Pak. J. Bot., 55(3): 1041-1049, 2023. DOI: http://dx.doi.org/10.30848/PJB2023-3(25)

EVALUATION OF THE ANTIOXIDANT PROPERTIES AND ANTIFUNGAL ACTIVITY OF METHANOLIC EXTRACT OBTAINED FROM *NASSAUVIA DENTATA* GRISEB. (ASTERACEAE)

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Abstract

Nassauvia dentata Griseb. (Asteraceae), is distributed from the mountain range in the Bío Bio region to Magallanes region in Chile. In this investigation, an antioxidant effect evaluation of *N. dentata* leaves and stems was performed with 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) cationic radical and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical, expressed in equivalents. The concentration of the methanolic extract obtained from *N. dentata* (MEN), required to inhibit 50% of ABTS absorbance and DPPH absorbance (IC_{50} value), was calculated..In a kinetic assay performed with ABTS and determined with the IC_{50} value of 0.737 mg mL^{-1}, the maximum effect was detected after 30 minutes (min), with an inhibition of 96.44 ± 2.44%. DPPH kinetic assay was made with the IC_{50} value of 1.562 mg mL^{-1} and after 20 min it was observed the maximum point of inhibition level, of 79.92 ± 3.39%. Consequently the assay of total polyphenols (TP) was determined according to the Folin-Ciocalteu method. Also phytochemical assays were made to recognize the secondary metabolites, present in the plant material and in MEN. A bigger presence of tannins, anthraquinones heterosides, steroid derivatives, coumarins, and flavonoids were found. Evaluation of the antifungal effect assay was made using different concentrations of MEN against *Rhizoctonia solani* Kühn (Ceratobasidiaceae). It was possible to determine the maximum inhibition level of growing of the mushroom at 20 mg mL^{-1} of MEN concentration, on average 77 percent. The results of the present study indicate the presence of polyphenols in this plant, and might be considered as a source of bioactive compounds.

Key words: *Nassauvia dentata* Griseb ● Extraction ● Oxidations ● Fungicides ● *Rhizoctonia solani* Kühn

Introduction

The genus *Nassauvia* belonging to the subtribe *Nassauviinae*, is distributed across the south of the Andes from Bolivia to the Tierra del Fuego, and also can be found on the Falkland Islands and in Antarctica (Cabrera, 1982; Lewis Smith & Richardson, 2011). In Chile, there exist 24 species of *Nassauvia*, and 10 of them have been investigated chemically (Bohlmann & Zdero, 1979; Zdero *et al*., 1986; Zdero *et al*., 1988; Bittner *et al*., 1988; Bittner *et al*., 1989; Zdero & Bohlmann, 1990; Pritschow *et al*., 1991; Kotowicz *et al*., 2005). The native species *Nassauvia dentata* Griseb., distributed to the south of the Andes mountain range, appearing in Chile and Argentina (Moreira *et al*., 2011), has not been chemically investigated, and the novelty of our research consists of a contribution on the knowledge of chemical compounds of *N. dentata*, which is a Chilean vascular plant with unknown and not investigated biological effects, up to the present day. The environmental and living conditions of *N. dentata,* contributes to a high stress environment where few species can adapt and survive (Hoffmann *et al*., 1998). Due to the living conditions of this species, it is of interest the study of its secondary metabolites and antioxidant activity.

Antioxidants are secondary metabolites that can delay or inhibit the oxidation of lipids or other molecules (Ozen & Demirtas, 2015). Many antioxidants of natural origin, show an ample range of biological effects, including antibacterial, antiviral, anti-inflammatory and cardioprotective effects (Kunnumakkara *et al.*, 2017; Davinelli *et al*., 2018).

The antioxidant activity and its relation to antibacterial properties are due to the presence of polyphenols and a variety of secondary metabolites present in distinct plant species (Martelli & Giacomini, 2018). In addition, some vegetable extracts due to their contents in secondary metabolites, possess antifungal effects specifically in the stages of post-harvest (Ncama *et al*., 2019). The natural antioxidants, including ascorbic acid and the plant extracts rich in polyphenols (Enko & Gliszczyńska-Świgło, 2015), are used as preservatives in foods, as beneficial agents for health, in pharmaceutical production and cosmetic products (Baenas *et al*., 2019; Vieira *et al*., 2019). Moreover it is possible to improve the activity and the preservative properties of the polyphenols in foods, using these secondary metabolites as antioxidant polymers (Gahruie & Niakousari, 2017).

The phenolic antioxidants of synthetic origin have been related to human live due to its use; however there is a growing concern due to its lack of biological security (Yang *et al*., 2018). Hence, an alternative is the use of biodegradable antioxidants (Hendel *et al*., 2016) of natural origin. Another aspect to consider in the selection of antioxidants of natural origin is their antioxidant activity in comparison with antioxidants of synthetic origin. In fact, there exist antioxidants of natural origin which are contained in plant extracts that possess a superior antioxidant activity in comparison with antioxidants of synthetic origin (Qasim *et al*., 2017; Taşkın *et al*., 2018). The application of chemical pesticides and the use of resistant plant species are methods used at controlling pathogenic fungi. The adverse effects of pesticides on human health and on the

environment, and the emergence of resistant pathogens to conventional pesticides, in addition to the observed changes in plants resistance, showed that the use of synthetic pesticides is not recommended for longer (Mamarabadi *et al*., 2018). An alternative to control the pathogenic organisms in plants are the plant extracts (Bineshian *et al*., 2018) which are the most effective and least damaging to the environment (Chowdhary *et al*., 2018). As mentioned above, it is relevant to focus on the search of new chemical compounds which are obtained from natural sources. The aim of this study is to determine the biological activity in methanolic extracts obtained from leaves and stalks of *N. dentata*, in order to contribute to the knowledge about the plant species that can provide antioxidant and antifungal effects.

Materials and Methods

Plant material: The leaf and stalk samples of the *N. dentata* were collected in summer time, at 1550 meters above sea level, on the west side of the Volcano Callaqui (37º55'0''S 71º27'0''O), located on the Andes mountain range, in the region of Bio-Bio, locality of Callaqui in Chile. The sample collection of the plants was conducted in a random format around the base of the volcano. The plant material was transported to the Laboratorio de Extractos Vegetales of the Universidad de Concepción. The plant was washed with water and oven dried (Memmert UFB 500) at 30°C. Then, the plant was crushed, using a grinder (Waring Commercial HGBT WT). After obtaining a homogenous sample, it was stored for its later use.

Preparation of the extract: The methanolic extract was obtained in a Soxhlet apparatus (Glassco 3049/8), with methanol (MeOH) as an extraction solvent, at 60ºC ± 5ºC, for a period of 6 hours (4 cycles). The methanol contained in methanolic extract obtained from *N. dentata* (MEN), was evaporated, utilizing a rotary evaporator (Heidolph Laborota 4001 efficient) at 50°C ± 5°C. The dry extract was weighed and stored at 4°C in complete darkness in the Laboratorio de Extractos Vegetales at the Universidad de Concepción, Campus Los Angeles.

Determination of antioxidant activity

ABTS radical-scavenging assay: According to the developed methodology (Huang *et al*., 2010) with modifications, the cation $ABTS^{\cdot+}$ was prepared after the reactions of ABTS (7 mM) (Sigma-Aldrich A1888) with potassium peroxodisulfate (2.45 mM) (Merck 1.05091.0250) incubated at room temperature and in complete darkness for 16 hours (h), using MeOH as solvent. Once the radical $ABTS^{\cdot+}$ was formed, it was diluted with MeOH until the absorbance value, measured at 734 nm, was between 0.70 ± 0.02. In this method 2 mL of $ABTS^{\cdot+}$ in MeOH with 0.175 mL of MEN were incubated at different concentrations during 1 minute (min) at room temperature and later the absorbance was measured using a spectrophotometer (UV/Vis Spectroquant Pharo 300). The antioxidant activity done via ABTS was evaluated at 734 nm, with various concentrations of MEN in a range of 0.125 to 6.000 mg mL^{-1}. Regarding the negative control, a mixture composed of 2.0 mL $ABTS^{\cdot+}$ plus 0.175 mL of MeOH was used, and MeOH was used for adjusting the zero point. The data of the absorbance was transformed in percentage decrease of absorbance of ABTS (Ruffo *et al*., 2017), in accordance with the following equation (1):

$$\text{Inhibition (\%)} = (A_0 - A_1 / A_0) \times 100 \quad (1)$$

where A_0 is the absorbance of the negative control and A_1 is the absorbance of the samples. The test was performed in triplicate and the IC_{50} concentration was calculated from a linear equation, obtained from the graphic of MEN concentration versus inhibition (%) of absorbance. The antioxidant capacity measured by means of ABTS was also expressed as trolox equivalents per gram of dry weight (TEAC) (Jorjong *et al*., 2015) and like Vitamin C (VitC) equivalents (VCEAC) per gram of dry weight (Kim *et al*., 2018). For the TEAC test and for the VCEAC test calibration curves with trolox (Merck 648471) and with VitC (Sigma-Aldrich 33034) were made, respectively. In the kinetic test performed using the radical $ABTS^{\cdot+}$, 2 mL of $ABTS^{\cdot+}$ were incubated with 0.175 mL of MEN at a concentration of IC_{50} and the absorbance was measured at 734 nm in a spectrophotometer (UV/Vis Pharo 300 Spectroquant) during 2h. A mixture of 2 mL of $ABTS^{\cdot+}$ plus 0.175 mL of MeOH was used as negative control. Additionally MeOH was used for adjusting the zero point. The absorbance values were registered in 5min intervals, and absorbances were transformed in percentage decrease of absorbance, using equation 1. The kinetic test was performed in triplicate.

DPPH radical-scavenging assay: The measurement of antioxidant activity was evaluated, measuring the capacity of MEN to reduce the free radical DPPH (Sigma-Aldrich D9132), according to the method previously described (Gargouri *et al*., 2013), with modifications. By means of this method, 4 mg of DPPH was measured and diluted with 100 mL MeOH; obtaining a concentration of 0.04 mg mL^{-1}. The methanolic solution of DPPH was diluted with MeOH until obtaining a absorbance near 0.90, measured at 517 nm. Two mL of the methanolic solution of DPPH (adjusted to 0.90 at 517 nm) with 0.175 mL of MEN at different concentrations were incubated during 1min at room temperature and later the absorbance was measured using a spectrophotometer (Thermo Scientific Genesys 10 U.V). In the test conducted using DPPH, concentrations of MEN were evaluated at 517 nm, in a range of 0.125 at 6.000 mg mL^{-1}. A solution composed of 2.0 mL of DPPH and 0.175 mL of MeOH was used as negative control, and MeOH was used for adjusting the zero point. The data of absorbance was transformed to a percentage inhibition of absorbance of DPPH in agreement with equation 1. The test was performed in triplicate and via the curve equation obtained from the graphic of MEN concentration versus inhibition (%) of absorbance, the IC_{50} concentration was determined. The antioxidant effect determined via DPPH was also expressed as TEAC (Devi *et al*., 2019) and as VCEAC (Zhang *et al*., 2018). In the kinetic test via the radical

DPPH, 2 mL of DPPH with 0.175 ml of MEN was incubated at the IC_{50} concentration and its absorbance was measured at 517 nm on the spectrophotometer (Thermo Scientific Genesys 10 UV) during 2h, and this test was perfomed in triplicate. As the negative control, a mixture of 2 mL of DPPH plus 0.175 mL of MeOH were used. Additionally MeOH was used for adjusting the zero point. The values of absorbance were registered in 5min intervals and were transformed in percentage inhibition of absorbance, using equation 1.

Determination of total polyphenols: The concentration of TP, was determined according to the Folin-Ciocalteu method (Grzegorczyk-Karolak *et al*., 2015), with modifications. Based in MEN, a diluted solution was prepared in MeOH to achieve a concentration of 0.125 mg mL^{-1} in 25 mL, after 1.25 mL of Folin-Ciocalteau's phenol reagent (Merck 1.09001.0500) was added, and mixed in a 25 mL volumetric flask for 1 min, afterwards 5 mL of Sodium carbonate was add at 20% m v^{-1} (Merck 1.06392.1000). Finnaly the flask of 25 mL was filled with methanol. MeOH was used instead of MEN to calibrate the zero and the assay was performed in triplicate. To express the results as Gallic acid equivalents (GAE) per gram of dry extract, a standard curve of Gallic acid was used. The readings were taken at 765 nm on the spectrophotometer UV/Vis (Thermo Scientific Genesys 10 UV).

Qualitative assays for detection of secondary metabolites: A test of secondary metabolites was performed to determine the presence of alkaloids (Maldoni, 1991), anthraquinones (Ayoola *et al*., 2008), coumarins (Yahyaoui *et al*., 2017) derived from the steroid nucleus, flavonoids, heterosides (anthraquinones, cyanogens and cardiotonics) (Tiwari *et al*., 2011), reducing sugars, saponins and tannins (Ayoola *et al*., 2008), in order to evaluate, distinguish and to be able to classify different metabolites present in the plant material (PM) and MEN, by means of tests, chemical reactions of coloration and precipitation. A qualitative scale was used to evaluate the presence of the absence of the secondary metabolites using the following symbols: the marked presence of the metabolite (+++), a normal presence (++), a weak presence of the metabolite (+) and the absence of a secondary metabolite to evaluate (–).

Antifungal effect of MEN: Antifungal activity of MEN was determined against the pathogenic fungus *Rhizoctonia solani* Kühn (LBH-Rs) and samples of the fungus were supplied by the Laboratorio de Biotecnología de Hongos of the Universidad de Concepción. In the test the proposed methodology (Radhakrishanan Sridhar *et al*., 2003), with modifications was applied. The fungus was activated using potato dextrose agar (PDA) (Liofilchem 610102) at 24°C, during a period of 72h in the incubator (Binder ED53). After the activation of the fungus four concentrations of MEN (100, 200, 300 and 400 mg mL^{-1}) were prepared, in a test tube diluted with distilled and purified water. The tubes were sealed and then mixed using a Vortex Agitator (Boeco v1 plus). Each solution was poured out on to a Petri dish with 19 mL of PDA solution and left to solidify (3–5min), and the process was perfomed in triplicate for the four concentrations (5, 10, 15, and 20 mg mL^{-1}, obtained as the final concentrations of MEN in the Petri dishes.

After the time period had lapsed, a piece of Agar was transferred that contained the hyphae (0.91 cm^2 and 5 mm of density) to the center of the dish, with the hyphae in contact with the new environment. The dishes were stored at 24°C for 72h in an incubator (Boeco v1 plus) and the measurement of the mycelium area was performed every 12h with Image J2x software. The same procedure was performed for the negative control samples composed of 19 mL of potato dextrose agar plus 1 mL of distilled and purified water. The results were expressed in a % inhibition using the proposed equation (Ruiz-Bustos *et al*., 2009).

To determine the Mycelium dry weight, the samples from the previous test were transferred to the glass petri dishes and taken to the autoclave. The paper filter was taken to the oven (Binder BD53) at 75°C for 24h to eliminate humidity. After finishing the autoclave process, the filtering process and the cleaning of the fungi with distilled water (hot) was continued (using a vacuum pump). The samples were stored in an oven (50°C) until they reached a constant mass free of humidity, and the assay was performed in triplicate. Then the inhibition was calculated from the dry weight of the mycelium (percentage inhibition of the dry mycelial weight) using the following equation (2):

$$\begin{matrix}\text{Mycelial mass}\\(\text{control and with MEN})\end{matrix} = \begin{matrix}\text{Mycelial mass (g)}\\(\text{With the filter pape})\end{matrix} - \begin{matrix}\text{Mass of the}\\\text{filter paper (g)}\end{matrix} \quad (2)$$

$$\begin{matrix}\%\ \text{Inhibition of mycelial}\\\text{dry weight}\end{matrix} = \frac{\begin{matrix}\text{Mycelial mass}\\\text{of the negative control (g)}\end{matrix} - \begin{matrix}\text{Mycelial mass}\\\text{with MEN(g)}\end{matrix}}{\begin{matrix}\text{Mycelial mass}\\\text{of the negative control (g)}\end{matrix}} \times 100$$

Statistical analysis

The data from the registered absorbance for the *In vitro* models for the radical DPPH, radical $ABTS^{\cdot+}$, TP, as well as for the antifungal effect were obtained using an analysis of variance (ANOVA) and multiple comparisons using the test of tukey ($p<0.05$) with the statistical software InfoStat.

Results and Discussion

Extraction process: Three extractions were performed with methanol on the Soxhlet extractor, employing a total mass of vegetal material of 30.78 g. The final mass obtained from the dry methanolic extract, was at 6.86 g. The methanolic extract obtained corresponds to the 22.29% of the dry weight of *N. dentata*.

Antioxidant capacity of MEN: The antioxidant activity of MEN, represented by the IC_{50} values, and equivalent values expressed as TEAC and VCEAC are shown in Table 1.

The obtained IC_{50} values fall within the acceptable ranges in accordance with previous investigations (Skenderidis *et al*., 2018; Li *et al*., 2021) and a high antioxidant activity is represented by a low IC_{50} value (Jiang *et al*., 2021). Previous studies show that there is a positive correlation between antioxidant activity and habitat conditions such us altitude (Suyal *et al*., 2019), drought stress (Espadas *et al*., 2019), and UV-B irradiation (Yildirim, 2020). As mentioned above *N. dentata* was collected at 1550 meters above sea level in summer time, under environmental conditions influencing the antioxidant activity. The type of solvents with different polarities and extraction conditions, used for the extraction of phenolic compounds, have an influence on the antioxidant activity, and the extraction of total phenolic content also varied according to the plant species (Ng *et al*., 2020). Methanol and ethanol are commonly used solvents, however acetone is also a good solvent for extracting polyphenols to observe the antioxidant activity (Nguyen *et al*., 2022). By comparing organic solvents such us methanol, ethanol and n-hexane, methanol was found to be the best solvent of choice for the extraction of phenolic compounds, and the methanol extract of mountain fennel (*Zaravschanica membranacea*) exhibited the highest antioxidant activities by DPPH and ABTS assays (Rezaei & Pirbalouti, 2019).

The reaction of reduction of ABTS was concentration dependent, reaching a 93.69 ± 3.58% in the inhibition of ABTS with a MEN concentration of 2.000 mg mL^{-1}. and increasing the concentrations of MEN maintained an average effect of 94.09 %. In the test conducted using DPPH, the main effect was observed in concentrations of 3 mg mL^{-1}, with a inhibition of 93.34 ± 3.93%, and on the other hand with MEN concentrations greater than 3 mg mL^{-1} the effect was atypically low, reaching a 68.82 ± 2.78% at a concentration of 6.000 mg mL^{-1}.

The IC_{50} values for MEN obtained from the graphics of extract concentration versus the inhibition percentage of absorbance, revealed the donation of electrons to reactive free radicals (Jiang *et al*., 2021).

By using the method based on ABTS, in the TEAC test, the measured absorbance at a MEN concentration of 0.5 mg mL^{-1} (500 µg mL^{-1}), showed an equivalence of 12.71 ± 0.99 µg mL^{-1} for trolox (25.42 ± 1.98 mg of trolox 1 g^{-1} of MEN). The VCEAC test based on the measured absorbance of ABTS at a MEN concentration of 0.5 mg mL^{-1} (500 µg mL^{-1}), showed an equivalence of 15.03 ± 1.12 µg mL^{-1} for VitC (30.06 ± 2.24 mg of Vit C 1 g^{-1} of MEN) (Table 1).

In the test conducted using DPPH, the TEAC test based on the measured absorbance of DPPH at a MEN concentration of 0.5 mg mL^{-1} (500 µg mL^{-1}) showed an equivalence of 15.87 ± 6.41 µg mL^{-1} for trolox (31.74 ± 12.82 mg of trolox 1 g^{-1} de MEN). In the VCEAC test based on the measured absorbance of DPPH at a MEN concentration of 0.5 mg mL^{-1} (500 µg mL^{-1}), the result was an equivalence of 49.45 ± 4.07 µg mL^{-1} for Vit C (98.90 ± 8.14 mg of Vit C 1 g^{-1} of MEN) (Table 1). The results of TEAC reveals the antioxidant capacity of MEN, in fact Trolox is a water soluble α-tocopherol derivative used as a reference related to the antioxidant capacity of other compounds (Said & Mekelleche, 2021). Ascorbic acid (Vitamin C) and tocopherols (Vitamin E) are nutrients, with antioxidant effect due to their free hydroxyl group (Im *et al*., 2014).

Table 1. Main data of antioxidant analysis.

Radical	IC_{50}	TEAC *	VCEAC **
ABTS	0.74 mg mL^{-1}	25.42 ± 1.98 mg	30.06 ± 2.24 mg
DPPH	1.56 mg mL^{-1}	31.74 ± 12.82 mg	98.90 ± 8.14 mg

* The expression of the results is 1 g of MEN, equivalent to mg of trolox

** The expression of the results is 1 g of MEN, equivalent to mg of Vitamin C

The antioxidant effect during the time was evaluated in 2h in a MEN concentration of 0.737 mg mL^{-1} (IC_{50}). Its initial effect was from 55.03 ± 5.99% at 0min, reaching a maximum effect at 30 min with an average of 96.44 ± 2.44%. After having reached a maximum of 30 min, the effect in time experiences a slight drop after each 5min. Upon finishing the test, it reached an effect of 93.91 ± 5.11% (Fig. 1).

The antioxidant effect during the time was evaluated in 2h using a concentration of 1.562 mg mL^{-1} (IC_{50}) of MEN. Its initial effect was at 53.90 ± 11.54% at 0min, reaching a maximum effect at 20min with an average of 79.92 ± 3.39%, after having reached the maximum effect at 20min, the effect in time lessened every 5min. finishing the test, and the final effect was measured at 74.42 ± 8.21% (Fig. 1). On both assays made using ABTS and DPPH, a steady state was observed, as shown in (Fig. 1) where the percentage decrease of absorbance reach a peak, in accordance with the largest percentage decrease of absorbance observed during the assays. The remaining DPPH percentage at the steady state at different concentrations of flower and leaf extracts of *Ferula gummosa* was determined by Nazari *et al.*, (2019), and these authors recorded the decrease in absorbance until 3 hours and thereafter 2 hours until the reaction reached a steady state. However, in our antioxidant effect of MEN during the time, a steady state was observed before 2 hours.

To determine the antioxidant activity, different methods and forms of result expression were utilized (Gülcin, 2012), because each method possess a distinct sensitivity to each compound with a determined chemical structure, just as it is necessary to express the antioxidant effect in equivalents to quantify and to compare the antioxidant compounds in the plant extracts in relation to the reference compound (Gupta, 2015). The antioxidant activity of *N. dentata* had not been studied, except in the present study and this effect is due to the secondary metabolites that are present in this species, like flavonoids (Fierascu *et al*., 2018) and tannins (Shirmohammadli *et al*., 2018).

Determination of TP: To determine the TP, using the method of Folin-Ciocalteu, a concentration of 0.125 mg mL^{-1} of Men was used, obtaining an average of absorbance of 0.460 for the MEN. The calculations indicated that the content of TP in MEN expressed as GAE in the extract, was 29.54 ± 0.81 mg g^{-1}, and this corresponds to a 2.95 ± 0.08%, and gallic acid (Jorjong *et al.*, 2015) is a good reference compound in comparision with other commercial standards, for determining total phenol content (Gao *et al*., 2019). However, the content of TP in MEN was also detected using qualitative chemical reactions.

The content of TP in MEN, was higher in comparison with other methanolic extracts of pants, as examples Jayswal *et al.*, (2021) detected 779.58 mg GAE 100 g^{-1} in *Basella alba* leaves, and Lee & Chang (2019) detected 0,37 mg GAE 100 g^{-1} in *Lepidium meyenii* leaves. On the other hand the content of TP in MEN was lower in comparison with methanolic extract of the aerial part of *Nepeta asterotricha*, with a total phenol content of 54, 6 mg g^{-1} (Mirjalili *et al*., 2021).

Secondary metabolites recognition assays: The results of the colorimetric analysis indicate a presence of various groups of secondary metabolites where steroid derivatives are highlighted. Reducing sugars were only present in PM and anthraquinones heterosides were present in PM and MEN. The presence of tannins was revealed, with a great amount of bluish-green precipitate, indicating the presence of not-hydrolysable tannins belonging to the catechol group and hydrolysable pyrogallic tannins. In the identification of flavonoids, an orange coloration indicating the presence of flavones (HCl/Zn) and flavonols (HCl/Mg) was obtained. In both samples, it was possible to identify the presence of de coumarins (Table 2).

Table 2. Phytochemical analysis of PM and of MEN to recognize secondary metabolites.

Secondary metabolites		PM			MEN		
Alkaloids	Dragendorff *	-			-		
	Lugol *	-			-		
	Mayer *	-			-		
Anthraquinones		-			-		
Coumarins		+	+		+	+	
Derivatives of the steroid nucleus		+	+	+	+	+	+
Flavonoids	HCl/Mg *	+	+		+	+	
	HCl/Zn *	+	+			-	
	Anthraquinones **	+	+	+	+	+	+
Heterosides	Cyanogens **		-			-	
	Cardiotonics **		-			-	
Reducing Sugars		+	+	+		-	
Saponins			-			-	
Tannins		+	+	+	+	+	+

Marked presence of the metabolite (+++), normal presence (++), Weak presence of metabolite (+) and (–) indicates absence of the secondary metabolite to evaluate. Reactive*, a classification of Heterosides**

In the MEN and the PM, the marked presence of the three metabolic groups was detected, using the phytochemical test of recognition, the presence of the derivatives of the steroid nucleus, anthraquinones heterosides and tannins were able to be identified. The derivatives of the steroid nucleus fulfill the hormonal functions of growth, development and also defense against herbivores (Stowe, 1998; Calf *et al*., 2018). It should be considered that the samples collected were in the process of flowering, and that implies hormonal changes (Arrom & Munné-Bosch, 2012; He *et al*., 2017). The tannins possess defensive properties against pathogenic fungi (Ullah *et al*., 2019) and insects, being the biochemical interactions between insects and plants the more important and effective than the morphological ones due to their dynamic nature (Rasheed War *et al*., 2018). Some studies have shown that the tannins play a main role for the defense of plants against abiotic and biotic stresses (Pinasseau *et al*., 2017; Top *et al*., 2017). In MEN and in the PM, a normal presence of coumarins and flavonoids were detected. The flavonoids possess multiple functions characterized for their defense mechanism against herbivores (Berardi *et al*., 2016), also participating in processes of pollination (Lan *et al*., 2017), and in the resistance of plants against the photo-oxidation (Falcone Ferreyra *et al*., 2012). In the genus *Nassauvia*, the identification studies and analysis of the coumarins stand out (Bohlmann & Zdero, 1979; Zdero *et al*., 1986; Zdero *et al*., 1988; Bittner *et al*., 1988; Bittner *et al*., 1989; Zdero & Bohlmann, 1990; Pritschow *et al*., 1991; Kotowicz *et al*., 2005). One of the functions of coumarins like phytoalexins is to act as antimicrobial agents (Jeandet *et al*., 2013) and their acumulation due to the presence of fungi, bacteria, and virus have been observed (Simkovitch & Hupppert, 2015).

Antifungal activity: The MEN presented fungistatic activity against the pathogenic fungus *R. solani* with a maximum percentage of inhibition of the radial mycelium growth of 76.95% at 20 mg mL^{-1} and at lower concentrations the effect decreased (Fig. 2).

The mycelia was measured upon finishing the antifungal test (at 72h). The mycelial mass was inversely proportional to the concentration of MEN. With a high concentration of MEN fungi mass was less. At a concentration of 5 mg mL^{-1} of MEN the percentage inhibition of dry weight was 28.98% and 56.99% at a concentrations of 20 mg mL^{-1} of MEN. Significant differences against the dry weight of the control were observed (Fig. 3).

The MEN presented antifungal activity (fungistatic) against *R. solani*, overall in high concentration (20 $mgmL^{-1}$). This activity is due to the morphological changes due to the destruction of the organelles in the endomembrane system. These alterations and damages in vegetative hyphae or conidia have been described previously (Hashem, 2011; Khan & Ahmad, 2011).

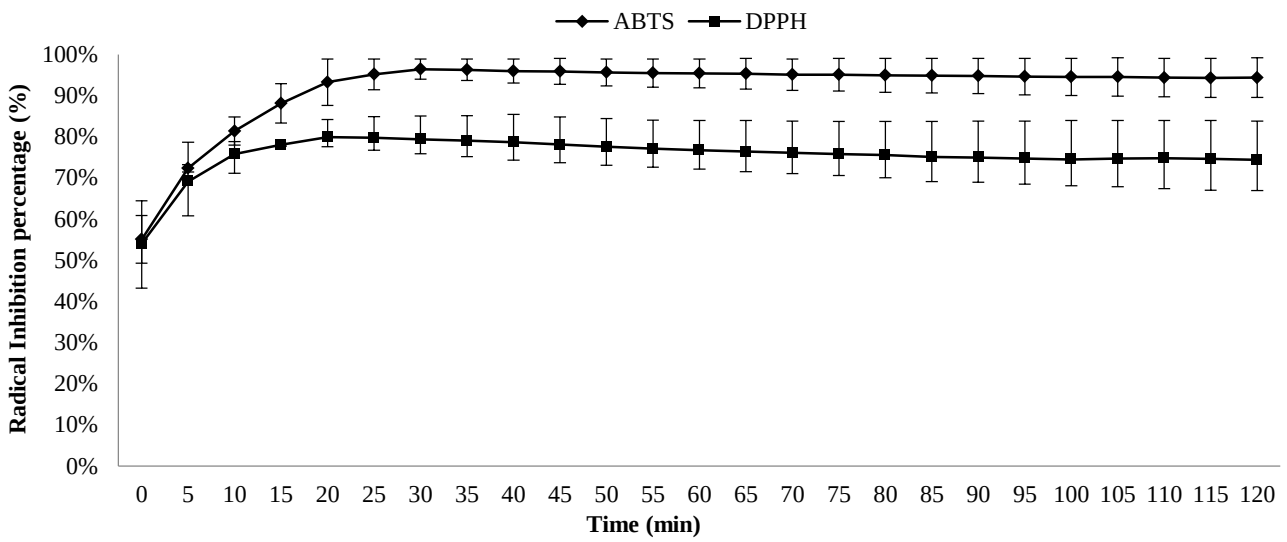

Fig. 1. Antioxidant effect of MEN in time, using the concentration IC_{50}. The values were expressed in the percentage inhibition of the radical, in a period of 120 min.

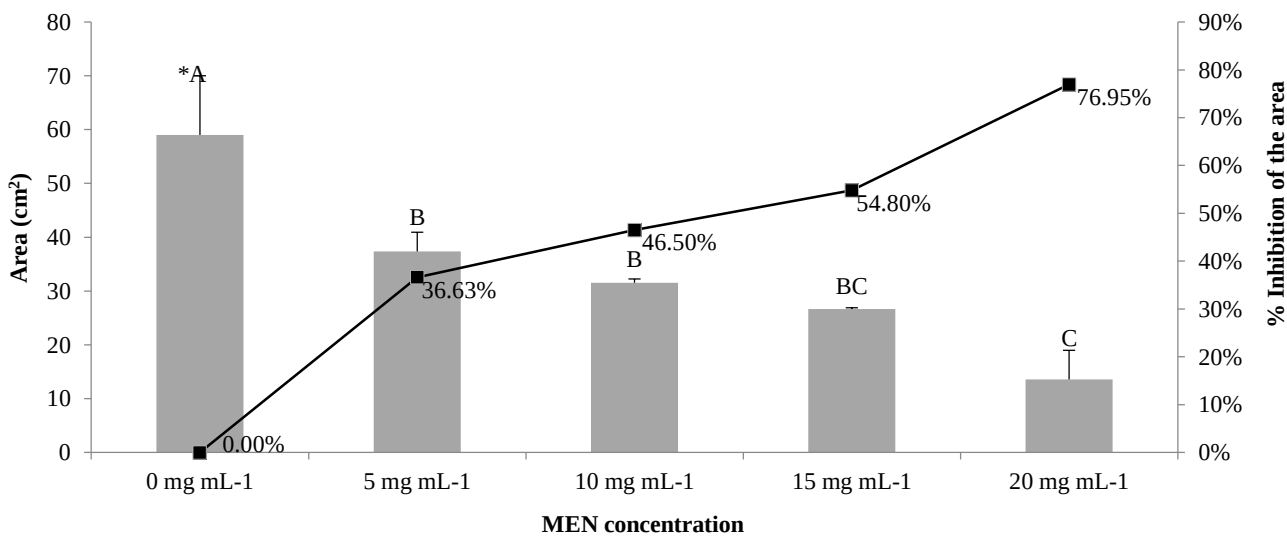

Fig. 2. Inhibition of the mycelial area of *R. solani* at different concentrations of MEN. The same letters indicate that the differences are not statistically significant (p<0.05) (Test Tukey). * Is a control sample without MEN.

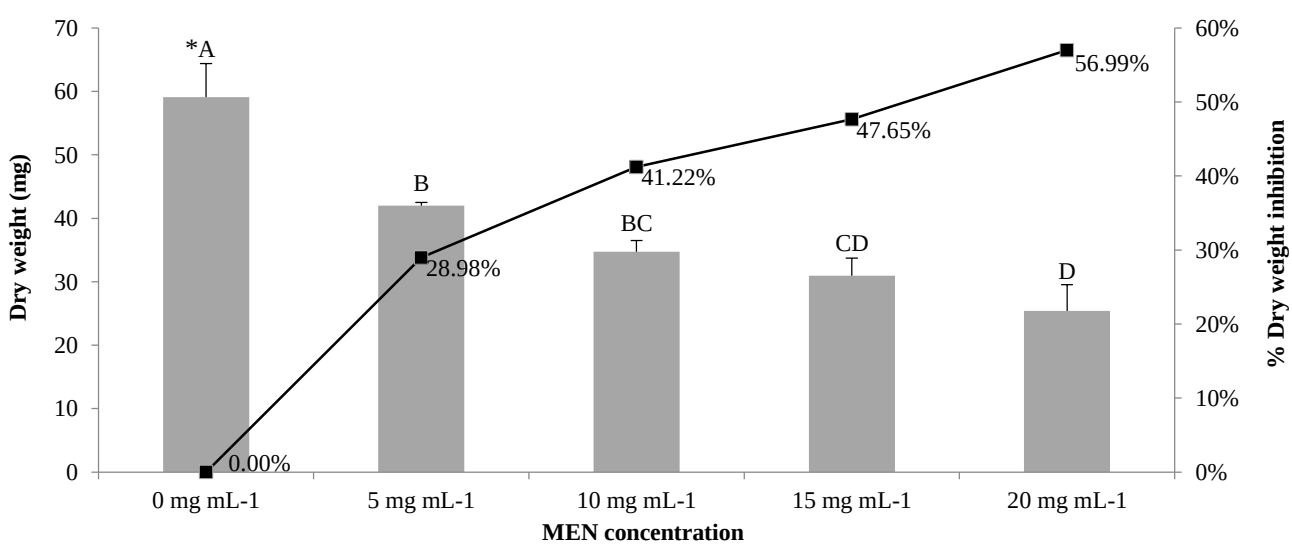

Fig. 3. Inhibition of the dry weight of mycelium *R. solani* at different concentrations of MEN. The same letters indicate that the differences are not statistically significant (p<0.05) (Test Tukey). * Is a control test without MEN.

Conclusion

N. dentata is a species that lives in extreme and dry conditions, it is exposed to the sun without any protection, and it can withstand high and low temperatures and can be covered with snow for months. In fact, *N. dentata* was collected in an area with a high solar radiation during summer time, and with snow and low temperatures in winter time. The above mentioned conditions indicate the existence of abiotic stress. These life conditions suggest that this species contains and produces compounds with stable antioxidant activity during the time, consisting of phenols, based on the results obtained in the present work. The observed antifungal activity of MEN could be related with the phenol content, especially with tannins and flavonoids, which were detected using qualitative methods. In summary, *N. dentata* opens possibilities for obtaining secondary metabolites, particularly phenols that could be applied as antifungal agents.

Acknowledgements

This work has been supported by the Vicerrectoría de Investigación y Desarrollo (Project Nr. 214.418.007-1.0) of the Universidad de Concepción. The autors thank Dr. Mrs. Alejandra Flores for her botanical identification and Mrs. Claudia Flores for her technical support.

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(Received for publication 8 November 2021)