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Biological Activities of Various Extracts from *Salvia cassia* Sam. ex Rech.f. and Chemical Composition of Its Most Active Extract

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Abstract: This study was conducted on the antibacterial, antimycobacterial and antifungal activities of various extracts (petroleum ether (PE), chloroform ($CHCl_3$) and ethanol (EtOH) extracts; infusion and decoction) and essential oil from the aerial parts of *S. cassia* Sam. ex Rech.f. and the chemical composition of the most active samples. The antibacterial activity was determined against *Bacillus subtilis*, *Staphylococcus aureus*, methicillin resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Escherichia coli*. The antimycobacterial activity was analyzed against five different *Mycobacterium tuberculosis* and the antifungal activity was compared with two dermotophytes and three Candida species. The composition of EO was analyzed by GC-FID and GC-MS. The EO had a very good activity against *B. subtilis* (0.3 µL/mL) and good activity against *S. aureus*, methicillin resistant *S. aureus* (MRSA) and *E. faecalis* (0.6 µL/mL). All extracts were active in the antimycobacterial and antifungal tests (MICs=50-400 µg/mL). The EO was the most active samples against all *Mycobacterium* strains, dermatophytes and *Candida* species (MICs=0.1-6.25 µL/mL). Because of the high activity of EO, its composition was analyzed, and the major compound was found caryophyllene oxide (22.3%), which was also found the major components of the essential oils from other antimicrobial plants such as *Salvia trichoclada* Benth.

Keywords: *Salvia cassia*; antimicrobial; antimycobacterial; antifungal; essential oil; caryophyllene oxide. © 2018 ACG Publications. All rights reserved.

1. Introduction

According to recent taxonomic revisions the genus *Salvia* is represented by 100 species in Turkey and 59 taxa are endemic [1-4]. Species of the genus are commonly used for rheumatism, warts, wounds, common cold, abdominal pain, flatulence, tonsillitis, stomachache and as antiseptic, stimulant, diuretic, laxative, antipyretic in traditional medicines [5-10]. The species have also economic value for local people;

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they collect the plants from nature and sell on local markets. It is commonly known as adaçayı, çalba, şalba, dağçayı in Turkish [11-12].

The studies on *Salvia* species exhibit the diterpene content and a number of biological activities such as antiseptic, antibacterial, astringent, anti-inflammatory, antiviral, cytotoxic, cardiovascular, sedative, tranquilizing, spasmolitic, anticonvulsant and carminative [13].

Salvia cassia Sam. ex Rech.f. is restricted to Amanos Mountains and the eastern Mediterranean region of Turkey and conservation status is "Endangered" [14]. There is an investigation on the ethanol extract of *S. cassia* (aerial parts). Its cytotoxicity against the human ovarian cancer cell line was compared with other 15 *Salvia* species and this extract showed a cytotoxicity with the IC_{50} value 29.4 μg/mL, while the extract of *S. hypargeia* exhibited highest cytotoxicity [13].

In spite of a number of studies on *Salvia* species, the previous pharmacognostical studies on *Salvia cassia* are quite few. Therefore, this study was conducted on the antibacterial, antimycobacterial and antifungal activities of various extracts (petroleum ether (PE), chloroform ($CHCl_3$) and ethanol (EtOH) extracts; infusion and decoction) and essential oil (EO) from the aerial parts of *S. cassia* and the chemical composition of the most active sample -its EO.

2. Materials and Methods

2.1. Plant Materials

Plant materials were collected from Hatay – Kırıkhan (South Turkey) in May 2015 within the scope of the Tubitak project (No: 114S734). Voucher specimen is kept in Herbarium of Faculty of Pharmacy, Istanbul University (ISTE 107532).

2.2. Preparation of Extracts and Essential Oil

The dried and powdered aerial parts (AP) of *Salvia cassia* (200 g) was extracted in a Soxhlet with petroleum ether (PE extracts), chloroform ($CHCl_3$ extracts), ethanol (EtOH extracts) and were concentrated. An infusion and a decoction were prepared from two other portions (5 g for each preparation) with water and lyophilized. The extracts were stored at ±4 °C after preparation. For isolation of essential oil (EO), the dried and powdered aerial parts (600 g) of *Salvia cassia* was hydro-distillated for 3 h using a Clevenger type apparatus.

The yields of PE, $CHCl_3$ and EtOH extracts, infusion, decoction and EO were found to be 0.91, 1.14, 6.79, 8.39, 8.09 and 0.083% (w/w for all extracts except EO; v/w for EO), respectively.

2.3. Analysis of Essential Oil

2.3.1. GC-MS Analysis

The GC-MS analysis was carried out with an Agilent 5975 GC-MSD system. Innowax FSC column (60 m x 0.25 mm, 0.25 mm film thickness) was used with helium as carrier gas (0.8 mL/min). GC oven temperature was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/min that was kept constant at 220°C for 10 min and followed by elevating the temperature to 240°C at a rate of 1°C/min. Split ratio was adjusted at 40:1. The injector temperature was set at 250°C. Mass spectra were recorded at 70 eV. Mass range was *m/z* 35 to 450.

2.3.2. GC-FID Analysis

The GC analysis was carried out using an Agilent 6890N GC system. FID detector temperature was 300°C. To obtain the same elution order with GC-MS, simultaneous auto-injection was done on a duplicate of the same column applying the same operational conditions. Relative percentage amounts of the separated compounds were calculated from FID chromatograms.

2.3.3. Identification of Components

Identification of the essential oil components were carried out by comparison of their relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) to series of *n*-alkanes. Computer matching against commercial (Wiley GC/MS Library, MassFinder 3 Library) [15,16] and in-house "Baser Library of Essential Oil Constituents" built up by genuine compounds and components of known oils. Additionally, MS literature data [17,18] was also used for the identification. A C9-C30 *n*-alkane standard solution was used for the calculation of retention indices.

2.4. Biological Assays

2.4.1. Antibacterial Assay

The antibacterial activity was evaluated in comparison against Gram positive and Gram negative reference standard microorganisms; *Staphylococcus aureus* ATCC 25923, methicillin resistant *Staphylococcus aureus* ATCC 43300 (MRSA)*, Entercoccus faecalis* ATCC 29212*, Bacillus subtilis* ATCC 6633, *Klebsiella pneumoniae* ATCC 4352*, Pseudomonas aeruginosa* ATCC 27853*, Proteus mirabilis* ATCC 7002 and *Escherichia coli* ATCC 25922) from the Institute of Microorganisms Culture Collection of Istanbul Medical Faculty/Istanbul/Turkey. Ciprofloxacin (Bayer Türk Kimya San. Istanbul /Turkey) and dimethylsulfoxide (DMSO-Merck) were purchased. Tryptic soy agar (TSA) from GBL, Istanbul/Turkey, Mueller Hinton Broth (MHB) TitanBiotek, Istanbul/Turkey and Sabouraud dextrose agar were obtained from GBL Istanbul/Turkey.

The extracts were dissolved in DMSO as a concentration of 10000µg/ml. The antibacterial activity of the extracts was evaluated using a standard microbroth dilution and disk diffusion methods regarding Clinical Laboratory Standards Institute [19,20] under Laminar Air Flow (aseptic conditions). Minimum inhibition concentration (MIC) and minimum bactericidal concentrations (MBCs) were determined for each. The positive control was taken ciprofloxacin for antibacterial activity and the DMSO was taken as vehicle control to determine the possible inhibitory activity of the diluting of extract.

2.4.1.1. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Bacterial inoculums were prepared from overnight grown cultures (24 hours at 37°C) in Mueller Hinton Broth and the turbidity was adjusted equivalent to 0.5 McFarland units approximately 10^8 CFU/mL using spectrophotometer (Multiscan Go) at 450 nm and diluted 1/100 to the final concentration 10^6 CFU/mL for each bacteria.

The MIC and MBC values of extracts were determined based on a microbroth dilution method in 96 multi-well microtitre plates. The 50 µg/mL MHB were added to the wells starting from the second until the 12th. The crude plant extracts of 10000 µg/mL were added to the first and second wells. Two-fold dilutions were made down to the desired minimum concentration starting from the highest concentration 10000 µg/mL in the first well, continuing with the second well which includes 5000 µg/mL down to the lowest concentration 19.52 µg/mL in the tenth well. Finally, 25 µL of bacterial suspensions and 0.001% resazurin solution were added to each well, except the last well and the range of 5000-9.76 µg/mL were achieved.

In each plate, one row with a broad-spectrum antibiotic was used as the positive control (ciprofloxacin in serial dilution 32 to 0,03 µg/mL). An additional row of DMSO was used as vehicle control to determine possible inhibitory activity of the dilutant of extract.

After incubation, at 37°C for 24 hours, the microtitre plates were examined visually for microbial growth which appeared as pink colored by resazurin dye. In each row, the well containing the least concentration and showed no visible growth was considered the MIC. To determine the MBC in each tested row in the MIC determination, a broth sample was collected from the wells which did not show visible growth and the samples were inoculated on TSA plates at 37°C for 24 hours. MBC's were determined with samples which did not show any growth.

2.4.2 Antimycobacterial Assay

Microdilution method was used according to a standard protocol by Clinical and Laboratory Standard Institute (CLSI) [21,22]. Three strains were tested including each of the following species: *M. tuberculosis* H37Rv ATCC 27294 (Susceptible all antimycobacterial drugs, American Type Culture Collection, USA), *M. tuberculosis* H37Rv ATCC 35838 (resistant to Rifampicin (R), American Type Culture Collection, USA) and multi-drug resistant (resistant to Isoniazid + Rifampicin (INH+R)) *M. tuberculosis* that was isolated from patient by Istanbul Faculty of Medicine, Department of Microbiology Laboratory.

Middlebrook 7H9 broth medium (Becton and Dickinson, USA) was used for microdilution method. The medium was adjusted to pH 7.0 at 25°C. Sterility control of each bottle was performed before it was used.

Rifampicin was provided by the Becton Dickinson (BD, USA) as standard lyophilized powders and dissolved in sterile distilled water. The extracts were dissolved in 100% dimethyl sulfoxide, EO were dissolved in Middlebrook 7H9 with 5% Tween80 [23] according to CLSI methods [21,22]. The final concentrations were 400 to 3,125µg/mL, and 50 to 0.1µL/mL for extracts and EO respectively. The critical concentration (1µg/mL) was used for rifampicin.

Preparation of inoculum suspensions of mycobacteria were based mainly according to the CLSI guidelines [21] and described previously [23]. The isolates were subcultured on to Löwenstein Jensen medium at 37°C, during 20-25 days. A few colonies from freshly grown *M. tuberculosis* were suspended in Middlebrook 7H9 broth medium to obtain 1.0 McFarland turbidity and then it was diluted ten times using the same medium.

The broth microdilution test was performed by using sterile, disposable microdilution plates (96 U-shaped wells from LP Italiano SPA, Milano, Italy). Rows from 1 to 11 contained the series of drug dilutions in 100 µL volumes and last row (number 12) contained 100 µL of drug-free medium, which served as the growth control. Each well was inoculated on the day of the test with 100 µL of the corresponding inoculum. This step brought the drug dilutions and inoculum size to the final test concentrations given above.

The microplates which contained including *M. tuberculosis* were incubated at 37°C until mycobacterial growth was clearly observed in positive control row as white sediment. Mycobacterial growth was confirmed by Ehrlich-Ziehl-Neelsen acid fast stain. For all drugs, the minimal inhibitory concentrations (MICs) was defined as the lowest concentration of the drug which resulted in a complete inhibition of visible growth compared to that one of drug-free growth control (100% inhibition) [21].

2.4.3 Antifungal Assay

Microdilution method was used according to a standard protocol by CLSI [24-26]. Five strains were tested each of the following species: *C. albicans* ATCC 90028 (American Type Culture Collection, USA), *Candida parapsilosis* ATCC 22019 (American Type Culture Collection, USA), *C. krusei* ATCC 6258 (American Type Culture Collection, USA), *Trichophyton mentagrophytes* var. *erinacei* NCPF 275 (National Collection of Pathogenic Fungi, Public Health England), *Microsporum gypseum* NCPF-580 (National Collection of Pathogenic Fungi, Public Health England).

RPMI 1640 broth with L-glutamine without sodium bicarbonate and 0.165 M MOPS buffer (34.54g/L) was used. The medium was adjusted to pH 7.0 at 25°C. Sterility control of each bottle was performed before it was used.

Amphotericin B was provided by the Sigma (Catalog number: A4888) as standard powder and itraconazole was provided by Johnson & Johnson (Johnson & Johnson Sıhhi Malzeme San. ve Tic. Ltd. Şti. Istanbul, Turkey). Amphotericin B, itraconazol and the extracts were dissolved in 100 % dimethyl sulfoxide and EO was dissolved in RPMI 1640 with 5% Tween80 [27] recommended as CLSI guidelines [24-26]. The final concentrations extracts were 400 to 3.13µg/mL, amphotericin B and itraconazol were 64 to 0.03µL/mL, for aqueous EO's were 50 to 0.1µL/mL.

Preparation of inoculum suspensions of dermatophytes was based according to the CLSI guidelines [24] and described previously [28]. The isolates were subcultured on to potato dextrose agar (PDA) plates at 30°C during 4-5 days. The fungal colonies were covered with 1 mL of sterile 0.85% saline and suspensions were made by gently probing the surface with the tip of Pasteur pipette. The resulting mixture

of conidia and hyphal fragments was withdrawn and transferred to a sterile tube. Heavy particles were allowed to settle for 5-10 min at room temperature; the upper suspension was mixed with a vortex for 15 sec. The turbidity of supernatants was measured spectrophotometrically (Pharmacia, LKB. Ultrospec II) at a wavelength of 530 nm, and transmission was adjusted to 65 to 75%. These stock suspensions were diluted 1:50 in RPMI medium to obtain the final inoculum sizes, which range from $1x10^3$ to $3x10^3$ CFU/mL. For dermatophytes; a constant volume (100 µL) of the inoculum was added to each microdilution well containing 100 µL of the serial dilution of drugs to reach final concentrations. The microplates which contained dermatophytes were incubated at 28°C during 7 days. The microplates were read visually with the aid to an inverted reading mirror after 7 days for dermatophytes. For all drugs except itraconazole (80% inhibition), the MICs were defined as the lowest concentration of the drug (100% inhibition) which resulted in a complete inhibition of visible growth compared to that one of drug-free growth control [24].

Preparation of inoculum suspensions of yeasts were based mainly on the CLSI guidelines [25,26]. The colonies of yeasts after 48 h at 35°C of incubation onto Sabouraud dextrose agar (BBL, Sparks, MD, USA) was subcultured to 5 mL sterile saline (0.85 %) and turbidity was adjusted spectrophotometrically at 530 nm 0.5 Mc Farland Standard, and firstly it was diluted 1:50 and then 1:20 in RPMI 1640 in order to obtain a final concentration of 0.5×10^3 to 2.5×10^3 CFU/mL.

The broth microdilution test was performed by using sterile, disposable microdilution plates (96 U-shaped wells from LP Italiano SPA, Milano, Italy). Rows from 1 to 11 contained the series of drug dilutions in 100 µL volumes and last row (row 12) contained 100 µL of drug-free medium, which served as the growth control. Each well was inoculated on the day of the test with 100 µL of the corresponding inoculum. This step brought the drug dilutions and inoculum size to the final test concentrations given above. For yeasts; a constant volume (100 µL) of the inoculum was added to each microdilution well containing 100 µL of the serial dilution of drugs to reach final concentrations. The microplates were incubated at 35°C for 48 h. For all drugs MICs except itraconazole (80% inhibition) were defined as the lowest concentration showing 100% inhibition of growth [25,26,29].

3. Results and Discussion

3.1. Antibacterial Activity Assay

Table 1. The results of the antibacterial tests.

	MICs /MBCs (µg/mL)							
	S.a.	**MRSA**	***E.f.***	***B.s.***	***K.p.***	***P.a.***	***P.m.***	***E.c.***
PE extract	625/ 1250	1250	1250	625/ 1250	1250	1250	1250	1250
$CHCl_3$ extract	625/ 1250	625/1250	1250	625/ 1250	1250	1250	1250	1250
EtOH extract	625/ 2500	1250	1250	625/ 1250	1250	625/ 2500	1250	1250
Infusion	> 5000	> 5000	> 5000	> 5000	> 5000	> 5000	> 5000	> 5000
Decoction	2500	> 5000	> 5000	> 5000	> 5000	2500/5000	> 5000	> 5000
Ciprofloxacin	0.5	0.5	0.5	0.25	0.5	0.25	0.25	0.06
DMSO	1250/2500	2500	1250	2500/5000	1250	1250	1250	1250/2500
	MICs /MBCs (µL/mL)							
EO	0.6/1.25	0.6/1.25	0.6/2.5	0.3/2.5	1.25/2.5	0.6/1.25	1.25/2.5	1.25

S.a.: Staphylococcus aureus ATCC 25923; MRSA: Methicillin resistant *Staphylococcus aureus* ATCC 43300; *E.f.*: *Enterococcus faecalis* ATCC 29212; *B.s.: Bacillus subtilis* ATCC 6633; *K.p.: Klebsiella pneumoniae* ATCC 4352; *P.a.: Pseudomonas aeruginosa* ATCC 27853; *P.m.: Proteus mirabilis* ATCC 7002; *E.c.: Escherichia coli* ATCC 25922; P.E: Petroleum ether

The antimicrobial activities of all samples were summarized by micro dilution method and disc diffusion method (Table 1). The tests were performed in duplicate and the results were signified as average values.

The EO showed more activity among all samples and it has weak activity against *S. aureus*, MRSA, *E. faecalis, P. aeruginosa*; and it has stronger activity against *B. subtilis*.

3.2. Antimycobacterial Assay

The results of antimycobacterial tests can be seen in the Table 2. In antimycobacterial activity analysis, the results of EO are very remarkable. It had stronger activity even against the multidrug resistant isolated strains. Also, it showed good activity against the other strains – susceptible; resistant to isoniazid and to rifampicin, too.

Table 2. The results of the antimycobacterial tests.

	MICs (µg/mL)				
	A	**B**	**C**	**D**	**E**
PE extract	50	100	100	100	100
$CHCl_3$ extract	100	200	200	50	200
EtOH extract	400	400	400	400	400
Infusion	400	400	400	400	400
Decoction	400	400	400	400	400
Rifampicin	< 1	< 1	> 1	> 1	> 1
Isoniazid	< 0.1	> 0.1	< 0.1	> 0.1	> 0.1
	MICs (µL/mL)				
EO	0.4	0.4	0.2	0.8	0.8

A: *M. tuberculosis* H37Rv ATCC 27294 (susceptible all antimycobacterial drugs); B: *M. tuberculosis* H37Rv ATCC 35822 (resistant to isoniazid); C: *M. tuberculosis* H37Rv ATCC 35838 (resistant to rifampicin); D: Multi-drug resistant *M. tuberculosis* (resistant to isoniazid and rifampicin – isolated from patient); E: Multi-drug resistant *M. tuberculosis* (resistant to streptomycin + isoniazid + rifampicin + ethambutol – isolated from patient) PE: Petroleum ether

3.3. Antifungal Assay

Table 3. The results of the antifungal tests.

	MICs (µg/mL)				
	F	**G**	**H**	**I**	**J**
PE extract	100	100	100	50	100
$CHCl_3$ extract	50	50	100	100	50
EtOH extract	200	200	400	100	200
Infusion	200	200	400	200	100
Decoction	200	200	400	200	200
Amphotericin B (100% inhibition)	0.5	0.5	4	2	1
Itraconazol (80% inhibition)	0.06	0.12	0.5	0.5	0.5
	MICs (µL/mL)				
EO	0.2	0.1	6.25	0.1	0.1

F: *Candida albicans* ATCC 90028; G: *Candida parapsilosis* ATCC 22019; H: *Candida krusei* ATCC 6258; I: *Trichophyton mentagrophytes* var. *erinacei* NCPF 275; J: *Microsporum gypseum* NCPF 580.

Similar results were obtained in antifungal tests (Table 3). The most active sample was EO. While it exhibited good activity against all *Candida* species, it had very good effect against dermatophytes with the MICs 0.1 µL/mL.

3.4. Isolation of the Essential Oil

As a result of all data, it can be seen that the EO was much more active than other samples, even than the standards. Thus, the study was continued with the determination of EO's chemical composition. The data and the chromatogram of the EO are seen in Figure S1 and Table 4. The major component group is oxygenated sesquiterpenes.

The plant kingdom provide over 100.000 secondary metabolites and the medicinal plants have an important place in the treatment of different diseases, also infectious diseases. Most of the compounds, isolated from natural sources, are determined as antimicrobial agents and some of them are the first step in drug discovery [33].

In a study, given the results in antimicrobial tests of EO from *Salvia tomentosa* Mill.. It exhibited an inhibition on the growth of *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Enterococcus faecalis* and *Enterobacter cloacae* [34]. Beside of this, the essential oil of *Salvia heldreichiana* Boiss. ex Benth. showed an activity on the growth of *E. coli, Salmonella typhimurium* and *Sarcina lutea*, while the EO of *Salvia cryptantha* Montbret et Aucher ex Bentham inhibited the growth of *S. lutea* [35]. In an other investigation, the reseachers demonstrated that the EOs of *Salvia multicaulis* Vahl*, S. palaestina* Benth*., S. syriaca* L. and *S. ceratophylla* L. showed a good effect on some bacteria such as *S. aureus, E. coli* and *Bacillus subtilis*; in addition, the EO of *S. multicaulis* exhibited an activity against *Pseudomonas aeruginosa* and the EO of *S. syriaca* against *P. aeruginosa* and *Streptococcus pyogenes* [36].

Beside of the studies on EO from *Salvia* species, the investigations on the antibacterial activity of their extracts in literature, too. For example, the methanol extract, gained from *Salvia libanotica* Labill., was found active against a clinical isolate of methicillin-resistant by the turkish reseachers [37].

The treatments of the tuberculosis and fungal diseases take a long time. Meanwhile, a resistance can develop against the known therapeutic agents or some toxicity problems can consist. Hereby, the treatment will not be useful. Because of the necessity of new therapeutic agents, the investigations for this aim increase day by day.

In a study in Turkey, the EOs from the aerial parts of five *Salvia tomentosa, S. aucheri* Benth., *S. fruticosa* Mill*., S. aramiensis* Rech.f. and *S. verticillata* L. subsp. *amasiaca* Freyn & Bornm. in Turkey showed an antimycobacterial activities [38,39].

16 *Salvia* species, preferred traditionally in South Africa against various infections, were investigated for their antibacterial and antimycobacterial activities and it is determined that three *Salvia* species (*S. verbenaca* L., *S. radula* Benth. and *S. dolomitica* Codd) had a remarkably effect on *M. tuberculosis* (MIC < 0.50 mg/mL) [40].

The antifungal activities of the extracts and EOs, prepared from *Salvia officinalis* L., *S. fruticosa* Miller and *S. tomentosa* were demonstrated with the previous studies [41-48].

Badiee et al. [23] compared the EO of *S. officinalis* and some antifungal agents for their antifungal effects on *C. parapsilosis, C. krusei, C. albicans* and *C. glabrata* and proposed that *S. officinalis* is as a natural antifungal agent. Furthermore, the EO from *S. sclarea* L. and the compounds, isolated from EO, (linalyl acetate and linalool), were studied against 30 different yeasts. The essential oil had stronger effect than the compounds [49]. Also, the antifungal activity of *S. officinalis* against yeasts, filamentous fungi was determined by macrodilution broth method. It showed an antifungal activity with the MIC values of 1.25-10 µL/mL; and against dermatophytes with MIC values of 0.63–2.5 µL/mL [46]. Additionally, its decoction, infusion and polar extract (prepared with methanol: water (80:20)) found effective on some *Candida* species as well as *C. parapsilosis*, by disc diffusion method [48]. In a study on the antifungal effect of the extracts, extracted with ethanol from the different parts of *Salvia tigrina* Hedge & Hub. Mor., all of them exhibited an effect, especially on *C. neoformans, C. albicans* and *Botrytis cinarea* [27]. The effects of the EO and the different extracts of *S. tomentosa* on *C. albicans* and *C. krusei* were determined [41]. Similar studies and data can be found on *S. fruticosa* in literature, too [42]. However, the antimycobacterial and antifungal activities of *S. cassia* has not been determined before.

Table 4. Composition of essential oil (EO).

KI[a]	RRI[b]	Compound	% [c]	IM[d]
1025[e]	1032	α-Pinene	0.1	tR, MS
1122[e]	1132	Sabinene	0.1	tR, MS
1245[e]	1255	γ-Terpinene	1.2	tR, MS
1282[e]	1280	*p*-Cymene	3.1	tR, MS
1480[e]	1466	α-Cubebene	0.1	MS
1488[e]	1497	α-Copaene	1.2	MS
	1515	Theaspirane A	0.1	MS
1523[e]	1535	β-Bourbonene	0.8	MS
1541[f]	1549	β-Cubebene	0.1	MS
	1556	Theaspirane B	0.3	MS
1588[f]	1589	Isocaryophyllene	1.0	MS
1590[f]	1600	β-Elemene	0.2	tR, MS
1601[f]	1611	Terpinen-4-ol	0.2	tR, MS
1608[e]	1612	**β-Caryophyllene**	**6.0**	tR, MS
1658[g]	1658	Sabinyl acetate	2.1	MS
1663[e]	1687	α-Humulene	t	tR, MS
1689[e]	1704	γ-Muurolene	t	MS
1708[e]	1726	**Germacrene D**	**4.6**	tR, MS
1723[e]	1740	α -Muurolene	tr	MS
1755[e]	1773	δ-Cadinene	0.3	MS
1820[f]	1838	(*E*)-β-Damascenone	0.2	MS
1945[g]	1945	1,5-Epoxy-salvial(4)14-ene	2.4	MS
2001[g]	2001	Isocaryophyllene oxide	1.1	MS
1986[e]	2008	**Caryophyllene oxide**	**22.3**	tR, MS
2016[e]	2037	Salvial-4(14)-en-1-one	1.7	MS
2047[e]	2071	Humulene epoxide-II	1.1	MS
2128[g]	2130	Salviadienol	1.6	MS
2124[e]	2131	Hexahydrofarnesyl acetone	0.2	MS
2126[e]	2144	**Spathulenol**	**3.1**	MS
2209[g]	2209	T-Muurolol	0.1	MS
2227[e]	2255	α-Cadinol	1.0	MS
2248[g]	2278	Torilenol	1.4	MS
	2287	**8,13-Epoxy-15,16-dinor-labd-12-ene**	**9.0**	MS
2289[g]	2289	Oxo-α-Ylangene	1.8	MS
2316[g]	2316	Caryophylla-2(12),6(13)-dien-5β-ol (=*Caryophylladienol I*)	1.0	MS
2366[e]	2369	Eudesma-4(15),7-dien-1β-ol	0.6	MS
	2373	14-Oxo-α-muurolene	0.5	MS
2392[f]	2392	Caryophylla-2(12),6-dien-5 β-ol (=*Caryophyllenol II*)	3.6	MS
	2396	13-*epi*-Manoyl oxide (=*8α-13-oxy-14-en-epilabdane*)	3.1	MS
2535[g]	2568	14-Hydroxy-α-muurolene	0.2	MS
2607[g]	2607	14-Hydroxy-δ-cadinene	0.3	MS
2613[f]	2622	Phytol	0.2	MS
2686[f]	2670	Tetradecanoic acid	1.2	tR, MS
2687[f]	2679	Manool	2.1	MS
	2735	Labda-7,14 dien-13-ol	1.6	MS
2913[e]	2931	**Hexadecanoic acid**	**5.7**	tR, MS
		Monoterpene Hydrocarbons	4.5	
		Oxygenated Monoterpenes	2.3	
		Sesquiterpene Hydrocarbons	14.3	
		Oxygenated Sesquiterpenes	43.8	
		Fatty acids	6.9	
		Diterpenes	16.0	
		Others	0.8	
		Total	**88.6**	

[a]KI Kovats indices from literature e) [30], f) [31], g) [32]; [b]RRI: Relative retention indices calculated against *n*-alkanes; [c]%: calculated from FID data; [d]IM: Identification Method; tR, identification based on the retention times (tR) of genuine standard compounds on the HP Innowax column; MS, tentatively identified on the basis of computer matching of the mass spectra with those of the Wiley and MassFinder libraries and comparison with literature data; t: Trace (< 0.1 %).

The investigations on the effects of the isolated compounds can be found in literature and are very notable to designate and evaluate the effectiveness of the plants [50,51]. As an example for these investigation, an *in vitro* study can be given, in which the usage of caryophyllene oxide (as antifungal activity) in an experimental model of onychomycosis was designated and a comparison between caryophyllene oxide, sulconazole and ciclopiroxolamine, two agents in the treatment of onychomycosis. In this study, the antifungal activity of caryophyllene oxide against dermatophytes was mentioned, too [52]. Also, on the effects of the isolated compounds, α-thujone, 1,8-cineole, β-thujone, camphor, linalool, linalyl acetate exhibited good activity against yeasts [43]. In addition, linalool and linalyl acetate have showed high effect on various *Candida* species (*C. albicans*, *Candida krusei*, *Candida parapsilosis*, etc.) [40,49]. Furthermore, two constituents from EO -camphor and 1,8-cineole- are distinguished chemicals having antimicrobial potentials [53,54].

In summary, the EO of *Salvia cassia* was most active sample in all activity tests, especially against *Bacillus subtilis*; *M. tuberculosis* strains -resistant to rifampicin and multidrug resistant-; also two dermatophytes (*Trichophyton mentagrophytes* var. *erinacei* and *Microsporum gypseum*). Therefore, the EO was investigated for its composition.

By GC-FID and GC-MS analysis of the EO of *S. cassia*, a species, belonging to the section of Aethiopis, the major compound was found caryophyllene oxide, followed by other compounds (8,13-epoxy-15,16-dinor-labd-12-ene, β-caryophyllene, hexadecanoic acid and germacrene D) [55,56]. Three species (*Salvia spinosa* L., *S. macrosiphon* Boiss. and *S. atropatana* Bunge) among 34 species in the section of Aethiopis, exhibited very different yields of caryophyllene oxide from each other in the studies on the samples collected from various localities. While the yield of caryophyllene oxide was 63% in the essential oil of *S. spinosa* collected from Iran, it was determined 0.1% in the samples from Jordan; also 26.9%, 19.3%, 11.3% and 0.7% in the essential oil of *S. macrosiphon* collected from four different localities in Iran; in the essential oil of *S. atropatana* 19.3% from Tehran, 0.7% from Fars province (Shiraz) in Iran [57-62]. According to the data in studies on the species, *S. cassia* has the highest amount of caryophyllene oxide (22.3%) after these species on the fourth place among the species in the section of Aethiopis. *S. frigida*, *S. cilici*a and *S. hypargeia* are other species, which contain caryophyllene oxide at the high yields, 19.4%, 14.9% and 10.7%, respectively. This compound was found as major component only in *S. frigida* among these three species [63-65]. There are also the species in the section of Aethiopis, in which caryophyllene oxide was determined. In the light of the studies in literature, some of these species and their content of caryophyllene oxide: *Salvia vermifolia* Hedge & Hub.-Mor. (8.69%), *S. microstegia* Boiss. & Balansa (6.2%), *S. modesta* Boiss. (5.31%), *S. aethiopis* L. (0.5-5%), *S. argentea* L. (3.28-4.11%), *S. chionantha* Boiss. (3.89%), *S. syriaca* L. (0.43-3%), *S. indica* L. (2%), *S. sclarea* L. (0.27-2.34%), *S. chrysophylla* Stapf (0.98%) and *S. limbata* C. A. Mey. (0.4%) [57, 61, 66-79].

As mentioned in "Flora of Turkey and the East Aegean Islands, VII", *S. cassia* is closely related to *S. cilicica*. As a matter of fact, *S. cilicica* contains a good ratio of caryophyllene oxide (14.9%). 8,13-epoxy-15,16-dinor-labd-12-ene, which is other major component in the essential oil of *S. cassia* (9%), was also found in *S. cilicica* (1.6%). In a study on the essential oils of *S. limbata* from two different localities (Van and Erzurum) in Turkey, their amounts were detected 0.4% and 3.7%, repectively. Additionally, this compound was determined in *S. glutinosa* (4.6%) and *S. sclarea* (1.02-3.03%), too. In brief, this compound was obtained from four species in the secton of Aethiopis [2,55,64,76,79,80].

Among isolated compounds from *S. cassia*; caryophyllene oxide, β-caryophyllene, hexadecanoic acid and germacrene D were found before in the plants, which showed antimicrobial activity, for example *Ballota pseudodictamnus* L. Bentham, *Lippia multiflora* Moldenke, *Origanum syriacum* L., *Lantana camara* L. and *Piper betle* L.. Beside of these species, three species in *Salvia* genus (*S. trichoclada* Benth., *S. multicaulis* and *S. frigida* Boiss.), recorded with their antimicrobial activities in literature, contain caryophyllene oxide as major compounds in their essential oil, with the similar yield (25.1%, 22.5%, 19.4%, respectively) as like as *S. cassia* (22.3%). These support that, these compounds may have a role in studied activities. In the light of all, it gives the impression that, this plant can be used as a source with its antimycobacterial and antifungal effects, for the aim to the treatment of various fungal diseases and tuberculosis, especially multidrug resistant tuberculosis, which is today very dangerous for the human health. It will be useful to continue the investigations on *Salvia* species for the aim to find new therapeutic agents against resistant tuberculosis and fungal diseases [36,63,64,81-89].

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/RNP

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