INTRODUCTION

Canine otitis externa (OE) is an inflammatory pathology commonly reported in veterinary clinical practice. During the early stages, inflammation results in erythema of the pinnae, external meatus, and lining of the external canal. Subsequently, there can be a wide range of clinical signs, such as head shaking, ear scratching, ceruminous, or purulent discharge, excoriations due to self-trauma, malodor, swelling, and pain. In recurrent or chronic cases, clinical signs may progress to proliferative changes leading to stenosis of external ear canal, and ultimately to occlusion (Guarda et al., 2013). In such a scenario bacteria or yeasts act as perpetuating causes of the inflammatory process, since they are not responsible for the initiation of the OE but permit to continue once established and can lead to pathology chronicity (Bajwa, 2019; Guaguère & Prèlaud, 2005). Staphylococcus and Pseudomonas are bacteria able to produce
biofilm that acts as protection and can lead to therapeutic failure (Bajwa, 2019), while among yeasts, the species by far more prevalent is *Malassezia pachydermatis* (Guillot & Bond, 2020).

In clinical practice, most cases of acute OE are managed using polyvalent topical ear products that include a glucocorticoid (used to control mild acute inflammation), and antimicrobial agents used to treat concurrent infections (Nuttall, 2016). Various detergents, such as ethylenediaminetetraacetic acid (TrizEDTA) and chlorhexidine, are largely used to clean the ear canal, remove debris, and excessive cerumen production and to disrupt biofilm (Guardabassi et al., 2009; Pye, 2018). Antibiotics, especially fluoroquinolones, aminoglycosides, and polymyxins, should be used only after the identification of etiological agents and only after ascertaining the integrity of the tympanic membrane (Ghibaudo et al., 2015). Treatments against *M. pachydermatis* include antifungal drugs like azole derivatives (thiabendazole, clotrimazole, miconazole, and itraconazole), nystatin, and terbinafine. The main causes of therapeutic failure are incorrect patient medication management, and the lack of identification of primary conditions (Nuttall, 2016) and progressing to otitis media force to switch to systemic therapy (Six et al., 2000).

Antimicrobial resistance (AMR) is a global threat for humans and animals, with a significant public health risk due to AMR transmission between these two populations considering the side-by-side style of life among humans and pets (WHO, 2015). Antimicrobial stewardships have been recently proposed in veterinary medicine and should be emphasized for frequently diagnosed pathologies (Vercelli et al., 2021), such as canine otitis externa (Chan et al., 2020). Resistance has been demonstrated for bacteria that can alter target sites, increased drug outflow, and enhance enzymatic degradation (Wright, 2005), and other mechanisms have been recently described also for *M. pachydermatis* (Angileri et al., 2018; Peano et al., 2017; Peano et al., 2020). Considering all the aforementioned factors, it does not surprise the increasing interest in alternative therapies. Medicinal plant-derived products represent today between 25% and 50% of pharmaceutical products (Gerwick, 2013; Gupta & Birdi, 2017).

The antimicrobial properties of medicinal plant extracts come from the large variety of secondary metabolites. These are intermediate or final products of plant metabolism, not fundamental for life plant processes, playing a defensive role toward bacteria, fungi, protozoa, and viruses (Gorlenko et al., 2020). Secondary metabolites include quinines, alkaloids, lecithins, polypeptides, flavones, flavonoids, coumarin, terpenoids, essential oils, and tannins (Chandra et al., 2017). The applications of plant-derived products are increasing, and mainly direct to treat parasitic disease and skin pathologies (Lopez et al., 2019; Tresch et al., 2019). Some studies have been performed in the last years to investigate the efficacy of essential oils (EOs) in otitis externa in dogs. Still, the majority are in vitro, and only one is in vivo (Nardoni et al., 2017; Sim et al., 2019; Sim, Khazandi, Pi, et al., 2019; Song et al., 2020).

The present study aimed to investigate the efficacy of a new phytotherapeutic blend containing essential oils with a double approach: first an in vitro evaluation on the most frequently diagnosticated microorganisms in case of canine otitis externa was performed. Then an in vivo trial was organized, evaluating the efficacy the same phytotherapeutic blend in dogs presenting with spontaneous acute otitis externa.

### MATERIALS AND METHODS

#### 2.1 | Blend composition

The commercially available Otogen® formulation was provided by the producing company (Nutrigen s.r.l., Prato, Italy). The commercial product includes essential oils of *Melaleuca alternifolia* (also named tea tree oil – TTO), *Thymus serpillum*, *Salvia officinalis*, *Eucalyptus officinalis*, *Rosmarinus officinalis*, *Macadamia alternifolia*, *Lavandula officinalis*, and *Helianthus annus*, as active compounds, and helianthus seed oil (HSO), isopropile miristate, isopropile adipate and a mixture of triglycerides as excipients. The different properties of the EOs

| Activity        | Natural component                  |
|-----------------|------------------------------------|
|                 | Malaleuca alternifolia| Thymus serpillum| Salvia officinalis| Eucaliptus officinalis| Rosmarinus officinalis| Anternifolia macadamia| Lavandula officinalis| Helianthus annus |
| Germicidal      | X                                   |
| Antimicrobial   | X                                   |
| Antibacterial   | X X X X X                     |
| Antifungal      | X X                               |
| Antiseptic      | X X X X                         |
| Antioxidant     | X                                  |
| Hydrating       | X                                  |
| Emollient       | X                                  |
| Skin-regenerating | X X X                     |
| Anti-inflammatory | X                             |

The present study aimed to investigate the efficacy of a new phytotherapeutic blend containing essential oils with a double approach: first an in vitro evaluation on the most frequently diagnosticated microorganisms in case of canine otitis externa was performed. Then an in vivo trial was organized, evaluating the efficacy the same phytotherapeutic blend in dogs presenting with spontaneous acute otitis externa.

**TABLE 1** properties of essential oils present in Otogen blend (Bozin et al., 2007; Carson et al., 2006; Rasooli & Mirmostafa, 2000; Woronuk et al., 2011)
are summarized in Table 1. The acidity of the blend was stated at 0.22% ± 0.02. The measurement was performed according to the European Regulatories 2568/1991 and 2016/1227 by a certified laboratory (Appendix S1).

### 2.2 In vitro assays

The in vitro efficacy of Otogen® blend was assessed following the method of the European Normative UNI EN 1275 for the evaluation of fungicidal or yeasticidal activity of chemical disinfectants and antiseptics (Anon., 2005), with some modifications. The method consists in evaluating the number of living microorganisms after the contact with antiseptics at different time points.

The organisms tested included one clinical strain of *M. pachydermatis*, *Pseudomonas aeruginosa*, and *Staphylococcus pseudintermedius* and an ATCC strain of *Candida albicans* (ATCC strain 90028).

The microorganism inoculums (Test Suspension - TS) were prepared by picking some bacterial or yeast colonies and suspending them into sterile tubes with HSO. The TS was vortexed for 5 min to obtain a preparation as homogeneous as possible. The use of HSO instead of distilled water recommended in the Normative (Anon., 2005), was necessary since the phytotherapic blend under test is a mix of oils. The possible effects of HSO on microorganisms were assessed in preliminary experiments, and it was established that HSO does not affect microorganism viability (data not shown).

As regards the inoculum size, we could not reach that indicated in the Normative (1–5 \( \times 10^7 \) colony-forming units [CFUs]/ml), due to the high viscosity of HSO. Thus, in our experiments, the inoculum sizes were: 4.5 \( \times 10^6 \) [CFUs]/ml (*M. pachydermatis*); 1.9 \( \times 10^5 \) CFUs/ml (*C. albicans*); 1.2 \( \times 10^6 \) CFUs/ml (*S. pseudintermedius*); 3.1 \( \times 10^5 \) CFUs/ml (*P. aeruginosa*).

For each test, 1 ml of TS was added in a tube with 9 ml of the blend (final concentration of the blend 90%, blend test suspension - BTS 90), and in a tube with 5 ml of the blend plus 4 ml of HSO (final concentration of the blend 50% - BTS 50). After 5, 15, and 60 min of contact, 50 µl of the different suspensions were seeded in neutralizing media: Sabouraud dextrose agar with Tween 80 30 g/L and lecithin 3 g/L was used for *M. pachydermatis* and *C. albicans*; Mueller Hinton agar with Tween 80 30 g/L and lecithin 3 g/L was used for

### Table 2

| n°   | Breed                  | Age (Year) | Sex | Neutered | Weight (kg) |
|------|------------------------|------------|-----|----------|-------------|
| 1    | Labrador retriever     | 1.5        | M   | No       | 28          |
| 2    | Golden retriever       | 12         | F   | Yes      | 43          |
| 3    | Mix breed              | 5          | M   | Yes      | 25          |
| 4    | Cavalier King Charles Spaniel | 5      | M   | No       | 10          |
| 5    | Newfoundland           | 14         | F   | Yes      | 50          |
| 6    | Bernese Mountain Dog   | 5          | F   | No       | 45          |
| 7    | German Shepherd        | 1          | F   | No       | 25          |
| 8    | Weimaraner             | 6          | F   | Yes      | 25          |
| 9    | Maremma shepherd       | 6          | M   | Yes      | 31          |
| 10   | German Shepherd        | 12         | F   | No       | 30          |
| 11   | Maremma shepherd       | 5          | F   | Yes      | 32          |
| 12   | Mixed breed            | 4          | F   | Yes      | 23          |

### Table 3

| Time of contact | 5 min | 15 min | 1 h |
|-----------------|-------|--------|-----|
| Microorganism   | CFUs/ml in the starting inoculum | Blend concentration under test | CFU | % of growth compared with starting inoculum | % of germ reduction |
| M. pachydermatis| 4.5 \( \times 10^6 \) | 90% | 20 | 0.0004 | 99.9996 |
|                 | 50%  |      | 246800 | 5.5303 | 94.4697 |
| C. albicans     | 1.9 \( \times 10^5 \) | 90% | 11400 | 6.0881 | 93.9119 |
|                 | 50%  |      | 10760 | 5.7463 | 94.2537 |
| S. pseudintermedius | 1.2 \( \times 10^6 \) | 90% | nc  | –       | – |
|                 | 50%  |      | nc  | –       | – |
| P. aeruginosa   | 3.1 \( \times 10^5 \) | 90% | nc  | –       | – |
|                 | 50%  |      | nc  | –       | – |
bacteria. After incubation at 37°C for 48–72 h and 24 h for yeasts and bacteria, respectively, CFUs in each Petri dish were counted and the reduction compared with the TS was calculated. According to the Normative (Anon., 2005) a product may be considered "effective" when causing at least a 4 decimal log reduction of the germ number (i.e., a reduction equal to 99.99%) after a 15-min contact time.

2.3 In vivo evaluation

To perform the in vivo evaluation of the phytotherapic blend, we enrolled owned dogs presenting with clinical symptoms of acute otitis externa. Owners signed an informed consent before the beginning of the trial. Dogs could be of any breed, weight, sex, or neuter status, provided that they were at least 8-week-old. Exclusion criteria consisted in the administration of systemic or topical drugs within the last 2 months. It was hypothesized to withdraw patients during the study for the following reasons: adverse events, administration of concomitant therapy, owner incompliance, or any other documented reason. Each ear was considered separately, as a single case: this is because each ear can have different anatomy and a unique microenvironment. Table 2 reports the description of the 12 dogs enrolled in the study.

At the first visit, a complete physical examination was performed by a veterinarian. All the information regarding the dog, past and recent anamnesis, general and objective examinations, signs of otitis and findings at the otoscopic evaluation were recorded. At the same time, a sample was collected for a cytological exam. Otogen® was administered once a day for seven consecutive days, using cotton soaked in the product. At the end of the treatment, a complete physical examination, including otological exam, and a new cytological exam were performed.

2.4 Ear examination

Both ears of each dog were examined. The investigator scored the severity of nine clinical signs – for each ear - of otitis externa divided into two main groups:

- Parameters investigated by clinical history and physical examination: head tilt, shaking/discomfort, pain, pruritus, and bad smell.
- Parameters investigated by otoscopic examination (following the method OTIS3 by Nuttall & Bensignor, 2014, with slight modifications): - erythema, edema/swelling, exudate, and quantification of earwax.

Scores for each parameter were given on a severity scale of 0–3 (0 = none; 1 = mild; 2 = moderate; 3 = marked). The sum of the scores yielded the total score for each ear (maximum score 27).

The presence of mites, ulcers, and foreign bodies was also recorded.

2.5 Cytological exam

Cerumen samples were collected using a swab prior to the first and after the last administration. Slides were prepared by rolling the swabs on their surface. They were stained by the Wright’s technique (Merchant, 2005) and observed microscopically for the presence of yeasts (Malassezia) and bacteria (coccis and rods).

Following a semiquantitative criterium (Merchant, 2005), the presence of microorganisms was evaluated, as follows:

- **Malassezia** (observation at 40X, mean count considering 10 fields):
  - Mean count ≤2: normal.
  - Mean count 3–4: intermediate growth.
  - Mean count ≥5: overgrowth.
- **Bacteria** (observation at 100X, mean count considering 10 fields):
  - Mean count ≤5: normal.
  - Mean count 6–24: intermediate growth.
  - Mean count ≥25: overgrowth.

The presence of inflammatory cells (neutrophils and macrophages), eventually with bacteria within them, was also recorded as an evidence of actual infection.
2.6 | Effectiveness evaluation criteria

At the control visit, a complete cure (ear "recovered") was defined as a return to normal of all parameters (sum of scores = 0).

Secondary criteria were also considered:

- an improvement between 80% and 100% of the initial severity score was considered as "strong improvement";
- between 60% and 80% "clear improvement";
- between 40% and 60% "improvement";
- between 0% and 40%: the dog was considered as "steady";
- if total score of the final visit was higher than that of the first examination the condition was considered as "worsened".

According to cytological findings, pathogens were considered normalized in case the score went to 0. Other evaluations were possible (improved, unmodified, worsened) basing on the comparison of the score pre- and post-treatment.

3 | RESULTS

3.1 | In vitro study

3.1.1 | Contact assays

The results obtained are presented in Table 3 and Figures 1-4. As regards the BTS 90, the highest efficacy was shown against M. pachydermatis (99.99% reduction already after 5 min of incubation) followed by C. albicans and P. aeruginosa (99.99% reduction after 1 h of incubation). For S. pseudintermedius, the activity was good, though the reduction did not reach 99.99%. The activity of the blend diluted at 50% was generally inferior, but anyway around 99% in many cases after 15 min or 1 h of contact (Table 3).

3.2 | In vivo study

Figure 5 presents the scores indicating the severity of clinical signs of otitis, considering single ears, before and after treatment. The figure reports, for each ear, the sum of scores regarding all the parameters considered (results for the individual parameters are available in Appendix S2). Table 4 shows data about the assessment of treatment efficacy. Main outcome (complete recovery) is considered together with secondary criteria. Mites, ulcers, and foreign bodies were not found either during the first visit or during the control visit.

No adverse effects were recorded and none of the cases was withdrawn.

3.3 | Cytological exam

Inflammatory cells were not seen in any of the cases. As regards microorganisms, the presence of cocci and Malassezia was noted in 18 and 12 cases, respectively. Rods were not present.

The number of microorganisms decreased after the treatment with Otogen: 11 cases were normalized and 3 were improved for cocci. Only 3 cases were unchanged and 1 was worsened out of 18. Malassezia
decreased till the normalization in 9 cases, unchanged in 2 cases and worsed only in 1 case out of 12 (details are available in Appendix S3).

4 | DISCUSSION

The present study permitted to evaluate the efficacy of a new phytotherapeutic blend during canine acute otitis externa, using a dual experimental approach. The results obtained by the in vitro assays are suggestive of high efficacy against *M. pachydermatis*. The BTS 90 was able to induce a significant (>99.99%) decrease of yeast growth after 5 min of incubation. The present results are in line with those obtained by other investigators, though these latter employed a different susceptibility test (Hammer et al., 2003): TTO MICs for all test fungi ranged from 0.004% to 0.25% and minimum fungicidal concentrations (MFCs) ranged from <0.03% to 8.0%. These data demonstrate that TTO has both inhibitory and fungicidal activity. Even if the BTS 50 was not able to reach the cutoff value, it was able to induce an appreciable reduction of the concentration of *Malassezia*. These data should be considered positively in the perspective of clinical application: an incomplete administration (i.e., a small amount administered or product accidental removal) might be sufficient to limit the yeast proliferation.

Contact assays with *C. albicans* and *P. aeruginosa* demonstrated a significant inhibition after 1 h of incubation in the presence of BTS 90. The BTS 50 was able to limit microorganism’s growth without reaching the cutoff value.
The results obtained with S. pseudintermedius demonstrated the incomplete efficacy of BST 90 and 50, though the reduction percentage approximated the significant value of 99.99%. Interestingly, the number of CFUs was higher after 1 h contact than 15 min. This phenomenon may be due to a bacteriostatic or slightly bactericidal action, that may hesitate in a partial inhibition of the pathogen.

Among the different EOs contained in the phytotherapeutic blend, Melaleuca alternifolia and Salvia officinalis have been considered as potent antibacterial agents and confirmed efficacy obtained in the present study (Tresch et al., 2019). Essential oils could thus be included in the treatment, as an alternative therapeutic option, alone or in combination with allotherapeutic approach (Ebani et al., 2017).

The essential oils are known to possess antimicrobial activity (Mickienė et al., 2011); particularly TTO is the most effective contrasting the growth of streptococci, enterococci, staphylococci, as well as having an antifungal effect against yeasts and dermatophytes (Carson et al., 2006). Some studies reported that TTO could have a sensitizing potential if used pure or in high concentrations, or in case of oxidation of its components terpinene-4-ol and α-terpinene (Groot & Schmidt, 2016). In the present study, the concentration of TTO in the blend did not cause any allergic reaction, indicating a good biocompatibility of the entire formulation. It seems also to possess a potent bactericidal activity toward multiresistant bacteria such as methicillin-resistant Staphylococcus aureus (MRSA), K. pneumoniae resistant to carbapenem, Acinetobacter baumannii and Pseudomonas aeruginosa (Oliveira et al., 2018). Salvia officinalis, Eucalyptus officinalis, and Lavandula officinalis are also able to control the growth of cocci and bacilli involved in the development of otitis externa. Salvia officinalis also shows an antifungal effect that can counteract the growth of Candida spp. (Oliveira, Vilela, et al., 2019) while lavender has a possible application against parasite infestations, including those caused by mites (Cavanagh & Wilkinson, 2002). The control of the inflammatory process can be mediated by the extracts of the Eucalyptus which, thanks to its high content of 1 – 8 cineole, is able to suppress the production of pro-inflammatory cytokines (Jerguson et al., 1998). To control pain, the essential oil of Rosmarinus officinalis is able to achieve an antinociceptive effect, and demonstrates antibacterial effect against Streptococcus spp., Staphylococcus spp., Pseudomonas aeruginosa, and Candida albicans, all involved in the development of the ear inflammatory process (Oliveira et al., 2019).

A limit of the present study may be the inoculum size employed in in vitro tests. The number of CFUs was indeed different from organism to organism, and in none of the experiments, we could reach the size indicated in the standard procedure followed (Anon., 2005). Due to the fact that we tested the final commercial product, we had to suspend the germs in oil, which did not allow to prepare a homogeneous and reproducible inoculum. Using “classical” broth-dilution methods (whose results are expressed as MIC values) would have allowed us to overcome this technical problem and obtain a more accurate and reproducible evaluation of the activity of the blend components. We decided to use contact tests instead, because we think that they are more predictive of in vivo outcome of topical treatment. Contact tests allow to take into account the main factors which influence the efficacy of antimicrobial topical products, namely the product formulation effects and the duration of contact (Russel & McDonnel, 2000). This in turn allows simulating what happens when the final marketed formulation is applied on skin or in the ears. Lloyd and Lamport (1999) demonstrated that formulation is an important factor affecting the antimicrobial efficacy of topical products (chlorhexidine in that case). In marketed products, other principles (i.e., surfactants) can interact with the active principles. Contact tests have been employed in another study on the activity of topical formulations employed to treat dermatitis and otitis in dogs (Nebbia et al., 2008).

Our study was performed to have a preliminary idea on the activity of the phytotherapeutic blend against the most representative microorganisms involved in canine OE. Therefore, only one isolate for each species was tested in vitro. A point of strength is that 3 out of 4 microorganisms were of clinical provenance, the results obtained in the in vitro experiments are easily comparable to real cases.

Considering the data obtained by the in vivo part it was possible to appreciate that several breeds presenting with signs of otitis externa were recruited, without concomitant pathologies. All the clinical signs of otitis externa were improved after a daily administration in most dogs, and owner’s compliance was high and mainly because they appreciated the pleasant scent, able to immediately reduce the unpleasant odor frequently occurring in course of otitis. The amelioration of typical signs of otitis, such as head shaking and pruritus, is an important point in the cure approach of this pathology, where auto-traumatism can worsen the clinical situation. Considering the cytological parameters, the macroscopic improvement is positively correlated with the decrease of the presence of cocci and Malassezia. A global consideration of all the above results can lead to the conclusion that the phytotherapeutic blend is able to induce a cleaning of the canal that permits to active substances to reestablish ear homeostasis, reducing the inflammation process and enhance global clinical conditions of the patient. According to the authors’ knowledge, few papers investigated the role of essential oils to treat otitis externa. None evaluated the efficacy of a complex blend like that used in the present study (Nardoni et al., 2017; Sim, Khazandi, Chan, et al., 2019).

The results obtained in the present study should be worthy of attention and can be considered a starting point for further

### Table 4: Outcome of treatment

|               | n (%) | n (cumulative) (%) |
|---------------|-------|--------------------|
| Cure          | 8 (33.3) | 8 (33.3) |
| Strong improvement | 5 (20.8) | 13 (54.2) |
| Clear improvement | 7 (29.2) | 20 (83.3) |
| Steady        | 2 (8.3)  | 22 (22)  |
| Worsened      | 2 (8.3)  | 24 (24)  |
| Total         | 24 | |
investigations on the efficacy of plant-derived compounds. The tested bacteria demonstrated in the last years increasing antimicrobial resistance phenomena. The total or partial inhibition obtained by the phytotherapeutic blend used in the present study suggests a good efficacy of EOs against these pathogens.

All the above considerations come from selected subjects that do not present chronic otitis, and it is not possible to extend these considerations to complicated otitis externa. The Authors’ advice is to consider Otogen to clean and regulate the wetting of the ear environment in case of complicated otitis externa but further studies are needed to collect information specifically addressed to this condition.

5 | CONCLUSION

The phytotherapeutic blend tested solution could be regarded as valuable support to cure acute otitis externa in dogs.

The improvement shown for both the clinical and cytological examinations in most of the enrolled cases leads to the hypothesis that the product has good effectiveness in restoring and maintaining homeostasis of the auricle environment.

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CONFLICT OF INTEREST

The authors declare that the study received financial support by Nutrigen LG Distribuzione S.r.l., necessary to purchase materials. No other conflicts of interest have to be declared in publishing this work.

AUTHOR CONTRIBUTIONS

CV and AP designed the experimental procedures, coordinate the different experimental phases and wrote the draft of the paper, and CV performed the visits, MP and SV performed the analysis, and enrolled patients, GG1, GG2, GR, and MG supervised all the procedures, revised the results, checked the draft of the paper.

DATA AVAILABILITY STATEMENT

All relevant data are within the paper and supporting information files.

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REFERENCES

Angileri, M., Pasquetti, M., De Lucia, M., & Peano, A. (2018). Azole resistance of Malassezia pachydermatis causing treatment failure in a dog. Medical Mycology Case Reports, 21(23), 58–61.

Anon. (2005) Chemical disinfectants and antiseptics - quantitative suspension test for the evaluation of basic fungicidal or basic yeasticidal activity of chemical disinfectants and antiseptics - test method and requirements (phase 1). EN 1275. Brussels: European Committee for Standardization. Retrieved from http://www.en-standard.eu/csn-en-1275-chemical-disinfectants-andantiseptics-quantitative-suspension-test-for-the-evaluation-of-basic-fungicidal-or-basic-yeasticidal-activity-of-chemical-disinfectants-and-antiseptics-test-method-and-requirements-phase-1/. Accessed Mar 20, 2015.

Bajwa, J. (2019). Canine otitis externa - Treatment and complications. The Canadian Veterinary Journal = La Revue Vétérinaire Canadienne, 60(1), 97–99.

Bozin, B., Mimica-Dukic, N., Samojlik, I., & Jovin, E. (2007). Antimicrobial and antioxidant properties of rosemary and sage (Rosmarinus officinalis L. and Salvia officinalis L., Lamiaceae) essential oils. Journal of Agricultural and Food Chemistry, 55(19), 7879–7885.

Carson, C. F., Hammer, K. A., & Riley, T. V. (2006). Malaleuca alternifolia (Tea Tree) Oil: A review of antimicrobial and other medicinal properties. Clinical Microbiology Reviews, 19, 50–62.

Cavanagh, H. M., & Wilkinson, J. M. (2002). Biological activities of lavender essential oil. Phytotherapy Research, 16, 301–308. https://doi.org/10.1002/ptr.1103

Chan, W. Y., Hickey, E. E., Khazandi, M., Page, S. W., Trott, D. J., & Hill, P. B. (2020). In vitro antimicrobial activity of narasin and monensin in combination with adjuvants against pathogens associated with canine otitis externa. Veterinary Dermatology, 31(2), 138–145.

Chandra, H., Bishnoi, P., Yadav, A., Patni, B., Mishra, A. P., & Nautiyal, A. R. (2017). Antimicrobial resistance and the alternative resources with special emphasis on plant-based antimicrobials - A review. Plants, 6, 1–11. https://doi.org/10.3390/plants6020016

de Groot, A. C., & Schmidt, E. (2016). Tea tree oil: Contact allergy and chemical composition. Contact Dermatitis, 75(3), 129–143. https://doi.org/10.1111/cod.12591

Ebani, V. V., Nardoni, S., Bertelloni, F., Najar, B., Pistelli, L., & Mancianti, F. (2017). Antibacterial and antifungal activity of essential oils against pathogens responsible for otitis externa in dogs and cats. Medicine, 4, 21. https://doi.org/10.3390/medicines4020021

Gerwick, W. H. (2013). Plant sources of drugs and chemicals. Encyclopedia of Biodiversity, 129–139. https://doi.org/10.1016/B978-0-12-809633-8.02306-2

Ghibaudo, G., Leone, F., Buracco, P., & Bernardini, M. (2015). Capitolo: Orecchio esterno, terapia medica in Malattie dell’orecchio esterno del cane e del gatto, Elanco, Sesto Fiorentino.

Gorlenko, C. L., Kiselev, H. Y., Budanova, E. V., Zamyatnin, A. A., & Ikryannikova, L. N. (2020). Plant secondary metabolites in the battle of drugs and drug-resistant bacteria: New heroes or worse combination of antibiotics? Antibiotics, 9, 170. https://doi.org/10.3390/antibiotics9040170

Guaguere, E., & Prélau, P. (2005). Capitolo: Dermatite e oiti da Malassezia in Gulla pratica di dermatologia canina, Merial, Parigi. (pp. 123–131).

Guarda, F., Mandelli, G., Biolatti, B., & Scanziani, E. (2013). Capitolo: Organi di senso, orecchio in Trattato di anatomia patologica veterinaria, UTET Scienze Mediche, Torino (pp. 655–657).

Guardabassi, L., Ghibaudo, G., & Damborg, P. (2009). In vitro antimicrobial activity of a commercial ear antiseptic containing chlorhexidine and Tris – EDTA. Veterinary Dermatology, 21, 282–286.

Guillot, J., & Bond, R. (2020). Malassezia yeasts in veterinary dermatology: An updated overview. Frontiers in Cellular and Infection Microbiology, 10, 79. https://doi.org/10.3389/fcimb.2020.00079
Gupta, P. D., & Birdi, T. J. (2017). Development of botanicals to combat antibiotic resistance. *Journal of Ayurveda and Integrative Medicine, 8*(4), 266–275. https://doi.org/10.1016/j.jaaim.2017.05.004

Hammer, K., Carson, C., & Riley, T. (2003). Antifungal activity of the components of Melaleuca alternifolia (tea tree) oil. *Journal of Applied Microbiology, 95*, 853–860. https://doi.org/10.1046/j.1365-2672.2003.02059.x

Juergens, U. R., Stober, M., & Vetter, H. (1998). Inhibition of cytokine production and arachidonic acid metabolism by eucalyptol (1,8-cineole) in human blood monocytes in vitro. *European Journal of Medical Research, 3*, 508–510

Lloyd, D. H., & Lampert, A. I. (1999). Activity of chlorhexidine shampoos in vitro against Staphylococcus intermedius, Pseudomonas aeruginosa and Malassezia pachydermatis. *The Veterinary Record, 144*(19), 536–537.

López, V., Pavela, R., Gómez-Rincón, C., Les, F., Bartolucci, F., Gallifa, V., Petrelli, R., Cappelletti, L., Maggi, F., Canale, A., Otranto, D., Sut, S., Dall’Acqua, S., & Benelli, G. (2019). Efficacy of origanum syriacum essential oil against the mosquito vector culex quinquefasciatus and the gastrointestinal parasite anisakis simplex, with insights on acetylcholinesterase inhibition. *Molecules (Basel, Switzerland), 24*(14), 2563. https://doi.org/10.3390/molecules24142563

Merchant, S. R. (2005). Chapter 9 - Microbiology of the ear of the dog and cat. In L. N. Gotthelf (Ed.), *Small animal ear diseases* (14), 256–262. W.B. Saunders.

Mickienė, R., Bakutis, B., & Baliūkonienė, V. (2011). Antimicrobial activity of two essential oils. *Annals of Agricultural and Environmental Medicine, 18*(1), 139–144.

Nardoni, S., Pistelli, L., Baronti, I., Najar, B., Pisseri, F., Bandeira Reidel, R. V., Papini, R., Perrucci, S., & Manciandi, F. (2017). Traditional Mediterranean plants: Characterisation and use of an essential oils mixture to treat Malassezia otitis externa in atopic dogs. *Natural Product Research, 31*(16), 1891–1894.

Nebbia, P., Robino, P., Tramuta, C., Beccati, M., & Peano, A. (2008). Stima dell’attività antimicrobica in vitro di due prodotti commerciali contenenti clorexidina e olio di melaleuca su ceppi batterici e lieviti isolati da cani. *Veterinaria, 22*(1), 63–67.

Nuttall, T. (2016). Successful management of otitis externa. *Practice, 38*, 17–21.

Nuttall, T., & Bensignor, E. (2014). A pilot study to develop an objective clinical score for canine otitis externa. *Veterinary Dermatology, 25*(6), S30–e92. https://doi.org/10.1111/vde.12163

Oliva, A., Costantini, S., De Angelis, M., Garzoli, S., Božović, M., Mascellino, M., Vullo, V., & Ragon, R. (2018). High potency of melaleuca alternifolia essential oil against multi-drug resistant gram-negative bacteria and methicillin-resistant staphylococcus aureus. *Molecules, 23*, 2584. https://doi.org/10.3390/molecules23102584

Oliveira, J. R., Camargo, S., & Oliveira, L. (2019). Rosmarinus officinalis L. (rosemary) as therapeutic and prophylactic agent. *Journal of Biomedical Science, 26*, 1–22. https://doi.org/10.1186/s12929-019-0499-8

Oliveira, J. R., Vilela, P., Almeida, R., Oliveira, F. E., Carvalho, C., Camargo, S., Jorge, A., & Oliveira, L. D. (2019). Antimicrobial activity of non-cytotoxic concentrations of Salvia officinalis extract against bacterial and fungal species from the oral cavity. *General Dentistry, 67*, 22–26.

Peano, A., Johnson, E., Chiavassa, E., Tizzani, P., Guillot, J., & Pasquetti, M. (2020). Antifungal Resistance Regarding Malassezia pachydermatis: Where Are We Now? *Journal of Fungi*, 6(2), E93.

Peano, A., Passquetti, M., Tizzani, P., Chiavassa, E., Guillot, J., & Johnson, E. (2017). Methodological Issues in Antifungal Susceptibility Testing of Malassezia pachydermatis. *Journal of Fungi (Basel, Switzerland), 3*(3), 37.

Pye, C. (2018). *Pseudomonas otitis externa in dogs*. The *Canadian Veterinary Journal = La Revue Vétérinaire Canadienne*, 59(11), 1231–1234.

Rasooli, I. & Mirmostafa, S. A. (2002). Antibacterial properties of Thymus pubescens and Thymus serpyllum essential oils. *Fitoterapia, 73*(3), 244–250.

Russell, A. D., & McDonnell, G. (2000). Concentration: a major factor in studying biocidal action. *The Journal of Hospital Infection, 44*(1), 1–3.

Sim, J., Khazandi, M., Chan, W. Y., Trott, D. J., & Deo, P. (2019). Antimicrobial activity of thyme oil, oregano oil, thymol and carvacrol against sensitive and resistant microbial isolates from dogs with otitis externa. *Veterinary Dermatology, 30*(6), 524–e159. https://doi.org/10.1111/vde.12794

Sim, J., Khazandi, M., Pi, H., Venter, H., Trott, D. J., & Deo, P. (2019). Antimicrobial effects of cinnamon essential oil and cinnamaldehyde combined with EDTA against canine otitis externa pathogens. *Journal of Applied Microbiology, 127*(1), 99–108.

Six, R., Clemence, R., Thomas, C., Behan, S., Boy, M., Watson, P., Benchouai, H., Clements, P., Rowan, T., & Jennigan, A. (2000). Efficacy and safety of selamectin against Sarcopetes scabiei on dogs and Otodectes cynotis on dogs and cat presented as veterinary patients. *Veterinary Parasitology, 91*, 291–309.

Song, S. Y., Hyun, J. E., Kang, J. H., & Hwang, C. Y. (2020). In vitro anti-bacterial activity of the manuka essential oil from Leptospermum scoparium combined with Tris-EDTA against Gram-negative bacterial isolates from dogs with otitis externa. *Veterinary Dermatology, 31*(2), 81–85.

Tresch, M., Mevissen, M., Ayre, H., Melzig, M., Roosje, P., & Walkenhorst, M. (2019). Medicinal plants as therapeutic options for topical treatment in canine dermatology? A systematic review. *BMC Veterinary Research, 15*, 174. https://doi.org/10.1186/s12917-019-1854-4

Vercelli, C., Della Ricca, M., Re, M., Gambino, G., & Re, G. (2021). Antibiotic stewardship for canine and feline acute urinary tract infection: An observational study in a small animal hospital in Northwest Italy. *Antibiotics, 10*, 562. https://doi.org/10.3390/antibiotics10050562

World Health Organization (WHO), (2015). *Global action plan on antimicrobial resistance*. WORONUK, G., Demissie, Z., Rheault, M., & Mahmoud, S. (2011). Biosynthesis and therapeutic properties of Lavandula essential oil constituents. *Planta Medica, 77*(1), 7–15.

Wright, G. (2005). Bacterial resistance to antibiotics: Enzymatic degradation and modification. *Advanced Drug Delivery Reviews, 57*(10), 1451–1470. https://doi.org/10.1016/j.addr.2005.04.002

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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