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INFLUÊNCIA DOS SOLVENTES DE EXTRAÇÃO NO CONTÉUDO EM COMPOSTOS BIOACTIVOS E ACTIVIDADE ANTIOXIDANTE DE FLORES DE AMORES-PERFEITOS

EXTRACTION SOLVENTS' INFLUENCE ON THE CONTENT OF BIOACTIVE COMPOUNDS AND ANTIOXIDANT ACTIVITY OF PANSIES

INFLUENCIA DE LOS SOLVENTES DE EXTRACCIÓN EN EL CONTENIDO DE COMPUESTOS BIOACTIVOS Y ACTIVIDAD ANTIOXIDANTE DE LAS FLORES DOS PENSAMIENTOS

Luana Fernandes[1, 2, 3]
José Alberto Pereira[4]
Jorge Saraiva[5]
Susana Casal[6]
Elsa Ramalhosa[7]

[1] Instituto Politécnico de Bragança, Escola Superior Agrária, Centro de Investigação de Montanha (CIMO), Bragança, Portugal.
[2] Universidade de Aveiro, Departamento de Química, Aveiro, Portugal.
[3] Universidade do Porto, Faculdade de Farmácia, Laboratório de Bromatologia e Hidrologia (LAQV@REQUIMTE) Porto, Portugal
[4] Instituto Politécnico de Bragança, Escola Superior Agrária, Bragança, Portugal
[5] Universidade de Aveiro, Departamento de Química, Aveiro, Portugal
[6] Universidade do Porto. Faculdade de Farmácia, Porto, Portugal
[7] Instituto Politécnico de Bragança, Escola Superior Agrária, Centro de Investigação de Montanha (CIMO), Bragança, Portugal

Luana Fernandes - luaopus@hotmail.com | José Alberto Pereira - jpereira@ipb.pt | Jorge Saraiva - jorgesaraiva@ua.pt | Susana Casal - sucasal@ff.up.pt | Elsa Ramalhosa - elsa@ipb.pt

Corresponding Author
Elsa Ramalhosa
Instituto Politécnico de Bragança
Campus de Santa Apolónia
5300-253 BRAGANÇA
elsa@ipb.pt

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RESUMO

Introdução: Amores-perfeitos (*Viola×wittrockiana*) são uma fonte rica de antioxidantes naturais com efeitos benéficos para a saúde humana.
Objetivos: O objetivo do presente estudo foi investigar a influência dos solventes (água, metanol e água:acetona (6:4, v/v)) na extracção de compostos bioactivos e actividade antioxidante de extractos de amores-perfeitos.
Métodos: Os compostos bioactivos analisados foram os seguintes: flavonóides, taninos hidrolisáveis e antocianinas monoméricas, bem como, os fenóis totais através da avaliação da capacidade redutora total. A actividade antioxidante foi avaliada pelos métodos de sequestro do radical livre 2,2-difenil-1-picrilhidrazilo (DPPH) e poder redutor. Uma análise de componentes principais (PCA) foi realizada para diferenciar os extractos dos amores-perfeitos.
Resultados: Os solventes que produziram extractos com os maiores teores de taninos hidrolisáveis e capacidade redutora total foram os de metanol e água: acetona (6:4, v/v). Para extrair os maiores teores de antocianinas monoméricas deve-se usar metanol (5.93 mg Cy 3-glu/ g de massa seca), enquanto que para extrair flavonóides, a água:acetona (6:4, v/v) é preferível (115 mg QE/g de massa seca). A água mostrou ser o solvente menos eficaz, obtendo-se extractos com a menor actividade antioxidante. Para além disso, os extractos de metanol e água:acetona foram claramente separados dos aquosos através da análise por PCA.
Conclusões: Os resultados mostraram que a extracção de compostos bioactivos e a actividade antioxidante de flores de amores-perfeitos são influenciadas pelo solvente usado.

Palavras-chave: Amores-perfeitos; Solventes; Actividade antioxidante; Compostos bioactivos.

ABSTRACT

Introduction: Pansies (*Viola×wittrockiana*) are a rich source of natural antioxidants with beneficial effects on human health.
Objetives: The aim of our study was to investigate solvents' influence (water, methanol, water:acetone (6:4, v/v)) on the extraction of bioactive compounds and antioxidant activity of pansies extracts.
Methods: The bioactive compounds analyzed were the following: flavonoids, hydrolysable tannins and monomeric anthocyanins, as well as total phenols by the total reducing capacity assay (TRC). The antioxidant activity was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and reducing power assays. A Principal Component Analysis (PCA) was performed to differentiate pansies extracts.
Results: The solvents that yielded extracts with the highest contents of hydrolysable tannins and TRC were methanol and water:acetone (6:4, v/v). To extract the highest contents of monomeric anthocyanins, methanol should be used (5.93 mg Cy 3-glu/g flower, d.w), while for flavonoids, water:acetone (6:4, v/v) was the preferred yielding an extract with 115 mg QE/g flower d.w. Water turned out to be the least effective solvent, giving extracts with the lowest antioxidant activity. In addition, methanol or water:acetone extracts were clearly distinguished from aqueous ones through a PCA analysis.
Conclusions: Our results show that the bioactive compounds and antioxidant activity of pansies' extracts are affected by the solvent used.

Keywords: Pansies; Solvents; Antioxidant activity; Bioactive compounds.

RESUMEN

Introducción: Los pensamientos (*Viola x wittrockiana*) son una rica fuente de antioxidantes naturales con efectos beneficiosos sobre la salud humana.
Objetivos: El objetivo del presente estudio fue evaluar la influencia de los solventes (agua, metanol y agua:acetona (6:4, v/v) en la extracción de compuestos bioactivos y actividad antioxidante de extractos de pensamientos.
Métodos: Los compuestos bioactivos analizados fueron flavonoides, taninos hidrolizados y antocianinas monoméricas. También se analizaron los fenoles totales por el ensayo de capacidad reductora total. La actividad antioxidante fue evaluada por los métodos: secuestro del radical libre 2,2-difenil-1-picrilhidrazilo (DPPH) y poder reductor. El análisis de componentes principales (PCA) fue realizado para diferenciar los extractos de los pensamientos.
Resultados: Los solventes que produjeron extractos con los mayores contenidos de taninos hidrolizados y capacidad reductora total fueron el metanol y agua:acetona (6:4, v/v). Los extractos con mayores contenidos de antocianinas monoméricas se obtuvieron utilizando metanol (5.93 mg Cy 3-glu/g peso seco). Para los flavonoides el agua: acetona (6:4, v / v) fue el solvente que hay dado los mejores valores de extracción (115 mg QE / g peso seco). El agua fue el solvente menos eficaz, obteniéndo se extractos con menor actividad antioxidante. Además, los extractos de metanol y agua:acetona se separaron claramente de los acuosos mediante el análisis por PCA.

Conclusións: Los resultados mostraron que la extracción de compuestos bioactivos y la actividad antioxidante de los pensamientos son influenciadas por el solvente utilizado.

Palabras Clave: Pensamientos; Solventes; Actividad antioxidante; Compuestos bioactivos

INTRODUCTION

Garden pansies (*Viola × wittrockiana Gams.*) are plants of complex hybrid origin, involving at least three species, namely *Viola tricolor*, *Viola altaica*, and *Viola lutea* (Vukics et al., 2008). They are popular ornamental plants, differing in size and color, depending on the variety (Weryszko-Chmielewska and Sulborska, 2012). These flowers are mentioned as edible, so they can be found on the market in special packages (Kelley et al. 2003), which are used for garnish, salads, soups, desserts and drinks.
Some studies have been performed in pansies, concerning their antioxidant activity and polyphenolic composition (Hase et al., 2005; Vukics et al., 2008; Gamsjaeger et al., 2011; Skowyra et al., 2014; González-Barrio et al., 2018). Nowadays, plant polyphenols (ex. flavonoids, tannins and anthocyanins) are increasing attention due to their antioxidant properties and documented effects in the prevention of various oxidative stress associated diseases, such as cancer, obesity and diabetes (Dai and Mumper, 2010). So, these active compounds from flowers can be used as drugs, as well as ingredients (to enhance flavor, color, etc.) in food and cosmetics (Jun, 2013).
The most common method to obtain these compounds from plants is solvent extraction. Polar solvents, such as methanol and ethanol, are frequently employed for the recovery of polyphenols from a plant matrix (Kuźma et al., 2014). In detail, reported solvents and extraction conditions used in pansies include: methanol (70%, v/v) in an ultrasonic bath at room temperature for 20 min (Vukics et al., 2008); methanol (100%, v/v) at 25 °C for 24 h (Rop et al., 2012); water (100%, v/v), ethanol (50%, v/v) and acidic ethanol (50%, v/v) at 4 °C for 24 h, under stirring (Skowyra et al., 2014); methanol containing 0.1% HCl at 4 °C for 24 h with gentle shaking (Li et al. 2014a); and methanol (80%, v/v) containing 1% HCl, at 4 °C for 12 h (Benvenuti et al., 2016). So, the aim of this work was to further analyze the effect of using solvents with a wide range of polarities on different bioactive compounds extraction and antioxidant activity of *Viola × wittrockiana*. The solvents studied were water, methanol, and water:acetone (6:4, v/v). The extracts obtained were compared with respect to their flavonoids, hydrolysable tannins and monomeric anthocyanins contents, as well as their total reducing capacity (TRC) and antioxidant activity evaluated by the reducing power and scavenging effect on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assays.

1. METHODS

1.1 Flower

Fresh pansies (*Viola × wittrockiana*) of different colors were collected from the greenhouse of School of Agriculture, Polytechnic Institute of Bragança, during the year 2015 (Figure 1). After harvest, flowers were transported immediately to the laboratory under refrigeration. On their arrival, flowers were immediately frozen and lyophilized (Scanvac, Coolsafe, Lynge, Denmark) for 2 days. Afterwards they were ground to a homogeneous powder, which was stored protected from light and moisture until further analyses.

Figure 1 – Pansies of different colors studied in the present work.

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1.2 Chemicals and reagents

Methanol and acetone were obtained from Fisher Scientific (Leicestershire, UK). Gallic acid and DPPH (2,2-diphenyl-1-picrylhydrazyl) were from Sigma–Aldrich (St. Louis, MO, USA), while Folin–Ciocalteu reagent and sodium carbonate were obtained from Panreac Quimica SA (Barcelona, Spain). All reagents were of analytical grade. Milli-Q system (Millipore Corp., Molsheim, France) ultra-pure water was used throughout this research.

1.3 Extraction conditions

The extraction with different solvents was based on the method described by Li et al. (2014b), with slight modifications. A 1:20 mass/volume ratio was used on all extractions. Dried powder (1 g) of a pansies' mixture was extracted with 20 mL of different solvents (methanol, water and water:acetone (6:4, v/v)) at 37 °C for 30 min under agitation (900 rpm) (IKA, RCT Model B, Staufen, Germany). The methanol and water:acetone extracts were filtered and concentrated in a rotary evaporator, in order to remove methanol and acetone, respectively. Then, all solutions were frozen and placed in the freeze-drier for 2 days to obtain the extracts. The extracts were weighted and dissolved in the same solvent used in the extractions to a concentration of 50 mg extract/mL and covered with aluminium foil under freezing until further analysis. All extractions were performed in triplicate.

1.4 Total flavonoids

Total flavonoids content was determined by the method described by Viuda-Martos et al. (2011), with slight modifications. To pansies extracts (1 mL) was added 0.3 mL $NaNO_2$ (5%, m/v) and, after 5 min, 0.3 mL $AlCl_3$ (10%, m/v) was mixed. After 6 minutes, 2 mL NaOH (1M) was added. The absorbance was read at 510 nm and flavonoids were quantified using a standard curve of quercetin (10-160 µg/mL). The results were expressed in mg of quercetin/g flower, dry weight (mg QE/g flower, dry weight (d.w.)). All measurements were performed in triplicate.

1.5 Hydrolysable tannins

The content of hydrolysable tannins was determined by the method described by Elfalleh et al. (2012). Flower extracts (1 mL), 5 mL 2.5% KIO_3 was added and stirred for 10 seconds. The absorbance was measured at 550 nm. The blank corresponded to the solvent used in each sample. Different concentrations of tannic acid (0.025 to 1.6 g/L) were used for calibration. Results were expressed in mg of tannic acid equivalent/g flower, dry weight (mg TAE/g flower, d.w.). All measurements were performed in triplicate.

1.6 Total monomeric anthocyanins

The total monomeric anthocyanins contents in the pansy extracts were estimated by the pH differential method, following the methodologies used by Bchir et al. (2012) and Rajasekar et al. (2012). The method consisted in using two buffer systems: potassium chloride buffer, pH 1.0 (0.025 M) and sodium acetate, pH 4.5 (0.4 M). The flower extracts (250 µL) were diluted with pH 1.0 and pH 4.5 buffers in 25 mL flasks and allowed to stand for 30 minutes at room temperature. Subsequently, the absorbance readings were made on a UV-Visible spectrophotometer (Thermo, Genesys 10 UV) at the wavelengths of 510 and 700 nm (A_{510} and A_{700}), being A determined by the equation: $A = (A_{510\ nm} - A_{700\ nm})_{pH\ 1.0} - (A_{510\ nm} - A_{700\ nm})_{pH\ 4.5}$. The monomeric anthocyanin pigment concentration was calculated as cyanidin-3-glucoside, being the concentration determined by the equation: Monomeric anthocyanin pigment (mg Cy 3-glu/L) = $A \times MW \times DF \times 1000/(\varepsilon \times 1)$, where A = absorbances difference, MW= molecular weight (449.2), DF = dilution factor, and ε = molar absorptivity (26,900). The results were then converted into flowers dry weight. All measurements were performed in triplicate.

1.7 Total Reducing Capacity

The Total Reducing Capacity (TRC) of each sample was determined by the Folin-Ciocalteu method, described by Falcão et al. (2007). To 8 mL of the different pansy extracts solutions was added 500 µL of Folin-Ciocalteu reagent. The blank and standards were prepared similarly, replacing the sample by the solvent used in the extraction and standards, respectively. After 3 to 8 minutes, 1.5 mL saturated sodium carbonate solution was added. After two hours, the absorbance values were read at 765 nm. A calibration curve was obtained with gallic acid (0.25 to 5 mg/L) and the results expressed on mg gallic acid equivalent (GAE)/g flower, d.w. All measurements were performed in triplicate.

1.8 Determination of Antioxidant Activity

1.8.1 DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity

DPPH radical scavenging activity was determined by the procedure described by Delgado et al. (2010), with some modifications. A 0.0024 g mass of DPPH was dissolved in 100 mL of methanol to obtain a solution 6.09×10^{-5} mol/L. The pansy extract solutions were diluted with the solvent used during extraction and 300 µL of these solutions was added to 2.7 mL of DPPH methanolic

solution. After 1 hour in the dark at room temperature, the absorbance was read at 517 nm. Antioxidant activity was expressed by the percentage of scavenging effect according to the formula in Eq. 1:

$$DPPH\ radical\ scavenging\ effect\ (\%) = \frac{A_{DPPH} - A_{Sample}}{A_{DPPH}} \times 100 \quad (1)$$

A_{DPPH} was the absorbance of the DPPH solution and A_{Sample} the absorbance in the presence of the sample. The blanks were made with the different solutions used in the extractions. The extract concentration providing 50% DPPH radical scavenging effect (EC_{50}) was calculated from the graph of DPPH radical scavenging effect (%) *versus* extract concentration. All measurements were performed in triplicate.

1.8.2 Reducing power

The reducing power of the extracts was determined by the procedure described by Delgado et al. (2010). To 1.0 mL of pansies extracts solutions at different concentrations was added 2.5 mL of phosphate buffer 0.2 M (pH 6.6) and 2.5 mL of $K_3[Fe(CN)_6]$ 1% (m/v). After shaking, the mixture was incubated at 50 °C for 20 minutes. After, 2.5 mL of 10% trichloroacetic acid (m/v) was added with further stirring. A volume of 2.5 mL of the mixture was transferred to another test tube, to which 2.5 mL distilled water and 0.5 mL $FeCl_3$ 0.1 % (m/v) were added. The absorbance values were read at 700 nm. From the graph Abs (700 nm) *versus* extract concentration, the EC_{50} values were determined corresponding to the extract concentration that gave an absorbance of 0.5. Each solution was analyzed in triplicate.

1.9 Statistical analysis

SPSS Statistic software, version 18.0 (SPSS Inc., Chicago, USA), was used for the statistical treatment of the data. Analyses of variance (ANOVA) or ANOVA Welch were carried out to evaluate if there were significant differences ($p < 0.05$) between samples. Additionally, significant post hoc analyses were performed (Tukey HSD test if variances in the different groups were identical or Games-Howell test if they were not). The homogeneity of variance was tested by Levene's test. The correlations between variables were determined by Pearson correlation coefficient. A Principal Component Analysis (PCA) was performed to differentiate pansies extracts. The variables considered were the Total Reducing Capacity, hydrolysable tannins, flavonoids and anthocyanin contents, as well as, the EC_{50} values of the DPPH and Reducing Power assays.

2. RESULTS AND DISCUSSION

2.1 Extraction yield

The extraction yields for pansies after applying different solvents are presented in Table 1. The extraction yield for methanol was higher (42.0%) than for the other two solutions, namely water (36.9%) and water:acetone (39.9%).

2.2 Flavonoids

Acording to Skowyra et al. (2014), flavonoids represent the main group of phenolic compounds in pansies. In the present work the flavonoid contents extracted from pansies ranged between 46.9 and 114.7 mg QE/g flower (d.w.) (Table 1), depending on the solvent. The highest content was obtained with water:acetone, approximately 2.4 times more than with water. These results were similar to Guinot et al. (2008), who reported that the organic solvent-water extracts (ethanol:water, 3:7, v/v) of marigold flowers had higher levels of total flavonoids than the solution obtained through the traditional decoction method (distilled water for 10 min). Moreover, Liu et al. (2009) also found that acetone extracts of lychee flowers had higher flavonoids contents than their water extracts.

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Table 1 - Flavonoids, hydrolysable tannins, monomeric anthocyanins, TRC, EC_{50} DPPH and EC_{50} Reducing Power values for pansies*.

Parameters	Solvent		
	Water	Water:acetone (6:4, v/v)	Methanol
Extraction yield (%)	36.9±0.1[a]	39.9±0.5[b]	42.0±0.3[c]
Flavonoids (mg QE/g flower, d.w.)	46.9±0.7[a]	114.7±1.0[c]	99.6±2.2[b]
Hydrolysable tannins (mg TAE/g flower, d.w.)	6.77±0.65[a]	12.8±1.9[b]	17.4±2.7[b]
Monomeric anthocyanins (mg Cy 3-glu/g flower, d.w.)	3.19±0.31[a]	2.59±0.09[a]	5.93±0.18[b]
TRC (mg GAE/g flower, d.w.)	27.2±2.5[a]	42.8±1.8[b]	39.3±2.7[b]
EC_{50} DPPH (mg extract/mL)	0.22±0.01[c]	0.15±0.01[a]	0.17±0.01[b]
EC_{50} Reducing power (mg extract/mL)	0.60±0.01[b]	0.33±0.01[a]	0.36±0.08[a]

*Values are expressed as: Mean±Standard deviation. Values with the same letter in the same line are not statistically different (p>0.05).

2.3 Hydrolysable tannins

Significant differences on hydrolysable tannins contents were detected between pansies extracts (Table 1), ranging from 6.77 to 17.4 mg TAE/g flower, d.w.. Methanol and water:acetone (6:4, v/v) were the best solvents to extract hydrolysable tannins in pansies. According to Mueller-Harvey (2001), methanol tends to be the best solvent for tannins of low molecular weight or if the tissues contain large amounts of enzymes; however, acetone is often the preferred solvent, as it is less likely to react with hydrolysable tannins than water or methanol.

Several solvent systems have been used, namely methanol, ethanol, acetone, water and their combination for tannins' extraction. For example, Hagerman (1988) studied the extraction of tannins from fresh and preserved leaves of three species of trees and reported that more tannins were extracted with aqueous acetone than with aqueous or acidic methanol. On the other hand, Elfalleh et al. (2012) investigated pomegranate flowers and concluded that methanol extracted more hydrolysable tannins than water (148.24 versus 57.04 mg TAE/g, d.w.). Both authors obtained similar results to ours. So, methanol and acetone:water combinations are good solvent systems to extract hydrolysable tannins.

2.4 Monomeric anthocyanins

The total monomeric anthocyanins contents of pansies extracts analyzed in the present study varied significantly among the solvents used in the extraction, ranging from 2.59 (water:acetone) to 5.93 (methanol) mg Cy 3-glu/g flower, d.w.

Skowyra et al. (2014) obtained values for total monomeric anthocyanins subjected to water extraction between 4.11 to 21.50 mg of malvidin glucoside equivalents (ME)/g of freeze-dried weight. Furthermore, the extracts obtained with the three solvents had different colors (Figure 2). This fact may be the result of different physical properties (solubility) and different types of co-extracted pigments, as suggested by Boonsong et al. (2011). Furthermore, it might be due to the extraction of different anthocyanins by the solvents with different polarities (water =1.00; methanol=0.76 and water:acetone (6:4, v/v)=0.74). Concerning pH, the three solvents presented similar values (water = 6.47; water:acetone (6:4, v/v) = 6.96 ; methanol= 6.41). Abarca-Vargas et al. (2016) also obtained extracts of *Bougainvillea × buttiana* Holttum and Standl, (var. Rose) with different colors when using solvents with different polarities, as well as Bertan et al. (2014) when studying the effect of the ethanol concentration in the solvent on the antioxidant properties of extracts of boldo (*Peumus boldus*).

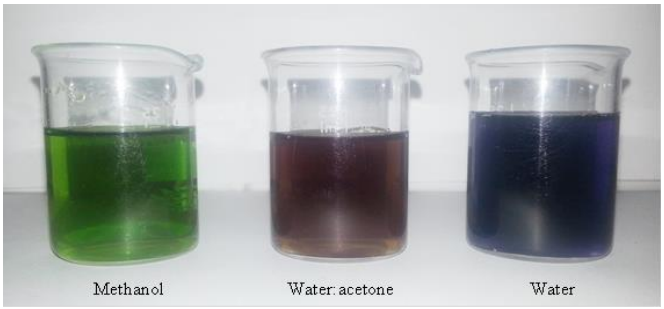

Figure 2 – Pansies extracts with different solvents: methanol, water:acetone (6:4, v/v) and water.

2.5 Total Reducing Capacity

The total reducing capacity (TRC) of the pansies extracts obtained in the present work are presented in Table 1, varying from 27.2 to 42.8 mg GAE/g flower, d.w.. Naczk and Shahidi (2006) mentioned that the extraction of phenolic compounds in plant materials is influenced by their chemical nature and interactions with other plant components such as carbohydrates and proteins. These interactions may lead to the formation of complexes that may be quite insoluble. In the present work, water:acetone and methanol yielded no statistically different amounts, but the highest value of TRC was obtained with water:acetone. According to Uma et al. (2010) and Kuźma et al. (2014), aqueous acetone solutions (48 and 70%, respectively) are the most effective solvent for extraction of polyphenols from henna leaves (*Lawsonia inermis*) and parsley leaves (*Petroselinum crispum*), which is in accordance with our results. On contrary, Shabir et al. (2011), evaluated different extraction solvents in Gold Mohar flowers and detected that 80% methanol gave the highest value of total phenolics contents, as well as Ahmad et al. (2011), who studied flowers of akk and reported that higher values were obtained with 80% ethanol. These different results can be explained because different flowers may synthesize and accumulate different compounds or different amounts of a particular compound, which in turn affects the TRC of the flowers extracts produced. Our results of water extraction was lower (27.2 mg GAE/g flower d.w.) than reported by Skowyra et al. (2014), who obtained a range of values between 120.56 (yellow pansies) to 419.28 (red) mg GAE/g of freeze-dried weight, after applying different extraction conditions.

2.6 Antioxidant activity

2.6.1 DPPH radical scavenging activity

The antioxidant activity determined by the DPPH method for the pansies extracts studied in present work showed significant differences between them (Figure 3A and Table 1). The DPPH free radical scavenging activity increased with extract concentration and the water:acetone (6:4, v/v) solvent was the one with the highest values (Figure 3A). The antioxidant activity was also expressed on EC_{50} values, which indicate the concentration of the extract required to decrease the DPPH radical concentration by 50% (Table 1). Thus, a low EC_{50} value is indicative of a high antioxidant activity. Water:acetone extracts exhibited the lowest EC_{50} value (0.15 mg extract/mL), while water extracts resulted in the highest (0.22 mg extract/mL). These results agree with Kuźma et al. (2014), who reported that higher antioxidant activity were obtained with aqueous organic solvents than with the respective absolute organic solvents. Furthermore, these results can be explained by the polarity because by increasing the proportion of water to the solvent, the polarity of the mixture also increases.

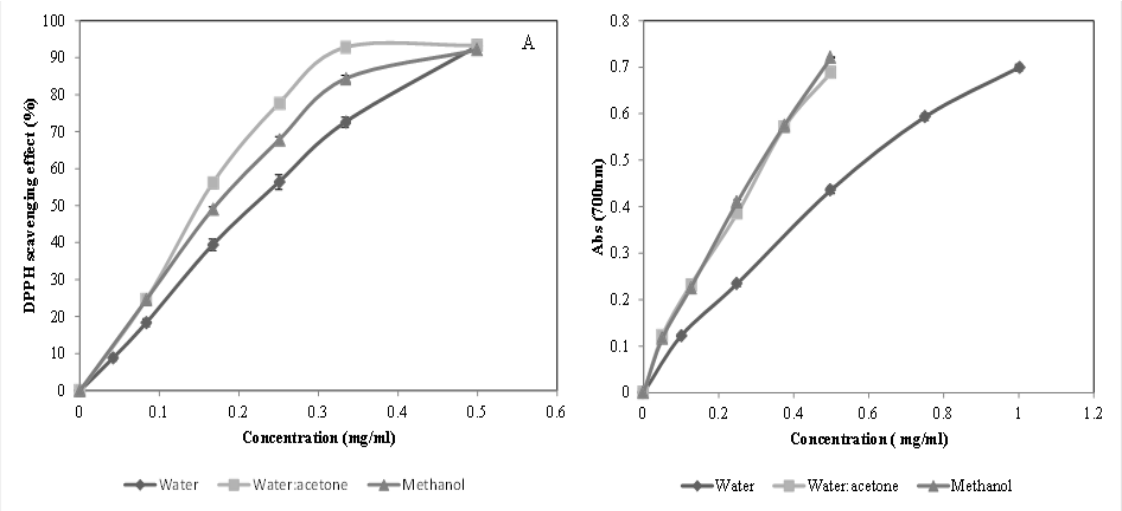

Figure 3 - DPPH radical scavenging activity (%) (A) and reducing power (Abs 700 nm) (B) *versus* pansies extracts concentration.

2.6.2 Reducing Power

The measurement of the reducing power can also be used to express the antioxidant activity of the plant extracts. In this assay, ferric ions are reduced to ferrous ions with change in color from yellow to bluish green (Ahmand et al., 2011). The intensity of the color depends on the reducing potential of the antioxidant compounds present in the extract. The reducing power data of the different extracts followed the similar trends as were observed for hydrolysable tannins and TRC assay, compounds responsible for the antioxidant activity in the extracts. So, methanol and water:acetone (6:4, v/v) extracts showed the lowest values of EC_{50} reducing power, while water extracts had the highest (Table 1). Furthermore, in the present work the reducing power was tested at different extract concentrations and reducing power increased with the extract concentration (Figure 3B).

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2.7 Correlations between Total Reducing Capacity, monomeric anthocyanins, flavonoids, hydrolysable tannins and antioxidant activity

The Pearson correlation coefficients determined between TRC, monomeric anthocyanins, flavonoids, hydrolysable tannins and antioxidant activity (EC_{50} values of DPPH and Reducing Power assays) are presented in Table 2. Significant positive correlations were found between TRC and flavonoids (0.968), as well as hydrolysable tannins (0.789), showing the important role of these compounds in TRC. As expected, negative correlations were detected between TRC and the EC_{50} values of DPPH (-0.971) and Reducing Power (-0.907), as these properties are inversely correlated. Concerning antioxidant activity, a significant negative correlations were found between flavonoids and EC_{50} DPPH values (-0.992) and EC_{50} reducing power (-0.953), related with the antioxidant potential of these compounds.

Table 2 - Pearson correlation coefficients for Total Reducing Capacity, monomeric anthocyanins, flavonoids, hydrolysable tannins and EC_{50} values of DPPH radical scavenging activity and Reducing Power assays.

	Monomeric anthocyanins	Flavonoids	Hydrolysable tannins	EC_{50} DPPH	EC_{50} Reducing Power
Total Reducing Capacity	0.003	0.968**	0.789*	- 0.971**	-0.907**
Monomeric anthocyanins	___	0.018	0.676	0.71	-0.014
Flavonoids	___	___	0.751*	- 0.992**	-0.953**
Hydrolysable tannins	___	___	___	-0.699*	-0.708**
EC_{50} DPPH	___	___	___	___	0.946**

Correlation is significant at **p < 0.01, *p < 0.05

2.8 Principal Component Analysis

A Principal Component Analysis (PCA) was applied to find possible clusters within the extracts prepared with the solvents studied in the present work, which may differ in bioactive compounds and antioxidant activity. The scores of the first two principal components are presented in Figure 4. The first two principal components took into account 99.6% (PC1 = 97.5% and PC2 = 2.1%, respectively) of the total variation. PC1 was mainly correlated positively to EC_{50} DPPH and EC_{50} Reducing Power, and negatively to TRC, hydrolysable tannins and flavonoids. PC2 was mainly correlated positively to monomeric anthocyanins. In PC1, aqueous extracts had positive scores due to their high values of EC_{50} DPPH and Reducing Power, as well as, low values of TRC, hydrolysable tannins and flavonoids, as stated in Table 1. So, when extracting pansies with water, the extracts obtained had the lowest antioxidant activity and the lowest contents of bioactive compounds. On contrary, the methanolic extracts had positive scores on PC2 due to their high values of monomeric anthocyanins. As stated in previous sections, the methanolic extracts were those with the highest values of hydrolysable tannins and monomeric anthocyanins. Concerning the water:acetone (6:4, v/v) solution, these extracts showed the highest antioxidant activity and flavonoids contents.

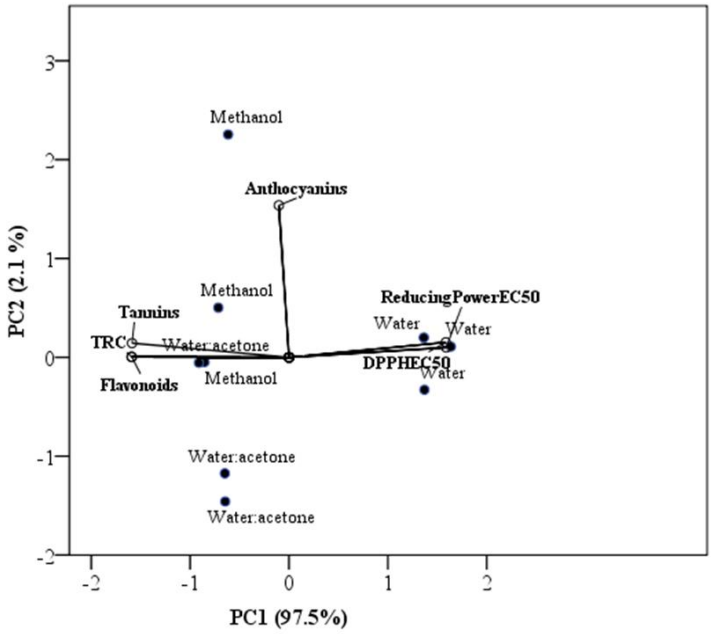

Figure 4 - Principal component analysis plot to flavonoids, monomeric anthocyanins, hydrolysable tannins, TRC and EC_{50} values of DPPH radical scavenging activity and Reducing Power assays.

CONCLUSIONS

The influence of the solvent (water, methanol, water:acetone (6:4, v/v)) on bioactive compounds extraction and antioxidant properties of pansies was demonstrated. The highest flavonoids content was obtained with water:acetone (6:4, v/v), while methanol extract had the highest value of monomeric anthocyanins. Concerning hydrolysable tannins and TRC, both methanol and water:acetone (6:4, v/v) extracts had similar contents. Aqueous extracts presented the lowest antioxidant activity. These results demonstrated the possibility of *Viola × wittrockiana* flowers to be a promising source of natural antioxidants for the food and pharmaceutical industries.

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