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# Micropropagation, antinociceptive and antioxidant activities of extracts of *Verbena litoralis* Kunth (Verbenaceae)

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## ABSTRACT

This work describes an efficient micropropagation protocol for *Verbena litoralis* and the study of the antinociceptive and antioxidant activities in extracts of this species. For the establishment *in vitro*, surface-sterilization procedures and PVPP showed high efficiency in fungal-bacterial contamination and phenol oxidation controls. Nodal segments cultivation in MS medium supplemented with 6-benzyladenine (7.5 µM)/α-naphthaleneacetic acid (NAA; 0.005 µM) induced multiple shoots. Elongated shoots were rooted with IAA (0.2 µM). Acclimatization rates were elevated and the plants showed the typical features of this species. The hexanic fraction (HF) of powdered leaves presented a radical scavenging activity with $IC_{50}$ = 169.3 µg mL$^{-1}$. HF showed a non-dose dependent analgesic activity in the writhing test; its antinociceptive activity in the hot plate test was restricted to 500 mg kg$^{-1}$, which is the highest dose. The results of this study showed the potential of tissue culture on conservation and large scale multiplication and confirmed the traditional folk medicine use of *V. litoralis*.

**Key words:** tissue culture, biodiversity conservation, biological effects, natural products.

## INTRODUCTION

Verbenaceae is found in practically all terrestrial ecosystems. It is one of the five most important families among the Eudicots of Brazilian Campos Rupestres (Giulietti et al. 1987). This family includes 1035 species and 36 genera with a pantropical distribution. Few species are found in temperate areas (Atkins 2004). In Brazil, the most important specific diversity centers are located at Cadeia do Espinhaço, Minas Gerais State, and at Chapada Diamantina, Bahia State (Salimena-Pires and Giulietti 1998).

Plant tissue culture techniques have been widely applied to medicinal and aromatic plants (Jain and Saxena 2009), and *in vitro* propagation appears as an

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alternative to conventional vegetative propagation and germplasm conservation, especially for endemic and endangered species (Passera and Ambrosetti 1999, Gupta et al. 2001, Peixoto et al. 2006).

Several Verbenaceae genera have being investigated due to their medicinal properties (Pascual et al. 2001, Costa et al. 2003, Penido et al. 2006, Saini et al. 2007). *Verbena litoralis* Kunth is currently used in traditional South and Central American folk medicine against diarrhea, fever, gastrointestinal disorders and some sexually transmitted diseases (Pérez-Arbeláez 1978, Ocampo and Maffioli 1987). Preliminary ethnobotanical and phytochemical information on *V. litoralis* has been published and various constituents were obtained (Umaña and Castro 1990a, b, Li et al. 2001a, b). The interest in this plant increased after the isolation of substances with nerve growth factor-potentiating activity (Li et al. 2001a, b, 2003a, b, c). The aerial parts of this plant produce an extract that revealed an interesting preliminary antibacterial potential (Castro-Gamboa and Castro 2004).

As the use of analgesic agents is somewhat criticized for its adverse side effects, induced tolerance and dependence, new analgesic drugs lacking these effects are being searched for all over the world (Dharmasiri et al. 2003). Further studies should be conducted with plants used in folk medicine since they have a low-cost and can be an effective alternative to the opiates currently used. Several studies have been carried out with *V. litoralis* due to the isolation of neuritogenically active substances, such as verbenachalcone (Li et al. 2001a), littoralisone (Li et al. 2001b) and littorachalcone (Li et al. 2003a). Moreover, Castro-Gamboa and Castro (2004) managed to isolate two new iridoids, 6*S*-hydroxy-8*S*-methyl-4-methylene-hexahydro-cyclopenta[*c*]pyran-3-one and 6*S*,9*S*-dihydroxy-8S-methyl-4-methylene-hexahydro-cyclopenta[*c*]pyran-3-one, which showed moderate antibacterial, intestinal peristaltic reflex and antioxidant activities.

The aim of this work was to develop a large scale micropropagation method and investigate the effectiveness of the antinociceptive and antioxidant activities of *V. litoralis* extracts.

## MATERIALS AND METHODS

### IN VITRO PLANT MATERIAL

*Verbena litoralis* Kunth plants were collected at Cadeia do Espinhaço, Minas Gerais State, and in Southeastern Brazil. The plants were further established by vegetative propagation and grown in an orchard at the Experimental Plant Station at the Universidade Federal de Juiz de Fora, Juiz de Fora, Minas Gerais State, Brazil. The donor plants were sprayed once a week with a Benomyl solution (0.172 mg $L^{-1}$; DuPont®, USA). Then, apical cuttings (2-3 cm height) with two to three axillary buds were removed from shrubs and the explants were washed in running tap water for 60 min, followed by immersion in ethanol 70% (v/v) for 30 s and with Benomyl solution (0.172 mg $L^{-1}$) for 10 min. The explants were soaked in 100 mL of a commercial bleach (2% active chlorine) 40% (v/v) solution added two drops (30 μL) of Tween-20 (Sigma®, USA) for 15 min, and finally rinsed five times in sterile distilled water. The explants were cultivated on a half-strength MS basal medium (Murashige and Skoog 1962) supplemented with sucrose (30 g $L^{-1}$; Vetec, Brazil), myo-inositol (100 mg $L^{-1}$; Sigma®), vitamins (nicotinic acid, pyridoxine-HCl and thiamin-HCl; all at 0.05 mg $L^{-1}$; Sigma®), glycine (0.05 mg $L^{-1}$; Sigma®) and Benomyl (0.172 mg $L^{-1}$).

Attempting phenol oxidation control, poly-N-vinylpyrrolidone (PVPP; 0.5 g $L^{-1}$; Sigma®) was included in a media culture. Phenol oxidation measurement was accessed by complete browning of nodal explants. All media were prepared with 7 g $L^{-1}$ agar (Sigma®) and the pH of the media was adjusted to 5.7 before autoclaving at 120°C, at 1 atm for 20 min. The excision of the explants was

carried out under sterile conditions in a laminar flow hood (VECO®, Brazil). The cultures were incubated in a growing room at 25-28°C under a 16-h photoperiod and photon flux density of 35 µmol m$^{-2}$ s$^{-1}$ provided by cool-white fluorescent tubes (20 W, Osram Sylvania Inc.). A single *in vitro* plantlet was used as the initial source of explants for subsequent experiments. Experiments were set up in a completely randomized design with 15 test tubes (25 x 150 mm) containing one explant per test tube per treatment.

SHOOT PROLIFERATION AND ROOTING

Full-strength MS-based medium supplemented with sucrose (30 g L$^{-1}$), myo-inositol (100 mg L$^{-1}$), vitamins (nicotinic acid, pyridoxine-HCl and thiamin-HCl; all at 0.05 mg L$^{-1}$) and glycine (0.05 mg L$^{-1}$) induced shoot proliferation from apical nodal segment cuttings with combinations of 6-benzyladenine (BA; 0, 2.5, 5.0 or 7.5 µM) and α-naphthaleneacetic acid (NAA; 0, 0.005, 0.05 or 0.5 µM) in a completely randomized design. Elongated apical shoots (average 2-cm height) from MS-based medium (without growth regulators) were rooted in a full-strength MS-based medium supplemented with sucrose (30 g L$^{-1}$), myo-inositol (100 mg L$^{-1}$), vitamins (nicotinic acid, pyridoxine-HCl and thiamin-HCl; all at 0.05 mg L$^{-1}$) and glycine (0.05 mg L$^{-1}$) added with either NAA, indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) at 0, 0.1, 0.2, 0.3 or 0.4 µM. All growth regulators were added before the autoclaving of the culture media. Evaluated parameters were the number of shoots, elongated shoots, callus formation, percentage of root induction, and roots length 45 d after culture initiation. The number of shoots and roots was normalized through the equation ($\sqrt{\chi + 0.5}$). The length of roots and shoots was normalized through the equation log (x + 1). The data were subjected to one-way analysis of variance (ANOVA) and Tukey and Scott-Knott multiple range tests. Statistical analyses were carried out using the Statistical Package for Social Sciences (SPSS) v.8.0 for Windows software.

ACCLIMATIZATION

Elongated rooted plants (average 5-cm height) from rooting medium supplemented with 0.2 mM IAA were individually transferred to plastic beakers (100-mL capacity) containing 25-30 mL of sterile distilled water, covered with a transparent plastic bag and kept for 2 days under the same growth room conditions. Then, the plants were transferred to plastic containers filled with horticulture organic substrate (Plantmax®, Paulínia, Brazil), soil and sand (2:2:1) under a shading house (50% light interception). Plastic bags were progressively opened by lateral cuts throughout the first 3 weeks of the hardening-off process. At the end of the fourth week, the containers were completely uncovered. Plants were irrigated on a daily basis up to the third week, and at every 2 d during the fourth week. After 2 months in the greenhouse, plants were successfully transferred to beds containing a mixture soil:sand:manure in the ratio 4:3:1 (v/v/v). The plants were irrigated twice a week.

ANTINOCICEPTIVE AND ANTIOXIDANT ACTIVITIES

Dried powdered leaves (50 g) of *V. litoralis* were macerated at room temperature. The dry powder was Soxhlet partitioned with hexane and yielded 0.7 g of hexanic fraction (HF). Only one fraction was prepared due to the small amount of material available. The choice was made to work with HF because previous studies reported that the most found activities are concentrated in the non-polar extracts (Li et al. 2003a, b).

RADICAL SCAVENGING ACTIVITY

The radical scavenging activity of DPPH (2,2-difenil-1,2-picrilhidrazil) was determined according to Sreejavan and Rao (1997) with a few

modifications. 1.5 mL of ethanolic (EtOH) solution of DPPH (0.05 mM) (Jagetia et al. 2003) was added to a 500 µL EtOH solution of HF in the following concentrations: 250; 125; 62.50; 31.25; 15.62; 7.81; 3.90; 1.95; 0.97 µg mL$^{-1}$. A 30 min reaction was allowed to happen in the dark at room temperature, after which the absorbance was monitored spectrophotometrically at 517 nm. A blank was used to ignore the influence of the color in the samples. An EtOH solution of DPPH 0.05 mM was used as negative control. Vitamin C was used as standard in the same concentrations. The reduction percentage of the DPPH absorbance gave the DPPH scavenging activity of the test samples and was used as a marker for their antioxidant activities. The IC$_{50}$ was given by the equation IC$_{50}$ (%) = 100 x (A$_0$ - A$_s$)/A$_0$, where A$_0$ and A$_s$ stand for the absorbance of the negative control and the absorbance of the sample, respectively. Tests were carried out in triplicate.

ANTINOCICEPTIVE ACTIVITY

Male Swiss mice (*Mus musculus*) (25-30g) from the Center of Reproductive Biology at the Universidade Federal de Juiz de Fora - UFJF - (Juiz de Fora, Minas Gerais State, Brazil) were used throughout the experiments. They were housed in a standard, room temperature cage under environmental conditions and fed with a rodent diet. This study was conducted in accordance with guidelines set forth by the Brazilian Association for Laboratory Animal Science (COBEA) and has been approved by the Research Commission for Ethics and Animal Experimentation of UFJF.

WRITHING AND HOT PLATE TESTS

For all tests described below, HF was solved in saline solution of 1% DMSO:Tween 80 (1:2, v/v). Writhing activity in mice was evaluated according to Koster et al. (1959). Eight (n = 8) animals were

induced in each of the five groups (standard, control and HF: 100 mg kg$^{-1}$, 300 mg kg$^{-1}$ and 500 mg kg$^{-1}$). The different concentrations of HF were orally administered. One hour later, 1.0 mL of 0.6% acetic acid was injected intraperitoneally. The number of writhes/mouse was counted during a 20 min period, starting 10 min after the administration of the acetic acid. Indometacin 5 mg kg$^{-1}$ and saline solution of 1% DMSO: Tween 80 (1:2, v/v) were respectively used as standard and control, and were also orally administered (0.3 mL animal$^{-1}$) one hour before the administration of the acetic acid.

The hot plate test was performed according to Franzotti et al. (2000), with a few modifications. Ten animals (n = 10) were included in each of the six groups (standard, control and HF: 100 mg kg$^{-1}$, 300 mg kg$^{-1}$ and 500 mg kg$^{-1}$). Animals were placed on a hot-plate set at 55 ± 0.5°C. Reaction time was recorded at different times after the different concentrations of HF had been administered (30, 60, 90 and 120 min.), when the animals licked their fore and hind paws or jumped. Animals that did not react after 30 s were taken off the plate to avoid damage to tissues, which could jeopardize further evaluations. A baseline was obtained immediately before the drug administration and was defined as a normal reaction of the animal to temperature increase. The control group was orally administered with 0.3 mL/30 g of saline solution of 1% DMSO:Tween 80 (1:2, v/v). In the standard group, morphine 5 mg Kg$^{-1}$ was administered subcutaneously (Kuraishi et al. 1983).

The data of antinociceptive and antioxidant activities are presented as the means ± S.E.M. for eight animals per group in writhing test and ten animals per group for the hot plate assay. One-way ANOVA was used to compare means followed by Bonferroni's test when appropriate. Values of p<0.05 were considered to be statistically significant. Statistical analyses were carried out using the Statistical Package for Social Sciences (SPSS) v.8.0 for Windows software.

## RESULTS AND DISCUSSION

TISSUE CULTURE INITIATION

As previously found for explants of *L. filifolia* (Peixoto et al. 2006), PVPP showed an effectiveness on phenol oxidation control during the *in vitro* establishment of apical microcuttings, reducing the number of brownish explants. The percentage of explants oxidized was less than 1%. Disinfection procedures showed high efficiency in the control of the fungal and bacterial contamination. The use of benomyl, ethanol and commercial bleach in the disinfection produced more than 95% of the free bacterial and fungal cultures during the *in vitro* establishment of apical cuttings.

SHOOT PROLIFERATION

After the establishment phase, different concentrations of plant growth regulators enabled the plant propagation via nodal segments, which were placed vertically on a surface of a solidified culture medium. BA and NAA interaction showed statistical differences for the number of shoots (Table I and Figure 1-AB) and roots (Table II and Figure 1-AB) produced in response to the increase of BA in the culture medium. For *V. litoralis*, the highest multiple shoot response was obtained after the treatment with BA (7.5 µM)/ NAA (0.005 µM; Table I), whereas in *L. filifolia* multiple shoots were observed with BA (4.5 µM)/NAA (0.005 µM) (Peixoto et al. 2006). BA combined with NAA produced a maximum of 4.8 shoots per explant (Table I), while NAA alone reduced the number of shoots, reinforcing the importance of cytokinins for shoot induction in *V. litoralis*. For *L. alba*, the addition of 0.23 µM IAA to MS medium significantly decreased plant regeneration, number of shoots per explant and number of nodes per plantlet, as compared to MS medium lacking growth regulators (Tavares et al. 2004). In general, higher rates of multiplication were observed in the presence of higher concentrations of BA (Table I). Similar results were observed for *Lippia junelliana* (Juliani et al. 1999), *L. alba* (Gupta et al. 2001) and *L. filifolia* (Peixoto et al. 2006).

**TABLE I**
**Average shoot numbers of *Verbena litoralis* as affected by BA and NAA combinations, 45 d after *in vitro* culture.**

| BA (µM) | NAA (µM) | | | | |
|---|---|---|---|---|---|
| | 0 | 0.005 | 0.05 | 0.5 | |
| 0 | 1.57 a[1] | 1.28 a | 1.09 a | 2.78 a | 1.68 B |
| 2.5 | 2.53 a | 1.37 ab | 0.74 b | 0.62 b | 1.32 B |
| 5.0 | 2.09 a | 2.31 a | 2.46 a | 1.25 b | 2.03 B |
| 7.5 | 4.62 a | 4.82 a | 3.39 a | 1.38 b | 3.55 A |

[1]Means followed by the same small letters (in each line; for NAA doses) and capital letters (in last row; for BA doses) are not different according to Tukey's test at 5% of probability.

**TABLE II**
**Average root numbers of *Verbena litoralis* as affected by BA and NAA combinations, 45 d after *in vitro* culture.**

| BA (µM) | NAA (µM) | | | | |
|---|---|---|---|---|---|
| | 0 | 0.005 | 0.05 | 0.5 | |
| 0 | 4.3 a[1] | 4.1 a | 8.5 a | 4.5 a | 5.4 A |
| 2.5 | 2.5 a | 3.2 ab | 3.7 b | 6.8 b | 4.1 A |
| 5.0 | 0 a | 0 a | 0 a | 0 b | 0 B |
| 7.5 | 0 a | 0 a | 0 a | 0 b | 0 B |

[1]Means followed by the same small letters (in each line; for NAA doses) and capital letters (in last row; for BA doses) are not different according to Tukey's test at 5% of probability.

During our experiments, we do not observed hyperhydricity, a negative effect commonly observed after BA treatment (Phan and Hegedus 1986). In this assay, rooting was observed. In MS media and in the small concentration of BA, 100% of explants showed rooting (Figure 1-A). At higher concentrations of BA, no rooting was observed even when NAA was included in the culture media

(Table II and Figure 1-B). Although not being important at this stage, rooting can increase the efficiency of the propagation process, since it reduces the time for reintroducing the plants under field conditions.
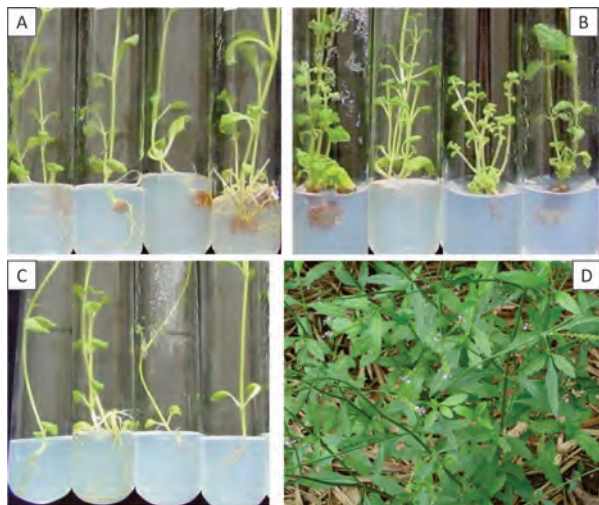


Fig. 1 - (**A** and **B**) Multiple rooting and shooting responses of *Verbena litoralis* nodal segments, 45 d after *in vitro* culture. Treatments (µM): A) BA 2.5 X NAA 0; BA 2.5 X NAA 0.005; BA 2.5 X NAA 0.05; BA 2.5 X NAA 0.5; B) BA 7.5 X NAA 0; BA 7.5 X NAA 0.005; BA 7.5 X NAA 0.05; BA 7.5 X NAA 0.5. (**C**) Rhizogenic responses of elongated shoots of *V. litoralis*, 45 days after inoculation. Treatments (µM): Control; IAA 0.2; IAA 0.3; IAA 0.4. (D) Acclimatized plants of *V. litoralis*.

ROOTING AND ACCLIMATIZATION

Shoots of *V. litoralis* rooted under all auxin treatments and in the control (Figure 1-C), as found for *L. alba* (Gupta et al. 2001) and *L. filifolia* (Peixoto et al. 2006). The best root system was observed in the presence of IAA at 0.2 µM, with nearly 11 roots per explant (Table III). This IAA concentration did not induce callus formation at shoots basis, which is a positive effect, since callus formation acts as a mechanical barrier to nutrient and water uptake (Thorpe et al. 1991, De Klerk 2002). IAA also induced the high shoot length production, although the length of the shoots was not an important factor for plant survival during the hardening-off phase. The roots length did not differ significantly among the different treatments (data not shown).

**TABLE III**

**Average root numbers in elongated shoot of *Verbena litoralis* as affected by NAA, IAA and IBA, 45 d after *in vitro* culture.**

| Concentration (µM) | Type of Auxin | | |
|---|---|---|---|
| | NAA | IAA | IBA |
| 0 | 2.8 e[1] | 2.8 e | 2.8 e |
| 0.1 | 6.5 c | 5.8 c | 6.3 c |
| 0.2 | 6.8 b | 10.8 a | 7.8 b |
| 0.3 | 7.5 b | 8.5 a | 8.1 a |
| 0.4 | 4.8 d | 9.0 a | 7.0 b |

[1]Means followed by the same letters are not different according to Scott-Knott's test at 5% of probability.

Plant survival rates were as high as 96% of the 128 acclimatized plants that were transferred to *ex vitro* conditions. The plants grew normally, and set flowers. Acclimatized plants displayed the typical features of this species (Figure 1-D).

ANTIOXIDANT ACTIVITY

The radical scavenging activity of the compounds can be measured as a decolorizing effect following the trapping of the unpaired electrons of DPPH. The lower value of $IC_{50}$ indicates a higher antioxidant activity. The HF and vitamin C demonstrated a scavenging activity of DPPH radicals with $IC_{50}$ values of 169.3 µg mL$^{-1}$ and 2.5 µg mL$^{-1}$, respectively. The antioxidant activity of FH from *V. litoralis* was lower than that found by Mensor et al. (2001), which can be attributed to different sources of plant material and also to the time to collect leaves in the field.

ANTINOCICEPTIVE ACTIVITY

In the abdominal writhing model the acetic acid addition is thought to cause inflammation due to the increase in the levels of cytokines, prostaglandins and bradykinins, and other endogenous substances that stimulate nociceptive endings (Pini et al. 1996). The HF 100 mg kg$^{-1}$, 300 mg kg$^{-1}$ and 500 mg kg$^{-1}$,

respectively, showed an average inhibition percentage of 52.6%, 52.6%, and 56.9%, therefore suggesting a good antinociceptive potential. Nevertheless, a significant dose-dependent relation could not be established (Figure 2). Indometacin, used as a standard, showed 76.22% of inhibition.
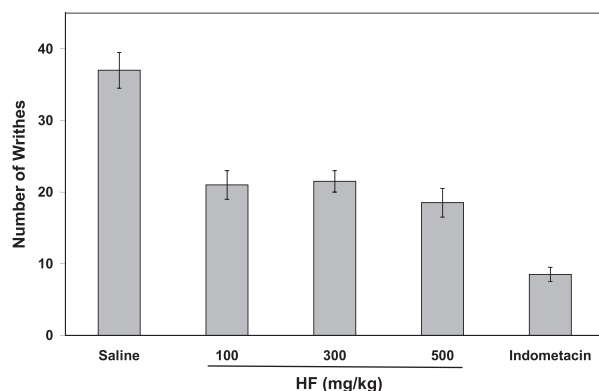


Fig. 3 - Analgesic effect (mg Kg$^{-1}$) of HF on the Hot Plate test. O time = baseline (Data expressed as mean ± S.E.M. n = 10 mice/group; ANOVA; Bonferroni's test).



Fig. 2 - Analgesic effect of HF on the writhing response induced by acetic acid 0.6% in mice. Indometacin 5 mg kg$^{-1}$ was used as standard. (Data expressed as mean ± S.E.M. n = 8 mice/group; ANOVA; Bonferroni's test)

Considering the fact that the writhing model involves several nociceptive mechanisms, such as amines release, related to the sympathetic system, and endogenous substances related to inflammation, the hot plate essay is important to exclusively evaluate the central analgesic effect of HF (Pini et al. 1996). In the hot plate test a significant difference from the saline control was found only for 500 mg kg$^{-1}$, therefore limiting the potential supra-spinal analgesic activity to a higher dose. Morphine reached its peak of action at 60 min and HF 500 mg kg$^{-1}$ showed its maximum potential activity 90 min after oral administration (Figure 3).

The difference between the results found by the hot plate and writhing test may be related to the gastroprotective effects and antioxidant activity of compounds of *V. litoralis* reported by Castro-Gamboa and Castro (2004), and the correlation between antioxidant and anti-inflammatory activities (Wang et al. 2004). Unlike the hot plate
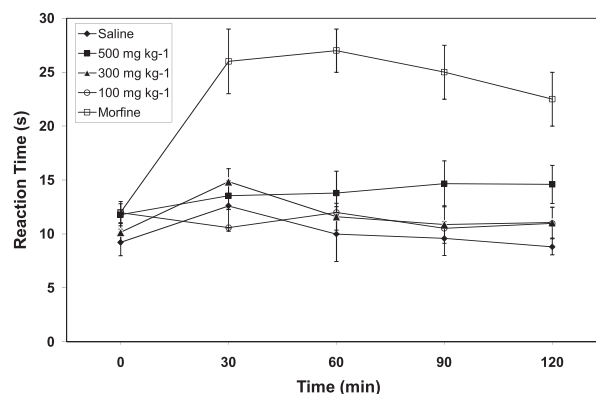
test, which is restricted to spinal nociception, the writhing test response can be affected by other processes involved. The administration of acetic acid addition in the writhing test induces inflammation, which leads to gastric irritation, bleeding and gastric mucosa damage (Andrade et al. 2007). Therefore, we suggest that anti-inflammatory and antinociceptive activities are acting together in the writhing test in order to reduce its response. These results may indicate potential anti-inflammatory activity of HF, which is already being tested. As the HF of *V. litoralis* produced a significant decrease in the number of writings in the antinociceptive essay and had some antioxidant effects, the traditional use in folk medicine was confirmed. HF analgesic activity can be increased by its potential anti-inflammatory activity.

The results of this study showed the potential importance of tissue culture on a large scale multiplication and conservation, and confirmed the traditional use of *V. litoralis* in folk medicine.

## RESUMO

Este trabalho descreve um protocolo eficiente de micropropagação para *Verbena litoralis* e estuda as atividades antinociceptiva e antioxidante de extratos desta espécie. Para o estabelecimento *in vitro*, os procedimentos de desinfecção e o PVPP mostraram alta eficiência no controle da contaminação por fungos e bactérias e da oxidação fenólica. O cultivo de segmentos nodais em meio MS suplementado com 6-benziladenina (7,5 μM) e ácido α-naftalenoacético (ANA; 0,005 μM) induziu múltiplos brotos. Brotos alongados foram enraizados com AIA (0,2 μM). As taxas de aclimatização foram elevadas e as plantas apresentaram características típicas da espécie. A fração hexânica (FH) de folhas trituradas apresentou atividade sequestradora de radicais livres com $IC_{50} = 169,3$ μg mL$^{-1}$. A FH mostrou atividade analgésica não dose-dependente no teste das contorções abdominais; sua atividade antinociceptiva no teste de placa quente foi restrita a 500 mg kg$^{-1}$, a dose mais elevada. Os resultados deste estudo mostraram o potencial da cultura de tecidos na conservação e multiplicação em larga escala de *V. litoralis* e confirmaram o uso tradicional dessa planta na medicina popular.

**Palavras-chave:** cultura de tecidos, conservação da biodiversidade, efeitos biológicos, produtos naturais.

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