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RESEARCH ARTICLE

# Evaluation of Antibacterial Potential of *Satureja montana* L., *Ocimum basilicum* L. and *Salvia officinalis* L. Essential Oils against Reproductive Tract Pathogens in Cattle and their Toxicity Impact on Endometrial and Kidney Cells

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**A B S T R A C T**

The abundant use of commercial anti-infective agents has led to the resistance development in bacterial strains, the presence of their residues in animal products and other negative consequences. The aim of the present work was to examine the antibacterial activity of EOs obtained from three plants (*Satureja montana* L., *Ocimum basilicum* L. and *Salvia officinalis* L.) against field isolates and reference bacterial strains of the most important pathogens in the cow endometrium. Their susceptibility was tested *in vitro* using a disk diffusion method, and the agar dilution test (ADT) and microdilution test (MDT) were used to determine the minimum inhibitory concentrations (MIC). In addition, the cytotoxic effect of the highest effective oil was tested by MTT, using the two cell lines, i.e. the BEND cell line (endometrium, 24h after exposure), and the MDBK cell line (kidney, 3, 6, 12 and 24h after exposure). The results of the antimicrobial assays suggest that the EO of *S. montana*, consisting mainly of p-cymene (42.8%) and carvacrol (28.1%), is a potent antimicrobial agent with high zones of inhibition determined on the disk diffusion method, where none of the individual isolates were resistant. Moreover, the calculated MIC values on both ADT and MDT were <1 mg/mL for bacterial isolates of all species. In addition, the oil exhibited low cytotoxic potential on both BEND ($IC_{50}$=1.27 mg/mL) and MDBK cell lines ($IC_{50}$=1.02-1.56 mg/mL). The obtained results indicate the possibility of the use of *S. montana* EO for the treatment of bacterial-induced reproductive diseases in cows.

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## INTRODUCTION

The increasing demand for food of animal origin, especially in developing countries, is one of the biggest challenges facing livestock farming worldwide today (Lupia *et al*., 2024). In this context, reproductive performance remains one of the main factors influencing the profitability of the cattle industry and making it economically viable (Yoo, 2010; Tóth *et al*., 2023). In cattle, uterine diseases can occur at all stages of the reproductive cycle, with the postpartum period being considered particularly risky (Drillich and Wagener, 2018). These diseases cause high economic losses in the dairy sector related to reduced milk production or discarding of milk due to antibiotics, increased use of drugs to treat diseases, reduced fertility and prolonged interval to pregnancy, as well as fatal outcome or early culling of cows in some cases (Eslami *et al*., 2015; Paiano *et al*., 2020). Therefore, they still represent a significant health problem in most commercial dairy farms (Várhidi *et al*., 2024). In industrialized countries, uterine infections in dairy cows cost approximately €1.4 billion in Europe and $650 million in the United States (Mekibib *et al*., 2024), while some estimates suggest that production losses due to metritis cost producers an average of $511 per case (Basbas *et al*., 2022).

Although several uterine diseases can occur, including puerperal metritis, clinical metritis and pyometra (Drillich and Wagener, 2018; Várhidi *et al*., 2024), endometritis is considered one of the most important, characterized by inflammation of the endometrium around 21 days postpartum with purulent or mucopurulent uterine discharge (Paiano *et al*., 2020). There may also be a subclinical form characterized by an increased proportion of polymorphonuclear leukocytes in the endometrium without the presence of clinical disease (Paiano *et al*., 2022). In both cases, most common pathogenic bacteria that can cause metritis include *Escherichia coli*, *Trueperella pyogenes*, *Fusobacterium necrophorum*, *Bacteroides* spp., *Staphylococcus* spp., *Pasteurella* spp., *Histophilus somni*, *Pseudomonas aeruginosa*, *Clostridium* spp. and *Streptococcus* spp. (Negasee, 2020). In the case of clinical endometritis, which is typically caused by a mixed bacterial infection, the most important pathogens are *Trueperella* (formerly known as *Actinomyces* or *Arcanobacterium*) pyogenes and *E. coli* (Paiano *et al*., 2023).

Treatment of infectious uterine diseases depends on the etiology, pathophysiology and severity and mainly involves the use of a combination of antibiotics, NSAIDs and hormones, including uterotonics. The most commonly used antibiotics are tetracycline, amoxicillin, ampicillin and sulfonamides, often with trimethoprim, cephapirin, ceftiofur, etc. (Várhidi *et al*., 2024). However, a major problem today is the development of antimicrobial resistance in many bacterial pathogens responsible for uterine diseases in cows, caused by the incorrect use of antibiotics, both in terms of dosage and timing of administration, or even use without proper diagnosis (Lupia *et al*., 2024). In addition, the widespread use of antibiotics in the treatment of uterine infections has led to the establishment of multidrug-resistant strains of bacteria that prolong the treatment of the infection and increase the risk of mortality (Amin *et al*., 2023). Public attention has also focused on the safety problems caused by residues of veterinary drugs, including antibiotics, in animal products, which can pose a risk to human health and the entire ecosystem (Mesfin *et al*., 2024; Bosco *et al*., 2025). By passing on the reservoir of resistance to humans and the environment, the use of antibiotics above or below the recommended dosage in animals can also lead to short- or long-term public health problems. These include the emergence of resistant bacterial strains, but also toxicity, allergy, mutagenesis, teratogenicity and carcinogenic effects. (Mesfin *et al*., 2024).

For these reasons, there is a worldwide consensus that alternative solutions should be implemented in the treatment of bovine metritis in order to reduce the use of antibiotics. Várhidi *et al*. (2024) noted that endometritis could heal spontaneously if the oestrus successfully clears the uterus, whereas metritis may require systemic treatment to restore the general welfare of the animals. However, if treatment is necessary, numerous alternative solutions have been investigated, including intrauterine application of proteolytic enzymes (Singh *et al*., 2020), dextrose solution (Lection *et al*., 2024) and/or chitosan nanoparticles (Okawa *et al*., 2021), the use of probiotics (Adnane *et al*., 2024), bacteriophages (Zduńczyk and Janowski, 2020), acetylsalicylic acid (Barragan *et al*., 2021), subcutaneous or intravaginal vaccination (Meira Jr. *et al*., 2020) and even acupuncture therapy (Pinedo *et al*., 2020). Numerous *in vitro* and some *in vivo* studies performed so far also demonstrated the positive impact of using medicinal plants and their products, such as essential oils and extracts, which are usually applied intrauterine in field studies for the treatment of bovine metritis and other uterine diseases (Paiano *et al*., 2020, 2023; Armansyah *et al*., 2023; Menoud *et al*., 2024). In addition to their efficacy against a wide range of diseases, these products have been reported to have minor adverse effects on reproduction and milk production, whereby these treatments are cost-effective (Kadivar *et al*., 2022).

Essential oils (EOs) are complex mixtures of volatile compounds extracted from aromatic plants (Ratajac *et al*., 2024; Štrbac *et al*., 2024). Due to the chemical diversity of their composition, they are a natural source of great pharmacological interest (de Sousa *et al*., 2023). Since EO have antibacterial and antifungal properties, some of them have already been shown potential to be used in the treatment of various reproductive diseases in cows, such as mastitis (Caneschi *et al*., 2023), vaginitis (Lee *et al*., 2023) and various uterine diseases including metritis, endometritis and pyometra (Lefebvre *et al*., 2021; Várhidi *et al*., 2024). For these reasons, EOs and herbal products in general are thought to improve the reproductive performance of cows, including an increase in conception and pregnancy rates, as well as fertility in general (Serbester *et al*., 2012; Swelum *et al*., 2021). However, further studies within this area of application of EOs are needed, as it is still associated with many unknowns. In this perspective, EOs from three plant species from Lamiaceae family – winter or mountain savory (*Satureja montana* L.), basil (*Ocimum basilicum* L.), and sage (*Salvia officinalis* L.) are widely recognized medicinal plants whose EOs have already shown a variety of pharmacological properties, including antioxidant, anti-inflammatory, antibacterial, antifungal, antiparasitic and anticancer potential among others (Ghorbani and Esmaeilizadeh, 2017; Maccelli *et al*., 2019; Azizah *et al*., 2023). On the other hand, their full potential for the treatment of reproductive disorders in cows is still unexplored. The aim of this study was therefore to investigate the antibacterial effect of these EOs against pathogens isolated from the uterus of cows with reproductive disorders, and their reference strains.

## MATERIALS AND METHODS

**Plant material and chemical analyses:** Flowers of winter savory (*Satureja montana* L.) and basil (*Ocimum basilicum* L.) were obtained from the Department for Organic Production and Biodiversity, Institute for Field and Vegetable Crops, Novi Sad, Serbia, while commercially available leaves of sage (*Salvia officinalis* L.) were used. The EOs were extracted from these plant materials by steam distillation according to the procedure described in Ph.Jug.V (2.8.12), (Jugoslovenska farmakopeja), 2000.

Gas chromatography, i.e., United Technologies Packard model 439 gas chromatograph, equipped with a CP-SIL 5 CB capillary column (10m x 0.25mm ID, 100% dimethyl polysiloxane), and a flame ionization detector (GC-FID) was used for the qualitative and quantitative

analysis of the EOs. The following parameters were used: Column temperature 60ºC-200ºC; Injector temperature 2000°C; Detector temperature 2900°C; Carrier gas nitrogen; Flow rate of 86 mL/min. The components were identified by comparing the retention times of the sample signal with the retention times of available standards (Sigma, Chemical CO., USA and Merck-Schuchardt, Germany), and their percentage composition was determined on the basis of peak areas, without using correction factors.

**Animals and isolation of pathogenic bacteria:** For the present study, only isolates from cattle were used. A detailed history and clinical examination of the genital organs (rectal and/or vaginoscopic examination) were performed to determine the health status of the reproductive organs of animals during the puerperium and individuals with an extended service period. After identification of the suspect cows, isolation and identification of potentially pathogenic bacterial species were performed using routine methods applied according to Quinn *et al*. (1994), using biological material from the genital organs of the suspect cows (n=68). The cows were of Holstein Friesian breed, with an average age of 5.24 years–and number of calvings - lactations of 2.9 (1-6). Three cows had abortions at 6-7 months. The average number of days from calving (abortion) to clinical examination and taking samples was 115.8 days. The average number of treatments was 5.6, and the number of inseminations was 1.32.

After seeding the uterine mucus samples (0.1 ml) on culture media (blood agar - HiMedia Laboratories Pvt. Ltd., India) (with the addition of 5% defibrinated sheep blood) and MacConkey agar - HiMedia Laboratories Pvt. Ltd., India) and incubation for 24-48h at 37°C, macroscopic examination of the colonies was performed. The result of the bacteriological examination was considered negative if there was no growth on the blood agar or only insignificant contamination (saprophytic flora) was isolated. Suspicious colonies were taken from solid substrates and multiplied in pure culture, after which (or simultaneously) microscopic, biochemical and serological identification was performed. The number of clinical isolates used in disk-diffusion and agar-dilution methods was as follows: *Truperella pyogenes* (n=15), *Escherichia coli* (n=10), *Pasteurella* spp. (n=8), *Staphylococcus aureus* (n=15) and *Streptococcus* spp. (n=10). In addition to the clinical isolates, the following reference strains were also used: *Trueperella pyogenes* (ATCC 19411), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and *Streptococcus agalactiae* (ATCC 13813) as well as *Fusobacterium necrophorum* subsp. *necrophorum* ATCC 25286.

**Evaluation of antibacterial activity**

**Disk-diffusion method:** The inoculum was prepared and standardized from 24-hour-old bacterial cultures. The isolates were cultivated on a nutrient medium (Nutrient agar, Torlak) at 37°C for 24h and 48h. Using the UV-VIS spectrophotometer ANTHELIE (Seccoman, France) and the DEN-1 McFarland densitometer, MF units (Biosan, EU), and comparison with the 0.5 McFarland standard, the desired concentration of approximately 1-2 x $10^8$ CFU/ml was achieved. The isolated bacterial strains were inoculated with a sterile cotton swab or by direct application (5 mL) on Mueller-Hinton agar (for some bacteria with 5% sheep blood). The tested EOs were dissolved in propylene glycol or dimethyl sulfoxide (DMSO, AppliChem, Germany) v/v (10%). Subsequently, 10 µL of these (1 mg active ingredient per disk) were applied to sterile paper disks ("Sterile Disks", HiMedia Laboratories Pvt. Ltd., India) with a diameter of 6 mm. Pure propylene glycol or DMSO were used as controls along with commercial antibiotic disks.

The disks and tablets were applied manually using sterile tweezers (distance between the disks and tablets ≥ 30 mm and the distance to the edge of the Petri dish ≥ 10 mm). The prepared "antibiogram" plates were incubated in a thermostat at a temperature of 35-37°C for 18-48h. The results were then read by measuring the growth inhibition zone around the disks or tablets (Société Française de Microbiologie, 1996). The therapeutic category S, R and I of the tested strains for antibiotics was determined by comparing the inhibition zones read according to the manufacturer's reference values (Bioanalysis, Torlak and Pfizer (Oxoid ltd.), Serbia).

**Agar-dilution method:** The minimum inhibitory concentration (MIC) of EOs was determined using the agar dilution method (NCCLS, 1990, 2000) with minor modifications. In the present study, an inoculum of 10 µL containing a total of $10^4$ bacteria (1-2 x $10^6$/mL) was used. Bacterial susceptibility testing was performed in sterile plastic Petri dishes (90 mm, Spektar, Serbia). From each antimicrobial dilution, from the lowest to the highest concentration, 2 mL were poured into the test tubes to achieve the following final concentrations: 0.02, 0.039, 0.078, 0.156, 0.313, 0.625, 1.25, 2.5 and 5 mg/mL. Propylene glycol or DMSO was used as a negative control. Then, 18 mL of melted Mueller-Hinton agar (or MH with 5% sheep blood) (Torlak, Serbia), cooled to a temperature of 46-48°C, was added to the test tubes with the addition of Tween 80 (Sigma, Serbia) (0.5% v/v) to better dissolve and diffuse the EOs. After slight homogenization, the contents of the test tubes were poured into Petri dishes and it was waited until the agar had solidified. The Petri dishes were then dried in a thermostat at a temperature of 35-37°C for 30 min.

The substrates with the incorporated antimicrobial agents were inoculated with 10 µL of a previously standardized bacterial suspension. After incubation of the inoculated Mueller-Hinton agar (or MN with 5% sheep blood) in a thermostat at a temperature of 35 to 37°C for 18-48h, the results were read. Before determining the MICs, the growth of the tested strains was checked on a substrate without antimicrobial agents. The lowest concentration of a particular antimicrobial agent at which no visible bacterial growth or only a few individual colonies occurred was taken as the MIC. All combinations of bacteria and concentrations of the tested oils were performed in triplicate and in three independent experiments.

**Microdilution method:** The determination of the concentration of bacterial cells in the inoculum was performed using a 48-hour-old bacterial culture, cultivated in a nutrient medium (Thioglycolate, Himedie laboratories

Pvt. Ltd., India) under anaerobic conditions at 37°C for 48h. A concentration of 1-2 x $10^8$ CFU/mL was achieved as described in subsection 2.3.1. The MICs of EOs were determined by serial dilution in 96-cell microtiter plates (TC MICROWELL 96F, Nunc, Denmark). Working solutions of EOs and standards were applied to the plate and serially diluted in Tryptose soy broth (TSB) medium (Himedie laboratories Pvt.Ltd., India) along the columns of the microtiter plate. A series of final concentrations were prepared in the base of 16.0, 8.0, 4.0, 2.0, 1.0, 0.5, 0.25, 0.125, 0.0625 and 0.0312 mg/mL. Pure TSB substrate was used as a blank, TSB + culture as positive and TSB + sample as negative control. At the end of plate preparation, the final concentration of bacteria in the suspension (obtained by dilution) of 1 x $10^6$ CFU/mL was added to the wells in an amount of 10 µL/well (1 x $10^5$ CFU/well). The reading was performed after incubation (72h, 37°C) under anaerobic conditions, where the MIC (mg/mL) was defined as that present in the first pool with the lowest concentration in which no growth of the microorganisms took place (Rotilie *et al*., 1975; Citron and Hecht, 2011).

**Evaluation of cytotoxicity**

**Cell cultures:** The cell line BEND ATCC-CRL-2398 (American Type Culture Collection, USA), which was obtained from the endometrium of the uterus of a cow (*Bos taurus*, breed: crossbred Angus) on day 14 of the sexual cycle in 1997, and the cell line MDBK (NBL-1) ATCC-CCL-22, which was obtained from the kidney of a clinically healthy adult male bovine (*Bos taurus*) in 1957, were used for cytotoxicity assays. Both cell lines morphologically represent epithelial tissue and were obtained directly from ATCC (American Type Culture Collection, Manassas, USA).

**MTT assay:** The EO of *Satureja montana* was selected for the cytotoxicity assay and dissolved in dimethyl sulfoxide (dimethyl sulfoxide (DMSO) hybri-max, Sigma, Serbia), v/v (50%). Nutrients were then added to the medium to obtain the final concentrations of the substances in the medium: 5.0, 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078 and 0.039 mg/mL. To perform the MTT assay, cells were seeded at 1-2 x $10^4$ cells/well by adding 200 µL of medium containing 5 x $10^4$ cells/mL to each central well of a microtiter plate. Cell-free medium was added to the peripheral wells at a rate of 200 µL. Flat-bottom microtiter plates (TC MICROWELL 96F, Nunc, Denmark) were used for cell adhesion. The plates were incubated for 24h at 37°C with 5% CO2. The substrate was replaced by a fresh substrate (200 µL) containing up to 0.1 mL of the tested EO, the carrier and the substrate (control). The whole procedure regarding cytotoxic activity was performed according to the Ehrich and Sharova (2000).

Plates were incubated (37°C, 5% $CO_2$) at set time intervals (BEND cells were incubated for 24h, and MDBK cells at time intervals of 3, 6, 12 and 24h). After that, the existing medium was discarded and a solution of MTT (0.5 mg/mL or 0.05/well) (Thiazolyl Blue Tetrazolium Bromide, Sigma, USA) was added, prepared in the medium immediately before the addition. The plates were then incubated again (3h, 37°C, 5% $SO_2$), after which the medium containing MTT was carefully removed. To dissolve the formazan product, 100 µL of 0.04 N hydrochloric acid (HCl) in isopropanol was added to each well. The plates were stirred occasionally at room temperature until the formazan crystals dissolved and readings were taken after 6h. Colorimetric detection of staining was performed by measuring absorbance at a working wavelength of 540 nm (A1) and a reference wavelength of 690 nm (A2) using a multichannel reader (Labsystems Multiscan, MCC/340, Finland). The final absorbance is calculated as A=A1 - A2. The test was repeated three times for each group and consisted of at least three trials. The absorbances in the wells containing the test substances were compared with the wells containing untreated control cells, and the cytotoxicity was calculated according to the following formula:

$IC = (1 – As / Ac) \times 100$

As - absorbance of the sample with the *S. montana* EO

Ac - absorbance of the control samples

**Statistical analyses:** All data obtained in these tests were processed using the descriptive statistics method, where all tests were performed in triplicates. In order to process the data in the context of quantitative analysis, the results were presented as mean±standard deviation of three determinations. Statistical analyzes were performed using Student's t-test and one-way analysis of variance (ANOVA). Multiple comparisons of means were determined using the least significant difference (LSD) test. A probability value of 0.05 was considered significant. All calculations were performed using a statistical program (SPSS, version 11.0). The $IC_{50}$ values were calculated from a sigmoidal dose-response curve using a non-linear regression analysis.

## RESULTS

**Chemical analyses:** GC-MS analyses revealed a rich chemical composition of the EOs tested, with a total number of compounds of 17, 42 and 27 in *S. montana*, *O. basilicum* and *S. officinalis* EO, respectively. The main representative compounds in each oil were p-cymene (42.8%), carvacrol (28.1%) and γ-terpinene (14.6%) in *S. montana*; linalool (62.8%), γ-muurolene (4.45%) linalool and estragole (3.78%) in *O. basilicum*, as well as α-thujone (38.8%), camphor (19.8%) and eucalyptol (8.40%) in *S. officinalis* EO (Table 1, Fig. 1-3).

**Bacteriological examination of biological material:** The bacteriological examination, which included aerobic, microaerophilic and anaerobic cultivation on solid and liquid media, yielded negative results in 18 or 26.5% of the examined samples of uterine swabs from cows with reproductive problems. Strict anaerobes causing endometritis were not isolated from the samples by anaerobic cultivation. One type of bacteria causing endometritis was isolated in 42 or 62%, and two types of bacteria that could cause endometritis were isolated and identified in 8 individuals or 12 of the samples. A total of 58 isolates were isolated, representing seven different types of bacteria: *Trueperella pyogenes* (25.9%), *Escherichia coli* (17.2%), *Pasteurella multocida* (13.8%), *Staphylococcus aureus* (25.9%), *Streptococcus uberis* (8.6%), *Streptococcus α haemolyticus* (6.9%) and *Streptococcus dysgalactiae* (1.7%).

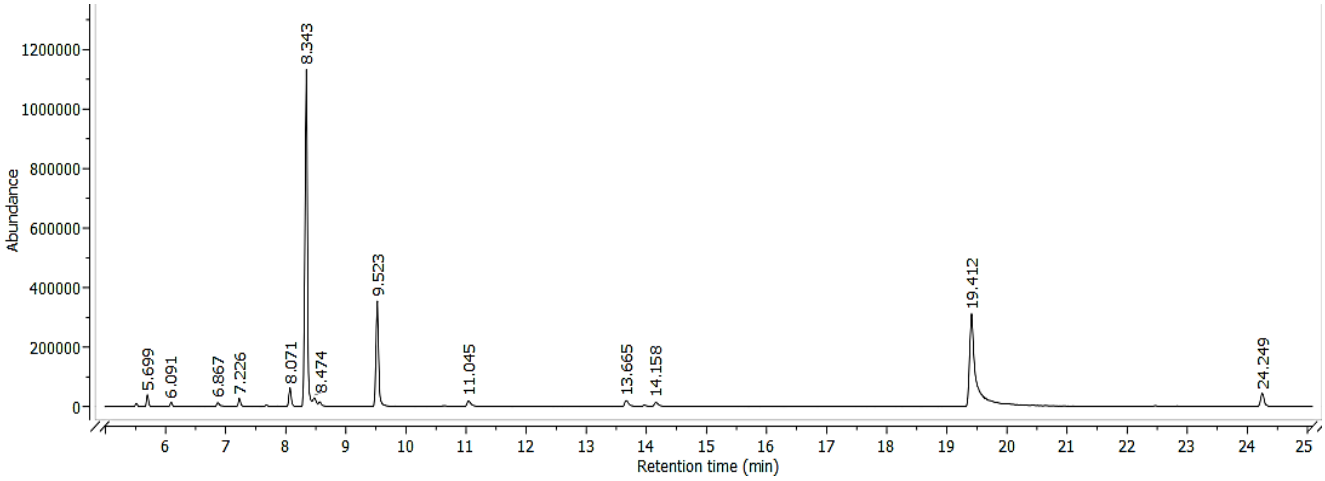


**Fig. 1:** GC–MS chromatogram of the *Satureja montana* essential oil.

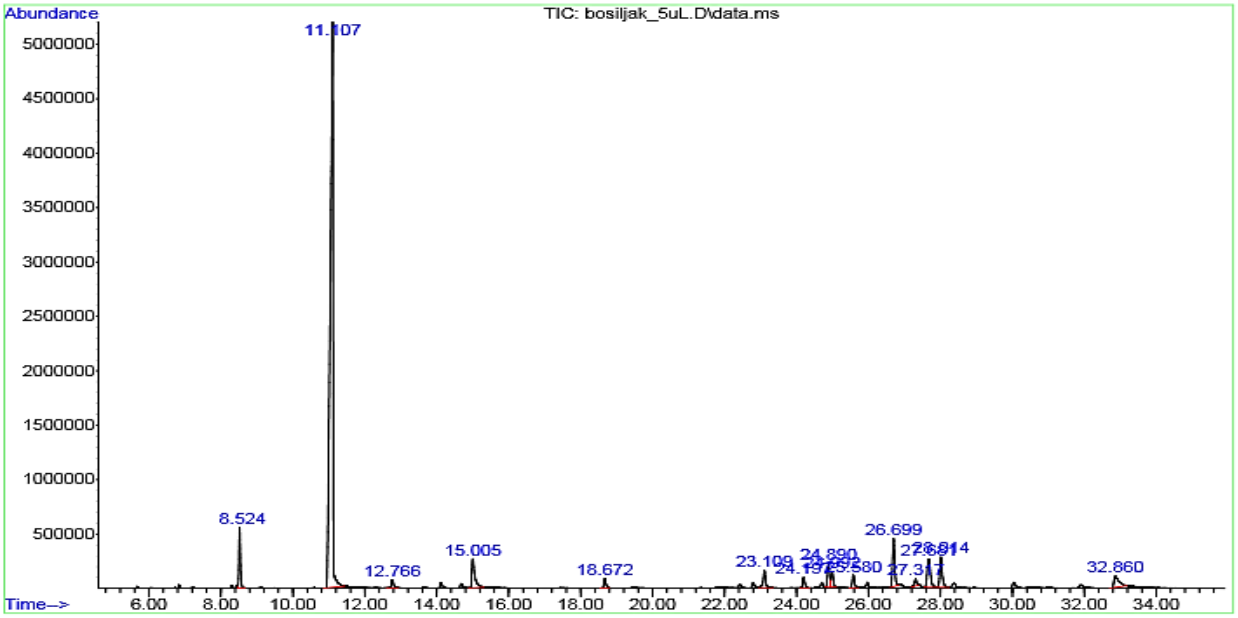


**Fig. 2:** GC–MS chromatogram of the *Ocimum basilicum* essential oil.

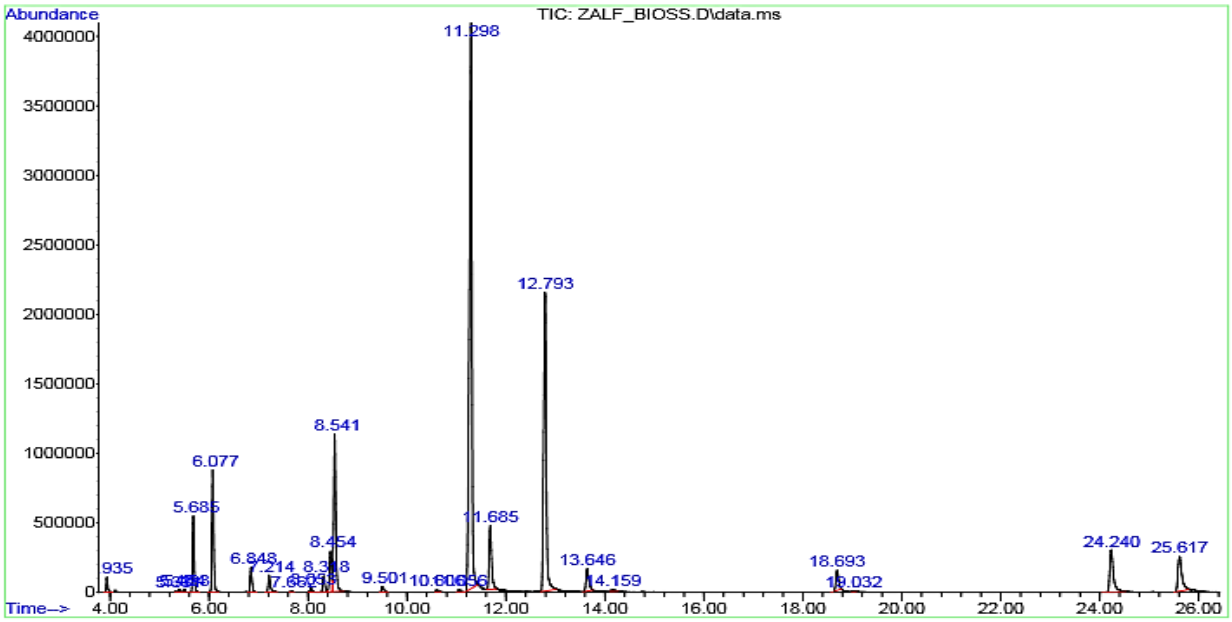


**Fig. 3:** GC–MS chromatogram of the *Salvia officinalis* essential oil.

**Evaluation of antibacterial activity**

**Disk-diffusion method:** In the first antibacterial test performed, *S. montana* EO showed the highest activity against the tested bacterial species, which included reference strains and clinical isolates (Table 2). The highest activity was obtained against *T. pyogenes* and *Pasteurella* spp. (zone of inhibition ≥18 mm), and none of the tested isolates of each bacterial species were resistant to this EO. In contrast, the EOs of *O. basilicum* and *S. officinalis* were inactive (inhibition zone ≤10 mm). In the same test, amoxicillin + clavulanic acid, ceftiofur and chloramphenicol were the most effective antibiotics, to which more than 90% of the isolates of the different bacterial species were sensitive. In contrast, streptomycin, neomycin and tetracycline showed the least activity with <50 susceptible and >25 resistant isolates. However, all reference strains were sensitive to all antibiotics tested, except in the case of erythromycin and penicillin (Table 3).

**Agar-dilution method:** Similar to the results of the disk diffusion test, the EO of *S. montana* was the most effective, and the EO of *S. officinalis* showed the least antibacterial activity ($P<0.05$) in agar-dilution method (Table 4). In each EOs tested, the effect against different bacterial species was similar ($p>0.05$), and in the case of *S. montana* and *O. basilicum*, the effect was significantly higher than that of the negative control for all bacterial species tested ($P<0.05$). The calculated mean MICs, including both the clinically isolated and the reference strains of all tested bacteria, were 0.60±0.15, 6.27±0.16 and 19.60±7.70 mg/mL for *S. montana*, *O. basilicum* and *S. officinalis* EO, respectively.

**Microdilution method:** The reference strain and the purchased sample of *F. necrophorum* showed the same susceptibility to the EOs tested in the microdilution method. The most effective oil was *S. montana* with a MIC of 0.125 mg/ml, and the least effective was *S. officinalis* with a determined MIC of over 16 mg/ml (Table 5).

**Evaluation of cytotoxicity**

The results of the MTT test clearly show the dose-dependent cytotoxic activity of *S. montana* EO, with the percentage of surviving cells decreasing with increasing concentration (Fig 4. and 5.). Namely, the tested EO showed no cytotoxic effect on the BEND cell line after 24h of incubation at the concentrations of 0.039 and 0.078 mg/mL, while concentrations of 0.156 (0.8%, STD=1.3) and 0.3126 (7.0%, STD=1.6) mg/mL showed a mild cytotoxic effect. Concentrations of 0.625, 1.25 and 2.5 mg/mL showed intermediate cytotoxic effects with 43.3% (STD=3.3), 44.5 (STD=3.8) and 54.8% (STD=1.6), respectively, whereas the concentration of 5.0 mg/mL showed a high cytotoxic effect of 92.1% (STD=0.9). The calculated $IC_{50}$ and $IC_{25}$ values are shown in Fig. 4.

In the MDBK cell culture, longer exposure and a higher concentration of *S. montana* EO caused higher cytotoxic activity (Fig. 5). Concentrations from 0.039 to 0.625 mg/mL caused no cytotoxic effect at any of the time points (3, 6, 12 and 24h after incubation), while concentrations of 1.25, 2.5 and 5 mg/mL caused cytotoxicity of 24.4-49%, 88.6-98.1% and 89.9-100%, respectively, depending on the time of exposure. The calculated $IC_{50}$ values were 1.56, 1.16, 1.07 and 1.02 mg/mL at 3, 6, 12 and 24h after incubation, respectively.

Finally, Fig. 6 shows the $IC_{50}$/MIC quotients calculated for both cell lines after different exposure times. The higher value of this quotient indicated a higher activity of the tested antimicrobial agent and/or its higher safety.

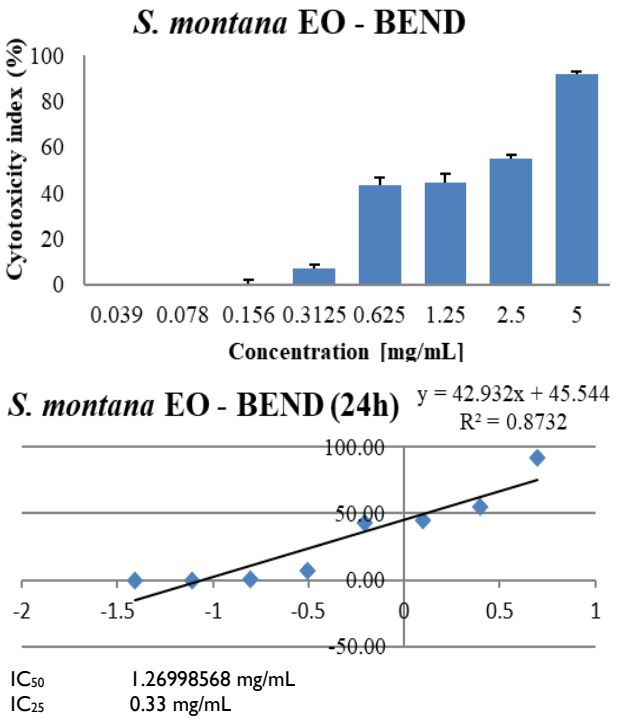


**Fig. 4:** The results of the MTT cytotoxicity test for *Satureja montana* EO obtained on the BEND cell line after 24 hours of incubation.

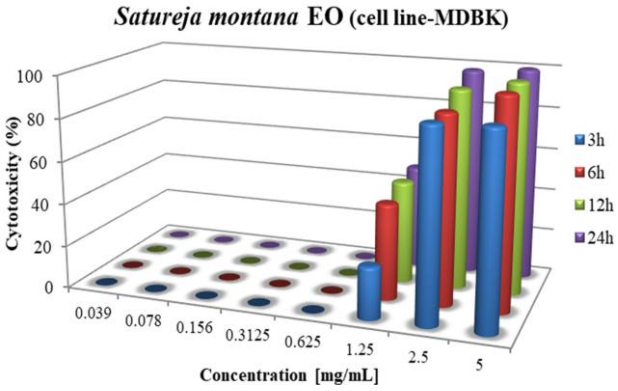


**Fig. 5:** Cytotoxicity of *Satureja montana* EO on the MDBK cell line depending on the time of exposure - incubation (3, 6, 12 and 24 hours).

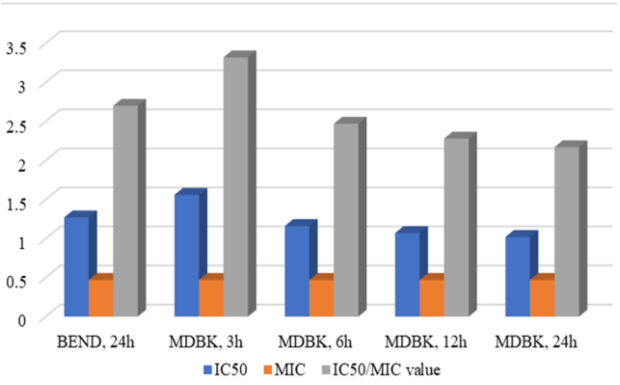


**Fig. 6:** $IC_{50}$/MIC quotient on both cell lines after different time of exposure.

**Table 1:** Chemical composition (% of total peak area) of tested essential oils determined by gas chromatography-mass spectrometry.

| AI | Compound/Essential oil | % of total peak area | | |
|---|---|---|---|---|
| | | *Satureja montana* | *Ocimum basilicum* | *Salvia officinalis* |
| 847 | cis-Salvene | - | - | 0.52 |
| 858 | trans-Salvene | - | - | 0.07 |
| 918 | n.i. | - | - | 0.04 |
| 921 | Tricyclene | - | - | 0.14 |
| 925 | α-Thujene | 0.33 | - | 0.14 |
| 932 | α-Pinene | **1.16**[a] | 0.1 | **3.16** |
| 947 | Camphene | 0.44 | 0.03 | **5.36** |
| 971 | Sabinene | - | 0.04 | 0.02 |
| 976 | β-Pinene | 0.66 | 0.19 | **1.16** |
| 990 | β-Myrcene | 0.96 | 0.10 | 0.91 |
| 1005 | α-Phellandrene | 0.18 | - | 0.09 |
| 1016 | α-Terpinene | **2.32** | - | 0.25 |
| 1024 | p-Cymene | **42.8** | 0.19 | 0.90 |
| 1027 | Limonene | **1.52** | 0.18 | **2.22** |
| 1030 | 1,8-Cineole | 0.73 | **3.52** | **8.40** |
| 1045 | n.i. | - | 0.10 | - |
| 1057 | γ-Terpinene | **14.6** | 0.02 | 0.36 |
| 1065 | n.i. | - | 0.01 | - |
| 1070 | n.i. | - | 0.02 | - |
| 1088 | α-Terpinolene | 0.17 | 0.05 | 0.20 |
| 1100 | Linalool | **1.20** | **62.8** | 0.20 |
| 1106 | α-Thujone | - | - | **38.8** |
| 1116 | β-Thujone | - | - | **5.07** |
| 1142 | Camphor | - | 0.73 | **19.8** |
| 1163 | n.d. | - | 0.16 | - |
| 1164 | Borneol | **1.27** | - | **1.89** |
| 1176 | Terpinen-4-ol | 0.78 | 0.46 | 0.34 |
| 1189 | α-Terpineol | - | 0.51 | - |
| 1197 | Methyl chavicol | - | **3.78** | - |
| 1283 | Isobornyl acetate | - | 0.74 | - |
| 1284 | Bornyl acetate | - | - | **1.86** |
| 1292 | Sabinyl-acetate | - | - | 0.11 |
| 1302 | Carvacrol | **28.1** | - | - |
| 1374 | α-Copaene | - | 0.32 | - |
| 1383 | β-Panasinsene | - | 0.53 | - |
| 1390 | β-Elemene | - | **1.72** | - |
| 1418 | β-caryophyllene | **2.46** | 0.92 | **3.95** |
| 1427 | β-Copaene | - | 0.09 | - |
| 1429 | β-Gurjunene | - | 0.46 | - |
| 1434 | α-trans-Bergamotene | - | **2.11** | - |
| 1436 | γ-Elemene | - | **1.42** | - |
| 1451 | α-Humulene | - | **1.13** | **4.15** |
| 1460 | cis-Cadina-1(6),4-diene | - | 0.46 | - |
| 1479 | γ-Muurolene | - | **4.45** | - |
| 1483 | α-Amorphene | - | 0.55 | - |
| 1494 | γ-Amorphene | - | 0.85 | - |
| 1496 | Viridiflorene | - | 0.30 | - |
| 1503 | α-Bulnesene | - | **3.66** | - |
| 1512 | γ-Cadinene | - | **2.98** | - |
| 1521 | δ-Cadinene | - | 0.64 | - |
| 1536 | α-Cadinene | - | 0.08 | - |
| 1564 | sesquiterpene | - | 0.63 | - |
| 1575 | Spathulenol | - | 0.19 | - |
| 1613 | sesquiterpene | - | 0.64 | - |
| 1639 | α-epi-Cadinol | - | **2.17** | - |
| Number of identified compounds | | 17 | 42 | 27 |

* AI - arithmetic retention index; a - compounds present in more than 1% are shown in bold.

**Table 2:** Susceptibility (zone of inhibition, mm) of isolates of different bacterial species obtained from infected animals and its reference strains to the tested essential oils

| Essential oil | | *T. pyogenes* | *E. coli* | *Pasteurella* spp. | *S. aureus* | *Streptococcus* spp. |
|---|---|---|---|---|---|---|
| *Satureja* | CI | ≥18 | 16-18 | ≥18 | 12-18 | 14-18 |
| *montana* | RS | ≥18 | 10-18 | - | 10-18 | 10-18 |
| *Ocimum* | CI | 8-12 | ≤10 | ≤10 | ≤10 | ≤10 |
| *basilicum* | RS | ≤10 | ≤10 | - | ≤10 | ≤10 |
| *Salvia* | CI | ≤10 | ≤10 | ≤10 | ≤10 | ≤10 |
| *officinalis* | RS | | | | | |
| Propylene | CI | ≤10 | ≤10 | ≤10 | ≤10 | ≤10 |
| glycol | RS | | | | | |

## DISCUSSION

The evaluation of the antimicrobial potential of an active substance requires the application of suitable methods. The disk diffusion method allows the simultaneous testing of a large number of antimicrobial substances in a relatively simple and flexible way, whereby the diameter of the inhibition zone and the CLSI interpretation criteria determine the classification of the results into three categories: sensitive, intermediate or resistant. However, the main disadvantage is the fact that this method is not quantitative and therefore cannot generate MIC values (Jiang, 2011). On the other hand, agar dilution is a quantitative susceptibility testing method that allows the determination of the lowest concentration, i.e. the MIC value of the tested agent, and it is suitable for evaluating the activity of new antimicrobial agents (Wiegand *et al*., 2008), although it can be time-consuming and labor-intensive (Jiang, 2011). Finally, a microdilution technique was developed to determine the MICs of antimicrobial agents for anaerobic bacterial species (Rotilie *et al*., 1975).

In the disk diffusion method, only the EO of *S. montana* showed an effect on the tested bacterial species (Table 2.). Moreover, this EO was the only antimicrobial agent, including the antibiotics tested, to which none of the bacterial isolates of any of the species tested were resistant. Thus, the effect of the oil was quite comparable to the antibiotics tested and showed the highest efficacy against *Pasteurella* spp. and, along with ampicillin, also against *T. pyogenes* of all antimicrobial agents tested. In addition, the differences in the zone of inhibition that occurred to some extent between the bacterial species suggest a different sensitivity of these species to the EOs, although the efficacy against all species tested was high. Regarding the results for antibiotics (Table 3.), the combination of amoxicillin + clavulanic acid, ceftiofur and chloramphenicol was the most active with a high percentage of susceptible clinical isolates (>90%). In addition, lincomycin + spectinomycin combination and enrofloxacin also showed high activity with more than 85% of susceptible isolates. In contrast, many of the antibiotics tested, including neomycin, streptomycin, tetracycline and the combination of sulfamethoxazole + trimethoprim, showed low activity with a high percentage of resistant isolates (>25%). This and the fact that, in contrast to the clinical isolates obtained from the animals, all antibiotics were highly effective against the reference strains suggest a high level of resistance development on the examined farms. Similar to the *S. montana* EO, the effect of most antibiotics tested clearly depended on the bacterial species, indicating that a combination of the tested EO and antibiotics can achieve the highest effect against all species.

The results of the agar-dilution method (Table 4.) confirmed the results of the disk-diffusion method, in which *S. montana* showed the strongest antimicrobial effect, which was significantly higher ($P<0.05$) than other EOs in all tested bacterial species. Namely, the average MIC value was below <1 mg/mL in all cases, and the overall average value was 0.54±0.09. In contrast, EOs of *O. basilicum* and *S. officinalis* were less effective with average values for all bacterial species of 6.27±0.16 and 19.60±7.70 mg/mL, respectively. Although not significant

**Table 3:** Susceptibility of isolates of different bacterial species obtained from infected animals and its reference strains to different antibiotics and propylene glycol

| Antibiotic and dose | | | *T. pyogenes*, number of CI and (%) | *E. coli*, number of CI and (%) | *Pasteurella* spp., number of CI and (%) | *St. aureus*, number of CI and (%) | *Streptococcus* spp., number of CI and (%) | *TOTAL, number of CI and (%)* |
|---|---|---|---|---|---|---|---|---|
| Amoxicillin + clavulanic acid, 20+10 µg | CI | S | 15 (100) | 8 (80) | 6 (75) | 14 (93.3) | 10 (100) | *57 (91.9)* |
| | | I | 0 (0) | 2 (20) | 1 (12.5) | 0 (0) | 0 (0) | *3 (4.9)* |
| | | R | 0 (0) | 0 (0) | 1 (12.5) | 1 (6.7) | 0 (0) | *2 (3.2)* |
| | RS | | S | S | - | S | S | |
| Ampicillin, 10 µg | CI | S | 15 (100) | 7 (70) | 5 (62.5) | 9 (60) | 10 (100) | *50 (80.6)* |
| | | I | 0 (0) | 3 (30) | 2 (25) | 0 (0) | 0 (0) | *5 (8.1)* |
| | | R | 0 (0) | 0 (0) | 1 (12.5) | 6 (40) | 0 (0) | *7 (11.3)* |
| | RS | | S | S | - | S | S | |
| Ceftiofur, 30 µg | | S | 14 (93.3) | 10 (100) | 8 (100) | 15 (100) | 10 (100) | *61 (98.4)* |
| | CI | I | 1 (6.7) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | *1 (1.6)* |
| | | R | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | *0 (0)* |
| | RS | | S | S | - | S | S | |
| Enrofloxacin, 5 µg | | S | 13 (86.7) | 10 (100) | 7 (87.5) | 15 (100) | 4 (40) | *53 (85.5)* |
| | CI | I | 2 (13.3) | 0 (0) | 1 (12.5) | 0 (0) | 4 (40) | *7 (11.3)* |
| | | R | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 2 (20) | *2 (3.2)* |
| | RS | | S | S | - | S | S | |
| Erythromycin, 15 µg | CI | S | 15 (100) | 0 (0) | 8 (100) | 15 (100) | 8 (80) | *49 (79.0)* |
| | | I | 0 (0) | 5 (50) | 0 (0) | 0 (0) | 2 (20) | *7 (11.3)* |
| | | R | 0 (0) | 5 (50) | 0 (0) | 0 (0) | 0 (0) | *6 (9.7)* |
| | RS | | S | R | - | S | S | |
| Gentamicin, 10 µg | CI | S | 14 (93.3) | 10 (100) | 1 (12.5) | 15 (100) | 1 (10) | *45 (72.6)* |
| | | I | 1 (6.7) | 0 (100) | 3 (37.5) | 0 (0) | 3 (30) | *7 (11.3)* |
| | | R | 0 (0) | 0 (100) | 4 (50) | 0 (0) | 6 (60) | *10 (16.1)* |
| | RS | | S | S | - | S | S | |
| Chloramphenicol, 30 µg | CI | S | 15 (100) | 9 (90) | 8 (100) | n.a. | 10 (100) | *46 (97.9)* |
| | | I | 0 (0) | 1 (10) | 0 (0) | n.a. | 0 (0) | *1 (2.1)* |
| | | R | 0 (0) | 0 (0) | 0 (0) | n.a. | 0 (0) | *0 (0)* |
| | RS | | S | S | - | S | S | |
| Kanamycin, 30 µg | CI | S | 12 (80) | 7 (70) | 7 (87.5) | n.a. | 0 (0) | *30 (63.8)* |
| | | I | 3 (20) | 3 (30) | 1 (12.5) | n.a. | 2 (20) | *9 (19.2)* |
| | | R | 0 (0) | 0 (0) | 0 (0) | n.a. | 8 (80) | *8 (17.0)* |
| | RS | | S | S | - | S | S | |
| Lincomycin + Spectinomycin, 15+200 µg | CI | S | 15 (100) | 2 (20) | 7 (87.5) | 15 (100) | 10 (100) | *53 (85.5)* |
| | | I | 0 (0) | 6 (60) | 1 (12.5) | 0 (0) | 0 (0) | *7 (11.3)* |
| | | R | 0 (0) | 2 (20) | 0 (0) | 0 (0) | 0 (0) | *2 (3.2)* |
| | RS | | S | S | - | S | S | |
| Neomycin, 30 µg | CI | S | 12 (80) | 2 (20) | 4 (40) | 1 (6.67) | 0 (0) | *23 (37.1)* |
| | | I | 3 (20) | 6 (60) | 4 (40) | 3 (20) | 6 (60) | *22 (35.5)* |
| | | R | 0 (0) | 2 (20) | 0 (0) | 11 (73.33) | 4 (40) | *17 (27.4)* |
| | RS | | S | S | - | S | S | |
| Penicillin, 10 µg | CI | S | 14 (93.3) | 0 (0) | 5 (60) | 5 (33.3) | 10 (100) | *37 (59.7)* |
| | | I | 1 (6.7) | 6 (60) | 3 (40) | 0 (0) | 0 (0) | *10 (16.1)* |
| | | R | 0 (0) | 4 (40) | 0 (0) | 10 (66.7) | 0 (0) | *15 (24.2)* |
| | | | S | R | - | S | S | |
| Streptomycin, 10 µg | CI | S | 4 (26.7) | 5 (50) | 0 (0) | 5 (33.3) | 0 (0) | *18 (29.0)* |
| | | I | 5 (33.3) | 5 (50) | 7 (87.5) | 3 (20) | 0 (0) | *20 (32.3)* |
| | | R | 6 (40) | 0 (0) | 1 (12.5) | 7 (46.7) | 10 (100) | *24 (38.7)* |
| | RS | | S | S | - | S | S | |
| Tetracycline, 30 µg | CI | S | 8 (53.4) | 0 (0) | 3 (37.5) | 12 (80) | 2 (20) | *29 (46.8)* |
| | | I | 5 (33.3) | 5 (50) | 5 (62.5) | 1 (6.7) | 1 (10) | *17 (27.4)* |
| | | R | 2 (13.3) | 5 (50) | 0 (0) | 2 (13.3) | 7 (70) | *16 (25.8)* |
| | RS | | S | S | - | S | S | |
| Sulfamethoxazole + trimethoprim, 23.75 +1.25 µg | CI | S | 4 (26.7) | 10 (100) | 1 (12.5) | 15 (100) | 2 (20) | *36 (58.1)* |
| | | I | 2 (13.3) | 0 (0) | 1 (12.5) | 0 (0) | 4 (40) | *7 (11.3)* |
| | | R | 9 (60) | 0 (0) | 6 (75) | 0 (0) | 4 (40) | *19 (30.6)* |
| | RS | | S | S | - | S | S | |
| Propylene glycol | CI | S | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | *0 (0)* |
| | | I | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | *0 (0)* |
| | | R | 15 (100) | 10 (100) | 8 (100) | 15 (100) | 10 (100) | *62 (100)* |
| | RS | | R | R | - | R | R | |

** S - susceptible; I - intermediate; R - resistant; CI - clinical isolates; RS - reference strains*

**Table 4:** Minimum inhibitory concentrations (MIC, mg/mL) of different essential oils to different bacterial species obtained from infected animals and their reference strains

| Essential oil | | *T. pyogenes* | *E. coli* | *Pasteurella* spp. | *S. aureus* | *Streptococcus* spp. | *Mean±st. dev. (n=62)* |
|---|---|---|---|---|---|---|---|
| *Satureja montana* | CI | $0.55\pm0.20^{Aa}$ | $0.47\pm0.16^{Aa}$ | $0.54\pm0.20^{Aa}$ | $0.68\pm0.18^{Aa}$ | $0.47\pm0.16^{Aa}$ | *0.60±0.15** |
| | RS | 0.78 | 0.78 | - | 0.78 | 0.39 | |
| *Ocimum basilicum* | CI | $6.67\pm1.61^{Ba}$ | $6.25\pm0.0^{Ba}$ | $6.25\pm0.0^{Ba}$ | $6.04\pm0.81^{Ba}$ | $6.25\pm0.0^{Ba}$ | *6.27±0.16*** |
| | RS | 6.25 | 6.25 | - | 6.25 | 6.25 | |
| *Salvia officinalis* | CI | $6.25\pm0.0^{Ba}$ | $22.5\pm5.27^{Cb}$ | $23.44\pm4.42^{Cb}$ | $21.67\pm5.72^{Cb}$ | $21.25\pm6.04^{Cb}$ | *19.60±7.70**** |
| | RS | 6.25 | 25 | - | 25 | 25 | |
| Propylene glycol | CI | $>25^{Ca}$ | $>25^{Ca}$ | $>25^{Ca}$ | $>25^{Ca}$ | $>25^{Ca}$ | *>25**** |
| | RS | | | | | | |

*Capital letters compare means between values obtained for different essential oils within one bacterial species. Lowercase letters compare means between values obtained for different bacterial species within one essential oil. In the last column, asterix compare means between average values of different essential oils. Different letters/asterix indicate significant differences (P<0.05); CI – clinical isoaltes; RS – reference strains.

**Table 5:** Minimum inhibitory concentrations (MIC, mg/mL) of different essential oils to the referent strain and isolate of *Fusobacterium necrophorum*

| Essential oil | *F. necrophorum* subsp. *Necrophorum* (ATCC 25286) | *F. necrophorum* isolate (V3 Subotica, Serbia) |
|---|---|---|
| *Satureja montana* | 0.125 | 0.125 |
| *Ocimum basilicum* | 8.0 | 8.0 |
| *Salvia officinalis* | >16 | >16 |
| Propylene glycol (32 mg/ml) | + (growth) | + (growth) |

(P<0.05), the effect was variable depending on the bacterial species in all tested EOs. This suggests that it may be important to mix different EOs with intermediate or low activity in a combination to achieve a higher effect in clinical cases of endometritis, although this needs to be confirmed in future studies.

To date, there have not been many studies investigating the efficacy of EOs against bacterial species isolated from clinical cases of bovine endometritis. In the present study, detection of the pathogenic bacteria *T. pyogenes* and *E. coli*, which are known to cause endometrial lesions, as well as other bacterial species recognized as pathogens (*P. multocida*, *S. aureus* (coagulase +) and *S. uberis*, was expected. The results of the microbiological analysis match the clinical picture, as the vaginal discharge in cows with mixed infection does not show marked suppuration, whereas in most cases of *T. pyogenes* infection there are pus spots or a mucopurulent composition of the discharge.

Paiano *et al*. (2020) evaluated the antimicrobial activity of seven different EOs against the reference strains of *E. coli* (ATCC 25922), *T. pyogenes* (19411), *F. necrophorum* (ATCC 25286) and *S. aureus* (ATCC 25293) using the agar disk diffusion method. The oils of *Cinnamonum cassia* (L.) J.Presl (29.67-38.33 mm), *Thymus vulgaris* L. (24.67-36.00 mm) and *Origanum vulgare* L. (20.67-36 mm) showed the largest diameter of the inhibition zone and thus the highest effect. For all oils tested, with the exception of cinnamon, the effect was significantly different for the different types of bacteria, similar to the present study. In another study, the same authors (Paiano *et al*., 2023) selected the most effective oils to test against *E. coli* and *T. pyogenes* isolated from clinical cases of endometritis in cows by determining the MICs, minimum bactericidal concentrations (MBC) and fractional inhibitory concentrations (FIC). The highest effect was obtained with the cinnamon EO, with the determined MIC and MBC values of 0.51 and 16.38 and 2.05 and 32.77 mg/mL against *T. pyogenes* and *E. coli*, respectively. In addition, the authors investigated the effect of different binary and ternary combinations of the tested oils. Although a synergistic effect was observed in some cases, the MIC values obtained show that cinnamon oil alone showed the best results.

Kadivar *et al*. (2022) evaluated the effect *in vivo* of mixed herbal EOs (*Satureja bachtiarica Bunge*, *Artemisia Aucheri Boiss* and *Syzygium aromaticum* L.) applied as an intrauterine injection (EOs + distilled water=50ml) in cows with clinical endometritis. The formulation used resulted in a higher cleansing rate, a higher first conception rate in cleansed and non-cleansed cows, as well as a lower number of open days and a lower number of conceptions per insemination compared to the oxytetracycline used (P<0.05). In a study by Lisboa *et al*. (2022), the effect of various EOs against endometritis-causing microorganisms in mares was evaluated, with serial concentrations of the EOs ranging from 30.00 mg/mL to 0.47 mg/mL being tested. The tested EOs (*Cymbopogon citratus* L., *O. basilicum* and *Rosmarinus officinalis* L.) were effective against all microorganisms (*S. equi*, *S. aureus*, *Klebsiella pneumoniae*, *E. coli*, and *Candida albicans*) except *Pseudomonas aeruginosa*, where *C. citratus* EO showed the highest effect, followed by *O. basilicum* EO.

In the other studies, the effect of plant extracts applied intrauterine alone or in combination was evaluated. For example, in a study by Gopikrishnan *et al*. (2022), the use of an ethanolic extract resulted in a better conception rate and a significant percentage reduction in the bacterial count than other treatments, including the control (streptopenicillin). Armansyah *et al*. (2023) demonstrated that the application of red betel leaf extract and moringa leaves could reduce the extent of endometritis and increase the pregnancy rate in cows in Aceh. Based on the results of their study, Menoud *et al*. (2024) concluded that the clinical cure of endometritis in dairy cows, after the intrauterine application of a herbal product containing alcoholic extracts of *Calendula officinalis* L., *Mellissa officinalis* L., *Origanum majorana* L. and *Eucalyptus globulus* Labill., was not inferior to the intrauterine application of the antibiotic cephapirin.

The use of cell cultures for the initial preclinical screening of potential therapeutic agents is now commonplace, and it is critical to obtain accurate and reliable results from the *in vitro* cytotoxicity assays used in the initial stages of preclinical research. This data can influence the success of a drug candidate in the development process (van Tonder *et al*., 2015). The MTT assay for cellular metabolic activity is almost ubiquitous in cell toxicity studies and is generally accepted as the "gold standard" for assessing cytotoxicity (van Tonder *et al*., 2015; Ghasemi *et al*., 2021). As a tool to measure cell viability, metabolic activity of cells and/or cytotoxicity of treatment, the MTT assay requires several considerations (number and density of cell seeding, MTT concentration, MTT incubation time, cell number, metabolism and secretome, background absorption and scattering, etc.). The limitation is that additional optimization experiments need to be performed sometimes, as well as complementary assays to aid interpretation, all of which can be time-consuming and tedious but important processes (Ghasemi *et al*., 2021).

In this study, a cytotoxicity test was performed on the most effective EO for antimicrobial testing, *S. montana* EO, to assess its safety potential, initially *in vitro*. The tested oil clearly showed a dose-dependent cytotoxic potential, showing no effect at the concentrations of 0.039 and 0.078 mg/ml for the BEND cell line (Fig. 4.), and at 0.039 to 0.625 mg/mL for the MDBK cell line (Fig. 5.). The calculated $IC_{50}$ values were 1.27 mg/mL and 1.02-1.56 mg/mL for the BEND and MDBK cell lines, respectively. It should be emphasized that the higher the $IC_{50}$ value in cytotoxic studies, implies that the higher the concentration required to achieve a cytotoxic effect, i.e. the tested substance is less cytotoxic. An antimicrobial agent with a higher ratio between the cytotoxic $IC_{50}$ values obtained and the antibacterial MIC values ($IC_{50}$/MIC ratio) will therefore have fewer adverse and toxic effects, i.e. its use will be

safer for epithelial cells in terms of toxicity. From this point of view, 2.7 (1.27/1.47) for BEND at 24 hours and 3.32 (1.56/0.47), 2.47 (1.16/0.47), 2.28 (1.07/0.47) and 2.17 (1.02/0.47) for the MDBK cell line at 3, 6, 12 and 24h after exposure, respectively (Fig. 6.), indicate the safety of the use of *S. montana* EO.

Cytotoxic studies, mainly performed with the MTT assay, were also part of other studies aimed at evaluating the antimicrobial and toxic potential of different EOs, including the EOs from the present study or the EOs from the same plant genera. However, in most of these studies, antimicrobial activity was evaluated using reference strains. For example, Mohammadpour *et al*. (2015) evaluated the antimicrobial and cytotoxic activities of *Satureja bakhtiarica* EO *in vitro*, where the MIC values calculated by agar well diffusion method were 0.5 and 0.7 mg/ml against *E. coli* (53218) and *S. aureus* (51153), respectively. The $IC_{50}$ value in the MTT assay tested on human embryonic kidney (HEK) cells was 0.1 mg/mL. Ribeiro *et al*. (2020) investigated the antibacterial and cytotoxic activities of *O. basilicum*, among other EOs. The oil was tested against various bacterial reference strains, including *E. coli* (LMG 8223, 15862) and *S. aureus* LMG 8064 (MSSA), 15975 (MRSA), LMG 16217 (MRSA), although it was not strongly effective in all cases with calculated MIC values of >1 mg/mL, similar to the present study. However, oil exhibited low cytotoxic potential for the keratinocyte cell line HaCaT.

In some cases, cytotoxic studies against tumor cells have been performed to evaluate their anticancer potential. In these cases, lower $IC_{50}$ means higher cytotoxic activity, i.e. higher antitumor potential. Thus, Miladi *et al*. (2013) investigated the cytotoxic activities of *Satureja montana* L. EO and its antibacterial potential using agar disk diffusion method against different species. The major inhibition zone in diameter for *S. aureus* and *E. coli* was 23.33±0.58 and 24.00±0.00, respectively, with MIC values of 0.78 mg/mL determined for both species. In the cytotoxic test performed on the human respiratory epithelial cell line (A549), the calculated $IC_{50}$ values were 0.4 mg/mL and 0.011 mg/mL at 48h and 72h after exposure. The antimicrobial activity of *S. officinalis* EO was evaluated by Abdulrashid and Bukhari (2023), where the MIC value of 16.6±0.5 mg/mL, against the reference strain of *E. coli* (ATCC25922), was determined using the broth dilution method. In the same study, the authors also evaluated the cytotoxic potential against African green monkey cells (VERO) and Caco-2 (colon cancer cells), although a different oil (*Mentha longifolia*) was selected for this study as it has a dominant effect compared to the sage EO.

In general, all of the above findings are consistent with the results of the present study, but only to a certain extent. Indeed, the qualitative and quantitative composition of EOs is responsible for their activities, especially their main ingredient(s) (Lupia *et al*., 2024; Ratajac *et al*., 2024; Štrbac *et al*., 2024). However, the chemical composition of EOs can be very different, even in cases where they are obtained from the same plant species, which can lead to differences in their effects. Thus, many abiotic factors such as the hydrology of the soil, the pH and salinity, the microclimate in which the plant grows, as well as biotic factors such as soil organisms and microorganisms, but also the plant itself (genetic characteristics of the plant, age, parts used for extraction, etc.) can influence the composition of the EOs. In addition, the post-harvest treatment of the plant material (drying, extraction method, storage method - excluding light and oxygen, etc.) can also play a role (Fokou *et al*., 2020; Lupia *et al*., 2024). The differences between the chemical composition and effects of EOs extracted from the same plant species have also been demonstrated for *S. montana*, which include antimicrobial properties (Skočibušić and Bezić, 2003; Aćimović *et al*., 2022). For the high antimicrobial potential of *S. montana* EO shown in this study, carvacrol with 42.12% and limonene with 24.57% are clearly mainly responsible (Table 1., Fig. 1.), although the entire composition, including the remaining ingredients represented in smaller percentages, contributes to the final effect through synergistic effects. In fact, carvacrol itself has already shown antibacterial activity against various species in different studies, as listed in a review by Mączka *et al*. (2023). Although the standardization of the composition of plant products can be a problem (Lupia *et al*., 2024), there have been some new approaches on how to standardize them, e.g., by fractional distillation (Nikkhah *et al*., 2024).

In contrast to the negative aspects of the exclusive use of antibiotics in the treatment of various microbial diseases in animals, EOs have many positive properties. As mentioned above, EOs are mixtures of compounds from different chemical groups (hydrocarbon terpenes, terpenoids – alcohols, phenols, aldehydes, ketones, esters, etc.), phenylpropanoids and some others (Fokou *et al*., 2020; Liang *et al*., 2023, Ratajac *et al*., 2024; Štrbac *et al*., 2024; 2025). This may lead to high activity through a synergistic effect within different pharmacological properties, including antimicrobial properties. In this regard, both the composition of the EO and the strain of the microbe utilize their antibacterial mode of action. In general, Gram-positive species are considered more susceptible to EO than Gram-negative species, whose outer membrane is more complex, rigid and rich in lipopolysaccharides that hinder the diffusion of hydrophobic compounds (Lupia *et al*., 2024). Although this was also confirmed in most cases in the present study, there were some exceptions (e.g. *E. coli* was one of the most susceptible species to *S. montana* EOs in the AD assay), suggesting that other factors also play a role. However, the main mechanisms of action of EOs and other plant products or isolated ingredients on the bacteria include: degradation of the cell wall and/or cell membrane (Elshafie *et al*., 2019; Owen *et al*, 2019), DNA fragmentation (Lee *et al*., 2016), inhibition of the FabZ enzyme (Geethalakshmi *et al*., 2018), degradation of the cell membrane with cell leakage or leakage of K+ from the cytosol (Tardugno *et al*., 2018; Lorenzo-Leal *et al*., 2019), etc. All these processes can affect the structure and metabolism, leading to cell death (Lupia *et al*., 2024).

In addition to high antimicrobial activity, the rich chemical composition of EOs may lead to lower susceptibility to antimicrobial resistance compared to commercial antibiotics. Moreover, as natural plant-based medicines, these products are often considered safer than chemical medicines, especially from the point of view of residues in animal products and in the environment which can affect public health and the ecosystem (Groot *et al*.,

2021; Štrbac *et al*., 2025). Another solution is to combine EOs with antibiotics, which can have many benefits: Reduction in their use, increase in activity when combined with drugs to which resistance has already developed, and lower toxicity by reducing the dose used (Drioiche *et al*., 2024). Despite the many advantages, the use of EO has some limitations, mainly related to the many unknowns due to the still limited number of studies, especially in terms of mechanism of action, pharmacokinetics, pharmacodynamics and potential toxicity. The other disadvantage is a still lower *in vivo* efficacy, possibly related to the instability of EO compounds (Lupia *et al*., 2024). In addition to conducting new studies which are increasing in number (Ebani and Mancianti, 2020; Lupia *et al*., 2024), nanoencapsulation of EOs, and/or exploiting the synergies between them, their constituents and antibiotics, have been recommended as an answer to this problem. However, less is known about the interactions leading to additive, synergistic or antagonistic effects. This knowledge could help to develop new and more effective antimicrobial mixtures and to understand the interplay between the components of crude EOs (Chouhan *et al*., 2017).

**Conclusions:** Antimicrobial resistance to commercially available antibiotics, and the residues of these drugs present in animal products and the environment, represent serious problems associated with the exclusive use of these drugs. The results of the disk diffusion test, ADT and MDT showed high antimicrobial potential of *S. montana* EO, consists of p-cymene, carvacrol and other bioactive compounds, against various pathogens of bacterial origin isolated from the uterus of cows. In addition, the oil showed low cytotoxic potential in two different cell lines in the MTT assay. Although further studies *in vivo* are required, these results suggest that the EO of *S. montana* has the potential to be used in the treatment of various diseases affecting the reproductive system of cows, including endometritis.

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**Authors contribution:** RR conceived of the presented idea, and the study was planned and designed in the cooperation with JP, IS, IP, DS, WAH and MMM. For obtaining bacterial isolates for the present study, IP was responsible. The experiment was conducted as follows: chemical analyses – NS, DO and TK; evaluation of antibacterial activity – RR, IS; evaluation of cytotoxicity – RR. The results obtained were interpreted by RR and DS and statistically proceeded by JP and FŠ. The original manuscript, including the preparation of tables and graphs, was written by FŠ, revised by RR, WAH and MMM and approved by all co-authors.

**Availability of data and materials:** The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests**: The authors declare that they have no competing interests.

## REFERENCES

Abdulrashid D and Bukhari A, 2023. Antimicrobial activity and multi-therapeutic potential of *Salvia officinalis* and *Mentha longifolia* essential oils in leaves. J Pure Appl Microbiol 17:826-37. https://doi.org/10.22207/JPAM.17.2.09

Aćimović M, Šovljanski O, Pezo L, *et al*., 2022. Variability in biological activities of *Satureja montana* subsp. *montana* and subsp. *variegata* based on different extraction methods. Antibiotics 11:1235. https://10.3390/antibiotics11091235

Adnane M, Whiston R, Tasara T, *et al*., 2024. Harnessing vaginal probiotics for enhanced management of uterine disease and reproductive performance in dairy cows: A conceptual review. Animals 14:1073. https://doi.org/10.3390/ani14071073

Amin YA, Abdelaziz SG and Said AH, 2023. Treatment of postpartum endometritis induced by multidrug-resistant bacterial infection in dairy cattle by green synthesized zinc oxide nanoparticles and *in vivo* evaluation of its broad spectrum antimicrobial activity in cow uteri. Res Vet Sci 165:105074. https://doi.org/10.1016/j.rvsc.2023.105074

Armansyah T, Siregar TN, Sutriana A, *et al*., 2023. Endometritis control and enhanced reproductive performance in Aceh cow using medicinal plants. Am J Anim Vet Sci 18:235-42. https://doi.org/10.3844/ajavsp.2023.235.242

Azizah NS, Irawan B, Kusmoro J, *et al*., 2023. Sweet basil (*Ocimum basilicum* L.) - A review of its botany, phytochemistry, pharmacological activities, and biotechnological development. Plants 12:4148. https://doi.org/10.3390/plants12244148

Barragan AA, Bas S, Hovingh E, *et al*., 2021. Effects of postpartum acetylsalicylic acid on uterine diseases and reproductive performance in dairy cattle. JDS Commun 2:67-72. https://doi.org/10.3168/jdsc.2020-0047.

Basbas C, Garzon A, Silva-del-Rio N, *et al*., 2022. Evaluation of antimicrobial resistance and risk factors for recovery of intrauterine *Escherichia coli* from cows with metritis on California commercial dairy farms. Sci Rep 12:13937. https://doi.org/10.1038/s41598-022-18347-w

Bosco A, Jesu G, Nappa A, *et al*., 2025. A novel *in vitro* repellent bioassay to evaluate the effect of wood vinegar against louse *Haematopinus tuberculatus*. Parasitology 2025:1-9. https://doi.org/10.1017/S0031182025000551

Caneschi A, Bardhi A, Barbarossa A, *et al*., 2023. Plant essential oils as a tool in the control of bovine mastitis: An update. Molecules 28:3425. https://doi.org/10.3390/molecules28083425

Chouhan S, Sharma K and Guleria S, 2017. Antimicrobial activity of some essential oils - present status and future perspectives. Medicines 4:58. https://10.3390/medicines4030058

Citron DM and Hecht DW, 2011. Susceptibility test methods: Anaerobic bacteria. In: Manual of Clinical Microbiology (Versalovic J, Carroll KC, Funke G, Jorgensen JH, Landry ML, Warnock DW, ed), 10th Ed, American Society for Microbiology (ASM) press, Washington DC, USA, Chapter 72., pp: 1204-14.

de Sousa DP, Damasceno ROS, Amorati R, *et al*., 2023. Essential oils: Chemistry and pharmacological activities. Biomolecules 13:1144. https://doi.org/10.3390/biom13071144.

Drillich M and Wagener K, 2018. Pathogenesis of uterine diseases in dairy cattle and implications for fertility. Anim Reprod 15:879-85. https://doi.org/10.21451/1984-3143-AR2018-0023

Drioiche A, Baammi S, Zibouh K, *et al*., 2024. A study of the synergistic effects of essential oils from *Origanum compactum* and *Origanum elongatum* with commercial antibiotics against highly prioritized multidrug-resistant bacteria for the world health organization. Metabolites 14:210. https://doi.org/10.3390/metabo14040210

Ebani VV and Mancianti F, 2020. Use of essential oils in veterinary medicine to combat bacterial and fungal infections. Vet Sci 7:193. https://10.3390/vetsci7040193

Ehrich M and Sharova L, 2000. *In vitro* methods for detecting cytotoxicity. Curr Protoc Toxic 3:2-6. https://doi.org/10.1002/0471140856.tx0206s03

Elshafie HS, Gruľová D, Baranová B, *et al*., 2019. Antimicrobial activity and chemical composition of essential oil extracted from *Solidago canadensis* L. growing wild in Slovakia. Molecules 24:1206. https://doi.org/10.3390/molecules24071206

Eslami M, Bolourchi M, Seifi HA, *et al*., 2015. Treatment of clinical endometritis in dairy cows by previously used controlled internal

drug release devices. Theriogenology 84:437-45. https://doi.org/10.1016/j.theriogenology.2015.03.036

Fokou JBH, Dongmo PMJ, and Boyom FF, 2020. Essential oil's chemical composition and pharmacological properties. In: Essential Oils–Oils of Nature (El-Shemy E, ed), IntechOpen, London, UK, Chapter 2.

Geethalakshmi R, Sundaramurthi JC and Sarada DVL, 2018. Antibacterial activity of flavonoid isolated from *Trianthema decandra* against *Pseudomonas aeruginosa* and molecular docking study of FabZ. Microb Pathog 121:87–92. https://10.1016/j.micpath.2018.05.016

Ghasemi M, Turnbull T, Sebastian S, *et al*., 2021. The MTT assay: utility, limitations, pitfalls, and interpretation in bulk and single-cell analysis. Int J Mol Sci 22:12827. https://10.3390/ijms222312827

Ghorbani A and Esmaeilizadeh M, 2017. Pharmacological properties of *Salvia officinalis* and its components. J Tradit Complement Med 7:433–40. https://doi.org/10.1016/j.jtcme.2016.12.014

Gopikrishnan D, Selvaraju M, Palanisamy M, *et al*., 2022. Therapeutic efficacy of garlic (*Allium sativum*), turmeric (*Curcuma longa*) and eucalyptus (*Eucalyptus globules*) extracts in the treatment of bovine endometritis. Indian J Anim Res 56:904-8. https://doi.org/10.18805/IJAR.B-4492

Groot MJ, Berendsen BJA and Cleton NB, 2021. The next step to further decrease veterinary antibiotic applications: Phytogenic alternatives and effective monitoring; the Dutch approach. Front Vet Sci 8:709750. https://doi.org/10.3389/fvets.2021.709750

Jiang L, 2011. Comparison of disk diffusion, agar dilution, and broth microdiultion for antimicrobial susceptibility testing of five chitosans. Louisiana State University and Agricultural and Mechanical College, LSU Master's Theses

Kadivar A, Davoodian N, Elahi R, *et al*., 2022. Evaluation of the efficacy of mixed herbal essential oils as a treatment option for clinical endometritis in dairy cattle. Iran Vet J 18:52-62. https://doi.org/10.22055/IVJ.2022.355231.2487

Lection J, Syoc EV, Miles A, *et al*., 2024. Use of intrauterine dextrose as an alternative to systemic antibiotics for treatment of clinical metritis in dairy cattle: a microbiome perspective. Front Vet Sci 11:1478288. https://doi.org/10.3389/fvets.2024.1478288

Lee H, Choi H, Lee JC, *et al*., 2016. Antibacterial activity of hibicuslide C on multidrug-resistant *Pseudomonas aeruginosa* isolates. Curr Microbiol 73:519–26. https://10.1007/s00284-016-1092-y.

Lefebvre RC, Torabi A, Bazzazan A, *et al*., 2021. Alternative treatments of postpartum uterine diseases in dairy cows. Integr J Vet Biosci 5:1-7. ISSN: 2577-4492

Lee Y, Park E, Jang B, *et al*., 2023. Antifungal activity of Bulgarian rose damascena oil against vaginitis-causing opportunistic fungi. Evid Based Complement Alternat Med 2023:5054865. https://doi.org/10.1155/2023/5054865

Liang J, Zhang Y, Chi P, *et al*., 2023. Essential oils: Chemical constituents, potential neuropharmacological effects and aromatherapy - A review. Pharmacol Res - Mod Chin Med 6:100210. https://doi.org/10.1016/j.prmcm.2022.100210

Lisboa FP, Silvestre WP, Castro JO, *et al*., 2022. *In vitro* antimicrobial activity of selected essential oils against endometritis-causing microorganisms in mares. J Equine Vet Sci 110:103840. https://doi.org/10.1016/j.jevs.2021.103840

Lorenzo-Leal AC, Palou E, López-Malo A, *et al*., 2019. Antimicrobial, cytotoxic, and anti-inflammatory activities of *Pimenta dioica* and *Rosmarinus officinalis* essential oils. Biomed Res 2019:1639726. https://doi.org/10.1155/2019/1639726

Lupia C, Castagna F, Bava R, *et al*., 2024. Use of essential oils to counteract the phenomena of antimicrobial resistance in livestock species. Antibiotics 13:163. https://doi.org/10.3390/antibiotics 13020163

Maccelli A, Vitanza L, Imbriano A, *et al*., 2019. *Satureja montana* L. essential oils: Chemical profiles/phytochemical screening, antimicrobial activity and O/W nanoemulsion formulations. Pharmaceutics 12:7. https://doi.org/10.3390/pharmaceutics 12010007

Mączka W, Twardawska M, Grabarczyk M, *et al*., 2023. Carvacrol - A natural phenolic compound with antimicrobial properties. Antibiotics 12:824. https://doi.org/10.3390/antibiotics12050824

Meira Jr. EBS, Ellington-Lawrence RD, Silva JCC, *et al*., 2020. Recombinant protein subunit vaccine reduces puerperal metritis incidence and modulates the genital tract microbiome. J Dairy S 103:7364-76. https://doi.org/10.3168/jds.2019-17006

Mekibib B, Belachew M, Asrade B, *et al*., 2024. Incidence of uterine infections, major bacteria and antimicrobial resistance in postpartum dairy cows in southern Ethiopia. BMC Microbiol 24:4. https://doi.org/10.1186/s12866-023-03160-w

Menoud V, Holinger M, Graf-Schiller S, *et al*., 2024. Comparison between intrauterine application of an antibiotic and an herbal product to treat clinical endometritis in dairy cattle – A randomized multicentre field study. Res Vet Sci 172:105250. https://doi.org/10.1016/j.rvsc.2024.105250

Mesfin YM, Mitiku BA and Admasu HT, 2024. Veterinary drug residues in food products of animal origin and their public health consequences: A review. Vet Med Sci 10:e70049. https://doi.org/10.1002/vms3.70049

Miladi H, Slama RB, Mili D, *et al*., 2013. Chemical composition and cytotoxic and antioxidant activities of *Satureja montana* L. essential oil and its antibacterial potential against *Salmonella* spp. strains. J Chem 2013:275698. https://dx.doi.org/10.1155/2013/275698

Mohammadpour G, Tahmasbpour R, Noureini SK, *et al*., 2015. *In vitro* antimicrobial and cytotoxicity assays of *Satureja bakhtiarica* and *Zataria multiflora* essential oils. Am J Phyto Clin Ther 3:502-11. ISSN 2321 – 2748.

NCCLS, 1990. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard NCCLS Publication M7/A2, Villanova, PA, USA

NCCLS, 2000. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved standard - Fifth edition. NCCLS document M7-A5. [ISBN 1-56238-394- 9] NCCLS, 940 West Valley Road, Suite 1400, Wayne, PA 19087-1898. USA.

Negasee KA, 2020. Clinical metritis and endometritis in diary cattle: A review. Vet Med Open J 5:51-6. https://doi.org/10.17140/VMOJ-5-149

Nikkhah A, Nikkhah H, Shahbazi A, *et al*., 2024. Cumin and eucalyptus essential oil standardization using fractional distillation: Data-driven optimization and techno-economic analysis. Food Bioprod Process 143:90-101. https://doi.org/10.1016/j.fbp.2023.10.005

Okawa H, Wijayagunawardane MMP, Vos PL, *et al*., 2021. Effects of intrauterine infusion of a chitosan solution on recovery and subsequent reproductive performance of early postpartum dairy cows with endometritis: A pilot field trial. Animals 11:197. https://doi.org/10.3390/ani11010197

Owen L, White AW and Laird K, 2019. Characterization and screening of antimicrobial essential oil components against clinically important antibiotic-resistant bacteria using thin layer chromatography-direct bioautography hyphenated with GC-MS, LC-MS and NMR. Phytochem Anal 30:121–31. https://10.1002/pca.2797.

Paiano RB, Bonilla J, Bonilla J, *et al*. 2020. Chemical composition and antibacterial activity of essential oils against pathogens often related to cattle endometritis. J Infect Dev Ctries 14:177-83. https://doi.org/10.3855/jidc.12076

Paiano RB, Moreno LZ, Gomes VTM, *et al*., 2022. Assessment of the main pathogens associated with clinical and subclinical endometritis in cows by culture and MALDI-TOF mass spectrometry identification. J Dairy Sci 105:3367-76. https://doi.org/10.3168/jds.2021-20642

Paiano RB, de Sousa RLM, Bonilla J, *et al*., 2023. *In vitro* effects of cinnamon, oregano, and thyme essential oils against *Escherichia coli* and *Trueperella pyogenes* isolated from dairy cows with clinical endometritis. Theriogenology 196:106-11. https://doi.org/10.1016/j.theriogenology.2022.11.010

Pinedo PJ, Caixeta LS, Barrell EA, *et al*., 2020. A randomized controlled clinical trial on the effect of acupuncture therapy in dairy cows affected by pyometra. Res Vet Sci 133:12–6. https://doi.org/10.1016/j.rvsc.2020.08.011.

Quinn PJ, Carter ME, Markey B, *et al*., 1994. Clinical Veterinary Microbiology. Wolf/Mosby, London, UK

Ratajac R, Pavličević A, Petrović J, *et al*., 2024. *In vitro* evaluation of acaricidal efficacy of selected essential oils against *Dermanyssus gallinae*. Pak Vet J 44:93-8. https://doi.org/10.29261/pakvetj/2023.123

Ribeiro SO, Fontaine V, Mathieu V, *et al*., 2020. Antibacterial and cytotoxic activities of ten commercially available essential oils. Antibiotics 9:0717. https://10.3390/antibiotics9100717

Rotilie CA, Fass RJ, Prior RB, *et al*., 1975. Microdilution technique for antimicrobial susceptibility testing for anaerobic bacteria. Antimicrob Agents Chemother 7:311-5. https://doi.org/10.1128/aac.7.3.311

Serbester U, Çınar M, Ceyhan A, *et al*., 2012. Effect of essential oil combination on performance, milk composition, blood parameters and pregnancy rate in early lactating dairy cows during heat exposure. J Anim Plant Sci 22:556-63. ISSN: 1018-7081

Singh H, Brar PS, Singh N, *et al*., 2020. Effects of intra-uterine infusion of proteolytic enzymes on selected cytokine concentrations, uterine inflammation, and fertility in postpartum water buffalo cows with

subclinical endometritis. Anim Reprod Sci 215:106335. https://doi.org/10.1016/j.anireprosci.2020.106335

Société Française de Microbiologie, 1996. Recommandations de la Comité de l'antibiogramme. Available at: https://www.sfm-microbiologie.org/ (01.08.2025.)

Skočibušić M and Bezić N, 2003. Chemical composition and antimicrobial variability of *Satureja montana* L. essential oils produced during ontogenesis. J Essent Oil Res 16:387-91. https://doi.org/10.1080/10412905.2004.9698751

Swelum AA, Hashem NM, Abdelnour SA, *et al*., 2021. Effects of phytogenic feed additives on the reproductive performance of animals. Saudi J Biol Sci 28:5816:22. https://doi.org/10.1016/j.sjbs.2021.06.045

Štrbac F, Krnjajić S, Simin N, *et al*., 2024. *In vitro* anthelmintic potential of selected essential oils against gastrointestinal nematodes of sheep. Pak Vet J 44:1053-62. http://dx.doi.org/10.29261/pakvetj/2024.295

Štrbac F, Krnjajić S, Ratajac R, *et al*., 2025. Anthelmintic activity of winter savory (Satureja montana L.) essential oil against gastrointestinal nematodes of sheep. BMC Vet Res 21:405. https://doi.org/10.1186/s12917-025-04771-3

Tardugno R, Pellati F, Iseppi R, *et al*., 2018. Phytochemical composition and in vitro screening of the antimicrobial activity of essential oils on oral pathogenic bacteria. Nat Prod Res 32:544–51. https://doi.org/10.1080/14786419.2017.1329730.

Tóth V, Heinc E, Mikó E, *et al*., 2023. Profitability optimization of dairy farms: The effect of pregnancy rate and culling decision. Animals 14:18. https://doi.org/10.3390/ani14010018

van Tonder, Joubert AM and Cromarty AD, 2015. Limitations of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays. BMC Res Notes, 8:47. https://doi.org/10.1186/s13104-015-1000-8

Várhidi Z, Csikó G, Bajcsy ÁC, *et al*., 2024. Uterine disease in dairy cows: A comprehensive review highlighting new research areas. Vet Sci 11:66. https://doi.org/10.3390/vetsci11020066

Wiegand I, Hilpert Kai and Hancock REW, 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc 3:163–75. https://doi.org/10.1038/nprot.2007.521.

Yoo HS, 2010. Infectious causes of reproductive disorders in cattle. J Reprod Dev 56:S53-S60. https://doi.org/10.1262/jrd.1056s53.

Jugoslovenska farmakopeja 2000. Beograd: Savremena Administracija, Ph.Jug.V, The European Pharmacopoeia, 5th ed. (Ph.Eur.5.) Strasbourg: Council of Europe, 2004

Zduńczyk S and Janowski T, 2020. Bacteriophages and associated endolysins in therapy and prevention of mastitis and metritis in cows: Current knowledge. Anim Reprod Sci 218:106504. https://doi.org/10.1016/j.anireprosci.2020.106504