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Full Length Research Paper

The effect of growing conditions on phenolic compounds and antimicrobial activity of *Myracrodruon urundeuva* Fr. Allemão

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***Myracrodruon urundeuva* is a naturally occurring species in the Brazilian semi-arid and is widely used for anti-inflammatory and healing properties. This study evaluates the level of phenolic compounds and the antimicrobial activity in extracts of *M. urundeuva* obtained from greenhouse seedlings grown from seeds that were previously submitted or not to osmotic stress treatment (osmoconditioning), and from seedlings and callus grown *in vitro*. The Folin and Ciocalteau method was used to determine the phenolic content, whereas antimicrobial activity was evaluated by agar well diffusion and bioautography. Phytochemical analysis revealed greater concentrations of phenolic compounds in greenhouse seedlings independent of whether they originated from osmoconditioned seeds or not. Chromatography was shown to be an efficient technique for the separation of compounds. However, it was not possible to detect activity by bioautography. On the other hand, growth inhibitory activity was detected by agar well diffusion in acetone-ethyl acetate extracts taken from leaves of seedlings grown in greenhouse conditions.**

Key words: *Myracrodruon urundeuva*, antimicrobial activity, chromatography, phenolic compounds.

INTRODUCTION

Myracrodruon urundeuva Fr. Allemão (Anacardiaceae) occurs naturally in the Caatinga and Cerrado savannas and fluvial forests of Brazil (Andrade et al., 2000). It has great economic value due to the presence in its bark of large quantities of tannins widely used in the leather industry and folk medicine. Its inner bark is rich in substances with anti-inflammatory, adstringent, anti-allergic and healing properties (Mors et al., 2000). Studies of the inner bark of this species have resulted in the identification of substances with pharmacological activity, such as A, B and C *Urundeuva* chalcone dimmers (Nobre-Júnior et al., 2009), condensed tannins and flavonoids (Souza et al., 2007). However, the low concentration of these substances in these tissues has limited their exploitation.

The induction of biotic and abiotic stress has been used as a strategy to stimulate the secondary metabolism of plants by increasing the content of bioactive compounds and their biological activity (Kaufman et al., 1999; Gobbo-Neto and Lopes, 2007). Among abiotic factors, osmotic stress induced by polyethylene glycol (PEG 6000) has been used as it promotes low toxicity in plants. Seeds pretreated by imbibition in osmoticum undergo water stress which may induce changes in metabolic pathways that may in turn induce seed priming and promote the acquisition of tolerance to other stresses during further germination and plant growth (Goellner and Conrath,

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2007; Li et al., 2008), resulting to changes in the profile and content of secondary metabolites in plant tissues (Pastori and Foyer, 2002).

Cultivation of cells, tissues and plants *in vitro* have also been used as a promising alternative for the production of secondary metabolites (Yu et al., 2005). However, the *in vitro* production of bioactive compounds on an industrial scale is still limited by several factors, among them the low crop yields, production instability and lack of knowledge about their originating plant metabolism (Briskin, 2000). However, there are several examples of high-value substances successfully produced from this technique (Chattopadhyay et al., 2002; Tripathi and Tripathi, 2003; Vanisree and Tsay, 2004). Several studies have shown that exposure of plants to stresses caused by the *in vitro* environment induces the expression of genes that often is preserved beyond the seed and seedling stages, lasting during its growth and development (Silva et al., 2007). However, for most species there is need for studies to evaluate the production of bioactive compounds of interest in the *in vitro* environment, which may contribute to their exploitation by means of biotechnological approaches

In the case of medicinal plants, research related to culture conditions must be accompanied by phytochemical and biological activity studies. Several methods were employed to determine the antimicrobial activity of the plant extracts (Vanden Berghe and Vlietinck, 1991). Diffusion tests are quantitative methods based on the diffusion of the substance to be tested (extract), which is then placed in contact with solid culture medium inoculated with the test microorganism. Diffusion of the extract causes the appearance of a halo in which there is no cellular growth, called the inhibition halo. The way in which this contact occurs defines the different types of diffusion, including disc diffusion (with paper discs) and agar well diffusion, through holes made in the culture medium. The low cost and fast response of diffusion in agar make it the most indicated technique when dealing with plant extracts and *in vitro* selection of its bioactive compounds. However, when a more thorough examination of the molecular structure is necessary, the selection of the compounds should be carried out using chromatographic methods (França, 2001).

Thin layer chromatography (TLC) is a physicochemical method for the separation of components within a mixture by means of differential migration using absorbent material on a flat surface in which the process of separation is based in adsorption. The gradual removal of components occurs as a result of a mobile phase with individual solute migration between the two phases (stagnant and moving), which results in the separation of different components. TLC is one of the simplest and most economical chromatographic techniques if the objective is the rapid separation and visual identification of possible bioactive compounds (Ciésla, 2011).

However, TLC only permits the separation of different classes of molecules, and should be coupled to a method for the detection of antimicrobial activity, such as bioautography, which is considered to be a simple and efficient technique for selecting extracts (Burkhead et al., 1995). During bioautography, after the separation of components by chromatography, a fine layer of culture medium containing the test microorganism is applied and the appearance of growth inhibition zones indicates the presence of substances with antimicrobial potential (Burkhead et al., 1995). In this technique, the inhibiting components are identified by retention indexes (RI), which allow bioactive components to be located, as such confirming the existence of anti-bioactive substances or fractions (Hostettmann and Marston, 1994).

Given the above, the objective of this study was to determine the phenolic compounds and the antimicrobial activity of extracts of *M. urundeuva* obtained from greenhouse seedlings grown from seeds previously submitted or not to osmotic stress treatment (osmoconditioning), and from seedlings and callus grown *in vitro.*

MATERIALS AND METHODS

In vitro and *ex vitro* seedlings were grown in the Laboratory of Plant Tissue Culture and Nursery of the State University of Feira de Santana. Seeds were in initially harvested from native stock *M. urundeuva* plants located in the municipality of Jutaí, nearby the municipality of Petrolina, within the Brazilian semiarid and Caatinga region of the state of Pernambuco, Brazil.

For the *in vitro* assays, seeds were previously sterilized as described by Andrade et al. (2000) and then inoculated in test tubes containing 10.0 ml of WPM medium (Lloyd and McCown, 1981). The cultures were kept in a growth room at 25 ± 2 °C, with a 16 h photoperiod and photosynthetic active radiation of 30 µmol m^{-2} s^{-1} for 60 days. After this period, leaves were collected, washed and dried to constant weight in an air circulation oven at 40 ± 5°C. Dried leaves were subsequently ground with a porcelain mortar and pestle and stored in a desiccator until obtaining the extracts. To obtain callus leaf, segments of *in vitro* established seedlings were inoculated in WPM medium supplemented with 2.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). After inoculation, the cultures were placed in a growth room with no exposure to light and at a temperature of 25 ± 2 °C. After 45 days, the callus were lyophilized for 24 h and then stored in a freezer at -20°C until obtaining the extracts.

For the greenhouse assays, seeds were previously subjected to osmotic stress treatment (osmoconditioned) modified from Virgens et al. (2008), by imbibing the seeds in PEG 6000 solution at -1.0 MPa under continuous forced aeration. After the osmotic treatment seeds were transferred to plastic tubes filled with soil supplemented with phosphate (18% P_2O_5) at a ratio of 1 l/m^3 of substrate. Tubes were then kept in a greenhouse under 50% shading with daily irrigation. Leaves were collected after 42 days in order to obtain the extracts. Seedlings grown from seeds not subjected to osmotic conditioning (pre-imbibed in water, 0 MPa) were set as control.

Quantification of phenolic compounds

The methodology put forward by Folin and Ciocalteau (1927) and modified by Arnaldos et al. (2001) was used to determine the concentration of phenolic compounds in the dry leaf samples taken from *in vitro* callus and seedlings, and from seedlings grown in

greenhouse conditions. Briefly, 10 g sample was weighed in triplicate followed up by five successive extractions with 2.0 ml 80% methanol at 105°C. After each extraction, the supernatant was collected in a Falcon tube and a final volume of 10.0 ml. Activity analysis was conducted using test tubes containing 1.0 ml of sodium carbonate (75 g L^{-1}), 0.25 ml of sample and 1.25 ml of Folin reagent (1:10 v/v) against a blank of distilled water. The reaction mixture was placed in a bain-marie at 50°C for 5 min before being cooled to room temperature before measurements in a spectrophotometer UV-VIS (Analyser 850M) at 760 nm, using gallic acid as standard.

Preliminary screening of antimicrobial activity

A preliminary screening aimed at evaluating antimicrobial activity was carried out on aqueous extracts from seedlings roots, stems and leaves, using bacterial and fungi lineages deposited in the American Type Culture Collection (ATCC). Samples made of 10 g of each seedling part were initially placed in 80 ml of water at 50°C for 1 h, subsequently ground to pass a 32 mesh screen and centrifuged for 20 min at 3.000 rpm, after which the supernatant was collected and filtered under vacuum in order to obtain the final aqueous extracts. The extracts were tested for antimicrobial activity against the following bacteria strains: *Escherichia coli* ATCC 10536, *Klebsiella pneumoniae* ATCC 70603, *Micrococcus luteus* ATCC 9341, *Salmonella choleraesuis* ATCC 10708, *Pseudomonas aeruginosa* ATCC 15442, *Staphylococcus aureus* ATCC 6538, and a strain of *Staphylococcus aureus* isolated from a clinical sample and referred to as SAIACLIN as the name of the clinical laboratory; and against the following isolates of fungi: *Candida albicans* ATCC14053, *Candida parapsilosis* ATCC 22019 and *Candida krusie* ATCC 6558.

The experiments were carried out using the disc diffusion method in Petri dishes containing Müller-Hinton agar (MHA) for the bacterial lineages and Sabouraud dextrose agar (SDA) for the fungi. Dishes were inoculated with cell suspension adjusted to 0.5 McFarland standard turbidity (1.5 ×10^8 UFC ml^{-1}), followed by the placement of filter paper discs soaked with the various extracts in the following concentrations: 1.0, 2.0 and 3.0 µL $disc^{-1}$. Control consisted of paper discs soaked with commercial antibiotics recommended for each type of microorganism (Oplustil et al., 2000). Following inoculation, discs were incubated in a bacteriological incubator at 37°C for 24 h and subsequent measurement of the diameter of the inhibition halos formed around the discs. The experiment was performed in triplicate and the results obtained by the average diameter of the halos (NCCLS, 2003).

Crude extracts and chromatographic analysis

According to preliminary screening, new extracts were obtained from fresh and dry leaves from greenhouse seedlings grown from both osmoconditioned and non-osmoconditioned seeds, as well as from *in vitro* callus and seedlings. The compounds were extracted by means of solvents with distinct polarities (acetone, ethyl acetate, ethanol and hexane) using 0.10 g of fresh leaves and 0.05 g of dry leaves or lyophilized callus, and then placed in 8.0 ml of solvent in conical Falcon tubes. After resting for 16 h, the extracts were concentrated by evaporating the solvent at room temperature for 24 h and resuspended in dimethylsulphoxide (DMSO) to a standard final volume of 3.0 ml (crude extract). Thin-layer chromatography (TLC) was conducted to fraction the extracts using 7.0 × 5.0 cm chromatographic aluminum sheets (Merck CCD 1.5 × 8.0 cm silica G60 F254-366, 0.25 mm film thickness) which were autoclaved at 121°C and 1 atm for 30 min, and then dried in an air circulation oven at 50°C for 48 h. It was applied 5.0 µL of each crude extract to the aluminum sheets, which were eluted with hexane: ethyl acetate (3:7). Bands were visualized under ultraviolet (UV) light (366 nm) and the migration distance measured based on Rf values (derived by dividing the distance from the center of the bands by the total distance of the solvent front elution).

Antimicrobial activity

The antibacterial activity was evaluated by the bioautographic method and agar well diffusion against *S. aureus* ATCC 6538, which was chosen due to the elevated activity observed during the preliminary screening and for the fact that it is the most common agent in pyogenic infections, with a high capacity to develop drug resistance. For bioautography, cell suspensions adjusted to McFarland scale 0.5 (1.5 × 10^8 UFC ml^{-1}) were diluted in Müeller-Hinton agar medium (1:100) added to an aqueous solution of 2,3,5 triphenyltetrazolium chloride at 1% and poured onto Petri dishes containing the eluted chromatograms. After 2 h at 4°C, the dishes were further incubated for 24 h at 37°C. The antibacterial activity was then evaluated based on the thickness of the colorless halos surrounding each band formed by groups of substances at determined retention factors (Rf) (Moulari et al., 2006).

Petri dishes containing Muller-Hinton Agar (MHA), and inoculated with *S. aureus* ATCC 6538 cell suspensions adjusted to 0.5 McFarland standard turbidity (1.5 × 10^8 UFC ml^{-1}), were used to evaluate antimicrobial activity through well diffusion. Each Petri dish solid medium with 6.0 mm diameter wells was added separately with 5.0 and 10.0 µL of each extract, 10 µL of DMSO (negative control), 50 µg of chloramphenicol and 30 µg of ciprofloxacin (positive control). After 2 h at 4°C, the dishes were subsequently incubated for 24 h at 37°C. A qualitative evaluation of antibacterial activity was conducted based on the thickness of the bacterial growth inhibition zone surrounding the wells.

RESULTS AND DISCUSSION

Phytochemical analysis showed that the concentration of phenolic compounds was markedly different in the four samples evaluated, being higher in the leaves taken from greenhouse seedlings, independently of whether or not they originated from seeds that underwent osmotic conditioning (Table 1). Phenolic compound concentrations of greenhouse seedlings were two times greater than those obtained in *in vitro* grown callus and seedlings (Table 1).

The present results demonstrate the great influence of growth environment on the concentration of phenolic compounds. The plants in the *in vitro* environment are exposed to low light, high humidity and aseptic conditions, which may have affected the concentration of phenolic compounds in the tissues. Positive correlation has been established between the intensity of radiation and production of phenolic compounds such as flavonoids, tannins and anthocyanins, providing photoprotection by absorbing and / or dissipating the solar energy, thus explaining the higher concentration of these compounds in leaves of plants grown in greenhouses (Kaufman et al., 1999; Gobbo-Neto and Lopes, 2007). Another aspect to note is the stress conditions to which the plants are subjected to in the *in vitro* environment, due to high salt concentrations, ethylene accumulation in

Table 1. Concentration of phenolic compounds in *Myracrodruon urundeuva* obtained from leaves of greenhouse seedlings grown from seeds that did or did not undergo osmoconditioning and from leaves of seedlings and callus grown *in vitro*.

Sample	Phenolic compound (mg of EAG g^{-1})
Leaves of seedlings grown from non-osmoconditioned seeds	103.89[a]
Leaves of seedlings grown from osmoconditioned seeds	106.02[a]
Leaves of seedlings grown *in vitro*	50.60[b]
Callus	47.11[c]

Averages followed by the same letter do not differ statistically by Tukey's Test at 5% probability.

Table 2. Diameter mean values of the inhibition halo by the aqueous extract from leaves of *M. urundeuva* against strains of *K. pneumoniae* ATCC 70603, *M. luteus* ATCC 9341, *S. choleraesuis* ATCC 10708, *S. aureus* ATCC 6538, *S. aureus* (SAIACLIN) and *P. aeruginosa* ATCC 15442 obtained in the preliminary screening.

Microorganism	Inhibition halo (mm)*				
	Extract (µL $dish^{-1}$)			Control[1] (50 µg $dish^{-1}$)	Control[2] (30 µg $dish^{-1}$)
	1.0	2.0	3.0		
K. pneumoniae	12.0	13.0	14.0	12.5	NT
M. luteus	-	8.0	10.0	NT	15.0
S. choleraesuis	23.3	24.0	26.0	NT	18.5
S. aureus	14.0	16.0	18.0	13.6	NT
S. aureus (SAIACLIN)	17.3	17.3	19.0	16.0	NT
P. aeruginosa	8.0	9.3	10.0	13.8	NT

*Average values for inhibition halo. Control[1] = Chloramphenicol; Control[2] = ciprofloxacin; NT = not tested.

the tubes and the presence of growth regulators. In general, plants under stress conditions invest in chemical strategies in an attempt to adjust to metabolic oscillations triggered by stresses, affecting the production of metabolites in tissues (Hadacek, 2002).

Phenolic compounds are associated with various medicinal effects and plant toxins, representing great ecological significance, especially in the protection of plants against pathogens and herbivores. Preliminary screening showed an absence of activity in the aqueous root and stem extracts of *M. urundeuva* against all bacterial strains tested. On the other hand, leaf aqueous extracts showed greater activity against strains of *K. pneumoniae*, *S. cholerae* and *S. aureus*, than that encountered in the antibiotic control independently of the concentration tested (Table 2). However, there was no activity detected against fungi in the aqueous extracts. Alves et al. (2009) also confirmed the activity of *M. urundeuva* extracts on oral cavity microorganisms such as *C. albicans* and *C. krusei*. However, only extracts taken from the bark of this species were tested. The positive antibacterial activity by *M. urundeuva* leaf extracts in the present study are compatible with the antibacterial activity found specifically in leaf extracts of *Baccharis trimera* and *Rosmarinus officinalis* against Gram positive bacteria (Avancini et al., 2000; Nascimento et al., 2000).

Chromatographic analysis showed an absence of bands for the different extracts taken from callus. On the other hand, one to two bands were formed with different extracts from fresh leaves, and two to three bands with extracts from dry leaves from greenhouse seedlings grown from seeds that were or were not submitted to osmoconditioning and from *in vitro* plants (Table 3). Similarity in retention factors (Rf) among the separated substances reflects the efficiency of the different extractors used. It is known that the fractionation of crude extracts with solvents of distinct polarity makes possible the separation of different classes of bioactive substances, extracted in accordance with their polarity and solubility (Simões et al., 2004).

Moreover, it was not possible under the extraction conditions tested to detect growth inhibition zones for *S. aureus* in autobiographic testing. On the other hand, inhibitory activity was detected by the agar well diffusion analysis where regions that cell growth did not occur was observed when using leaf acetone and ethyl acetate extracts from greenhouse seedlings grown from seeds subjected or not to osmoconditioning (Table 4). Acetone extracts presented low activity as inhibition was detected only when applying 10.0 µL of the extract, while ethyl acetate extracts presented higher activity as inhibition

Table 3. Retention indices values obtained with extracts from leaves of *M. urundeuva*, obtained using acetone, ethyl acetate, ethanol and hexane, and subsequently eluted with hexane/ethyl acetate 3:1 (v/v) and visualized under UV light (366 nm).

Sample	Retention factor (Rf)									
	Acetone			Ethyl acetate		Ethanol			Hexane		
Fresh leaves from seedlings grown from osmoconditioned seeds	31	-		37	31	35	-	-	28	-	-
Dry leaves from seedlings grown from osmoconditioned seeds	31	35		33	30	34	39	-	28	31	37
Fresh leaves from seedlings grown from non-osmoconditioned seeds	31	-		36	32	34	-	-	28	-	-
Dry leaves from seedlings grown from osmoconditioned seeds	30	35		34	32	33	37	-	27	31	38
Dry leaves from seedlings grown *in vitro*	-	-		32	28	18	20	25	36	40	42
Lyophilized callus	-	-		-	-	-	-	-	-	-	-

Table 4. Qualitative analysis of the formation of inhibition halos from crude extracts of *M. urundeuva* leaves, extracted with ethanol, hexane, acetone and ethyl acetate, against *S. aureus* ATCC 6538, through the agar well diffusion method.

Sample	Volume used (µL dish^{-1})							
	Ethanol		Hexane		Acetone		Ethyl acetate	
	5.0	10.0	5.0	10.0	5.0	10.0	5.0	10.0
Fresh leaves from seedlings grown from osmoconditioned seeds	n/a	n /a	n /a	n /a	n /a	+	+	+++
Dry leaves from seedlings grown from osmoconditioned seeds	n /a	n /a	n /a	n /a	n /a	+	+	+++
Fresh leaves from seedlings grown from non-osmoconditioned seeds	n /a	n /a	n /a	n /a	n /a	+	+	+++
Dry leaves from seedlings grown from non-osmoconditioned seeds	n /a	n /a	n /a	n /a	n /a	+	+	+++
Dry leaves from seedlings grown *in vitro*	n /a	n /a	n /a	n /a	n /a	n /a	n /a	+++
Lyophilized callus	n /a	n /a	n /a	n /a	n /a	n /a	n /a	n /a

*n/a, No activity; +, low activity; +++, high activity.

was detected applying 5.0 µL and even higher when applying 10.0 µL of the extract. This demonstrates that ethyl acetate is the most adequate solvent for the extraction of compounds with microbial activity from the leaves of *M. urundeuva.* The ethanol and hexane extracts did not present activity, independently of the sample used (Table 4). The lack of activity in the compounds separated by bioautography could be related to their low concentration after fractionation or due to possible synergism, that is - compounds do not present or present lower activity when separated into its isolated form as detected in compounds from *Cynara scolymus* (Bonati, 1980). Such synergistic effect has been observed also in *Hypericum perforatum* in which the inhibitory affects was detected only in pooled fractions from leaves. Vanden Berghe and Vlietinck (1991) reported that bioautography is a method of low sensitivity to test plant extracts, which contain antimicrobial agents often much less potent than the antibiotics in use.

Although this work has not identified the classes of substances in plant extracts, it is suggested that the highest antimicrobial activity observed in the ethyl acetate extract was due to the presence of polyphenols, given the high solvency power of polar extractant for this class of compound. Furthermore, polyphenols have proven antimicrobial effects, breaking down the microbial cell wall and inhibiting the enzyme systems responsible for its formation (Silva et al., 2007). Considering the relevance of the results obtained in the present study for the sustainable production of compounds of pharmaceutical interest from this species which is native to the northeastern semiarid region of Brazil, it is suggested that new tests should be carried out aiming at the identification and isolation of the compounds present in each type of extract for further confirmation of its bioactivities.

Conclusion

The results obtained in this study indicate the possible use of extracts taken from young *M. urundeuva* plants (seedlings) for the production of bioactive compounds and prospection of antibiotic substances, especially those from leaves. Further studies should be undertaken in order to purify and identify the bioactive compounds extracted from the leaves of *Myracrodruon urundeuva*.

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