human monocyte-like histiocytic lymphoma cells U937 (from ATCC), the lipopolysaccharides stimulation, cell treatment with wine extracts, and cell viability assay, was done and explained in detail in our previous report (Ali et al., 2012).

2.3. NMR spectroscopy, data analysis, and statistics

The three fractions eluted from SPE were redissolved in 1 mL of methanol-d4. An aliquot of 800 μL of sample was transferred to the 5-mm NMR tube and used for the NMR analysis. The parameters for NMR analysis (1D and 2D), data treatment, and statistics are used as previously explained (Ali, Maltese, Fortes, et al., 2011; Ali, Maltese, Toepfer, Choi, & Verpoorte, 2011; Ali et al., 2012). The TNFα content was arbitrarily set as 100 in the positive control and all the other values are normalised to this (% activity) and shown in results.

3. Results and discussion

3.1. Visual analysis of ^{1}H NMR spectra

Solid phase extraction (SPE) in combination with NMR spectroscopy was applied for the metabolic profiling of different red wines. The ^{1}H NMR spectra of water, methanol–water (1:1), and methanol fractions is shown in Supplementary data (Fig. SF1). It is quite obvious from the figure that the metabolic contents in each SPE fraction are very different and dominated by distinct classes of metabolites. The water fraction shows mostly sugars, organic acids, with few signals related to phenolics. The methanol fraction shows high signal intensity in amino acids and fatty acids regions with relatively low amount of sugars and no phenolics. The highest phenolic contents were observed in methanol–water fraction with relatively less sugars and amino acids contents. The distribution of specific metabolites in SPE fractions will be discussed in Sections 3.3 and 3.4.

3.2. Identification of metabolites

Different metabolites have been identified using ^{1}H NMR with the help of the above mentioned 2D techniques and cover a wide range of diversity and include amino acids, organic acids, carbohydrates, hydroxycinnamates, hydroxybenzoates, stilbenes, and flavonoids. Phenolics belong to one of the major classes of wine metabolites and many characteristic wine phenolics are identified in this study. Among the flavonoids, quercetin, and myricetin 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 also showed ^{1}H–^{1}H COSY correlations. Compounds like catechin and epicatechin were also identified. For 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 the reduced coupling constants (from 16.1 to 13.2 Hz). These olefinic protons are also found 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 also between H-6′ (δ 7.18) and 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. The derivatives of these two cinnamic acids, along with *trans*-feruloyl derivative, were also identified conjugated with tartaric acid through an ester linkage i.e. *trans*-caftaric acid, *trans*-fertaric acid, and *trans*-coutaric acid. Along with the *trans*-forms, the *cis*-forms, i.e. *cis*-caftaric acid and *cis*-coutaric acid, were also detected as the reduction in the coupling constant from 16.0 to 13.0 Hz was observed in the *J*-resolved spectrum. The *J*-resolved and COSY spectra with resonances correspond to different phenolics identified in wine are shown in Supplementary data (Fig. SF2).

The amino acids 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. The carbohydrate regions showed the signals of β-glucose, α-glucose, and sucrose. Other metabolites, including choline, 2,3-butanediol, and γ-amino butyric acid (GABA) were also identified in the same region. Organic acids like acetic acid, succinic acid, fumaric acid, formic acid, citric acid, lactic acid, malic acid, and tartaric acid are also identified. All the assignments were performed by comparing the spectra with previous reports (Ali et al., 2009; Ali, Maltese, Fortes, et al., 2011; Ali, Maltese, Toepfer, et al., 2011) as well as 1D and 2D NMR spectra of common plant metabolites in our in-house library. The chemical shifts and coupling constants of the identified metabolites are shown in the supplementary data (Table ST1).

3.3. Wine type, vintage, and anti-TNFα activity

Anti-TNFα activity resulted from the SPE fractions of different wine samples are shown in Fig. 1. Among the water fractions, the lowest activity is shown by the samples of Petit Verdot 2008 while the most active water fraction is of Aragonês 2010 wine. Different

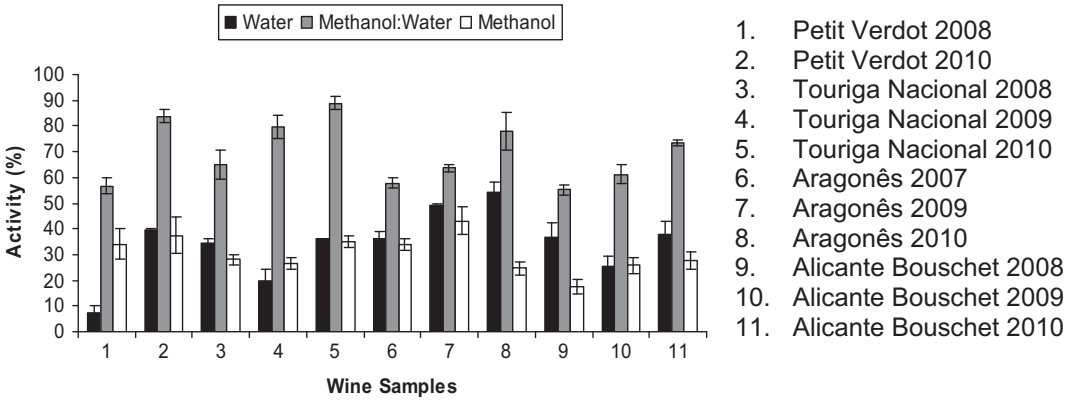

Fig. 1. Anti-TNFα activity (%) shown by SPE fractions of different wine samples at the concentration of 100 μg mL^{-1}. Bars represent mean ± standard deviation ($n = 3$, $p < 0.01$).

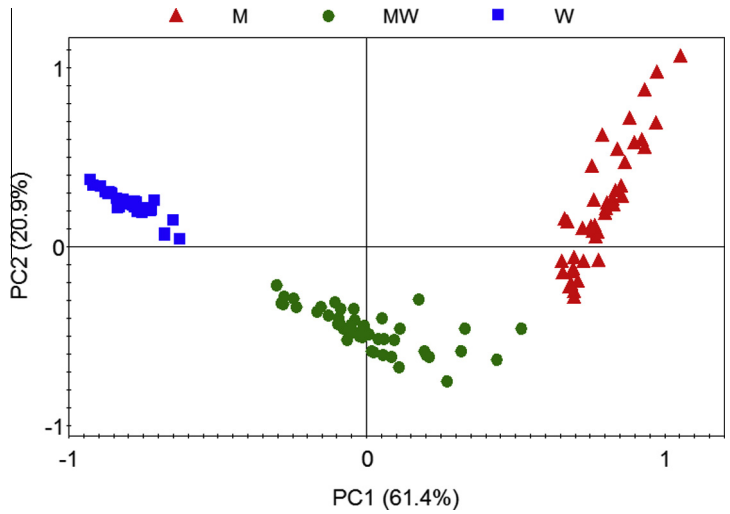

Fig. 2. Principal component analysis (PCA) score plot of SPE fractions of all the wine samples. All the three fractions are clearly separated from each other. Samples in blue, green, and red represent water, methanol–water (1:1), and methanol fractions, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

vintages from the wines like Petit Verdot (2008 and 2010), Touriga Nacional (2009 and 2010), and Aragonês (2007 and 2010) shown significantly different TNFα inhibition by the water fraction. By comparing the water fraction from the 2010 vintage of all wine types, it is evident that Aragonês is the most active against TNFα production than the other three wines.

The methanol fraction of SPE showed nearly equal activity in the case of different vintages of the same wine except for Aragonês, as its 2009 vintage showed significantly higher in activity than 2010 vintage. The most active methanol fraction is also from the Aragonês 2009 while the least active fraction is from Alicante Bouschet 2008. In most cases the activity shown by the methanol fractions are similar to their respective water fractions.

Methanol–water (1:1) fraction is the most active among the SPE fractions and showed the significantly higher anti-TNFα activity than the other two fractions. The vintage effect is very obvious in methanol–water fractions as Petit Verdot, Touriga Nacional, Aragonês, and Alicante from 2010 vintage are significantly higher in inhibiting TNFα production than the vintages of 2008 (Petit Verdot, Touriga Nacional, and Alicante Bouschet) and 2007 (Aragonês). Among the different wine types, the Touriga Nacional (2010) showed the maximum anti-TNFα activity, but not significantly higher than the other wines from the same 2010 vintage.

3.4. Principal component analysis (PCA)

Multivariate data analysis methods, either supervised or unsupervised, used to reduce the dimensionality of multivariate dataset and provide aid to identify differences or similarities among the samples. Principal component analysis (PCA) is a primary tool among the various multivariate data analysis methods. It is an unsupervised method and samples are clustered or separated due to metabolic profiles. The NMR data from the SPE fractions of all the samples have been subjected to PCA, Fig. 2 shows the score plots of PCA where different colors represent SPE fractions.

The PCA score plot shows clear distinction of all three fractions of SPE with tight clustering among the samples of same fraction. It is evident that water fractions are clustered on the negative side of PC1 (61.4%) and positive side of PC2 (20.9%) while the methanol fractions are grouped on the positive side of PC1 and PC2, with few exceptions. The methanol–water fractions are assembled on the negative side of PC2 while nearly distributed on both positive and negative sides of PC1. By examining the corresponding loadings plot, metabolites responsible for this separation are revealed. As shown by NMR spectra (Supplementary Fig. SF1), the methanol fractions are concentrated with fatty acids and amino acids including alanine, threonine, valine, arginine, and glutamic acid while the

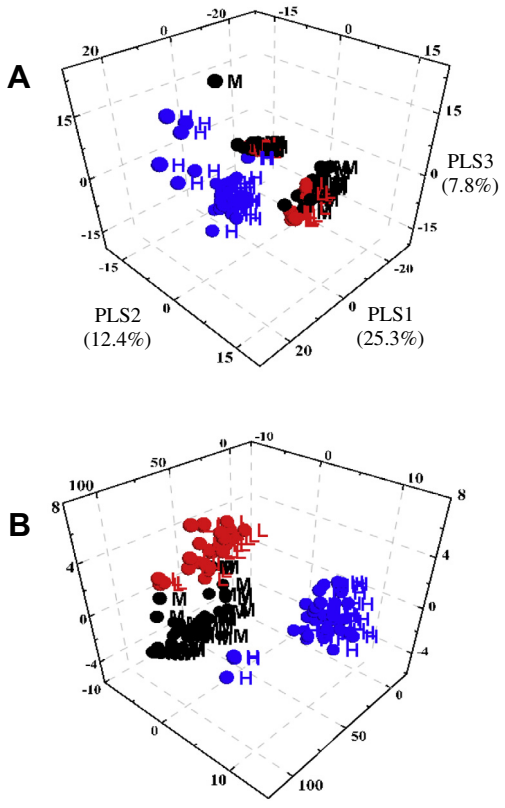

Fig. 3. Score plots of PLS-DA (A) and O2PLS-DA (B). Samples with high anti-TNFα activity are shown with 'H', while samples with medium and low anti-TNFα activity are shown as 'M' and 'L', respectively.

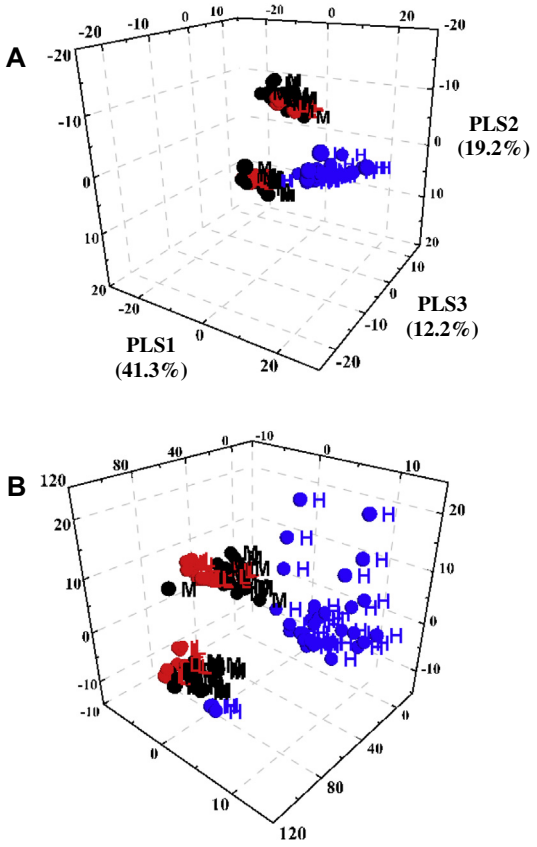

Fig. 4. Score plots of PLS (A) and O2PLS (B). Samples with high anti-TNFα activity are shown with 'H', while samples with medium and low anti-TNFα activity are shown as 'M' and 'L', respectively.

water fraction contain higher levels of glucose and sucrose with major organic acids like malic acid, tartaric acids, and succinic acids. The water–methanol fractions are accumulated with relatively higher amounts phenolics like quercetin, caftaric acid, coutaric acid, and resveratrol, as compared to other fractions of solid phase extraction.

3.5. Projection to latent structures-discriminant analysis (PLS-DA)

PLS-DA is a supervised multivariate data analysis method, performed with a pre-input data regarding the analysed samples i.e. classification of samples by creating dummy *Y*-variables. The 3D-score plot of PLS-DA (Fig. 3A) shows good separation of samples with high activity from the others. Not a clear distinction between the samples with medium and low activity is observed.

To discriminate the low and medium activity samples, another supervised algorithm, bidirectional orthogonal PLS-DA (O2PLS-DA), was used. As shown by the score plot (Fig. 3B), a very nice separation among all the three classes of samples is achieved. By examining the corresponding loadings plot, metabolites responsible for the separation are identified. 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 medium activity samples.

3.6. Projection to latent structures (PLS)

PLS is another supervised data reduction algorithm in which instead of creating dummy *Y*-variables, actual data from other analysis (in this case anti-TNFα activity) is used as a *Y*-data set. Fig. 4A shows the 3D-score plot of PLS analysis and similar to PLS-DA, samples with high activity are well separated but this is not observed with samples of low and medium activity. Bidirectional orthogonal PLS was also used in order to separate all the three classes of samples but as shown by the score plot (Fig. 4B), only high activity samples are clearly separated with many overlaps between the samples of low and medium activity. The loadings plot shows similar classes of metabolites accumulates in samples with high, medium and low activity, as already shown by PLS-DA.

3.7. Validation of PLS and PLS-DA

One of the key aspects of a supervised regression algorithm is model validation, for which permutation test is often used. The R2 and Q2 values of PLS and PLS-DA were calculated using six components for both analyses. For anti-TNFα 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. 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. 5A and B).

3.8. Variable importance in the projection (VIP)

It has been indicated that it is directly proportional with the influence of factor on the separation on score plot, meaning, factors with higher VIP values are more influential for the samples separation. It has been reported that factors with VIP values more than

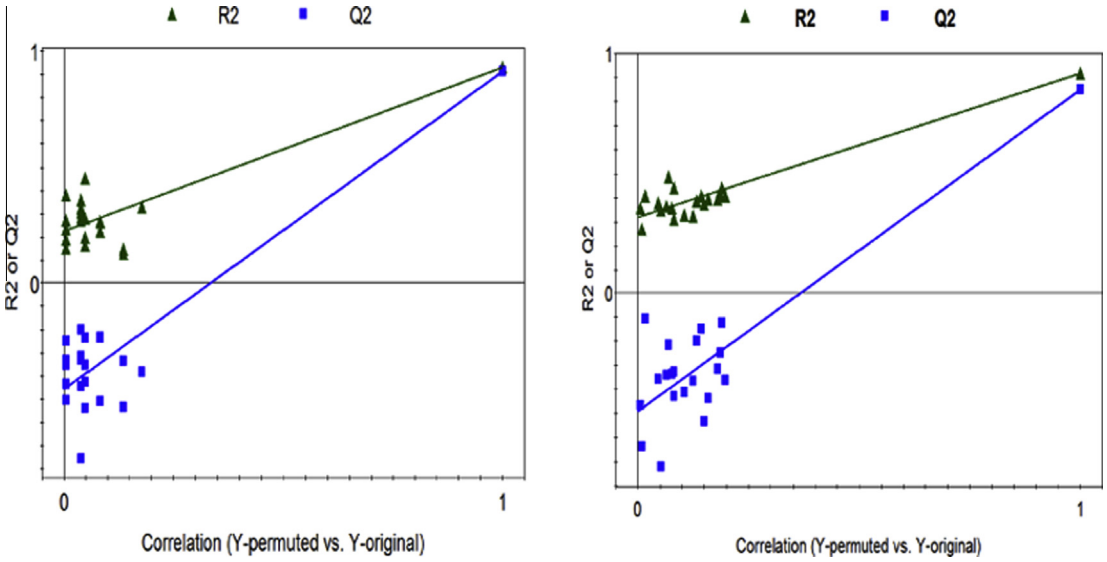

Fig. 5. The validation plot of permutation test for PLS-DA (A) and PLS (B) models using ^{1}H NMR resonances and anti-TNFα based on high, medium, and low activity classes.

0.7 could be regarded influential for the separation of samples (Eriksson et al., 2006). For O2PLS-DA and O2PLS analyses, VIP values for several phenolic compounds are calculated. Among the identified phenolics in wine during this study the VIP values of the major contributing metabolites are as follows; caftaric acid at δ 7.02: 1.91, quercetin at δ 7.71: 1.74, coutaric acid at δ 7.59: 1.42, and catechin at δ 5.89: 1.18. This high VIP scores authenticate phenolics involvement in the separation of samples different TNFα production inhibition potential.

Plants metabolites generally known to have some bioactivities related to their structure and function. 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 analysis of the extracts from different plants (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 with the combination of chemometrics methods. Recently our group published a significant advancement in the approach by using multivariate regression methods in combination with NMR spectroscopy of *Orthosiphon stamineus* (Yuliana et al., 2011). This methodology is very effective in the screening of various plant extracts in order to identify the plants with some medicinal activities without any laborious fractionation and chromatographic separation of the crude extract. Fractions from SPE of various red wines from Portugal were analysed for anti-TNFα activity and the combination of NMR spectroscopy and chemometrics successfully applied to identify the active ingredients.

The vintage effect on metabolic profile of grapes and wine has been extensively studied (Lee, Hwang, Berg, Lee, & Hong, 2009; Pereira et al., 2006). It is known that the amino acids and phenolics are highly affected by the environment of a grape production area. It reported 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 TNFα 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 either in 2010 vintage the berries experienced hot and dry climate which ultimately resulted in higher phenolic contents or and more potency towards TNFα inhibition. Another strong possibility of low activity in older wine samples is with the passage of time (ageing), the phenolics tends to bind together to form their polymeric form (for instance, catechin monomers combine to form their polymer known as tannins) which significantly reduced their effectiveness as an antioxidants (Waterhouse, 2002).

The ageing effect on wine phenolics is well documented in numerous reports (Fang et al., 2008; Gambuti, Rinaldi, Ugliano, & Moio, 2013). The wines tested in this study are well studied for their antioxidant properties and phenolic composition (Castillo-Munoz, Gomez-Alonso et al., 2009; Paixao, Perestelo, Marques, & Camara, 2007). Our previous report on grape berry development documented the fact that cultivars like Touriga Nacional contains higher phenolics (Ali, Maltese, Fortes, et al., 2011; Paixao et al., 2007), and it is also already reported that the phenolic content in berries very closely reflect the phenolic content of the wines made from those berries (Castillo-Munoz, Fernandez-Gonzalez, Gomez-Alonso, Garcia-Romero, & Hermosin-Gutierrez, 2009). These observations were well supported by the findings in this study as fractions of Touriga Nacional are most active to inhibit TNFα production. Other samples from Petit Verdot (Castillo-Munoz, Gomez-Alonso et al., 2009) and Alicante Bouschet (Castillo-Munoz, Fernandez-Gonzalez, et al., 2009) are also well known to have higher levels of flavonoids like quercetin and myricetin along with their 3-*O*-glucoside derivatives, which are reported for their antioxidant activity (Pekkarinen, Heinonen, & Hopia, 1999). The presented work also showed the presence of these highly active metabolites among the studied wine samples due to which the inhibition of TNFα production is observed.

It is a fact that diet could have beneficial effects and the consumption of antioxidant rich food (fruits, vegetables, tea, and wine) could show health-promoting effects. The medicinal importance of moderate wine consumption has been proven by many studies. Several health promoting activities associated to wine polyphenols are comprehensively reviewed (Cordova & Sumpio, 2009; Opie & Lecour, 2007) and are now well known against cardio vascular diseases, renal disorders, Alzheimer's disease, cancer, and also against bacteria, and virus (Cordova, Jackson, Berk-Schlessel, & Sumpio, 2005; Marambaud, Zhao, & Davies, 2005). Phenolics, in general, are also well known for their potency against TNFα production as they are widely accepted to have anti-oxidative and anti-inflammatory properties (Baur et al., 2006; Chuang et al., 2010). Phenolics

like resveratrol (Stewart et al., 2008) and quercetin (Rivera, Morón, Sánchez, Zarzuelo, & Galisteo, 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, Yi, Pearson, Waterhouse, & Frankel, 1997).

4. Conclusions

The present study, NMR spectroscopy-based metabolomic approach, is an attempt to analyse different wine types and vintages for TNFα inhibition. The proposed approach is found very effective in discriminating the SPE fractions from different wine types and vintage based on the efficacy to reduce TNFα production. Solid phase extraction in combination with different chemometrics methods proved that the active ingredients in an extract could be identified using a PLS-based regression models with ^{1}H NMR and anti-TNFα activity data set. Vintages and types of wines proved to be influential on their TNFα inhibition potency. Phenolics like quercetin, caftaric acid, and catechin are identified as most influential in inhibiting TNFα production among the other wine metabolites. It is suggested that the similar approach can be applied for the prediction of anti-TNFα activity of crude plant extract using NMR and multivariate data analysis. The methodology proposed here can also be applied to infer the various other bioactivities associated to wine or other food items without any laborious chromatographic separation of metabolites.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2013.06.001.

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