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Full Length Research Paper

Antimicrobial properties of *Sacrocephalus latifolius* (Sm.) Bruce

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***Sacrocephalus latifolius* (Sm.) Bruce (Rubiaceae) formerly known as *Nauclea latifolia* (Sm.), is a highly valued medicinal plant used in ethnomedicine in the treatment of various infectious diseases due to its insecticidal and antiparasitic properties. The proximate, mineral composition and antimicrobial properties of the leaves were analyzed with a view to evaluate its nutritional and medicinal potentials. The proximate analysis revealed that the moisture content, ash, lipid, crude fibre, crude protein and the carbohydrate content of the leaves were 56.36 ± 0.50, 5.93 ± 0.2, 0.28 ± 0.50, 4.48 ± 0.20, 6.55 ± 0.10 and 3.21 ± 0.30%, respectively. Mineral compositions of the leaves showed that the vitamins content, potassium, calcium, iron and zinc content of the leaves were 3.41 + 0.30, 1.52 + 0.20, 2.28 + 0.30, 3.51 + 0.20 and 2.71 + 0.20%, respectively. The phytochemical screening revealed the presence of glycosides, alkaloids, flavonoids, tannins, triterpenoids and saponins in the methanolic extracts of the sample. Aqueous, choloroform and methanol extracts exhibited broad spectrum activity against *Staphyococcus aureus* and *Escherichia coli* by using disc diffusion method. The results indicate that *S. latifolius* contains substantial amounts of organic constituents and minerals, suggesting its potential for nutrient composition. As such, the results of phytochemical screening could justify observed antimicrobial herbal medicine.**

Key words: *Sacrocephalus latifolius,* nutrients, ethnomedicine, Antimicrobial, phytochemicals.

INTRODUCTION

Medicinal plants are valuable and renewable sources of active substances in pharmacology. Approximately 20% of the plants in the world have been subjected to pharmacological and/biological evaluation and as such substantial number of new antibiotics are being introduced into the market obtained from natural or semi synthetic sources (Mothana and Lindequist, 2005; Aliyu et al., 2011). Many species in the genus *Sacrocephalus* have been reported to have extensive history in traditional medicine system, widely used for the treatment of malaria,

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of malaria, hypertension, diarrhea, tuberculosis, dysentery and also as a laxative (Benoit-Vicalet.al., 1998). A number of researchers have reported the anti-bacterial (Fakae et-al., 2001) and antidiabetic properties of the plant. Phytochemical analysis revealed that the *Sacrocephalus latifolius*. contain alkaloids saponins, polyhenols and tannins (Tona et al., 2000; Boakye-Yiadom et al., 1977).

S. latifolius (Sm.) Bruce formerly known as *Nauclea latifolia* is a savanna shrub sometimes found in undisturbed fringing forest and closed savanna woodland. It belongs to the family Rubiaceae commonly called pin cushion tree, locally known as 'Egbo egbesi in Yoruba, 'Ubalu inu in Igbo and 'Tabasiya' in Hausa in Nigeria. It has many branches varying up to a height of 12 m with an open canopy. Flowers possess terminal spherical head like cymes of small whitish Flowers (Benoit-Vical et al., 1998). The flowers are found conjoined with their calyces. The fruit is syncarp and ripens during July to September. However, the baboons eat the fruits and disperse the seeds. It is further reported that livestock eat shoots and the leaves (Deeni and Hussain, 1991).

In West and Central Africa, *S. latifolius* is prominent for its insecticidal and antiparasitic properties. In Gabon, Congo and Nigeria, infusions of the leaves and bark are used to cure fevers (Di Giorgio et al., 2006). In Kinshasa, DR Congo extracts and preparations, along with other plants, are administered for diagnosing diarrhea (Tona et al., 2000). *S. latifolius* is used in medicine and as food, thus serving the dual purpose of food and medicine (Etkin, 1996). This study evaluates the nutritional and medicinal properties of *S. latifolius* since it serves both as food and medicine.

MATERIALS AND METHODS

Plant

Fresh leaves of *S. latifolius* were collected in bushes located around, Abuja, Nigeria. The plant was authenticated at the Herbarium unit of the Department of Biological Sciences, University of Abuja, Abuja, Nigeria. The leaves were air-dried at room temperature and grounded to fine powder, using a laboratory mill and stored in air-tight containers for laboratory analysis. All analysis was carried out in triplicates.

Proximate and mineral analysis

The proximate analysis of *S. latifolius* powdered leaf sample was tested to obtain values for the moisture content, crude fat, crude protein, crude fibre, ash and carbohydrates following the procedures as described by official methods of Analysis of Official Analytical chemists (AOAC, 1995). The moisture content was determined by air oven drying that exhibited a weight difference at 130°C for 1 h. The crude protein content by micro Kjeldahl method is given as 1% total nitrogen × 6.25 (AOAC, 1990). The crude fibre content was determined using dilute acid and alkali hydrolysis. Crude fat was extracted by exhaustively extracting 10 g of each sample in a soxhlet apparatus using dimethyl ether (boiling range 30 to 60°C) as the solvent. Ash was also determined by the incineration of 10 g of each sample placed in a muffle furnace maintained at 550°C for 5 h. The total carbohydrates content (on dry weight basis) was calculated by the difference: 100 - (crude protein + crude fat + ash + crude fiber).

The mineral constituents and vitamin analysis

The ash solutions were prepared by weighing 5 g of each of the powdered samples and dried at 550°C in muffle furnace for about 5 h and the resultant residues were dissolved in 100 ml of deionized water. A standard solution of the minerals (potassium, calcium, iron and zinc) to be analyzed were prepared. The atomic absorption spectrophotometer (model 200 – A, Buck Scientific) was set with power on for ten minutes to stabilize and the standard mineral solutions were injected to calibrate the atomic absorption spectrophotometer (AAS) using acetylene gas. Finally, an aliquot of ash solutions were injected and the concentrations obtained from the AAS.

Vitamin analysis

Vitamin C in the sample was determined as follows; 100 g of the fresh leaves of *S. latifolius* were cut into small pieces and grounded with a mortar and pestle. 10 cm^3 of distilled water was added several times and decanted into a 100 ml. volumetric flask. The grounded leaves was strained through a cheese cloth, filtrate were collected and washed in volumetric flask. The extracted solution was made up to 100 ml with distilled water. 20 ml aliquots of the sample solution were pipette into a 250 ml conical flask, 150 ml distilled water and 1 ml starch solution was added. The sample was titrated with 0.005 m iodine solution. The end point of the titration was identified as the first permanent trace of a dark blue colour due to the starch iodine complex. The titrations were repeated two times with further aliquot of sample solution, with a constant result obtained (Adeboye, 2008).

Phytochemical analysis

Extraction of plant material: In order to extract plant material, an equal amount of aqueous, chloroform and methanol were used as solvents for the extraction of bioactive compounds. The extracts so obtained were completely evaporated by using vacuum rotary evaporator. The resultant concentrated extracts were used for antibacterial activity.

Phytochemical screening: The phytochemical properties of the dried powdered plant leaf parts were determined using standard methods described by Harborne (1993) and Nabavi et al. (2008). The leaf extract was screened for glycoside, anthraqiunones alkaloids, tannins, terpenoids, flavonoids and saponins.

Test microorganisms: The test organisms used to detect the presence of antibacterial assay are *Staphylococcus aureus* and *Escherichia coli*. Cultures of these bacteria were maintained on the slopes of the nutrient agar at 37°C which were collected from Microbiology Laboratory of University of Abuja Teaching Hospital,

Table 1. Proximate composition of *S. latifolius*.

Organic composition	Value (%) ± SD
Moisture	56.36 ± 0.50
Ash	5.93 ± 0.20
Crude fat	0.28 ± 0.50
Crude fibre	4.48 ± 0.20
Crude protein	6.55 ± 0.10
Carbohydrate	3.21 ± 0.30

Table 2. Mineral composition of the leaves.

Mineral composition	Value (%) ± SD
Vitamin C	3.41±0.30
Potassium	1.52±0.20
Calcium	2.28±0.30
Iron	3.51±0.20
Zinc	2.71±0.20

Gwagwalada, Abuja, FCT. Nigeria.

Antibacterial assay

The agar well diffusion technique as described by Jombo and Enebebeaku (2007) was employed for the determination of the zone of inhibition of the extract. The minimum inhibiting concentration (MIC) was determined after Baron and Finegold (1990) while the minimum bactericidal concentration was determined as described by Jombo and Enebebeaku (2007). The same procedure was followed to know the frequency of occurrence of standard antibiotics ampiclox for *Staphylococcus aureus* while chloramphenical was used for *Escherichia coli* to know the zone of inhibition as controls.

RESULTS AND DISCUSSION

Table 1 shows the result of the proximate analysis of *S. latifolius.* The concentration of organic compounds analyzed followed a sequential order of moisture > protein > ash > fibre > carbohydrate > fat. In *Sacrocephalus* species, the moisture content of the leaves (56.30%) is higher than the reported value of *Phyllanthus pentandrus* (Aliero and Shehu, 2010). A high moisture content of *Sacrocephalus* indicate that the plant is susceptible to microbial attacks during storage. The low ash content of 5.93% reflects low mineral and high organic content which are comparable to those recorded by Dike (2010). A very low content of fat (0.28%) was found similar when compared with the leaves of some higher plants by Dike (2010). However, the low fat content of the leaves of *Sacrocephalus* revealed that this plant is not an oil species and as such cannot be used either in industrial or domestic purposes, for industrial or domestic purposes. The fibre content of *S. latifolius* leaves was found to be 4.48% which validates the findings of Ujowundu et al. (2010) who reported the presence of fibre content in the leaves of higher plants. It is revealed that the protein content (6.55%) was found to be lower than the values of *Vernonia amygdalina* (Dike, 2006) carbohydrates content. However, the value of *S. latifolius* was 3.21% similar to those recorded for some leafy vegetables (Mensah et al., 2008).

The results showing presence of mineral composition are presented in Table 2. The presence of major elements like potassium, calcium, iron and zinc in the leaves correlates with the reports of Gafar et al. (2011). Potassium along with sodium is responsible for maintaining proper acid-base balance and nerve transmissions in the body (Akinyeye et al., 2010). Calcium plays an important role in building strong and healthy bones and teeth throughout life (Ojo and Ajayi, 2009). Iron helps in the formation of hemoglobin and myoglobin (Gafar et al., 2011). Zinc speeds up the healing process after injury and vitamin C content was relatively high when compared to lettuce (USDA, 2005). However, the deficiency in vitamin C leads to the disease scurvy and the primary cause of vitamin C deficiency is a poor diet (king, 2008). Thus the vegetable leaves can be used as a supplement for vitamin C.

Table 3 shows the qualitative phytochemical analysis of leaves of *S. latifolius.* The results showed that the aqueous leaf extract of S. *latifolius* contains tannins, terpenoids, flavonoids and saponins. The chloroform leaf extract contains glycoside, tannin, terpenoids flavonoid and saponins. The methanolic leaf extract contains glycoside, alkaloids, tannins terpenoids, falvonoids and saponins.

The antimicrobial activities of higher plants are attributed to the presence of alkanoids and flavonoids (Cordell et al., 2001). The role of tannins and other compounds of phenolic nature as active antimicrobial compounds has been reported (Rojas et al., 1992). The presence of these phytochemicals could to some extent justify the observed antimicrobial activities exhibited by the extracts in the present study. The result of antibacterial assay of aqueous, chloroform and methanol extracts are presented in the Table 4. The extracts of *S. latifolius* exhibited varying activities against *S. aureus* and *E. coli* as evidenced by its zone of inhibition when compared with the standard antibiotics of ampliclox for *S. aureus* and choramphenical of *E. coli.*

The highest zone of inhibition was observed at 200 mg/ml against *S. aureus* (18.0 mm) with methanol extract, while *E. coli* exhibited highest zone of inhibition at 21.0 mm with methanol extract. Aqueous extract has the least inhibition for both organisms at all concentrations.

Table 3. Phytochemical compositions of leaves of *S. latifolius*.

Phytochemical composition	Aqueous	Chloroform	Methanol
Glycoside	-	+	-
Anthraquinones	-	-	-
Alkaloids	-	-	+
Tannins	+	+	+
Terpenoids	+	+	+
Flavonoids	+	+	+
Saponins	+	+	+

Table 4. Zone of inhibition of the aqueous choroform and methanolic leaf extract of *S. latifolius* against the microorganisms.

Test organism	Conc. (mg/ml)	Mean diameter of zones of inhibition (mm)			
		Aqueous	Chloroform	Methanol	Control
	200	11±0.6	14±1.4	18±1.3	22±2.4
Staphylococcus aureus	100	10±1.5	12±1.3	15±1.5	19±3.6
	50	9±1.4	11±1.6	13±1.4	16±2.1
	200	13±1.3	16±1.3	21±2.8	24±2.5
Escherichia coli	100	11±1.8	15±1.2	19±1.5	22±3.3
	50	9±0.5	12±2.2	14±1.7	18±2.7

For Control: Ampliclox was used for *Staphylococcus aureus* while choramphenicol was used for *E. coli.*

Table 5. Minimum inhibitory concentration (MIC), minimum bacterial concentration (MBC) of *S. latifolius* leaf extracts of the micro-organisms (mg/ml).

Microorganism	Solvent	MIC	MBC
	Aqueous	100	200
Staphylococcus aureus	Choroform	25	50
	Methanol	12.5	25
	Aqueous	50	100
Escherichia Coli	Choroform	12.5	25
	Methanol	6.3	12.5

Although, gram-negative bacteria tend to have higher intrinsic resistance to most antimicrobial agents (Ndukwe et al., 2005). However, there is impressive activity against this gram negative bacterium in gastrointestinal and urinary tract of the human body. The susceptibility of *E. coil* to the extracts is an indication to the therapeutic potentials of these extracts against such disease in Table 5. The growth of *S. aureus* was inhibited by the extracts with greatest inhibition in methanol. *S. aureus* is a human pathogen whose infection is difficult to treat with conventional antibiotics. In animals, *S. aureus* is encapsulated by fibrotic tissue and forms microabsceses to protect it from the usual antibiotics (Giesecke et al., 1994). Therefore, the activity of *S. latifolius* against *S. aureus* may prove to be a worthy alternative in search of new antibiotics.

The phyotochemical screening of *S. latifolius* extracts shows the presence of glycoside, alkanoids, tannins, terpenoid, flavonoids and saponins in Table 3. Differences in antimicrobial activity of medicinal plants have been compared with the contents of active compound (Boakye-Tiadom and Konning, 1975). However, the antimicrobial activities of higher plants are attributed to the presence of alkaloids and flavonoids (Cordell et al., 2001). The role of tannins and other compounds of phenolic nature as active antimicrobial compound has been reported (Rojas et al., 1992). As such, presence of these phytochemicals could therefore justify the observed antimicrobial activities exhibited by the extracts of *S. latifolius*.

However, it was concluded from the present study that *S. latifoliu* contain substantial amounts of organic substances and minerals, representing the prospective nutrient composition. The result of phytochemical screening justified the antimicrobial activities and strongly confirm its use in herbal medicine for the treatment of bacterial infection

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Conflict of Interest

Authors have not declared any conflict of interest.

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Full Length Research Paper

Arbuscular mycorrhizal fungi increase gallic acid production in leaves of field grown *Libidibia ferrea* (Mart. *ex* Tul.) L. P. Queiroz

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Because arbuscular mycorrhizal fungi (AMF) has been shown to induce concentration increases of pharmaceutically useful phytochemicals in some Brazilian semi-arid native plants, the current study examined whether mycorrhizal inoculation increased the production of bioactive compounds, especially gallic acid, in field grown *Libidibia ferrea* (Mart. ex Tul.) L. P. Queiroz plants. Seedlings were inoculated with *Claroideoglomus etunicatum* (W.N. Becker & Gerd.) C. Walker & A. Schüßler*, Acaulospora longula* Spain & N. C. Schenck*, Gigaspora albida* N. C. Schenck & G. S. Sm. or non-inoculated (control) and, seven months after transplanting, examined for growth parameters, chlorophyll, phenols, tannins and gallic acid concentrations, mycorrhizal colonization and rhizosphere AMF spore density. Plants inoculated with *C. etunicatum* had 21% higher gallic acid concentrations than control plants while those inoculated with *G. albida* had higher total chlorophyll concentrations. Mycorrhizal technology employing *C. etunicatum* can constitute an alternative to increase gallic acid production in field grown *L. ferrea* plants.

Key words: Glomeromycota, secondary compounds, ironwood, *pau ferro*, Caatinga, semi-arid.

INTRODUCTION

Arbuscular mycorrhizal fungi (AMF), which belong to the phylum Glomeromycota, associate in a symbiotic way to the majority of plant species. The association benefits the plants because the AMF increase nutrient absorption in poor soils, in particular phosphorus (Yao et al., 2008), and alleviate the stress caused by biotic and abiotic factors (Smith and Read, 2008). AMF has been shown to stimulate growth of seedlings of economically important species (Tristão et al., 2006), including medicinal species native of the Brazilian semi-arid region (Oliveira et al*.,* 2013), which can accumulate high concentrations of primary and secondary metabolites (Mandal et al*.,* 2013; Pedone-Bonfim et al*.,* 2013).

Mycorrhizal biotechnology can constitute an alternative to increase production of secondary compounds in some species: essential oils in *Anethum graveolens* L., *Ocimum basilicum* L. and *Salvia officinalis* L. (Copetta et al., 2006; Geneva et al., 2010; Kapoor et al., 2002), caffeic acid in *O. basilicum* (Toussaint et al., 2007); artemisinin in *Artemisia annua* L. (Chaudary et al., 2008); and alkaloids in *Castanospermum australe* A. cunn. & C. Fraser and *Catharanthus roseus* L.G. Don (Abu-Zeyad et al., 1999; Ratti et al., 2010). However, there are no reports on AMF effect on gallic acid concentrations, and this is an important compound that belongs to the group of phenols and possesses antibacterial and antioxidant properties (Broinizi et al., 2007; Chanwitheesuk et al*.,* 2007).

In the Brazilian semi-arid region, inhabitants benefit from various medicinal plant species with therapeutic properties (Agra et al., 2007). Among these, *Libidibia ferrea* (Mart. ex Tul.) L. P. Queiroz (*pau ferro* or ironwood), a species of the Fabaceae family, which has proven to be mycotrophic (Gattai et al., 2011), possesses therapeutic characteristics with antiulcerogenic, anti-inflammatory, analgesic, hypoglycemic, anti-cancerogenic, anti-histaminic, antimicrobial, anticoagulant, larvicidal against *Aedes aegypti*, and cicatrizing activities (Bacchi et al., 1995; Carvalho et al., 1996; Cavalheiro et al., 2009; Gonzalez, 2005; Oliveira et al., 2010; Sampaio et al., 2009). Such characteristics are related to the presence of secondary compounds (Gonzalez, 2005; Nozaki et al., 2007; Souza et al., 2006; Ueda et al., 2001), especially gallic acid (Nakamura et al., 2002). Therefore, alternatives to increase the production of gallic acid and other compounds in ironwood leaves are of interest to the phytotherapeutic industry.

There are no registers of cultivation of mycorrhized ironwood plants under field conditions with the objective of increasing the production of secondary compounds, especially gallic acid. Therefore, in this study hypothesis that inoculation of seedlings established in the field with AMF increases the growth of the plants and the production of gallic acid were tested, but these increases vary according to the AMF species used. The objective of this study was to determine whether mycorrhizal inoculation increases plant growth and production of gallic acid in mycorrhizal ironwood plants established in the field.

MATERIALS AND METHODS

Experimental field

An experiment was set up at the Experimental Field of the University of Pernambuco at Petrolina Campus, Northeast Brazil. The region has a climate of the Bswh type, according to the Köppen classification. During the period, the experiment was carried out (February to September, 2013). Averages of the maximum and minimum temperature were 33.2 and 21.7°C, respectively, relative humidity was 55.2% and total rainfall was 9.5 mm. The experiment consisted of four treatments, in a random block design, with six replicates: inoculation of *L. ferrea* seedlings with the arbuscular mycorrhizal fungi *Claroideoglomus etunicatum* (W.N. Becker & Gerd.) C. Walker & A. Schüßler (UFPE 06), *Gigaspora albida* N. C. Schenck & G. S. Sm. (UFPE 01) or *Acaulospora longula* Spain & N. C. Schenck (UFPE 21) and a non-inoculated control.

Ironwood seeds were collected from healthy plants in the Caatinga region of Juazeiro municipality, Bahia State. The seeds were treated with concentrated sulfuric acid for 20 min to break dormancy (Biruel et al., 2007), washed in running and distilled water, and were placed to germinate in 50 ml recipients containing vermiculite (medium granulation). When the plantlets had four definite leaves, they were transferred to polyethylene bags containing 1.2 kg of substrate composed of soil (not sterilized) + 5% vermicompost and inoculate or not with soil-inoculate containing 200 spores of each AMF.

The soil was collected from a native Caatinga area in a perpendicular path starting in KM 152 of the BR 428 road that departs from Petrolina. The collected soil had a density of 96 glomerospores in 100 g of soil and the presence of the following AMF species: *Scutellospora* species 1, *Glomus macrocarpum* Tul. & Tul., *Ambispora appendicula* (Spain, Sieverd. & N.C. Schenck) C. Walker, *Acaulospora* species 1, *Glomus* species 1 and *Acaulospora scrobiculata* Trappe (Lima, 2014). The soil that was mixed with vermicompost had the following 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}; and $pH_{H2O\ (1:2,5)}$ 5.2.

The inocula of *C. etunicatum*, *G. albida* and *A. longula* were obtained from the UFPE, multiplied in soil + 10% vermicompost, using millet (*Panicum miliaceum* L.) as host and stored for 22 months at 4°C until the moment of use.

After 225 days under protected roofing, the seedlings were transplanted to the experimental field. On this occasion, they had, on average, 72 cm of height, 5.1 mm of stem diameter, 14 leaves, and a foliar concentration of total chlorophyll of 50.07 FCI (Falker Chlorophyll Index). In the field, the seedlings were planted in holes of 0.4 × 0.4 × 0.4 m, with a spacing of 5 m of one to the next, in rows with four plants, with a distance of 5 m between the rows, with four rows per experimental plot, resulting in a density equivalent to 400 plants ha^{-1}. Since each of the four treatments was replicated in six plots and each plot had 4 plants, the total number of transplanted seedlings was 96.

Before transplanting, 10 points were marked zigzagging in the experimental area, from which soil samples were collected, in the layers from 0 to 20 and 20 to 40 cm depth. The samples were mixed to form two composite samples that were analyzed for their

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Table 1. Chemical characteristics of the soil of the experimental area at depths of 0 to 20 and 20 to 40 cm.

Soil depth	P (mg dm^{-3})	cmol$_c$ dm^{-3}					O.M. (g kg^{-1})	pH (H_2O[1:2,5])	C.E. (mS cm^{-1})
		K	Ca	Mg	Na	Al			
0-20	10.38	0.24	1.4	0.5	0.03	0.00	0.41	6.2	0.21
20-40	9.61	0.14	0.7	0.3	0.01	0.00	0.23	5.6	0.21

O.M.: Organic matter; E.C.: electric conductivity.

chemical characteristics (Table 1). The average density of the AMF spores in the samples was 54 spores per 100 g of soil. The area was plowed, disked and holes were made in each of which 5 L of vermicompost and 150 g of simple superphosphate were applied. The vermicompost had the following characteristics: P, 4.8 g kg^{-1}; C, 279 g kg^{-1}; N, 32.3 g kg^{-1}; K, 0.8 g kg^{-1}; Ca, 34.2 g kg^{-1}; pH$_{(H2O-1:2,5)}$ 6.65**;** Mg, 4.14g kg^{-1}; Cu, 36 mg kg^{-1}; and Mn, 823 mg kg^{-1}.

After transplanting, the area was irrigated with a semi-automatic drip system for 15 min on alternate days (8.4 L plant^{-1} h^{-1}). The plants received daily care such as weeding and manual picking of herbivorous insects until evaluations were done seven months after transplanting. The following characteristics were evaluated: height, stem diameter, number of leaves, concentrations of chlorophyll, phenols, tannins and gallic acid in the leaves; number of glomerospores in the soil; and proportion of mycorrhizal colonization in the roots. Measurements were taken from the two central plants of each plot.

Phytochemicals analysis

Chlorophyll concentrations were measured with a clorofiLOG CFL1030 electronic meter. The total phenols and tannins were quantified by the Fiolin-Ciocalteu method and that of casein precipitation, respectively (Monteiro et al., 2006), using a foliar ethanol extract obtained by maceration (Oliveira et al., 2013).

Quantitative analysis by high performance liquid chromatography-photodiode array (HPLC-PDA)

Sample preparation

The herbal drug of leaves from *L. ferrea* was prepared by reflux in water bath (85 to 90°C) and using water as solvent. The extraction was performed in the round-bottom flask containing 0.5 g of drug and 15 ml of water by 30 min. The extractive was cooled to room temperature (25°C), filtered through cotton and transferred to 25 ml volumetric flask and the volume was adjusted with the same solvent. An aliquot of 5 ml of that solution was diluted in a 10 ml volumetric flask and the volume was completed with distilled water. The samples were filtered through membrane of polyvinylidene fluoride (PVDF) with a diameter of 25 mm and a pore size of 0.45 µm and transferred to vials.

Experimental conditions

The analyses was performed in HPLC-PDA (Ultimate 3000, Dionex®). Optimum separation was achieved with a column Dionex® (C_{18}, 250 × 4.6 mm, 5 µm) and pre-column (C_{18}; 4 mm; 3.9 µm). The mobile phase consisted of phase A: water ultrapure and phase B: metanol HPLC grade, both acidified with trifluoroacetic acid (0.05 %) and degasified in an ultrasound bath (Ultracleaner®). The phases were filtered through a membrane with 0.45 µm pore size before use. A gradient elution was carried out as follows: 0 to 10 min, 25% phase B; 10 to 11 min, 40% phase B and 11 to 12 min, 25% phase B; and at flow rate of 0.8 ml/min. The sample injection volume was 20 µl and the column was kept under controlled room temperature (24°C). The eluent was monitored by a PDA, and the detection wavelength was set at 280 nm. Data were analyzed using Chromeleon® software. A gallic acid standard solution was used as reference (>96%, Sigma Aldrich®). The gallic acid contents were expressed in gram percent, calculated in accordance to a standard calibration curve.

AMF analysis

The rizospheric soil was collected from the surface layer at a depth of 0 to 20 cm in three equidistant points from the stem and the glomerospores were quantified according to the methodologies of Gerdemann and Nicolson (1963) and Jenkins (1964). The roots were separated from the soil, diaphanized with KOH (10%, w/v) and H_2O_2 (10%, v/v), stained with Trypan blue in lactoglycerol, at 0.05% (Phillips and Hayman, 1970), and the mycorrhizal colonization was determined using the intersection of quadrants by the gridline intersecting method, according to Giovannetti and Mosse (1980).

Data analysis

The data were submitted to analysis of variance (ANOVA) and the means compared by the Tukey test (5%), using the Assistat 7.6 program (Assistat, 2011).

RESULTS AND DISCUSSION

The ironwood plants associated with *C. etunicatum* as well as with *A. longula*, were significantly taller than those of the non-inoculated control, while those inoculated with *G. albida* did not differ from the control (Table 2). The stem diameters, in spite of being 13% larger in plants inoculated with *C. etunicatum* and 10% larger in those associated with *A. longula*, did not differ significantly from the diameter of the control plants, nor did the number of total leaves differ significantly. Benefits of inoculation with AMF are not always observed in arboreal species transplanted to the field since the response differs according to the measured parameter and the species in question (Smith and Read, 2008). Vandresen et al. (2007), for example, did not observe any benefits of

Table 2. Height, number of leaves, stem diameter, colonization and spore density in *Libidibia ferrea* plants, inoculated or non-inoculated with arbuscular mycorrhizal fungi (AMF), seven months after transplanting to the field, in Petrolina, PE.

Variable	Inoculation treatment			
	Control	***Gigaspora albida***	***Claroideoglomus etunicatum***	***Acaulospora longula***
Height (m)	1.49[b]	1.52[b]	1.74[a]	1.65[a]
Number of leaves	45.75[a]	42.70[a]	41.42[a]	39.67[a]
Stem diameter (mm)	16.16[a]	16.55[a]	18.22[a]	17.76[a]
Colonization (%)	64.43[ab]	53.58[b]	73.97[a]	65.28[ab]
Spore density (100 g solo^{-1})	38.80[a]	44.33[a]	34.25[a]	34.25[a]

Averages (*n*= 6) on the line followed by the same letter do not differ according to the Tukey test (5%).

Table 3. Concentrations of chlorophyll, total phenols and tannins and gallic acid in *Libidibia ferrea* plants, inoculated or non-inoculated with arbuscular mycorrhizal fungi (AMF), seven months after transplanting to the field, in Petrolina, PE.

Variable	Inoculation treatment			
	Control	***Gigaspora albida***	***Claroideoglomus etunicatum***	***Acaulospora longula***
Chlorophyll (Falker Index)	65.3[b]	77.5[a]	66.7[b]	67.8[b]
Phenols (mg g planta^{-1})	2.11[ab]	2.16[a]	2.13[ab]	2.08[b]
Tannins (mg g planta^{-1})	2.02[ab]	2.09[a]	2.08[a]	1.99[b]
Gallic acid (g %)	0.038[b]	0.027[d]	0.046[a]	0.033[c]

Averages (*n*=6) on the line followed by the same letter do not differ from the Tukey test (5%).

inoculation in the height of five native tree species that were transplanted to the field in Paraná State, South of Brazil.

Although the plants that were inoculated with *G. albida* did not grow more than those of the control, the inoculation favored the accumulation of total chlorophyll (19%) in relation to the control (Table 3). Therefore, the symbiosis with the inoculated species or the change in composition of the symbiosis caused by the inoculation was efficient in increasing the levels of photosynthesizing pigments, which are important to the plant anabolism. Reports on the effects of AMF inoculation on leaf chlorophyll contents of Caatinga species were not found, but increased contents have been observed in inoculated species of other regions, such as those of *Gloriosa superba* L. inoculated with *Acaulospora laevis* (Yadav et al., 2013).

The density of glomerospores in the rizosphere and mycorrhizal colonization in plants of the inoculated treatments did not differ significantly from the density and colonization of the control treatment (Table 2). Positive responses have been reported by other researchers, like Gupta et al. (2002) who registered a higher rate of mycorrhizal colonization in plants of *Mentha arvensis* L. that were inoculated and kept in the field than in the control treatment. It is likely that the duration of our experiment was not sufficient to provoke detectable alterations in AMF colonization and reproduction. Higher colonization is usually reported following inoculation in pot experiments with sterilized substrates since the native AMF species are eliminated or substantially decreased in the control treatment (Oliveira et al., 2013).

There were no increases in the concentrations of total phenols and tannins in the inoculated plants in relation to the control (Table 3). Although no other measurements were found with ironwood plants, increases were registered in the contents of total phenols in *Myracrodruon urundeuva* (Engl.) Fr. All. (Oliveira et al., 2013), *C. roseus* L.G. Don (Rosa-Mera et al., 2011), *Cynara cardunculus* L. (Ceccarelli et al., 2010), *Echinacea purpurea* L. (Araim et al., 2009) and also in the contents of tannins in *Wedilla chinensis* (Osbeck) Merril. (Nisha and Rajeshkumar, 2010) and *Plectranthus amboinicus* (Lour) Spreng (Rajeshkumar et al., 2008). However, these patterns of increase are not always found and Geneva et al. (2010) and Nell et al. (2009), for example, also did not observe any increase in the contents of total phenols in *S. officinalis* L. High levels of photosynthetic pigments are proposed mechanisms to explain the influence of AMF on the increased concentrations of secondary compounds in medicinal plants (Dave and Tarafdar, 2011), but such a mechanism does not seem to have influenced the concentration of secondary compounds in ironwood plants that were inoculated with AMF.

Although the inoculation of AMF did not significantly increase the concentration of total phenols, inoculation with *C. etunicatum* increased the production of gallic acid (21%) in relation to the non-inoculated control treatment and also in relation to the other inoculation treatments (Table 3). A proposed mechanism to explain the AMF influence on the increases of secondary compound concentrations is the activation of metabolic pathways (Zimare et al., 2013). Among these, the shikimic acid pathway, a precursor of phenolic compounds (Heldt, 2005) is one of the relevant ones and could justify the increase in foliar gallic acid concentration in plants inoculated with *C. etunicatum*. Furthermore, this increase was dependent on the AMF used (Copetta et al., 2006), which confirms the initial working hypothesis. Experiments carried out in the field with other species validate the application of mycorrhizal fungi with the objective of increasing the biosynthesis of secondary compounds with therapeutic characteristics as was observed by Ceccarelli et al. (2010) in plants of *C. cardunculus*, with an increased production of total phenols, and by Gupta et al. (2002), in cultivars of *M. arvensis*, with a high concentration of essential oils.

Leaves of ironwood are used by the local population because of their medicinal properties (Albuquerque et al., 2007). Mycorrhizal technology, applying *C. etunicatum*, can aggregate value to the phytomass of ironwood commercialized for the phytotherapeutic industry, considering the production of gallic acid. Farmers of the São Francisco Valley, where the study was performed, could cultivate ironwood and sell the leaves to the pharmaceutical industry, contributing to a reduction in the extractivism of this species, which is not abundant in the native caatinga vegetation.

Future studies must analyze the effect of mycorrhizal inoculation on the production of other foliar compounds of pharmaceutical importance in ironwood such as ellagic acid and pauferrol A.

Conclusion

Mycorrhizal inoculation can favor the growth of *L. ferrea* established under field conditions and increase the production of gallic acid, with the benefits varying according to the inoculated AMF. Cultivation of inoculated *L. ferrea* could be an alternative, instead of collecting from the native vegetation, in order to supply raw materials with a better quality to the phytotherapeutic industry.

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Conflict of Interest

Authors have not declare any conflict of interest.

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