# Antibacterial Activity of *Rosmarinus officinalis* and *Lavandula angustifolia* Essential Oils Against Selected Poultry Pathogenic Bacteria

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**Abstract-** Poultry is the host of many species of bacteria and the intestine is the privileged place of their colonization and their persistence. These bacteria are the cause of several cases of food poisoning in humans through the consumption of eggs or soiled chicken meat. In addition, these bacteria develop resistance to antibiotics that are adjusted to poultry feed as growth promoters. Essential oils are considered as important secondary metabolites for plant defense by their antimicrobial and antioxidant properties. These essential oils may be considered as a source of natural antimicrobials for the conservation of poultry food. The aim of our study is to isolate and identify bacterial strains isolated from poultry and to determine the antibacterial and antioxidant activities of *Rosmarinus officinalis* and *Lavandula angustifolia* essential oils on these bacterial strains.Essential oils of Moroccan *Rosmarinus officinalis* and *Lavandula angustifolia* were extracted by hydrodistillation. The identification of their chemical composition are performed by gas chromatography-mass spectrometry. Antimicrobial activity of extracted essential oils against *Staphylococcus* aureus, *Clostridium perfringens*, *Escherichia coli* and *Salmonella entiritidis* was evaluated by aromatogram test and Microdilution in a liquid medium. The identification of strains are performed by several test: Gram staining, Kligler test, Catalase test, test Mobility and Api Gallery 20E. The results show that essential oils tested have a considerable antibacterial activity against all isolated bacterial strains.

**Keywords:** Poultry feed, Essential oils, Bacterial strains, Antibiotic resistance, Antimicrobial activity.

## Introduction

Poultry has been domesticated by humans, it represents a great stake in the food, economic and scientific fields. However, the consumption of eggs or contaminated poultry meat by humans leads to their intoxication[1]. Today, it is clearly established that pathogens isolated from the colonic flora of poultry are linked to gastrointestinal diseases. These are mainly aerobic sporulating Gram-negative or positive bacteria such as *Enterobacteriaceae, Staphylococcus aureus* and *Clostridium perfringens*[2].

In addition, these bacteria are pathogenic by their ability to infect humans and cause serious diseases such as Salmonella which causes salmonellosis[3]. The risk of Salmonella infection is an important factor to consider when preparing poultry.

Furthermore, several strains of Salmonella develop resistance to antibiotics added to poultry feed as growth promoters. This bacterial resistance constitutes a major problem leading researchers to the suppression of antibiotics and to propose new effective strategies of substitution of the latter[4].

Advances in scientific techniques have allowed the identification of components of volatile plant extracts (notably essential oils) that have multiple antimicrobial activities. Essential oils can therefore be a natural substitute for antibiotics[5].

Morocco, through its geographic and climatic diversity benefits from favorable conditions for the development of a rich and varied flora (often endemic) with significant potential in aromatic and medicinal plants (MAP). The exploitation of this rich and diverse floral source has made the country one of the main producing countries of essential oils, the quality of which is in great demand on the international market and more particularly in Europe and the United States of America. The main essential oils produced in Morocco come from the following plants: *Lavandula angustifolia*, *Rosmarinus officinalis* [6].

The medicinal and biological properties of these essential oils obtained from the plants mentioned above are promising[6]. Today, research continues for the antimicrobial activity of these essential oils by testing them on microorganisms that cause several diseases.

However, the widespread use and the benefits described in the literature of these essential oils by the Moroccan population in chicken farming still remain unsupported by scientific studies and hence the interest of our work.

The objective of this work is to test in vitro the antibacterial effect of essential oils obtained from the medicinal plants *Rosmarinus officinalis* and *Lavandula angustifolia*, on various virulent bacteria isolated from poultry.

## Material and Methods

### Isolation

The isolates comprising of *Staphylococcus aureus, Clostridium perfringens, Escherichia coli,* and *Salmonella entiritidis* were isolated from the stool of broiler chickens picked up from the Casablanca-Settat region (Morocco).

### Morphological and biochemical tests of isolates

Bacterial strains were identified to genus level on the basis of colony morphology (appearance, size, margin, shape, elevation), microscopic examination (Gram stain), physiological tests (Kligler test, mannitol mobility, EMB) and biochemical tests (catalase, oxidase,) using API 20E (bioMerieux), adopting standard procedures.

### Hydrodistillation by Clevenger

For the extraction of *Lavandula angustifolia* and *Rosmarinus officinalis* essential oils by hydro distillation under optimal operating conditions, a quantity of 100 g of rosemary and lavendulan was added to 1000 ml of distilled water in a 2-litre flask. The set was placed in a balloon heater attached to a refrigerator to ensure condensation of essential oils for 3 hours. At the end of the distillation, two phases were observed, an aqueous phase (aromatic water) and an organic phase (essential oil), less dense than water[7]. The essential oil was collected, dried under magnesium sulfate and stored in sealed vials in the dark at $4^0$C, until used [8]. Experiments were conducted twice for each condition.

### Chromatographic analysis of essential oils

The separation and identification of the different chemical compounds of the extracted essential oils were performed by gas chromatography coupled with mass spectrometer GCMS-QP2010 Plus.

**Evaluation of antibacterial activity**

**Aromatogram**

In this step 100 µl of each bacterial strain in nutrient broth corresponding to 106 UFC/ml were inoculated by flooding in Muller-Hinton agar. Then disks soaked with 10 µl of essential oil were deposed on the agar. After incubation at 37°C for 24 hours, zones of inhibition appeared around these disks[7].

**Microdilution test**

The determination of the minimum inhibitory concentration (MIC) of the oils is performed according to the protocol described by[9]. The minimum inhibitory concentration (MIC) of each essential oil was determined by the microdilution method on 96-well plates as described by[4].

50µl of Mueller-Hinton broth were added to each microplate well and different concentrations of EO dissolved in 10% DMSO were added to all wells. Then, 50µl of bacterial suspension corresponding to 106 CFU/ml were added to all the wells, then the microplates were incubated for 24h at 37°C. Microbial growth in each well was determined using the Resazurin technique. Resazurin working solution was prepared at a concentration of 0.01% (w/v) in distilled water and sterilized by filtration through a 0.45 mm membrane. 20µl of the Resazurin solution were added to each well, and the microplates were incubated for 2h at 37°C. The growth was indicated by change in colour from purple to pink color.

**Antibiogram**

Instead of discs soaked with essential oils, antibiotic discs were used. After incubation at 37°C for 24 hours, results were analyzed. The antibiotics used were penicillin P10, Chloramphenicol C30, Gentamycin GM, Tetracycline TE30, Amoxicillin AML, Sulfamides SSS, Ciprofloxacin CIP, Trimethoprim TMP.

## Results and Discussion

**Chemical composition of *L. angustifolia* and *R. officinalis* essential oils**

The chemical composition of the two essential oils; *L.angustifolia* and *R.officinalis*, by gas chromatography mass spectrometry (GC-MS) is presented in Table-1. The results identified 53 components that vary in percentage from one essential oil to another.

The essential oil of *L.angustifolia* has major monoterpenic compounds such as 1,8-cineol (61.76%) and α-pinene (17.35%), limonene (4.65%), p-cymene (2.47%) and aromadendrene (2.63%). Previous studies have shown that the chemical composition of the essential oil of *L.angustifolia* cultivated in many regions of the world presents a predominance of monoterpene compounds in most of the cases, but with different percentages[10] which is in agreement with our study. In addition, we observed that α-pinene (24.5%), camphor (22.8%) and 1,8-cineole (22.6%) were the major components of *R.officinalis* essential oil. The same results were obtained from analysis of *R.officinalis* essential oil from the region of Tlemcen - Algeria[11]. However, other studies from Morocco and Tunisia have shown a similar composition but with very high concentrations of α-pinene (37-40%), cineole(58.7-63%) and camphor (41.7-53.8%) and cineole (40.1-55.1%), known as monoterpenes[12,13].

The variations encountered in the chemical composition of essential oils, from quantitative and qualitative point of view, may be due to certain ecological factors, the part of the plant used, the age of the plant and the period of the vegetative cycle or even to genetic factors[14].

Table-1 Percentage compositions of two essential oil.

| Compounds | *L.angustifolia* | *R.officinalis* |
|---|---|---|
| (E)-α-Ocimene | 0.07 | 0 |
| (z)-α-Ocimene | 0.06 | 0 |
| 1,8 -Cineole | 61.76 | 22.6 |
| 3-Carene | 0 | 0.19 |
| 3-Methylbutanal | 0.11 | 0 |
| 3-Octanone | 0 | 0.67 |
| Alloaromadendrene | 0.51 | 0 |
| Aromadendrene | 2.63 | 0 |
| Borneol | 0.09 | 1.77 |
| Bornylacetate | 0 | 1.34 |
| $C_{15}H_{26}O$ | 0.12 | 0 |
| Camphene | 0.06 | 7.8 |
| Camphor | 0.27 | 22.8 |
| cis-p-Menth -1(7),8 -dien -2-ol | 0.1 | 0 |
| Epi-Globulol | 0.09 | 0 |
| Geraniol/p -cymen -8-ol | 0.18 | 0 |
| Gerany lacetate | 0.19 | 0 |
| Globulol | 0.36 | 0 |
| Isoamyl isobutyrate | 0.07 | 0 |
| Isoborneol | 0 | 0.06 |
| Isoborny lacetate | 0 | 0.04 |
| Limonene | 4.65 | 4.7 |
| Linalool | 0.07 | 0.47 |

| | | |
|---|---|---|
| Linaly lacetate | 0 | 0.02 |
| Myrcene | 0.33 | 1.71 |
| p-Cymene | 2.47 | 2.62 |
| p-Cymenene | 0.07 | 0 |
| Pinocarvone | 0.18 | 0 |
| Sabinene | 0 | 0.01 |
| Terpinen -4-ol | 0.48 | 1.01 |
| Terpinolene | 0.09 | 0.01 |
| trans -Carveol | 0.05 | 0 |
| trans -Pinacarvol | 1 | 0 |
| trans -p-Menth -1(7),8 -dien -2-ol | 0.17 | 0 |
| Tricyclene | 0 | 0.34 |
| Verbenene | 0.3 | 0 |
| α-Copaene | 0.03 | 0 |
| α-Fenchene | 0.02 | 0 |
| α-Gurjuine | 0.26 | 0 |
| α-Humulene | 0 | 0.02 |
| α-Phellandrene | 0.24 | 0.01 |
| α-Pinene | 17.35 | 24.5 |
| α-Terpinene | 0.04 | 0.01 |
| α-Terpineol | 1.04 | 0 |
| α-terpineol | 0 | 1.93 |
| α-Terpinylacetate | 1.36 | 0 |
| α -Caryophyllene | 0.07 | 0 |

| | | |
|---|---|---|
| α-Eudesmol | 0.05 | 0 |
| β-Guiene | 0.14 | 0 |
| β-Phellandrene | 0.13 | 0 |
| β-Pinene | 0.53 | 2.96 |
| γ-Terpinene | 0.59 | 0.01 |
| δ-Elemene | 0.05 | 0 |

**Evaluation of antibacterial activity**

In disk diffusion assay, the antibacterial activity of essential oils has shown a potential inhibition zones. This last one differs from bacterium to another and from essential oil to another. The results of the experiments on the antibacterial effect of essential oils are presented in (Table-2 and 3). Based on the antibiotic resistance profiles, we determined a range of antibiotics in this study, most isolates were clearly multi drug resistant.

Screening of the antibacterial properties of the two essential oils revealed that these oils had antimicrobial activity against all the strains tested with a slight difference in sensitivity between gram positive and gram negative (Table-3 and 4). In our study the *L.angustifolia* essential oil showed an effect on all bacteria except *Clostridium perfringens*. The diameters of inhibition were between 16 and 30mm. On the other hand, the *R.officinalis* essential oil seem to have less effect on the tested bacterial strains compared to *L.angustifolia* essential oil. *Salmonella entiritidis* seems to be the most sensitive to this essential oil compared to *E. coli,* although they are Gram negative. As reported, this important bioactivity of these essential oils is due to their richness in phenolic derivatives (1,8-cineol, alpha-pinene, camphor)[10,15,16]. It is also reported that the different components of essential oils show different degrees of activity against gram-negative and gram-positive bacteria and this is largely due to the percentage of the phenolic components of essential oils[17]. Other studies revealed no selective antimicrobial activity against gram-positive or gram-negative bacteria [18].

The MIC of essential oils is in agreement with the results obtained in the aromatogram, i.e. the larger the diameter around the disc, the more interesting the MIC. Generally, the MIC values obtained from two essential oil for the strains studied vary between 0.26 and 4.61%. These results correspond to the results obtained by other [11].

Table-2 Resistance of studied strains to antibacterial agents

| Strains | Resistance profile |
|---|---|
| *Staphylococcus aureus* | P,C,SSS,GM,TMP,AML,TE,CIP |
| *Clostridium perfringens* | P ,C,SSS,GM,TMP,AML,TE,CIP |
| *Escherichia coli* | P, SSS, TMP, AML, TE, CIP |
| *Salmonella enteritidis* | AML, P, SSS, TMP |

AML : *Amoxicillin 25µg; TE : Tétracycline 30µg ; P : Pénicilline 6 µg ; G : Gentamicine 15 µg ; C : Chloramphénicol 30 µg ; CIP : Ciprofloxacine 5 µg ; SSS : Sulfamide 200 µg ; TMP : Triméthoprime 5µg.*

Table-3 Antibacterial activity (inhibition zone measured in mm) of essential oils against selected strains of bacteria

| Strains | *R.officinalis* | *L.angustifolia* | *Mean* ± SD |
|---|---|---|---|
| *Clostridium perfringens* | - | - | |
| *Escherichia coli* | 18± 0.05[a] | 20 ± 0.0[a] | |
| *Staphylococcus aureus* | - | 20 ±1.0[c] | |
| *Salmonella entiritidis* | 30 ± 2.26[a] | 33± 0.3[b] | |

In the same column with different superscript letters differ significantly (P < 0.05).
Diameter of inhibition zone (mm) including disc diameter of 6 mm.

Table-4 Minimum inhibitory concentration (MIC; %) and Minimum bactericidal concentration (MBC; %) of two essential oils against selected strains of bacteria

| **Microorganisms** | ***L.angustifolia*** | | ***R.officinalis*** | |
|---|---|---|---|---|
| | MIC | MBC | MIC | MBC |
| *Staphylococcus aureus* | 0.52 | 2.08 | 0.52 | - |
| *Clostridium perfringens* | 4.61 | 8.33 | - | - |
| *Escherichia coli* | 0.52 | 4.61 | 8.33 | - |
| *Salmonella entiritidis* | 1.04 | 4.61 | 0.52 | 8.33 |

- : not detected.

The ratio MBC/MIC allows to define the bacteriostatic or bactericidal character of an essential oil. When this ratio is less than 4, the essential oil is considered bactericidal [19]. In our study, the MBC/MIC ratios of the different essential oils tested are between 1 and 4 for all the bacterial strains studied. Thus, the essential oil (*L.angustifolia*) seems to have on the one hand a bactericidal activity on *Salmonella entiritidis, Escherichia coli* and *Staphy lococcus aureus* and on the other hand a bacteriostatic activity on *Clostridium perfringens*. It is reported that the antibacterial activity of essential oil is due to phenolic components. However, the compounds present in the highest proportions are not necessarily responsible for the total activity; the involvement of less abundant constituents should also be considered [21] and therefore the activity comes back due to the presence of components such as p-cymene, 1,8-Cineole, linalool, borneol[22,23,24,25]. In addition, the particular structure of the external membrane of this genus, particularly impermeable to hydrophobic molecules, combined with the mechanisms of proton efflux reducing the pH gradient across the cytoplasmic membrane, ultimately leading to cell death [20].

## Conclusion

Essential oils are aromatic substances with a complex chemical composition which give them very interesting antibacterial properties. In the present study, we have shown that the essential oils of *Rosmarinus officinalis* and *Lavandula angustifolia* have an effective antibacterial activity against *Staphylococcus aureus, Clostridium perfringens, Escherichia coli* and *Salmonella entiritidis* isolated from poultry in Morocco. This effectiveness is due to high presence of monoterpenoids, since the analysis by gas chromatography coupled with mass spectrometer showed the presence of 1,8-Cineole (61,76) and α-Pinene (17,35), as major components of *Lavandula* essential oil, and 1,8-Cineole (22.6), Camphor (22.8) and α-Pinene (24,5) in essential oil of *Rosmarinus*. These components are responsible for attacking the bacterial wall which mainly destabilizes the cell architecture leading to the degradation of the integrity of the membrane and increased permeability, this disrupts many cellular activities. The studies described here establish the utility of the tested essential oils to open new horizons towards their use as new alternative strategies to the antibiotics used in poultry food. Further exploration are needed to understand the mode of action of these essential oils to inhibit or kill bacteria.

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