Analele Ştiinţifice ale Universităţii „Al. I. Cuza” Iaşi
s. II a. Biologie vegetală, 2012, **58**, 1: 51-58
http://www.bio.uaic.ro/publicatii/anale_vegetala/anale_veg_index.html
ISSN: 1223-6578, E-ISSN: 2247-2711

CHEMICAL COMPOSITION AND ANTIBACTERIAL ACTIVITY OF ESSENTIAL OILS OF THREE *SALVIA* SPECIES, WIDESPREAD IN EASTERN ROMANIA

Magda COISIN[1*], Ioan BURZO[2], Marius ŞTEFAN[1], Elida ROSENHECH[1], Maria Magdalena ZAMFIRACHE[1]

Abstract: Essential oils of *Salvia aethiopis* L., *S. nemorosa* L. and *S. verticillata* L. were investigated in terms of chemical composition and antibacterial activity and tested on two pathogen bacterial strains: *Staphylococcus aureus* and *Escherichia coli*. Essential oils obtained by hydro-distillation were analyzed using GC-MS method. Chemical composition revealed 45 components present in the three species: 29 compounds in *S. verticillata* sample, 12 in *S. aethiopis* ones and 24 respectively in *S. nemorosa* sample, representing 99.7% of the oil composition, 99.3% and 95% respectively. The volatile compounds identification was made using the NIST spectral bank and Kovats indexes. Then, the percentages of compounds of each oil are summarized. The minimal inhibitory concentration (MIC) was determined by diffusion method and successive micro-dilutions. It was noticed that the dilution method is much more precise. Test results have shown a greater antibacterial effect of *Salvia* oil samples against Gram positive, than the Gram negative bacteria.
Keywords: *Salvia*, GC-MS, essential oils, antimicrobial activity.

Introduction

In the recent decades, antimicrobial plant products have gained a special attention because of increase resistance to antibiotics acquired of some microorganisms (Al-Sheddi, 2009). With the growing interest in the use of essential oils in both the food and the pharmaceutical industries, a systematic examination of plant extracts for these properties has become increasingly important. The use of natural antimicrobial compounds is important not only in the preservation of food but also in the control of human and plant diseases of microbial origin (Baratta et al., 1998). Many species and herbs exert antimicrobial activity due to their essential oil fractions. Some scientists reported the antimicrobial activity of essential oils from oregano, thyme, sage, rosemary, clove, coriander, garlic, and onion against both bacteria and molds. The composition, structure, as well as functional groups of the oils play an important role in determining their antimicrobial activity (Celikel and Kavas, 2008).

Despite the medicinal potential of plants being considerable in our country, knowledge and studies on wild growing *Salvia* species from this area are scarce; accounts for their therapeutic effects were found especially in other sources (Anonymus, 1997; Bagci and Koçaka, 2007; Tepe et al., 2007). Like most of the representative species of *Lamiaceae*, *Salvia* species are aromatic plants, rich in essential oils which have been used in food, cosmetics, perfumes and pharmaceutical products (Baratta et al., 1998). *Salvia* species are used in traditional medicine to treat various conditions like sore throats, colds, digestive disorders or as an insect repellent (Kamatou, 2006).

1* Faculty of Biology, “Alexandru Ioan Cuza” University of Iasi. Bd. Carol I, no 20 A, 700506. magda_coisin@yahoo.com (corresponding author)
2 Faculty of Horticulture, U. S. A. M. V. Bucharest

Essential oils are a complex mixture of compounds, mainly monoterpenes, sesquiterpenes, and their oxygenated derivatives (alcohols, aldehydes, esters, ethers, ketones, phenols and oxides). Other volatile compounds include phenylpropenes and specific sulphur- or nitrogen-containing substances (Longaray Delamare et al., 2007).

Microorganisms are involved in the pathogenesis of many diseases and cause deterioration of a variety of products (Kamatou, 2006). A survey of the literature revealed that *Salvia* species from different places in the world have been widely tested against both Gram-positive and Gram-negative bacteria (Marino et al., 2001; Kamatou, 2006; Longaray Delamare et al., 2007; Al-Sheddi, 2009, Sultanbawa et al., 2009). *Staphylococcus aureus* is one of the most important pathogens that cause staphylococcal food poisoning in meat products and contamination being generally associated with highly manual-handled food (Rasooli, 1999). Staphylococcal food poisoning is a major public health risk all over the world (Hall, 1997). Researchers and food manufacturers are looking for new plant material with antimicrobial activity, therefore it is important to have a rapid screening method to identify and compare plant material with antimicrobial activity (Sultanbawa et al., 2009).

The aim of this study was to determine the chemical composition of essential oils for three spontaneous *Salvia* species from eastern Romania and then to evaluate their antimicrobial potential in vitro, by testing them on two pathogen bacterial strains.

Materials and methods

Aerial parts of *Salvia aethiopis* L., *S. nemorosa* L. and *S. verticillata* L. (*Lamiaceae*) species, were collected at flowering phase for analysis. All investigated taxa were collected from natural populations in different locations from eastern Romania, in the year 2010. Voucher specimens of each species were deposited in the Herbarium of the Faculty of Biology from "Alexandru Ioan Cuza" University of Iasi.

Essential oil samples were individually tested against two pathogenic microorganisms represented by *Staphylococcus aureus ATCC 25923* and *Escherichia coli ATCC 25922* bacterial strains.

Isolation of the essential oils was made in the Plant Physiology Laboratory of the Faculty of Biology in "Al. I. Cuza" University of Iasi. A portion (100g) of fresh vegetal material was submitted for 3 hours to water-distillation, using a Clevenger-type apparatus, according to the method recommended by European Pharmacopoeia.

Identification of the oil components was performed by gas-chromatography using a 6890N Agilent GC/MS. The volatile compounds identification was made using the NIST spectral bank and Kovats indexes, at the Faculty of Horticulture, U.S.A.M.V. Bucharest.

For the antimicrobial assays all essential oils were mixed with DMSO 99.9% (dimetyl sulphoxide). Two different methods were applied:

- Diffusimetric method on Muller Hinton Agar (MHA) – The inoculum used for this method, was adjusted to achieve the McFarland standard. 1ml of inoculum was placed onto the surface of pre-dried MHA in the Petri plates. After 1 minute, the excess inoculum was removed, and the plates were allowed to dry at room temperature for almost 20 minutes. Four wells were made on each plate, with a sterile perforator. In each plate were added three oil concentrations (5%, 10% and 20%). Gentamicin was use as bioactivity control. The plates were incubated at 37^{o}C for 24 hours.

- Microplates method – A broth microdilution susceptibility assay was used, as recommended by NCCLS, for the determination of MIC (NCCLS, 1999). Flat bottom 96-well sterile microtiter plates with lids to prevent cross contamination were used for the study. Bacterial strains were cultured overnight at 37^{o} C on Mueller Hinton Broth (MHB), followed by the adjustment of the culture to achieve a final density of approximately 0,5 McFarland standard, and used to inoculate (1/10) 96-well micro liter plates containing serial twofold dilutions of the essential oils (10-0.01 mg/ml) on MHB supplemented with 0.5% (v/v) Tween 80. Plates were incubated under normal atmospheric conditions at 37^{0}C, for 24 hours. A color change from blue to pink, using resazurine, was indicative of bacterial growth. The micro plate assay also included a negative control, a sterility and a positive control of the test organism without essential oil (Fig. 2). All tests were performed in duplicate at Microbiology Laboratory of The Faculty of Biology from "Alexandru Ioan Cuza" University of Iasi.

Results and discussions

The essential oils of sage, complex mixtures, often containing more than 100 individual components (Waterman, 1993), although terpenoid molecules are predominant. They have low boiling points and can be recovered from the plant tissues by steam distillation. In addition to flavouring foods, volatile oils can also act as antioxidants and preservatives against food spoilage, while a broad range of applications in aromatherapy and health care has been observed during the last fifteen years (Hay and Waterman 1993).

The essential oil compositions determined by GC-MS revealed 45 components present in the three species: 29 chemical compounds in *S. verticillata* sample, 12 in *S. aethiopis* ones and 24 respectively in *S. nemorosa* sample, representing 99.7% of the oil composition, 99.3% and 95% respectively. In Table 1 are displayed all volatile compounds identified in the essential oils (EOs), with their respective percentage composition in each species.

The following were the major components, having a great percentage in the composition of the three oils:

- *Salvia verticillata* essential oil sample: camphene (16.03%), β-pinene (15.24%), sabinene (14.54%).
- *Salvia aethiopis* essential oil sample: germacrene D (31.68%), β-caryophyllene (24.8%), α-copaene (17.1%).
- *Salvia nemorosa* essential oil sample: sabinene (38.2%), β-caryophyllene (19.03%), germacrene D (18.62%).

Alfa-caryophyllene, beta-caryophyllene, caryophyllene oxide, germacrene D and γ-cadinene were detected in all the three species (Table 1), forming the major profile for EOs of investigated species (caryophyllene compounds, especially).

There are many components identified in one plant, but absent in the others (Table 1), showing the high variation which exists in EOs composition between different species, factors such as the time of harvesting, the method of extraction, the ecological parameters etc. explaining such variations, in addition to the genetic map of each species (Kamatou, 2006).

The essential oil composition data has provided extra information needed to better understand the biological activities of these oils, in the antimicrobial assays further carried out.

Table 1. Percentage of EO components of three *Salvia* sp.

No	Compounds	*S. verticillata*	*S. aethiopis*	*S. nemorosa*
			%	
1	α-thujene	-	-	2.71
2	α-pinene	0.29	-	0.15
3	Sabinene	0.17	-	38.2
4	β-pinene	0.33	-	0.48
5	Myrcene	0.16	-	-
6	β-myrcene	-	-	0.84
7	α-terpinene	-	-	0.39
8	p-cymene	-	-	0.71
9	Limonene	1.75	-	0.39
10	γ-terpinene	-	-	1.28
11	α-terpinolene	-	-	0.22
12	α-copaene	-	17.34	0.25
13	α-bourbonene	0.20	-	0.2
14	β-caryophyllene	16.03	24.8	19.03
15	z-β-farnesene	0.46	-	0.44
16	α-caryophyllene	14.54	5.55	0.72
17	γ-muurolene	0.30	-	0.4
18	Germacrene D	2.29	31.68	18.62
19	Elixene	-		2.98
20	γ-cadinene	0.36	0.89	0.44
20	β-cadinene	-	-	0.72
21	Spatulenol	8.64	-	0.81
22	Caryophyllene oxide	15.24	1.11	3.53
23	τ-cadinol	-	-	0.96
24	β-cubebene	-	7.03	-
25	Bicyclogermacrene	-	4.14	-
26	δ-cadinol	-	0.45	0.63
27	δ-cadinene	-	5.29	-
28	Aromadendrene	-	0.46	-
29	α-cadinol	-	0.6	-
30	Camphene	-	0.16	-
31	Ocimene	0.16	-	-
32	trans β-ocimen	1.95	-	-
33	cis β-ocimen	0.17	-	-
34	Borneol	0.17	-	-
35	Borneol acetat	0.17	-	-
36	Terpenil acetat	3.63	-	-
37	γ-Elemene	2.47	-	-
38	Izoaromadendrene oxide	1.67	-	-
39	ent Spatulenol	6.91	-	-
40	τ neurolol	1.30	-	-
41	izo-longifolol	1.54	-	-
42	Patchoulol	1.77	-	-
43	Ledenoxide	0.93	-	-
44	Hexahydroxy-farnesyl-acetone	0.72	-	-
45	Phytol	0.79	-	-
	% Identification	99.71	99.33	95.01

For testing and quantifying antibacterial activity, numerous standard microbial protocols are available, which can be classified as diffusion, dilution or bioautographic methods (Kovats et al., 2011). In these assays, two different methods were applied for the evaluation of the antimicrobial activity: diffusion method and minimum inhibitory concentration (MIC) by the method of serial dilution in microplates (NCCLS, 1997). The MIC was defined as the lowest concentration of essential oil that completely inhibited the growth of the organism as detected by them. MIC_0 is defined as the highest concentration of plant extract which results in no inhibition of growth, MIC_{50} is the concentration of plant extract which results in 50% inhibition of growth and MIC_{100} is the lowest concentration of plant extract which results in 100% inhibition of growth.

Using agar diffusion method, we noticed very little antimicrobial activity concerning both bacteria tested; especially against *Escherichia coli* where the inhibitory effect was insignificant, with normally bacterial increase at 5% and 10% oil concentration. A small inhibition zone at 20% concentration can be observed (Fig. 1). The second bacterial strain, *S. aureus,* has shown a greater sensibility at essential oils action, in all three samples, comparatively with *E. coli*, which manifest an increased degree of resistance. Irregular inhibition zone is noticeable and staphylococcal effect of *Salvia* oil being evident at 5% oil concentration yet, with well progress to 20%. Also, is well visible for the plates inoculated with *Staphylococcus* that some compounds of the oils exhibit their inhibitory effect after volatilization, being necessary to perform the volatilization test for detected them.

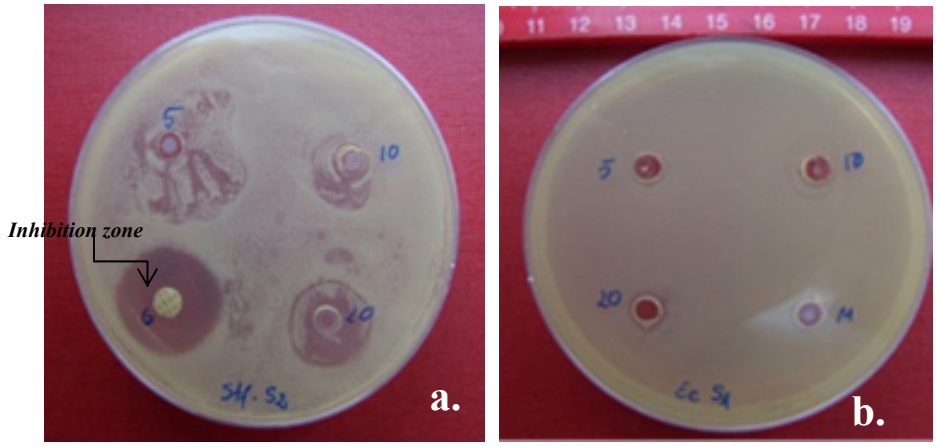

Figure 1. Inhibition zone of bacterial growth: a. *S. aethiopis* EOs against *Staphylococcus aureus;* b. *S. verticillata* EOs against *Escherichia coli*

G – Gentamycine; M – Control; Ec – *Escherichia coli*; Stf– *Staphylococcus aureus*; S1 – *Salvia verticillata*; S2 – *Salvia aethiopis*

Regarding the second method that was used, this very sensitive method exposes the lowest concentration of the bacterial cells to the highest concentration of plant extract, thus maximizing the potential to detect antimicrobial activity. Development of the micro plate assay is based on correlating the bacterial growth with the inhibitory effect of the plant extract (Sultanbawa et al., 2009). We can certainly conclude that some antimicrobial

activity of investigated *Salvia* oils occur and the minimal inhibitory concentration can be determinate easily. MIC was assessed at only 0.5 % for all essential oils (Fig. 2) against *E. coli*. For the plates inoculated with *Staphylococcus* strain we noticed that for the essential oil obtained from *S. verticilata,* the MIC was assessed at 0.5 % oil concentration. Regarding the EO extracted from *S. aetiopis* and *S. nemorosa* the MIC assessed for the *Staphylococcus* strain was at 0.25% oil concentration. Literature reports 0.02–1% as the range for antibacterial activity for essential oils (Burt, 2004).

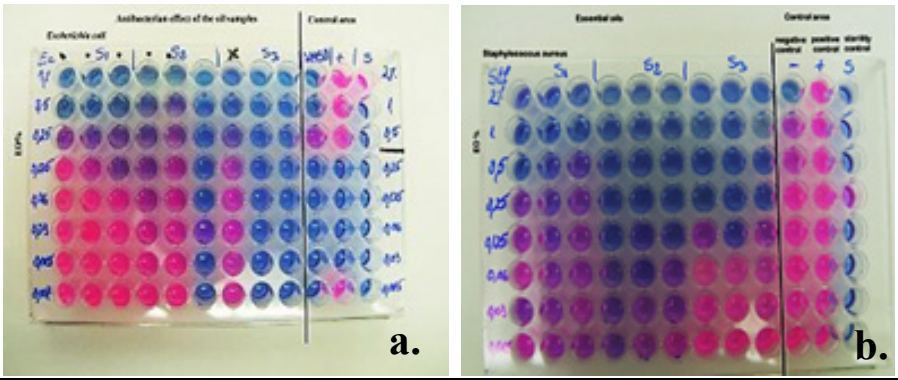

Figure 2. Minimum inhibitory concentration (MIC) values of the 3 EOs against *E. coli* (a.) and against *Staphylococcus aureus* (b.)
S1 – *Salvia verticillata* Eos; S2 – *Salvia aethiopis* Eos; S3 – *Salvia nemorosa* Eos; Ec. – *Escherichia coli*; Stf. – *Staphylococcus aureus*; DMSO = dymetil sulfoxide

The mechanisms by which essential oils can inhibit microorganisms involve different modes of action, and in part may be due to their hydrophobicity. They get partitioned into the lipid bilayer of the cell membrane, rending it more permeable, leading to leakage of vital cell contents (Burt, 2004; Edris, 2007). Aromatic and phenolic compounds exert their antimicrobial effects at the cytoplasmic membrane by altering its structure and function (Sikkema et al., 1995). The loss of the differential permeability character of the cytoplasmic membrane is frequently identified as the cause of cell death.

We found, in agreement with other study (Kamatou, 2006), that the Gram positive bacteria be more sensitive than the Gram negative. Current hypotheses for the resistance of many Gram negative bacteria, favor the adverse effect on the integrity of the bacterial cell membrane (Delaquis et al., 2002). The sensitivity of bacteria was related to the morphological structure and chemical composition of their membrane. Therefore, those bacteria possessing an outer membrane composed mainly of polysaccharides rather impermeable can prevent the inhibitors molecules from passing through (Kamatou, 2006). Given the differences in the cell wall of Gram positive bacteria, it is plausible that access through the cell membrane is more restricted. Although the investigation into the biological activities of chemical mixtures are difficult, it is important to fully understand the mechanism by which these naturally occurring compounds are effective and interact in the treatment of various diseases (Liu, 2005).

In nowadays Europe, where industrial development, pollution and urban agglomerations are continuously extending, the rich medicinal flora of Romania represents a priceless asset (Antal and Peev, 2006). Demand for organic products is still increasing in

UE as well as the consumption of natural substances. In connection, pharmaceutical potential possessed of spontaneous flora is need to be specified, like representing a prerequisite for its valorization and protection. Therefore, *S. verticillata*, *S. nemorosa* and *S. aethiopis*, which have not been applied in human medicine, these can be considered and should be further checked for their active ingredients and for possible use.

Conclusions

The monoterpenes and sesquiterpenes confere the chemical profile of analyzed essential oil *Salvia* samples.

Caryophyllene compounds and germacren D are representative, identified in all 3 species in high percentage.

This study has shown that essential oils from *S. verticillata, S. aethiopis* and *S. nemorosa,* displayed inhibitory activity against the tested bacteria to varying degree, higher against *S. aureus* than *E. coli*. The MIC values of EOs obtained of these wild growing species indicated promising activities against *S. aureus* during incubation at 37^0C for 22 hours.

The bioassay confirm that Gram positive bacteria are more sensitive compared to Gram negative ones, *E. coli* being in general the most resistant strain. Especially in low concentrations, essential oils of *Salvia* sp. have failed in their inhibitory action against this pathogen.

To evaluate the MIC, the micro-dilution method was much more precise than diffusion one.

Acknowledgements

This work was made possible with financial support within the POSDRU/88/1.5/S/47646 project, co-funded from the Social European Fund, via Human Resources Development Operational Programme 2007-2013.

REFERENCES

Al-Sheddi, E.S., 2009. *Pharmacognostical Study of Salvia merjamie Forsk Growing in Saudi Arabia,* PhD thesis. King Saud University.

Anonymous, 1997. Draft International Standard ISO/DIS 11024-1.2/2.2, Essential oils – General guidance on chromatographic profiles. International Organization for Standardization, Geneva.

Antal, D.S., Peev C.I., 2006. The spectrum of bioforms and the areal geographic structure of the medicinal plants from the western part of the Banatian Mountains (Romania). In: *Proc. of the 4th Conf. Med. and Aromatic Plants of South-East European Countries, 28-31 May 2006, Iasi (Romania)*: 4-10.

Bagci, E., Koçaka, A., 2007. Study on the analysis and evaluation of essential oils of two *Salvia* L. (*S. ceratophylla* L., *S. aethiopis* L.) species. Science and Eng. J of Firat Univ. **19**, 4: 435-442.

Baratta, M.T., Dorman, H.J.D., Deans, S.G., Figueiredo, A.C., Barroso, J.G., Ruberto, G., 1998. Antimicrobial and antioxidant properties of some commercial essential oils. Flavour and Fragrance Journal **13**: 235–244.

Burt, S., 2004. Essential oils: their antibacterial properties and potential applications in foods – a review. Int. J. Food Microbiol. **94**: 223–253.

Celikel, N., Kavas, G., 2008. Antimicrobial properties of some essential oils against some pathogenic microorganisms. Czech J. Food Sci. **26**: 174–181.

Delaquis, P.H., Stanichm, K., Girard, B., Mazza, G., 2002. Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. International Journal of Food Microbiology **74**: 101-109.

Edris, A.E., 2007. Pharmaceutical and therapeutic potentials of essential oils and their individual volatile constituents – a review. Phytotherapy Research **21**: 308–323

Hall, R.L., 1997. Food-borne illness: Implications for the future. Emerg. Infect. Dis. **3**: 555-559.

Hay, R.K.M., Waterman, P.G. (Eds.), 1993. *Volatile Oil Crops: Their biology, biochemistry and production*. Longman Scientific and Technical, Essex, UK.

Longaray Delamare, A.P., Moschen-Pistorello, I.T., Atti-Serafini, L., Echeverrigaray, S., 2007. Antibacterial activity of the essential oils of *Salvia officinalis* L. and *Salvia triloba* L. cultivated in South Brazil. Food Chemistry **100**, 2: 603–608.

Kamatou, G.P.P., 2006. *Indigenous Salvia species – an investigation of their pharmacological activities and phytochemistry*. PhD thesis, University of the Witwatersrand, Johannesburg.

Kovats, N., Aks, A., Gölöncsér, F., Barabás, A., 2011. Quantifying of bactericide properties of medicinal plants. Plant Signaling & Behavior **6**, 6: 777-779.

Liu, J., 2005. Oleanolic acid and ursolic acid: research perspectives. Journal of Etnopharmacology **100**: 92-105.

Marino, M., Bersani, C., Comi, G., 2001. Impedance measurement to study antimicrobial activity of essential oils from *Lamiaceae* and *Compositae*. Int. J. Food Microbiol. **67**: 187–195.

National Committee for Clinical Laboratory Standards, 1997. *NCCLS. Performance standards for antimicrobial disk susceptibility test*. 6 ed. Wayne PA. Approved Standard M2-A6.

Rasooli, B., 1999. *Study of antimicrobial activity of thyme, mint, sumac and wild pistachio by in vitro method*. Msc Thesis of Urmia University.

Sikkema, J., De Bont, J.A.M., Poolman, B., 1995. Mechanism of membrane toxicity of hydrocarbons. Microbiological Reviews, **259**: 2201-2222

Sultanbawa, Y., Cusack, A., Currie, M., Davis, C., 2009. An innovative microplate assay to facilitate the detection of antimicrobial activity in plant extracts. Journal of Rapid Methods & Automation in Microbiology **17**: 519–534

Tepe, B., Eminagaoglu, O., Akpulat, H.A., Aydin, E., 2007. Antioxidant potentials and rosmarinic acid levels of the methanolic extracts of *Salvia verticillata* (L.) subsp. *verticillata* and *S. verticillata* (L.) subsp. *amasiaca* (Freyn & Bornm.).Bornm. Food Chem. **100**: 985-989.

Waterman, P.G., 1993. *The chemistry of volatile oils*. In: Hay, R.K.M, Waterman, P.G., (eds.), *Volatile oils: their biology, biochemistry and production*. Longman Scientific and Technical, Essex, UK: p. 47-61.