

PLANT ELICITATION WITH SALICYLIC ACID INCREASES BIOACTIVE COMPOUNDS CONTENT AND ANTIOXIDANT ACTIVITY IN THE INFUSION OF *Achillea millefolium* L.

ELICITAÇÃO DE PLANTAS DE *Achillea millefolium* L. COM ÁCIDO SALICÍLICO AUMENTA O TEOR DE COMPOSTOS ATIVOS E A ATIVIDADE ANTIOXIDANTE DA INFUSÃO

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ABSTRACT: This study evaluated the total polyphenols content and the antioxidant activity of the infusion prepared with leaves from *Achillea millefolium* L. plants treated with salicylic acid (SA). Field cultivated plants received SA foliar applications (T1: control; T2: 1.0 mmol L⁻¹ applications at 20, 60 and 100 days after planting - DAP and T3: 1.0 mmol L⁻¹ applications at 100 DAP during three days). The infusions from SA treated plants showed higher levels of total polyphenols and flavonoids compared to the control one. T2 and T3 infusions showed increases in the antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH), nitric oxide (NO) and ferric-reducing antioxidant power (FRAP) tests. However, only T2 treated plants had higher antioxidant activity by inhibition of lipid peroxidation (TBARS). It was concluded that elicitation of *A. millefolium* plants with SA can be considered an adequate strategy to increase the production of bioactive compounds and the antioxidant capacity of infusions.

KEYWORDS: Phenolic compounds. Nitric oxide. DPPH. FRAP. TBARS.

INTRODUCTION

Achillea millefolium L. - Asteraceae - is a plant used in traditional medicine for the treatment of gastrointestinal and hepatobiliary disorders and externally in case of skin inflammations and wound healing. In addition, this species is an appetite-inducing drug because of its bitter taste (BENEDEK; KOPP, 2007). Popularly known as yarrow, *A. millefolium* L. is commercially produced as raw material for the beverage (tea composition) and phytomedicines industries (WILLUHN, 2002). The aerial parts of yarrow plants are used in the form of aqueous or alcoholic extracts, due to the high solubility of their polar phenolic substances (BENEDEK; KOPP, 2007).

The profile of phenolic compounds presents in the tea, extracted during the infusion process, causes this beverage to present high antioxidant activity. In recent years, increasing attention has been devoted to the role of diet in human health, where the ingestion of plant foods rich in antioxidant compounds (fruits, vegetables and herbs) is essential for resistance to cellular oxidative

stress, which causes degenerative diseases and aging (SARKAR; SHETTY, 2014).

Elicitor application can be used to increase metabolite production in the plant and to enhance its qualitative value for fresh produce, enriched food, or as a raw ingredient for feed/food and pharmaceutical products (POULEV et al., 2003). The elicitation technique is one of the strategies employed in the cultivation of medicinal plants to increase the content of bioactive compounds (PÉREZ et al., 2014). Elicitors are defined as natural or synthetic substances that, when applied to plants in small concentrations, initiate or increase the synthesis of specific bioactive compounds (NAMLI et al., 2014). Salicylic acid (SA) is a phenolic compound of hormonal nature present in plants, also classified as an elicitor (ANGELOVA et al., 2006). The eliciting action of SA has been proven both by its application in intact plants (PÉREZ et al., 2014; GORNI et al., 2017) and in cell and tissue culture. SA induces the expression of plant genes involved in secondary metabolites production like phenolic compounds, flavonoids, glucosinolates, anthocyanins, carotenoids and

others. Plant-related factors such as species, cultivar and physiological status influence SA elicitation responses, as well as SA doses and forms of application (GORNI; PACHECO, 2016).

Studies have shown that teas antioxidant activity is higher than popularly fresh consumed vegetables, such as spinach and onion. Such studies demonstrate the importance of tea as a potentially functional food for health (KARORI et al., 2007). The potential antioxidant activity of ethanolic and aqueous extracts of leaves, flowers and seeds of *A. millefolium* was tested by elimination of ferric thiocyanate and H₂O₂ radicals. All the extracts showed a high antioxidant activity (KESER et al., 2013).

This study evaluated the effect of foliar application of salicylic acid in yarrow plants on total polyphenol and flavonoid contents and the antioxidant capacity of the resulting infusions.

MATERIAL AND METHODS

The research was carried out from May to September 2016 under field conditions in Presidente Prudente (22°07'04" S, 51°27'05" W, 471 m.a.s.l.), São Paulo, Brazil. According to Köppen, the climate of the region is characterized as Mesothermal AW, with hot summers and dry winters. The soil is classified as Dystrophic Red Argisol with 10-12% of clay in the 0 - 20 cm layer (EMBRAPA, 2006).

The soil of the experimental area was analyzed according to Raji et al. (2001) The soil of the experimental area was analyzed and corrected by applying limestone for elevate soil base saturation to 80% according to Boletim 100 (perennial herbaceous species). The organic fertilization was based on tanned bovine manure (10 kg of manure per square meter of plot, which is higher than the recommended due to the sandy soil).

The seedlings were obtained by vegetative propagation from *A. millefolium* parent plants, whose exsiccates are deposited in the herbarium of the Federal University of Uberlândia-MG (HUFU 74428). After a period of thirty days, seedlings were transplanted to the field. The adopted spacing was 70 x 50 cm (NETTO; RAFFAELLI, 2004). Plants were manually irrigated twice a day during the experimental period.

The SA were applied in the leaves by CO₂ pressurized sprayer (40 PSI) (50 mL per plant) as follows: T1 - control (plants sprayed only with water); T2 - 1.0 mmol L⁻¹ SA applications at 20, 60 and 100 days after planting (DAP) and T3 - 1.0 mmol L⁻¹ SA applications only at 100 DAP, spraying the plants for three consecutive days. The

experimental design was in randomized blocks, with 5 plots (10.5m²) per treatment in a total of 300 plants (20 plant per plot). The harvest was carried out at 120 days after planting. The 6 central plants of each plot (useful area) were collected.

Preparation of the infusion of *Achillea millefolium* L.

The harvested leaves were dried in an air drier at 40° C for 48 hours. After drying, the leaves went grind. The infusion was obtained by the addition of 100 mL of distilled water (98°C) infused with 1g of dry leaves of *A. millefolium*. The infusion was maintained at room temperature for 10 minutes under agitation (SU et al., 2006). After this period, infusions were filtered and stored in amber glass bottles and kept under refrigeration (4°C).

Total polyphenols content (µg/mL)

The total polyphenols content in the different infusion of *A. millefolium* was assessed using Folin-Ciocalteu reagent and gallic acid as a standard (STAGOS et al., 2012). 25 µL of each infusion was added to 1.25 mL of distilled water, 125 µL of Folin-Ciocalteu reagent. The homogenized tubes were resting for 3 min and then 350 µL of 25% sodium carbonate (Na₂CO₃) and 750 µL of distilled water and then tubes were incubated for 1 h. After that, their absorbance was recorded at 765 nm. The total polyphenols contents of the infusion were reported based on micrograms of gallic acid equivalents per milliliters (µg GAE/mL). Gallic acid solutions were prepared with concentrations of 25 to 500 µg mL⁻¹ in absolute ethyl alcohol.

Total flavonoid content (µg/mL)

The dosage of flavonoids was performed according to Yao et al. (2013). To quantify the total flavonoid content in *A. millefolium* infusion, a 100 µL aliquot of the extract was added to the test tube together with 400 µL of 70% alcohol and 50 µL of 5% NaNO₂. After 6 minutes 50 µL of the 10% aluminium chloride (AlCl₃) solution, 300 µL of NaOH (1 M) and 100 µL of distilled water were added. The spectrophotometer was read at 510 nm. The total flavonoids contents of the infusion were reported based on micrograms of rutin equivalents per milliliters (µg RE/mL). Rutin solutions were prepared with concentrations of 25 to 500 µg mL⁻¹ in absolute ethyl alcohol.

Antioxidant activity

Free radical scavenger activity DPPH (2,2-diphenyl-1-picryl-hydrazyl)

In this method, DPPH solution (500 μM) was prepared in ethanol, 250 μL of this solution was added to 50 μL of sample solution (at concentrations of 250, 500 and 1000 $\mu\text{g} / \text{mL}$), 1 mL of acetate buffer (100 $\text{mmol L}^{-1} / \text{pH}$ 5.5) and 1.25 mL of absolute ethanol and then incubated at room temperature for 30 min, the absorbance was measured at 518 nm (BLOIS, 1958). The percentage of radical scavenging activity was calculated from Equation: AA (%) = $[(A_c - A_s)/A_c] \times 100$ where A_c is the absorbance of the control, and A_s is the absorbance of the sample and expressed as IC_{50} value.

Iron Reduction Activity (FRAP)

The total antioxidant potential of the tea samples was determined using a modification of the ferric reducing ability of plasma (FRAP) assay of Kukić et al. (2008). 90 μL of each sample was added to 2.7 mL of FRAP reagent (0.3 M sodium acetate buffer pH 3.6, tripyridyl triazine (TPTZ) 10 mmol L^{-1} and ferric chloride 20 mmol L^{-1}) and 270 μL of distilled water. The mixture was vortexed and incubated at 37°C for 30 minutes. Absorbance of the solutions was read at 595 nm. The calibration curve was obtained with ferrous sulfate (100 - 2000 μM) and the results expressed in $\mu\text{mol Fe}^{2+} \text{mg}^{-1}$ sample.

Nitric Oxide (NO) sequestering activity

For the evaluation of nitric oxide (NO) scavenging assay was carried by using sodium nitroprusside, it was used the methodology described by Marcocci et al. (1994) was measured with the Griess reagent. 320 μL of each sample was added to 360 μL sodium nitroprusside (25 $\text{mmol L}^{-1} / \text{phosphate}$ buffer saline pH 7.4) and 215 μL of Griess reagent was added in test tubes and incubated in a 37°C water bath for 2 hours, in the absence of light. The absorbance of the test was determined in a spectrophotometer at 540 nm. The calibration curve was obtained with sodium nitrite (2.5 – 80 μM) and the results expressed in the amount of nitrite formed ($\mu\text{M mL}^{-1}$).

Inhibition of lipid peroxidation (TBARS)

The evaluation of antioxidant capacity by the inhibition of lipid peroxidation was based on TBARS (Tiobarbituric Acid Reactive Species) methodology, described by Guimarães et al. (2010) with some modifications. Egg homogenate (1 mL) of 1%, v/v in phosphate buffered saline (20 $\text{mmol L}^{-1} / \text{pH}$ 7.4) and 100 μL of sample were added to 100 μL of the 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) (0.12M) to induce lipid peroxidation. The mixture was incubated for 30 minutes at 37°C. After incubating and cooling at room temperature, it was added 500 μL of trichloroacetic acid (15%) and 500 μL of

thiobarbituric acid (0.67%). The mixture was incubated at 97°C for 15 minutes. After incubation, centrifugation 1200 rpm for 10 minutes. Then, the reading of the supernatant was performed at 532 nm. Inhibition (%) of lipid peroxidation was calculated using the equation: Inhibitory activity (%) = $[(A_c - A_s)/A_c] \times 100$ where A_c is the absorbance of the control, and A_s is the absorbance of the sample.

Statistical analysis

All determinations (total polyphenols, flavonoids and antioxidant tests) were repeated five times. Data were tabulated and submitted to analysis of variance ($p \leq 0.05$), and the Tukey test using the Sisvar software. The results were presented by means of the treatments and standard error.

RESULTS AND DISCUSSION

The chemical composition of teas and infusions is complex and include polyphenols, alkaloids (caffeine, theophylline and theobromine), amino acids, carbohydrates, proteins, chlorophylls, volatile compounds, minerals, trace elements and other unidentified compounds (KARORI et al., 2007). Among these, polyphenols are the most interesting group and are the main bioactive molecules in teas and infusions, corresponding to about 250-350 mg of the soluble solids present in this beverage (CABRERA; GIMENEZ; LOPEZ, 2003). Several studies have demonstrated that the teas biological activity is related to the phenolic composition of the plant species that are used for the preparation of infusions (GRAMZA; KORCZAK, 2005). Among these biological activities, is cited the ability of teas act as antioxidants in the human body.

It can be verified that there was variation in the content of total polyphenols and flavonoids in the infusion of yarrow as a function of SA application to the plants (Table 1). Infusions from treated plants showed increases of 43.3% (T2 treatment) and 54.2% (T3 treatment) in polyphenols, compared to the infusion obtained from the control plants. According to Ghasemzadeh and Jaafar (2012), when applied exogenously to plants at low concentrations, the SA interacts with stress-signaling mechanisms and induces the synthesis of phenolic compounds. The elicitor signal transduction starts on the surface of the plasma membrane and includes events like the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), changes in the potential of plasma membrane cell and enhanced ion fluxes (Cl^- and K^+ efflux and Ca^{2+} influx), rapid changes in

protein phosphorylation, lipid oxidation, salicylic acid and jasmonic acid accumulation and the *de novo* biosynthesis of transcription factors, which directly regulate the expression of genes involved in several bioactive compounds (FERRARI, 2010 ; FRANKFATER et al., 2009).

The treatments with SA resulted in significant increases of 27.5% (T2 treatment) and 45.4% (T3 treatment) in the total flavonoid contents present in the infusions, when compared to the control (Table 1). This result agrees with Pérez et al. (2014), where SA treatments increased the flavonoids rutin and naringin in peppermint infusion (*Mentha piperita*).

The presence of phenolic compounds in *A. millefolium* has been associated with its health-promoting properties, such as choleric activities, antioxidant, anti-inflammatory and antimutagenicity (GEORGIEVA et al., 2015; HUO et al., 2013). The phenolic compounds present in *A. millefolium* are flavonoids and phenolic acids. The flavonoids mainly occur as apigenin, luteolin and quercetin. Regarding the phenolic acids, the ubiquitous plant compound chlorogenic acid is accompanied by dicaffeoylquinic acids (DCQAs), namely 1,5-, 3,4-, 3,5- and 4,5 -DCQA (BENEDEK; KOOP, 2007).

Table 1. Total compounds in the tea of *Achillea millefolium* L. plants treated with salicylic acid. Different letters indicate significant difference by the Tukey test ** ($p \leq 0.01$).

Salicylic acid application	Total compounds ($\mu\text{g/mL}$)	
	Polyphenols	Flavonoids
Control	69.25 \pm 0.00b	239.42 \pm 6.01c
20, 60 and 100 DAP	99.25 \pm 1.44a	305.25 \pm 4.33b
100 DAP	106.75 \pm 6.03a**	348.175 \pm 3.25a**

Due to the different types of free radicals and their different forms of action in living organisms (ALVES et al., 2010), it is necessary to use other complementary methods to evaluate the antioxidant activity of plants. The antioxidant substances can present different protective properties and act in several stages of the oxidative process through different mechanisms (SILVA et al., 2010). DPPH is the most commonly used standard method in the world for this purpose. However, DPPH is a synthetic radical, not present in living beings. In this way, other methods like FRAP (based on the antioxidant activity via ferric reducer and iron ion chelation capacity), TBARS (based on

antioxidant activity via enzymatic action to reduce lipid peroxidation) and NO - nitric oxide (based on activity antioxidant via free radical sequestration action) are used for antioxidant activity determination. Georgieva et al. (2015) studied the antioxidant activity in *A. millefolium* (leaves and stems) in different tests, observing a greater activity of elimination of free radicals in the DPPH and FRAP tests.

Infusions of *A. millefolium* plants treated with SA showed high antioxidant activity, both in terms of inhibition of oxidation and elimination of free radicals (Table 2).

Table 2. Antioxidant activity in tea of *Achillea millefolium* L. plants treated with salicylic acid. Different letters indicate significant difference by the Tukey test ** ($p \leq 0.01$).

Salicylic acid application	Antioxidant activity		
	DPPH (IC ₅₀)	FRAP ($\mu\text{mol Fe}^{2+}/\text{mg}$)	
Control	1283,652 \pm 14,849c	0.396 \pm 0.0001c	
20, 60 and 100 DAP	1037,098 \pm 7,805b	0.507 \pm 0.0001b	
100 DAP	936,729 \pm 14,565a**	0.517 \pm 0.0001a**	
Salicylic acid application	TBARS (%)		
	NO (μM nitrite/mL)		
	Control	61.38 \pm 0.038b	10.778 \pm 0.161c
	20, 60 and 100 DAP	69.31 \pm 0.028a**	13.222 \pm 0.184b
100 DAP	53.60 \pm 0.018c	13.863 \pm 0.024a**	

Using the DPPH method, there was a reduction in the IC₅₀ value of 37% in T3 treatment when compared to control plants infusion. This increase in antioxidant activity as a result of SA plant elicitation was also verified when the FRAP (28 and 30.5% for T2 and T3 treatments, respectively) and NO (22.7 and 28.6% for T2 and T3 treatments, respectively) tests were used. Regarding the TBARS method, there was a 13% increase in antioxidant activity only in the infusion of the plants from T2 treatment, compared to the control infusion.

The phenolic compounds have, in their structure, several characteristic benzene groups, having as substituents hydroxyl groups (HERNÁNDEZ; PRIETO GONZÁLES, 1999). The hydrogen atoms of the adjacent hydroxyl groups (ortho-diphenols), located at various positions of rings A, B and C, the double bonds of the benzene rings and the double bonding of the oxo function (-C = O) of some flavonoid molecules guarantee to these compounds their high antioxidant activity (RICE-EVANS; MILLER; PAGANGA, 1996).

There is a high correlation between vegetal species antioxidant effects and total phenolic contents (PIZZALE et al., 2002; PÉREZ et al., 2014). In this way, SA elicitation is a tool for improving the antioxidant activity of herbal and spices, due to improvements in phenolic compounds accumulations.

CONCLUSION

The results obtained in this study showed that the foliar application of salicylic acid in *Achillea millefolium* L. plants resulted in increases in total polyphenols and flavonoids present in the infusion, with a consequent increase in their antioxidant activity.

ACKNOWLEDGEMENTS

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

RESUMO: Este estudo avaliou o teor total de polifenóis e a atividade antioxidante da infusão preparada com folhas de plantas de *Achillea millefolium* L. tratadas com ácido salicílico (AS). As plantas cultivadas em campo receberam aplicações foliares de AS (T1 – controle; T2 - aplicação de 1,0 mmol L⁻¹ aos 20, 60 e 100 dias após o plantio (DAP) e T3 – aplicações de 1,0 mmol L⁻¹ aos 100 DAP durante três dias consecutivos). As infusões de plantas tratadas com AS apresentaram níveis mais elevados de polifenóis totais e flavonóides em comparação ao controle. As infusões T2 e T3 mostraram aumentos na atividade antioxidante por meio de testes de 2,2- diphenyl-1-picrylhydrazyl (DPPH), óxido nítrico (NO) e poder antioxidante de redução férrica (FRAP). No entanto, apenas plantas do tratamento T2 apresentaram maior atividade antioxidante por inibição da peroxidação lipídica (TBARS). Concluiu-se que a elicitação de plantas de *A. millefolium* com AS pode ser considerada uma estratégia adequada para aumentar a produção de compostos bioativos e a capacidade antioxidante das infusões.

PALAVRAS-CHAVE: Compostos fenólicos. Óxido nítrico. DPPH. FRAP. TBARS.

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