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Growth promotion and elicitor activity of salicylic acid in *Achillea millefolium* L.

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Salicylic acid (SA) is a phenolic compound present in plants and has multiple functions, including hormonal effect on stimulus of plant growth and development and induction of plant defense responses under conditions of biotic and abiotic stresses. Studies related to SA's elicitor action on the synthesis of secondary metabolite in medicinal plants have been conducted in order to increase the economic value of these species. The objective of this study was to determine the effect of SA foliar application on biomass production and the synthesis of secondary compounds in yarrow (*Achillea millefolium* L. - Asteraceae). The experiment was conducted in potted plants under greenhouse conditions. The SA application was done at concentrations of 0, 0.25, 0.50 and 1.00 mM 20 days after transplanting the seedlings to pots. The effect of SA on the metabolism of yarrow plants was evaluated through biometric parameters of growth and biochemical parameters. The SA at 0.50 mM resulted in linear increases in biomass accumulation of roots, total dry mass, ratio root/shoot and chlorophyll *a* and chlorophyll *a+b* content in yarrow plants.The application of SA at 0.50 and 1.00 mM was most effective in eliciting the production of essential oils and total phenols, with a consequent improvement of the antioxidant activity of the plant extract. It can be concluded that SA application constitutes an advantageous management practice for commercial production of *Achillea millefolium*, increasing the nutraceutical and medicinal values of this species.

Key words: Photosynthetic pigments, essential oil, phenolic compounds, antioxidant activity.

INTRODUCTION

Achillea millefolium L. (Asteraceae), known as yarrow is a perennial species used in folk medicine against various diseases, including skin inflammation and gastrointestinal and hepatobiliary disorders. In addition to the traditional use, yarrow is also used as raw material in tea-producing industries (Chandler et al., 1982; Benedek and Kopp, 2007). Among the active compounds of yarrow, the presence of essential oil with terpenes, tannins, mucilages, coumarins, resins, saponins, steroids, fatty acids, alkaloids and bitter principle can be highlighted (Simões et al., 1998).

Salicylic acid (SA) is a phenolic compound, benzoic

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acid produced by the plants with important roles in plant defense responses to biotic and abiotic stress (Popova et al., 1997; He et al., 2002; Noreen et al., 2009; Hayat et al., 2005). SA also acts as a regulator of many physiological processes related to growth and development of plants, this is the reason why this compound was referred to as a plant hormone (Hegazi and El-Shrayi, 2007; Singh and Usha, 2003; Liu et al., 2011).

Recently, the emphasis on SA elicitor activity has been gaining attention in the literature. Elicitors can be defined as substances that initiate or increase the biosynthesis of secondary compounds in plants, both in medicinal plant species and the species categorized as functional foods (Taguchi et al., 2001; Radman et al., 2003). The SA is classified by the Food and Drug Administration as "substance generally recognized as safe", which enables its use in commercial cultivation of medicinal species targeting the market of phytomedicines (Poulev et al., 2003; Divya et al., 2014).

Marigold plants (*Calendula officinalis* L.) treated with SA (0, 0.25, 0.50 and 1.00 mM) showed linear increases in the accumulation of biomass, number of inflorescences and flavonoid content; without changes in photosynthetic activity and transpiration however (Pacheco et al., 2013). In mint (*Mentha piperita*), the foliar application of SA (2 mM) resulted in increases in growth parameters and changes in the metabolic profile of carbohydrates and amino acids of the resulting infusions, therefore lower concentrations increased content of phenolic compounds in leaves (Pérez et al., 2014). The concentration of betacyanin and total phenols in *Alternathera tenella* leaves cultivated *in vitro* increased after 36 h of treatment with SA. In contrast, the increase in exposure time caused a slight decrease in the contents of total flavonoids and decreased antioxidant activity (Brandão et al., 2014). Divya et al. (2014) studied the application of different levels of methyl jasmonate and salicylic acid on pre-harvest of coriander (*Coriandrum sativum* L.). Plants treated with SA 0.5 mM showed increases of 5.4 and 3.5% in the levels of carotenoids and phenolic compounds, besides additional increases in compounds such chlorophyll and lutein. There have been no reports in the literature on the exogenous application of SA in *Achillea milefolium* so far. Thus, the aim of this study was to evaluate the effect of different concentrations of SA in *A. millefolium* in order to promote growth and simultaneously increase the synthesis of secondary compounds in this medicinal species.

MATERIALS AND METHODS

Study site and plant material

The experiment was conducted under controlled greenhouse conditions (temperature of 26°C and 70% of humidity) in Presidente Prudente (22°7'39" S, 51°23'8" W, 471 ma.s.l.), São Paulo, Brazil. The seedlings were obtained from mother plants of yarrow (*Achillea millefolium* L.) from the Garden of Medicinal Plants of UNOESTE. Botanical identification was performet at the Universidade Federal de Uberlândia Herbarium (voucher specimen number HUFU 46844).

The seedlings were propagated by clump division and after the training period (30 days) they were planted in 18 L pots containing soil. The soil was corrected as Boletim 100 (IAC) recommendations for perennial herbaceous species. Dolomitic lime 85% PRNT (31.86 $g.pots^{-1}$), potassium chloride (2.7 $g.pots^{-1}$) and simple super phosphate (18 $g.pots^{-1}$) were added. For fertilization with micronutrients, FTEBR12® (S: 3.9%; B: 1.8%; Mn: 2.0% and Zn: 9.0%) (0.9 $g.pots^{-1}$) was added. The pots were irrigated by sprinklers twice daily at 6 a.m. and 18 p.m. The irrigation blade was 6.4 mm to maintain high soil humidity during all the period of the experiment.

The application of salicylic acid (SA) was performed 20 days after transplanting the seedlings to pots, with three consecutive applications of SA solutions (40 mL per plant) in the early hours of the morning. The different concentrations of SA (0.00, 0.25, 0.50 and 1.00 mM) were prepared from a master solution of 1.00 mM. The product, as in powder formulation (Sigma Aldrich, PM= 138.1 g) was weighed in analytical balance, dissolved in 10 mL ethanol 90° and finally dissolved in distilled water 1000 mL. SA treatments were carried out by spraying the aerial part of the plants with waterbased solutions supplemented with Agral® (50 $\mu L.L^{-1}$ of solution) until drip point. Control plants were sprayed with only distilled water (1000 mL) mixed with ethanol (10 mL).

Plants were harvested at 120 days after transplanting the seedlings to pots, picking up youth and adult leaves. The leaves were dried in an oven with air circulation at 40°C until they achieved constant weight to determine the dry mass.

Plant growth

The effect of SA on the plants was evaluated using the following variables: total leaf area (LA - cm^2), number of leaves per plant (NL), dry weight of leaves and roots ($g.plant^{-1}$), leaf area/number of leaves ratio and biomass partitioning.

Total leaf area was determined with a LI-3000A portable area meter (LI-COR, Lincoln, NE, USA) in five plants per treatment. Leave number per plant was determined considering the young and adult leaves. Leaf and root dry mass ($g.plant^{-1}$) was measured after drying samples at 40 (leaves) and 60°C (roots) for 72 h until they achieved constant weight. The biomass partitioning in the plants was determined for the root/shoot and leaf area/number of leaves ratios.

Pigments and secondary metabolites content

The levels of chlorophylls, carotenoids and anthocyanins ($\mu mol.g^{-1}$) were determined spectrophotometricly following extraction on TRIS-acetone buffered solution (hydroximetil-aminomethan), according to the method of Sims and Gamon (2002).

The determinations of the content of total phenolic compounds ($\mu g.mL$) and antioxidant capacity ($IC_{50\%}$) were made from ethanol extract of the leaves. A mass of 50 g of dried and crushed leaves was subjected to extraction with 1.5 L of ethanol at room temperature and protected from light. The process of maceration and filtration of the supernatant was carried out in three consecutive stages and each extraction lasted 30 for min. The extracts obtained were combined and concentrated by evaporation *in vacuo*. Extracts were then dried at 30°C in a forced air circulation oven (Costa, 2002; Simões et al., 2007). The total phenolics content was determined according to Folin-Ciocalteu reagent (Singleton et al., 1999) with modifications, using gallic acid as a standard in ethanol, and sodium carbonate solution. The antioxidant capacity was

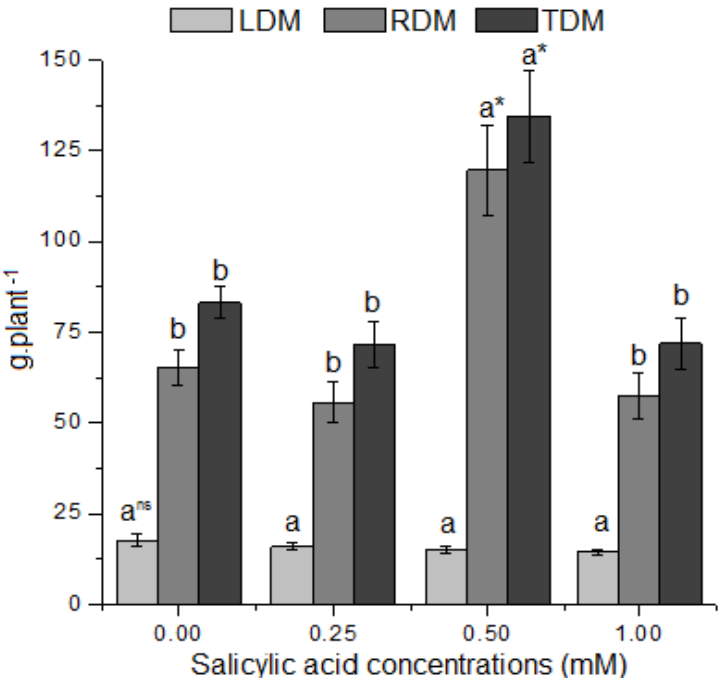

Figure 1. Determination of the leaf (LDM), root (RDM) and total (TDM) dry mass of *Achillea millefolium* L. plants, under different concentrations of salicylic acid. Different letters indicate significant differences by Tukey's test ($p \leq 0.05$) and ns (not signicant) (p=0.1736, p=0.0001, p=0.0001).

assessed via free radical scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH). The amount of antioxidants required to decrease the initial concentration of DPPH by 50%, termed "inhibitory concentration (IC_{50})" (Brand-Williams et al., 1995) was determined. The absorbance values at all concentrations tested were also converted to percentage of antioxidant activity (% AA), determined by the equation: AA (%) = [($A_{control}$ - A_{sample})/$A_{control}$] x 100 (Moreira et al., 2005).

Essential oil content (%) and yield (g) were estimated by hydrodistillation in Clevenger apparatus, from 10 g of dried leaves samples. Oil mass was obtained after removal of the salt (anhydrous magnesium sulfate) and evaporation of the solvent (dichloromethane), according to Brant et al. (2009).

Data analysis

The experiment was arranged in a completely randomized design with four treatments (SA concentrations) and 10 replicates (individual plants) per treatment. ANOVA was performed on all experimental data using the statistical program ASSISTAT (Silva, 2010) and mean differences were assessed at a 5% level by Tukey's test. Regression equations were used to express the behavior of the variables as a function of increased AS doses just when a high correlation coefficient (R^2) value was observed.

RESULTS AND DISCUSSION

Plant growth

The exogenous application of SA did not affect significantly the shoot dry mass of the yarrow plants (Figure 1). However, an increment of 83.11% in the dry mass of roots was observed in plants treated with 0.50 mM SA, which in turn contributed to the higher total dry mass of the plants (up to 61.93% as compared to control plants). Parashar et al. (2014) reported increases in both root dry mass (26%) and the shoot (51%) in *Brassica juncea* plants treated with 0.01 mM SA. In our case, the SA promoted changes in mass distribution between the different organs of the plant, prioritizing the root system over the shoots.

It is well known that SA promotes both cell division and elongation (Hayat et al., 2005; Ahmad et al., 2014). SA has been reported to increase plant growth parameters (number of branches, number of leaves and leaf area) in other medicinal plant species, like marigold (Pacheco et al., 2013), marjoram and oregano (Gharib, 2007). In this study, the number of leaves and leaf area in yarrow plants treated with 0.25 and 1.00 mM SA did not differ from control plants (Figures 2A and B). However, the application of the SA at 0.50 mM resulted in a significant reduction in the number of leaves per plant (28%) and in the total leaf area (46.2%). It is tempting to speculate that this result may be a consequence of a higher export of carbohydrates to the large roots system exhibited by plants treated with this SA concentration.

There was an increase in the root/shoot ratio of yarrow plants in the concentration of 0.50 mM SA (Figure 3A). In

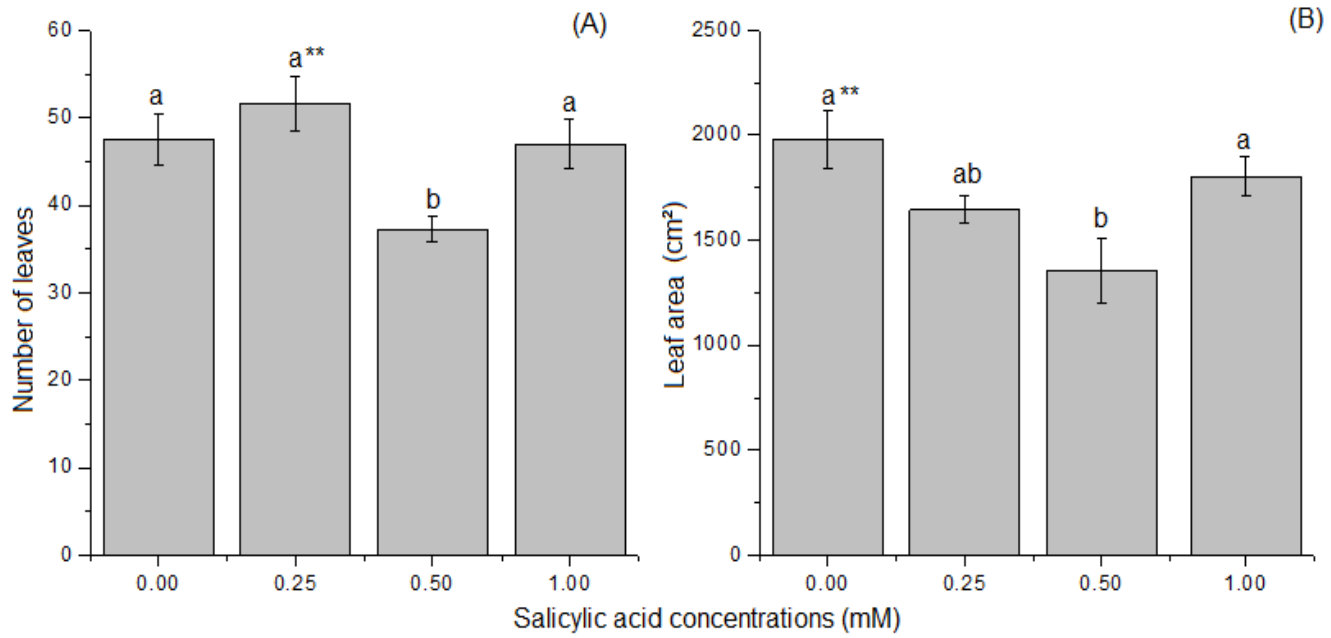

Figure 2. Number of leaves and leaf area of *Achillea millefolium* L. plants under different concentrations of salicylic acid. Different letters indicate significant differences by Tukey's test ($p \leq 0.01$) (p=0.0191, p=0.0082).

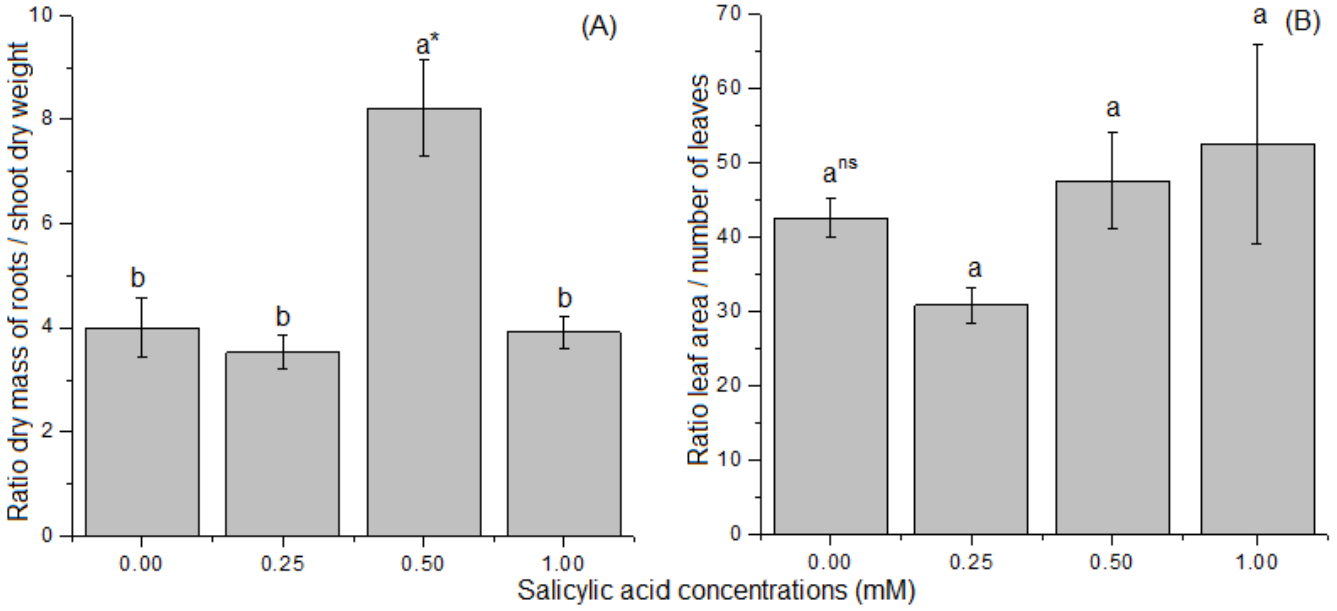

Figure 3. Root/shoot and leaf area/number of leaves ratios of *Achillea millefolium* L. under different concentrations of salicylic acid. Different letters indicate significant differences by Tukey's test ($p \leq 0.05$) and ns (not significant) (p=0.0001, p=0.2516).

contrast, the leaf area/number of leaves ratio observed in SA treated plants did not differ significantly from the control plants (Figure 3B). The root/shoot ratio seems to be controlled by a functional balance between water uptake by roots and photosynthesis by the shoot. In this way, the shoot can eventually grow to its full size until the water uptake by the roots becomes limiting and the roots until the demand for shoot photosynthates is equal to the supply. This functional balance is changed if the water supply decreases (Blum, 2005) or by the application of plant growth regulators, as occurred in this experiment. However, the observation of a higher root/shoot ratio was

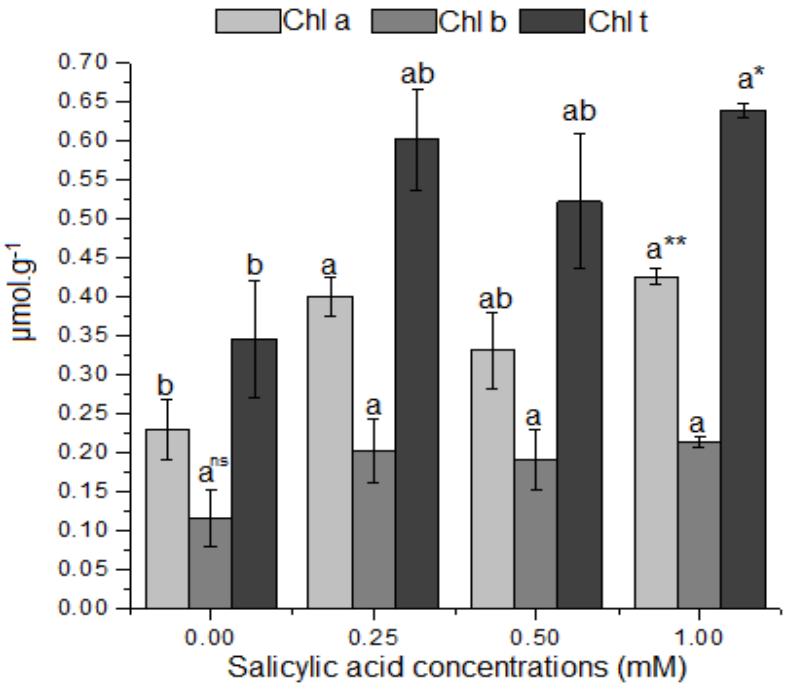

Figure 4. Chlorophyll a (Chl a), chlorophyll b (Chl b) and total chlorophyll (Chl t) contents of *Achillea millefolium* L. under different concentrations of salicylic acid. Different letters in each column indicate significant differences by Tukey's test ($p \leq 0.01$ and $p \leq 0.05$) and (ns - not significant) (p=0.0036, p=0.2134, p=0.0287).

not repeated in the highest SA concentration applied (1.00 mM SA). Different results were found in maize plants treated with 20 and 40 mg.L^{-1} SA, which presented lower root/shoot ratio than the untreated plants (Ahmad et al., 2014). Thus, our results confirm that the SA exerts species-specific and concentration-dependent responses in plants (Divya et al., 2014; Pérez et al., 2014).

Pigments content

The chlorophyll content is an important physiological index directly related to the photosynthetic performance of plants (Abdollahi et al., 2011; Parashar et al., 2014). The *Chl a* content (Figure 4) observed in yarrow plants treated with 0.25 and 1.00 mM SA was significantly higher (74.46 and 85.77%, respectively) as compared to control plants, although no effect was seen for *Chl* b content (Figure 4). The total chlorophyll content (*Chl t*) significantly increased up to 84.83% in 1.00 mM SA treated plants when compared with the control plants. Similar results were observed in *Coriandrum sativum* plants treated with 0.50 mM SA (Divya et al., 2014).

Increased levels of chlorophylls after SA applications observed in both stressed and the control plants and were explained by the positive action of this hormone in the plant nutrient uptake (Shakirova and Sakhabutdinova, 2003) as greater contents of Fe, Mg and Ca can stimulate the biosynthesis of chlorophylls (Parashar et al., 2014). Further, the increase of photosynthetic pigments can be a result of SA stimulatory effect on the activity of the Rubisco enzyme and photosynthesis (Idrees et al., 2010).

Higher contents of other pigments, such as carotenoids and anthocyanins, in response to SA application have been reported in recent studies (Divya et al., 2014; Baenas et al., 2015). However, here we did not observe significant differences in these two pigments classes between the SA-treated and control yarrow plants (Figure 5).

Essential oil, phenols and antioxidant activity

In plants, SA also acts as a chemical elicitor which can enhance the production of different groups of secondary metabolites, such as terpenes, alkaloids, flavonoids, phenolic compounds and phytoalexins (Ali et al., 2006; Silva et al., 2014). In this study, the application of 0.50 mM SA resulted in significant increases in the content (94.86%) and yield (58.24%) of essential oil in the yarrow plants as compared to the control (Figure 6). Nourafcan et al. (2014) reported similar results with *Lippia citriodora,* as they showed that the application of 10 mM SA caused an improvement in the yield and quality of the essential oil.

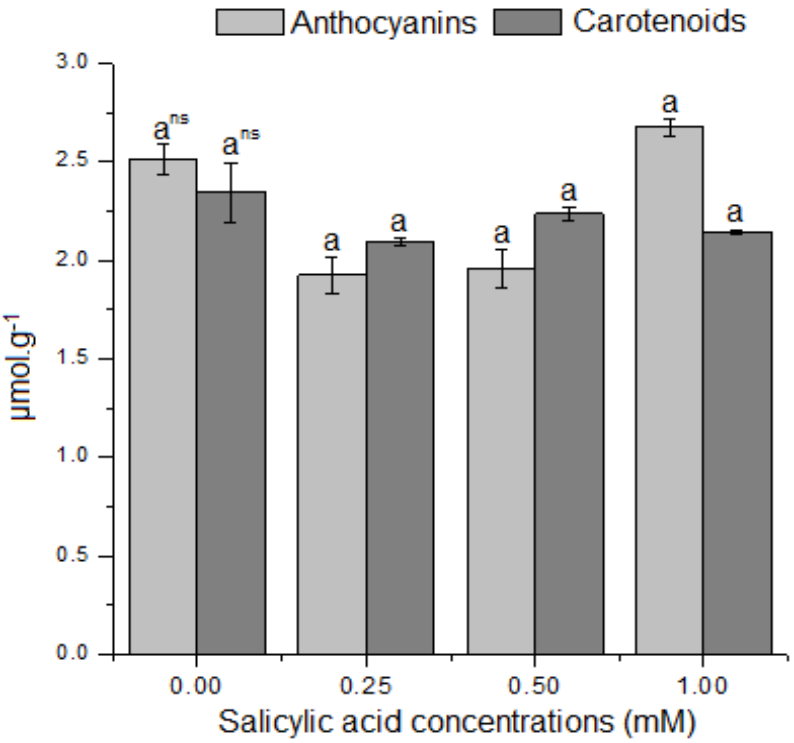

Figure 5. Anthocyanins and carotenoid contents of *Achillea millefolium* L. under different concentrations of salicylic acid. Equal letters in each column indicate no significant differences by Tukey's test (p=0.2189, p=0.8311).

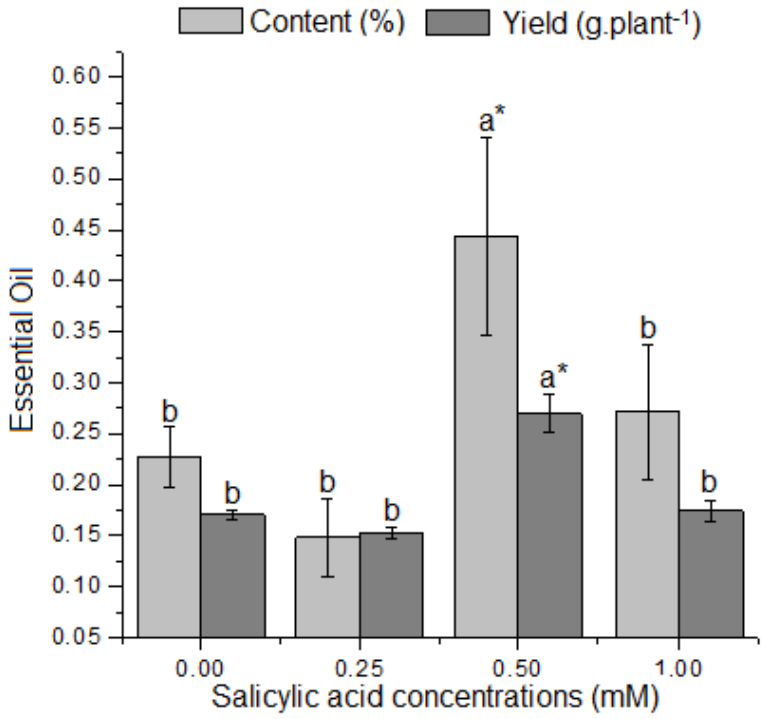

Figure 6. Content and yield of essential oil of *Achillea millefolium* L. under different concentrations of salicylic acid. Different letters in each column indicate significant differences by Tukey's test ($p \leq 0.05$) (p=0.0308, p=0.0104).

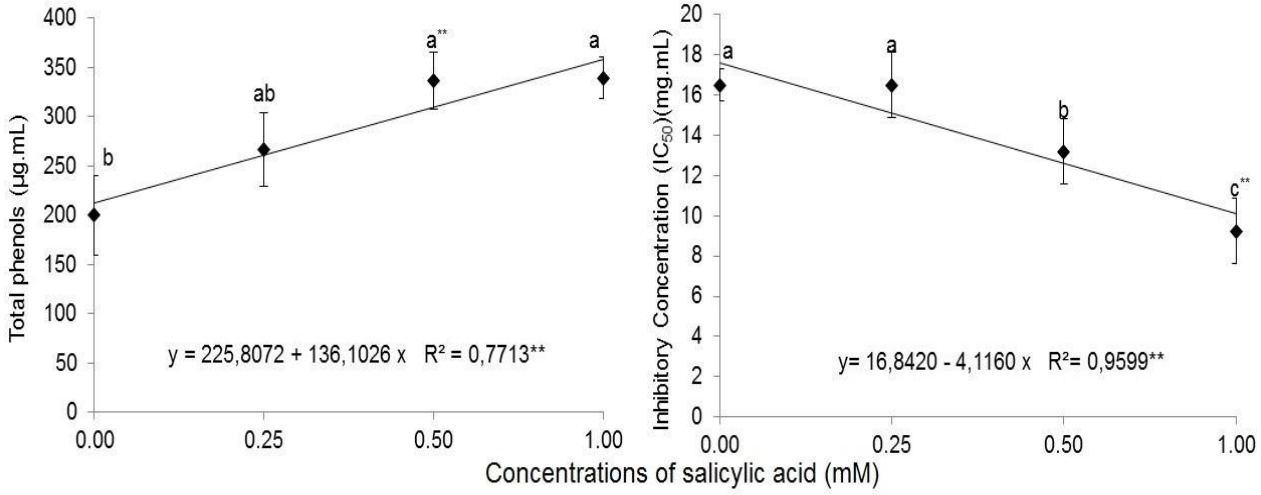

Figure 7. Content of total phenols and antioxidant activity of *Achillea millefolium* L., under different concentrations of salicylic acid. Different letters indicate significant differences by Tukey's test ($p \leq 0.05$) (p=0.0246, p=0.0001).

Regarding phenolic compounds, it was observed that exogenous application of SA exerted significant effects on these secondary metabolites, as shown by the positive linear relationship between phenolic content and SA concentrations (Figure 7). Plants treated with 0.50 and 1.00 mM SA showed a marked increase in the total phenolic content (68.11 and 69.74%, respectively) as compared to control plants. The results of this study are in agreement with those obtained by Perez et al. (2014) in mint plants treated with SA, where increases up to 65% in the total phenolic content were detected, depending on the applied SA concentrations.

Enhancement of the synthesis of various phenolic compounds in response to elicitors application, especially SA, may be explained by the induction of a state of oxidative stress in plants (Perez et al., 2014); as exogenous SA, even at low concentrations, interacts with signaling mechanisms of stresses. One example is the concentration-dependent inhibition of the antioxidant enzymes ascorbate peroxidase and catalase by SA, generating an increase in the cellular content of H_2O_2 (Moharekar et al., 2003; Askari and Ehsanzadeh, 2015). Thus, the applied concentration and duration of treatment with elicitors' substances must be carefully considered in order to avoid harmful effects to the plant (Cheeseman, 2007). Still, biotic and abiotic elicitors may have different effects on different and even the same plant species, making it also necessary to consider the type of elicitor to be used (Brandão et al., 2014).

The antioxidant activity of the leaf extract of yarrow plants was analyzed according to the IC_{50} values (Figure 7B), where lower values indicate high antioxidant activity (Sousa et al., 2007). The concentrations of 0.50 and 1.00 mM SA resulted in significant reductions in IC_{50} values relative to control plants (25 and 79%, respectively). Similar results have been reported in mint plants (*M. piperita*) treated with SA (Perez et al., 2014), where the concentrations of 0.5 and 1.0 mM SA resulted in significant decrease in IC_{50} values as compared to control plants. According to these authors, this effect can be attributed to the increase of phenolic compounds (such as flavonoids and hydroxycinnamic acids) in plants treated with SA, which are considered the major antioxidant compounds in the plant. Phenolic compounds act in the removal of singlet oxygen and other free radicals in cells, and contribute to the stabilization of oxidative stress (Rice-Evans et al., 1995). Antioxidant properties of phenolic compounds are mainly due to its ability to donate hydrogen from hydroxyl groups positioned along the aromatic ring, in order to avoid oxidation of lipids and other biomolecules (Foti et al., 1994). However, other organic compounds whose levels in the plant are increased by the SA application may also act as antioxidants, such as carbohydrates (Bohnert and Jensen, 1996), betacyanins (Brandão et al., 2014) and terpenes such as carotenoids and tocopherol (Janda et al., 2014).

Yarrow plants treated with 0.50 and 1.00 mM AS showed higher antioxidant activities for the same ethanol extracts quantities as compared to control plants (Figure 8). It is suggested that increased endogenous levels of SA in treated plants can trigger cell signaling responses that regulates the expression of defense genes encoding enzymes related to phenylpropanoids production pathway (Ruiz-Garcia and Gómez-Plaza, 2013). The increase in the activity of key enzymes in this metabolic pathway, such as phenylalanine ammonia lyase and chalcone synthase, is as a result of the SA application

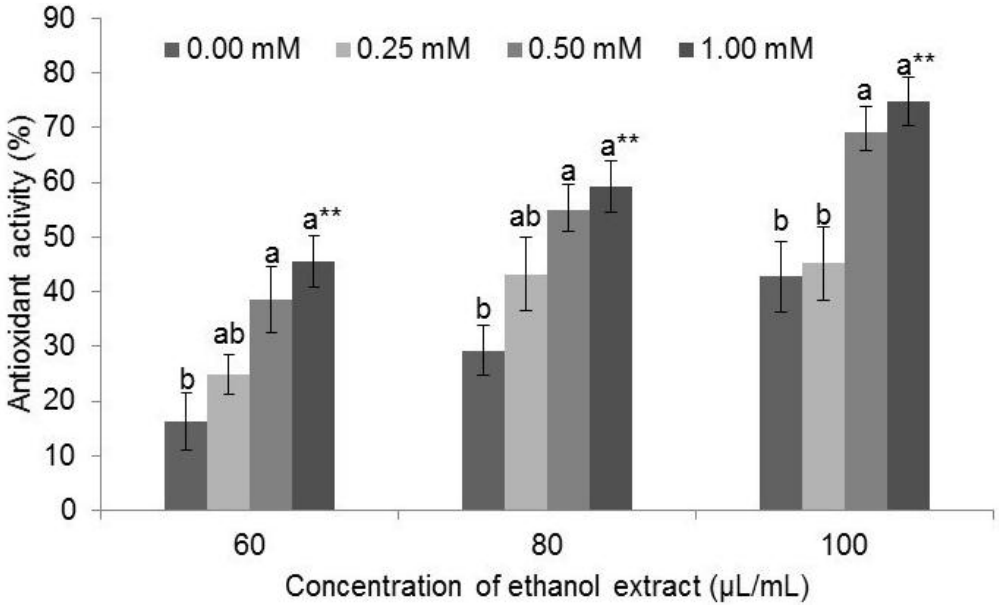

Figure 8. Percentage of antioxidant activity of ethanol extracts of *Achillea millefolium* L., under different concentrations of salicylic acid. Different letters indicate significant differences between the salicylic acid concentrations for each extract concentration, by Tukey test (p≤0.01) (p=0.0055, p=0.0054, p=0.0020).

(Ghasemzadeh et al., 2012; Obinata et al., 2003). Another mode of action of SA would be through its interference in the activity of antioxidant enzymes, where inhibition of the enzyme catalase (CAT) would cause an increase in cellular levels of H_2O_2, which in turn can increase the production of secondary metabolites in the plant for acting as a second messenger (Askari and Ehsanzadeh, 2015).

Conclusions

The global market along with the use of medicinal plants has been growing in recent years and generating an alternative income source for small farmers. Given the results obtained in this study, it can be concluded that the application of SA constitutes an advantageous management practice for commercial production of *A. millefolium*. Plants treated with 0.50 mM SA presented increased root growth, enabling producers to cultivate a greater number of plants per area which can generate better economic results than that obtained with lower density. In relation to the synthesis of secondary metabolites, the application of SA at 0.50 and 1.00 mM was most effective in eliciting the production of essential oils and total phenols, with a consequent improvement of the antioxidant activity of the plant extract.

Conflict of Interests

The authors have not declared any conflict of interest.

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