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Original Contribution

ANTIMICROBIAL ACTIVITIES OF TWELVE ESSENTIAL OILS AGAINST MICROORGANISMS OF VETERINARY IMPORTANCE

N. Rusenova*, P. Parvanov

Department of Veterinary Microbiology, Infectious and Parasitic Diseases, Section of Microbiology, Trakia University, Stara Zagora, Bulgaria

SUMMARY

Twelve essential oils were tested for their inhibitory activity against some microorganisms of veterinary interest using disc diffusion procedure and the most active were selected for further study in agar dilution method. Disc diffusion technique showed variation in the antimicrobial activity of selected essential oils. According to agar dilution method, the most potent essential oils were cinnamon, oregano, lemongrass and thyme. MICs were tested at concentrations ranging from 2.0 to 0.008% (v/v). These inhibitory effects are interesting in relation to treatment of bacterial and yeast infections in animals.

Key words: essential oils, antimicrobial activity, veterinary medicine.

INTRODUCTION

Essential oils are distillates of the volatile compounds of a plant's secondary metabolism and may act as phytoprotective agents (1). Their curative effect has been known since antiquity. It is based on a variety of pharmacological properties which are specific for each plant species. Therefore, essential oils are used in the pharmaceutical industry as active ingredients or constituents of drugs, soaps, shampoos, perfumes and cosmetics (2, 3, 4). They are also applied as food conservatives in the food industry (5). Therefore, interest in essential oils and other extracts of plants as sources of natural products has increased during the last years. They have been screened for their potential uses as alternative remedies for treatment of many infectious diseases (6). Since essential oils possess complex chemical constituents, which vary depending on the amount of rainfall and daylight to which plants are exposed, and the soil conditions, humidity, elevation, even the time of day at which the plants are harvested (7,8), resistance among bacteria is not yet detected (9).

A major problem in antimicrobial chemotherapy is the increasing occurrence of resistance to antibiotics and chemotherapeutics, which leads to the insufficiency of antimicrobial treatment (10). The overuse of antibiotics and consequent antibiotic selection pressure is thought to be the most important factor contributing to the appearance of different kinds of resistant microbes (11,12). There is a strong necessity for the development of new drugs for the cure of infections provoked by resistant and multi-resistant bacterial species.

It has been recognized that some essential oils have different antimicrobial activities against individual strains of microorganisms (13, 14). Besides antibacterial properties, essential oils also have insecticidal (15), antiparasitic (16), antiviral and antifungal activities (17, 18), which are important both for food preservation and the control of human and plant diseases that are of microbial origin (19). Therefore, our interest was directed toward antimicrobial activity of some essential oils and possibilities for their application in veterinary medicine.

The aim of the current investigation was to study the antimicrobial activities of

*** Correspondence to**: *Nikolina Velizarova Rusenova. Assist. Prof., Department of Veterinary microbiology, infectious and parasitic diseases, Section of Microbiology, Trakia University, Stara Zagora, Bulgaria; Tel.: (+359) 42 699-604; E-mail: n_v_n_v@abv.bg*

twelve essential oils against some bacteria and fungi of veterinary importance.

MATERIALS AND METHODS

Essential oils

Essential oils from thyme (*Thymus vulgaris*), clove (*Syzygium aromaticum*), cinnamon (*Cinnamomum aromaticum*), marjoram (*Origanum marjorana*), tea tree (*Malaleuka alternifolia*), clary sage (*Salvia sclarea*), peppermint (*Mentha x piperita*), lemon (*Citrus limon*), grapefruit (*Citrus paradisi*), lemongrass (*Cymbopogon citratus*), mandarin (*Citrus reticulate var. madurensis*) and oregano (*Origanum vulgare*) were obtained from ArtMedix Ltd, Sofia and from Lavena Ltd, Shumen, Bulgaria.

Microbial strains

Microorganisms included in the current investigation were obtained from the microbial collection of section "Veterinary Microbiology" at Faculty of Veterinary Medicine, Trakia University-Stara Zagora. The following Gram-positive strains were studied: *Staphylococcus aureus* ATCC 25923, *Listeria monocyto*genes (clinical isolate), *Bacillus licheniformis* ATCC 14575, *Enterococcus faecalis* ATCC 29212; Gram negative bacteria: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella* Enteritidis (clinical isolate), *Yersinia pseudotuberculosis* (clinical isolate), *Proteus vulgaris* (clinical isolate), *Proteus mirabilis* (clinical isolate), *Klebsiella pneumoniae* ATCC 13883, *Enterobacter aerogenes* ATCC 13048 and yeasts: *Candida albicans* and *Malassezia pachydermatis* (clinical isolates). Microbial strains were stored on beads at -20°C. Prior to use, they were grown freshly on tryptone soya blood agar (Emapol, Poland).

Antimicrobial screening

Disc diffusion method was employed for the determination of antimicrobial activities of the essential oils, following the method described by Bauer et al. (20).

The MICs of the essential oils against the test microorganisms were determined by agar dilution method applying the procedures as recommended by the CLSI (NCCLS) (21).

Disc diffusion assay

Bacterial and fungal inoculums were prepared from overnight culture (24h) on tryptone soya blood agar. Colonies were directly suspended in saline to obtain turbidity comparable to that of the 0.5 McFarland standards (approximately $1.5x10^8$ CFU/ml).
Aliquots (100µl) of inoculums were spread over the surface of pre-dried Mueller-Hinton agar (NCIPD, Sofia, Bulgaria) plates with a sterile glass spreader. Sterile 6 mm filter paper discs (NCIPD) were placed on the plates and immediately 10 µl portions of the essential oils were added. Sterile PS was used as control. The plates were left for 30 min at room temperature to allow the diffusion of oil and then they were incubated at 35°C for 24h. The inhibition zone was measured in millimetre and the assay was carried out in triplicate. The scale of measurement was the following (disk diameter included): ≥20mm zone of inhibition is strongly inhibitory; <20-12mm zone of inhibition is moderately and <12mm is no inhibitory. Values are presented as means ± S D of three parallel measurements.

Agar dilution method

The agar dilution method recommended by CLSI was used with the following modification: a final concentration of 1% (v/v) Tween-20 (Sigma) was incorporated into the agar medium after autoclaving to enhance oil solubility. A series of twofold dilutions of each oils, ranging from 2 % (v/v) to 0.008 (v/v), was prepared in Mueller-Hinton agar with 1% (v/v) Tween-20 at 50°C. Plates were dried at 35°C for 30 min prior to spot inoculation with 2 µl aliquots of culture containing approximately 10^4 cfu of each organism. MHA with 1% (v/v) Tween-20 but no oil was used as a positive growth control. Inoculated plates were incubated at 35°C for 24h for bacteria and 48h for yeasts. The MICs were determined as the lowest concentration of oil inhibiting the visible growth of each organism on the agar plate. The presence of 1 or 2 colonies was disregarded.

RESULTS

The antimicrobial activity of twelve selected essential oils against fourteen microorganisms

with veterinary interest using disc diffusion technique is summarized on **Table 1**. The results revealed variation in the antimicrobial properties of selected essential oils. The essential oils showed strong activity (inhibition zone ≥20mm), moderate activity (inhibition zone <20-12mm) and no inhibition-(zone < 12mm). The most potent oils were thyme, followed by oregano, cinnamon and tea tree. Good antimicrobial activities were exhibited by lemongrass, clary sage and peppermint oils. Lemon, grapefruit and mandarine oils did not show antimicrobial activity against the microorganisms and were not tested in agar dilution method.

The MICs of tested essential oils by the agar dilution method are presented on **Table 2**. The MIC values confirmed the results obtained with the agar diffusion method, with some exceptions. Cinnamon oil did not show inhibitory effect against *L. monocytogenes*, but with agar dilution method MIC was 0.06 % (v/v); lemongrass oil did not give inhibition against some of the tested microorganisms but MICs were ≤0.25% (v/v). Some deviations between inhibitory zones and expected MICs were observed in clove, clary sage and peppermint oils. Thyme, cinnamon, peppermint, lemongrass and oregano oils inhibited all organisms at ≤2.0% (v/v). *P. aeruginosa* was the most resistant among tested strains. It was inhibited by 5 oils, followed by *S.* Enteritidis and *E. aerogenes*, which were inhibited by 8 oils. Tested yeasts were more sensitive to cinnamon, oregano and lemongrass with MICs of 0.03-0.06% (v/v).

DISCUSSION

Essential oils used in veterinary medicine may be classified as follows: oils attracting and repelling animals; insecticidal, pest repellent and antiparasitic oils; oils used in animal feed and oils used in treatment of diseases in animals. Studies on antimicrobial properties of essential oils against microorganisms with veterinary importance *in vitro* and *in vivo* are still limited.

Essential oils were widely used, although mechanism of their action was not well understood. They were mainly applied to treat infections because of their antimicrobial activity (9). Results from *in vitro* studies in this work showed that the essential oils inhibited bacterial and fungal growth but their effectiveness varied.

Bampidis at al. (22) indicates that dried oregano leaves administered as an oral solution to calves with diarrhoea may be as effective in the treatment of colibacillosis as neomycin. At the moment, when the antibiotics, as growth promoters, are being replaced, the essential oils could be a very reasonable choice (23). Horošová et al. (24) determined that essential oils after oral administration significantly increased MIC values of amikacin, apramycin, streptomycin and neomycin of *E. coli* chicken isolates. In contrast, Docic and Bilkei (25) found in pigs that oregano feeding caused decrease in MIC values against *E. coli* of ampicillin, enrofloxacin, gentamicin and doxycyclin.

In our study cinnamon, oregano, lemongrass, and thyme exhibit the strongest activity (based on data of agar dilution method as more sensitive) against the selected strains with veterinary interest. This work is in agreement with the study of Prabuseenivasan et al. (26) which found that the essential oil of cinnamon was the most effective as an antibacterial agent. Our results for lemon, grapefruit and mandarine are confirmed with other publications (27). *P. aeruginosa* was the most sensitive to cinnamon and lemongrass oils with MICs of 0.125% (v/v). Our results are the most comparable to those of Hammer et al. (28), since we used the same method for determination of MIC with a little modification. We found differences in MICs of two-eightfold. It is possibly due to local climatic and environmental conditions (29, 30).

Oregano, cinnamon and clove oils were judged “very active” by examining their inhibitory effects on *Clostridium botulinum* 33A (31). In addition to effects against *K. pneumoniae* and *S. aureus*, oregano oil is fungicidal (32). Also, antiviral actions of oregano and clove oils against DNA and RNA viruses have been reported (33).

In summary, this study confirms antimicrobial (antibacterial and antifungal) activity of selected essential oils. Cinnamon, oregano, lemongrass and thyme oils, according to agar dilution method were the most effective. They could be used in the treatment of infections and may potentiate the efficacy of chemotherapeutics. This work needs further *in vivo* extension to find appropriate doses of essential oils showing both antimicrobial activity and very low detrimental effect on eukaryotic cells.

Table 1. *Antimicrobial activity of 12 essential oils against tested microorganisms using disc diffusion method. Values are mean inhibition zone (mm) ± S.D of three replicates.* Legend: *NI- means No inhibition (zone< 12mm).*

Test organism	***Essential oils***											
	Thymus vulgaris	Syzygium aromaticum	Cinnamomum aromaticum	Origanum majorana	Malaleuca alternifolia	Salvia sclarea	Mentha x piperita	Citrus limon	Citrus paradisi	Cymbopogon citratus	Citrus reticulata var madurensis	Origanum vulgare
S. aureus ATCC 25923	30.7±2.3	18.0±2.6	36.0±1.7	17.7±1.5	27.3±1.5	37.7±3.1	22.0±1.0	NI	NI	38.3±2.1	NI	28.0±4.0
L. monocytogenes	29.7±0.6	15.3±0.6	NI	14.3±1.5	21.7±4.0	16.7±0.6	18.0±1.7	NI	NI	30.7±3.5	NI	25.0±2.0
B. licheniformis	43.0±2.6	22.3±2.5	25.0±2.0	19.3±2.5	25.6±0.6	23.3±0.6	24.7±4.0	NI	NI	37.0±8.9	NI	40.3±10.6
E. faecalis ATCC 29212	31.7±0.6	NI	20.7±1.2	13.7±2.5	26.7±0.6	16.0±2.0	15.0±2.0	NI	NI	NI	NI	19.7±0.6
E. coli ATCC 25922	39.3±1.2	29.3±3.5	35.0±1.0	28.3±1.5	34.0±6.2	26.3±5.5	21.0±2.6	NI	NI	40.7±1.5	NI	24.0±2.0
P. aeruginosa ATCC 27853	14.0±2.6	NI	20.0±1.7	NI	NI	NI	NI	NI	NI	NI	NI	13.3±0.6
S. Enteritidis	38.0±1.0	20.3±1.5	28.7±1.1	19.3±2.1	25.0±2.6	NI	NI	NI	NI	15.0±2.0	NI	20.0±2.0
Y. pseudotuberculosis	33.7±6.4	12.7±2.1	25.7±1.2	20.7±3.1	31.3±1.2	13.0±1.0	13.0±1.0	NI	NI	16.3±1.5	NI	35.0±1.0
P. vulgaris	37.0±3.0	19.0±1.0	33.3±3.1	19.3±3.5	31.7±1.5	30.0±2.0	25.0±1.0	NI	NI	35.7±1.2	NI	37.7±3.1
P. mirabilis	39.0±3.6	14.3±1.5	30.3±5.9	25.0±2.6	30.3±0.6	26.7±1.5	29.3±2.1	NI	NI	35.7±1.5	NI	34.0±2.0
K. pneumoniae ATCC 13883	43.3±2.1	15.0±1.7	26.7±2.5	29.3±2.1	17.3±0.6	NI	NI	NI	NI	NI	NI	25.3±0.6
E. aerogenes ATCC 13048	22.0±2.6	NI	19.7±2.1	13.0±1.0	NI	NI	NI	NI	NI	NI	NI	17.3±0.6
C. albicans	35.3±1.2	23.0±1.7	30.7±0.6	18.0±3.6	36.0±4.0	34.3±1.2	23.7±0.6	NI	NI	37.0±2.6	NI	47.0±3.0
M. pachydermatis	33.7±0.6	23.0±00	37.0±1.0	20.7±0.6	17.7±1.2	24.0±2.0	29.7±0.6	NI	NI	32.3±2.1	NI	34.7±0.6

Table 2 *Minimum inhibitory concentrations (MICs) of selected essential oils (% v/v) against 14 different microorganisms. Legend:* ***1****-Thyme,* ***2****-Clove,* ***3****-Cinnamon,* ***4****-Marjoram,* ***5****-Tea tree,* ***6****-Clary sage,* ***7****-Peppermint,* ***8****-Lemongrass,* ***9****-Oregano*

Microorganisms	Essential oils								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
S. aureus ATCC 25923	0.5	0.25	0.03	0.5	1.0	0.5	0.25	0.03	0.06
L. monocytogenes	1.0	0.5	0.06	1.0	1.0	2.0	0.5	0.125	0.06
B. licheniformis	0.25	0.25	0.03	0.5	0.5	0.5	0.25	0.06	0.03
E. faecalis ATCC 29212	1.0	0.5	0.125	1.0	1.0	2.0	1.0	0.125	0.125
E. coli ATCC 25922	0.25	0.125	0.015	0.25	0.5	0.5	0.25	0.06	0.015
P. aeruginosa ATCC 27853	2.0	>2.0	0.125	>2.0	>2.0	> 2.0	1.0	0.125	2.0
S. Enteritidis	0.5	0.25	0.06	1.0	0.5	> 2.0	1.0	0.125	0.03
Y. pseudotuberculosis	0.25	0.125	0.03	0.5	0.5	1.0	0.125	0.06	0.015
P. vulgaris	0.5	0.25	0.03	0.5	0.5	2.0	0.25	0.06	0.03
P. mirabilis	0.25	0.25	0.03	0.25	0.5	2.0	0.25	0.125	0.06
K. pneumoniae ATCC 13883	0.5	0.25	0.03	0.25	0.5	1.0	0.25	0.125	0.03
E. aerogenes ATCC 13048	0.5	0.25	0.06	0.5	0.5	> 2.0	1.0	0.25	0.06
C. albicans	0.5	0.25	0.03	1.0	1.0	0.5	0.5	0.06	0.06
M. pachydermatis	0.5	0.25	0.03	1.0	1.0	0.5	0.5	0.06	0.03

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