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In Vitro and In Vivo Antimicrobial Activity of two Essential Oils *Thymus Vulgaris* and *Lavandula Angustifolia* against Bovine Staphylococcus and Streptococcus Mastitis Pathogen

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ABSTRACT

This work was conducted in order to investigate the antimicrobial activities of essential oils of *Thymus vulgaris* and *Lavandula angustifolia* as alternatives for antibiotics against bovine staphylococcus and streptococcus mastitis pathogen. In four farms, milking cows were examined weekly for 5 months for the presence of clinical mastitis using California Mastitis Test (CMT). Meanwhile essential oils of *Thymus vulgaris a*nd *Lavandula angustifolia* were steam extracted and were applied alone or in combination in 3 different concentration 10, 20 and 30%. Data showed that In vitro, the 2 essential oils of Thymus and Lavender have shown strong antibacterial activity against the main strains of mastitis Staphylococcus and Streptococcus with potency similar to the control antibiotic. Intramammary application of 10% solution of Thymus and Lavender essential oils and of mixture thymus lavender has driven to a drastic decrease in the bacterial colony count in the different sample of milk after 4 consecutive treatments. External application or massage of a solution of thymus or lavender in Vaseline showed a strong antibacterial activity against the 2 bacterial pathogens Staphylococcus and Streptococcus. This activity is stronger than intramammary application with a rate of recovery of 100% with thymus essential oils.

Key words: *Thymus vulgaris*, *Lavandula angustifolia*, staphylococcus, streptococcus, essential oils.

Introduction

Mastitis continues to be among the most common and the costliest diseases to the dairy industry throughout the world (Kinabo and Assey, 1983; Lightner *et al.,* 1988; Kaneene and Hurd, 1990; Miller *et al.,* 1993; Kossaibati *et al.,* 1998). Among cattle diseases, bovine mastitis is a serious problem which affects the basic income of the farmers, depleting their dairy resources (Mubarack *et al.,* 2001). The disease affects not only the physical, chemical, bacteriological, technological and organoleptic properties of milk but also the quantity of milk. According to Mayer (1990), the worldwide cost of this disease was 35 billion US dollars annually. The estimated economical losses differed from one country to another where in the US were found to be more than 2 billions dollars per annum (Jasper *et al.,* 1982); in the UK dairy industry faced a loss of 93 million pounds per year, while Australian dairy industry faced more than 100 million dollars annually (Hogeveen, 2005). In a recent study, Huijps *et al.,* (2008) reported an average economic losses assessed by the Dutch dairy farmers were 78 euros/cow per year with a range of 17 to 198 euros/cow per year. According to Philpot and Nickerson (1991), worldwide one third of all dairy cows were infected with some forms of mastitis in one or more quarters. *Staphylococcus aureus* is often considered to be the most contagious mastitis pathogen (Fox *et al.,* 2000; Mubarack *et al.,* 2001; Fagundes *et al.,* 2010; Dal Pozzo *et al.,* 2011).

According to Mubarack *et al*. (2001) losses due to subclinical mastitis are more severe than those due to clinical cases. Controlling subclinical mastitis can reduce the losses in milk production substantially. Decreased milk production and quality, as well as veterinary expenses, all contributes to these economic losses. The most common treatment is based on intramammary infusion of antibacterial agents. However, cure rates obtained with such drugs are not always effective, because it may determine the emergence of resistant bacteria (Zafalon *et al.*, 2007) as well increase amounts of antibiotic residues in milk (Fagundes *et al.*, 2010). Nevertheless, the treatment of bovine subclinical mastitis caused by S. aureus in the lactation can be economically unviable (Zafalon *et al.,* 2007). On the other hand, concerns regarding the perceived or potential harmful effects of antibiotics residues in milk have driven the consumer demand for organically produced dairy products (McEwen *et al.*, 1991), where the use of antibiotics in cows supplying milk for organic production is prohibited (Pol and Ruegg, 2007). Worldwide dairy products are only second (16%) to fruits and vegetables (40%) in organic food purchase (Organic Trade Association, 2007). The demand for organic milk is predicted to grow and potentially may result in a dramatic increase in proportion of dairy farms transitioning to organic practices (Rueeg, 2009).

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Alternative treatments to bovine mastitis with bacteriocins (Pieterse *et al*., 2010) and plant derived compounds (Baskaran *et al*., 2009; Mubarack *et al.,* 2001) have been described. Essential oils (EO) are classified as GRAS (generally regarded as safe), show antibacterial proprieties and resistance has not been reported after prolonged exposure. Then, the investigation of their antimicrobial activity against bacterial agents of mastitis is justifiable. According to Ruegg (2009) there are many natural remedies that are topical and pose little risk for contamination of milk and meat. However, research is lacking to support the efficacy of these compounds. Natural antimicrobials such as caprylic acid, eugenol, trans-cinnamaldehyde, carvacrol and thymol have been found to be inhibitory, in vitro, towards an array of pathogenic microorganisms and may be potential candidates for efficacious non antibiotics treatments for mastitis (Baskaran *et al.,* 2009; Nair *et al.,* 2005). If so, evaluation of these natural products or essential oils in vivo can be justifiable. Other Concern about these products, even if there are no human health hazards, is that these essential oils can have impacts on the taste of the milk or they may interfere with the culturing process for cheese and yoghurt which can be subjects for further study.

In this context, the aims of this study are a)) to evaluate the in vitro activity of two essential oils thymus and lavender against the 2 major bacteria of mastitis Staphylococcus and Streptococcus; and b) will be to study in vivo the effect of these 2 essential oils in the control of bovine mastitis.

Materials and Methods

The experiment was conducted in West Bekaa valley in order to evaluate the in-vitro and in vivo activities of two essential oils thymus and lavandula against two major bacteria of mastitis *staphylococcus* and *streptococcus* in control of dairy cattle mastitis. The five-months study was performed in four different farms of Friesian- Holstein cow in a mountains region in West Bekaa valley about 1000 m above sea level. The experiment was done in the laboratory of microbiology of the Faculty of Agriculture Engineer and Veterinary Medicine, Lebanese University. The weather is cold in winter (-1°C) and hot in summer (30°C). The rainfall pattern is characterized as annual with an average of 600 mm.

Four intensive dairy cow farms belonging to Friesian- Holstein breed were selected randomly during the experiment. The first flock counts 50 lactating cows; the second flock counts 74 lactating cows; the third flock counts 27 lactating cows; while the fourth flock counts 47 lactating cows. The cows are raised mainly in closed farms, sometimes not properly ventilated. All cows have access for external pastures during specific hours of the day. Milk samples were collected from each clinical cases and isolates identified the streptococcus and staphylococcus where tested with antibiotics and essential oil extracts to determine the sensitivity profile.

Milking cows were examined every week during the 5 months of the experiment. All milking cows were checked for the presence of clinical mastitis. Clinical mastitis was defined as the presence of abnormal milk secretion by using California Mastitis test. The California Mastitis Test (CMT) is a rapid, accurate, cow-side test to help determine somatic cell counts (SCC) in a specific cow. The test was developed to sample individual quarters to determine the presence of subclinical mastitis.

The CMT reagent reacts with the white blood cells and the mixture thickens or gels in proportion to the amount of infection present as shown in table 1.

Table 1: Reaction of California Mastitis Test with the white blood cells

CMT Score	Average Somatic Count (Cells per milliliter)	Description of reaction
N (negative)	100,000	No thickening, homogeneous.
T (trace)	300,000	Slight thickening. Reaction disappears in 10 seconds.
1	900,000	Distinct thickening, no gel formation.
2	2,700,000	Thickens immediately, begins to gel, levels in the bottom of cup.
3	8,100,000	Gel is formed, surface elevates, with a central peak above the mass.

Plants used in the experiment:

Two plants were used in the experiment for the extraction of the essential oil: *Thymus vulgaris* and *Lavandula angustifolia.*

*Lavender: " Lavandula angustifolia":*is a temperate plant and it is native to the Mediterranean regions. Lavender is distributed in Syria, Palestine, and Lebanon. In Lebanon it is mainly present in Haramoun, South Lebanon and Bekaa (Hilal, 1988). It belongs to the *Labiatae* family. Most of the essential oils accumulate in the oil-holding glands, which are around the ridges of the calyx (Hay and Waterman, 1993). Oil of Lavender possesses refreshing and sweet odour. It is used in perfumery industry. It is also an excellent insecticide and antiseptic (Akhtar *et al.,* 1988).

Thymus: Thymus vulgaris: is an aromatic plant belong to the *labiatea* family. The aromatic herb grows wild in almost all countries bordering the Mediterranean and also over much of Asia and in all parts of central Europe. It is well distributed in Turkey, Syria, Egypt and Lebanon where it is found on great area (Sivropoulou *et al.*, 1996) and is usually collected for human consumption (Sivropoulou *et al.*, 1996). The oil is used in food products, cosmetics and alcoholic lequeurs. It is used as carminative, diaphoretic, and stimulant. Thymus vulgaris oil is a powerful disinfectant and caravacrol thymol are considered to be anthelmintic and antifungal agents (Sivropoulou *et al.,* 1996).

Preparation of essential oil:

Two essential oils *Thymus vulgaris* and *Lavandula angustifolia* were used either alone or in combination in this study; The extraction of essential oils was obtained by using steam distillation.

Microbiological identification of mastitic agents:

Positive milk samples (22 samples) were collected from the 4 different farms in a sterile beaker of 100 ml. To avoid growth of the bacteria found in the milk before arrival to the lab the beakers were saved in a refrigerator – 10 °C to freeze the milk. We have to note that milk was collected from infected cows that were not treated with intramammary antibiotics.

Streptococcus and Staphylococcus bacterial pathogens were isolated from the different milk samples through milk culture in 2 different media: Manitol Salt Agar for Staphylococcus and bile esculin agar for streptococcus.

In vitro microbiological experiments:

The two essential oils from *Thymus vulgaris* and *Lavender angustifolia* and their combination were diluted in methanol at 3 different concentrations 10, 20 and 30%.

Previously weighed paper disks were immersed for half an hour in the solutions of different concentrations of the extracts and were dried out. Negative control disks were prepared using the solvent of the extracts (methanol) in the same way. These (together with positive control disks (multiject IMM antibiotic that is composed of streptomycin sulfate, neomycin sulfate, prednisolone, procaine penicillin; Norbrook factory) have been used for microbiological assay in new plates containing the appropriate microorganisms. The sensitivity of bacterial pathogens to the different concentrations of the essential oils was assessed based on the diameter of the inhibition zone as follows:

S1: (high sensitivity) diameter of the inhibition zone>20mm

S2: (low sensitivity) diameter of the inhibition zone varies between 10mm and 20mm

R: (resistance) diameter of the inhibiton zone less than 10mm

In Vivo activity of thymus and lavender essential oils against bovine mastitis:

In farm 1: the 5 mastitis positive cows were submitted to intramammary injection of 10% thymus essential oils in methanol. Treatment is repeated for 4 consecutive days. Milk was submitted to California tests and samples were recollected.

In farm 2: The 6 mastitis positive cows were submitted to intrammamary injection of 10% lavender essential oils in methanol. Treatment is repeated for 4 consecutive days.

In farm 3: The 4 cases of mastitis were submitted to intramammary injection of a combination 10% EO from thymus and 10% EO from Lavender in methanol for the same period of time.

In Farm 4: 4 cases of infected cows were submitted to external application of thymus EO in Vaseline, while the other 3 cases were submitted to external treatment of Lavender EO in Vaseline for 4 consecutive days and milk samples were collected.

Data analysis:

The data obtained were stored in excel spread sheet. Paired t-test was used for the statistical analysis of the results of the in vitro study. Analysis of the in vivo study was carried out using ANOVA test. Results were recorded as mean ± standard deviation.

Results

Prevalence of cow mastitis in the 4 farms during the experiment

The number of mastitis positive cows in the 4 farms of the study is summarized in table (2).

According to table (2), the prevalence of subclinical cases of mastitis in the 4 farms under study is generally very high ranging between 8.1% in farm 2 and 14.8% in farm 3 and 4. The California score test reveal a serious case of mastitis in farm 3 and 4. The high number of cases is an indicator of bad milking practices in this farm which indicate that the hygiene of the milking process in this farm under study should be revised.

Table 2: Prevalence of subclinical cases of mastitis in the 4 farms of West Bekaa-Lebanon

Farm	Number of cows	NO of cases	percentage	California test score			
				T	1	2	3
1	50	5	10%	2		3	
2	74	6	8.1%	1	3	2	
3	27	4	14.8%			2	2
4	47	7	14.8%		2	3	2

Bacteriological counts of the different milk samples from the 4 farms under study

The average number of colony of Streptococcus and Staphylococcus from the different sample of milk is summarized in table (3).

The number of colony of *Staphylococcus* and *Streptococcus* isolates is very high in the milk samples collected from farm 3 and 4. This high number of isolates could be an explanation for the values of California Score test recorded in these 2 farms. Farms 1 and 2 isolates were also staphylococcus and Streptococcus contaminated but the rate of contamination is less pronounced than farms 3 and 4.

Table 3: Average colony count (in 1 ml*1000) of milk in the 4 farms under study

Farm	*Staphylococcus sp.*	*Streptococcus sp.*
1	19.2±8.4 [a]	37.3±5.64 [a b]
2	38.2±9.1 [b]	27.34±10.12 [a]
3	51.5±11.9 [c]	45.87±8.98 [b]
4	62±10.8 [c]	45±8.3 [b]

a, b,c: between farm, p<0.01

In vitro antibacterial effect of the 2 essential oils against the Staphylococcus and Streptococcus isolates from the 4 farms:

The antimicrobial activity of different dilutions of thymus and lavender essential oils against mastitis pathogenic bacteria*: Staphylococcus sp.* and *Streptococcus* species were measured by measuring the zone of inhibition in disc diffusion method.

Table (4) shows the zone of inhibition for the different concentration of the 2 extracts (Thymus and Lavender) against collected samples of *Streptococcus* species in the 4 farms of the study.

Table 4: Zone of inhibition produced by the different dilution of essential oils against Streptococcus species isolates from different farm

E.O. Dilution	Zone of inhibition (mm)			
	Farm 1	Farm 2	Farm 3	Farm 4
Thymus solution (10%)	19.2±3.8 [a]	20.1±1.9 [a]	19.6±1.9 [a]	21.6±2.2 [a]
Thymus solution (20%)	24.7±3.6 [b]	27.6±1.9 [b]	27.3±4 [b]	27.6±1.9 [b]
Thymus solution (30%)	29.8±1.1 [c]	30.9±1.6 [c]	32±3.2 [c]	28.7±5 [b]
Lavender solution (10%)	10.7±0.26 [a]	11.6±0.55 [a]	13.7±0.25 [a]	14.1±0.1 [a]
Lavender solution (20%)	17.8±0.2 [b]	18.7±0.3 [b]	19.8±0.28 [b]	16.7±0.25 [a]
Lavender solution 30%)	20±0.5 [b]	24.2±0.52 [c]	21.7±0.25 [b]	17.8±0.26 [a]
Lavender +Thymus solution (10%)	30.7±2.3 [a]	31.7±0.6 [a]	33.7±6.4 [a]	30.7±8.9 [a]
Lavender +Thymus solution (20%)	29.7±0.6 [a]	39.3±1.2 [b]	37±3 [b]	37±8.9 [b]
Lavender +Thymus solution (30%)	43±2.6 [a]	38±1 [b]	39±3.6 [b]	38.3±2.1 [b]
Multiject antibiotics	29.8±4.7	28.4±4	30.01±0.9	31.3±2.3

a,b,c,d: Within farm, between E.O. dilution, p<0.01

According to table (4), the results of experiments showed that the oil from *T. vulgaris* exhibited extremely strong activity against *Streptococcus* isolates. At 10% dilution, the antibacterial activity of thymus essential oils against Streptococcus isolates is generally moderate with an inhibition zone ranging between 19.2±3.8 mm in farm 1 and 21.6±2.2 mm in farm 4. At 20% dilution, *Thymus vulgaris* essential oils showed a strong antibacterial activity against Streptococcus with an inhibition zone ranging between 24.7±3.6mm in farm 1 and 27.6±1.9 mm in farm 3 and 4. At 30% dilution, the antibacterial activity of thymus essential oils against streptococcus in the 4 farms of the study is higher than the control Multiject antibiotic.

At 10% and 20% dilution rate, the antibacterial activity of lavender essential oils is generally moderate with zone of inhibition between 10 and 20 mm. At 30% dilution rate, lavender essential oils showed high antibacterial effect against streptococcus isolates with an inhibition zone ranging between 17.8±0.26 mm in farm 4 and 24.2±0.52 mm in farm 2. At different concentration rate, lavender essential oil has been less effective against Streptococcus isolates than thymus essential oil and the control antibiotic.

The mixture of lavender and thymus essential oil at different concentration showed a strong antibacterial activity against Streptococcus isolates. The zone of inhibition values in reaction to this mixture are higher than the control antibiotic. Active compounds present in the *Thymus vulgaris* and *Lavender* essential oils show the antibacterial activity against *Streptococcus* with the dose dependent manner. Table (5) shows the zone of

inhibition for the different concentration of the 2 extracts (Thymus and Lavender) against collected samples of Staphylococcus species in the 4 farms of the study.

Table 5: Zone of inhibition produced by different dilution of the essential oils against Staphylococcus isolates

E.O. Dilution	Zone of inhibition (mm)			
	Farm 1	Farm 2	Farm 3	Farm 4
Thymus solution (10%)	18±2.6 [a]	15.3±0.6 [a]	18±3.6 [a]	19.3±3.5 [a]
Thymus solution (20%)	22.3±2.5 [b]	20.3±1.5 [b]	23±1.7 [b]	20.7±1.2 [a]
Thymus solution (30%)	25±2 [c]	28.7±1.1 [c]	25.7±1.2 [b]	26.7±2.5 [b]
Lavender solution (10%)	13±1 [a]	14.3±1.5 [a]	15±1.7 [a]	13.7±2.5 [a]
Lavender solution (20%)	12.7±2.1 [a]	19±1 [b]	20.7±1.2 [b]	20±1.7 [b]
Lavender solution 30%)	19.7±2.1 [b]	25±2.6 [c]	29.3±2.6 [c]	28.3±1.5 [c]
Lavender +Thymus solution (10%)	29.5±1.3 [a]	28.6±1.4 [a]	27.4±3 [a]	23.6±5.9 [a]
Lavender +Thymus solution (20%)	30.5±1.2 [a]	29.5±1.5 [b]	29.5±0.96 [b]	27.5±3.7 [b]
Lavender +Thymus solution 30%)	34.5±2.3 [a]	31±1 [b]	30.1±3.3 [b]	30.9±2.3 [c]
Multiject antibiotics	25.7±3	27.2±1.3	25.3±2.3	26.4±5

a, b,c: within farm, between treatment, p<0.01

According to table (5), at 10% dilution rate, thymus essential oil showed a moderate antibacterial activity against Staphylococcus with an inhibition zone less than 20 mm. At 20% and 30% dilution rate, thymus essential oil showed a strong antibacterial activity against Staphylococcus isolates (inhibition zone>20 mm). However, the antibacterial activity of thymus essential oil against Staphylococcus species is less effective than the control antibiotic.

At 10% and 20% dilution rate, the antibacterial activity of lavender essential oils is moderate with an average inhibition values less than 20 mm. At 30% dilution rate, lavender essential oils showed a strong antibacterial activity against Staphylococcus isolates. This activity is less pronounced than the control antibiotic.

The different dilution of the mixture lavender and thymus showed a strong inhibition activity against Staphylococcus isolates. This combination seems more potent for the control off Staphylococcus than the control antibiotic.

In vivo antibacterial effect of the 2 essential oils against the Staphylococcus and Streptococcus isolates from the 4 farms:

Clinical experiments collected samples of microorganism from the four farms are shown in Tables 6, 7, 8 and 9.

Farm 1:

The results of colony count before and after once daily treatment of mastitis with 5 ml of 10% solutions of thymus essential oils by IMM injection are shown in Table (6). The results indicate that the extract had a strong effect against Streptococcus and Staphylococcus. At day 4 post IMM injection, collected milk samples showed a reduction of 71% and 75% of their Staphylococcus and Streptococcus colonies count.

Table 6: Staphylococcus and Streptococcus colony count (in 1mL*1000 of milk) 4 days after IMM of 5mL (10%) solution of Thymus

Pathogens	24 hours after				
	Before IMM	1st IMM	2nd IMM	3rd IMM	4th IMM
Staphylococcus	19.2±8.4 [a]	18.7±6.8 [a]	16.4±1.2 [b]	10.5±4 [c]	5.6±1.2 [d]
Streptococcus	37.3±5.64 [a]	34±9.1 [b]	29.4±3.2 [c]	17.8±3.7 [d]	9.45±3.1 [e]

a,b,c,d: between days of treatment, p<0.01

Farm 2:

The results of colony count before and after once daily treatment of mastitis with 5 ml of 10% solutions of lavender essential oils by IMM injection are shown in Table 7. The results indicate that lavender essential oils have moderate antibacterial activity in vitro against Streptococcus and Staphylococcus infection.

Table 7: Staphylococcus colony count (in 1ml*1000 of milk) after intramammary injection of 5 mL (10%) solution of Lavender

Farm	24 hours after				
	Before IMM	1st IMM	2nd IMM	3rd IMM	4th IMM
Staphylococcus	38.2±9.1 [b]	37.6±7.4 [a]	35.4±7.8 [a b]	32.1±2.4 [b]	28.7±5.6 [b]
Streptococcus	27.34±10.12 [a]	25.2±2.6 [a b]	23.4±3.7 [b c]	21.7±3.7 [c d]	18.7±5 [d]

a,b,c,d: between days of treatment, p<0.01

Farm 3:

The results of colony count before and after once daily treatment of mastitis with 5 ml of 10% mixture of lavender and thymus essential oils by IMM injection are shown in Table 8. The mixture thymus-lavender essential oils showed a strong in vivo antibacterial activity against the 2 pathogens of mastitis Staphylococcus and Streptococcus.

Table 8: Staphylococcus colony count (in 1 ml*1000) after intramammary injection of 5ml of a mixture (10%) Thymus and (10%) Lavender

Farm	24 hours after				
	Before IMM	1st IMM	2nd IMM	3rd IMM	4th IMM
3	51.5±11.9 [a]	45.6±8.3 [b]	33.4±7.6 [c]	14.7±2.7 [d]	5.4±2.3 [e]
3	45.87±8.98 [a]	38.3±3.9 [b]	27.4±8.4 [c]	13.7±3.8 [d]	7.9±2.1 [e]

a,b,c,d,e: Between days of treatment, p<0.01

Farm 4:

The results of colony count before and after once daily treatment of mastitis with 5 ml of 10% solution of thymus essential oils by external palpation are shown in Table 9. The external application of the thymus essential oils through palpation and massage of the udder seems very efficient for the control of subclinical mastitis and leads to complete recovery of the cows at day 4 post treatment.

Table 9: Staphylococcus and Streptococcus colony count (in 1 ml *1000 of milk) after external massage with Vaseline impregnated Thymus E.O

Farm	24 hours after				
	Before IMM	1st IMM	2nd IMM	3rd IMM	4th IMM
4	59±5.4 [a]	25±7.1 [b]	12.8±8.7 [c]	6.7±4.5 [d]	0±0 [e]
4	40±8.3 [a]	17.5±3.4 [b]	12.6±9.1 [c]	5.4±2.7 [d]	0±0 [e]

a,b,c,d,e: Between days of treatment, p<0.01

The external application of lavender essential oils by massages is also efficient for the control of mastitis and leads to drastic decrease in the number of Staphylococcus and Streptococcus colonies at day 4 post treatment (table 10). However, the antibacterial activity of lavender essential oil is less efficient than the thymus essential oils.

Table 10: Streptococcus and Staphylococcus colony count (in 1 mL*1000 of milk) after external massage with Vaseline impregnated Lavender E.O

Farm	24 hours after				
	Before IMM	1st IMM	2nd IMM	3rd IMM	4th IMM
4	65±8.3 [a]	27.9±3.3 [b]	12.6±4.7 [c]	5.4±1.4 [d]	2.1±0.5 [e]
4	50±8.3 [a]	27.8±3.1 [b]	12.6±5.4 [c]	5.3±1.7 [d]	4.5±1.2 [e]

a,b,c,d,e: Between days of treatment, p<0.01

Rate of recovery in the different farm of the study

The rate of recovery from mastitis following the application of different essential oils was determined through the help of California score test at day 4 post treatment. This recovery rate in the different farm is summarized in table (11).

Table 11: Recovery case in the different farm of the study

Farm	Treatment	Number of cases	NO of cases	percentage
1	IMM Thymus E.O.	5	2	40%
2	IMM Lavender E.O.	6	1	16.7%
3	IMM thymus +lavender E.O.	4	2	50%
4	Massage Thymus E.O.	4	4	100%
4	Massage Lavender E.O.	3	1	33%

Among all the combination used in this study, external application of thymus essential oils seems to be the most efficient treatment of subclinical mastitis leading to 100% recovery rate at day 4 post treatment. Despite, the high antibacterial potency of the mixture thymus lavender both in vitro and vivo, the rate of recovery at day 4 post treatment is still low (50%). Intramammary application of lavender essential oils is not efficient for the control of mastitis and the rate of recovery is very low 16.7%.

Discussion

Statistical analysis of the results showed that the different dilution of the 2 essential oils Thymus and Lavender had significant in vivo antimicrobial activity in the 4 farms of the study and this effect was dose-dependent where it was positively related to the applied concentration. These results are in agreements with the

results obtained by Sienkiewicz *et al.* (2011) who reported a strong antibacterial activity against many pathogenic bacteria in human beings such as Staphylococcus, Enterococcus, Escherichia and Pseudomonas. Lavender essential oils according to the same study have been less effective against the same pathogenic bacteria. The same results were also reported by Sokovic *et al.* (2007) who reported essential oils from *thymus vulgaris* have better inhibition zone (16-30 mm) than Streptomycin (20 mm) against some pathogenic bacteria in human beings such as *Staphylococcus aureus*, Staphylococcus epidermis and *Escherichia coli*. Lavender essential oils according to the same study have lower inhibition zone (6-22 mm) compared to Thymus essential oils and the control antibiotic (Streptomycin). The difference in the antibacterial activity of different essential oil extracts is mainly linked to their composition. For example, hydrocarbon monoterpenes show the lowest antibacterial activity, while oxygenated compounds possess a higher potential, especially phenol-type compounds as thymol and carvacrol. Sokovic *et al*. (2007) showed that oxygenated monoterpenes, exhibit strong antimicrobial activity, especially pronounced on whole cells, while hydrocarbon derivatives possess lower antimicrobial properties, as their low water solubility limits their diffusion through the medium. Therefore the high antibacterial activity of Thymus essential oils could be due to their high content of thymol and carvacrol. In fact, in a study done in Yemen, Almahtari *et al.* (2011) reported that thymus essential oils showed a high content of oxygenated monoterpenes (56.53%) and low contents of monoterpene hydrocarbons (28.69%), sesquiterpene hydrocarbons (5.04%) and oxygenated sesquiterpenes (1.84%). The predominant compound among the EO components was thymol (51.34%) while the amount of all other components of the oil was less than (19%). According to the same authors, thymus essential oils had stronger antibacterial activity against *Staphylococcus aureus* than ciprofloxacin. This latter one however possesses a better activity against E. coli.

The mixture of the 2 essential oils (thymus and lavender) showed a high antibacterial in vitro activity at the 3 dilutions level (10%, 20% and 30%) against the 2 bacterial isolates Streptococcus and Staphylococcus. This mixture showed a higher inhibition zone against the 2 bacterial pathogens than the control antibiotic.

In vivo, intramammary injection of 10% thymus essential oils has driven to reduction of 71 and 75% of Staphylococcus and Streptococcus colony count in milk 4 days post treatment. However, the percentage of recovery in cows is 40%. The same percentage of recovery was reported by Lefevre (2006) following IMM administration of 1.5% solution of thymus essential oils. The author reported the low rate of healing to the inhibitory effect of milk content mainly casein in the activity of the essential oils. In a study by Lefevre (2008), the effect of intramammary injection of a combination of the same 2 essential oils at a dilution of 10% for Thymus vulgaris and 5% for Rosmarinus officinalis, the number of cured cows remained low (33%). In vitro, this combination was able to eliminate the growth of Streptococcus species in a nutritive media. While in the presence of milk, the growth of Streptococcus species was only reduced. On the other hand, in vivo, intramammary injection of 10% solution of lavender essential oils has driven to reduction of 24.8% and 31.6% in the Staphylococcus and Streptococcus colony count in cow milk 4 days post treatment. In vitro and in vivo, lavender essential oils have shown less antibacterial activity than thymus essential oils. These results are in agreements with the results obtained by Sienkiewicz *et al.* (2011) and Sokovic *et al.* (2007).

Meanwhile, in vivo, intramammary injection of 10% mixture of thymus and lavender essential oils has driven to reduction of 89.5% and 82.7% in the Staphylococcus and Streptococcus colony count in cow milk 4 days post treatment. The rate of recovery recorded was 50%. This rate of recovery is higher than that obtained by Lefevre (2007) following intramammary administration of a mixture Thymus-Rosmarinus at 6% dilution. The rate of recovery recorded in this study was 22% after 4 injections and it rose to 56% after 6 injections. According to Rakhshandeh *et al.* (2011), the antibacterial activity of essential oils differs from samples to samples. The amount of active ingredients of the same plant can be affected by the area and the season of collection. According to the same authors in vivo effects of antimicrobial agents may be different from their in vitro effects, because of the effects of the immune system and the interference of some milk ingredients such as casein.

External application of 10% solution of thymus essential oils in vaseline has proven more efficient in the control of mastitis than the IMM administration with a reduction rate of 100% in the Staphylococcus and Streptococcus colony count in milk with a recovery rate of 100% 4 days post treatment. This rate of recovery is higher than that recorded by Boutin (2008) who reported a recovery rate of 68.6% in cows following extramammary application of a mixture of E.O. *Eucalyptus Citriodora*, E.O. *Thymus satureoides*, E.O. *Rosmarinus officinalis*. Meanwhile, external application by massage of 10% solution of lavender essential oils in Vaseline showed a strong antibacterial activity against the 2 strains of bacteria and the rate of reduction recorded was 96.7% and 91% for both Staphylococcus and Streptococcus colony count in milk.

Conclusion

In vitro, the 2 essential oils of Thymus and Lavender have shown strong antibacterial activity against the main strains of mastitis Staphylococcus and Streptococcus with potency similar to the control antibiotic. Intramammary application of 10% solution of Thymus and Lavender essential oils and of mixture thymus

lavender has driven to a drastic decrease in the bacterial colony count in the different sample of milk after 4 consecutive treatments.

External application or massage of a solution of thymus or lavender in Vaseline showed a strong antibacterial activity against the 2 bacterial pathogens Staphylococcus and Streptococcus. This activity is stronger than intramammary application with a rate of recovery of 100% with thymus essential oils.

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