Bactericidal activity of macela (*Achyrocline satureioides* (Lam.) DC.) and jaborandi-falso (*Piper aduncum* L.) against strains of *Staphylococcus aureus* isolated from subclinical bovine mastitis

Lemos, Glória C. S.[1]; Oliveira, Luiz O.[2]; Eberli, Bianca B.[1]; Motta, Olney V.[3]; Folly, Márcio M.[3]

[1]Laboratório de Melhoramento Genético Vegetal, UENF, 28015-620, Campos, RJ. [2] Departamento de Bioquímica e Biologia Molecular, UFV, 36571-000, Viçosa-MG, E-mail: lorlando@mail.ufv.br. [3]Laboratório de Sanidade Animal, UENF, 28015-620, Campos, RJ.

RESUMO: **Atividade bactericida de macela (*Achyrocline satureioides* (Lam.) DC.) e de jaborandi-falso (*Piper aduncum* L.) sobre cepas de *Staphylococcus aureus* isoladas de mastite bovina subclínica.** A atividade bactericida de extratos obtidos por maceração fracionada de inflorescências de *Achyrocline satureioides* e de inflorescências e folhas de *Piper aduncum* foi testada em 8 cepas de *Staphylococcus aureus* isoladas de amostras de leite coletados de vacas portadoras de mastite bovina subclínica. Os valores da concentração mínima bactericida apresentados pelos extratos hexânico e clorofórmico de *Achyrocline satureioides* variaram entre 14 a 2400 mg/ml e entre 56 a 675 mg/ml, respectivamente. *Piper aduncum* produziu extrato hexânico que foi inativo, porém seu extrato clorofórmico apresentou valores de concentração mínima bactericida que variaram entre 237-3800 mg/ml. Os extratos obtidos a partir de acetato de etila, metanol e agua não apresentaram atividade bactericida nas concentrações testadas.

Palavras-chave: Extratos vegetais; bactericidas; *Staphylococcus aureus*; mastite bovina

ABSTRACT: The bactericidal activities of *Achyrocline satureioides* (inflorescences) and *Piper aduncum* (inflorescences and leaves) extracts were tested against 8 strains of *Staphylococcus aureus*. These were isolated from milk samples taken from cows with subclinical bovine mastitis. The hexanic and chloroformic extracts of *Achyrocline satureioides* gave minimum bactericidal concentrations ranging from 14 to 2,400 µg/ml and from 56 to 675 µg/ml, respectively. *Piper aduncum* yielded a non-active hexanic extract, however its chloroformic extract gave minimum bactericidal concentrations from 237 to 3,800 µg/ml. Ethyl acetate, methanolic, and aqueous extracts did not show bactericidal activity within the range of concentrations tested.

Key Words: Plant extracts; bactericides; *Staphylococcus aureus*; bovine mastitis.

INTRODUCTION

The Brazilian Atlantic Coastal Forest ("Mata Atlântica") was an extensive heterogeneous forest that uses to occupy an area larger than 1,000,000 Km² along the Brazilian cost and covered about 12% of the country territory. As a consequence of progressive destruction and degradation brought about largely by anthropogenic activities, the remnant of the original forest occupies now a fragmented area that is less than 9% of the original cover (Fundação SOS Mata Atlântica, 1992). A direct consequence of deforestation is the reduction of plant genetic diversity, which decreases the possibilities for discovering novel natural compounds with useful biological activities. Current information about the traditional ethnomedicine employed by the vanishing indigenous people from South-eastern Brazil is particularly deficient and needs to be recorded with urgency. Sustained economic growth and conservation of biodiversity may be brought together more efficiently when the local communities get aware of the past, present, and future value of their surrounding natural resources from which valuable compounds may be derived.

Previous studies have shown that aqueous and hydroalcoholic preparations of macela (*Achyrocline satureioides*) have been used in South America mainly for their antispasmodic, antiinflamatory, and analgesic activities in the treatment of gastrointestinal problems (Pio Corrêa, 1969). These traditional applications have received scientific support of several pharmacological investigations, which attributes much of these

Recebido para publicação em 23.08.00 e aceito para publicação em 14.10.00

https://doi.org/10.70151/gm1yjk33

properties to the presence of flavonoids and terpenes (Ferraro *et al.*, 1981; Simões *et al.*, 1988; Filot da Silva & Langeloh, 1994; Rocha *et al.*, 1994).

It is not known to us whether or not jaborandi-falso (*Piper aduncum*) was once used by indigenous people of South-eastern Brazil for its antimicrobial properties. However, ethnomedical information from traditional communities of Papua New Guinea and Peru reported that drug preparation from this plant species has been used as external antiseptic and as antidiarrheac (Orjala et al., 1994; Macedo & Oviedo, 1987). Biological screenings of extracts from leaves led to the isolation and characterization of several antibacterial compounds (Okunade *et al.*, 1997; Orjala *et al.*, 1994).

The present study has a twofold objective: (1) to determine the bactericidal activities present in extracts obtained from *Achyrocline satureioides* and from *Piper aduncum*, two native plant species from the Atlantic Coastal domain; and (2) to compare the efficacy of the most active extracts when tested against strains of *Staphylococcus aureus* isolated from subclinical bovine mastitis.

MATERIAL AND METHOD

Plant material

Plants were collected in September/October 1995 from the Desengano State Park, State of Rio de Janeiro, Brazil. Approximate geographic position of the park is shown in Figure 1. *Achyrocline satureioides* (Lam.) DC. (Asteraceae) also known as "macela", was identified by Jean Kleber de Abreu Mattos (UnB, Brazil) and voucher specimen has being maintained at State University of Norte Fluminense (UENF) for future references. *Piper aduncum* L. (Piperaceae), commonly known as "jaborandi falso", was identified by Elsie Guimarães (Rio de Janeiro Botanical Garden, Brazil) and voucher specimens have been deposited at VIC Herbarium (Federal University of Viçosa, Brazil) with code number VIC-22700.

Extraction procedure

Inflorescences of *Achyrocline satureioides* were harvested at mid-maturation and exposed to 80 ºC for 30 minutes and then dried at 45 ºC and powdered. 96g of dried plant material was extracted by maceration at room temperature with hexane (1) and subsequently with chloroform (2), ethyl acetate (3), methanol (4) and water (5). After concentration under reduced pressure to dryness the extracts yielded residua AS1 (2.15 %), AS2 (1.89 %), AS3 (0.83 %), AS4 (1.90 %), and AS5 (8.12 %). Inflorescences and leaves of *Piper aduncum* were prepared using the procedure of maceration described above. In this case, 103 g of dried plant material was used to finally yield residua PA1 (3.43%), PA2 (6.13 %), PA3 (1.95 %), PA4 (2.24 %), and PA5 (12.38 %). All residua were kept at 4ºC until further use. A known amount of the each residuum was dispersed by agitation with sterile 0.2 % Tween 20. pH of each supernatant was determined (AS1, PA1, AS2, PA2, AS5 and PA5, 5,0; AS3 and PA3, 3,0; AS4 and PA4, 2,0) and adjusted to 7.0 with 0,1N NaOH. After pH neutralization the dispersions were homogenized by sonication in bath sonicator (50 Hz for 3 minutes) and by probe sonicator (60 Hz for 2 minutes) and filtered. For sterilization purposes the supernatants were then exposed to 3 cycles of heating (80 ºC) and cooling (8.0 ºC). The concentration of each dispersion was determined: AS1, 9.67 mg/ml; AS2, 11.33 mg/ml; AS3, 14.00 mg/ml; AS4, 39.67 mg/ml; AS5, 332.67 mg/ml; PA1, 46.00 mg/ml; PA2, 3.67 mg/ml; PA3, 11.67 mg/ml; PA4, 77.67 mg/ml; PA5, 348.00 mg/ml. The dispersions were then kept at 4 ºC until used in the bactericidal assay.

Bacterial strains

Two sets of bacteria were used in this study. The first set was composed of 4 reference strains. Three of them (*Staphylococcus aureus* 25923, *Escherichia coli* 25922, and *Pseudomonas aeruginosa* 27583) were obtained from the American type culture collection (ATCC). The forth reference strain (*Escherichia coli* DH-α), commonly used in genetic engineering, was obtained from the Laboratory for Biotechnology, at the Norte Fluminense State University, Campos dos Goytacazes (RJ). The second set was composed of 8 strains of *Staphylococcus aureus* isolated from milk samples of cows with bovine subclinical mastitis. Approximate origin of farms where milk samples were collected is depicted in Figure 1. The mastitic strains are now part of a collection stored in the Laboratory for Animal Science, at the Norte Fluminense State University, Campos dos Goytacazes (RJ).

Bactericidal assay

The assay was performed using the standard twofold dilution technique, according to Lennette et al. (1985). For microbiological tests, dispersions obtained from each plant extract were serially diluted in Muller-Hinton broth (Difco, USA). Inocula were prepared using bacterial suspension previously incubated in Muller-Hinton broth for 20 h at 37 ºC. The suspensions were adjusted to 0.5 Mac Farland Standard [about 10^8 colony forming units (CFU)/ml]. Testing tubes contained inocula and

serially diluted dispersions. Positive control tubes received inocula and 0.2 % Tween 20 aqueous solution instead of dispersion. Negative control tubes received dispersion at the highest concentration and broth, but no inocula. Control tubes and testing tubes were incubated at 37 ºC for 24 h and then visually observed for the presence or absence of turbidity. Bacterial suspension of each tube was then subcultured onto either a fresh blood agar media (for Gram positive bacteria) or eosin methylene-blue lactose sucrose media (for Gram negative bacteria). These Petri dishes were incubated at 37 ºC for 24 h in order to evaluate the possibility of further bacterial growth in the absence of plant-derived antibacterial agents. When growth was observed on subcultures, a smear was prepared and Gram staining was used for a preliminary evaluation of possible contaminant organisms in the sample. The minimum bactericidal concentration for each plant extract was recorded observing the subcultures in the Petri dishes and corresponded to the lowest concentration of dispersion that did not allowed the growth of any bacterial colony. All experiments were carried out in duplicates and the mean was recorded. All doses refer to the weight of the correspondent dried plant extract.

Susceptibility to antibiotics

Antibiograms of 12 standard antibiotics were obtained from Laborclin Ltda (Paraná, Brazil) and used to test the susceptibility of the strains *of S. aureus* according to the manufacture's instruction. Multidisc set A contained penicillin G (10 units), oxacillin (1 µg), tetracyclin (30 µg), amoxycillin (10 µg), clindamycin (2 µg), cephalothin (30 µg). Multidisc set B contained vancomycin (30 µg), ampicillin (10 µg), erythromycin (15 µg), sulfazotrin (25 µg), gentamycin (10 µg), cefoxitin (30 µg). All bacterial strains were grown in Muller-Hinton broth for 24 h at 37 ºC. Bacterial suspensions were adjusted to 0.5 Mac Farland Standard [about 10^8 colony forming units (CFU)/ml] with Muller-Hinton broth and used as inocula for the test. Multidisc sets were placed equidistantly in each Petri dish (12 cm) containing Mueller-Hinton agar media inoculated with the test organisms using sterile cotton swabs. The plates were incubated at 37 ºC for 24 h and then the zones of growth inhibition were measured (expressed in mm).

Statistical analysis

Data were subjected to conventional analysis of variance (ANOVA) using the SAS (1990) statistical package. Means separation was based either on F-tests or Tukey method of multiple comparations. Data were considered statistically significant at $P<0.05$.

RESULT AND DISCUSSION

A preliminary experiment was carried out to investigate the antibacterial activity of the plant extracts against four reference strains. Positive as well as negative control tubes were prepared throughout all the experiments to monitor, respectively, the presence and absence of microbial growth in the test system used. No antibacterial activity was detected for 0.2 % Tween 20 aqueous solution in positive control tubes. No bacterial growth was observed when dispersion at the highest concentration was incubated with broth in negative control tubes (data not shown). Table 1 shows the minimum bactericidal concentration obtained for the ten extracts tested in this experiment. All extracts were basically ineffective against the Gram-negative micro-organisms. The most active extracts against the Gram positive *Staphylococcus aureus* were the non-polar AS1, AS2 and PA2 that showed minimum bactericidal concentration values of 38, 354 and 459 µg/ml, respectively.

We have then investigated the anti-*Staphylococcus* activity of the two most non-polar extracts from each plant species. This investigation was carried out using samples from the same origin than those employed in the preliminary experiments. In order to prepare the extracts, the new samples were subjected this time to maceration with hexane followed by chloroform only. When newly prepared AS1; AS2; PA1; and PA2 were tested against the standard strain ATCC 25923 the new minimum bactericidal concentration values were 110; 112; 9,150; and 459 µg/ml; respectively (Table 2). Utilization of strain ATCC 25923 was very useful for comparative purposes. Despite the difference in absolute values from the results presented in Table 1, the relative effectiveness was kept unchanged. Hence, we were able to confirm the bactericidal activity of AS1, AS2 and PA2 we found in the preliminary experiment and once more demonstrated that PA1 is inactive. Reasons for the observed variation between minimum bactericidal concentrations obtained with different extracts from the same origin remain unclear. We attributed this variation to the methodology we employed during the preparation of plant extracts and to the bactericidal assay adopted. In this regard, the final concentration of active compounds in the

dispersions we prepared may be controled by a combination of diverse factors such as: efficiency of extraction during maceration, degradation of labile, active molecules during pH neutralization and cycles of cooling and heating, and non-uniform homogenization during preparation of aqueous dispersions.

TABLE 1. Minimal bactericidal concentration (µg/ml) of crude extracts of *Achyrocline satureioides* and *Piper aduncum* against standard bacterial strains.

Extract[1]	Reference strains			
	S.a. 25923	E.c. 25922	E.c. DH5-α	P. a. 27583
AS1	38	4,835	604	>4,835
AS2	354	2,833	708	>5,665
AS3	3,500	>7,000	875	>7,000
AS4	1,240	9,918	4,959	19,835
AS5	5,198	>166.3	20,790	166,330
PA1	2,875	>23,000	>23,000	>23,000
PA2	459	>1,835	>1,835	>1,835
PA3	2,920	>5,840	>5,840	>5,840
PA4	2,427	>38,830	>38,830	38,830
PA5	87,000	>174,000	>174,000	5,438

[1]Extracts obtained from *Achyrocline satureioides* (AS) and *Piper aduncum* (PA) by maceration with hexane (1) and subsequently with chloroform (2), ethyl acetate (3), methanol (4) and water (5). S.a., *Staphylococcus aureus*; E.c., *Escherichia coli*; P.a., *Pseudomonas aeruginosa*.

TABLE 2. Comparison of minimum bactericidal concentration (µg/ml) of hexanic and chloroformic extracts of *Achyrocline satureioides* and *Piper aduncum* against *Staphylococcus aureus* strains isolated from bovine subclinical mastitis.

Bacterial strains	Plant extract[1]			
	AS1	AS2	PA1	PA2
LSA007	450	112	>12,200	>3,800
LSA015	112	112	>12,200	950
LSA047	262	450	>12,200	3,800
LSA059	2,400	675	>12,200	950
LSA063	600	675	>12,200	475
LSA075	375	56	>12,200	237
LSA233	47	450	>12,200	475
LSA269	14	84	>12,200	237
ATCC 25923	110	112	9,150	237
LSD[2]	368.5	596.3	5,647	2,508

[1]Extracts obtained from *Achyrocline satureioides* (AS) and *Piper aduncum* (PA) by maceration with hexane (1) and subsequently with chloroform (2). [2]Least Significative Difference between means (Tukey, $P<0.05$).

The anti-*Staphylococcus* activity was evaluated against a set of 8 strains of *S. aureus* isolated from milk samples taken from cows with subclinical mastitis (Table 2). Approximate geographical origin of the mastitic strains is indicated in Figure 1. PA1 was completely unable to inhibit the growth of all mastitic strains even at the highest concentration used (12,200 µg/ml) and therefore its inactivity was established. Except to the inactive PA1, there was a high degree of variability among the sensitivity of the 8 mastitic strains to a given plant extract. Interestingly, PA2 was less effective against strains LSA007 and LSA047 then against any other mastitic strain. AS1 and AS2 were the most potent extracts and showed minimum bactericidal concentrations that ranged between 14 to 2,400 and between 56 to 675 µg/ml, respectively.

FIGURE 1. Geographical origin of the 8 mastitic strains of *Staphylococcus aureus*. Locations in northern Rio de Janeiro state and strains are respectively: 1, Macaé (LSA007); 2, São João da Barra (LSA015); 3, São Francisco do Itapaboana (LSA047); 4, Itaperuna (LSA059); 5, Santo Antônio de Pádua (LSA063); 6, Cardoso Moreira (LSA075); 7, Campos dos Goytacazes (LSA233); and 8, Bom Jesus do Itabapoana (LSA269). Desengano State Park where plant material for microbiological tests was collected is also shown.

We found during liquid dilution experiments that the employment of non-sterile dispersions of plant extracts resulted eventually in the proliferation of micro-organisms possessing unusual morphological characteristics (Gram staining not shown). We regarded the growth of this unexpected organism as the result of contamination. Sterilization of plant extracts by autoclaving or other strenuous methods should be avoided because of the risk of destroying of thermo-labile antimicrobial agents (Vanden Berghe & Vlietinck, 1991). Nevertheless we found that the contamination problem was eliminated when the aqueous dispersions were subjected to cycles of heating and cooling before testing. Therefore, our data demonstrated the bactericidal activity that remained after this treatment and that should be brought about by constituents that are either totally or at least partially heat-resistant. We also should take into account that the supernatant of each extract had its acidic pH adjusted to neutrality, which may have limited further the spectrum of active compounds responsible for the activity we have demonstrated. The main reason for pH adjustment is based on the fact that therapeutic agents that still remained active at physiological pH may be more attractive in approaches that relies on experimentation *in vivo*. The possibility that the killing action of any of our plant extract may be modulated by either heat treatment or pH deserves investigation.

An antibiogram was carried out to characterize further the susceptibility of the staphylococci strains to 12 pharmacological antibiotics (Table 3). At the concentration tested, all strains showed no resistance against agents that disrupt protein synthesis (tetracycline, erythromycin, gentamycin, and clindamycin) and against an inhibitor of DNA synthesis and cell growth (sulfazotrin, a sequential blocker that combines sulfamethoxazole and trimethoprim).

TABLE 3. Sensitivity of *Staphylococcus aureus* strains isolated from bovine subclinical mastitis to antibiotics (inhibition zone diameter in mm).

Antibiotic (µg/disc)	Strains								
	LSA 007	LSA 015	LSA 047	LSA 059	LSA 063	LSA 075	LSA 233	LSA 269	ATCC 25923
Polydiscs Set A									
Penicillin G 10*	ND	24.30^{R}	ND	22.30^{R}	21.00^{R}	ND	20.90^{R}	21.10^{R}	ND
Oxacillin 1	25.50^{S}	24.70^{S}	25.95^{S}	29.85^{S}	26.95^{S}	25.30^{S}	27.05^{S}	24.20^{S}	ND
Tetracyclin 30	33.50^{S}	37.00^{S}	35.20^{S}	29.75^{S}	30.45^{S}	32.85^{S}	31.70^{S}	28.40^{S}	37.90^{S}
Amoxycillin 10	23.95^{I}	17.45^{R}	30.20^{S}	17.35^{R}	16.35^{R}	29.35^{S}	17.20^{R}	13.95^{R}	40.10^{S}
Clindamycin 2	ND	38.05^{S}	ND	ND	33.40^{S}	33.35^{S}	36.20^{S}	33.90^{S}	36.75^{S}
Cephalothin 30	ND	35.40^{S}	ND	3220^{S}	33.70^{S}	ND	35.85^{S}	31.70^{S}	ND
Polydiscs Set B									
Vancomycin 30	19.55^{S}	23.40^{S}	21.45^{S}	2160^{S}	20.45^{S}	21.20^{S}	21.50^{S}	20.50^{S}	21.70^{S}
Ampicillin 10	29.20^{S}	23.85^{R}	38.85^{S}	22.95^{R}	23.10^{R}	41.70^{S}	24.50^{R}	20.10^{R}	46.55^{S}
Erythromycin 15	28.30^{S}	33.80^{S}	33.55^{S}	30.30^{S}	29.90^{S}	29.75^{S}	31.40^{S}	31.60^{S}	26.10^{S}
Sulfazotrin 25**	32.30^{S}	36.40^{S}	38.70^{S}	36.60^{S}	33.30^{S}	32.75^{S}	36.80^{S}	36.75^{S}	35.00^{S}
Gentamycin 10	25.50^{S}	29.70^{S}	30.40^{S}	25.85^{S}	26.80^{S}	27.35^{S}	28.10^{S}	38.25^{S}	26.35^{S}
Cefoxitin 30	31.45^{S}	37.10^{S}	53.05^{S}	37.75^{S}	38.65^{S}	34.90^{S}	33.60^{S}	30.85^{S}	44.95^{S}

*Penicillin G, 10 units/disc; **Sulfazotrin = Sulfamethoxazole + trimethoprim. ND, not determined due to overlapping of growth inhibition zones. ^{S}Susceptible, ^{R}Resistant, and ^{I}Intermediate, according do manufacture's instructions.

However, the susceptibility pattern to inhibitors of bacterial cell wall synthesis showed a high degree of variability among the strains. All of the mastitic strains were susceptible to β-lactamase-resistant antibiotics (the cephalosporin analogs cephalothin and cefoxitin, and the penicillinase-resistant penicillin, oxacillin). On the other hand, 5 mastitic strains were resistant to antibiotics that can be deactivated by the β-lactamase enzyme (penicillin G and the class I broad-spectrum agents, amoxycillin and ampicillin). Similar association of penicillin-G and ampicillin resistance and of tetracycline, erythromycin and sulfazotrin resistance has been previously reported in coagulase-negative staphylococci strains of bovine mastitis (Honkanen-Buzalski *et al.*, 1994). Among the strains resistant to penicillin G and other related antibiotics are LSA233 and LSA269, which were two of the most sensitive strains to AS1 and AS2. By other hand, strain LSA059 was considered resistant to these antibiotics and showed the highest level of resistance to the plant extracts tested. Taken together, these data suggest that the bactericidal activity found in the plant extracts may possess a mechanism of action that is not related to deactivation by β-lactamase enzymes. For this reason identification and further characterization of the active constituents from these plant species may contribute to the discovery of new anti-infective drugs with minimum cross-resistance to widely used antibiotics.

In conclusion, results from these experiments established that bactericidal activity is present in *Achyrocline satureioides* and in *Pipper aduncum*. The killing action of the non-polar extracts AS1, AS2 and PA2 was very effective against *Staphylococcus aureus* strains of veterinary importance and suggests the presence of either very potent anti-*Staphylococcus* agents or of high concentration of active compounds in specific organs of these two plant species.

ACKNOWLEDGMENT

This investigation received financial support from CNPq (Grant No 52.4030/94-4). G.C.S.L. and B.B.E. are indebted to FENORTE and CNPq, respectively, for fellowships. The authors are grateful to NAPRALERT for data search on *Achyrocline satureioides* and *Piper aduncum*.

REFERENCES

FERRARO, G.E., NORBERTO, C., COUSSIO, J.D. Polyphenols from *Achyrocline satureioides*. **Phytochemistry,** v.20, p.2053-54, 1981.

FILOT-DA-SILVA, L., LANGELOH, A. A comparative study of antispasmodic activity of hydroalcoholic 80% (V/V) extracts of *Achyrocline satureioides* (Lam.) DC. (Asteraceae) with papaverine and atropine on rat isolated jejunum. **Acta Farmacéutica Bonaerense,** v.13, p.35-40, 1994.

FUNDAÇÃO SOS MATA ATLÂNTICA. **Dossier:** Atlantic Rain Forest (Mata Atlântica). São Paulo, 1992.

HONKANEN-BUZALSKI, T., MYLLYS, V., PYÖRÄLÄ, S. Bovine clinical mastitis due to coagulase-negative Staphylococci and their susceptibility to antimicrobials. **Journal of Veterinary Medicine Bacteriology**, v.41, n. 5, p.344-50, 1994.

LENNETTE, E.H., BALOWS, A., HAUSLER, W.J., SHADOMY, J. H. **Manual of Clinical Microbiology.** Washington(DC): American Society of Microbiology, 1985.1149p.

MACEDO, J.C.B., OVIEDO, S.G. El aceite esencial de *Piper aduncum* L. 'Matico Hembra'. **Boletin de la Sociedad Química de Peru**, v.53, p.228-33. 1987

OKUNADE, A. L., HUFFORD C.D., CLARK A.M., LENTZ D. Antimicrobial properties of the constituents of *Piper aduncum*. **Phytotherapy Research,** v.11, p.142-4, 1997.

ORJALA, J., WRIGHT, A. D., BEHRENDS, H., FOLKERS G., STICHER, O., RÜEGGER, H., RALI, T. Cytotoxic and antibacterial dihydrochalcones from *Piper aduncum*. **Journal of Natural Products,** v.57, p.18-26, 1994.

PIO CORRÊA, M. **Dicionário das plantas úteis do Brasil e das exóticas cultivadas.** Rio de Janeiro: Imprensa Nacional, 1969. v.5

ROCHA, M.J.A., FULGENCIO, S.F., RABETTI, A.C., NICOLAU, M., POLI, A., SIMÕES, C.M.O., RIBEIRO-DO-VALLE, R.M. Effects of Hydroalcoholic extracts of *Portulaca pilosa* and *Achyrocline satureioides* on urinary sodium and potassium excretion. **Journal of Ethnopharmacology,** v.43, p.179-83, 1994.

SAS INSTITUTE. **SAS/STAT user´s guide**. Cary(NC): SAS Institute, 1990.

SIMÕES, C.M.O., SCHENKEL, E.P., BAUER, L., LANGELOH, A. Pharmacological investigations on *Achyrocline satureioides* (Lam.) DC. Compositae. **Journal of Ethnopharmacology,** v.22, n.3, p.281-93, 1988.

VAN DEN BERGHE, D.A., VLIETINCK, A.J. Screening methods for antibacterial and antiviral agents from higher plants. In: DEY, P.M., HARBORNE, J.B. (Ed.) **Methods in Plant Biochemistry.** London: Academic Press, 1991. v.6, p.47-69.