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In vitro antimicrobial activity of the organic extract of *Cladonia substellata* Vainio and usnic acid against *Staphylococcus* spp. obtained from cats and dogs[1]

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ABSTRACT.- Moura J.B., Vargas A.C., Gouveia G.V., Gouveia J.J.S., Ramos-Júnior J.C., Botton S.A., Pereira E.C. & Costa M.M. 2017. ***In vitro* antimicrobial activity of the organic extract of *Cladonia substellata* Vainio and usnic acid against *Staphylococcus* spp. obtained from cats and dogs.** *Pesquisa Veterinária Brasileira 37(4):368-378.* Laboratório de Microbiologia e Imunologia Animal, Universidade Federal do Vale do São Francisco, Campus de Ciências Rurais, BR-407 Km 12, Lote 543, Projeto de Irrigação Nilo Coelho s/n, "C1", Petrolina, PE 56300-990, Brazil. E-mail: juscikoelho@hotmail.com

Cladonia substellata Vainio is a lichen found in different regions of the world, including the Northeast of Brazil. It contains several secondary metabolites with biological activity, including usnic acid, which has exhibited a wide range of biological activities. The aim of this study was to evaluate the *in vitro* antimicrobial activity of the organic extract of *C. substellata* and purified usnic acid. Initially, *Staphylococcus* spp., derived from samples of skin and ears of dogs and cats with suspected pyoderma and otitis, were isolated and analyzed. In antimicrobial susceptibility testing against *Staphylococcus* spp., 77% (105/136) of the isolates were resistant to the antimicrobials tested. In the assessment of biofilm production, 83% (113/136) were classified as producing biofilm. In genetic characterization, 32% (44/136) were positive for *bla*Z, no isolate (0/136) was positive for the *mec*A gene, and 2% (3/136) were positive for the *ica*D gene. The *in vitro* antimicrobial activity of the organic extract of *C. substellata* and purified usnic acid against *Staphylococcus* spp. ranged from 0.25mg/mL to 0.0019mg/mL, inhibiting bacterial growth at low concentrations. The substances were more effective against biofilm-producing bacteria (0.65mg/mL-0.42mg/mL) when compared to non-biofilm producing bacteria (2.52mg/mL-2.71mg/mL). Usnic acid and the organic extract of *C. substellata* can be effective in the treatment of pyoderma and otitis in dogs and cats caused by *Staphylococcus* spp.

INDEX TERMS: Antimicrobial activity, *Cladonia substellata,* lichens, usnic acid, *Staphylococcus* spp., cats, dogs, bacteria, biofilm, genes.

RESUMO.- [**Atividade antimicrobiana *in vitro* do extrato orgânico de *Cladonia substellata* Vainio e ácido úsnico frente *Staphylococcus* spp. obtidos de cães e gatos.**] *Cladonia substellata* Vainio é um líquen encontrado em diversos continentes do mundo, inclusive no nordeste do Brasil, possui vários metabólitos secundários com atividade biológica, entre eles, o ácido úsnico, que tem apresentado uma vasta gama de atividades biológicas. O objetivo deste trabalho foi avaliar a atividade antimicrobiana *in vitro* do extrato orgânico de *C. substellata* e do ácido úsnico purificado.

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Para isto, foram isolados *Staphylococcus* spp. de amostras de pele e orelha de cães e gatos com suspeita de piodermatite e otite. No teste de sensibilidade aos antimicrobianos frente *Staphylococcus* spp., 77% (105/136) foram resistentes. Na avaliação da produção de biofilme 83% (113/136) foram classificadas como produtoras de biofilme. Na caracterização genotípica, 32% (44/136) foram positivos para o gene *bla*Z, nenhum isolado (n=136) foi positivo para o gene *mec*A, e 2% (3/136) foram positivos para o gene *ica*D. A atividade antimicrobiana *in vitro* do extrato orgânico de *C. substellata* e do ácido úsnico purificado para *Staphylococcus* spp. variou de 0,25mg/ml a 0,0019mg/ml, inibindo o crescimento bacteriano em baixas concentrações. Foram mais eficazes contra bactérias produtoras de biofilme (0,65mg/ml–0,42mg/ml) quando comparadas às não produtoras de biofilme (2,52mg/ml-2,71mg/ml). Viabilizando a utilização do ácido úsnico e do extrato orgânico de *C. substellata,* no tratamento de otite e piodermatite em cães e gatos com o envolvimento de *Staphylococcus* spp.

TERMOS DE INDEXAÇÃO: Atividade antimicrobiana, *Cladonia substellata,* liquens, ácido úsnico, *Staphylococcus* spp., cães, gatos, antimicrobianos, bactérias, biofilme, genes.

INTRODUCTION

Usnic acid is a yellow substance found only in lichens (Carvalho et al. 2005) and has a strong hydrophobic nature, being derived from lichen secondary metabolites. It also provides lichen with protection against several agents that cause injuries including microorganisms, UV rays, and dryness (Cochietto et al. 2002). Furthermore, it is abundant in many phylogenetically distinct genera of lichens, such as *Usnea*, *Cladonia*, *Cetraria*, *Parmelia*, and *Ramalina* (Venkataramana & Krishna 1992, Müller 2001, Cochietto et al. 2002, Ingólfsdóttir 2002, Carvalho et al. 2005). Usnic acid was first isolated in1834 from *Ramalina calicaris,* of *Usnea barbata* and other species of lichens (Rochleder & Heldt 1996).

Usnic acid has exhibited a wide range of biological activities, such as antimicrobial, antifungal, anticancer, antiviral, antipyretic, anti-inflammatory, analgesic, and anti-protozoal activities. It also acts as an insecticide, moisturizer, antihistamine, and agent that offers protection from the harmful effects of lightning UV (Fournet et al. 1997, Cochietto et al. 2002, Ingólfsdóttir 2002, Falcão et al. 2004, Ribeiro et al. 2006, Honda et al. 2010). In humans, a hepatotoxic effect was observed when it was used systemically (Moura et al. 2008). However, it had already been in use topically for medicinal purposes, perfumery, and ecological applications in several formulations, as well as for creams, toothpaste, oral antiseptics, deodorants, and sunscreen products (Ingólfsdóttir 2002). These many possible topical applications suggest future studies to verify the use of usnic acid in animal dermatology.

Veterinary dermatology is responsible for 20 to 75% of veterinary care (Scott et al. 2001, Cardoso et al. 2011) and otological problems represent 8 to 15% of disorders in veterinary clinic in Brazil (Leite 2000). In addition, dermatopathology cases may include primary and secondary bacterial infections (Meneses et al. 2000, Cardoso et al. 2011, Muller et al. 1986) commonly associated with *Staphylococccus pseudintermedius*, *S. aureus*, and *S. schleiferi* (Huerta et al. 2011).

In this study, we evaluated antimicrobial susceptibility, biofilm production, and identification of *Bla*Z *mec*A and *ica*D genes in *Staphylococcus* spp. isolated from the skin and ears of dogs and cats with otitis and pyodermitis. In addition, we assessed the *in vitro* antimicrobial activity of the extract of *C. substellata* and usnic acid against all the *Staphylococcus* spp. isolates.

MATERIALS AND METHODS

Bacterial isolates. One hundred thirty-six isolates of *Staphylococcus* spp. were obtained from pyoderma and otitis in dogs and cats in the Brazilian states of Pernambuco (PE) and Rio Grande do Sul (RS) from 1990 to 2012 (Table 1).

From a total of 136 isolates of *Staphylococcus* spp., 110 were obtained in Petrolina, PE, from private clinics and the veterinary hospital at Univasf in 2011 to 2012. The samples were collected with sterile swabs, kept in a modified Stuart medium under refri-

Table 1. Characteristics of isolates of *Staphylococcus* spp. (n=136) of dogs and cats

Species - n[a] (%)	Source (n)	Antimicrobial resistance	*mec*A[b,f] n (%)	blaZ[c] n (%)	Biofilm n (%) IcaD[d]	Biofilm n (%) BPP[e]
Staphylococcus aureus 119 (87.5%)	Skin of cats (06) Ear of dogs (46) Skin of dogs (67)	AMP: 61, PEN: 72, AMC: 01, OXA: 05, EST: 31, GEN: 15, ENO, 12, ERI: 36, SUT: 41, TET: 47, CFE: 10, CFL: 09, CRO: 12, CLIND: 30, CLO: 07	-	41 (30%)	03 (2%)	97 (71%)
Staphylococcus intermedius 11 (8%)	Skin of cats (01) Skin of dogs (07) Ear of dogs (03)	AMP: 07, PEN: 07, EST: 02, ERI: 02, SUT: 04, TET: 06, CFE: 01, CFL: 01, CRO: 01, CLIND: 02, CLO:02	-	03 (2%)	-	10 (7%)
Staphylococcus spp. 06 (4.5%)	Ear of cats (01) Skin of cats (03) Skin of dogs (02)	AMP: 04, PEN: 05, EST: 01, GEN: 01, ERI: 02, SUT: 04, TET: 04, CFE: 01, CFL: 01, CRO: 02, CLIND: 02	-	-	-	06 (4%)
TOTAL 136 (100%)	125 / dogs 11 / cats	AMP: 72, PEN: 84, AMC: 01, OXA: 05, EST: 34, GEN:16, ENO: 12, ERI: 40, SUT: 49, TET: 57, CFE: 11, CFL: 11, CRO: 15, CLIND: 34, CLO: 11	-	44 (32%)	03 (2%)	113 (83%)

[a] Total number of each isolate, [b,c] genes related to the bacterial virulence factor, [d] gene associated with bacterial biofilm production, [e] quantitative technique to assess biofilm production in microplates, [f] Not detected/absent- AMP (10µg) ampicillin, PEN (10µg) penicillin, AMC (30µg) amoxicillin+clavulanic acid, OXA (1µg) oxacillin, EST (10µg) streptomycin, GEN (10µg) gentamicin, ENO (5µg) enrofloxacin, ERI (15µg) eritromicin, SUT (25µg) Sulfamethoxazole, TET (30µg) tetracycline, CFE (30µg) cephalexin, CFL (30µg) cephalothin, CRO (30µg) ceftriaxone, CLIND (2µg) clindamycin, CLO (30µg) chloramphenicol.

geration, and sent within 24 hours after collection to the Animal Microbiology and Immunology Laboratory at Univasf (Petrolina/PE). The other 26 isolates were provided by the Laboratory of Bacteriology (Labac) of the Department of Preventive Veterinary Medicine, Universidade Federal de Santa Maria (UFSM); these samples were collected from the years 1990 to 1995 in three different municipalities in southern Brazil, as Santa Maria (RS), Santa Cruz do Sul (RS), and Caçapava do Sul (RS). The isolates were first identified, freeze dried, and refrigerated at Labac, and then sent chilled to the Animal Microbiology and Immunology Laboratory at Univasf in 2011.

Source of the lichen *Cladonia substellata* and usnic acid. The lichen *C. substellata*, source of the organic extract and usnic acid, was collected on sandy soils of the coastal tablelands of Mamanguape of Paraíba, which corresponds to the northeastern Coastal Plains area. It was identified through the morphological and chemical characteristics of its stem. Subsequently, organic extracts and purified usnic acid were obtained in the Laboratório de Produtos Naturais (Natural Products Laboratory) at UFPE (Universidade Federal de Pernambuco).

Preparation of organic extract of *C. substellata* and isolation of usnic acid. Organic extracts were obtained by an exhaust system at room temperature (28±3°C) from dry lichen thallus (20g) following the ether, chloroform, and acetone series. The material was ground in a mortar and extracted with 100mL of ethyl ether under stirring for 1h, and then maintained at 6°C for 24h. After this period, the material was filtered and the residue extracted with the same volume of chloroform. Then, after filtration, the residue was extracted again with acetone under the same conditions. The extracts were then evaporated at room temperature and kept in a desiccator until constant weight (Nóbrega et al. 2012). The usnic acid was isolated and purified by the method of recrystallization from diethyl ether extract (Pereira 1998) in the Natural Products Laboratory of the UFPE.

Thin Layer Chromatography (TLC). The ethereal extract, chloroform, and acetone were subjected to ascending chromatography on silica gel plates 60 F254 + 366 (Merck), 20×20 cm, and were developed in the solvent systems A (toluene/dioxane/acetic acid system 180: 45: 5v/v) and B (hexane/diethyl ether/formic acid 130: 80: 20v/v) (Culberson, 1972). The bands obtained were visualized under UV light at 254nm and 366nm. The chromatoplates were then stained with 10% H_2SO_4 and heated at 100°C for 10 min. The highlighted bands were compared by staining reaction and values of Rf (Retention Factor), with patterns of usnic acid applied as standards.

Bacterial identification

Morphological and biochemical identification. Bacterial isolates from samples of pyoderma and otitis in dogs and cats were kept in the Animal Microbiology and Immunology Laboratory of Univasf in Petrolina/PE, Brazil. The samples were smeared on 5% blood agar using a platinum loop and incubated at 37°C, and the plates were read at 24h and 48h. Bacterial agents were identified by colonial, biochemical, and morphological characteristics (Mannitol semi-solid, semi-solid glucose, maltose Purple Agar Base, urease, catalase, oxidase, coagulase with DNase and rabbit plasma in a tube), and stained (Gram Stain) (Quinn et al. 2005). Coagulase enzyme production divided staphylococci into two groups: coagulase-positive and coagulase-negative (Ziebuhr et al. 2006).

Molecular identification. Total bacterial DNA was extracted and purified following the protocols described by Wade et al. (2005) and Ausubel et al. (1987) with modifications. An inoculum of each strain was placed in 300µL of TE (Tris-EDTA) and heat inactivated (80°C/10 min) in dry bath. After that, we added 70µL of 10% SDS, with subsequent addition of 100µL of 5M $NaCl_2$ and 80µL CTAB/NaCl. This solution was incubated at 65°C for 10 min. Then, 700µL of chloroform/isoamyl alcohol (24:1) was added, homogenized by inversion, and centrifuged at 11,750g for 5 min. Subsequently, the liquid from the 1st stage was transferred to another clear tube and 450µl of isopropanol was added. The tube was kept on ice for 20 min and centrifuged at 11,750g for 15 min, discarding the supernatant and adding 500µL of 70% ethanol, spun at 11,750g for 10 min. The supernatant was discarded and the pellet was dried at room temperature. It was then resuspended in 80µL of TE (pH 8.0) and incubated at 65°C for 1 min. Total DNA was stored at -20°C.

Staphilococcus aureus molecular characterization was performed by identifying a fragment of the nuc gene (279pb) with the primers F-GCGATTGATGGTGATACGGTT 5'-3' and 5'-R--AGCCAAGCCTTGACGAACTAAAGC-3' (Kateete et al. 2010). The non-quantified DNA template (4µL) extracted from bacterial strains was added to 11µL of mix containing 0.66µM of each primer, 0.4mM dNTPs, 1X diluted enzyme buffer, 2mM MgCl2, and 1.5U Taq DNA polymerase. Amplifications were performed in a thermocycler (AMPLITHERM TX96 Plus + G) with an initial denaturation at 94°C for 5 min, followed by 37 cycles, each consisting of 94°C for 1 min, primer annealing at 55°C for 30 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min (adapted from Kateete et al. 2010). *S. epidermidis* was identified by a fragment of 130pb of rdr gene with the primers SERF-5'AAGAGCGTGGAGAAAAGTATCAAG3' and SERR--5'TCGATACCATCAAAAAGTTGG3' (Shome et al. 2011). The non--quantified DNA template (5µL) extracted from bacterial strains was added to 20µL of mix containing 0.5µM primer, 0.4mM dNTPs, 1X diluted enzyme buffer, 1.5mM $MgCl_2$, and 2.5U Taq DNA polymerase. Amplifications were performed in a thermocycler (AMPLITHERM TX96 Plus + G) with an initial denaturation at 94°C for 5 min, followed by 30 cycles, each consisting of denaturation at 94°C for 30 s, primer annealing at 60 °C for 30 s and extension at 72°C for 45 s, followed by a final extension at 72°C for 10 min (adapted from Shome et al. 2011). The PCR products (5 µL) were verified in 1.5% agarose gel, stained with ethidium bromide, and documented by the Kodak Digital Science 1D Capture imaging system. The controls used were *S. aureus* ATCC 25923 (nuc) and *S. epidermids* ATCC 12228 (rdr) (positive control DNA) and nuclease-free water (negative control).

Antimicrobial susceptibility testing

Phenotypic test. The sensitivity profile of the microorganisms was determined by the modified Kirby-Bauer disk diffusion method (Bauer et al. 1996). The isolates were seeded into Müller-Hinton broth and incubated at 37°C to obtain a turbidity of 0.5 McFarland scale. The isolates were smeared on Petri dishes containing Müller-Hinton agar. The disks were then impregnated with the following groups/antimicrobial drugs: i) beta-lactams: ampicillin (AMP 10µg), penicillin (PEN 10µg), amoxicillin-clavulanic acid (AMC 30µg) and oxacillin (OXA 1µg); ii) aminoglycosides: streptomycin (10µg EST), and gentamicin (GEN 10µg); iii) quinolones: enrofloxacin (ENO 5µg); iv) Macrolides: erythromycin (15µg ERI); v) sulfonamides: sulfametaxona (SUT 25µg); vi) tetracyclines: tetracycline (30µg TET); vii) cephalexin: cephalexin (30µg CFE), cephalothin (30µg CFL), ceftriaxone (CRO 30µg); viii) lincosamides: clindamycin (CLIND 2µg); and ix) amphenicols: chloramphenicol (30µg CLO) (CLSI 2008). The plates were incubated at 37°C for 24 h. The halos were then measured and the sensitivity profile of the isolates was determined following the classifications of sensitive, intermediate, and resistant, according to the document M31-A3 (CLSI, 2008). The Multiple Antibiotic Resistance Index (MARI) was calculated according to the methodol-

ogy described by Schwarz et al. (2010). This index is determined by the ratio between the number of antimicrobials that the sample was resistant to and the total number of drugs from the group of β-lactams tested.

Genotypic test. Molecular confirmation of the presence of genes linked to resistance to the β-lactam group was assessed by amplification of *mec*A and *bla*Z gene fragments. The primers used for amplification of 217pb of the *mec*A gene were F-5'AAAATCGATGGTAAAGGTTGGC3' and R-5'AGTTCTGCAGTACCGGATTTGC3' (Coelho et al. 2007); and for amplification of 517pb of the *bla*Z gene were F-5'AAGAGATTTGCCTATGCTTC3' and R-5'GCTTGACCACTTTTATCAGC3' (Sawant et al. 2009). The PCR reaction of the *mecA* gene was DNA template (~100-200ng), 0.4μM of each primer, 0.4mM of dNTP, 1X enzyme buffer, 2mM $MgCl_2$, and 1.5U Taq in a final volume of 15μL. This reaction was placed in a thermocycler (AMPLITHERM TX96 Plus + G) and subjected to initial denaturation at 94°C for 1 min, followed by 15 cycles at 94°C for 30s, 68°C for 30s, and 72°C for 30s, followed by 20 cycles at 94ºC for 30s, 60ºC for 30s, 72°C for 30s, and a final extension at 72°C for 2 min (adapted from Coelho et al. 2007). The PCR for the *bla*Z gene was the same used for the *mecA* gene. However, the thermal conditions were initial denaturation at 94°C for 4min, followed by 30 cycles at 94°C for 1 min, 50.5ºC for 30s, 72°C for 30s, and a final extension at 72°C for 5 min (Sawant et al. 2009).

The PCR products were analyzed in 1.5% agarose gel, stained with ethidium bromide, and documented by the image capturing system (Kodak 1D Digital Science) (adapted from Coelho et al. 2007, Sawant et al. 2009).

Characterization of biofilm production in *Staphylococcus* spp.

Phenotypic characterization. Quantitative analysis of *Staphylococcus* spp. biofilm formation (n=136) in the 96-well polystyrene microplates was performed according to Merino et al. (2009) with modifications. In the phenotypic characterization of biofilm formation, *Staphylococcus* spp. isolates were previously identified and chilled. The bacteria were seeded into Tryptic Soy Agar (TSA) and incubated at 37°C for 24h. Single colonies were inoculated in 3mL of Trypticase Soy Broth (TSB) until a turbidity of 0.5 McFarland, and incubated at 37°C for 24h. Then, 5μl were transferred to microdilution plates containing TSB (195μL) and incubated at 37°C for 24h. They were rinsed three times with 200μL of distilled water and dried at room temperature (RT). The plates were then stained with 0.25% violet crystal (100μL) for 3 min at RT. After that, the plates were rinsed three times with distilled water. To dissolve the dye, an alcohol-acetone (80:20) solution (200μL) was used. Absorbance was measured in an ELISA plate reader using a 620nm filter. All analyses were performed in triplicate, using positive and negative controls. *S. aureus* (ATCC 25923), previously characterized genotypically for biofilm production, was used for positive control. After reading, the samples were classified into four categories: non-adherent (0) when the OD (Optical Density) obtained is less than the OD of the negative control (ODc); weakly adherent (+):ODc <OD ≤2x ODc; moderately adherent (+ +): 2x ODc <OD ≤4x Doc; and strongly adherent (+ + +): 4xODc ≤OD (adapted from Merino et al. 2003). All assays were performed three times.

Genotypic characterization. To amplify the *Ica*D gene, we used the primers ICADF (5'AAA AAG AGA CGT GGT GG3 ') and ICADR (5'GGC ATC ATG AAT ATA AAG C3'), which amplified a fragment of 381pb (Vasudevan et al. 2003). The PCR reaction consisted of 1.33mM $MgCl_2$, 0.2mM dNTP, 1μM of each primer, 1.5U Taq polymerase, and 2μL of DNA template (~100ng) ("bacterial and molecular identification") in a final volume of 15μL. This reaction was placed in a thermocycler (AMPLITHERM TX96 Plus + G) and the thermal conditions were: initial denaturation at 94°C for 2min, followed by 30 cycles at 94°C for 2 min, at 92°C for 45s, and at 72°C for 1 min, with a final extension at 72°C for 7 min. The PCR products were analyzed in 1.5% agarose gel, stained with ethidium bromide, and documented by the image capturing system (Kodak 1D Digital Science) (adapted from Vasudevan et al. 2003). *S. aureus* ATCC 25923 was used as a positive control and nuclease-free water as a negative control.

Determination of the antibacterial activity of the organic extract of *C. substellata* and usnic acid against *Staphylococcus* spp. To determinate the *in vitro* antibacterial activity of the organic extract of *C. substellata* and purified usnic acid against 136 *Staphylococcus* spp. isolates, the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were established based on the CLSI document, using the following concentrations: 0.25mg/mL, 0.125mg/mL, 0.0625mg/mL, 0.0312mg/mL, 0.0156mg/mL, 0.0078mg/mL, 0.0039mg/mL, and 0.0019mg/mL, in triplicate, in accordance with the document M100-S20 (CLSI 2010).

An inoculum of a bacterial suspension was prepared in Mueller-Hinton agar with turbidity equivalent to 0.5 on the McFarland scale. The lichen extracts were diluted to a concentration of 0.50mg/mL in 200μL (1:2) of Mueller-Hinton Broth (MH). This suspension was inoculated (10μL) ($1x10^4$ UFC/mL) in each well containing a dilution of each extract. The plates were incubated at 37°C for 24h under aerobic conditions and the sequence was determined; with MIC_{50} and MIC_{100} being considered as partial and complete visual inhibition of bacterial growth. For the dilutions where no visible bacterial growth was observed, an aliquot (10μL) was removed and seeded on Mueller-Hinton agar (MH agar). The material was incubated at 37°C for 24h. The MBC was determined as the lowest concentration of the extract under study capable of causing the death of the microorganism tested. All assays were performed in triplicate.

Interaction of the organic extract of *C. substellata* and purified usnic acid with the consolidated bacterial biofilm. Nineteen isolates of *Staphylococcus* spp. previously identified as biofilm producers were evaluated in this study. Biofilm formation in microplates was obtained from incubation of the bacterial suspension (inoculum) (100μl) in each well of the 96-well plate. The plates were incubated at 37°C for 24h. After that, the wells were rinsed three times with distilled water. Then, 100μl of the organic extract of *C. substellata* and purified usnic acid at a concentration of 0.25mg/mL were added. This concentration was capable of inhibiting bacterial growth of all 136 *Staphylococcus* spp. tested (MIC_{100} and MBC_{100}). The optical density (OD) was determined immediately after addition of the extract (0 h) and after 24h. Interference in the consolidated biofilm was defined by the equation: OD_{0h} mean/OD_{24h} mean x 100 (Adapted from Nostro et al. 2007). All assays were performed in triplicate.

Interaction of the organic extract of *C. substellata* and purified usnic acid with bacterial biofilm formation. A concentration of 0.25mg/mL of organic extract of *C. substellata* and purified usnic acid (concentration able to inhibit the bacterial growth of 136 *Staphylococcus* spp. tested at MIC_{100} and MBC_{100}) was used on the biofilm in formation. This assay was performed in 96-well microplates. Bacterial inoculum were cultured in 10mL of TSB with 1% of glucose at 1% and kept at 37°C for 24h. A quantity of 100μl was added to the wells of the plate, to which 100μl of extract and 100μl of culture medium in the controls had previously been added. After 24h of incubation at 37°C, the plates were stained with crystal violet. The effectiveness of the extract in interfering with biofilm formation was defined by the equation: mean OD of treated well/ mean OD of control wells x 100 (adapted from Nostro et al. 2007). Nineteen samples of biofilm-producing *Staphylococcus* spp. (which were collected from pyodermatitis in dogs (16) and

cats (03) in 2011-2013 in Petrolina, PE, Brazil) were evaluated in regard to interaction of the consolidated biofilm against organic extract of *C. substellata* and purified usnic acid at a concentration of 0.25 mg/mL. All assays were performed in triplicate.

Statistical analyses. Statistical analysis was performed using the Fisher exact test for analysis of correlation between biofilm production and the presence of the *ica*D gene, as well as the relationship between the presence or absence of the *bla*Z gene for resistance or susceptibility to β-lactam antibiotics (ampicillin, penicillin, amoxicillin+clavulanic acid, and oxacillin) (Reis 1998). The Wilcoxon test was used to verify differences between ordered pairs for comparison of the antimicrobial effect of the organic extract of *C. substelatta* and purified usnic acid (Sampaio 2007). All analyses were performed using the R statistical software (R Core Team 2012).

RESULTS AND DISCUSSION

Bacterial identification

One hundred and thirty-six isolates of *Staphylococcus* spp. (Table 2) were classified as Gram-positive, catalase-positive and oxidase-negative. From these, 116 were classified as coagulase-positive and 20 were coagulase-negative. Through biochemical tests, it was possible to identify 70 *S. aureus*, 11 *S. intermedius*, 46 *Staphylococcus* spp. coagulase-positive and 20 *Staphylococcus* spp. coagulase-negative isolates. To identify *S. aureus* and *S. epidermids*, *nuc* and *rdr* genes, respectively, were evaluated by PCR. One hundred and nineteen isolates were positive for the *nuc* gene (*S. aureus*), in the absence of amplification of the *rdr* gene (*S. epidermidis*); five isolates were classified as *Staphylococcus* coagulase-positive, and one as *Staphylococcus* coagulase-negative.

From the 119 *S. aureus* isolates, 18 were coagulase-negative and six DNase-negative by biochemical testing. PCR showed greater sensitivity and specificity in the identification of *S. aureus* by *nuc* gene amplification as compared to biochemical testing. Furthermore, 15% (18/119) were negative in the coagulase test, similar to the findings of Koneman et al. 1997. It should be noted that rare strains of *S. aureus* might be coagulase-negative. In addition, 5% (6/119) of the isolates were negative by the DNase test, which was also reported by Rao et al. (2002) and Kateete et al. (2010). Other biochemical tests included mannitol semi-solid medium, semi-solid glucose medium, maltose plus purple agar base, urease, catalase, and oxidase. There was no difference in identification of *S. aureus* through biochemical results as compared to PCR.

The isolates from pyodermatitis and otitis of dogs and cats were predominantely *S. aureus* 87.5% (119/136) and *S. intermedius* 8% (11/136). According to Frank et al. (2003), *S. aureus* is more prevalent in humans, while *S. intermedius* and *S. schleiferi* are more prevalent in dogs. In this study, among the 50 isolates of otitis (49 dogs), 6% were identified as *S. intermedius* and 94% were *S. aureus*. The results obtainded in this study also disagree with results from Cole et al. (1998) and Lilenbaum et al. (2000), which showed that *S. intermedius* was the main bacterial agent isolated from otopathologies in dogs. The possible occurrence of a zoonotic transmission of *Staphylococcus* spp. strains from pets to humans has already been described in the literature (Tanner et al. 2000, Cizman 2003, Kikuchi & Ohshima 2004, Oliveira et al. 2005). However, a possible bacterial inverse migration from man to animal could explain the high occurrence of *S. aureus* in skin and otologic diseases described in this study.

Table 2. Interaction of organic extract (*Cladonia substellata* Vainio) and purified usnic acid with biofilm (*Staphylococcus* spp.)

Extract/Fraction[a]	Inhibition of biofilm in formation - % (n)	Inhibition of consolidated biofilm - % (n)
Organic extract	42 (8/19)	47 (9/19)
Usnic acid	37(7/19)	58 (11/19)

[a] Concentration evaluated 0.25mg/mL.

Antimicrobial susceptibility tests

Phenotypic test. Nine classes of antimicrobials were tested: β-lactams, aminoglycosides, quinolones, macrolides, sulfonamides, tetracyclines, cephalexin, lincosamides, and amphenicols. Thirty-two percent of the isolates (44/136) were resistant to more than four classes of antimicrobials, 10% (13/136) to three classes, 16% (22/136) to two classes, 19% (26/136) to only one class, and 23% (31/136) showed no resistance to any antimicrobial tested.

From the 136 isolates, 70 were resistant to more than two antimicrobial groups of β-lactam antibiotics (ampicillin, penicillin, amoxicillin+ clavulanic acid, and oxacillin) (Multiple Antibiotic Resistance Index, MARI ≥ 0.5), 13 isolates showed resistance only to penicillin, two isolates were resistant to ampicillin, and 51 isolates showed no resistance to any antimicrobial tested *in vitro*. The highest resistance of the isolates was observed against β-lactams; the sensitivity of the isolates to these drugs was verified in 37% (50/136) of the bacteria. Within this group, amoxicillin associated with clavulanate potassium and oxacillin showed efficiency exceeding 96%. In contrast, the antimicrobial groups with greatest activity against *Staphylococcus* spp. were amphenicols, quinolones, and cephalexin, to which the isolates exhibited sensitivity greater than or equal to 80% (109/136). Meng & Doyle (1998) cited in their study that drug resistance may be related to the excessive and oftentimes irresponsible use of antibiotics. This fact could explain the high resistance to the β-lactam antibiotics, which have been used in veterinary medicine, especially for treatment of diseases involving respiratory, orthopedic, urinary, respiratory, and dermatological systems.

Low resistance against quinolones was also described in previous studies (Junco & Barrasa 2002, Hoekstra & Paulton 2002). Junco & Barrasa (2002) warned that indiscriminate use of fluoroquinolones and, in particular, enrofloxacin, could result in an increase in bacterial resistance. Concerning the cephalosporin group, other authors reported over 90% susceptibility of *Staphylococcus* spp. isolated from pets (Bornand 1992, Cole et al. 1998, Oliveira et al. 2006, Santos 2007). Over 80% susceptibility of *Staphylococcus* spp. to chloramphenicol is reported by Oliveira et al. (2000) and Batista et al. (1998). However, Vandžurová et al. (2013) described strains of *S. nepalensis* resistant to chloramphenicol.

Antimicrobial susceptibility tests

Genotypic test. From the 136 isolates of *Staphylococcus* spp. assessed by PCR, 32% (44/136) were positive for the *bla*Z gene. Forty were identified as *S. aureus*, three as *S. intermedius*, and only one as coagulase positive *Staphylococcus*. All were negative in amplification of the *mec*A gene. The Fisher exact test did not identify any relationship 0% (0/136) between the presence of the *bla*Z gene and *in vitro* resistance to the beta-lactam group of antibiotics (Reis 1998).

In those isolates that were positive for the *bla*Z gene, there may be the presence of a bacterial resistance mechanism to produce β-lactamases, enzymes that hydrolytically destroy β-lactam compounds (Fuda et al. 2005) on the sites where β-lactam antimicrobials bind to bacterial PBPs (Penicillin Binding Protein), exercising the antimicrobial effect (Oliveira 2011). In this study, using PCR, it was possible to identify a large number of *Staphylococcus* spp. resistant to β-lactam antimicrobials through the presence of the *bla*Z gene. These values are higher than those found by Schlotter et al. (2013). The authors detected 11.1% (21 samples isolated from bovine milk) of *Staphylococcus* spp. that were penicillin-resistant *in vitro* and positive for the *bla*Z gene in a PCR assay. Livorsi et al. (2012) isolated 77% of *S. aureus* (human bloodstream) resistant to the β-lactam group, although susceptible to methicillin (Methicillin-Sensitive *Staphylococcus aureus* - MSSA), and which had the *bla*Z gene, confirmed by the PCR technique in the *S. aureus* isolates.

From the isolates positive for the *bla*Z gene, 12 were sensitive to β-lactam antibiotics *in vitro* and 32 samples were multiresistant (penicillin and ampicillin). From these, one sample of *S. aureus* was also resistant to amoxicillin associated with potassium clavulanate. In this case, clavulanic acid irreversibly binds to β-lactamases and inhibits their effect (Oliveira 2011). This raises the possibility that other factors may be involved in resistance in addition to the presence of the *bla*Z gene and production of β-lactamases. The presence of the *mec*A gene was also negative in this sample, reinforcing the possibility of involvement of one or more other resistance genes, outside the *mec*A gene, as well as the possibility of other resistance mechanisms.

One of the multidrug-resistant *S. aureus* isolates (positive for the *bla*Z gene) was resistant to oxacillin *in vitro*, and negative for the *mec*A gene. However Aarestrup et al. (2001) describe the modification of PBPs, synthesized by the *mec*A gene, as one of the major mechanisms of resistance described against oxacillin, there are other genes that were not evaluated in this study and that may be responsible for this resistance. Such genes include the presence of the chromosomal cassette SCCmec (Hiramatsu 1995), responsible for the transport of *mec*A genes (Katayama et al. 2000), the presence of mecI-mecR1 genes, responsible for transcriptional control of *mec*A (Oliveira 2011), and other genes and/or resistance mechanisms.

From the 136 isolates, five (4%) were resistant to oxacillin *in vitro*, three isolates of *S. aureus*, and a coagulase positive and a coagulase negative sample; however, no sample was positive for the *mec*A gene. Only one of them was positive for the *bla*Z gene and showed resistance to all groups of antimicrobials tested. Bemis et al. (2006) compared some tests to determine resistance to oxacillin and detection of the *mec*A gene. As result, PCR and disk diffusion tests were satisfactory for determining resistance in *S. aureus* isolates from canine hosts. Oliveira (2011) stated that MRSA (Methicillin-Resistant *Staphylococcus aureus*) is inherently cross-resistant to virtually all β-lactam antimicrobials. In this study, none of the samples showed that the resistance was positive for the *mec*A gene, suggesting the presence of other resistance mechanisms, possibly associated with BORSA (Borderline Oxacilin-Resistant *Staphylococcus aureus*) which may occurr due to overproduction of beta-lactamases (Perillo et al. 2012). This could explain the resistance to amoxicillin associated with clavulanate potassium exhibited by one of the strains of *S. aureus*.

The isolates that were resistant to oxacillin were also negative for the *mec*A gene, and they may be meticillinase producers since they were inhibited by clavulanic acid or have other resistance factors, such as the presence of PBPs (penicillin binding proteins) mutants (Borderline Resistant *Staphylococcus aureus* - Borsa), among other resistance mechanisms linked to oxacillin. Previous reports showed the prevalence of 42% (Coelho et al. 2007), 57.6% (Duijkeren et al. 2004), and 99% (Grisold et al. 2002) of the *mec*A gene in *Staphylococcus* spp. multidrug resistance *in vitro* to oxacillin. For Schlotter et al. (2013), only four isolates (2.1%) showed the presence of the *mec*A gene, although they exhibited *in vitro* oxacillin resistance. Frey et al. (2013) reported 9.7% of isolates as oxacillin resistant, and this resistance was attributed to the presence of the *mec*A gene, whereas it was also detected in *S. fleurettii*, *S. epidermidis*, *S. haemolyticus*, and *S. xylosus*. Furthermore, penicillin-resistance was attributed to the presence of the *bla*Z gene. In Japan, Kawakami et al. (2010) reported the *mec*A gene in 66.5% (113/170) of *S. pseudintermedius* and 30.0% (6/20) of *S. schleiferi* isolated from canine pyoderma, suggesting a high degree of resistance to methicillin. In Italy, Ghidini et al. (2011) isolated *Staphylococcus* spp. from skin lesions in dogs - 28 staphylococci strains were methicillin and/or oxacillin resistant, with confirmation of the *mec*A gene in 14 samples. Further studies are required involving unidentified factors and additional determinants reputedly controlling transcription of the *mec*A gene, as well as the *mec*A regulatory mechanism in contemporary MRSA, as suggested by Oliveira (2011).

Characterization of biofilm production by *Staphylococcus* spp.

Phenotypic characterization. Among the 136 isolates evaluated for biofilm production by the quantitative technique of biofilm formation in microplates, 57% (78/136) were classified as strongly adherent, 10% (13/136) as moderately adherent, 16% (22/136) as weakly adherent, and 17% (23/136) as non-adherent. Biofilm formation provides for bacterial protection against dehydration and colonization by bacteriophages. It also promotes antimicrobial resistance and avoids phagocytosis (Gilbert et al. 2003, Zhao et al. 2008). In this study, 83% of the isolates were biofilm producers and this feature may be one im-

portant mechanism of resistance that prevents the action of antimicrobial drugs routinely used in control of microorganisms. Yeon-Soo et al. (2008) highlighted the public health significance of involvement of biofilm-producing *Staphylococcus* spp. isolated from the skin and ears of dogs and cats in direct contact with humans.

Characterization of biofilm production by *Staphylococcus* spp.

Genotypic characterization. One hundred thirty-six isolates of *Staphylococcus* spp. were analyzed for the presence of the *ica*D gene (Table 2), which may be related to the production of biofilm. Three (2%) isolates were positive for the *ica*D gene - one isolate was classified as weakly adherent and the other two as strongly adherent by the quantitative technique of biofilm formation in microplates (phenotypical test).

The Fisher exact test did identify any correlation between the presence of the *ica*D gene and the production of biofilm by *Staphylococcus* spp. (Reis 2003). Arciola et al. (2001) found the presence of the *ica*D gene in 61% of 23 strains of *S. aureus* isolated from humans, a rate higher than the results from this study. Eftekhar & Speert (2009) reported that *ica*ADBC operon, which is included in the *ica*D gene, cannot be used as the only indication of the biofilm phenotype since the conditions for bacterial growth play an important role in this formation, or there may be biofilm formation by other mechanisms (e.g. exopolysaccharide intercellular adhesion, PIA) that are not associated with the *ica*D gene expression. This could help explain our findings, since 83% of bacteria were identified as biofilm producing by the phenotypic test and only 2% were positive for the presence of the *ica*D gene.

Determination of antibacterial activity of organic extract of *C. substellata* and purified usnic acid

The concentration of 0.0019 mg/mL for both the organic extract of *C. substellata* and purified usnic acid was able to inhibit the growth of most strains of *Staphylococcus* spp. (Fig.1). The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) for all 136 isolates ranged from 0.25 mg/mL - 0.0019 mg/mL, with an average of 0.0796 mg/mL for both the organic extract of *C. substellata* and purified usnic acid, and antimicrobial activity was 100% for all 136 isolates.

There was no significant difference in comparison of the antibacterial effect of the organic extract of *C. substellata* and purified usnic acid against *Staphylococcus* spp. when using the Wilcoxon test (Sampaio 2007). This can be explained by the composition of the organic extract of *C. substellata* originating from the Northeast of Brazil, with approximately 98% usnic acid in its composition (Huovinen & Ahti 1986, Ahti et al. 1993, Yano-Melo et al. 1999). This may be primarily responsible for the antimicrobial activity demonstrated in this study and others (Burkholder et al. 1944, Carrazzoni 1975, Huovinen & Ahti 1986, Ahti et al. 1993, Lauterwein et al. 1995, Pereira et al. 1997, Honda & Vilegas 1998, Yano-Melo et al. 1999, Donlan 2001, Falcão et al. 2004, Ribeiro et al. 2006, Moura et al. 2008).

Ranković et al. (2012) tested the antibacterial activity of usnic acid compared to streptomycin for *Bacillus mycoides* (ATCC 6462), *B. subtilis* (ATCC 6633), *S. aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), and *Klebsiella pneumoniae* (ATCC 13883). They observed that usnic acid obtained similar or stronger activity than the isolates tested with streptomycin. The MIC with *Staphylococcus* spp. were from 0.0008mg/mL - 0.5mg/mL, and with *S. aureus* (ATCC 25923), the MIC was 0.125mg/mL. The range found in this study was 0.0019mg/mL to 0.25mg/mL (n=136). Manojlovic et al. (2012) determined MIC against *B. mycoides* (ATCC 6462), *B. subtilis* (ATCC 6633), *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922), and *K. pneumoniae* (ATCC 13883), with values from 0.015mg/mL to 1mg/mL. They also found the concentration of 0.125mg/mL for *S. aureus* (ATCC 25923). Honda et al. (2010) determined MIC using usnic acid against *Mycobacterium tuberculosis*, with a value of 0.625mg/mL. Gupta et al. (2012) determined the MIC of L (-)-usnic acid against clinical isolates of MRSA (*S. aureus* MTCC-96 (SA-96)), and found inhibition from 25mg/mL to 50mg/mL. Those values are much higher than those found in this study, as well as by Manojlovic et al. (2012) and Honda et al. (2010). Gupta et al. (2012) showed that the antimicrobial activity of usnic acid against MRSA occurs by rupturing the cell membrane. In regarding to toxicity, the authors also showed that usnic acid is safe in mice at concentrations of up to 100mg/kg. Segatore et al. (2012) evaluated usnic acid in combination with antimicrobials and observed the following: synergism with gentamicin, antag-

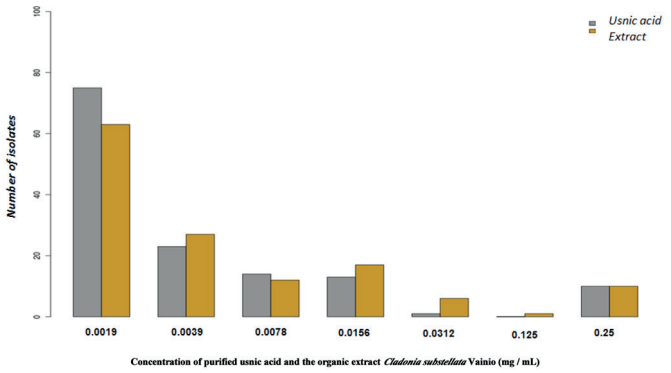

Fig.1. Antimicrobial effect. Comparison of the antimicrobial effect of the extract of *Cladonia substelatta* Vainio and purified usnic acid at different concentrations of mg/mL for determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), according to the Wilcoxon statistical test (Sampaio 2007).

Table 3. MIC[a] and MBC[b] of the organic extract *Cladonia substellata* Vainio and purified usnic acid

Extract/fraction	Mean of MIC and MBC in biofilm-producing *Staphylococcus* spp.[c,d]	Mean of MIC and MBC in non-biofilm-producing *Staphylococcus* spp.[c,e]
Organic extract	0.65mg/mL	2.52mg/mL
Usnic acid	0.42mg/mL	2.71mg/mL

[a] Minimum inhibitory concentration, [b] minimum bactericidal concentration, [c] Phenotypic test; the assay was performed in 96-well polystyrene microplates, [d] n=19 isolates, [e] n=23 isolates.

onism with levofloxacin, unresponsiveness when used in combination with erythromycin, and variability when associated with oxacillin and clindamycin. The authors suggested usnic acid as a good candidate for an antimicrobial agent when used in combination with other commercially available drugs.

Interaction of the organic extract of *C. substellata* and purified usnic acid with the biofilm of *Staphylococcus* spp. consolidated and in formation and their relationship to the MIC and MBC

Of the 19 isolates of biofilm-producing *Staphylococcus* spp., one isolate was classified as coagulase-positive *Staphylococcus,* two as coagulase-negative *Staphylococcus,* two as *S. intermedius,* and 14 as *S. aureus*. The interaction of biofilm in formation and consolidated biofilm of *Staphylococcus* spp. against the organic extract of *C. substellata* and purified usnic acid was also evaluated (Table 3).

The antibacterial activity (MIC and MBC) of the organic extract of C*. substellata* and purified usnic acid against 19 isolates of biofilm-producing *Staphylococcus* spp. (one coagulase-positive *Staphylococcus*, two coagulase-negative *Staphylococci*, two *S. intermedius*, and 14 *S. aureus*) and 23 isolates of non-biofilm-producing *S. aureus* was determined (Table 4). It was observed that the organic extract of *C. substellata* and purified usnic acid had antimicrobial activity at lower concentrations (0.65mg/mL-0.42mg/mL) against biofilm-producing bacteria as compared to non--biofilm-producing isolates (2.52mg/mL – 2.71mg/mL).

The organic extract of *C. substellata* and purified usnic acid at a concentration of 0.25mg/mL inhibited biofilm formation in more than 37% of the *Staphylococcus* spp. strains. Furthermore, they interacted with the already established biofilm, reducing or destroying more than 47% of the isolates. Chifiriuc et al. (2009), evaluating *in vitro* inhibition of biofilm production in dental plaque by usnic acid, found that it selectively inhibited the development of biofilm by Gram positive bacteria and also the hemolytic properties of the bacteria, exhibiting the action of usnic acid with the intra- and inter-bacterial interface, signaling mechanisms based on quorum sensing and response dependent on the density of bacterial cells. This recommends usnic acid as a new active ingredient for a pharmaceutical formulation that could be used in the prevention and treatment of gingival and periodontal diseases.

Kim et al. (2011) also examined the inhibition of biofilm by usnic acid, as well as the antimicrobial efficacy and cytotoxicity of the compound. The authors showed the effectiveness of usnic acid as an antimicrobial to be used in the prevention of biofilm production by *Staphylococcus* spp. A study by Franconi et al. (2004) showed the ability of (+)-usnic acid to control biofilm formation by *S. aureus* and *P. aeruginosa* in laminar flow conditions by image analysis. The polymers loaded with (+)-usnic acid did not inhibit the initial cell attachment of *S. aureus*, but killed the bound cells, resulting in inhibition of biofilm production. Regarding *P. aeruginosa*, with use of the same polymers of (+)-usnic acid, there was biofilm formation in the laminar surface, but the morphology of the biofilm was altered, possibly indicating that (+)-usnic acid may interfere in the signaling pathways of bacteria and thereby inhibit or modify production of bacterial biofilm. Pompilio et al. (2013) evaluated the *in vitro* usnic acid antibacterial and antibiofilm activity against three strains of MSSA and three MRSA isolates from human patients with cystic fibrosis. They concluded that usnic acid shows activity against planktonic cells, is active against MRSA, and is capable of inhibiting biofilm formation. Grumezescu et al. (2011) also evaluated the antibiofilm activity of usnic acid incorporated into nanoparticles, and showed that they could be used as coating agents for different medical devices to prevent biofilm formation, including formation in patients with cystic fibrosis.

In this study, organic extract of *C. substellata* and usnic acid inhibited biofilm formation of *Staphylococcus* spp. This may have occurred due to establish bacterial death. Usnic acid and the organic extract of *C. substellata*, containing more than 90% usnic acid in its composition, inhibited biofilm already established for *Staphylococcus* spp. Usnic acid may also contain mechanisms that interact directly with already consolidated bacterial biofilms. In addition, it was possible to determine antimicrobial activity through bacterial death and/or bacterial inhibition (MIC and MBC), and to quantify the interaction of usnic acid directly with biofilm (in formation and that already established), which showed better antimicrobial activity against *Staphylococcus* spp. biofilm producers. In this study, it was observed that lower concentrations of usnic acid are needed to inhibit bacterial growth when compared to *Staphylococcus* spp. non-biofilm producers. This might enhance the action of usnic acid since biofilm does not impede its mechanism of action. The formation of bacterial biofilm provides the bacteria with resistant to antimicrobial agents, and this has

Table 4. Description of one hundred thirty-six *Staphylococcus* spp. isolates obtained in Brazil

Clinical source (n[a])	Animal species (n)	Isolates (n)	City (State)	Period/Year
Skin/pyodermatitis (86)	Dogs[b] (76)	75	Petrolina (PE)	2011 to 2012
		01	Santa Maria (RS)	1991
	Cats[c] (10)	10	Petrolina (PE)	2012
External ear/otitis (50)				
	Dogs (49)	24	Petrolina (PE)	2012
		23	Santa Maria (RS)	1990 to 1995
		01	Santa Cruz do Sul (RS)	1990
		01	Caçapava do Sul (RS)	1990
	Cats (01)	01	Petrolina (PE)	2012

[a] Total number of samples according to clinical source, [b] *Canis familiaris*, [c] *Felis domesticus.*

been a concern in the search for new antimicrobials (Gilbert et al. 2003, Zhao et al. 2008). Usnic acid has great potential as a pharmaceutical and/or a pharmacological adjuvant for use in prevention of *Staphylococcus* spp. infections.

CONCLUSIONS

The organic extract of *Cladonia substellata* and purified usnic acid exhibited antimicrobial activity at low concentrations against *Staphylococcus* spp. that is resistant to conventional antibiotics.

Additionally, the substances inhibited biofilm formation by *Staphylococcus* spp. and showed greater efficacy against biofilm-producing isolates.

These characteristics are important and ought to be considered in regard to the drugs routinely used in clinical medicine, especially in the search for substances with pharmacological action on biofilm-producing bacteria and those resistant to various pharmacological groups.

We highlight the great potential of the organic extract of *C. substellata* and purified usnic acid in veterinary dermatology, mainly in the treatment of otitis and pyoderma caused by *Staphylococcus* spp. in dogs and cats.

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