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Ascophyllum nodosum extract improves phenolic compound content and antioxidant activity of medicinal and functional food plant *Achillea millefolium* L.

Ana Cláudia Pacheco*[1], Leonardo Araujo Sobral[1], Pedro Henrique Gorni[1], Marcia Eugenia Amaral Carvalho[2]

[1]Universidade do Oeste Paulista, Presidente Prudente – SP, Brazil
[2]Escola Superior de Agricultura "Luiz de Queiroz", Piracicaba – SP, Brazil

***Corresponding author: anaclau@unoeste.br**

Abstract

Genetic, biochemical and physiological parameters of plants can be changed by applying seaweed extract-based products. However, there is scarce information about the influence of seaweed extract on yarrow performance (*Achillea millefolium* L.), which is widely used in the folk medicine. Therefore, this study aimed to evaluate the effects of *Ascophyllum nodosum* extract on plant development (leaf and root biomass, and leaf area), physiological indexes (leaf weight ratio, and root: leaf ratio), secondary metabolite synthesis (phenolic compounds) and antioxidant activity of yarrow. The experiment was carried out in a completely randomized design with 4 treatments (seaweed extract concentrations 0, 3, 6 and 9 mL L^{-1}) and 10 replications. The higher concentration of seaweed extract caused higher total dry weight of plants (from 17.8 to 19%), especially due to increases in the root biomass (up to 28.5%). Only plants that received the highest concentration of seaweed-based product presented increments in the number of leaves when compared to the control plants (18.3 %). Furthermore, the use of *A. nodosum* extract 9 mL L^{-1} provided increases in the antioxidant activity and synthesis of phenolic compounds in leaves (up to 30.44%). In conclusion, application of *A. nodosum* is a potential tool to improve the quality of raw material from yarrow plants, since it increased the phenolic compound content and antioxidant activity in leaves, which are the plant organs commonly used in folk medicine.

Keywords: biostimulant, elicitor, medicinal plant, leaf pigments, plant defense, seaweed extract, secondary compounds.
Abbreviations: ANE_*Ascophyllum nodosum* extract, LDM_leaf dry mass, LWR_leaf weight ratio, LDLs_low-density lipoproteins, NL_number of leaves, ROS_reactive oxygen species, RDM_root dry mass, TDM_total dry mass, LA_total leaf area, LA/NL_individual leaf area.

Introduction

For millenniums, medicinal plants have been employed by humans in diverse areas that include medicine, nutrition, flavouring, beverages, dyeing, repellents, fragrances and cosmetics (Najafi and Deokule, 2010). *Achillea millefolium* L. (yarrow) belongs to Asteraceae family that is represented by about 85 species, which are mostly found in Europe and Asia (Turner and Wasson, 1997). Antioxidant properties of *A. millefolium* have previously been reported in hydroalcoholic, methanolic and aqueous extracts, as also in its essential oils (Trumbeckaite et al., 2011; Vitalini et al., 2011). This property is related to presence of phenolic compounds in leaves, especially flavonoids (hyperoside as the major class) and phenolic acids (chlorogenic acid and and p-coumaric acid are the most abundant compounds) (Georgieva et al., 2015).

In order to supply the pharmaceuticals, food additives, flavours and herbal industries with high-quality raw materials, and add value to these products, strategies such as treating with elicitors have been used in both plant cell culture and in intact plants (Zhao et al., 2005). Elicitors refer to chemicals or natural products that can trigger physiological and morphological responses and accumulation of secondary metabolites, such as polyphenols in plants (Dong et al., 2010). For instance, the use of salicylic acid or utilization of a product based on a mix of hormones (cytokinins, auxins and gibberellic acid) elicited the production of phenolic compounds in marigold, yarrow, and fennel leaves, so increasing the quality of their raw material (Machado et al., 2014; Gorni and Pacheco, 2016; Gorni et al., 2017).

Medicinal plant farming should be carried out under organic system (Mógor et al., 2008). In this context, the use of seaweed extract is an environmentally friendly alternative to replace some fertilizers and biostimulants (Craigie, 2011). *Ascophyllum nodosum* (L.) Le Jolis extract contains several hormones, and also other compounds (such as amino acids and polysaccharides) that stimulate plant growth and yield, also improving tolerance to biotic and abiotic stresses through elicitor mechanisms (Rayorath et al., 2008; Craigie, 2011; Vera et al., 2011; Carvalho et al., 2013, 2014; Carvalho and Castro, 2014; Battacharyya et al., 2015; Castro et al., 2017). One of these mechanisms is related to the presence of polysaccharides and its derivated-oligosaccharides that trigger oxidative stress in plants, which is able to activated

defense pathways, hence increasing gene expression and synthesis of secondary metabolites (Vera et al., 2011).
Previously, an increased production of phenolic compounds and flavonoids were reported in cauliflower inflorescence (*Brassica oleracea* cv. Caraflex) after seaweed-based product application (Lola-Luz et al., 2013). Taking account this information, the use of seaweed extracts may potentially enhance both synthesis of bioactive compounds and biomass production of medicinal and functional food plants. However, there are scarce information about the influence of seaweed-based products on yarrow development or on quality of its leaves, which are used as raw material in phytomedicine and beverage industries. Therefore, this work aimed to evaluate the effects of *A. nodosum* extract on plant growth, as well as on phenolic compounds and antioxidant activity in leaves of *A. millefolium*.

Results and Discussion

Yarrow development

The use of seaweed extract in agriculture have increased in the last decades due to its positive effects on plant development and quality of edible portions (Craigie, 2011; Lola-Luz et al., 2013; Carvalho and Castro, 2014; Battacharyya et al., 2015). In this study, root dry weight increased linearly to the increases of seaweed extract concentration (up to 28.5% when compared to control, Fig 1). Application of *A. nodosum* extract also improved the root architecture of wheat cv. Pusa Gold, which presented an increased lateral root formation and, consequently, a higher root volume than the control plants (Kumar and Sahoo, 2011). This response can be related to the fact that *A. nodosum* extract modulates concentration and localization of auxin, which is the main hormone that regulates root development (Rayorath et al., 2008; Taiz and Zeiger, 2010). Therefore, seaweed extract potentially enhances nutrient uptake by plants through stimulus of root development, as reported by Mancuso et al. (2006).
Santos et al. (2013) also shown an increased root dry weight in maize (15.59%), in addition to increments in the leaf dry weight (up to 33.9%) after application of seaweed-based product. However, there were no statistical differences among treated and non-treated plants for both parameters (Santos et al., 2013). Application of seaweed extract on *A. millefolium* did not change the leaf dry weight, when compared to the control (Fig 1). Therefore, use of seaweed extract increased the total dry mass (from 17.8 to 19%) (Fig 1) and root: shoot ratio (Fig 2), since root biomass represented the highest proportion of plant weight. For the same reason, a decreased leaf weight ratio (Table 1) was observed in seaweed-treated plants when compared to control ones. On the other hand, wheat cv. IAC 364 exhibited an improved shoot development after application of *A. nodosum* extract as either seed treatment or soil irrigation (Carvalho et al., 2014), reinforcing reports that seaweed extract effects depend on several factors, such as dose, application mode and frequency, plant species and even cultivars (Carvalho and Castro, 2014).
In this study, yarrow plants presented an increased number of leaves (18.3%) after seaweed extract application (Fig 2), corroborating Silva et al. (2012) who reported an improved yield in cabbage (*Brassica oleracea* cv. collard greens of Georgia) due to the increments in both number and biomass of leaves after seaweed application. These same authors also observed a higher number of leaves (16.6%) in 'Vera' curly lettuce that was treated with seaweed extracts (at instance, from *Sargassum* and *Laminaria* genus) than non-treated plants. However, use of *A. nodosum* extract trends to reduce the leaf area per yarrow plant (from 33.85 to 40.8%), due to decreases in the individual leaf area after the use of seaweed-based product (Table 2). These responses can be related to the alterations in cytokinin production by plants, a hormone that drives the leaf development (Taiz and Zeiger, 2010), since *A. nodosum* extract can modifies its endogenous synthesis (Zhang and Ervin, 2004). Taking together these results, the effects of *A. nodosum* on yarrow plants is probably due to the changes in hormonal synthesis that disturbs photoassimilate production and partitioning, inducing biomass accumulation in roots rather than in shoots (Fig 1).

Chlorophyll, carotenoid and anthocyanin contents

Seaweed extract application did not affect anthocyanin, carotenoid, total chlorophyll and chlorophyll *b* contents, but decreased chlorophyll *a* content when applied at 6 mL L^{-1} (Table 2). The biosynthesis of chlorophylls *a* and *b* in 'IAC 364' wheat was not changed after use of *A. nodosum* extract either on seeds or as soil application (Carvalho et al., 2014). However, Blunden et al. (1996) reported that application *A. nodosum* extract as foliar spray resulted in higher chlorophyll concentration in tomato, wheat, barley, and maize leaves. Moreover, these authors associated the increased chlorophyll production in treated plants to the presence of betaines in seaweed extract. However, according to the literature, there is a close relationship between chlorophyll synthesis and the applied dose of seaweed extract; where lower doses would be the most effective in promoting increases in chlorophyll content (Jothinayagi and Anbazhagan, 2009). The method of application is also referred as crucial factor to trigger increases in the chlorophyll content (Matysiak et al., 2011).

Seaweed extract effects on plant defense

The total phenolic content increased linearly to the increases in the seaweed extract concentration (Fig 3). Phenolic compounds are the most widely distributed secondary metabolites, ubiquitously present in the plant kingdom, that fulfill a very broad range of physiological roles in plants related to growth and survival (Cheynier et al., 2013). The expression "plant phenolics" encompasses a highly diverse group with an extremely large structural diversity: tens of thousands of diverse structures have been identified, with the number continually increasing (Quideau et al., 2011). Lola-Luz et al. (2013) also reported an increased (1.3 fold) production of phenolic compounds in cauliflower's inflorescence that was treated with two different seaweed-based products. Usually, these products contain polysaccharides and its derivated-oligosaccharides, which are able to induce synthesis of secondary metabolites (such as terpenes, terpenoids, phenols and/or alkaloids) through change in the gene expression (Vera et al., 2011).
The most remarkable feature of polyphenols is their capability to scavenge reactive oxygen species (ROS), which

Table 1. Total leaf area (LA, cm² plant^{-1}), superficial area per leaf unit (LA/NL, cm² leaf^{-1}), and leaf weight ratio (LWR) of *Achillea millefolium* that was treated with different concentrations (mL L^{-1}) of *Ascophyllum nodosum* extract (ANE).

ANE	LA^{ns}	LA/NL*	LWR**
0	1315.15 ± 106.80 a	32.51 ± 3.49 a	0.22 ± 0.011 a
3	1080.03 ± 90.31 a	25.84 ± 2.96 ab	0.16 ± 0.010 b
6	1015.91 ± 48.06 a	29.51 ± 1.41 ab	0.17 ± 0.009 b
9	1091.72 ± 101.66 a	24.65 ± 5.91 b	0.16 ± 0.009 b

NL = number of leaves. Means followed by distinct letters differ by Tukey's test. Bars represent the standard deviation of the mean (** significant at 1%, * significant at 5% and ns not significant).

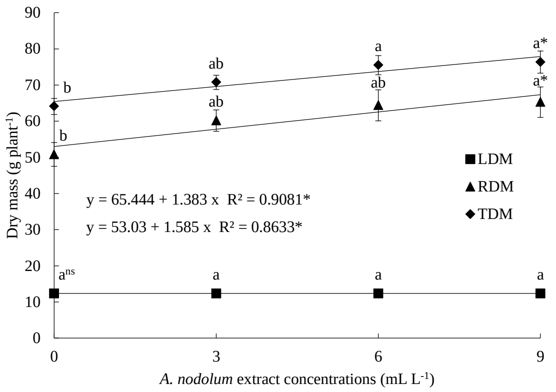

Fig 1. Leaf (LDM), root (RDM) and total dry mass (TDM) of *Achillea millefolium* that was treated with different concentrations of *Ascophyllum nodosum* extract. Distinct letters differ by Tukey's test at $p \leq 0.05$. Bars represent the standard deviation of the mean. Regression equation significant at $p \leq 0.05$ (*).

Table 2. Chlorophyll a (Chl a), chlorophyll b (Chl b) total chlorophy (Chl t), anthocyanins (Ant), and carotenoid (Car) contents of *Achillea millefolium* L. that was treated with different concentrations (mL L^{-1}) of *Ascophyllum nodosum* extract (ANE).

ANE	Chl a*	Chl b*	Chl t*	Ant^{ns}	Car^{ns}
	(µg ml^{-1})				
0	3.2619 ± 0.3377 a	2.2637 ± 0.1726 ab	5.2843 ± 0.4967 ab	3.2138 ± 0.1632 a	0.6005 ± 0.0783 a
3	3.1212 ± 0.4060 ab	3.1898 ± 0.5823 a	6.3111 ± 0.9284 a	4.4297 ± 1.1222 a	0.7089 ± 0.1080 a
6	1.8515 ± 0.1207 b	1.6554 ± 0.1331 b	3.5069 ± 0.2520 b	2.5050 ± 0.2202 a	0.4694 ± 0.0257 a
9	2.5465 ± 0.3056 ab	2.1895 ± 0.1624 ab	4.7361 ± 0.4655 ab	2.8044 ± 0.1140 a	0.6664 ± 0.0915 a

Means followed by distinct letters differ by Tukey's test. Bars represent the standard deviation of the mean (* significant at 5% and ns not significant).

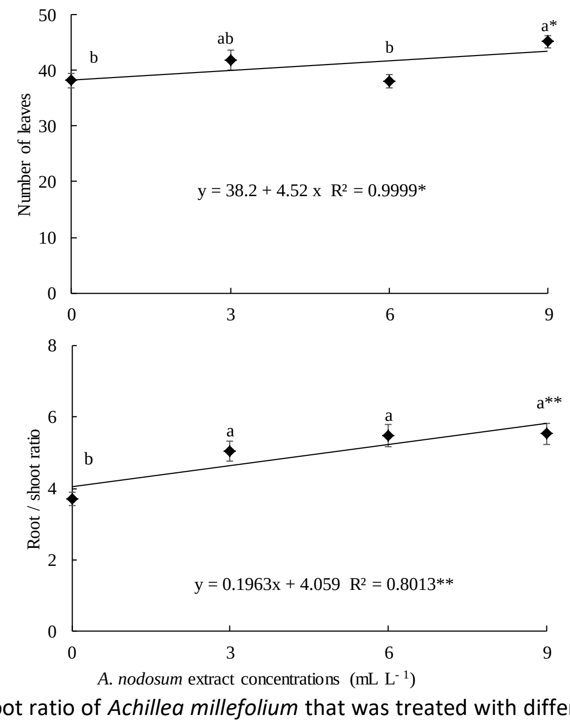

Fig 2. Number of leaves and root: shoot ratio of *Achillea millefolium* that was treated with different concentrations of *Ascophyllum nodosum* extract. Distinct letters differ by Tukey's test at $p \leq 0.05$. Bars represent the standard deviation of the mean. Regression equation significant at $p \leq 0.05$ (*) and $p \leq 0.01$ (**).

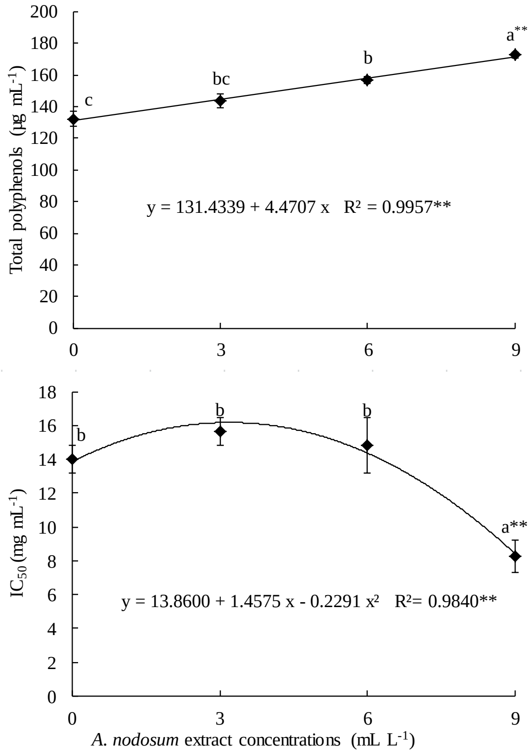

Fig 3. Content of total phenols and antioxidant activity of *Achillea millefolium* that was treated with different concentrations of *Ascophyllum nodosum* extract. Distinct letters differ by Tukey's test at $p \leq 0.05$. Bars represent the standard deviation of the mean. Regression equation significant at $p \leq 0.01$(**).

include radical and nonradical oxygen species such as $O_2^{\cdot-}$ HO·, NO·, H_2O_2, $^1O_2$, and HOCl , as well as oxidatively generated free radicals RO and ROO derived from biomolecules like low-density lipoproteins (LDLs) (Neudorffer, 2006) proteins, and oligonucleic acids (Shi et al., 2000). All these species can have deleterious effects on human health (Ferguson, 2001). The antioxidant activity in ethanolic leaf extract of seaweed-treated yarrow exhibited lower IC50 values, which indicate a higher antioxidant activity than control plants. It can be noted that *A. nodosum* extract 9 mL L^{-1} significantly decreased IC50 (70%), when compared to the non-treated plants (Fig 3). Significant increases in the antioxidant activities were associated with the increased phenolic and flavonoid content in plants treated with *A. nodosum* extract (Elansary et al., 2016; Lola-Luz et al., 2014).

Although phenolic compounds are the major antioxidant class in plants, other compounds (such as betacyanins, α-tocopherol, ascorbic acid and β-carotene) may act as free radical scavengers, hence contributing to the oxidative stress stabilization (Janda et al., 2014; Brandão et al., 2014). Several studies have indicated that the regular intake of plant-origin products [such as fruits, vegetables, spices and medicinal herbs (as tea, for example)] prevents and /or reduces the risk of chronic and degenerative diseases that are triggered by cellular oxidative stress, particularly due to presence of substances with antioxidant activity like as phenolic compounds (Rumbaoa et al., 2009; Cardoso Silva et al., 2010; Engel et al., 2016). Despite their structural diversity, phenolic compounds are categorized into several classes. Among them, phenolic acids, flavonoids and tannins are regarded as the main dietary phenolic compounds (Balasundram et al., 2006). In this context, water extracts of *A. millefolium* (obtained from infusion or decoction methods) can be intake in the everyday life, since this extract maintain total polyphenolic content and antioxidant capacity (Georgieva et al., 2015).

Materials and Methods

Plant materials and growth conditions

The experiment was carried out in greenhouse with controlled temperature (26 ºC) and humidity (70%), in Presidente Prudente, São Paulo state, Brazil (22° 7' 39" S, 51° 23 '8" W, 471 ma.s.l.). The seedlings were collected from yarrow plant matrices that were grown at Medicinal Plant Garden of Universidade do Oeste do Estado de São Paulo. Specimens were deposited in the Universidade Federal de Uberlândia's Herbarium (voucher # 74428 HUFU). Seedlings with three completely expanded leaves were transplanted to 20-dm³ vessels that were filled with 18 kg of soil (Table 1), and plants were managed as recommended by Bulletim 100 – IAC for perennial herbaceous species. Foliar sprays with solutions containing 0 (only water); 3; 6 and 9 mL L^{-1} of seaweed extract were applied three times - 20, 30 and 100 days after seedlings transplanting (DAT). As surfactant agent, nonyl phenoxy poly (ethyleneoxy) ethanol (Agral®) 50 uL L^{-1} was added to the solutions that were sprayed through a hand sprayer. Physical and chemical properties of the soil that was used in the experiment are described as following: pH ($CaCl_2$) 4.3, Ca 5.5 ($mmol_c\ dm^{-3}$), Mg 4.2 ($mmol_c\ dm^{-3}$), K 3.6, P 3.0, S 24.5, Mn 0.4, Fe 3.8, Cu 1.0, Zn 0.5 and B 0.14 ($mg\ dm^{-3}$).

Growth analyses and physiological indexes

At 120 DAT, leaves and roots (rhizomes + radicles) were collected. Next, the number of leaves (NL) and leaf area (LA – cm^2) per plant were evaluated. All organs were placed into an oven with air circulation at 40 ºC, in order to obtain their dry mass (g). With these data, (i) leaves, roots and total dry mass per plant (LDM, RDM and TDM, respectively), (ii) root: leaf ratio (RDM/LDM), (iii) leaf weight ratio (LDM/TDM), and (iv) individual leaf area (LA/NL) were calculated.

Quantification of the leaf pigments

Chlorophyll (*a*, *b* and total), carotenoid and anthocyanin contents were spectrophotometrically determined following the extraction on TRIS-acetone buffered solution (hydroximetil-aminomethan), according to the method of Sims and Gamon (2002).

Determination of the total phenol content and antioxidant capacity

Total phenol content in leaves was measured by Folin-Ciocalteu method, using gallic acid as standard in sodium carbonate solution (Stagos et al., 2012). In order to evaluate the antioxidant activity of yarrow, different volumes of leave extract, which were required to decrease initial concentration of 2,2-diphenyl-1-picrylhydrazyl (DPPH) by 50% - inhibitory concentration (IC_{50}), were used, as described by Brand-Williams et al. (1995). For assessments of total phenol content and antioxidant activity, ethanolic extracts from dried leaves were used.

Statistical analyses

The experiment was carried out in a completely randomized design with 4 treatments (seaweed extract doses) with 10 and 5 replications (*i.e.* one plant per experimental unit) for biometric and biochemical analyses, respectively. The obtained data were submitted to analysis of variance ($p \leq 0.05$), and then to both regression analysis and Tukey's test (all them considering $p \leq 0.05$).

Conclusion

Application of *A. nodosum* is a potential tool to improve the quality of raw material from yarrow, since it increased the phenolic compound content and antioxidant activity in leaves. Rises in the content of phenolic compounds may be related to both seaweed extract-induced production of new young leaves and presence of elicitor substance in *A. nodosum* extract, which are able to change secondary metabolism of plants. Data also revealed that this seaweed extract affects photoassimilate partitioning by inducting biomass allocations to roots without changing leaf dry weight.

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