# Study of the antimicrobial activity and synergistic effect of some plant extracts and essential oils

## Studiul activităţii antimicrobiene şi efectul sinergistic al unor extracte de plante şi uleiuri esenţiale

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**Abstract**

*Known for their antiseptic and medicinal properties and their fragrance, essential oils are used in the preservation of foods, and as antimicrobial, analgesic, sedative, anti-inflammatory, spasmolytic and locally anesthetic remedies. In the present study the antimicrobial effect of the water extract, the tincture and the essential oil of Foeniculum vulgare Mill., Thymus vulgaris L. and Salvia officinalis L. was assessed in the case of some bacteria important from the sanitary point of view: Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 25923, Listeria monocytogenes, Bacillus subtilis and Candida albicans CBS 562. At the same time, in the case of some volatile oil combinations we studied the phenomena of synergism and antagonism. Of the examined microorganisms, the most resistant appeared to be Escherichia coli ATCC 25922, while Bacillus subtilis was the most sensitive. Of the chosen plant species the most pronounced antimicrobial effect was detected in the case of Thymus vulgaris. When associating the essential oils of different plant species, the synergism was the most obvious in the case of the association of Salvia officinalis and Foeniculum vulgare.*

***Keywords:*** *essential oils, antimicrobial effects, minimum inhibitory concentration, synergism*

**Rezumat**

*Cunoscând proprietăţile antiseptice şi medicinale, precum şi aroma lor, uleiurile volatile sunt utilizate în conservarea alimentelor, importanţă majoră prezintă mai ales prin acţiunea lor antimicrobiană, analgezică, sedativă, antiinflamatorie, spasmolitică şi în remedieri de anestezie locală. În acest studiu am determinat efectul antimicrobian a extractului apos, a tincturii şi a uleiului volatil de Thymus vulgaris L., Salvia officinalis şi Foeniculum vulgare Mill. în cazul unor bacterii importante din punct de vedere sanitar: Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 25923, Listeria monocytogenes, Bacillus subtilis şi faţă de Candida albicans CBS 562. Totodată am urmărit fenomenul de sinergism şi antagonism prin utilizarea unor combinaţii de ulei volatil. Dintre microorganismele studiate cel mai rezistent a fost tulpina de Escherichia coli ATCC 25922, iar cel*

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*mai sensibil Bacillus subtilis. Dintre speciile de plante alese, efect antimicrobian cel mai pronunțat am determinat la Thymus vulgaris. Prin asocierea uleiurilor volatile, sinergism se contată în primul rând în cazul combinației Salvia officinalis şi Foeniculum vulgare.*

***Cuvinte cheie:*** *uleiuri esentiale, efecte antimicrobiene, concentrație inhibitorie minimă, sinergism*

## Introduction

The antimicrobial activity of plant oils and extracts has formed the basis of many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies (1). The cytotoxic capacity of the essential oils based on pro-oxidant activity can render them excellent antiseptic and antimicrobial agents for personal use, i.e. for purifying air, personal hygiene or even internal use via oral consumption. A big advantage of essential oils is the fact that they are usually devoid of long-term genotoxic risks. Some of them show a very clear antimutagenic capacity which could well be linked to an anticarcinogenic activity (2).

Illnesses caused by the consumption of food contaminated by pathogens have a worldwide importance from the point of view of public health. Plant essential oils are gaining interest for their potential as preservative ingredients or decontaminating treatments. Essential oils and their constituents are extensively used as flavor ingredients in a wide variety of foods, beverages and confectionery products, their application in controlling pathogens could reduce the risk of food borne outbreaks and assure consumers safe food products. Certain plants and extracts used as flavoring agents are known to possess antimicrobial activity offering a potential alternative to synthetic preservatives (3).

The biological activity of essential oils depends on their chemical composition which is determined by genotype and influenced by environmental and agronomic conditions (4). Generally, environmental conditions influence the nature of plant chemical composition, which has an important role in plant adaptation and speciation (5).

Generally, the essential oils possessing the strongest antibacterial properties against food borne pathogens contain a high percentage of phenolic compounds such as carvacrol, eugenol and thymol. Carvacrol and thymol are able to disintegrate the outer membrane of Gram-negative bacteria, releasing lipopolysaccharides and increasing the permeability of the cytoplasmic membrane to ATP. Besides the inhibition of the growth of the vegetative bacterial cells, the inhibition of toxin production is also of interest to food microbiologists (6).

In many cases the combinations of volatile oils are used in the study of the antimicrobial effect in order to determine synergism and to increase efficacy (7). Many phytomedicines exert their beneficial effects through the additive or synergistic action of several chemical compounds acting at single or multiple target sites (8).

Plant essential oils have been shown to be active against *Aeromonas hydrophila, Listeria monocytogenes, Clostridium botulinum, Enterococcus faecalis, Staphylococcus spp., Micrococcus spp., Bacillus spp., Enterobacteriaceae, Campylobacter jejuni, Vibrio parahaemolyticus, Pseudomonas fluorescens, Bacillus cereus, Shigella spp., Salmonella enterica* Typhimurium and Enteritidis, and *Escherichia coli* as well as yeasts and moulds (9). The extent of the sensitivity of a test organism varies with the strain studied, the environmental conditions imposed, and the reaction to the Gram stain (10).

The most interesting area of application for plant extracts and essential oils is the inhibition of growth and reduction in numbers of the more serious food borne pathogens such as *Salmonella spp., Escherichia coli* 0157:H7 and *Listeria monocytogenes* (3). Great importance can be attributed to those vegetal essential oils that possess antifungal and mycotoxin production blocking effect, too (11). The thyme essential oil may be recommended for large sale application as a plant-based preservative for stored food items because of its strong antifungal as well as anti-aflatoxigenic efficacy (12).

The aim of this study was to determine the efficacy of water extracts, tinctures and respectively essential oils obtained from some medicinal herbs as antimicrobials against some sanitarily important bacteria and against *Candida albicans*.

## Material and methods

In the present study three plant species were chosen with the aim to examine the antimicrobial effect: *Thymus vulgaris* L., *Salvia officinalis* L. and *Foeniculum vulgare* Mill. The dry vegetal material, tinctures and essential oils were acquired from the market. Different dilutions were prepared from the essential oils using ethanol with a concentration of 96% until no antimicrobial effect could be detected.

The main component of the essential oil of *Thymus vulgaris* is thymol, which amounts to 20-60% of its composition. The second principal component, with a proportion of 15% is carvacrol. The principal components of the essential oil of *Salvia officinalis* L are thujone 30-50%, borneol 6-15%, cineol 1-15%, camphor 6-1% and pinen 1-2%. The principal active substances found in the oil of *Foeniculum vulgare* are anethol (50-80%) and fenchone.

Water extracts were prepared from the dry vegetal material. In an Erlenmeyer flask 10 g of vegetal material was introduced and 100 ml hot distilled water was poured. The flask, plugged with a sterile cork, was put away for 15 minutes. After cooling, the infusion was filtered and used for microbiological studies.

The antimicrobial effects of the vegetal extracts were examined in the case of the following microorganisms: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes*, *Bacillus subtilis* and *Candida albicans* CBS 562. 24 hour-old microorganism cultures were used. They were obtained on Nutrient agar medium (meat extract 1 g, yeast extract 2 g, peptone 5 g, NaCl 5 g, agar 15 g, distilled water 1000 ml, pH=7,4±0,2), of which suspensions were made in 0,9% physiological NaCl solution.

The sensitivity of the microorganisms towards antimicrobial substances is tested „in vitro", under optimum and standardized cultivation conditions (culture medium, inoculum, incubation period), in the presence of decreasing quantities of antimicrobial substances.

In the case of the agar diffusion method, in the sterilized Petri dish 20 ml Nutrient agar medium is poured. After solidification the surface of the medium is inoculated with 0.1 ml suspension of microorganisms (106 CFU/ml). In the center of all of the inoculated mediums a 10 mm diameter hole is cut with the help of a sterile test-tube. 0.15 ml of vegetal extract or essential oil is dropped in this hole. The incubation takes place at the temperature of 37 ºC, for 24-48 hours. The dimensions of the inhibitory area are measured (7).

In the case of the essential oils the phenomena of synergism and antagonism can be determined within the agar diffusion method. In the holes made in the inoculated mediums, essential oils (0.75 ml) are added, obtained from two studied species of medicinal plants. After incubation the results are read and formulated in accordance with the size of the inhibition zone.

The disc diffusion method is similar to the agar diffusion method. By placing discs impregnated with antimicrobial solutions on the surface of the solid medium inoculated with a certain culture of microorganisms, the active antimicrobial substance will diffuse in the medium, presenting a constant decrease of concentration from the edges of the discs to the margins of the medium. 20 ml Nutrient agar medium is poured in sterile Petri dishes. After solidification the surface of the solid medium is inoculated with 0.1 ml from the microbial suspension (106 CFU/ml) studied. Discs of filter paper (with a diameter of 10 mm)

**Table 1. Effect of the *Thymus vulgaris* tincture considering the chosen microorganisms, well diameter 10 mm**

| Studied microorganisms | *Staphylococcus aureus* ATCC 25923 | *Listeria monocytogenes* | *Bacillus subtilis* | *Escherichia coli* ATCC 25922 | *Candida albicans* CBS 562 |
|---|---|---|---|---|---|
| Dimension of inhibition zone in mm (average ± S.D., n=10) | 15.8±1.54 | 13.2±1.48 | 13.5±1.65 | 11± 0.67 | 15±2.40 |

**Table 2. Effect of the *Salvia officinalis* tincture considering the chosen microorganisms, well diameter 10 mm**

| Studied microorganisms | *Staphylococcus aureus* ATCC 25923 | *Listeria monocytogenes* | *Bacillus subtilis* | *Escherichia coli* ATCC 25922 | *Candida albicans* CBS 562 |
|---|---|---|---|---|---|
| Dimension of inhibition zone in mm (average ± S.D., n=10) | 12.2±0.42 | 20.6±1.17 | 12.4±0.52 | No inhibition | No inhibition |

impregnated with the studied vegetal extracts are placed on the surface of the inoculated medium. The discs are prepared in advance by sterilization. Impregnation and drying are performed under sterile conditions. The incubation is carried out for 24-48 hours at the temperature of 37 ºC. Only those plates that present adequate cultures concerning purity and density are taken in study. Assessment is made by measuring the diameter of the inhibition zone in milimeters (4, 13-15).

## Results

### *1. Study results of the antimicrobial effect obtained after the application of the agar diffusion method*

Water extracts prepared from *Salvia officinalis* and *Foeniculum vulgare* do not present antimicrobial effect on the studied microorganisms: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes*, *Bacillus subtilis* and *Candida albicans* CBS 562. In the case of water extracts of Thymus vulgaris bacteriostatic effect can be observed only in *Bacillus subtilis* with inhibition zone sizes of 14.6 ± 0.7 mm (average ± S.D., n=10). The bacteriostatic effect does not appear in other microorganisms.

The tincture of *Thymus vulgaris* has antimicrobial effect in all of the microorganisms that were studied. Concerning the inhibition zone size, on the basis of the obtained data, the most resistant strain is *Escherichia coli* ATCC 25922, respectively *Staphylococcus aureus* ATCC 25923 and *Candida albicans* CBS 562 are the most sensitive microorganisms (*Table 1*). The tincture of *Foeniculum vulgare* has bacteriostatic effect only in the case of *Bacillus subtilis*, with an inhibition zone size of 12.8 ± 1.03 mm (average ± S.D., n=10). In the case of *Salvia officinalis* tincture bacteriostatic effect can be observed in *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* and *Listeria monocytogenes*, *L. monocytogenes* being the most sensitive. The strains of *Escherichia coli* ATCC 25922 and *Candida albicans* CBS 562 are resistant against the tincture of *Salvia officinalis (Table 2).*

The ethanol (96%) used as solvent at the preparation of the dilution of essential oils does not have any antimicrobial effect on the studied microorganisms. By using only alcohol during testing the antimicrobial effect, inhibition zones did not appear.

The essential oil of *Thymus vulgaris* has a pronounced antimicrobial effect upon the studied microorganisms (*Table 3*). An inhibition zone can still be detected in the case of *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 at 0.1% essential oil concentration,

**Table 3. The effect of the essential oil of *Thymus vulgaris* at the studied microorganisms, well diameter 10 mm**

| Concentration of the volatile oil (%) | Inhibition zone in mm (average± S.D., n=10) | | | | |
|---|---|---|---|---|---|
| | ***Staphylococcus aureus* ATCC 25923** | ***Listeria monocytogenes*** | ***Bacillus subtilis*** | ***Escherichia coli* ATCC 25922** | ***Candida albicans* CBS 562** |
| 100 | 31.8±1.75 | total inhibition | total inhibition | 24.6±1.87 | total inhibition |
| 90 | 31.4±2.80 | 24.1±4.04 | total inhibition | 19.4±0.84 | total inhibition |
| 80 | 31.2±1.62 | 22.6±3.27 | 33.9±2.73 | 19.3±3.40 | total inhibition |
| 70 | 25.9±2.77 | 24.2±3.19 | 35.6±2.27 | 19.5±2.12 | total inhibition |
| 60 | 26.2±2.62 | 24.8±2.90 | 34.1±3.00 | 19.4±1.43 | total inhibition |
| 50 | 23.8±3.05 | 20.8±0.79 | 30.7±1.64 | 19.0±1.15 | total inhibition |
| 40 | 24.3±0.95 | 20.6±0.70 | 29.5±2.07 | 19.5±2.46 | total inhibition |
| 30 | 22.9±0.57 | 20.3±0.95 | 28.0±2.98 | 17.4±0.84 | 33.2±1.75 |
| 20 | 19.4±1.51 | 19.3±0.82 | 25.8±1.03 | 16.3±1.16 | 31.8±1.48 |
| 10 | 17.4±0.97 | 17.9±1.10 | 24.5±0.85 | 16.2±0.42 | 29.9±0.88 |
| 5 | 16.9±1.20 | 16.2±0.92 | 23.5±1.08 | 16.0±0.47 | 24.0±1.15 |
| 1 | 14.5±0.85 | 13.1±0.88 | 18.5±0.71 | 13.4±0.70 | 16.8±1.48 |
| 0.5 | 12.4±0.70 | 12.9±0.57 | 15.1±0.57 | 13.0±0.47 | 16.0±0.67 |
| 0.3 | 12.3±0.46 | 12.8±0.63 | 14.6±0.52 | 12.4±0.52 | 15.4±0.52 |
| 0.1 | 11.3±0.49 | 12.2±0.42 | 12.5±0.53 | 11.4±0.52 | 14.2±0.63 |

**Table 4. The effect of the essential oil of *Salvia officinalis* at the studied microorganisms, well diameter 10 mm**

| Concentration of the volatile oil (%) | Inhibition zone in mm (average± S.D., n=10) | | | | |
|---|---|---|---|---|---|
| | ***Staphylococcus aureus* ATCC 25923** | ***Listeria monocytogenes*** | ***Bacillus subtilis*** | ***Escherichia coli* ATCC 25922** | ***Candida albicans* CBS 562** |
| 100 | 12.7±0.48 | 19.7±0.48 | 20.5±0.85 | 13.3±0.48 | 15.2±0.63 |
| 90 | 12.2±0.42 | 19.1±0.57 | 20.8±0.79 | 13.2±0.42 | 15.1±0.57 |
| 80 | 12.1±0.32 | 15.7±0.67 | 17.4±1.26 | 13.1±0.57 | 15±0.47 |
| 70 | 11.5±0.53 | 15.4±0.70 | 16.6±0.70 | 12.9±0.57 | 15.1±0.32 |
| 60 | 11.4±0.53 | 15.2±0.42 | 15.7±0.95 | 11.9±0.32 | 14.7±0.48 |
| 50 | 11.2±0.42 | 15.1±0.32 | 14.9±0.57 | 11.8±0.42 | 13.5±0.71 |
| 40 | 10.97±0.32 | 14.4±0.52 | 14.3±0.48 | 11.4±0.52 | 13.2±0.42 |
| 30 | 11 | 13.5±0.53 | 12.6±0.70 | 11.3±0.48 | 11.8±0.79 |
| 20 | 11 | 12.6±0.52 | 11.4±0.53 | No inhibition | No inhibition |
| 10 | NT* | 12.3±0.48 | 11 | NT | NT |

while in *Listeria monocytogenes* and *Bacillus subtilis* at 0.05%. *Candida albicans* CBS 562 is the most sensitive towards the essential oil of *Thymus vulgaris*, as the inhibitory effect can still be observed at 0.01% essential oil concentration.

The essential oil of *Salvia officinalis* presents a stronger bacteriostatic effect in *Listeria monocytogenes* and *Bacillus subtilis (Table 4)*. *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 are more resistant to-

**Table 5. The effect of the essential oil of *Foeniculum vulgare* at the studied microorganisms, well diameter 10 mm**

| Concentration of the volatile oil (%) | Inhibition zone in mm (average± S.D., n=10) | | | | |
|---|---|---|---|---|---|
| | ***Staphylococcus aureus* ATCC 25923** | ***Listeria monocytogenes*** | ***Bacillus subtilis*** | ***Escherichia coli* ATCC 25922** | ***Candida albicans* CBS 562** |
| 100 | No inhibition | 13.1±0.57 | 13.2±0.42 | No inhibition | 14.2±0.63 |
| 90 | No inhibition | 12.4±0.52 | 12.8±0.42 | No inhibition | 13.7±0.48 |
| 80 | No inhibition | 11.5±0.53 | 12.6±0.70 | No inhibition | 13.4±0.52 |
| 70 | No inhibition | 11.3±0.48 | 12.3±0.48 | No inhibition | 12.4±0.52 |
| 60 | No inhibition | No inhibition | 12.2±0.42 | No inhibition | No inhibition |
| 50 | NT* | NT | 11.8±0.42 | NT | NT |
| 40 | NT | NT | 11.3±0.48 | NT | NT |

** NT = not tested*

**Table 6. Intensity of the antimicrobial effect in the case of essential oil combinations, well diameter 10 mm**

| Studied microorganisms | Inhibition zone in mm (average± S.D., n=10) | | |
|---|---|---|---|
| | ***Salvia officinalis Thymus vulgaris*** | ***Salvia officinalis Foeniculum vulgare*** | ***Thymus vulgaris*** *Foeniculum vulgare* |
| *Staphylococcus aureus* ATCC 25923 | 13.7±0.67 | 11.4±0.52 | 14.3±1.42 |
| *Listeria monocytogenes* | 26.4±4.97 | 13.9±1.10 | 22.6±1.96 |
| *Bacillus subtilis* | 21.3±0.94 | 15.6±0.52 | 19.8±1.14 |
| *Escherichia coli* ATCC 25922 | 13.0±0.67 | 11.6±0.52 | 11.8±0.63 |
| *Candida albicans* CBS 562 | 23.9±1.66 | 15.1±0.32 | 24.6±3.72 |

wards the effects of this essential oil. The lowest applied essential oil concentrations with perceptible antimicrobial effect in the case of *Escherichia coli* ATCC 25922 and *Candida albicans* CBS 562 is 25%, in *Staphylococcus aureus* ATCC 25923 20%, in *Bacillus subtilis* 10% and in *Listeria monocytogenes* 5%.

The essential oil of *Foeniculum vulgare* does not have any bacteriostatic effect on *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 *(Table 5)*. The most sensitive bacteria appeared to be *Bacillus subtilis*, but the size of the inhibition zone is relatively small. The lowest essential oil concentration where an inhibitory zone still develops is 70% in the case of *Listeria monocytogenes* and 65% in the case of *Candida albicans* CBS 562.

In determining synergism and antagonism, combinations of 50% solutions of the chosen essential oils were used. Antagonism is observed when the effect of both compounds is lower when applied together than when applied individually. Synergism is observed when the effect of the combined substances is greater than the sum of the individual effects. On the basis of the results obtained in two of the three essential oil combinations (*Salvia officinalis-Thymus vulgaris*, *Thymus vulgaris-Foeniculum vulgare*) the phenomenon of synergism can be observed in the case of *Listeria monocytogenes (Table 6)*. At *Staphylococcus aureus* ATCC 25973, *Bacillus subtilis* and *Candida albicans* CBS 562 only in the case of the combination of *Salvia officinalis-Foeniculum vulgare* is there synergism, in the case of the other two combinations (*Salvia officinalis - Thymus vulgaris*, *Thymus vulgaris - Foeniculum vulgare*) antagonism can be demonstrated. In the case of the *Escherichia coli* ATCC 25922 strain only antagonism can be observed in all of the essential oil combinations.

***2. Study results of the antimicrobial effect obtained by application of the disc diffusion method***

There are no significant differences between the results of the disc diffusion and agar diffusion methods, with these two methods being similar. As an example, in neither of the methods does the essential oil of *Foeniculum vulgare* present an inhibition zone in *Escherichia coli* ATCC25922, while in *Bacillus subtilis* total inhibition can be observed under the action of the essential oil of *Thymus vulgaris*. Under the action of the essential oil of *Salvia officinalis* the size of the inhibition zone (mm) in the case of the agar diffusion method is 12.7±0.48 mm (average ± S.D., n=10), and in the disc diffusion method 12.5±0.71 mm (media ± S.D., n=10, the disc diameter was 10 mm).

## Discussions and conclusion

Of the studied microorganisms the *Escherichia coli* ATCC 25922 strain was the most resistant towards the effects of vegetal substances and *Bacillus subtilis* was the most sensitive. Gram-negative bacteria were shown to be generally more resistant than Gram-positive ones to the antagonistic effects of essential oils because of the lipopolysaccharide present in the outer membrane but this was not always true (9, 16, 17). In our studies the same phenomenon could be observed taking as an example the effect of the oil of *Foeniculum vulgare*. *F. vulgare* does not present bacteriostatic action on strains of *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. The oil of *F. vulgare* showed lower antimicrobial activity, in spite of studies describing stronger antimicrobial effects in certain cases (14). Of the studied medicinal plants, *Thymus vulgaris* presents the most pronounced antimicrobial effect, since formation of inhibitory zones could be observed even in the case of low concentrations. *Thymus vulgaris* presents increased antimicrobial effect towards many microorganisms with sanitary importance (18, 19). The results of the antimicrobial effect studies concerning the essential oil of *Thymus vulgaris* are similar to the data available in literature (1, 15, 20).

This fact is important both from a therapeutic point of view and from that of food conservation. Thus, the substances studied could take the place of synthetic condiments unfavorable for a healthy alimentation.

After associating essential oils, synergism can be found primarily in the case of the combination of *Salvia officinalis* and *Foeniculum vulgare*.

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