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Seasonal variation in the antioxidant phytocompounds production from the *Morus nigra* leaves

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ABSTRACT

Morus nigra L. has demonstrated a wide range of biological properties potentially related to its antioxidant capacity. However, there are a few investigations on climate seasonal variation impacting on the phytocompounds yield. The monitoring of seasonal influences is a promising research area to optimize the specific production of metabolites. This study evaluated the seasonal phytochemical profile and antioxidant activity of the *M. nigra* leaves (MN), thereby correlating with climatic data during the period of two years. Furthermore, the cytotoxicity and the antioxidant effect of MN extract and its major compound, syringic acid (SA), against H_2O_2 were determined. The MN recorded diverse seasonal tendencies in their phytocompounds production, since the higher concentration of total phenolics was observed in the Summer/2016, flavonoids and carotenoids in the Spring/2014 and 2015, respectively, whilst the ascorbic acid was more abundant in the Autumn/2014. However, the Pearson's correlation revealed weak to moderate influence of the climate parameters on the phytoconstituents measured. Indeed, both DPPH-scavenging and β-carotene/linoleic acid co-oxidation assays demonstrated a high antioxidant activity of MN correlated to the polyphenolic index. The MN and SA did not present cytotoxicity, since L929 cells treated with different concentrations maintain the cell viability. Moreover, the doses of the MN (3–30 μg/mL) and SA (60–250 μg/mL) were able to reduce the mortality induced by H_2O_2 demonstrated in both, MTT and LDH assays. The leaves of MN could be an antioxidant source of potential use in food and supplements for either human or animal production due to its attractive biomass yield, palatability and nutritional value.

1. Introduction

Plants constantly need to develop approaches to deal with the environmental adversities leading to the adaptive strategies. In this way, it is necessary to have a set of metabolic adjustments, structural and phenodynamic changes to resist a determined environment knowing that the favorable season is never perennial (Agrawal and Fishbein, 2006). Some approaches come from the secondary metabolism, for example: (i) polyphenols such as anthocyanins, confer coloration to the flowers and fruits, therefore playing a fundamental role in attraction of pollinators and dispersers (Kong et al., 2003); (ii) alkaloids, cyanides and, tannins and other phenolics, serving to defend the plant, protecting against pathogens and herbivores (see Agrawal, 2007). However, other factors such as the soil, atmospheric pollution, environment which the plant is inserted – considering neighboring and/or opportunistic species, climatic differences, stress levels, water and light available (Gobbo-Neto and Lopes, 2007).

Moreover, phenolics have gained attention in the last years due to their antioxidant activity. This effect is mainly due to reducing properties, where its chemical characteristics play an important role in the neutralization and scavenger of free radicals, protecting the human body against oxidative stress retarding the progress of chronic diseases (Gourine et al., 2010a, 2010b). Some studies have already demonstrated that polyphenolic-rich plant extracts have beneficial pharmacological activities such as antidepressant, neuroprotective, antihyperglycemic and hypolipidemic effects (Zeni et al., 2011; Cruz et al., 2016; Sharma et al., 2015). Despite the phenolics have been widely used due to their positive impact on human health there are a few scientific studies regarding their seasonality influence in the production, mainly in leaves (Wu et al., 2016; Tálos-Nebehaj et al., 2017;

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Nascimento et al., 2014; Zeni et al., 2013; Sommavilla et al., 2012; Zhang et al., 2016). Therefore, the analysis of the seasonal impact on the metabolites might direct the potential best harvest to the assessment of phenolics, since each species has its highest antioxidant values in distinct seasons and, consequently, the harvesting time of the leaves should be carefully selected when considering the future use of the leaves (Tálos-Nebehaj et al., 2017).

Morus nigra L. (Moraceae) known as black mulberry is a tree distributed worldwide famous for silk production although has been an increasingly interest in its fruits due to its highest nutritional value (Gundogdu et al., 2011). In addition, it was one of the first domesticated forages around the world. In this century its utilization for animal production has been intensified because of its attractive biomass yields, palatability and high nutritional value (Sánchez, 2002; Patnaik, 2008). Furthermore, some studies indicated that *M. nigra* leaves is a rich source of phenolic compounds which provides a potential antioxidant activity (Araújo et al., 2015; Memon et al., 2010; Malhi et al., 2014; Sánchez-Salcedo et al., 2015). The chlorogenic and syringic acids were the majorities of phenolics constituents in *Morus nigra* leaves (Memon et al., 2010; Dalmagro et al., 2017). In previous studies, we demonstrated that *M. nigra* leaves extract (MN) have lipid-lowering effect and, antidepressant-like and neuroprotective effects accompanied of the oxidative stress decrement (Zeni et al., 2017; Dalmagro et al., 2017).

Notwithstanding, in the only seasonal study available involving *Morus nigra* stem, the anthocyanins and antioxidant activity were higher in October and lower in February in Switzerland (Syvacy and Sokmen, 2004). Therefore, the main purpose of this study was to evaluate the seasonal phytochemical profile and antioxidant activity of the MN extract, correlating with climatic data. As well as, the cytotoxicity and the antioxidant effect of MN extract and its major compound, syringic acid (SA), against H_2O_2 were assessed.

2. Materials and methods

2.1. Collection, preparation of plant material and extract from Morus nigra leaves (MN)

The leaves of *Morus nigra* L. harvested in the city of Blumenau (Santa Catarina State, Southern Brazil – latitude 26°54′10″S, longitude 49°04′44″W) starting in Spring 2013 through Autumn 2015. The specie was identified, taxonomically authenticated and a voucher specimen (nr. 42,265) deposited at the Regional University of Blumenau's herbarium, Santa Catarina, Brazil. A healthy tree was selected, harvested around 200 g of random fresh leaves exposed to sun and shade. The material collected was enough to enable phytochemical tests and to avoid an impact on the plant. The harvest was performed forty-five days after the start of the season, around 1 p.m. in order to assess the metabolic production in response to each characteristic climatic season.

The plant material dryed at 45 °C with forced ventilation, grinded, and stored at −10 °C. The MN extract was performed according to Dalmagro et al. (2017), using 100 mL of boiled distilled water at 100 °C resting for a time period of 15 min with 2 g of the plant material, named infusion.

2.2. Phytochemicals determination of MN

The concentration of total phenolics (TP) in the MN extract was measured using the Folin-Ciocalteau assay described by Singleton and Rossi (1965) reading at the absorbance of 725 nm. The TP content was calculated through a gallic acid curve ($y = 0.1893x - 0.1429$, $r^2 = 0.99$) and expressed as gallic acid equivalent (GAE) mg/g dry weight.

The total flavonoids (TF) quantification was performed by mixing the 300 mg of plant sample in 10 mL of methanol. After, used 0.5 ml of the extracted material with $AlCl_3$ (2%, w/v) and 2.5 mL ethanol. The absorbance was determined at 420 nm and the TF content was calculated with a standard curve of quercetin ($y = 0.1755x - 0.3139$, $r^2 = 0.99$). The results expressed as quercetin equivalent (QE) μg/g dry weight (Woisky and Salatino, 1998).

For the total carotenoids (TC) analysis, a hexane:acetone solution (1:1, v/v) containing 100 mg of butylhydroxytoluene (BHT) was added 300 mg of biomass sample. After this, the absorbance was determined at 450 nm and the quantification based on the absorption coefficient ($A_1^{1\%}{}_{cm}2300$, hexane – 450 nm). The results defined as β-carotene equivalent (β-caroteneE) mg/g dry weight (Britton et al., 1995).

The quantification of ascorbic acid carried out with 20 mL of MN extract titrated by potassium iodide solution (KIO_3 0.01 N). The titrations of ascorbic acid in the samples using the starch solution indicator (1%, w/v) and the results expressed as mg/100 g dry weight (Alexéiev, 1983; Rebollo et al., 2005). All tests were performed in triplicate.

2.3. Correlation between MN phytochemicals and climate data

The data referring to the minimum and maximum temperature, precipitation index (accumulated rainfall and rainy days) and frost incidence was requested to the Centro de Informações de Recursos Ambientais e de Hidrometeorologia de Santa Catarina (CIRAM/EPAGRI), corresponding to the harvest period of the samples (from Spring 2013 to Autumn 2015).

2.4. High-performance liquid chromatography (HPLC) profile of MN

Firstly, the infusion (Section 2.1) underwent a liquid-liquid extraction with ethyl acetate (1:3) during two hours, according to Zeni et al. (2013). The supernatant was concentrated using rotary evaporator at 40 °C and dissolved in 5 mL of methanol. A high-performance liquid chromatograph (Flexar™, PerkinElmer®, USA) was equipped with a PDA detector and a reversed-phase C18 column (Nucleodur® RP, Macherey-Nagel, 250 × 4.6 mm, Ø 5 μm). The detection was achieved at 200–400 nm and the samples were eluted in isocratic mode using water acidified with 0.5% acetic acid:methanol (70:30, v/v) (Zeni et al., 2017). The flow rate was 0.4 ml min^{-1}, the injection volume 10 μL and the duration of each run was 40 min. The analysis carried out in triplicates and all solvents were HPLC grade. Phenolic compounds, quercetin, rutin and syringic acid used as standards for identification were purchased from Sigma-Aldrich, Steinheim, Germany. Preliminary quantification was estimated by % area per peak.

2.5. Antioxidant capacity analysis

The antioxidant activity determined spectrophotometrically using the 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH – Sigma Chemical Co., St Louis, MO, USA). After, storage at room temperature during 30 min in the dark, the absorbance of the samples was determined at 515 nm (Brand-Williams et al., 1995). DPPH radical-scavenging activity was calculated according to the following equation:

$$\text{DPPH discoloration \%} = 1 - (A_{sample}/A_{blank}) \times 100;$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound.

$$\text{\% inhibition} = \{(Ca - Sa)/Ca\} \cdot 100$$

Where, *Ca* is control absorbance and *Sa* is sample absorbance.

Furthermore, the β-carotene/linoleic acid co-oxidation system was carried according to Marco (1968) and Miller (1971), which is based on inhibition of the cellular lipid compounds oxidation by presence of antioxidant molecules due to degradation of linoleic acid. The β-carotene (20 mg) was dissolved in 1 mL of chloroform and, 40 μL of linoleic acid and 20 mg of Tween 80 was added. The chloroform was evaporated in nitrogen gas and added 30 mL Milli-Q water. This emulsion