The Assessment of the Antibacterial Activity of some Plant Extracts on Normal and Pathogenic Microflora from Milk

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Abstract
This paper presents the evaluation of antibacterial activity of some plant alcoholic extracts over bacteria in milk as an alternative to current antibiotics. Tests were conducted on alcoholic extracts from sage (*Salvia officinalis)*, two hop species *Brewers Gold* and *Perle* and two essential oils (rosemary - *Rosmarinus officinalis* and lavender - *Lavandula angustifolia).* The antibacterial susceptibility has been evaluated over 24 strains isolated from normal milk and mastitis milk, belonging to *Staphylococcus, Vibrio, Serratia, Aeromonas* and *Bacillus* genera. The obtained results show the best antibacterial effect with rosemary essential oil, having areas of inhibition as big as 28 mm, with an average of 16.4 mm. Weaker antimicrobial effect was obtained with alcoholic extract of sage where the average of inhibition diameters were 12.2 mm. Bacterial species isolated from mastitis were more sensitive to treatment with plant extracts compared with species isolated from normal milk. Among the tested bacteria, the strains from *Staphylococcus* type had the highest sensitivity, while *Vibrio* strains were more resistant. The results obtained open the prospect of using these herbal extracts as an alternative to the use of antibiotics in the treatment of mastitis and also aim at getting safer products from a microbiological point of view, with less antibiotic residues in milk.

Keywords: alcoholic extract, antimicrobial activity, bacteria in milk, essential oils, milk quality

1. Introduction

Milk is one of the most nutritious food products, with a chemical composition rich in carbohydrates, proteins, fats, vitamins and minerals [1]. Milk quality is influenced by a number of factors such as: water, air, food, soil, surfaces, equipment, udder, teats, feces, etc. [2,3]. Qualitative and quantitative losses of milk associated with mastitis in cows can be attributed to pathogenic effects or to an immune response produced by intramammary infection [1,4]. Thus, the bacteria in milk multiply and produce toxins that attack the tissues and other parts of the mammary glands secreting milk [4].

Mastitis directly affects milk quality by increasing the number of bacteria in raw milk and the inclusion of dead cells from infected udders [5]. While healthy udders usually release a low number of somatic cells, mastitis affected udders release 10^6 somatic cells/ml [6]. One way to control these intramammary infections is the administration of antibiotics.

Increasing concern about antibiotic residues in milk caused seeking alternatives to current antibiotics, to reduce or eliminate this problem, namely plant extracts therapy [7,8].

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Salvia officinalis L. is an aromatic plant that belongs to *Lamiaceae* family. The active principles are based on the volatile oils from 0.385 to 2.54% (monoterpenes, sequiterpene and their derivatives), flavones 1.66 to 1.92% and tannins 13.7%. Studies have shown that compounds from sage have strong antifungal and antibacterial activity [9], against gram-positive cocci and bacilli, like *Staphylococcus aureus* and *Bacillus subtilis*, against Gram-negative *Escherichia coli*, against *Candida albicans* and *Candida glabrata.*

Humulus lupulus L. (variety *Brewers Gold* and *Pearls*) belongs to the family *Cannabaceae* [10,11]. Inflorescences contain glandular structures (lupulin gland) which secrete a powder, lupulina, rich in secondary metabolites. Over 1,000 compounds have been identified in hop cones mature. They can be classified as volatile oils, polyphenols and bitter acids and have antibacterial properties, antifungal, antiviral and antiparasitic activities [12].

The two herbs *Rosmarinus officinalis L.* and *Lavandula angustifolia L.* belong to the family *Lamiaceae*. *Rosmarinus officinalis L.* essential oil possesses antioxidant and antimicrobial properties due to the presence of secondary metabolites namely phenolic diterpenes [13,14].

In *Lavandula angustifolia L.* essential oil 100 components were identified of which esters (ethyl linalool 17.6-53%), alcohols: linalool, α-terpineol, sesquiterpene, β-caryophyllene, monoterpenes, oxides. Due to the presence of esters and alcohols in lavender it exerts antifungal properties, antioxidant and antibacterial properties against certain strains of *Staphylococcus aureus* and *Enterococcus faecium* [15].

The study was conducted to investigate the *in vitro* effects of ethanolic extracts of plants: *Salvia officinalis* and *Humulus Lupulus* (variety *Brewers Gold* and *Pearls*) and essential oils of *Rosmarinus officinalis* and *Lavandula officinalis* on normal and pathogenic microflora of milk.

2. Materials and methods

2.1. Plant materials and bacterial strains:

Plant material. Sage, rosemary and lavender were obtained from Experimental fields of UASMV Cluj. Plants were harvested in their maximum amount of bioactive principles period, dried in the shade and in airy place. Dried cones of hops were given by Romanian Hop Production Association. Plant materials were grounded in fine powder and stored in paper bags until preparation.

The bacterial strains used in the experiment derived from both normal microflora, and the pathogenic milk. 12 species were isolated from normal milk: 2 strains of *Staphylococcus xylosus*, 2 strains of *Serratia liquefaciens*, 6 strains of *Vibrio fluvialis*, *Aeromonas hydrophila*, one strain of *Staphylococcus chromogenes* and 12 strains from mastitis milk: 4 strains of *Staphylococcus xylosus*, *Kytococcus sedentarius*, *Bacillus cereus*, *Staphylococcus hominis*, 4 strains of *Staphylococcus intermedius* and *Staphylococcus hyicus*.

2.2. Preparation of alcoholic extracts from plants

To obtain an alcoholic extract of sage 5 g of plant (aerial parts) were weighed and 100 ml ethanol 96^{o}C was added. The flask was kept for 14 days, protected from light at room temperature, stirring the content daily, after which the extracts were filtered through filter paper and contents was brought to 100 ml with ethanol [8].

Preparation of alcoholic extract of hops:

The process of making hops extract normally involves drying and powdering the hops cones, then adding the powder (50g) to solvent (500ml, ethanol 96^{o}C) then combination is sealed and continuously stirred on magnetic stirrer. After 4h, the extracts were centrifuged and filtered, collected and concentrated up to 10 ml using a vacuum evaporator at 40°C [16].

Preparation of essential oils:

Essential oils are obtained by steam distillation [17], from 50 g of plant inflorescence and 200 ml water.

2.3. California Mastitis Test

The test was performed for all milk samples and depending on the type of reaction, samples were classified with the following score: (0) = negative, (±) = trace (+) = weak positive (++) = positive (+++) = strongly positive (**table 1**).

Table 1. Interpretation of California mastitis test

Interpretation	Reaction
It does not form gel	(0) = negative
slight precipitation	(±) = trace
Gel formation	(+) = weak positive
Gel thicker, with central arrangement	(++) = positive
Gel thick in all the background	(+++)=strongly positive

The determination of colony forming units (CFU/ml)/ml milk was made using a culture medium with glucose and yeast extract (Standard Methods agar according to American Public Health Association and the Association of Official Analytical Chemists). From each positive sample at the California Mastitis Test, an amount of 1 ml of milk was dispersed on the surface of the medium following by incubation at 30^0C for 48 hours [18].

2.4. Determination of antibacterial activity

To determine the antimicrobial susceptibility of isolates from normal and pathogenic microflora, diffusion test was used for finding the minimum inhibitory concentration of plant extracts against microorganisms isolated, using Muller Hilton agar, poured on a perfectly flat surface so as to obtain an average thickness of 5 mm. Bacterial suspension was prepared by inoculating a quantity of bacterial strain grown on an agar plate with sheep blood, in 10 ml of saline until bacterial density coincides to 0.5 McFarland standard. On the Petri plates previously prepared, strains of interest were seeded by flooding technique with bacterial solutions prepared, and after sowing, the excess solution was removed. After the medium surface was dried, 8-10 microcomprimats with plant extracts were distributed around each plate. Incubation was performed at 37 ± 2^0C for 24 hours and the results were expressed in mm zone of inhibition [7, 19].

3. Results and discussion

After conducting the California mastitis test, 12 samples of milk were diagnosed with mastitis. After processing the milk samples in terms of determining the number of bacteria involved, results found were as follows (**table 2**):

Table 2. The test to detect mastitis and results obtained

Nr.of samples	California Mastitis test	Total number of germs (UFC/ml)
1	++	2189
2	++	1117
3	+	2326
4	++	3260
5	+++	7721
6	+	850
7	++	3560
8	+	1130
9	++	2325
10	+	1254
11	+	890
12	++	4577

All 12 milk samples were classified differently in California Mastitis Test, between weakly positive and strongly positive [18, 20]. There is a direct correlation between the intensity of reaction in California Mastitis test and the number of CFU/ml. After determining the sensitivity of bacterial strains to plant extracts, it is shown that there are differences between the antibacterial effect of extracts and bacterial sensitivity between species (**table 3**). The largest inhibition zones were obtained from rosemary essential oil, with an average of 16.38 mm. Zones of inhibition obtained in the treatment with essential oils are much higher compared to areas with alcoholic extracts. This shows that essential oils have a good capacity to extract the antimicrobial active principles from these plants [21].

Table 3. Results of the test for the determination of the susceptibility of bacterial plant extracts (mm, zone of inhibition)

Nr. crt.	Bacterial strains	Alcoholic Extract *Salvia officinalis*	Alcoholic Extract Hops *Perle*	Alcoholic Extract Hops *Brewers Gold*	Essential Oil *Lavandula angustifolia*	Essential Oil *Rosmarinus officinalis*	Negative control (alcohol 96°C)
	Bacterial strains from the normal microflora						
1.	*Staphylococcus xylosus*	6.0 17.0	19.5 8.0	26.0 6.0	18.5 9.0	25.0 17.0	7.5
2.	*Serratia liquefaciens*	11.0 15.0	9.0 20.0	7.0 18.0	12.0 6.0	22.0 9.0	7.5
3.	*Vibrio fluvialis*	10.0 10.0 16.0 23.0 19.0 9.0	7.0 7.0 7.0 7.0 0.0 0.0	6.0 8.0 6.0 6.0 6.0 8.0	7.0 8.0 9.0 6.0 7.0 7.0	7.0 8.0 11.0 12.0 12.0 22.0	6.9
4.	*Aeromonas hydrophila*	8.5	0.0	8.0	7.0	22.0	8.5
5.	*Staphylococcus chromogenes*	7.5	8.0	11.0	25.5	28.0	12
	Average, mm	12.67	7.71	9.67	10.17	16.25	8.48
	Standard Deviation	±5.23	±6.55	±6.18	±5.96	±7.29	±2.05
	Bacterial strains of pathogenic microflora						
6.	*Staphylococcus xylosus*	16.0 7.0 12.5 8.5	20.0 14.0 24.0 18.0	28.0 19.0 28.0 21.5	14.5 19.0 16.0 9.0	21.0 18.0 17.0 12.0	6.5
7.	*Kytococcus sedentarius*	17.0	14.5	18.0	21.5	25.0	8.0
8.	*Bacillus cereus*	15.0	20.0	24.0	11.0	8.5	8.5
9.	*Staphylococcus intermedius*	11.5 11.5 11.5 10.0	24.0 8.5 12.0 24.0	29.0 14.0 9.0 30.0	10.0 25.0 18.0 9.0	10.0 28.0 26.0 10.5	10.16
10.	*Staphylococcus hominis*	9.0	23.5	22.5	15.0	8.5	8.5
11.	*Staphylococcus hyicus*	10.0	21.0	27.0	14.0	13.5	8.0
	Average, mm	11.63	18.63	22.50	15.17	16.50	8.28
	Standard Deviation	±3.07	±5.27	±6.53	±5.06	±7.09	±1.18
	Average, mm	12.15	13.17	16.08	12.67	16.38	8.38
	Total Standard deviation	±4.23	±8.06	±9.04	±5.98	±7.03	±1.61

Both the alcoholic extracts and the essential oils, exert a greater antibacterial activity on Gram-positive and Gram-negative bacteria. The most sensitive Gram-positive bacteria are: *Staphylococcus chromogenes* tested from the normal microflora of milk (16 mm, zone of inhibition) and *Kytococcus sedentarius* tested from the pathogenic microflora of milk (19.2 mm, zone of inhibition). The most resistant gram negative bacterium is *Vibrio fluvialis* (9.03 mm, zone of inhibition). Comparison of antibacterial activity of plants extracts tested on Gram positive and Gram negative bacteria versus positive control (Amoxicillin AX 25) is reported in the figure 1.

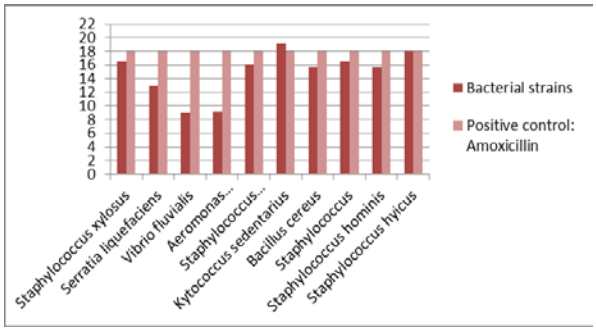

Figure 1. Graphic representation of studied bacterial strains compared to control positive (**Amoxicillin AX 25**).

The most susceptible bacterial strains used in this antimicrobial test on plant extracts are *Kytococcus sedentarius* and *Staphylococcus hycus*. Therefore bacterial strains taken into consideration in this study, show a sensitivity similar to other bacterial strains, in particular from *Staphylococcus* genus [22-25].

Rosmarinus officinalis essential oil analyzed on normal milk microorganisms shows the highest efficiency, with an average of 16.25 mm zone of inhibition, while the alcoholic extract of hops (*Perle*), have the weakest efficiency to these microorganisms, with an average of 7.71 mm inhibition zone.

In **Figure 2** is shown the *Rosmarinus officinalis* essential oil effect on *Staphylococcus intermedius*, with an inhibition diameter of 28 mm [7].

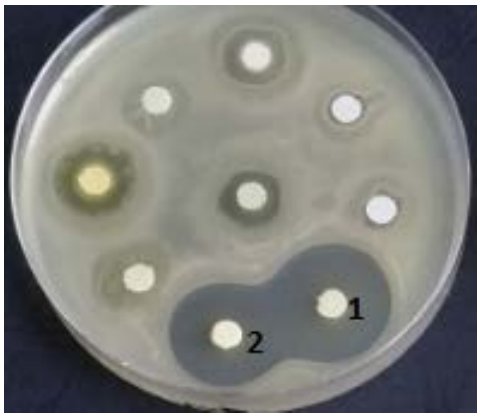

Figure 2. Areas of inhibition for essential oils 1. Essential oil *Rosmarinus officinalis*; 2. *Lavandula angustifolia* essential oil

From the analyzed alcoholic extracts it is shown that the extract of hops (*Brewers Gold*) present very statistically significant values (16.08 mm) compared to the negative control (8.38 mm), instead, *Salvia officinalis* ethanol extract is statistically insignificant (12.15 mm) compared to controls. Hops (*Brewers Gold*) tested on milk mastitis microorganisms shows a high antibacterial activity, with an average of 18.63 mm zone of inhibition versus alcoholic extract of *Salvia officinalis* (11.63 mm).

Table 4. Summary results of the inhibition zones on plant extracts

Alcoholic Extract / Essential Oil	Mean (mm)	Control positive(mm)	±d[1]	t[2]	Significance of difference
Salvia officinalis	12.67	18	5.33	2.5	**n.s.**[3]
Perle Hop	7.71	18	10.29	3.85	**xx**[4]
Brewers Gold Hop	9.67	18	8.33	3.3	**xx**
Lavandula angustifolia	10.17	18	7.83	3.22	**xx**
Rosmarinus officinalis	16.25	18	1.75	0.59	**n.s.**

[1]**d**=standard deviation; [2]**t**=`t` test; [3]**n.s.=**insignificant; [4]**xx**= significant distinct;

Using `t` Student test, it was shown that the significance of differences between means of *Rosmarinus officinalis* essential oil and *Salvia officinalis* (6 mg active substance) is not statistically significant for the value of` `t`and transgression probability p <0.1% compared to the positive control 25 AX amoxicillin (25 mg active substance), while the hop extracts and essential oil

of *Lavandula angustifolia* are distinctly significant ± d* (**Table 4**) [22-25].

The results obtained in the present study show that the tested plant extracts have antibacterial activity against *Staphylococcus ssp*., *Serratia liquefaciens*, *Vibrio fluvialis*, *Aeromonas hydrophila*, *Bacillus cereus* and *Kytococcus sedentarius* [26, 27].

The use of antibiotic substances over a long period of time resulted in larger doses of residues in milk, representing a potential biohazard. The results from this study show that the use of alcoholic extracts of herbs and essential oils is a real alternative in the treatment of mastitis in cows [25, 28-32].

4. Conclusions

The study demonstrates good antimicrobial activities of *Rosmarinus officinalis* essential oil and hops *Brewers Glod* similar to amoxicillin AX 25, strengthening the belief of using these products in the treatment of cow's mastitis with positive impact on reducing contaminants in milk and emergence of antibiotic resistance.

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