Original Paper

In vitro Antimicrobial Activity of Some Extracts of *Salvia* spp Harvested from the Oltenia Flora Using Different Solvents

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ABSTRACT: In this study, the antimicrobial activity of three *Salvia* spp. (*S. glutinosa, S. splendens, S. verticillata*) extracts prepared with different solvents was assessed using the diffusion method and the quantification of the minimum inhibitory concentration for each extract on *S. aureus, E. coli* and *C. albicans* standard strains. The results showed that the extracts of the three *Salvia* spp. are suppressing the growth of the bacteria tested with variable potency. Among the different solvent extracts, n-butanolic extracts of all the three species of *Salvia* spp. revealed the most important antibacterial activity against *S. aureus and E. coli. S. splendens* extracts proved to be the most efficient on *C. albicans* regardless of the solvent used.

***KEYWORDS**: Herbal extracts, Salvia glutinosa, Salvia splendens, Salvia verticillata, antimicrobial activity.*

Introduction

Multidrug resistant microorganisms have become an ever-increasing menace to public health and a major global issue, due to both inaccurate and excessive use of antibiotics not only in human treatment, but also in agriculture [1].

As a consequence, improving public awareness on appropriate antibiotic use and mostly, development of new efficient antibacterial molecules represents an urgent matter [2].

Plants synthetize secondary metabolites and naturally occurring phytochemicals are regarded as promising therapeutic agents [3].

Salvia L. is a representative genus of the *Lamiaceae* family and it comprises almost 1000 species cultivated worldwide, while being native to the Americas and Eurasia, with 15 species included in the Romanian flora [4,5].

The antibacterial activity of *Salvia* spp. is being increasingly documented, due to the high content of biological compounds predominantly found in the genus, such as polyphenols and terpenes, which are acknowledged for exerting antioxidant, antimicrobial, anti-inflammatory activities [6,7].

Species from the genus *Salvia*, such as *S. splendens*, *S. discolor*, and *S. microphylla* 'Hot Lips' are widely cultivated as ornamental herbs, having a variety of scents and colors [8,9].

Furthermore, sage, as the genus is widely known, is used in cosmetics, perfumery and as a flavor in the food industry, enhancing both taste and nutritional values of food [8].

Ethnopharmacological uses of *Salvia* spp. are reported worldwide and include treating loss of appetite, stomach aches, indigestion, diarrhea, constipation, flatulence, insomnia, common colds, fever, cough, postpartum pain, dysmenorrhea, malaria, anxiety, stomatitis, freshening breath, hepatitis, diabetes, nephropathy [10-13].

The main aim of this research was to investigate and compare the possible antimicrobial activity of the extracts obtained using various solvent systems from three *Salvia* spp. (*S. glutinosa*, *S. splendens* and *S. verticillata*), which are part of Banat and Oltenia flora (southwestern Romania).

Material and Methods

Plants origin. The specimens of *Salvia* spp. were harvested during the flowering period from three different places of the South-West region of

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Romania: *S. glutinosa* L. samples from Băile Olănești, Vâlcea County in June 2020, *S. splendens* Sellow ex J.A. Schultes samples from Craiova, Dolj County, in September 2021 and *S. verticillata* L. samples were collected from Băile Herculane, Caraș-Severin County in June 2020.

Our study did not concern endangered or protected species. Voucher specimens (SaG-20200626/1, SaS-20210910/1 and SaV-20200606/1) are deposited in the Herbarium of the Department of Pharmaceutical Botany, University of Medicine and Pharmacy of Craiova, Romania.

Chemicals and solvents

The solvents needed for extraction (water, methanol 70%, ethanol 70%, chloroform, n-butanol, and ethyl acetate) were purchased from Merck (Darmstadt, Germany) and met chromatographic purity conditions (LiChrosolv®).

Extraction procedure

The fresh flowering aerial parts were washed with purified water, and left to dry in the shade. The dried plants were crushed in powder form.

All samples were prepared individually, using 1g of dry powder for every sample in 5mL of each solvent (water, methanol 70%, ethanol 70%, chloroform, n-butanol, and ethyl acetate).

The extractions were carried out in a Bandelin Sonorex DL102H ultrasound bath (Bandelin Electronic GmbH&Co. KG, Germany), followed by centrifugation at 10000rpm for 10 minutes using an Eppendorf 5804 equipment (Eppendorf, Hamburg, Germany).

The extracts of all samples were filtered and afterwards stored in dark-colored glass bottles at 4ºC until analysis was performed.

For the microbiological analysis, the extracts were filtered using syringe filters with pore sizes of 0.2µm (Acrodisc MS Syringe Filters WWPTFE Membrane-Fisher Scientific, Sweden).

The antibacterial activity of *Salvia* extracts was evaluated against both Gram-positive and Gram-negative bacteria strains, *Staphylococcus aureus* ATCC®25923 and *Escherichia coli* ATCC®25922 and the antifungal activity on *Candida albicans* ATCC®10231 standard strain, using the disc diffusion method.

The inoculation medium was represented by Mueller-Hinton (MH) agar and inoculums collected from direct colony suspension equivalent to a 0.5 standard on the McFarland scale were used within 15 minutes of preparation [14].

Blank antimicrobial susceptibility paper discs, 6mm diameter (Thermo Scientific, UK), were slowly infused with 40µL of each sample extract (S1-S3, in A-F solvents) and manually placed with a sterilized forceps, each disk one at a time, to the surface of exponentially growing cultures of previously mentioned bacterial strains plated in MH agar.

The containers were incubated at 37ºC for 24 hours. Ciprofloxacin and disks containing each solvent were used as controls.

The antibacterial activity was evaluated according to the diameter of the areas of growth inhibition.

All tests were conducted in triplicate.

The antifungal activity of *Salvia* spp. extracts was evaluated against *Candida albicans* ATCC®10231 standard strain according to the disc diffusion assay.

C. albicans was maintained in Sabouraud dextrose agar medium.

The disks were then slowly infused with 40µL of each sample extract (S1-S3, in A-F solvents) and placed with sterilized forceps to the surface of the growing culture.

The incubation of the plates lasted for 48 hours, within a constant temperature of 37ºC.

The results of antifungal activity consisted in the measurement of the diameter of complete inhibition area, including disk diameter.

Clotrimazole and disks with each solvent were used as standard and blank, respectively.

All the experiments were carried out in triplicate.

Determination of minimum inhibitory concentration (MIC)

To determine MIC values, the stock extracts (80mg/mL) were prepared in Mueller-Hinton agar.

A two-fold serial dilution of the stock solution leads to various other concentrations until the final 1.25mg/mL solution is reached.

Using a sterile 96-wells plate, 180µL of each diluted extract was inoculated with 20µL of 10^6 colony-forming units (CFU)/mL culture.

After an overnight incubation at 37°C, the microbial growth was evaluated turbidimetrically.

The measurements for each microorganism were repeated in duplicate.

The MIC was determined as the first concentration with minimum turbidity.

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Statistical analysis

Data were analyzed using GraphPad Prism software (GraphPad, USA). Calculated variables were reported as means±standard deviations (SD).

The evaluation of differences between studied samples was performed using one-way analysis of variance (ANOVA) test, a p value<0.05 being considered statistically significant.

Results

Tables 1 and 2 display the diameters for the inhibition zones of bacterial growth for the three *Salvia* spp. extracts.

Table 1. Antibacterial activity of Salvia spp. extracts against Staphylococcus aureus.

Tested strain *Staphylococcus aureus* ATCC®25923						
Diameter of inhibition areas [mm] (mean±SD)						
	A	**B**	**C**	**D**	**E**	**F**
S_1	N	10.33±0.57	11.66±0.57	14±1	16±1	11±1
S_2	N	10.33±0.57	13.16±0.28	15.33±0.57	15.33±1.15	11.66±0.57
S_3	N	9±1	11.66±0.57	14.66±0.57	17±1	12±0

Note: N-no zone of inhibition was observed. S1-S. glutinosa, S2-S. splendens, S3-S. verticillata. Solvents used for extraction: A-water, B-methanol, C-ethanol, D-ethyl acetate, E-n-butanol, F-chloroform.

Table 2. Antibacterial activity of Salvia spp. extracts against Escherichia coli.

Tested strain *Escherichia coli* ATCC®25922						
Diameter of inhibition areas [mm] (mean±SD)						
	A	**B**	**C**	**D**	**E**	**F**
S_1	N	9±1	17±1	13±1	20.66±0.57	20±1
S_2	N	N	16±1	13±1	15.66±0.57	11±1
S_3	N	N	10±1	9±1	15±1	10±1

Note: N-no zone of inhibition was observed. S1-S. glutinosa, S2-S. splendens, S3-S. verticillata. Solvents used for extraction: A-water, B-methanol, C-ethanol, D - ethyl acetate, E-n-butanol, F-chloroform.

As one can notice, aqueous *extracts of Salvia spp. had no antibacterial activity* against both tested bacterial strains.

Regarding the activity on *S. aureus* strain, the extracts in highly polar solvents (methanol, ethanol) were less active than those in other solvents.

For all *Salvia* spp., the most efficient proved to be tested the extracts in n-butanol (E), a solvent less polar than the alcohols mentioned.

n-Butanol extracts of *S. glutinosa* and *S. verticillata* had the strongest activity against *S. aureus* while for *S. splendens* ethyl acetate extract was more efficient with an inhibition area comparable with that of the n-butanol extract.

Concerning the activity on *E. coli* strain, the extracts in ethanol and n-butanol of all the three *Salvia* spp. showed potency compared to those in other solvents.

The most efficient were the alcoholic extracts of *S. glutinosa* and *S. splendens*. Surprisingly, chloroformic extract of *S. glutinosa* presented the same activity as that in n-butanol.

In addition, *Salvia* extracts exhibited a growth inhibitory effect even on *C. albicans* strain (Table 3).

Table 3. Antifungal activity of Salvia spp. extracts against Candida albicans.

Tested strain *Candida albicans* ATCC®10231						
Diameter of inhibition areas [mm] (mean±SD)						
	A	**B**	**C**	**D**	**E**	**F**
S_1	N	9±1	13.33±1.15	13±1	16±1	17.66±0.57
S_2	N	10±1	14±1	14.66±0.57	20±1	18.33±0.57
S_3	N	8±1	9±1	9±1	15±1	14±1

Note: N-no zone of inhibition was observed. S1-S. glutinosa, S2-S. splendens, S3-S. verticillata. Solvents used for extraction: A-water, B-methanol, C-ethanol, D - ethyl acetate, E-n-butanol, F-chloroform.

As stated in Table 3, aqueous extracts had no activity on this fungus.

At the same time, *S. splendens* extracts proved to be the most effective for all tested solvents compared to the other species.

And this time, the extract in n-butanol had the largest inhibition zone for all the three species. Chloroformic extracts of *S. glutinosa* and *S. splendens* also showed a relatively high potency.

Because the tolerated solvent was ethanol, we performed an analysis to compare the minimum inhibitory concentration (MIC) against each bacterium for different alcohols extracts.

MIC varied as follows: *S. glutinosa* on both bacteria had the lowest MIC (20mg/mL) for n-butanol extract; *S. splendens* had the same MIC of 20mg/mL for ethanol and n-butanol extracts on *S. aureus* and the lowest MIC of 10mg/mL for n-butanol extract on *E. coli* strain; for *S. verticillata*, the lowest MIC values were obtained for n-butanol extract, 20mg/mL on *S. aureus* and 5mg/mL on *E. coli*.

ANOVA comparison of *Salvia spp.* extracts activity in all the solvents used revealed significant differences for *S. aureus* ($p<0.0001$), *E. coli* ($p=0.0226$) and *C. albicans* ($p=0.0016$), suggesting the importance of an appropriate solvent selection depending on the targeted activity.

Discussion

The antibacterial and antifungal properties of *S. glutinosa*, *S. splendens* and *S. verticillata* extracts in polar (water, methanol, ethanol), semi-polar (n-butanol, ethyl acetate) and non-polar (chloroform) solvents at a concentration of 20% have been assessed in this study.

Multiple previous studies have shown antibacterial activity of *Salvia* spp. predominantly against Gram-positive strains, generally attributed to the rich content of polyphenolic active compounds, which supposedly mechanism of action consists in damaging the structure of the cellular membrane and its functions, consequently [15-17].

A possible explanation for the poor susceptibility of the Gram-negative bacteria to various extracts and antibiotics would be the barrier formed against lipophilic compounds, due to the lipopolysaccharides and lipoproteins present in their cellular walls [18,19].

Subsequently, *S. spinosa* exhibited good inhibitory activity on Gram-negative bacteria when extracted in methanol, while the dichloromethane and *n*-hexane proved less active [20].

Another such example is the Iranian *S. nemorosa* extracted in methanol, dichlormethane and n-hexane, that were all capable of inhibiting the growth of Gram-positive strains, *Bacillus cereus* and *S. aureus*, while only the methanolic extract had mild effects on Gram-negative bacteria strains tested (*Pseudomonas aeruginosa* and *E. coli*) [21].

Furthermore, methanolic extract of *S. sclarea* showed powerful inhibitory capacity against both Gram-negative (*E. coli, Klebsiella spp.* and *Salmonella typhi*) and Gram-positive microorganisms (*S. aureus, Streptococcus epidermidis* and *Bacillus subtilis*).

The antimicrobial effect was in strong correlation with the composition of the methanolic extract, characterized by high amounts of caffeic acid and its dimer, rosmarinic acid, along with quercetin.

In contrast, *S. aethiopis* and *S. nemorosa* did not inhibit the growth of either *E. coli* or *S. aureus* [22].

Antibacterial effects of the ethanolic extract were discussed in yet another study. *S. glutinosa* demonstrated antibacterial effect on all strains included, notably proving more active against *E. coli* (MIC 0.6 mg/mL), rather than *S. aureus* (1.5 mg/mL), which partially stands in line with our findings [23].

In our study, *S. glutinosa* similarly demonstrated the highest inhibition zone against *E. coli* out of the three tested species, whilst the n-butanol extract of sticky sage had a bigger inhibition zone (20.66±0.57mm) than the ethanolic extract (17±1mm).

Furthermore, *S. glutinosa* extracted in ethyl acetate together with *S. verticillata* also had a remarkable inhibitory effect on *S. aureus*.

The ethanolic extract of *S. verticillata* also proved antibacterial activity against Gram-positive *B. cereus* and antifungal activity against *C. albicans* and *Penicillium canescens* [7].

According to our results, the extract of *S. verticillata* showed fair inhibitory effect against *C. albicans* when extracted in chloroform and n-butanol, respectively, while the ethanolic extract had low area of inhibition.

A study including petroleum-ether, methanol and chloroform extracts derived from *S. splendens* leaves, all of which were tested against several bacterial strains, identified methanol as the most effective solvent for the extraction, with *S. aureus* being inhibited with a MIC of 200±2.57µg/mL.

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While withstanding the highest flavonoidic and phenolic content, the methanolic fraction also possessed a fair amount of terpenoids and tannins.

Low concentrations of terpenoids and tannins were found also in the chloroformic extract, opposed to the petroleum ether extract, which did not contain any amounts of the afore-mentioned active compounds [24].

The results of our study are consistent with the data regarding the chemical composition of *Salvia* spp. [13,17,23], with special mention on some natural compounds that are soluble in semi-polar (*n*-butanol, ethyl acetate) and non-polar (chloroform) solvents, such as essential oil rich in bicyclic monoterpenes, flavonoid aglycones, diterpenoids, pentacyclic triterpenes, resins and oleo-resins, fatty acids, and which justifies the antimicrobial action (with highest inhibition area) of the corresponding extracts.

Conclusions

Our results revealed that the extracts of the three *Salvia* spp. tested in this study suppress the growth of the bacteria selected with variable potency.

With an inhibition zone ranging from 10.33±0.57 to 20.66±0.5mm and a MIC from 5 to 40mg/mL for the alcoholic extracts, the extracts of *S. glutinosa* and *S. splendens* revealed notable antibacterial activity against *S. aureus* and *E. coli*.

Moreover *S. splendens* extracts proved to be the most efficient on *C. albicans* irrespective of the solvent used.

The findings of our study suggest that the complex composition of these *Salvia* spp. could be responsible for their potency of action in different solvent conditions and support the use of ethanolic extracts of these plants as adjuvant medicines in various diseases.

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Conflict of interests

The authors declare no conflict of interests.

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