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Chapter 8

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

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Abstract

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

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

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

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

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

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

*Eugenia uniflora*, also known as “arrayán” in Argentina, “Surinam cherry”, “cerezo Brazileño”, “cereza de Cayena”, “pitanga” in Brazil, “pendanga” in Venezuela, “guinda” in El Salvador, ñanga-piré and “cereza cuadrada” in Colombia, belongs to the family Myrtaceae, indigenous to...
Argentina, Southern Brazil, Surinam, Guyana, Uruguay and also commonly dispersed in other South American countries. People consume this fruit as fresh fruit, juice, frozen pulp, or jam. It is also used to make typical liquor in Northeastern Brazil (Porcu and Rodriguez-Amaya, 2008). It is a 7- to 10-ribbed berry that ranges between 1.5 and 5.0 cm long and is known for its exotic flavor. While ripening, its taste varies from very acid to sweet (Malaman et al., 2011). Epicarp of the fruit changes from green to yellow, orange, dark red and finally almost black in order from (Celli et al., 2011).

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

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

Transgenic line of zebrafish expressing green fluorescent protein in neutrophils was used to study in-vivo. Due to the transparency of zebrafish embryo, it is possible to visualize the movement of neutrophils towards the
affected site. Other advantages of zebrafish embryo are their limitless availability, low cost and ease of handling. Apart from this they require very little medium for growth, so that they are cost effective if compared with other mammalian models (Kari et al., 2007).

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

### 8.2. Materials and Methods

#### 8.2.1. Sampling

Fruits of \textit{Eugenia uniflora} L from 4 different ripening stages were used. Fruits were classified as followed: green (immature), orange, red and purple (mature). The berries were collected around 10 a.m. in November of 2009 at Nogalito’s wood located in El Siambón, Tucumán (Northwestern Argentina). Samples were immediately transported to the laboratory in dry ice. Five biological replicates (each including 80-100 berries) were realized. Each replicate contained berries from a single plant, and from the sunny and shady
sides of the plants. Berries were grinded in liquid nitrogen, seeds removed and kept at -20 °C until use.

8.2.2. Extraction

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

8.2.3. Cell Culture

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

8.2.4. TNF-α assay

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

8.2.5. MTT assay

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

8.2.6. Zebrafish culture

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

8.2.7. Chemical induced inflammation assay (ChIn)

Assay was performed as described by (d’Alencon et al., 2010). Briefly, E3 medium was used to grow zebrafish larvae of the GFP strain. They were kept in Petri dish until 56 hours post fertilization. Spontaneously hatched larvae were transferred to 48-well plates at the rate of 1 larva/well in a volume of 500 µL of E3 solution. Fruit extracts and controls were pipette to the wells containing embryos 1 hour before the addition of CuSO₄. Plates were incubated for 40 minutes at 28 °C. E3 medium was replaced with 4% paraformaldehyde in PBS buffer which was used to fix the embryo and further incubation was carried out for 1 hour at room temperature. Fixing and subsequent steps normally carried out in dark to evade fading of the fluorescent protein signal. Larvae were washed with PBS-Tween20. Fluorescent cells were examined and counted within the next 48 hours after fixation using a Leica (Wetzlar, Germany) MZ-12 fluorescent stereoscope. Labeled cells were within a specific area known as myoseptum which consist of between the first somite and the end of the tail on one side of each larva. Sixteen embryos were used for each concentration and cells were counted by two independent observers.

8.2.8. ¹H NMR spectroscopy

50 mg lyophilized sample of berries was extracted according to (Kim et al., 2010). Briefly samples were transferred to 2 ml eppendorf tubes, 1ml of MeOD and D₂O 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. ¹H. 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- d₄ was used as the internal lock. Each ¹H 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$_2$O signal with low power selective irradiation at the H$_2$O frequency during the recycle delay. FIDs were Fourier transformed with LB = 0.3 Hz. The resulting spectra were manually phased and baseline corrected, and calibrated to TSP at 0 ppm, using XWIN NMR (version 3.5, Bruker). 2D NMR techniques were performed by using parameters described by (Ali et al., 2012).

8.2.9. Data analysis

The $^1$H NMR spectra were reduced to ASCII files. Bucketing was performed by AMIX software (Bruker) with scaling to total intensity. Spectral intensities were scaled to the TSP signal (δ 0.0) and reduced to integrated regions of equal width (0.04) corresponding to the region of δ 0.3–10.0. During analysis, regions between δ 4.75–4.9 and δ 3.28–3.40 were excluded because of the residual signal of water and methanol-d$_4$, respectively. SIMCA-P software (v. 12.0, Umetrics, Umeå, Sweden) was used to perform principal component analysis (PCA) with scaling based on Pareto while partial least square (PLS) with scaling based on Unit Variance. Means and standard deviations were calculated. ANOVA was performed for comparison with means and significance level was set at <0.05.

8.3. Results and discussion

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

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

To analyze and identify the most important metabolites characteristic for the developmental process of *Eugenia uniflora*, we subjected NMR data to PCA. The obtained results are displayed in Fig. 2. The PCA score plot reveals a pattern in which green stages tend to cluster on the negative side of PC1 along with the yellow stages. Few replicates of the yellow stages lie closer to the red stage in the positive side of PC1. Both Red and purple ripened stages are on the positive side of PC1. This result indicates good separation of the four developmental stages on the basis of the time of development. The loading
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column plot which is the projection of the variables in our case metabolites of the cherries revealed that green and yellow stages are rich in phenolics, organic acids and amino acids while the more ripened stage obviously has high content of sugars like sucrose, fructose and glucose.

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

![Figure 2: Scatter plot of stage development. G stands for green, P stands for purple, Y stands for yellow and R stands for red.](image)

*In-vivo* analysis was done by using mutant zebrafish embryos as a model. A representative figure of control and zebrafish embryo treated with CuSO₄ with and without fruit extract is shown in Fig 3B. The fluorescents cells represent the sites of injury after treating the embryo with 10 µM CuSO₄. The zebrafish embryo treated with CuSO₄ in presence of fruit extract exhibit less number of leukocytes movement as compared to embryo treated with CuSO₄ without fruit extract. Fig 3C shows results similar to the *in-vitro* TNF-α inhibition. The green stage of fruits would seem to be dominantly significant from other stages in term of controlling or inhibiting the injury. To identify
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compounds related to activity activity for TNF-α inhibition, a supervised method (Partial least square model, PLS) was applied. TNF-α inhibition value was added as \( Y \)-input to the model. Activity values were divided into two classes: active (>60%) and non active (<60%). The score plot for the resulting model is shown in Fig 4 A. The red dots show the stage responsible for high TNF-α inhibition activity, while the black triangles show samples with less activity for TNF-α inhibition. One replicate from the study has been excluded due to some experimental error. As the PLS score plot shows, a nice separation between active and non active groups have been achieved with PLS1 (65%) and

Figure 3 A: TNF-α inhibition (%) exhibited by four different developmental stages of *Eugenia uniflora* at the dose rate of 100 µg/ml. Bars represent standard error of Means \((n=3)\). 3B (a): 56hpf zebrafish embryo with fluorescent leukocytes. (b) myoseptum area of 10 µM CuSO\(_4\) treated embryo (yellow arrows shows the area of injury with fluorescent clusters of leukocytes). (c) myoseptum area of extract treated embryo exhibit less number of leukocytes movement as compare to CuSO\(_4\) treated embryo. 3 C. Quantification of migrating leukocytes after treatment with extract at the dose rate of 100 µg/ml. The higher the number of cells the lower the activity of extracts.
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and PLS2 (13%). The stage related to high activity is on the positive side of vector t1 of the PLS scatter plot while stages correlating with less activity cluster on the negative side of t1. By examining the corresponding Y-coefficient loadings plot Fig 4.b, we find high content of phenolics like quercetin, myricetin, kaempferol, cinnamic acid, chlorogenic acid, and amino acids like glutamate and glutamine which are corresponding with the inhibition of TNF-α.

For *in-vivo* analysis cell counts were used as Y-input value. Fruit extracts able to reduce the cell count to less than 10 were assigned as active while extracts unable to reduce the cell count less than 10 were assigned as non active. The resulting model does not show a clear distinction among the active and non-active samples (data not shown) which can be due to noise in the spectra. To improve the correlation of activity with metabolites, a built-in filter was used called orthogonal signal correction (OSC). This filter removes the unwanted and uncorrelated signals by using orthogonal logarithm (Ali et al., 2012; Eriksson et al., 2006). After applying their filter the remaining data were again subjected to PLS modeling, the resulting model was highly improved with PLS1 (37%) and PLS2 (32%) values. From the score scatter plot (Fig.5 A), we can see that the active stages are on the negative side of the score plot or PLS1 while the non active stages are on the positive side of the PLS1 plot. The relevant coefficient plot (Fig.5.B) showed a high content of phenolics compounds like quercetin, myricetin, kaempferol, organic acids like cinnamic

Figure 4A: PLS score plot for *in vitro* activity based on whole range of 1H NMR spectra. Triangle shows non active extracts, while red dots represent active extracts for *in vitro* anti inflammatory activity. 5B. Coefficient plot for *in vitro* PLS score plot. Black bars on negative side of coefficient plot relate with the non active extracts while red bars on the positive side of coefficient plot relate with active extract for *in vitro* activity.
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acid, chlorogenic acid, malic acid and amino acid like glutamate and glutamine for the active stages the PLS1 while high sugar content is on the non active side of PLS1. These results confirm the findings that high phenolic contents are responsible for antiinflammatory activities both *in-vivo* as *in-vitro*.

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

Flavonoids present in the plants either simple or complex glycosides. These polyphenolic compounds and their sugar derivatives display a remarkable spectrum of biological activities including anti-inflammation (Miles et al., 2005; Pietta, 2000). Several reports have been published related to activities of the mentioned flavonoids against TNF-α production (Chuang et al., 2010; Park et al., 2008). There are reports that quercetin inhibits TNF-α secretion selectively in different cell studies (Wadsworth and Koop, 1999; Wadsworth et al., 2001). Myricetin, another flavonoids have been reported to inhibit TNF-α production in LPS stimulated J774.1 cell lines (Herath et al., 2003). Similarly, phenolics

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**Figure 5A:** OSC-PLS scatter plot for *in-vivo* activity. Triangle shows non active extract, while red dots represent active extract for *in-vivo* anti inflammatory activity. 6B. Coefficient plot for *in-vivo* OSC- PLS score plot. Black bars on positive side of coefficient plot relate with the non active extracts while red bars on the negative side of coefficient plot relate with active extract for *in vitro* activity.
acids including caffeic acid, gallic acid, ferulic acid, p-coumaric acid and chlorogenic acids are well known inhibiting TNF-α production (Chauhan et al., 2011; Kim et al., 2006; Sakai et al., 1997; Shin et al., 2004).

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

8.4. Conclusion

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

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