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In vitro and *in vivo* efficacy of tea tree essential oil for bacterial and yeast ear infections in dogs[1]

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ABSTRACT.- Neves R.C.S.M., Makino H., Cruz T.P.P.S., Silveira M.M., Sousa V.R.F., Dutra V., Lima M.E.K.M. & Belli C.B. 2018. ***In vitro* and *in vivo* efficacy of tea tree essential oil for bacterial and yeast ear infections in dogs.** *Pesquisa Veterinária Brasileira 38(8):1597-1607*. Departamento de Clínica Médica Veterinária, Universidade Federal de Mato Grosso, Av. Fernando Corrêa da Costa 2367, Bairro Boa Esperança, Cuiabá, MT 78060-900, Brazil. E-mail: nevesrita@hotmail.com.br

Otitis externa is a common complaint in dogs. Bacteria and yeasts are commonly involved and may perpetuate inflammatory reactions inside the ear canal. Otoscopy, cytological examination of secretion and microbiological culture embody forms of diagnosis. Cytology also has great use in accessing treatment evolution. Therapy usually consists of cleaning ear canals and subsequent use of antibiotics or antifungal products. As some of them may cause hypersensitivity and even ototoxicity, searching for new pharmacological bases is currently necessary and justifies this study, which aimed to evaluate in vitro and in vivo efficacy of tea tree essential oil for bacterial and yeast ear infections in dogs. Twenty-eight dogs from a particular shelter in Cuiabá (Mato Grosso, Brazil), presenting clinical signs of otitis externa, were enrolled in this clinical trial. In all of them, clinical and cytological evaluations, as well as culture and susceptibility testing of the affected ears were carried out. From each dog, one ear was treated with 5% tea tree essential oil lotion and the other with standard otic formulation, according to the type of infection (bacterial, yeast or both). *In vitro* susceptibility testings of all ear cultures, to the same drugs used in treatment, were also carried out. Culture results showed 62.5% bacterial and fungal infection, 33.9% bacterial infection and 3.6% fungal infection, from the 56 ear samples collected. The most common microorganisms isolated were *Staphylococcus intermedius, Staphylococcus aureus, Proteus mirabilis* and *Malassezia pachydermatis*. Gram-positive bacteria were susceptible to gentamycin in 60.5% and resistant in 16.3% of the samples. Five percent tea tree essential oil formulation produced a 5mm clear zone of inhibition around the disks in one of the 63 samples evaluated. Pure (100%) tea tree essential oil formulation produced a 10mm clear zone of inhibition around the disks in four of the 63 samples evaluated, a 9mm zone in three samples, an 8mm zone in 16 samples, a 7mm zone in seven samples, a 6mm zone in two samples and there was no clear zone in 31 samples. Inhibition zones were produced by strains of *Staphylococcus intermedius, Staphylococcus hyicus, Corynebacterium* sp., *Proteus mirabilis* and *Enterobacter* sp. tea tree essential oil ear solution significantly induced remission of clinical signs both in bacterial and yeast ear infections. It also reduced as much *Malassezia pachydermatis* ear infection as the nystatin solution used in this study, while gentamycin solution showed better antibacterial effect. More studies should be conducted to evaluate in vitro diffusion properties of tea tree essential oil. Good antimicrobial spectrum and the absence of adverse reactions confirm the importance of developing a tea tree formulation as an alternative therapy for ear infections in dogs.

INDEX TERMS: Tea tree essential oil, yeast, bacteria, dog, ear diseases, phytotherapy, clinics.

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RESUMO.- [**Uso *in vitro* e *in vivo* do óleo essencial de Melaleuca (*Melaleuca alternifolia*) em otites bacterianas e por leveduras**]. Otite externa é queixa frequente em cães. Bactérias e leveduras estão comumente envolvidas e podem perpetuar as reações inflamatórias dentro do canal auditivo. Dentre as formas de diagnóstico, encontram-se a otoscopia, o exame citológico da secreção e a cultura microbiológica. Citologia também tem grande utilidade no acesso à evolução do tratamento. A terapia consiste de limpeza dos canais auditivos e posterior utilização de antibióticos ou produtos antifúngicos. Como alguns antimicrobianos utilizados no tratamento podem causar hipersensibilidade e até mesmo ototoxicidade, a busca por novas bases farmacológicas justifica a existência deste estudo, que teve como objetivo avaliar *in vitro* e *in vivo* a eficiência do óleo de *Melaleuca alternifolia* em otites bacterianas e fúngicas de cães. Vinte e oito cães, de um abrigo particular, apresentando sinais clínicos de otite externa, foram incluídos neste estudo clínico. Todos passaram por avaliação clínica, citologia e cultura de material das orelhas afetadas. De cada animal, uma orelha foi tratada com óleo de Melaleuca 5% e a outra com formulação ótica padrão, de acordo com a afecção (bacteriana, fúngica ou mista). As culturas também foram submetidas a testes de susceptibilidade *in vitro* aos mesmos agentes utilizados no tratamento *in vivo*. Os resultados da cultura mostraram 62,5% de infecção mista (bacteriana e fúngica), 33,9% de infecção bacteriana e 3,6%, de infecção fúngica a partir das 56 orelhas. Os micro-organismos mais isolados foram *Staphylococcus intermedius*, *Staphylococcus aureus, Proteus mirabilis e Malassezia pachydermatis*. As bactérias GRAM-positivas foram sensíveis à gentamicina em 60,5% e resistentes em 16,3% das amostras. A formulação com 5% de óleo essencial de Melaleuca produziu uma zona de inibição de 5mm em torno dos discos em uma das 63 amostras avaliadas. A formulação pura (100%) do mesmo produto produziu uma zona de 10mm de inibição em quatro das 63 amostras analisadas, uma zona de 9 mm em três amostras, uma zona de 8mm em 16 amostras, uma zona de 7mm em sete amostras, uma zona de 6mm em duas amostras e não havia nenhuma zona clara em 31 amostras. Zonas de inibição foram produzidas por estirpes de *Staphylococcus intermedius, Staphylococcus hyicus, Corynebacterium* sp., *Proteus mirabilis e Enterobacter* sp. Clinicamente, a formulação com o óleo essencial de melaleuca induziu significativamente uma melhora nas manifestações, tanto nas otites bacterianas quanto nas causadas por leveduras, sendo estatisticamente semelhante ao tratamento com nistatina (nas otites por levedura), mas menos eficaz que a solução de gentamicina nas otites bacterianas. Mais estudos devem ser realizados para avaliar as propriedades de difusão *in vitro* do óleo essencial de melaleuca. O bom espectro antimicrobiano, a boa resposta clínica e a ausência de reações adversas confirmam a possibilidade de desenvolvimento de formulação ótica com o óleo essencial de melaleuca, como uma alternativa para a terapia de infecções do ouvido em cães.

TERMOS DE INDEXAÇÃO: Bactéria, cão, doenças de ouvido, fitoterapia, levedura, óleo essencial de melaleuca, caninos, clínica.

INTRODUCTION

Otitis is defined as the inflammation of the ear canal (Tuleski 2007), and can be classified as external, media and internal otitis, according to the anatomical structures affected (Harvey et al. 2004). Commonly observed in dogs, otitis externa is the inflammation of the external ear canal, which includes the pinnae and both vertical and horizontal auditory canals (August 1988, Murphy 2001).

Multifactorial aethiologic features of external otitis include inflammation caused by predisposing factors, such as anatomic and physiological (skin folds, excessive hair inside the ear canal and neoplasias), allergic, autoimmune, parasitic, and keratinization disorders (Gotthelf 2007); but also by perpetuating factors, such as yeast and bacterial (*Pseudomonas* spp., *Escherichia* spp., *Proteus* spp.) infections (August 1988, Lyskova et al. 2007, Zamankhan Malayeri et al. 2010).

As the external ear of dogs is covered by skin, it shares the same physiopathologic features as the rest of the body surface. So, microorganisms usually found in the skin are also present in the ears, like *Staphylococcus* spp., *Bacillus* spp. and *Malassezia* spp. (August 1988), and chronic inflammation of the external ear epithelium often results in bacterial and yeast proliferation (Lucas et al. 2016). Besides, depending on the severity of infection and on which micro-organisms are involved, tympanic rupture can occur, leading to otitis media, and consequently, even internal otitis.

Clinical features of external otitis are inflammation, pain, bad smell, exudate, pruritus and head shaking. Chronic cases may also show resistance to antimicrobial agents, ear canal stenosis, polyps and tympanic rupture, which causes chronic pain and can lead to deafness (August 1988, Angus et al. 2002).

Canine ear infections are a common dermatological complaint. *Staphylococcus* spp. and *Malassezia pachydermatis* are more frequently isolated from dogs ears, with or without external otitis, while *Proteus sp*. and *Pseudomonas sp* are isolated only in dogs with otitis externa (Yoshida et al. 2002), especially in chronic ear infections (Dickson & Love 1983).

Several publications emphasize the need to consider susceptibility of bacteria to the products used in the treatment of otitis, by culture and susceptibility testing (Oliveira et al. 2005, Lyskova et al. 2007). However, Morris (2004) alerts that identification of bacterial susceptibility is not applicable in all kinds of ear infections, considering the bad diffusion of systemic antibiotics through external ear epithelial tissue, and also the fact that susceptibility testing is performed based on serum, not tissue concentrations. Moreover, when performing susceptibility tests for otitis externa in dogs, using standard serum concentration discs, *in vitro* and *in vivo* results can be different, as topical therapy can achieve much higher concentrations.

Treatment includes identification of the underlying causes of the ear infection, and should be initiated after cytological evaluation (Mueller et al. 2007), so that a proper ear cleaner and topical antimicrobials can be used. Excess earwax or exudate prevent contact of topical medication to the ear epithelium and infectious agents, making topical therapy ineffective.

Therapeutic protocol for otitis externa can cause systemic effects, especially when there is perforation in the eardrum and/or ear canal ulcerations (Nuttall & Cole 2004, Tuleski 2007). Also, the majority of ear cleaners and antimicrobial agents may be ototoxic to the structures of the middle and/or internal ear, causing balance disorders and hearing loss in dogs (Merchant 1994).

People have become increasingly demanding and more discerning about the quality of life of their pets and the quality

of the product they consume. There is a growing concern in making use of less aggressive and of natural origin products (Packer & Luz 2007), such as essential oils formulations.

Essential oils are volatile compounds produced by plants for their survival, both for defense or to attract pollinators. These oils are complex mixtures of liquids, of intense and pleasant odor, which main characteristic is volatility, that differentiates them from fixed oils, extracted from lipid seed (Jesus et al. 2007).

Melaleuca alternifolia, also known as tea tree plant, is native from Australia, (Silva et al. 2002), and, such as *Myrtaceae* thin skin trees, it can reach 5-7 meters tall, and have long thin pointed leaves that, when broken, loose a strong aroma (Simões et al. 2002).

There is evidence that the Australian aborigines crushed *M. alternifolia* leaves to make antibacterial poultices, centuries before the scientific knowledge about microorganisms (Simões et al. 2002). Scientific data shows that essential oil extracted from the leaves by hydrodistillation has proven antibacterial, antifungal and antiviral properties (Silva et al. 2002).

Monoterpenes, sesquiterpenes and their corresponding alcohols are present in *M. alternifolia* oil (Carson et al. 2006), but its main constituents are α-terpinene (10%), γ-terpinene (23%) and terpinen-4-ol (40%), with antimicrobial effect (Oliva et al. 2003). Reichling et al. (2004) showed the efficacy of 10% tea tree oil cream in dogs presenting yeast and bacterial isolates from pruriginous cutaneous lesions, caused by localized acute and chronic pyoderma. Another study demonstrated that some strains of *Pseudomonas aeruginosa* were resistant to tea tree oil, while 51% of the pathogenic microorganisms, including yeasts and bacteria, found in humans with otitis were susceptible to this oil (Farnan et al. 2005). In Canada, a case report indicated that tea tree oil was effective against fleas and otitis in dogs and cats (Lans et al. 2008). According to Baldissera et al. (2016), α-terpinen from tea tree oil showed *in vitro* tripanocid effect, extending longevity of mice infested by *Trypanosoma evansi*.

Identification of each component of volatile oils is widely performed by gas chromatography, because of its high selectivity in comparing retention time of the components with their corresponding patterns (Jesus et al. 2007). Since 1996, *M. alternifolia* oil composition has been standardized by the International Standard for tea tree oil entitled "Oil of Melaleuca - Terpinen-4-ol type (tea tree oil)" (Carson & Riley 2001). The oil obtained from the leaves may contain varying amounts of terpenes (pinene, terpinene and cymene), terpineol (terpinen-4-ol), sesquiterpenes and cineole, which are the most important constituents with antimicrobial activity. The Australian committee of standardization (Australian Standard AS 2782-85) states that the oil should contain cineol, a skin irritant (Simões et al. 2002) below 15% and terpinen-4-ol between 30-40%, in order to have any antiseptic efficacy (Simões et al. 2002, Walton et al. 2004).

Terpinen-4-ol is touted as the largest contributor of antimicrobial activity among the components (Simões et al. 2002). The higher the terpinen-4-ol content, the better its antimicrobial activity (Jesus et al. 2007), and when used alone, it shows similar results to pure oil.

In addition to its antibacterial properties, even against bacteria resistant to antibiotics, tea tree essential oil is also effective against pathogenic molds, some viruses, and has been used as a strong repellent against mosquitoes, fleas, lice (Silva et al. 2002) and mites, such as *Otodectes cynotis* (Neves et al. 2012, 2013).

There is a low risk of allergic reactions to tea tree essential oil 100% (Hammer et al. 2000). A study of its use to treat otoacariasis showed no adverse reactions in 30 dogs (Neves et al. 2012). And there was no sign of phototoxic effect of an undiluted oil formulation on mice skin (Gao et al. 2005).

Although pet segment is the fastest growing in Brazil, there are only a few products containing tea tree essential oil available for veterinary use, as only 1% of phytotherapeutic investment is directed to the pet market in this country (Ozaki & Duarte 2006).

The objective of this study was to evaluate *in vitro* and *in vivo* efficacy of tea tree essential oil for bacterial and yeast ear infections in dogs.

MATERIALS AND METHODS

Ethics statement

The protocol and study procedures complied with Ethical Principles in Animal Experimentation guidelines and were conducted with approval of the Ethics Committee for Animal Research of Mato Grosso Federal University, no. 23108.030616/09-3. A Free and Informed Consent Form was signed by the owner.

Animal houses

Twenty-eight dogs from a particular shelter in Cuiabá (Mato Grosso, Brazil) presenting clinical signs of otitis externa were eligible for enrollment in this clinical trial, regardless of size, sex, breed or age. Patients who have received antibiotics, antifungal or anti-inflammatory as topical or systemic treatment in the previous 15 days were excluded from the study, as well as infants and the ones presenting signs of pregnancy, parasitic ear infection, tumors or foreign bodies in the ears.

In vivo

Diagnosis of otitis externa was established by otoscopic and cytological evaluations, before culture and susceptibility testing and treatment. Otoscopy was carried out in three days: -5, +7, +15 and treatment began in day zero (D zero). Otoscopic evaluation was carried out using an optic zoom veterinary otoscope (*MacroView* de 3,5V; *Welch Allyn*®, Skaneateles Falls, New York, USA).

In order to define an individual score of each ear canal, the following clinical parameters were evaluated, by a single examiner: degree of inflammation, pruritus (through history) and exudation of both external ear canals. These questions were scored on a scale of 0 to 3, according to its intensity (0 = absent, 1 = mild, 2 = moderate, 3 = severe). Maximum score for these three parameters did not exceed nine points for each ear and each valuation day, according to Lozina et al. (2010).

Efficacy of treatments was evaluated comparing the scores obtained in D-5, D+7 and D+15, using analysis of variance for repeated measures (Sokal & Roohlf 1995), considering that the same individuals were evaluated over time and that each experimental group was tested separately.

In vitro

Specimens for cytological evaluation were collected in D-5 and D+15. These days were determined by the duration of cleansing pre-treatment and treatment of the ears, and also determined according to standard clinical recommendations to initiate treatment of otitis externa

by cleansing the ear canals for three to seven days, until complete elimination of exceeding material, and then start specific therapy (Lucas et al. 2016). In this study, cleansing period extended for 5 days and treatment period for 14 days. Therefore, specimens for evaluation were collected five days before and 14 days after treatment of the ears.

Two samples, collected with non-sterile swabs, were rolled onto microscope slides, with gentle extension movements of the swab, then, dried in free air and were stained for cytological evaluation. In the lab, one of the samples was successively immersed for one minute in three flasks containing solutions 1, 2 and 3 of Romanowsky stain, which includes Wright, Giemsa and Diff-Quik - Panótico rápido (Laborclin®, Pinhais, PR, Brazil). After the last solution, the sample was washed in deionized water and left to dry in vertical position. Then, microscopic evaluation was carried out, in order to observe the presence, classification and quantification of micro-organisms and cell types, specially the inflammatory ones (Angus 2004). The second sample was immersed in solution 1 (gentian violet) for one minute and washed with distillated water, then immersed in solution 2 (lugol) for another minute, washed, and received some drops of solution 3 (ketone alcohol) for 15 seconds, before being washed again. After that, it was immersed in phenol fuchsin for 30 seconds and then washed in distillated water and left in vertical position to dry.

Cytological evaluation of the microscope slides, using GRAM stain (Laborclin®, Pinhais, PR, Brazil) to differentiate GRAM-positive (violet blue) from GRAM-negative bacteria (red), was made by observation in a 100x magnification, using an optic microscope (Eclipse E200, Nikon®, New York, USA), and a drop of immersion oil (Laborclin®, Pinhais, PR, Brazil).

Specimens for culture and susceptibility evaluation were obtained in D-5, from the proximal horizontal ear canal, after shaving and cleaning the entrance of each external ear, using small scissors and gauze soaked in saline. Autoclavable plastic otoscope speculums helped to shroud a sterile pediatric swab, and after, a non-sterile small cotton-tipped swab, from both right and left ear canals of all dogs enrolled.

Sterile swabs were stored in medium under cooling and sent to a Microbiology Laboratory. Ear swab samples were seeded in blood agar (5% sheep blood), MacConkey agar and Sabouraud dextrose agar (Acumedia®, Lansing, Michigan, USA), deposited in an incubator chamber (BOD SPLabor®, Presidente Prudente, SP, Brazil) at 37°C for 7 to 15 days in aerobic condition. Isolates were identified by morphological and dye characteristics, and biochemical tests were performed (staining of gram, catalase, oxidase, glucose, sucrose, lactose, motility, oxidation / fermentation, citrate) according to Quinn et al. (1994).

Susceptibility tests of the bacterial isolates obtained were performed by the agar diffusion method (Bauer et al. 1966). These isolates were cultured in Mueller Hinton broth and after reaching turbidity were transferred with sterile swab to Mueller Hinton agar plates using seven discs. Five were paper discs (6mm diameter) impregnated (Edwards-Jones et al. 2004, Gupta & Saxena 2012) with 10uL (Ostrosky et al. 2008) of otic solution from a commercial compounding pharmacy. Disc 1 was impregnated with 5% tea tree essential oil, disc 2 with 100% tea tree essential oil, disc 3 with 0,3% gentamicin (positive control), disc 4 with 0,3% gentamicin and 0,15% nystatin, disc 5 with vehicle (negative control), disc 6 was a commercially available standard gentamicin disc (10mcg) and disc 7 was a paper disc not impregnated.

In vivo

Treatment. Treatment of otitis externa began with flushing the ears twice a day, from D-5 to D-1, using N-acetylcysteine (Fluimucil®, Laboratory Zambon, São Paulo, SP, Brazil) (Alves et al. 2008) when there was purulent discharge, and a ceruminolytic formula (Vetriderm Ceruminolítico®, Laboratory Bayer, Belford Roxo, RJ, Brazil) (Leite 2008) in ear canals with waxy material.

After cleansing phase, the 28 enrolled dogs (56 ears) were divided into three groups: fungal otitis, bacterial ear infections and mixed ear infections (bacterial and fungal). All right ears received 5% tea tree essential oil lotion, while left ears received treatment according to group classification: 0,15% nystatin lotion for fungal otitis (Lorenzini et al. 1985, Leite 2008); 0,3% gentamicin lotion (Leite 2008) for bacterial ear infection and both of them when there was mixed otitis.

Each ear was regarded as an experimental unit, thereby minimizing, anatomical, genetic or immune interference. Depending on the size of auditory canals, 0.25 to 1ml of otologic solution were applied (Lozina et al. 2010), using a disposable sterile 1ml syringe, every 12 hours for 14 days.

Dog owners were advised not to clean the auditory canals and not to use any other topical nor systemic treatments during the experimental protocol.

Efficacy of treatment was assessed through clinical findings and cytology and compared to susceptibility testing results. A good clinical response was defined as a return to normal of all parameters (score = zero), according to Lozina et al. (2010). Early diagnosis and follow up during treatment depended on the symptoms reported by the owner, such as head shaking, pruritus, pain, otorrhea, unpleasant odor and liquid collections located in the acoustic meatus, characterizing external otitis (Lucas et al. 2016). The presence or absence of this symptomatology, added to improve cytological examination of the secretion, based the good results obtained in this study.

Chromatography. Tea tree essential oil (Galen®, Campinas, SP, Brazil) used to compound the lotion was chemically analysed using gas chromatography, by the Chromatography Laboratory of the Department of Chemistry at Minas Gerais Federal University (UFMG). Both qualitative and quantitative analysis of the essential oils were performed using gas chromatography high resolution, with flame ionization detector (GC-FID). The GC analysis was carried out on a HP 7820A (Agilent®, Santa Clara, California, USA) equipped with HP-5 column (30m 9 0.32mm 9 0.25mm) at an initial temperature of 70°C with addition of 3°C min-1 up to 240°C. The temperature of the injector and of the FID detector was 250 and 260°C, respectively. The flow rate of hydrogen, used as carrier gas, was 3 ml min-1 and the split ratio 1:30. The oils were diluted in chloroform (1%), and 1 ll was injected into the chromatograph. The identification of oil componentes was performed by comparing their mass spectra with the Kovats retention index (R.I.). Data were acquired and processed by EZChrom Elite Compact software (Agilent®, Santa Clara, California, USA). The identified and quantified compound found in the tea tree oil used were: α-thujeno 0.7, α-pineno 2.5, β-pineno 0.7, mirceno 0.7, α-terpineno 9.1, p-cimeno 4.3, limoneno 3.1, 1.8-cineol 1.7, γ-terpineno 21.2, terpinoleno 3.5, terpinen-4-ol 42.9, α-terpineol 1.9, β-gurjuneno 1.3, viridiflorino 1.4, and cis-calamenocalabcariofilenom 1.1%.

Tea tree essential oil treatment efficacy for ear infections was evaluated through a paired T-test, applied separately to each lotion, considering the score before and after its administration. Tea tree essential oil treatment efficacy, comparing to nystatin and gentamycin, was evaluated through a paired T-test, considering the difference in score for each product and the number of leukocytes

before and after treatment. All analyses were conducted with the R 2.15.1 software (R Core Team 2014).

After 14 days of treatment, the ears that did not show improvement with the therapeutic protocols used, would receive a standard protocol.

RESULTS AND DISCUSSION

Otitis externa may occur in any dog irrespective of sex or age, although some predisposition, depending on geographic region, has been recognized in some breeds. In this study, Cocker Spaniel (n=9) and mixed-breed dogs (9) were over-represented, while there were also four American Pit Bull Terriers, three Poodles, two Labrador Retrievers, two Basset Hounds and one Yorkshire Terrier. There were 13 males, 11 females, 17 adults and 11 elderly dogs. Considering the 28 dogs enrolled in this study, culture results (Table 1) showed 62.5% bacterial and fungal infection, 33.9% bacterial infection and 3.6% fungal infection, from the 56 ear samples collected.

Cytology and culture results correlated well and bacterial otitis were predominantly caused by Gram-positive bacteria, as published by Petersen et al. (2002) and Tuleski (2007), especially *Staphylococcus intermedius* (Blue & Wooley 1977, Megid et al. 1990, Logas 1994, Kiss et al. 1997, Petersen et al. 2002, Tuleski 2007), followed by *Staphylococcus aureus* (Lilenbaum et al. 2000). Culture-based investigation showed that *Pseudomonas aeruginosa* was not the most frequent cause of ear infection, as suggested by many authors (Blue & Wooley 1977, Kiss et al. 1997, Petersen et al. 2002, Tuleski 2007). Instead, this study found *Proteus mirabilis* in most Gram-negative samples.

Fungal otitis predominantly involved Malassezia pachydermatis and less frequently Candida sp, repeating Kiss et al. (1997) and Tuleski (2007).

Most otitis was polymicrobial and caused both by *Malassezia pachydermatis* and Staphylococcus sp, supporting the existence of a symbiotic relation between these two agents (Kowalski 1988, Rougier et al. 2005, Lyskova et al. 2007, Marques 2010). Furthermore, when there was Proteus mirabilis, *M. pachydermatis* was only found in three samples, confirming a possible inhibitory influence of those bacteria, as suggested by Gotthelf (2007).

As Pseudomonas aeruginosa was detected only a couple of times in this study, and was accompanied by yeast only once, it was not possible to make any assumptions in this regard.

Susceptibility testing results considered the size of a clear zone of inhibition around the disks. Vehicle solution impregnated disks showed no inhibition zone formation, and therefore represented the negative control in this experiment, according to Karaman et al. (2003) and Springfield et al. (2003). Paper disks not impregnated did not create inhibition zone.

Gentamycin disk was used as biological positive control because it is a first choice antimicrobial in cases of otitis externa, according to Kiss et al. (1997), Leite (2008) and Ostrosky et al. (2008), frequently used for bacterial ear infection in dogs (Leite 2008) and easily found in ear products in Brazil.

Gram-positive bacteria were susceptible to gentamycin in 60.5%, resistant in 16.3% and less susceptible in 23.2% of the samples, while other studies showed no resistance to aminoglycosides (Oliveira et al. 2005, 2006).

Gram-negative bacteria were susceptible to gentamycin in 70%, resistant in 20% and less susceptible in 10% of the samples, repeating the findings of Hariharan et al. (2006), and opposing the results showed by Vargas et al. (2004).

Table 1. Microorganisms isolated from dogs with mixed, bacterial and fungal otitis

Microbial species isolated in ear canals	Absolute value	Relative value
Staphylococcus intermedius **	4	7.14
*Staphylococcus intermedius + Malassezia pachidermatis**	20	35.7
*Staphylococcus intermedius + Malassezia pachidermatis + Candida**	1	1.8
*Staphylococcus intermedius + Candida**	5	8.9
*Staphylococcus intermedius + Proteus mirabilis + Malassezia pachidermatis**	1	1.8
*Staphylococcus aureus***	3	5.3
*Staphylococcus aureus + Proteus mirabilis+ Candida**	1	1.8
*Staphylococcus aureus + Proteus mirabilis + Escherichia coli***	1	1.8
*Staphylococcus aureus + Proteus mirabilis + Escherichia coli + M. pachidermatis**	1	1.8
*Staphylococcus hyicus + Malassezia pachidermatis**	1	1.8
*Streptococcus + Malassezia pachidermatis + Candida**	1	1.8
*Corynebacterium + Proteus***	2	3.6
Corynebacterium sp. + *Malassezia pachidermatis**	1	1.8
Proteus mirabilis **	8	14.3
*Proteus mirabilis + Malassezia pachidermatis**	1	1.8
*Pseudomonas aeruginosa***	1	1.8
*Pseudomonas aeruginosa + Malassezia pachidermatis**	1	1.8
Enterobacter sp.*+ Candida sp**	1	1.8
*Malassezia pachidermatis****	2	3.6

* Mixed otitis, ** bacterial otitis, *** fungal otitis.

Both commercial and impregnated gentamycin disks presented similar susceptibility properties, showing how effective it is *in vitro.*

An in vitro study showed bacteriostatic and fungistatic activity of tea tree essential oil to strains of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans* (Packer & Luz 2007). In our research this activity on the bacteria was also observed, being proportional to the concentration of the product: 5% tea tree essential oil formulation produced a 5mm clear zone of inhibition around the disks in 1 of the 63 samples evaluated (1/63); while pure (100%) tea tree essential oil formulation produced a 10mm clear zone of inhibition around the disks in 4 of the 63 samples evaluated (4/63), a 9mm zone in 3 samples (3/63), an 8mm zone in 16 samples (16/63), a 7mm zone in 7 samples (7/63), a 6mm zone in 2 samples (2/63) and there was no clear zone in 31 samples (31/63).

Inhibition zones were produced by strains of *Staphylococcus intermedius, Staphylococcus hyicus, Corynebacterium* sp., *Proteus mirabilis* and *Enterobacter* sp., and were not compared between them, or with the standard solution, because the former is not a pure substance and the latter is a complex mixture of chemical compounds. However, growth of certain micro-organisms was inhibited in the presence of pure oil. Micro-organisms requiring concentrations greater than 4% are considered resistant to tea tree essential oil by Farnan et al. (2005).

Simões at al. (2002) showed that tea tree essential oil has *in vitro* antimicrobial properties against human resistant staphylococcal strains. Another study, involving human subjects ranging from two to 77 years-old presenting otitis externa, concluded that tea tree essential oil, in concentrations lower than 2%, was effective against 72% of pathogens, including *Staphylococcus aureus* and MRSA (Methicillin-resistant *Staphylococcus aureus*) strains. The same research showed that coliform bacteria, Candida, Streptococcus and Proteus were highly sensitive to tea tree oil, in a concentration of up to 0.5%. *Pseudomonas aeruginosa* was the only micro-organism that showed some resistance.

Though it is recommended by some authors, as Edwards-Jones et al. (2004), Ostrosky et al. (2008), Gupta & Saxena (2012), results found herein suggest that agar diffusion technique may not be suitable for tea tree oil susceptibility testing, due to the bad diffusion of the oil fractions through the hydrophilic culture medium used. According to Farnan et al. (2005), such oils need an emulsifier agent, such as Tween 80, to increase solubility. Thus, more studies should be conducted to decide the most suitable technique to perform tea tree oil susceptibility testing.

N-acetylcysteine was used to reduce viscosity in 7 ears that presented mucopurulent discharge, according to Alves et al. (2008), while the ceruminolytic formula was used in the remaining ears, presenting waxy secretion. All dogs showed obvious discomfort minutes after instillation of both flushing formulations.

Although topical route is the method of choice for treatment of otitis externa, as systemic drugs have very low epithelial distribution, physical and chemical properties of vehicles in topical ear formulations should be taken into account in order not to affect treatment outcome (Leite 2008). All three otologic lotions used in this study showed adequate viscosity to allow good permanence of the product inside the ears, despite frequent head shaking.

An experienced clinician may predict which types of micro-organisms are present in a particular type of otitis, however the identification of such population is essential to an effective treatment (Leite 2008). According to cytological results, the 56 ear samples were divided into three different groups: yeast and bacterial otitis, yeast otitis, and bacterial otitis.

In the yeast group, a 5% tea tree essential oil lotion was used in all right ears, while a 0,15% nystatin lotion was used in all left ears, as positive control, according to Lorenzini et al. (1985), Larsson et al. (1988) and Pereira (2000). There was no significant difference between treatments ($F_{1;30}$=0,19; p=0,66) and both of them had a smaller score after 15 days ($F_{2;30}$=72,38; p<0,001), suggesting clinical improvement (Fig.1). Regarding the efficacy as an anti-infection agent (Fig.2), tea tree oil showed a significant reduction for *Malassezia* (t=8.33, gl=13, p<0.001). This finding agrees with the antifungal hypothesis of tea tree and its components (Hammer et al. 2000), when *Malassezia* species presented similar sensitivities as to ketoconazole, econazole and miconazole. The same authors showed, some years later, in 2004, the tea tree oil ability to change yeast membrane. This antifungal property was noted against *Candida* sp. by Nenoff et al. (1996), Catalán et al. (2008) and Noumi et al. (2010).

In the bacterial group, a 0,3% gentamycin lotion was used in all left ears, as positive control, and a 5% tea tree essential oil lotion was used in all right ears. There was significant differences between treatments ($F_{1;30}$=6,97; p=0,012), though both of them had a smaller score after 15 days $F_{2;30}$=20,08, p<0,001), suggesting clinical improvement (Fig.1). Effectiveness against bacterial infection showed herein (Fig.3) by a significant reduction in ear bacteria (t=2,52; gl=6; p=0,045) and leukocytes (t=2,83; gl=6; p=0,03), was also published in a previous *in vivo* study in mice with staphylococcal infection, that improved after receiving a single dose of 700mg/kg of tea tree oil intra-gastrically (Simões et al. 2002). Sherry et al. (2003) noted resolution of wound infected with MRSA in 92% of 25 patients, without any other associated antibiotic. Gram-negative bacteria were more resistant to both treatments used in this study, though the number of micro-organisms and leukocytes were clearly declining in Panotic stained cytological samples (before and after treatments). A possible explanation for such resistance could be the higher and more complex lipid cell wall contents of Gram-negative than Gram-positive bacteria (Sforcin et al. 2000).

In the yeast and bacterial group, a 5% tea tree essential oil lotion was used in all right ears, while a 0,15% nystatin and 0,3% gentamycin lotion was used in all left ears, as positive control. There was no significant difference between treatments ($F_{1;30}$=0,84; p=0,36) and both of them had a smaller score after 15 days ($F_{2;30}$=15,81; p<0,001), suggesting clinical improvement (Fig.1). There was reduction in the number of yeasts (t=2,5; gl=6; p=0,046) and of bacteria (t=3,65; gl=6; p=0,01), but not of leukocytes (Fig.4) (t=2,12; gl=6; p=0,08), what can be attributed to the speed at which the

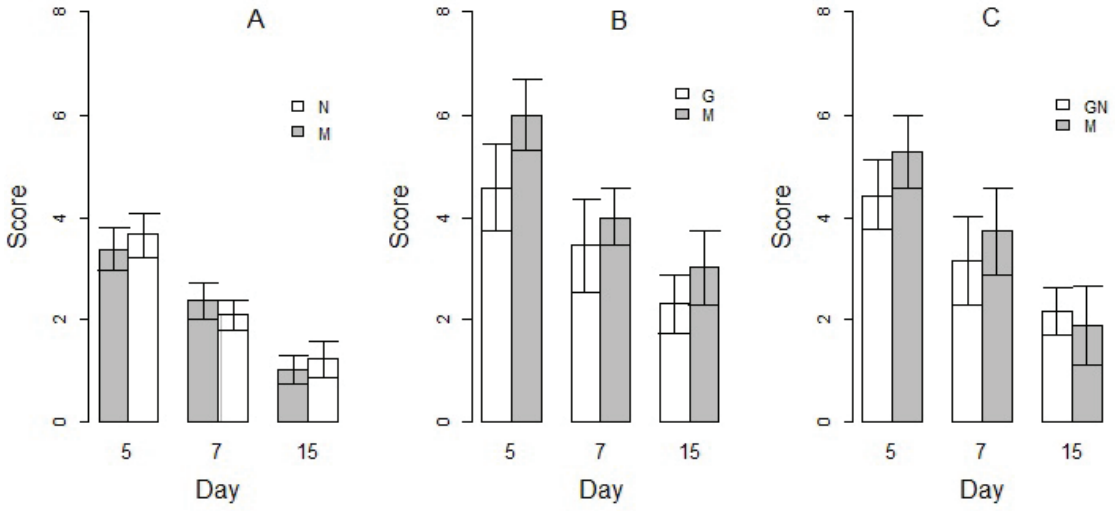


Fig.1. Clinical examination score, before, during and after treatment with (**A**) tea tree oil (M) and Nystatin (N); (**B**) tea tree Oil (M) and gentamicin (G); (**C**) tea tree oil (M) and gentamicin + Nystatin (GN), to the groups: yeast otitis, bacterial otitis and yeast and bacterial otitis. The bars represent a standard error of the mean.

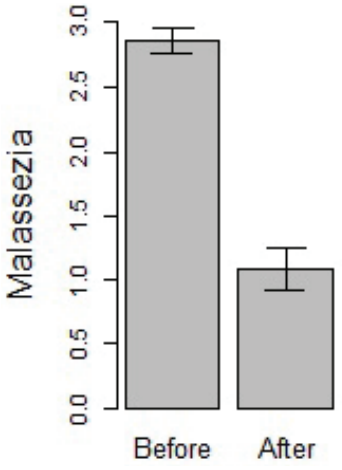


Fig.2. Results of cytological examination before and after treatment with tea tree oil (M) and Nystatin (N) of the yeast otitis group. The bars represent a standard error of the mean.

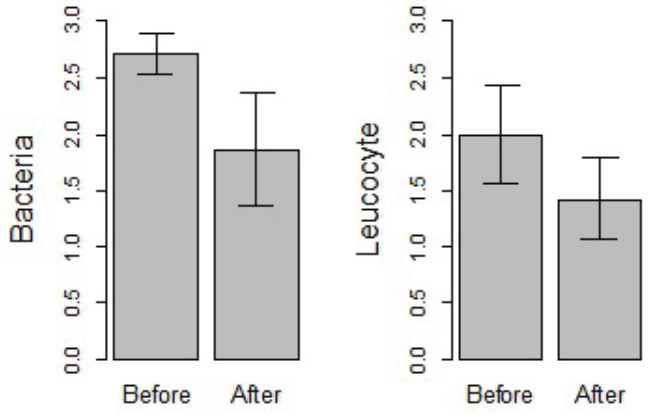


Fig.3. Results of cytological examination before and after treatment with tea tree oil (M) and Gentamicyn of the bacterial otitis group. The bars represent a standard error of the mean.

infection was uninstalling, since the number of inflammatory cells was decreased with the use of tea tree oil. Packer e Luz (2007) proposed the hypothesis of bacteriostatic and fungistatic effectiveness of tea tree essential oil for strains of *Staphylococcus aureus*, *Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans*, depending on the individual immune response.

Despite not all dogs treated with tea tree essential oil have healed completely in this 14 days trial, a significant improvement of clinical and cytological were evident in all patients on days +7 and +14, and all 28 dogs treated with tea tree essential oil showed significant improvement in clinical parameters (inflammation, pruritus, ear discharge) and in cytological evaluation (quantification of micro-organisms and inflammatory cells), when comparing findings before and after treatment. Decrease in inflammation in 21 human volunteer after application of tea tree oil was described by Koh et al. (2002). Similar results were obtained in human patients with acne, when comparing tea tree oil and benzoyl peroxide at the same concentration (Bassett et al. 1990). Hart et al. (2000) suggest that terpinen-4-ol (42%), alpha-terpineol (3%) and 1,8-ceneole (2%) are the mainly responsible for this proinflammatory suppression effect, attributed to tea tree oil.

UFMG chromatography laboratory certified that the tea tree oil used was within established standards, with concentrations (Fig.5) of 42,9% of terpinen-4-ol and 6,1% of cineol, which determined the low incidence of adverse effects (as for the quality levels above the established by the Australian Committee for Standardization) and also the expected effect against the micro-organisms in this study (as the higher concentration of terpinol-4, the higher its antimicrobial and anti-inflamatory activities) (Australian Standard AS2782-85).

Considering tea tree essential oil lotion used, there were no adverse effects, topical or systemic, as suggested by Gao et al. (2005) and Neves et al. (2012).

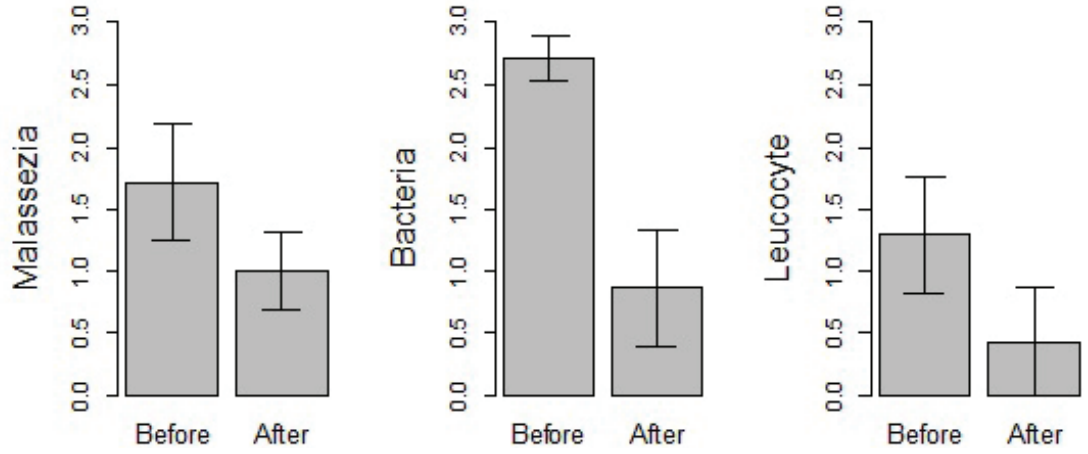
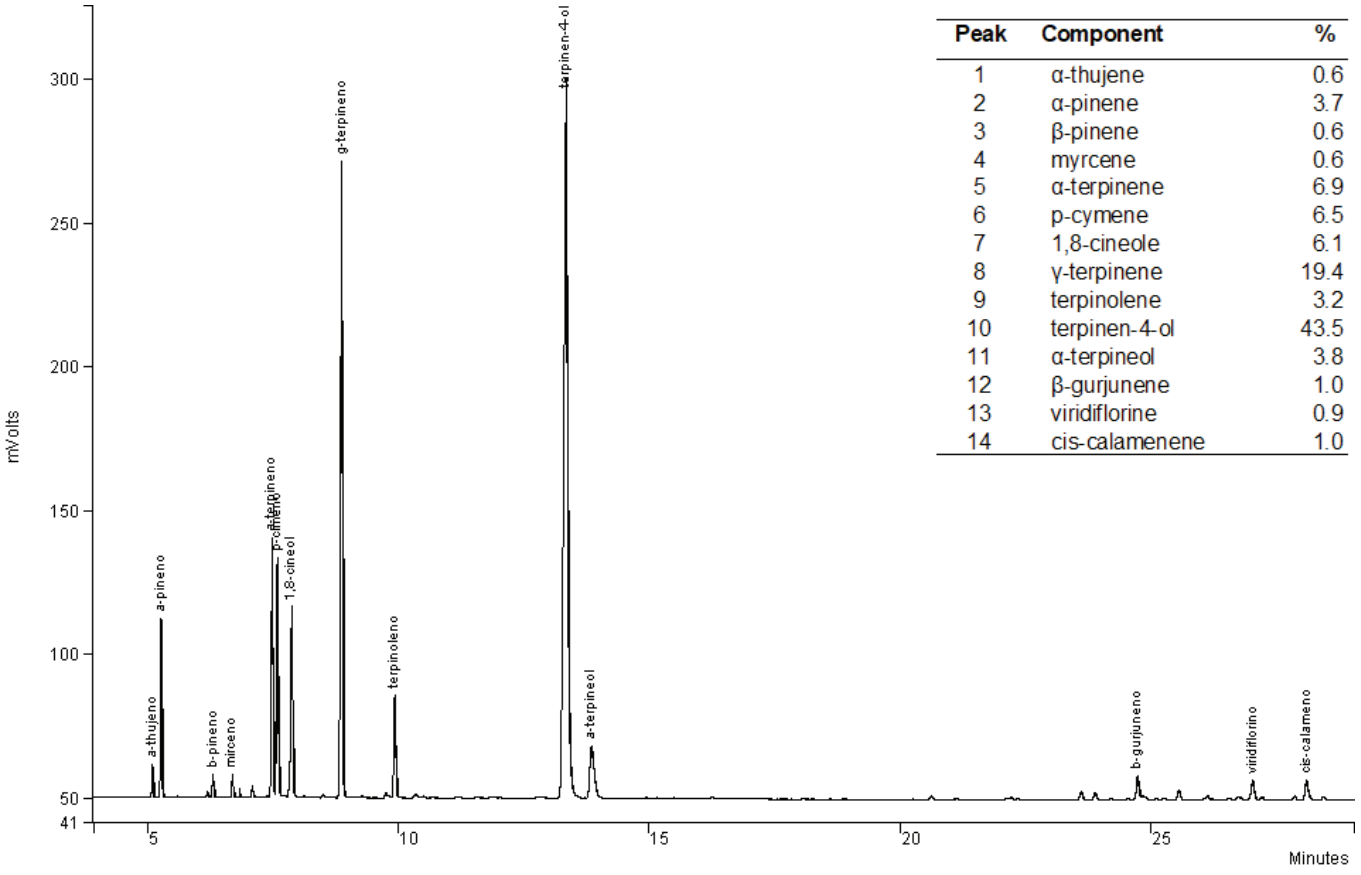


Fig.4. Results of cytological examination before and after treatment with tea tree oil (M) and Gentamicyn + Nystatin (GN) of the yeast and bacterial otitis group. The bars represent a standard error of the mean.



Peak	Component	%
1	α-thujene	0.6
2	α-pinene	3.7
3	β-pinene	0.6
4	myrcene	0.6
5	α-terpinene	6.9
6	p-cymene	6.5
7	1,8-cineole	6.1
8	γ-terpinene	19.4
9	terpinolene	3.2
10	terpinen-4-ol	43.5
11	α-terpineol	3.8
12	β-gurjunene	1.0
13	viridiflorine	0.9
14	cis-calamenene	1.0

Fig.5. Chromatography laboratory certified that the tea tree oil used was within established standards, with concentrations of 42.9% of terpinen-4-ol, 1.9% of alpha-terpineol and 1.7% of 1,8 cineol.

CONCLUSIONS

Tea tree essential oil ear solution significantly induced remission of clinical signs both in bacterial and yeast ear infections.

It also reduced as much *Malassezia pachydermatis* ear infection as the nystatin solution used in this study, while gentamycin solution showed better antibacterial effect.

More studies should be conducted to evaluate *in vitro* diffusion properties of tea tree essential oil.

Good antimicrobial spectrum and the absence of adverse reactions confirm the importance of developing a tea tree formulation as an alternative therapy for ear infections in dogs.

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