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**HPLC-SPE-NMR characterization of major metabolites in *Salvia fruticosa* Mill. extract
with antifungal potential: Relevance of carnosic acid, carnosol and hispidulin**

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ABSTRACT

Plant pathogenic fungi are considered of significant economic importance, for adversely affecting both quantitatively and qualitatively the fresh and the processed produce. Extracts of *S. fruticosa* were initially screened for their antifungal activity and the ethyl acetate fraction was further analyzed using HPLC-SPE-NMR hyphenation, as being the most active. The methoxylated flavones hispidulin, salvigenin and cirsimaritin and the diterpenes carnosic acid, carnosol and 12-methoxy-carnosic acid were identified as the major components of the extract. In addition, the concentration levels of all identified components were determined using q-NMR. The antifungal activity of the crude extract and selected phytochemicals was estimated against the fungal species *Aspergillus tubingensis*, *Botrytis cinerea* and *Penicillium digitatum*. The estimated MIC and MFC values of the ethyl acetate extract of *S. fruticosa*, as well as three of its major constituents, carnosic acid, carnosol and hispidulin, support their antifungal activity, especially against *B. cinerea* and *P. digitatum*, suggesting their potential use in food and agricultural systems.

Keywords: *Aspergillus tubingensis*; *Botrytis cinerea*; diterpenes; flavonoids; Greek sage; *Penicillium digitatum*; phytochemicals; q-NMR

INTRODUCTION

The genus *Salvia* is one of the largest genera in the Lamiaceae family. It comprises approximately 900 species that grow throughout the world, some of which are economically important considering their extensive use as spices, flavoring agents and herbal drugs.^{1,2} Ethnopharmacological studies reported that *Salvia* species have been used for more than sixty different ailments ranging from aches to epilepsy, and mainly to treat colds, bronchitis, tuberculosis, hemorrhage, and menstrual disorders.³ *Salvia officinalis* L. (sage), *S. miltiorrhiza* (red sage), *S. divinorum*, and *S. fruticosa* (Greek sage) are the most well-known and studied *Salvia* species.³⁻⁶

S. fruticosa is cultivated and/or grown wild in macquis and phrygana ecosystems in the Mediterranean basin.⁴ Traditionally, aerial parts of *S. fruticosa* were used to treat many diseases such as malaria, fever, pneumonia, gastro-intestinal tract disorders, cholera, plague, and common cold.⁷ Numerous studies have revealed several physiological responses to *S. fruticosa*, which may be relevant to the promotion of health and the prevention or treatment of some chronic diseases. Antiproliferative activity against cancer cells⁸ cholinesterase inhibitory activity,⁹ protection from oxidative damage of DNA,¹⁰ anti-inflammatory¹¹ and antimicrobial activity^{1,2} of *S. fruticosa* have been highlighted. Furthermore, research has manifested the inhibitory effect of *S. fruticosa* against plant pathogenic fungi, such as *Aspergillus terreus*, *Fusarium oxysporum*, *Penicillium expansum*, *Verticillium dahliae*, *Botrytis cinerea*, *Alternaria solani*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum*. It is noteworthy to mention that the antifungal potency of *S. fruticosa* has been mainly focused towards the efficacy of their essential oils, while their polar extracts have not been studied yet.¹²⁻¹⁵

Salvia species are known to be rich in a number of natural products such as essential oils, diterpenoids, triterpenoids and flavonoids.¹⁶ Phytochemical studies showed that *S. fruticosa* extracts are complex mixtures of diterpenes, phenolic acids and flavonoids, but their phytochemical composition has not been studied thoroughly yet. Previous works on *Salvia* focused on targeted phytochemicals, using mainly chromatographic techniques highlighting the characteristic presence of carnosic acid, carnosol and flavonoids.¹⁷⁻²¹

Direct crop losses caused by plant pathogenic fungi, as well as indirect product deterioration imposed by such contaminants, pose significant health and economic problems. In the agricultural sector, an array of approaches is employed to encounter the causal agents, with the use of conventional fungicides as the core component. However, concerns about environmental and human health risks associated with fungicide residues, in addition to issues regarding fungicide resistance due to their overuse, underline an increasing need to find and implement an alternative to synthetic fungicides approach, such as biofungicides.²² Natural products, crude or isolated from aromatic and medicinal plants can be envisaged as new antifungal ingredients.

In the search for naturally occurring antimicrobial agents, the antifungal activity of *S. fruticosa* extracts against the plant pathogenic fungi *A. tubingensis*, *B. cinerea*, and *P. digitatum* was assessed. The ethyl acetate extract of *S. fruticosa* was studied for first time using HPLC-SPE-NMR set-up and q-NMR in order to reveal its phytochemical composition. Then, the antifungal activity of major identified phytochemicals, namely carnosic acid, carnosol, and hispidulin was determined against plant pathogenic fungi using disk diffusion and broth microdilution assays since there are no data on their antifungal potency.

MATERIALS AND METHODS

Plant material, chemicals and fungal microorganisms

S. fruticosa Mill. was collected from the Athalassa botanical gardens in Nicosia, Cyprus (Department of Agriculture, Ministry of Agriculture, Natural Resources and Environment) and was air-dried and pulverized into fine powder. Carnosic acid ($\geq 91\%$), carnosol ($\geq 98.0\%$) and hispidulin ($\geq 98.0\%$) were obtained from Sigma-Aldrich in order to test their antifungal potency. Formulated fungicides containing the active ingredients fenhexamid (Teldor[®] 50WG; Bayer CropScience) and fludioxonil (Geoxe[®] 50WG; Syngenta Crop Protection) were purchased from local authorized suppliers. Analytical grade solvents were obtained from Acros Organics. Water was prepared by reversed osmosis (Rios, Millipore) and for HPLC MilliQ water (Millipore) was used. CH₃CN *d*₃, 99.8% was purchased from Sigma-Aldrich.

Pure cultures of *A. tubingensis*, *B. cinerea* and *P. digitatum* on Sabouraud agar, identified by molecular and standard microbiological methods were used in the antimicrobial assays. Culture media, Sabouraud dextrose agar (SDA) and Sabouraud dextrose broth (SDB) were obtained from Oxoid.

Extraction and sample preparation

The extraction was carried out on a semi-automated Soxtec system 2055 (Foss[®] Analytical, Hilleroed, Denmark) as described in previous work.²³ Three grams of plant material were placed into extraction thimbles and 80 mL of ethyl acetate were transferred into each Soxtec extraction cup. Phytochemicals were extracted by setting the unit at the boiling position for 30 min and then at the rising position for 1 h. The ethyl acetate extracts were allowed to cool down at room

temperature and evaporated to dryness in a rotary evaporator at 45°C. The dry extracts were stored at -20°C until needed.

The ethyl acetate sage extract (27.2 mg) was dissolved in 1.5 mL of acetonitrile-water (2:1, v/v) and centrifuged. The supernatant was subjected to HPLC-SPE-NMR analysis.

HPLC-SPE-NMR

HPLC-SPE-NMR measurements were carried out on a chromatographic separation system consisting of an Agilent 1200 series HPLC with an in-line solvent degasser, quaternary pump, autosampler, column compartment with thermostat, and a diode-array detector. The optimized HPLC-SPE-NMR parameters were used to analyze *S. fruticosa* extracts.²⁴ The Bruker/Spark Prospect 2 solid phase extraction unit (Bruker BioSpin and Spark, Emmen, The Netherlands) was used to trap the chromatographic peaks on Hysphere General Phase cartridges (2 mm i.d, 10-12 µm) after post-column addition of water by a Knauer K 120 HPLC pump (Berlin, Germany). The chromatographic peaks were multiply trapped from repeated HPLC injections on the same cartridge to increase their concentration, dried with dry nitrogen gas and eluted with acetonitrile-*d*₃ into 3 mm NMR tubes using a Gilson Liquid Handler 215. 1D and 2D NMR spectra were recorded on a Bruker DRX-400 MHz instrument equipped with a 3 mm inverse broadband (BBI) probe using standard Bruker pulse sequences. The NMR system was controlled by software TopSpin 1.3 (Bruker BioSpin, Rheinstetten, Germany). Chemical shifts were expressed in ppm (parts per million) referenced to the solvent peak (1.94 ppm for CD₃CN).

The chromatographic separation was carried out a Luna C₁₈, 5µm analytical column (25 cm x 4.6 mm i.d., Phenomenex). The flow rate was 0.6 mL min⁻¹, and the injection volume was

50 μ L. Gradient elution was performed using 0.1% formic acid (solvent A) and acetonitrile (solvent B) with the following linear gradient combination: at t=0 min A=90 %, at t=10 min A=70 %, t=20 min A=50 %, t=35 min A=20 %, t=50 min A=0 %, t=55 min A=0 %, and t=60 min A=90 %.

Quantitative NMR for major phytochemicals

For the quantitative analysis three independent samples were prepared by weighting 5 mg of the extract adding 300 μ L of acetonitrile- d_3 and transferred to 3 mm NMR tubes. All chemical shifts were measured with reference to the internal standard, 3-trimethylsilyl-2, 2', 3', 3'-tetra deuteriopropionic acid (TMSP- d_4) (δ_H =0.000 ppm), of known concentration (0.3 mmol L⁻¹). NMR measurements were recorded on a Bruker DRX400 MHz instrument equipped with a 3 mm inverse broadband (BBI) probe (BrukerBiospin, Rheinstetten, Germany). The following acquisition parameters were used: $\pi/2$ excitation pulse, spectral width 4006.4 Hz, 32K real data points, acquisition time 4 s, relaxation delay 15 s, number of transient accumulations 256. Data were processed by using Topspin 1.3 (Bruker) software including manual phase correction and baseline correction over the entire spectral range.

Antifungal properties

Disk diffusion assay

The inhibitory activity of the *S. fruticosa* extract and its main constituents on the mycelial growth of the plant pathogenic fungi *A. tubingensis*, *B. cinerea* and *P. digitatum* was assessed by the disk diffusion assay.²⁵ The ethyl acetate extract of *S. fruticosa*, as well as its pure carnosic acid, carnosol and hispidulin were dissolved in an 2% and 5% of aqueous dimethyl sulfoxide (DMSO) solutions, respectively and stock solutions of 1000 μ g mL⁻¹ were prepared. Conidial suspensions

(10^6 conidia mL^{-1}) of each fungus were evenly distributed on the surface of SDA Petri dishes and were allowed to dry. Subsequently, sterile 6-mm in diameter Whatman filter paper disks were impregnated with 20 mL of each antimicrobial agent and were placed in the center of the inoculated Petri dishes. The natural products were tested at concentrations of 400 and 200 $\mu\text{g mL}^{-1}$, while aqueous solutions of synthetic fungicides were used as positive reference standards at the concentration of 10 $\mu\text{g mL}^{-1}$. More specifically, the hydroxyaniline fenhexamid was used as a reference botryticide, while the phenylpyrrole fludioxonil was used against *A. tubingensis* and *P. digitatum*. Negative controls were prepared using the solvents employed to dissolve the antimicrobial agents at their highest concentration. The plates were then sealed and incubated in the dark for 72 h at 25°C. Mean inhibition zone diameters (IZD) were calculated against each microorganism by means of a ruler. Experiments were conducted three times, with two replicates per experiment.

Broth microdilution assay

A serial broth microdilution method was used to determine the minimum inhibitory concentrations (MIC) values of the ethyl acetate extract of *S. fruticosa* and its major constituents against the aforementioned plant pathogenic fungi.^{25,26} Briefly, stock solutions of the natural antimicrobials were prepared in DMSO and serially diluted to obtain the desired final concentrations ranging from 300 to 10 $\mu\text{g mL}^{-1}$. The final DMSO content was 5% (v/v), and this solution was used as negative control. It is noteworthy to mention that the DMSO controls did not show any inhibitory effects on the growth of the microorganisms tested (data not shown). One hundred μL of each tested concentration were added per well (96-well microplate) containing 80 μL of SDB and 20 μL of inoculum (10^6 conidia mL^{-1}). The final volume in each well was 200 μL . Aqueous solutions of fenhexamid and fludioxonil were used as standard

fungicides for positive control (0.5, 0.1, 0.05, 0.01, 0.005, and 0.001 $\mu\text{g mL}^{-1}$). For each microorganism a sterility control that contained only the culture medium and a growth control for the respective conidial suspension without the tested antimicrobial agents were included. The plates were covered with a sterile plate sealer, and incubated at 25°C for 72 h in an orbital shaking incubator at 120 rpm. The assay was conducted three times, with two replicates per experiment. MIC was defined as the lowest concentrations of the compound that completely inhibited visible growth of the tested fungi. After the MICs were estimated, 20 mL samples from all the optical clear wells, plus one negative control that shown growth, were spread onto the surface of SDB Petri dishes and incubated at 25°C for a minimum of 72 h until mycelial growth was visible in the control plates. The minimum fungicidal concentration (MFC) value was determined as the lowest concentration of the antimicrobial agent resulting in no growth on subculture, indicating 99.5% killing of the original inoculum.^{26,27} Each experiment was repeated three times and mean MFC values were calculated.

Statistical Analysis

The relative inhibition data for each fungal microorganism were compared using analysis of variance (ANOVA) and least significant difference (LSD) separation procedures of SAS version 9.1 (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Phytochemical analysis of *S. fruticosa* extract

Solvents of different polarity such as hexane, ethyl acetate, methanol and water were used to prepare extracts of *S. fruticosa*. Preliminary data showed that ethyl acetate extract had the most potent antifungal potency among the tested extracts (data not shown). Therefore the ethyl acetate extract of *S. fruticosa* was selected to be further analyzed. The chromatographic separation of the ethyl acetate extract of *S. fruticosa* was optimized and displayed six major peaks. The first three chromatographic peaks showed a UV spectrum (UV maxima at 260–270 nm and 340–350 nm) similar to those of flavonoids.²⁴ Their ¹H NMR spectra confirmed the flavonoid skeleton due to the presence of a deshielded signal in the region of 12–13 ppm attributed to hydroxyl proton OH-5 and the distinctive spin system in the aromatic region.²⁸ All three compounds exhibited a AA'XX' pattern with two doublets resonating at the region of ~7.9 and 6.9 ppm respectively with a *J*-coupling of 8.8 Hz, and a number of singlets at the region of 3.7–3.9 ppm indicating the presence of methoxy groups. Comparing the data obtained herein with those previously reported, compounds **1**, **2** and **3** were identified as hispidulin (4', 5, 7-trihydroxy-6-methoxyflavone), cirsimaritin (4', 5-dihydroxy-6, 7-dimethoxyflavone) and salvigenin (5-hydroxy-6, 7, 4'-trimethoxyflavone).^{29–31} The identified flavones differ in the degree of methylation of their hydroxyl groups. All compounds were unambiguously identified using 2D NMR experiments (COSY, HSQC and HMBC) and their structures are described in **Table 1** and **Figure 1**. Hispidulin and cirsimaritin were found for first time in *S. fruticosa*, although they were detected in other *Salvia* species.^{32,33} The salvigenin in *S. fruticosa* has been recently identified.³⁴

Compound **4** showed a methyl singlet at 0.85 integrating to six protons, two methyl doublets at 1.15 and 1.16 ppm respectively integrating to six protons, a 1H multiplet at 3.18 ppm, a 1H doublet at 5.39 and 1H singlet at 6.75 ppm. This combination of signals indicates a carnosol type diterpene. A comparison with literature NMR data allowed the identification of compound **4**

as carnosol. The lack of signals in the region of 4-6 ppm and the similarity observed in almost all signals indicated the presence of carnosic acid (**5**). Compound **6** was easily attributed to 12-methoxy carnosic acid, due to the additional signal at 3.64 ppm integrating to three protons that suggested the presence of a methoxy group. All results are in agreement with previously reported NMR data and listed in **Table 1** and **Figure 1**.^{35,36} Carnosic acid and carnosol are the most well-known diterpenes in *Salvia* species and they have been found in *S. fruticosa*.¹⁹ The methoxy derivative of carnosic acid (compound **6**) is widely distributed in *Salvia* species,³⁷ but its presence in *S. fruticosa* has not been reported.

Quantitative ¹H NMR (q-NMR) is an extremely useful tool for the quantification of constituents in complex plant extracts without the need of laborious and time-consuming fractionation and isolation procedures. The area (referred to as the intensity or the integral) of a NMR signal is directly proportional to the number of corresponding nuclei and thus, at variance with other techniques, the response factor is not dependent on the molecular structure. To date, q-NMR is considered to be at least as reliable and precise as the more commonly used chromatography-based techniques, while providing several advantages such as simple method development, easy sample preparation, relatively short analysis time, and multiple calibration options without the need for identical reference materials.³⁸ The quantification of polyphenols and terpenes in complex plant extracts using q-NMR has been described comprehensively.^{39,40} In **Table 1**, the concentration levels of the identified phytochemicals are displayed and shown that carnosic acid and its 12-methoxy derivative were the main components of plant extract. Carnosol and hispidulin derivatives were found at lower concentrations ranged from 8.0 mg g⁻¹ to 27.1 mg g⁻¹. Overall, the amount of diterpenes (381.3 mg g⁻¹) was significantly higher than flavonoids (45.6 mg g⁻¹) in *S. fruticosa* extract.

Antifungal potential of *S. fruticosa* extract and its constituents

It is difficult to correlate the antimicrobial activity of an extract to a specific compound due to their complexity and variability. Nevertheless, researchers have reported on the relationship between the chemical composition of the most abundant components of the extracts and their antimicrobial activity.⁴¹ In addition, a structure-activity relationship for antimicrobial activity on a plethora of chemical classes is well documented.⁴²⁻⁴³ Among the six major phytochemicals described in the present study, the selection of those that were further assessed for their antifungal activity was based on their abundance in the extract and their structural attributes. Thus, the diterpene carnosic acid was selected, since it was the predominant compound of the extract, and carnosol, for its structural dissimilarity among the other diterpenes found. With regard to the flavonoids, hispidulin was chosen because the other two, cirsimaritin and salvigenin, are hispidulin methoxy-derivatives. The antifungal activity of *S. fruticosa* was initially qualitatively assessed against *A. tubingensis*, *B. cinerea*, and *P. digitatum*. The IZD was measured and the (%) relative inhibition (RI) was estimated as the quotient of each antimicrobial agent of natural origin to that of the synthetic fungicide used per microorganism, considering the inhibition caused by each synthetic fungicide to be 100%. Overall, the inhibitory effect of all tested compounds was better at the concentration of 400 $\mu\text{g mL}^{-1}$, while among them the *S. fruticosa* extract was the most active against all fungal species, exhibiting moderate to high antifungal activity (**Table 2**). The least inhibited species by the extract was *A. tubingensis* with an RI of 49%, while for *B. cinerea* and *P. digitatum* the RI was 74.3% and 76.7%, respectively. Regarding the natural compounds present in the extract, carnosic acid was the only one that caused inhibition across all tested microorganisms, ranging from the high 62.3% RI for *B. cinerea* to the lower 31.7% RI for *A. tubingensis*, while hispidulin performed the best against *P.*

digitatum. Their antifungal potency was also quantitatively evaluated by means of MIC and MFC values (**Table 3**). The results confirmed that overall *A. tubingensis* was the least inhibited among the tested fungal species, as shown by the disk diffusion assay, with MIC values of 300 $\mu\text{g mL}^{-1}$ for all *S. fruticosa* compounds, but carnosic acid with an MIC value of 200 $\mu\text{g mL}^{-1}$. Similarly, MFC values recorded where $\geq 300 \mu\text{g mL}^{-1}$. On the other hand, MIC values of *B. cinerea* and *P. digitatum* in the presence of the extract after 72 h were 100 and 150 $\mu\text{g mL}^{-1}$, respectively. Overall, based on the MIC values of the pure phytochemicals, it can be suggested that overall *B. cinerea* was better inhibited (**Table 3**). *P. digitatum* MIC values were also in the same range. *B. cinerea* and *P. digitatum* exhibited the lowest MIC values in carnosol (100 $\mu\text{g mL}^{-1}$), followed by carnosic acid and hispidulin.

Cos et al. (2006) defined stringent endpoint criteria for “activity”, suggesting that plant extracts should be considered efficacious, if they exhibit IC_{50} values $\leq 100 \mu\text{g mL}^{-1}$ ⁴⁴. Considering that for the same active ingredient/microorganism system the MFC values, indicating 99.5% killing, should be by definition lower than its IC_{50} values, the ethyl acetate extract of *S. fruticosa* possesses antimicrobial properties worthy of further investigation, since the calculated MFC values for *B. cinerea* and *P. digitatum* were not far from the cut-off “activity” value.

The calculated MFC values for carnosic acid, carnosol and hispidulin are in the same range as the extract itself, suggesting that probably its antifungal activity cannot be solely attributed to a single constituent. The antifungal potency of the extract also cannot be correlated with cirsimatin, salvigenin, and 12-methoxy carnosic acid, since they were found at low concentrations and their chemical structures are closely similar to the tested phytochemicals.

A plethora of experimental studies have documented the promising biological activities of diterpenoids isolated from *Salvia* species, showing that most of them possess cytotoxic activity.⁴⁵ Carnosic acid and carnosol, as well as rosmanol and ferruginol have been considered to be responsible for the biological activity of *Salvia* spp.⁴⁶ Collins and Charles (1987) manifest the significant inhibition of a range of food spoilage bacteria and yeasts by carnosol,⁴⁷ while carnosic acid and carnosol have been suggested to be responsible for the antimicrobial activity of *Rosmarinus officinalis* leaf extract against selected oral, cariogenic bacteria. The potent antifungal activity of carnosic acid against an array of yeast and molds was also documented by Gigante and co-workers (2003).⁴⁸ Regarding to hispidulin, no strong antimicrobial activity against *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Salmonella typhimurium* and *Candida albicans* were found since its MIC was $> 250 \mu\text{g mL}^{-1}$.⁴⁹ The antifungal activity of cirsimaritin and salvigenin is maybe weaker than hispidulin as these compounds contain only one hydroxyl group on ring A, whereas a high antifungal activity of flavones is guaranteed by at least two hydroxyl groups on ring A.⁵⁰

The action of different classes of natural products with diverse modes may be responsible for the antifungal activity of an extract. In regards to their mode of action, carnosic acid has exhibited the capacity to act as a natural free radical scavenger with a potential chemopreventive activity, while diterpenoids with antifungal activity on *B. cinerea* have been reported to induce permeabilization of the fungal cell membrane.⁵¹⁻⁵² Flavonoids inhibit the growth of *Fusarium* spp. by different mechanisms, such as cross-linking of microbial enzymes, inhibition of microbial cellulases, xylanases, and pectinases, chelation of metals necessary for enzyme activity, and/or formation of a hard, almost crystalline, structure as a physical barrier against pathogen attack.⁵³

Overall, results obtained herein suggest the ability of the ethyl acetate extract of *S. fruticosa* to control a panel of fungal species, particularly responsible in postharvest fruit spoilage. In addition, MIC and MFC values of this extract meet the endpoint criteria for “activity”. Research could be conducted towards the application of the extract alone or in combination with synthetic fungicides in the food industry, since it could be incorporated to edible coatings in order to control decay in organic production of horticultural crops. In addition, the documented antifungal activity of the *S. fruticosa* presented in this study should be explored also in other pathosystems, in order to further elucidate its promising antimicrobial potential. HPLC-SPE-NMR and q-NMR experiments showed that diterpenes (carnosol, carnosic acid, 12-methoxy carnosic acid) and flavonoids (hispidulin, cirsimaritin, salvigenin) are the main constituents of ethyl acetate extract. Although the antifungal potency of carnosic acid, carnosol and hispidulin could be characterized as moderate, they could be used as lead compounds towards the discovery of new antifungal agents.

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472 **Figure Captions**

473 Figure 1. Structures of the major phytochemicals present in *Salvia fruticosa* Mill. extract

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475

Table 1. Retention times, ^1H NMR Chemical Shifts, and Concentration of the Chromatographic Peaks of *S. fruticosa* Ethyl Acetate Extract

Peak	Retention time (min)	Compound	^1H NMR (400 MHz, CD_3CN), δ (ppm)	Concentration (mg g $^{-1}$ extract)
1	26.1	Hispidulin	δ 13.12 (s, 1, 5-OH), 7.86 (d, 2, J=8.8 Hz, 2',6'-CH), 6.96 (d, 2, J=8.8 Hz, 3',5'-CH), 6.59 (s, 2, overlapping of 8-CH and 3-CH), 3.87 (s, 3, 6-OCH ₃)	8.0
2	29.8	Cirsimaritin	δ 12.88 (s, 1, 5-OH), 7.88 (d, 2, J=8.7 Hz, 2',6'-CH), 6.97 (d, 2, J=8.7 Hz, 3',5'-CH), 6.77 (s, 1, 8-CH) 6.63(s, 1, 3-CH), 3.93 (s, 3, 7-OCH ₃), 3.78 (s, 3, 6-OCH ₃)	10.4
3	37.0	Salvigenin	δ 12.86 (s, 1, 5-OH), 7.97 (d, 2, J=9.0 Hz, 2',6'-CH), 7.10 (d, 2, J=9.0 Hz, 3',5'-CH), 6.79 (s, 1, 8-CH) 6.67(s, 1, 3-CH), 3.94 (s, 3, 7-OCH ₃), 3.88 (s, 3, 4'-OCH ₃), 3.79 (s, 3, 6-OCH ₃)	27.1
4	38.8	Carnosol	δ 6.75 (s, 1, 14-CH), 5.39 (d, 1, J=2.8 Hz, 7-CHOC), 3.18 (m, 1, 15-	20.7

			CH(CH ₃) ₂), 2.78 (d, 1, J=13.5 Hz, 1 α -CH ₂), 2.69 (dd, 1, J=14.1 and 3.5 Hz, 1 β -CH ₂), 2.18 (m, 1, 6 β -CH ₂), 1.87 (m, 1, 2 α -CH ₂), 1.84 (m, 1, 6 α -CH ₂), 1.69 (dd, 1, J=10.6 and 5.0 Hz 5-CH), 1.60 (dt, 1, J=13.6 Hz, 2 β -CH ₂), 1.50 (d, 1, J=12.8 Hz 3 α -CH), 1.31 (ddd, 1, J=12.0, 13.6 and 3.6 Hz, 3 β -CH ₂), 1.18 (d, 3, J=6.8 Hz, 16-CH ₃), 1.16 (d, 3, J=6.7 Hz, 16-CH ₃), 0.85 (s, 6, 17-CH ₃ and 18-CH ₃)	
5	43.5	Carnosic acid	δ 6.53 (s, 1, 14-CH), 3.36 (bd, 1, J=11.6 Hz, 1 β -CH ₂), 3.14 (m, 1, 15-CH(CH ₃) ₂), 2.77 (m, 2, 7-CH ₂), 2.27 (m, 1, 6 β -CH ₂), 2.08 (d, 1, J=13.0 Hz, 2 β -CH ₂), 1.83 (bd, 1, J=12 Hz, 6 α -CH ₂), 1.45-1.56 (m, 3, overlapping of 3 α -CH ₂ , 2 β -CH ₂ and 5-CH), 1.32 (ddd, 1, J=12.8, 13.2 and 4.0 Hz, 3 β -CH ₂), 1.17 (d, 3, J=7.2 Hz, 17-CH ₃), 1.15 (d, 3, J=7.0 Hz, 16-CH ₃), 1.08 (m, 1, 1 α -CH ₂), 0.98 (s, 3, 18-CH ₃), 0.88 (s, 3, 19-CH ₃)	257.2
6	45.8	12-methoxy carnosic acid	δ 6.53 (s, 1, 14-CH), 3.51 (m, 1, 1 β -CH ₂), 3.17 (m, 1, 15-CH(CH ₃) ₂), 2.79 (m, 2, 7-CH ₂), 2.20 (m, 1, 6 β -CH ₂), 2.20 (m, 1, 2 β -CH ₂), 1.80 (bd, 1, J=13.2 Hz, 6 α -CH ₂), 1.51 (m, 3, overlapping of 3 α -CH ₂ , 2 β -	103.4

			CH ₂ and 5-CH), 1.30 (ddd, 1, J=12.8, 13.6 and 4.8 Hz, 3 β -CH ₂), 1.18 (d, 3, J=6.8 Hz, 17-CH ₃), 1.15 (d, 3, J=7.0 Hz, 16-CH ₃), 1.05 (m, 1, 1 α -CH ₂), 0.96 (s, 3, 18-CH ₃), 0.86 (s, 3, 19-CH ₃), 3.64 (s, 3, 12-OCH ₃)	
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Table 2. Antimicrobial Activity of *Salvia fruticosa* Ethyl Acetate Extract and its Constituents by Measuring the Diameter of the Inhibition Zone (IZD) Against the Tested Fungal Species, Using the Disk Diffusion Assay.

Antimicrobial agents	Concentration ($\mu\text{g mL}^{-1}$)	Fungal microorganisms					
		<i>Aspergillus tubingensis</i>		<i>Botrytis cinerea</i>		<i>Penicillium digitatum</i>	
		IZD (mm) ^a	% Relative inhibition ^b	IZD (mm)	% Relative inhibition	IZD (mm)	% Relative inhibition
<i>Salvia fruticosa</i> ^c	400	8.1	49 B ^h	13.6	74.3 B	19.8	76.7 B
	200	NI ^f	NI	7.8	42.6 E	13.1	50.1 C
carnosic acid ^d	400	5.2	31.7 C	11.4	62.3 C	7.3	28.3 F
	200	NI	NI	1.8	9.8 G	1	3.9 I
carnosol ^d	400	NI	NI	7.9	43.2 E	8.8	34.1 E
	200	NI	NI	4.4	24 F	6.9	26.7 G
hispidulin ^d	400	NI	NI	10.1	54.3 D	10.7	41.5 D
	200	NI	NI	1.8	9.8 G	2.8	12.5 H
fludioxonil ^e	10	16.4	100 A	NA	NA	25.8	100 A
fenhexamid ^e	10	NA ^g	NA	18.3 A	100 A	NA	NA

a Antimicrobial activity was evaluated by measuring the diameter of the inhibition zone (IZD) against the tested fungal species, using the disk diffusion assay. The inoculated plates were incubated at 25°C for 72 hours.

b Relative inhibition (%) was expressed as the quotient of the IZD of each antimicrobial agent of natural origin to that of the synthetic fungicide used per microorganism. The inhibition caused by the synthetic fungicides was considered 100%.

c Ethyl acetate extract of *Salvia fruticosa* in 2% (v/v) dimethyl sulfoxide (DMSO).

d Stock solutions of the pure compounds of the main constituents of *S. fruticosa* tested in 5% (v/v) DMSO.

e Aqueous solutions of the synthetic fungicides fenhexamid and fludioxonil were used as positive controls.

f NI: No inhibition zone was observed.

g NA: The antimicrobial agent was not applied.

h Relative inhibition values (%) followed by different letters within the same column were significantly different in Least Significant Difference tests at $P = 0.01$.

Figure 3. The Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of *Salvia fruticosa* Ethyl Acetate Extract and its Constituents was Evaluated Using the Broth Microdilution Method in Sabouraud Dextrose Broth

Antimicrobial agents	Fungal microorganisms					
	<i>Aspergillus tubingensis</i>		<i>Botrytis cinerea</i>		<i>Penicillium digitatum</i>	
	MIC ($\mu\text{g mL}^{-1}$) ^a	MFC ($\mu\text{g mL}^{-1}$) ^b	MIC ($\mu\text{g mL}^{-1}$)	MFC ($\mu\text{g mL}^{-1}$)	MIC ($\mu\text{g mL}^{-1}$)	MFC ($\mu\text{g mL}^{-1}$)
<i>Salvia fruticosa</i> ^c	300	<300	100	162.5	150	212
carnosic acid ^d	200	300	150	200	200	285
carnosol ^d	300	<300	100	150	100	156
hispidulin ^d	300	<300	150	200	200	275
fenhexamid ^e	NA ^f	0.5	NA	NA	NA	NA
fludioxonil ^e	NA	NA	NA	0.5	NA	0.05

a The minimum inhibitory concentration (MIC) was evaluated using the broth microdilution method in Sabouraud dextrose broth. MICs were defined as the lowest concentrations causing no visible growth. Results were read after 72 hours of incubation at 25°C.

b After the MIC values were determined, samples from all optically clear tubes (complete growth inhibition), plus a negative control showing growth were subcultured on Sabouraud dextrose agar. The dishes were incubated at 25°C for 3 days, until growth was clearly visible in the control, and the minimum fungicidal concentration (MFC) value was determined as the lowest concentration with no visible growth.

c Ethyl acetate extract of *Salvia fruticosa* in 2% (v/v) dimethyl sulfoxide (DMSO).

d Stock solutions of the pure compounds of the main constituents of *S. fruticosa* tested in 5% (v/v) DMSO.

e Aqueous solutions of the synthetic fungicides fenhexamid and fludioxonil were used as positive controls.

f NA: The antimicrobial agent was not applied.

Figure 1

Table of Contents Graphic (TOC)