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Antimicrobial Activity *in vitro* of Aqueous Extracts of Oregano (*Origanum vulgare* L.) and Thyme (*Thymus vulgaris* L.)

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A B S T R A C T

The antimicrobial effect of hot and cold water extracts and infusions of herbs oregano (*Origanum vulgare* L.) and thyme (*Thymus vulgaris* L.), prepared in concentrations of 20%, was investigated using the classical agar-gel diffusion method. Pure cultures of 20 pathogenic strains were tested (by 4 strains of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Pasteurella multocida*, *Enterococcus faecalis* and *Candida albicans*). The highest antibacterial and antimycotic effect *in vitro* exhibited the infusions of both herbs, similar to that of the control broad-spectrum antibiotic thiamphenicol. The inhibitory effect of the infusion of thyme was slightly higher than that of the infusion of oregano. Highest sensitivity to both infusions showed the tested strains of *P. aeruginosa* and *C. albicans*, and the lowest - these of *P. multocida*. The cold water extracts of both studied herbs showed less pronounced antimicrobial effect, which was slightly higher in thyme. The cold extract of oregano showed pronounced antibacterial effect against the strains of *P. aeruginosa* and *K. pneumoniae*, but not to those of *P. multocida*. The hot aqueous extracts of the two herbs manifested weakest antimicrobial activity *in vitro*. The inhibitory effect of hot water extract of oregano was slightly higher than that of thyme.

Introduction

The wide use of different antibiotic agents for therapy and prophylaxis of bacterial diseases in animals led to selection of resistant microorganisms. Because of this problem Committee for Veterinary Medicinal Products of the European Union stopped the use of some antibiotics and banned their use as growth promoters from 01.01.2006 by Regulation 1831/2003 (Federation, 2003). This decision was justified by the emergence of multy resistant bacterial strains and danger antibiotics become ineffective in therapeutic use in animals and humans. Therefore increasingly emerging trend of looking for new means for use in veterinary medicine, to which it is difficult to build resistance, including biologically active substances of plant origin (Yordanov *et al.*, 2002). Plant extracts are becoming increasingly popular as feed additives for animals replacing antibiotics. They act as antibacterial, antioxidant, anti-cancer means, antifungals, analgesics, insecticides, such as anticoccidial agents and as growth promoters (Levic *et al.*, 2011). There are also a number of studies on antibacterial effect of herbal spices and their essential oils against different types of microorganisms, including food pathogens.

Herbs are used in many foods as natural supplements because of their antibacterial, antifungal, antioxidant and anticancer properties. These alternative preservatives do not show adverse effect on health (Özkalp *et al.,* 2010).

It is known that since ancient time plants have been used by folk medicine for prophylaxis and therapy in humans. Today the use of plant extracts for pharmaceutical purposes is increasing worldwide. This necessitates a thorough examination of their composition, properties, efficiency and safety (Gopinath and Prakash, 2013). Due to the increasing antibiotic resistance of microorganisms in recent years intensive studies have conducted on the biological effects of many herbs, including on their antimicrobial action. Plants contain a wide variety of secondary metabolites such as tannins, alkaloids, phenols, flavonoids, etc., which exhibit antimicrobial properties *in vitro*. A number of plants with strong antioxidant effect are rich also of antimicrobial components that can be used to treat bacterial infections (Lewis and Ausubel, 2006; Shirzad *et al.,* 2011; Tabassum *et al.,* 2013; Shahnama *et al.,* 2015).

There are a number of data about inhibitory action of water, alcohol and other extracts of various herbs on the development of pathogens, giving rise to hopes for efficient use of plant extracts in a variety of infections (Islam *et al.,* 2008; Pichardo *et al.,* 2013; Shahnama *et al.,* 2015; Sarbadhikary *et al.,* 2015). For example, studies of Tabassum *et al.,* (2013) on coriander, cinnamon and other plants showed that the ethanol plant extracts have the best antimicrobial effect, but the aqueous and and methanol extracts also exert a good antimicrobial activity on cause agents of urinary tract infections such as *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterobacter faecalis* and *Proteus mirabilis*. Plant extracts have great potential as antimicrobial agents even against microorganisms, resistant to antibiotics and can be applied with success in the treatment of infectious diseases caused by resistant microbes, and even by multy resistant enterococci (Gopinath and Prakash, 2013). Shohayeb *et al.,* (2014) pointed out that aqueous, ethanol and hexane extract from petals of *Rosa damascena* MIL posses moderately broad antimicrobial activity against Gram-positive (*Staphylococcus aureus, Bacillus subtilis, Streptococcus pyogenes),* Gram-negative (*Acinetobacter baumannii, Klebsiella pneumoniae),* acid-resistant (*Mycobacterium phlei*) bacteria and fungi.

The purpose of the present work was to establish in vitro the extent of the inhibitory effect of aqueous extracts of herbs oregano and thyme, prepared by different technologies on the development of pathogenic microorganisms isolated from patients treated with antibiotics.

Materials and Methods

Microorganisms

Pure cultures of 20 pathogenic strains of five different species were tested - 4 bacterial (by 4 strains of *Pseudomonas aeruginosa, Klebsiella pneumoniae, Pasteurella multocida, Enterococcus faecalis*) and 4 strains of the oval fungus *Candida albicans*. Microorganisms were isolated in the Microbiological laboratory of Faculty of Veterinary Medicine at the University of Forestry from clinical specimens of pet dogs with chronic otitis and pyodermatitis, and *Pasteurella multocida* - from aviary bred pheasants died of cholera. The bacterial strains demonstrated *in vitro* poly-resistance to β-lactam, aminoglycoside and tetracycline antibiotics.

Antibacterial means

Dried drugs (loose dry leaves and flowers) for herbal tea of oregano (*Origanum vulgare* L.) and thyme (*Thymus vulgaris* L.) from BILEK company, Troyan - 5600, Bulgaria were tested. The following aqueous extracts from each of the herbs were prepared immediately prior to testing:

Hot extracts

20% of oregano and thyme were prepared within a tightly closed vial by soaking (maceration) of 10 g dry drug in 100 ml of sterile distilled water heated to 100ºC for 2 h under frequent shaking, followed by filtration through double sterile gauze.

Infusions

20% of each of the herbs were prepared by boiling in a water bath at 100ºC for 15 min of 10 g dry drug in 100 ml of sterile distilled water at periodic shaking, filtration through sterile double gauze after cooling (45 minutes) and refilling with sterile distilled water up to restoring original volume.

Cold extracts

20% of oregano and thyme were prepared by soaking (maceration) of 10 g dry drug in 100 ml of sterile distilled water in a sterile vial for 24 h at room temperature with frequent shaking and filtration through sterile double gauze.

The antimicrobial effect

The antimicrobial effect was tested using the classical agar-diffusion method of Bauer *et al.,* (1966) intended for fast-growing under aerobic conditions microorganisms on Mueller Hinton agar with pH 7.2-7.4 and layer thickness of 4 mm. The preparations were administered by instillation of 0.1 ml (5 mg) in wells with a diameter of 9 mm. In parallel, a solution in physiological saline of the control antibiotic thiamphenicol was used by dropping in the wells of 0.1 ml contained the standard concentration of 30 μg. The inoculation of the microbial suspensions at dose of 2.10^6 cells/ml was performed immediately before the introduction of the herbal extracts and the control in the wells. Then incubation for 2 hours at room temperature was carried out for diffusion of the compounds in the agar. Incubation was conducted at 37°C for 18–24 hours. Reporting of results was carried out by measuring the diameters of the inhibitory zones in millimeters accurate to the nearest whole millimeter including the wells diameter, with a transparent ruler and caliper on the outside of the plate's bottoms. The zones of inhibition were interpreted by the three-degree system for categorizing of Bauer *et al.,* (1966). According to this system of the Bauer-Kirby and NCCLS (1997, 1999), inhibitory effect of the herbal extracts is reported at zones > 12 mm, and of the antibiotic - at > 17 mm. The sensitivity of the test microorganisms to the extracts was determined as follows: resistant (R) - at zones <12 mm, intermediate sensitive (I) - 13 to 16 mm and sensitive (S) - at zones > 17 mm. For thiamphenicol the relevant borders are: R - <12 mm, I - 13–17 mm and S - > 18 mm.

Statistical analysis

Statistical analysis of the results was carried out by the classical method of Student-Fisher.

Results and Discussion

The results of studies of the effect of the aqueous extracts of both herbs on *P. aeruginosa* are presented in table 1. Some of them can be seen in figure 1.

From the data in the table is seen that the highest inhibitory effect on the investigated strains of *P. aeruginosa* showed the infusion of oregano. The cold extract also inhibited the growth of the tested strains, while hot extract had an effect only to one of these strains. Similar data were obtained and for extracts of thyme. The antimicrobial effect of the infusion of thyme was even slightly superior to that of the infusion of oregano while cold extract of oregano had a slightly higher activity than that of thyme. The results in most of the strains were similar to those obtained in the control antibiotic, as in some of them the effect of the extracts was higher than that of the antibiotic.

The results obtained in studies of the effects of extracts from both herbs against *K. pneumoniae* are presented in table 2 and figure 2.

From the data in the table it is seen that the infusions of both herbs showed the highest inhibitory effect on the tested strains of *K. pneumoniae*, particularly that of thyme. The cold extracts of the two drugs also suppressed the growth of the test strains and the effect of oregano was a more pronounced compared with that of thyme. The hot extracts exhibited such an effect only against individual strains. The results in many of the strains were close to those of the control antibiotic, such as in some of them the effect of the extracts was superior to that of the antibiotic.

The results of the investigations of the effect of the extracts from both herbs against *P. multocida* are presented in table 3 and figure 3.

The data in the table show that the highest inhibitory effect on the tested strains of *P. multocida* exhibited again the infusions of the two herbs, such as a slightly higher was the effect of that of oregano. In the extracts of the two drugs received by hot and cold way, the results were weaker than those in infusions, as well as than the obtained when testing the control antibiotic.

The data obtained in the studies of the effect of the extracts of both herbs for against *E. faecalis* are presented in table 4. Some of them can be seen in figure 4.

The results show that the highest inhibitory effect on the tested strains of *E. faecalis* again exhibited the infusions, as that of thyme showed a slightly higher effect in comparison with the infusion of oregano. The antimicrobial effect of the other two types extracts was less pronounced, as the weakest was the action of the hot extract of thyme. The results in most of the strains were similar to those obtained in the control antibiotic.

The results of studies of the effect of water extracts of both herbs against *C. albicans* are presented in table 5 and some of them can be seen in figure 5.

From the data in the table it can be seen that like against the tested bacterial strains, also against the studied strains of these oval fungi, the highest inhibitory effect exhibited the infusions of both herbs, such as here the higher activity indicated this of thyme. The cold extract of thyme also exhibited significant antifungal effect. The cold and hot extracts of oregano and the hot extract of thyme showed less pronounced antimycotic effect.

From the summarised results in table 6 is seen that the infusions exhibited the highest antimicrobial effect among all tested types of extracts from the two herbs. They inhibited to a significant extent the multiplication of all strains tested positive and negative by Gram bacteria and that of the oval fungi. Average diameters of the inhibitory zones of *P.*

aeruginosa were similar to those of thiamphenicol (P>0.05), as well as those of *E. faecalis* (P>0.05), while the zones of *K. pneumoniae* were larger than the areas recorded in the control antibiotic (P>0.05). The inhibitory action of the infusion of thyme was slightly higher than that of the infusion of oregano (P>0.05). The highest sensitivity to the infusion of thyme showed the tested strains of *C. albicans* (average diameters of the inhibitory zones 19.25±3.77), and of *P. aeruginosa* (zones of inhibition of growth x = 18.00±3.08), and lowest - those of *P. multocida* (zones of inhibition x = 14.50±1.50), as the differences between them were not statistically significant (P>0.05).

The cold extracts of the two studied herbs also exhibited antimicrobial activity, which was slightly higher in thyme. Thyme extract turned out to be more effective against the tested Gram-positive microorganisms (enterococci and oval fungi). The cold extract of oregano showed pronounced inhibitory effect on *K. pneumoniae* (average diameter of inhibitory zone 14.75±1.29) and on *P. aeruginosa* (inhibitory zone x = 14.50±1.12). The sensitivity of the strains of *P. multocida* to this extract turned out to be the lowest (11.67±0.47), significantly lower than that of the other tested Gram-negative bacteria *K. pneumoniae* and *P. aeruginosa* (P<0.05).

The hot extracts of the two studied herbs showed weakest antimicrobial effect. However, the hot extract of oregano showed slightly higher inhibitory activity than that of thyme. The highest sensitivity to the hot extract of oregano showed the tested strains of *K. pneumoniae* (inhibitory zones x = 14.25±1.92), as well as those of *C. albicans* (x=14.25±1.92), and the lowest - of *P. aeruginosa* (barely x=11.75±1.48). The differences in the sensitivity of the strains of the different tested microbial species, however, were not statistically significant (P>0.05). The Gram-positive species tested microorganisms (oval fungi and enterococci) showed higher sensitivity to the hot extract of thyme in comparison with the Gram-negative bacteria, but the differences also were not significant (P>0.05).

The results of these studies show that the water extracts of thyme and oregano prepared at a concentration of 20% exhibit inhibitory action against poly-resistant pathogenic microorganisms isolated from treated with various antibiotics patients. Considering their safety and the row favorable biological effects on the body at their external and internal application, these results are promising for practice.

Table.1 Inhibitory effect of aqueous extracts of oregano and thyme against 4 strains of *Pseudomonas aeruginosa*

Herbal drugs	Type of the extract	Inhibitory zones in mm			
		Strain No 1	Strain No 2	Strain No 3	Strain No 4
Oregano	Hot extract	12	11	10	14
Oregano	Infusion	20	15	15	14
Oregano	Cold extract	16	14	13	15
Thyme	Hot extract	15	10	12	12
Thyme	Infusion	20	22	15	15
Thyme	Cold extract	13	14	10	15
Control	Thiamphenicol	17	16	28	15

Table.2 Inhibitory effect of aqueous extracts of oregano and thyme against 4 strains of *Klebsiella pneumoniae*

Herbal drugs	Type of the extract	Inhibitory zones in mm			
		Strain No 5	Strain No 6	Strain No 7	Strain No 8
Oregano	Hot extract	16	11	15	15
Oregano	Infusion	18	15	15	19
Oregano	Cold extract	16	16	13	14
Thyme	Hot extract	15	12	12	12
Thyme	Infusion	15	17	17	14
Thyme	Cold extract	14	12	12	13
Control	Thiamphenicol	16	18	9	15

Table.3 Inhibitory effect of aqueous extracts of oregano and of thyme against 4 strains of *Pasteurella multocida*

Herbal drugs	Type of the extract	Inhibitory zones in mm			
		Strain No 9	Strain No 10	Strain No 11	Strain No 12
Oregano	Hot extract	10	15	11	15
Oregano	Infusion	14	16	15	22
Oregano	Cold extract	11	12	12	16
Thyme	Hot extract	10	9	11	12
Thyme	Infusion	12	15	16	15
Thyme	Cold extract	11	16	12	12
Control	Thiamphenicol	22	21	23	22

Table.4 Inhibitory effect of aqueous extracts of oregano and of thyme against 4 strains of *Enterococcus faecalis*

Herbal drugs	Type of the extract	Inhibitory zones in mm			
		Strain No 13	Strain No 14	Strain No 15	Strain No 16
Oregano	Hot extract	13	14	13	15
Oregano	Infusion	15	16	15	18
Oregano	Cold extract	13	12	13	17
Thyme	Hot extract	11	12	15	15
Thyme	Infusion	16	18	16	17
Thyme	Cold extract	12	17	15	15
Control	Thiamphenicol	20	21	14	22

Table.5 Inhibitory effect of aqueous extracts of oregano and of thyme against 4 strains of *Candida albicans*

Herbal drugs	Type of the extract	Inhibitory zones in mm			
		Strain No 17	Strain No 18	Strain No 19	Strain No 20
Oregano	Hot extract	17	12	15	13
Oregano	Infusion	18	15	17	17
Oregano	Cold extract	11	15	12	15
Thyme	Hot extract	15	11	12	14
Thyme	Infusion	17	15	20	25
Thyme	Cold extract	16	14	15	18
Control	Thiamphenicol	16	24	9	25

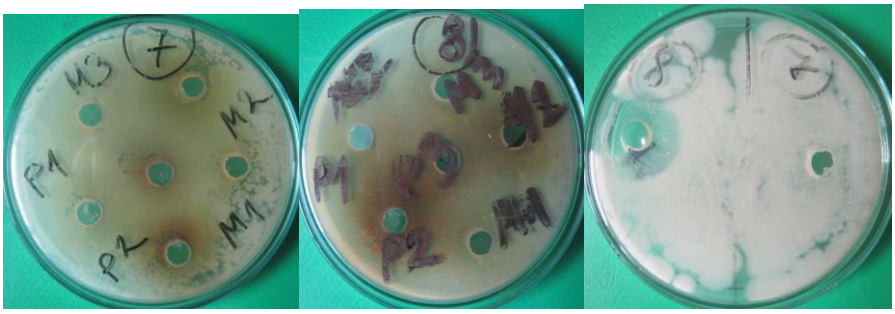
Table.6 Inhibitory effect of aqueous extracts of oregano and of thyme against pathogenic microorganisms in the agar diffusion method

Micro-organisms	Number of tested strains	Mean zone of inhibition in mm ± Standard deviation						
		Hot extract		Infusion		Cold extract		Thiam-phenicol
		Oregano	Thyme	Oregano	Thyme	Oregano	Thyme	
Pseudomonas aeruginosa	**4**	11,75± 1,48	12,25± 1,78	16,00± 2,34	18,00± 3,08	14,50± 1,12	13,00± 1,87	19,00± 5,24
Klebsiella pneumoniae	**4**	14,25± 1,92	12,75± 1,29	16,75± 1,78	15,75± 1,29	14,75± 1,29	12,75± 0,83	14,50± 3,35
Pasteurella multocida	**4**	12,75± 2,28	10,50± 1,12	16,75± 3,11	14,50± 1,50	11,67± 0,47	12,75± 1,92	22,00± 0,71
Enterococcus faecalis	**4**	13,75± 0,83	13,25± 1,78	16,00± 1,22	16,75± 0,83	13,75± 1,92	14,75± 1,78	19,25± 3,11
Candida albicans	**4**	14,25± 1,92	13,00± 1,58	16,75± 1,09	19,25± 3,77	13,25± 1,78	15,75± 1,48	18,50± 6,50
Total	**20**	13,35± 0,97	12,35± 0,98	16,45± 0,37	16,85± 1,66	13,58± 1,09	13,80± 1,23	18,65± 2,41

Fig.1 Antibacterial effect of aqueous extracts of oregano (P 1 – hot extract; P 2 – infusion; P 3 – cold extract) and of thyme (M 1 - hot extract; M 2 – infusion; M 3 – cold extract) against *Pseudomonas aeruginosa*: strain No 1 (a), strain No 2 (b), and of the control antibiotic thiamphenicol against the same strains No 1 and 2 (c)

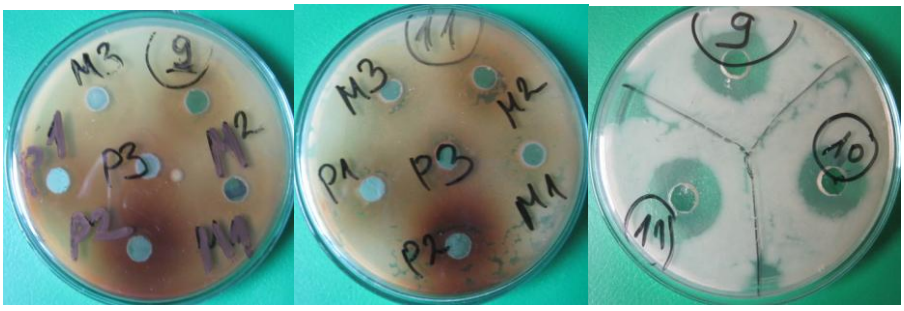
a b c

Fig.2 Antibacterial effect of aqueous extracts of oregano (P 1 – hot extract; P 2 – infusion; P 3 – cold extract) and of thyme (M 1 - hot extract; M 2 – infusion; M 3 – cold extract) against *Klebsiella pneumoniae*: strain No 7 (a), strain No 8 (b), and of the control antibiotic thiamphenicol against the same strains No 7 and 8 (c)



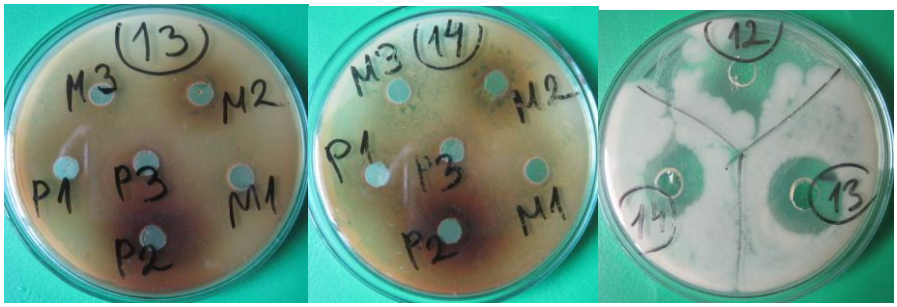
a b c

Fig.3 Antibacterial effect of aqueous extracts of oregano (P 1 – hot extract; P 2 – infusion; P 3 – cold extract) and of thyme (M 1 - hot extract; M 2 – infusion; M 3 – cold extract) against *Pasteurella multocida*: strain No 9 (a), strain No 11 (b), and of the control antibiotic thiamphenicol against the strains No 9,10 and 11 (c)



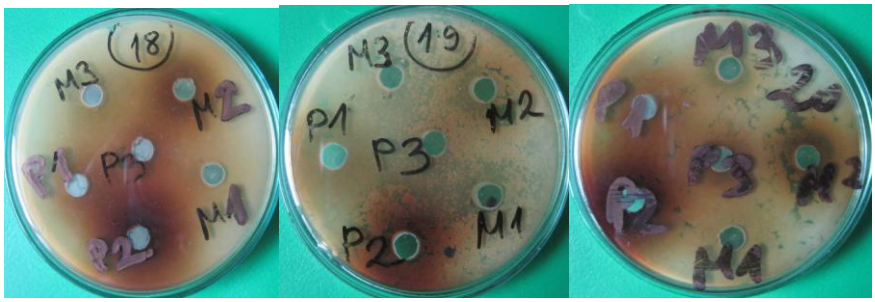
a b c

Fig.4 Antibacterial effect of aqueous extracts of oregano (P 1 – hot extract; P 2 – infusion; P 3 – cold extract) and of thyme (M 1 - hot extract; M 2 – infusion; M 3 – cold extract) against *Enterococcus faecalis*: strain No 13 (a), strain No 14 (b), and of the control antibiotic thiamphenicol against the strains No 12,13 and 14 (c)



a b c

Fig.5 Antifungal effect of aqueous extracts of oregano (P 1 – hot extract; P 2 – infusion; P 3 – cold extract) and of thyme (M 1 - hot extract; M 2 – infusion; M 3 – cold extract) against *Candida albicans*: strain No 18 (a), strain No 19 (b), and strain No 20 (c)



a b c

The most pronounced antimicrobial properties in our tests showed the infusions, while the lowest - the hot extracts from the studied herbs. The inhibitory effect of the cold extracts superior to that of the hot made. These our data to some extent correspond to the results obtained by Saeed and Tariq (2009), who reported significant activity of cold extract and essential oil of oregano (*O. vulgare*) against Gram-positive bacteria (staphylococci, streptococci and bacilli). These organisms, however, have been resistant to the effects of decoction of the plant. According to the authors this may be due to the thermolability of the active ingredients of oregano. Naim and Tariq (2006) reported antibacterial activity of essential oil of oregano against *Staphylococcus aureus* and resistance of the same strains to decoction and infusion of oregano. Higher than that found by us inhibitory activity against Gram-negative bacteria of the genera of the family *Enterobacteriaceae*, including *E. coli*, as well as to *Aeromonas* sp. and *Pseudomonas aeruginosa*, has been established by Chaudhry *et al.,* (2007) at testing of extracts from seeds of oregano (*O. vulgare*). They found pronounced inhibitory effect of cold extract and the absence of such at testing of hot infusion. Obviously the seeds contain higher concentrations of active ingredients, including such with antimicrobial effect. Also, their double prolonged cold extraction

compared to that applied by us, seems to provide a better extraction of the active ingredients.

In this study, important from a practical point of view is that even bacteria known with rapid development of resistance to antibiotics showed sensitivity, such as *P. aeruginosa*, *K. pneumoniae* and *E. faecalis*. Essential for the practice is also the well defined antifungal activity of the infusions against the oval fungus *C. albicans*. Obviously, the boiling of the drug at the preparation of the decoctions is essential for better extraction of the antimicrobial ingredients from the herbs. It seems that the technology of getting hot extracts prevents complete extraction of these substances from the dried drugs. Our studies show that at the cold extraction good results are also achieved, but weaker than those in the extracts obtained by the hot methods.

According Teixeira *et al.,* (2013) the extracts and essential oil of *O. vulgare* have significant potential to be used as an alternative to synthetic chemicals in the food industry where the oxidation and microbial contamination are problems. The authors have investigated the antibacterial activity of extracts and essential oil of oregano to various pathogenic bacteria causing food spoilage. Most of them have shown resistance to hot and cold aqueous oregano extracts, while the ethanol extract had been able to inhibit the growth of most bacteria including *E. coli* and *L. monocytogenes.* The essential oil also had been effective in inhibiting the growth of all tested bacterial strains. The results of other studies also show that the methanol extracts have a higher activity in this respect than other types of extracts (Ejikeugwu *et al.,* 2012; Shahnama *et al.,* 2015). According Shahnama *et al.,* (2015) The bactericidal effect against Gram-positive and Gram-negative clinical isolates as *S. aureus, S. agalactiae, B. cereus, L. monocytogenes, E. coli, K. pneumoniae, P. mirabilis, Salmonella enterica* and *P. aeruginosa* was due to the high concentration of pinene in methanol extracts.

Martins *et al.,* (2014) reported the efficiency of water extracts of oregano as an infusion (obtained in hot water for 5 min) and decoction (by boiling for 5 min) against Gram-positive (*Staphylococci*) and Gram-negative (*Enterobacteria* and *P. aeruginosa*) microorganisms. The authors point out that the use of these aqueous extracts for internal or external use can avoid the toxic effects shown by the oregano essential oil. Our results also show that a particularly promising would be the application of infusions of both herbs against *P. aeruginosa*, whose strains are characterized by poly-resistance to antimicrobial agents and rapid development of resistance to antibiotics and disinfectants. Taking into account the small number of effective antifungal agents, the infusions of thyme and oregano can successfully be applied topically on the skin and mucous membranes in fungal infections.

In conclusion, water extracts of the dried herbs oregano and thyme, prepared at a concentration of 20% through warm and cold extraction of active substances, exhibit antimicrobial activity *in vitro***.** It is highest against the tested Gram-positive microorganisms - the strains of *Enterococcus faecalis* and *Candida albicans*, and the lowest - to those of *Pasteurella multocida*. The infusions of both herbs exhibit the most pronounced inhibitory effect on the multiplication of bacteria and oval fungi. This effect is high and against Gram-negative bacteria of the species *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The cold extracts of both studied herbs exhibit less pronounced antimicrobial action, which is slightly higher in this of thyme. The hot extracts from each of both drugs exhibit

negligible antimicrobial activity *in vitro*. The inhibitory effect of the hot extract of oregano is slightly higher than that of thyme.

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