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The Open Microbiology Journal

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DOI: 10.2174/1874285801812010412, 2018, *12*, 412-418

SHORT COMMUNICATION

Bark of the Stem of *Libidibia Ferrea* Associated with Mycorrhizal Fungi: An Alternative to Produce High Levels of Phenolic Acids

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Received: September 13, 2018 Revised: December 09, 2018 Accepted: December 10, 2018

Abstract:

Background:

The use of microorganisms such as Arbuscular Mycorrhizal Fungi (AMF) may represent a sustainable biotechnological alternative for the cultivation of medicinal plants to facilitate plant growth, in addition to increasing the production of secondary compounds. These fungi are associated with *Libidibia ferrea*, a species which produces gallic and ellagic acid, compounds with preventive properties against cancer and diabetes complications.

Objective:

The objective of this paper was to verify whether the stem bark of *L. ferrea* concentrates higher amounts of gallic and ellagic acids when inoculated with *Claroideoglomus etunicatum*, *Gigaspora albida* and *Acaulospora longula*.

Methods:

The extractive methanolic solutions from the barks of *L. ferrea* were analyzed by RP-HPLC in order to establish the contents of gallic and ellagic acids.

Results:

The application of fungus *Claroideoglumus etunicatum* was more efficient at increasing the concentration of gallic acid (18%) and ellagic acid (45.2%) in the stem bark of *L. ferrea* in comparison to the control. In contrast, plants inoculated with *Acaulospora longula* benefited only with the increase in the amount of gallic acid if compared with the non-inoculated plants.

Conclusion:

The mycorrhizal technology may be an alternative to the cultivation of *L. ferrea* with higher concentrations of both gallic and ellagic

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acids in the stem bark, providing a promissory strategy to produce high quality herbal materials for the production of herbal medicines.

Keywords: Phenolic content, Mycorrhizal association, Glomeromycotina, *Libidibia ferrea*, Gallic acid, Ellagic acid.

1. INTRODUCTION

The World Health Organization (WHO) periodically establishes recommendations for the countries to formulate national policies and regulations for the use of herbal medicines which were proven efficient at treating diseases. In Brazil, in 2008, the National Program of Medicinal Plants and Herbal Medicines was created to assure the access of population to herbal medicines and medicinal plants in a safe and sustainable way for biodiversity [1]. However, the raw material used to manufacture herbal medicines is majorly obtained through extractivism. In addition, the plant can produce a low amount of some therapeutically important compounds. This scenario has developed the necessity to employ methods of cultivation of medicinal plants species with higher amount of phytochemicals to provide the herbal medicines industry with raw material [2].

Among the alternatives to increase the production of phytochemicals, we highlight the Arbuscular Mycorrhizal Fungi (AMF), microorganisms that are able to benefit from the nutritional status of the host, growth, protection against pathogens [3], and the increase in the concentration of specific compounds of the secondary metabolism [4]. In this context, Mandal *et al*. [5] registered that a higher expression of key genes of the metabolic routes and intensive density of glandular trichomes in plants associated with *Rhizophagus intraradices* justified the raise in the production of artemisinin in *Artemisia annua*.

Libidibia ferrea (Mart. ex Tul.) L. P. Queiroz var. *ferrea*, popularly known as "*pau-ferro*", was included in the National Listing of Medicinal Plants of interest of the *Sistema Único de Saúde* (SUS - Basic Health System) (RENISUS) in Brazil [6], due to ethnopharmacological studies [7] and pharmacological properties related, such as anti-inflammatory [8] and wound healing [9]. Phytochemical studies with *pau-ferro* ascertain the presence of gallic and ellagic acids, compounds which have anticancer activity and work against diabetes complications [10, 11]. Even though *pau-ferro* forms mycorrhizal association [12], no register is found on the influence of the AMF on the production of gallic and ellagic acids in the stem bark, one of the parts used by the population for medicinal purposes.

Gallic acid is a phenolic acid produced from shikimic acid and the ellagic acid formed by the dimer condensation of the gallic acid [13]. In addition to the therapeutic function, these phenolic compounds are used as patterns for the quantitative research of phenolic compounds [14] as well as in the industry of dyes and inks [13].

In this context, we carried out a field testing of the hypothesis that the mycorrhizal association may benefit the production of gallic and ellagic acids in the stem bark of *pau-ferro*. Therefore, the objective was to verify whether the stem bark of *pau-ferro* accumulated higher amount of these molecules upon inoculation with AMF.

2. MATERIALS AND METHODS

2.1. Field Experiment

The experimental field was set in February 2013 [15] at the University of Pernambuco - Campus of Petrolina, Brazil. The planted area was 2.400 m^2 and was divided in six blocks including the distribution of 96 plants. In the experimental area 20 soil sample (composite sample of each depth) were collected that were analysed for chemical characteristics: layer 0-20 (P 10.38 mg/dm^3; K 0.24 mg/dm^3; Ca 1.4 mg/dm^3; Mg 0.5 cmolc dm^{-3}; Na 0.03 cmolc dm^{-3}; Al 0.00 cmolc dm^{-3}; organic matter 0.41 g/kg; pH 6.2 H_2O (1:2,5); C.E 0.21 mS/cm) and layer 20-40 (P 9.61 mg/dm^3; K 0.14 mg/dm^3; Ca 0.7 mg/dm^3; Mg 0.3cmolc dm^{-3}; Na 0.01 cmolc dm^{-3}; Al 0.00 cmolc dm^{-3}; organic matter. 0.23 g/kg; pH 5.6 H_2O (1:2.5); C.E 0,21 mS/cm). Each block had four mycorrhizal treatments [control, *Claroideoglomus etunicatum* (W.N. Becker & Gerd.) C. Walker & A. Schüßler (UFPE 06), *Gigaspora albida* N. C. Schenck & G. S. Sm. (UFPE 01), and *Acaulospora longula* Spain & N. C. Schenck (UFPE 21)].

The seeds of *L. ferrea* were set to germinate in small cups (50 mL) filled with vermiculite with mean granulation followed by a 20-minute treatment with concentrated sulfuric acid to break the numbness [16]. The transference of seedlings to black polyethylene bags was carried out upon the presentation of four definitive leaves and was laid on the root or not, inoculum soil (containing 200 spores, hypha and colonized roots) of *A. longula, C.etunicatum e G. albida*. The bags contained 1.2 Kg of soil + 5% of vermicomposting (Chemical characteristics: P, 12.68 mg dm^{-3}; K, 0.26

$cmol_c dm^{-3}$; Ca, 2.7 $cmol_c dm^{-3}$; Mg, 1.8 $cmol_c dm^{-3}$; Na, 0.49 $cmol_c dm^{-3}$; Al, 0.05 $cmol_c dm^{-3}$; organic matter, 3.21 g kg^{-1}; e $pH_{H2O\ (1:2,5)}$ 5.2). The multiplication of FMA (obtained from UFPE) was observed in soil + 10% vermicompost, using *Panicum miliaceum* L. (host) andstored (-4ºC) for 22 months [17].

The seedlings (on avarage: 14 leaves, 5.1 mm of steam diameter, 72 cm of height) were transferred to the experimental field 225 days later, using experimental netting. The irrigation was been for semi-automatic dripe system 15 min on alternate days (8.4 L H_2O $plant^{-1}$ h^{-1}).

The stem bark was removed from the material collected from the pruning of the two central plants of each plot, 13 months after inoculation with the AMF. The plants inoculated with *C. etunicatum*, *G. albida, A. longula* and control had stem diameter of 2.97 cm, 3.32 cm, 3.2 cm and 2.46 cm, respectively [18]. Subsequently, the material was dried in an oven (Biopar, Porto Alegre, RS, Brazil) at 45ºC along three consecutive days, the contents of gallic and ellagic acids were established.

2.2. Quantification of Gallic Acid and Ellagic Acid

2.2.1. Sample Solution

We weighted 0.5 g of the stem bark samples, transferred to a round-bottom flask of 100 mL and added with 15 mL of distilled water. The solution was subjected to water reflux (85-90 ºC, during 30 min). Following, the flask was cooled and its content was transferred to a volumetric flask of 25 mL, filtered in cotton, and the volume was assessed with distilled water, constituting the Stock Solution (**SS**). From the **SS**, an aliquot of 5 mL was removed and transferred to a volumetric flask of 10 mL; subsequently, the volume was assessed with distilled water, constituting a sample solution (**SaS**). After this procedure, the **SaS** was filtered in a PVDF filter (25 mm; 0.45 µm) for the vials.

2.2.2. Standard Solutions

Gallic acid (98%, Sigma-Aldrich®): 1.0 mg of gallic acid was dissolved with water in a volumetric flask of 10 mL (100 µg/mL) followed by the performance of the required dilutions in order to obtain the calibration form.

Ellagic acid (96%, SigmaAldrich®): 1.0 mg of ellagic acid was dissolved with methanol:water (3:1) in a volumetric flask of 10 mL (100 µg/mL) followed by the performance of the required dilutions in order to obtain the calibration curve.

2.2.3. Chromatographic Analysis

The analysis was conducted using a liquid chromatograph (Ultimate 3000, Dionex®) with a detector of photodiode arrays (wavelength of 254 and 280 nm); column C_{18} (250 mm x 4.6 mm, 5 µm; Dionex®) protected by a security guard C_{18} (3.9 µm x 4.6 mm, 5 µm, Phenomenex®), maintained under room temperature (24°C); and the flow of the mobile phase of 0.8 mL/minute. The volume of injection was 20 µL.

The mobile phase was constituted of purified water (Purelab Classic UV, Elga®) as solvent A and methanol as solvent B both acidified with trifluoroacetic acid at 0.05%, and degassed in ultrasonic bath (Unique®) and filtered through the membrane of PVDF (47 mm; 0.45 µm). The separation was conducted through linear gradient: 0-10 min, 20-22.5% B; 10-20 min, 22.5-40% B; 20-25 min, 40-75% B; 25-28 min, 75-20% B; 28-32 min, 20% B. The results were expressed in g % of gallic acid and ellagic acid.

2.3. Statistical Analysis

The data were subjected to ANOVA and the measures compared using Tukey test (5%) through Assistat 7.7 [19].

3. RESULTS AND DISCUSSION

The inoculation with AMF benefited the accumulation of gallic and ellagic acids in the stem bark (Fig. **1**). Plants inoculated with *C. etunicatum* had an increase in the concentrations of gallic acid and ellagic acid of 18% and 45.2%, respectively, in relation to the control (Table **1**). In contrast, fungus *A. longula* provided an increase of 22% in the concentration of gallic acid in comparison with the control (Table **1**). The inoculation with *Gigaspora albida* did not bring additional benefits to the concentration of these phenolic acids in relation to the control plants (Table **1**). Similar results were observed in the aerial part of *Hypericum perforatum* L., which was inoculated with different species of AMF [4]. The authors verified that the fungi influenced the increase in the concentration of hypericin and

pseudohypericin in relation to the control. Benefits of the application of AMF on the production of bioactive compounds were also evidenced in other legumes [15, 20 - 22].

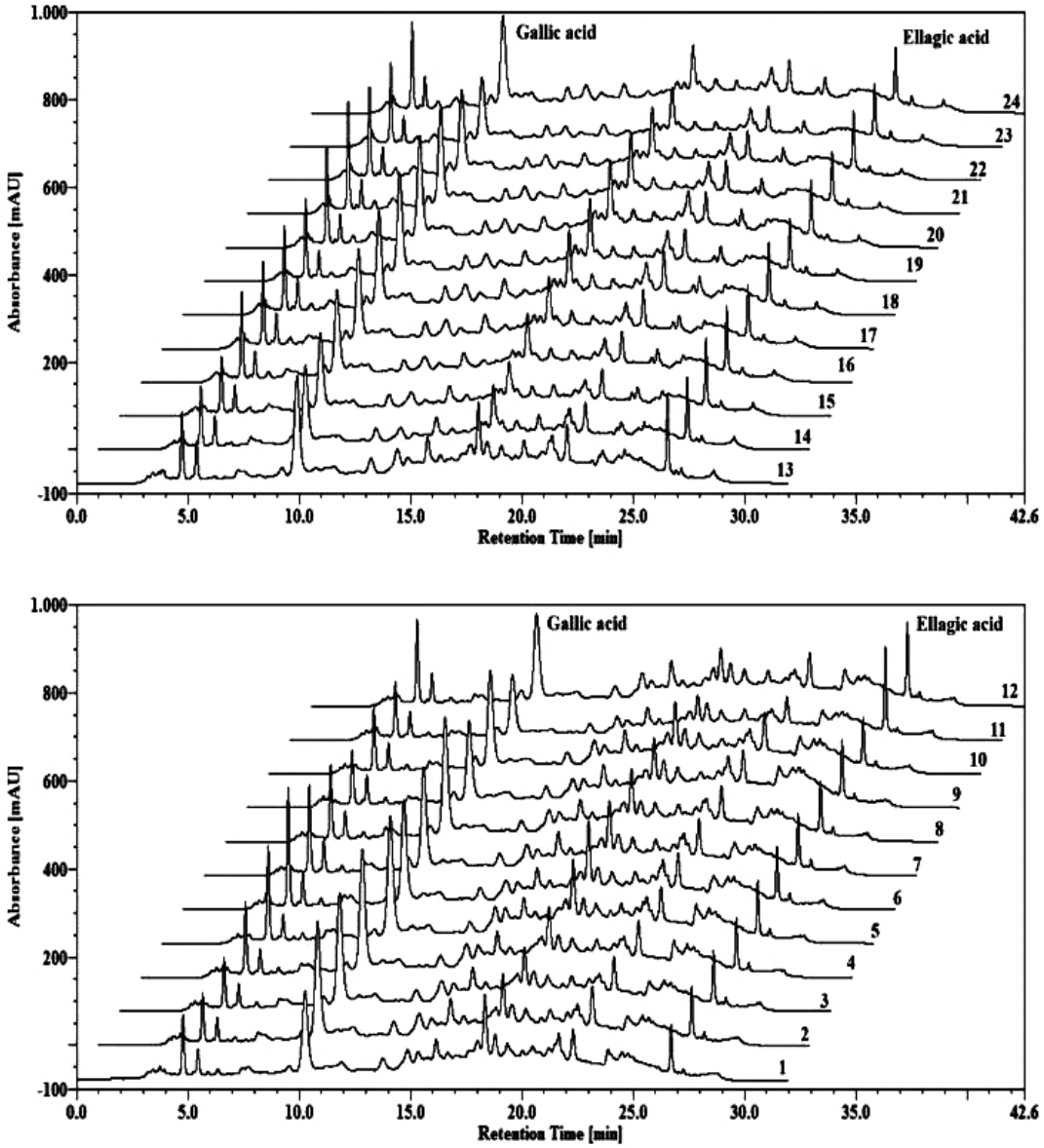

Fig. (1). Chromatographic profile of samples from stem bark of *L. ferrea*. 1- 6 (control without AMF); 7-12 (inoculated wih *Acaulospora longula*); 13-18 (inoculated with *Claroideoglomus etunicatum*); 19-24 (inoculated with *Gigaspora albida*).

Table 1. Concentrations of gallic and ellagic acids (g %) in the stem bark of *Libidibia ferrea*, inoculated or no with arbuscular mycorrhizal fungi, and cultivated in the field 13 months after transplanting, Petrolina, Brazil.

Variable	Inoculation treatment			
	Control	*Acaulospora longula*	*Claroideoglomus etunicatum*	*Gigaspora albida*
Gallic acid	0.050 ± 0.000 b	0.061 ± 0.001 a	0.059 ± 0.002 a	0.056 ± 0.000 ab
Ellagic acid	0.0104 ± 0.000 b	0.0127 ± 0.001 ab	0.0151 ± 0.001 a	0.0122 ± 0.000 ab

Averages (n= 6) followed by the same letter on the line do not differ by the Tukey test ($P < 0.05$).

In plants inoculated with *A. longula*, the fact of the gallic acid is the precursor of the ellagic acid did not imply a direct relationship of the increase in the concentration of the latter in detriment of the former when compared with the non-inoculated control (Table **1**). This demonstrates the necessity of conducting studies with molecules that have a relation in the biosynthetic route, such as gallic and ellagic acids, in plants associated with AMF. A similar result was

verified by Silva *et al*. [15] in leaves of mycorrhizal *pau-ferro* established in field for seven months. Plants inoculated with *C. etunicatum* revealed an increase of 21% in the concentration of gallic acid, without difference in the concentration of total phenols and total tannins in relation to the control.

The interest of the pharmaceutical industry in the secondary compounds produced by medicinal plants has encouraged studies involving AMF seeking an increase in the concentration of these molecules, especially specific ones, such as recorded in this study (Table **1**). There is a register of higher concentration of anticancer alkaloids vinblastine and vincristine in *Catharanthus roseus* in response to the inoculation with *G. mosseae* [23]; increased production of caffeic acid, an antioxidant compound, on the aerial part of *Ocimum basilicum* L. associated with fungi *Glomus caledonium* and *Glomus mosseae* was recorded by Toussaint *et al*. [24], finally, a higher concentration of artemisinin, an antimalarial compound, in the species *Artemisia annua* inoculated with *Rhizophagus intraradices* had also been documented by Mandal *et al*. [5].

Vasconcelos *et al*. [25] quantified, using HPLC, 112.76 mg gallic acid/g stem bark and 12.00 mg ellagic acid/g stem bark of *L. ferrea*. The data obtained in this paper reveal the highest concentrations of gallic acid as 59 and 61 mg/g of stem bark, induced by fungi *C. etunicatum* and *A. longula*, respectively. In contrast, the production of ellagic acid was 15.1 mg of ellagic acid/g of stem bark when inoculated with *C. etunicatum*. Based on these data, the use of the stem bark of *pau-ferro* of mycorrhizal plants with *C. etunicatum* (three-month cultivation) may be more profitable for the herbal medicines industry, considering that a 25% increase will occur in the contents of ellagic acid. Similarly, the use of a technology employing *C. etunicatum* would be profitable to the chemical industry that commercializes 1 g of ellagic acid from the biological source (powder of the stem bark of the tree) for R$ 838.00 [26], that is, it would be extracted 25% more ellagic acid from the same amount of plant material.

CONCLUSION

A technology applying *C. etunicatum* may be an alternative to increase the production of gallic and ellagic acids in the stem bark of *L. ferrea* by using a sustainable system which may encourage the establishment of plantations, contributing to reduce the extractive use of this legume.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise

ACKNOWLEDGMENTS

The authors would like to thank the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq – National Council for Scientific and Technological Development) for the financial support (308386/2015-9) and for having granted the Research Productivity Scholarships to FSB Silva (307749/2015-0) and LA Lira, and finally to the *Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco* (FACEPE – Foundation of Support to Science and Technology of the State of Pernambuco) (APQ-0493-4.03/14).

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