

**Anti-Acetylcholinesterase and Synergistic Antifungal Activities of Selected
Salvia Species: Correlation with Metabolic Profiles**

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Abstract

Salvia species are known for their anti-fungal and anti-acetylcholinesterase (AChE) activity. Metabolomics is defined as a comprehensive quantitative and qualitative analysis of large-scale metabolites. In this study, besides determining anti-acetylcholinesterase and synergistic antifungal activities of selected *Salvia* species (*S. cryptantha*, *S. tchihatcheffii*, *S. officinalis*, *S. virgata*), the metabolite profiles were clarified and correlation analyzes between the activity results and profiles were carried out. Aerial parts of all plant materials were extracted by methanol to determine the metabolic profile by using GC-MS (Gas Chromatography-Mass Spectrometry) and LC-QTOF-MS (Liquid Chromatography Quadrupole Time of Flight Mass Spectrometry). Also, Ellman's spectrophotometric method for anti-acetylcholinesterase activity and the checkerboard method for synergistic antifungal activity between extracts and fluconazole were performed. 295 known and 1408 unknown metabolites were detected in GC-MS while 346 known and 69008 unknown metabolites in LC-QTOF-MS. *S. cryptantha*, *S. tchihatcheffii*, *S. officinalis*, and *S. virgata* demonstrated inhibitory activities on AChE with the ratio of 32.72%, 5.9%, 43.96%, and 12.1% at the concentration of 200 µg/m and a synergism was found for all *Salvia* sp. against *Candida tropicalis* ATCC 750 strain with the FICI values of 0.28, 0.15, 0.10, 0.5 respectively. In addition, as a result of correlation analysis, 73 metabolites positively and 31 metabolites negatively correlated with anti-acetylcholinesterase activity, while 18 metabolites positively, and 117 metabolites negatively correlated with synergistic activity were found. All *Salvia* species exhibited synergic activity with fluconazole while having weak AChE inhibitory activity. Combining natural products with synthetic drugs is important in order to increase efficacy. At the same time, herbal products must be used carefully at the point of herb-drug interactions. Further studies are needed to investigate active metabolites according to correlation results.

Keywords: Acetylcholinesterase inhibitory activity, Metabolomics, *Salvia*, Synergism, *Candida*

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1. Introduction

Since ancient times, people have utilized medicinal plants for their therapeutic effects.

Previous studies have revealed that these plants have a definite impact on several human biological systems, including the

nervous, urinary, and respiratory systems, and are significant sources of potential therapeutic agents [Kumar et al., 2013; Mamun et al., 2014]. Current methods among which omics technology has an important place, are being developed in the research of effective compounds in the content of medicinal plants [Verpoorte et al., 2005; Nemutlu et al., 2012].

Metabolomics (phytomics for plants) is an omics step used to determine the metabolite profile in plant extracts. GC-MS (Gas Chromatography-Mass Spectrometry) and LC-QTOF-MS (Liquid Chromatography Quadrupole Time of Flight Mass Spectrometry) are the chromatographic methods used effectively in metabolomics studies [Gonulalan et al., 2020].

In plant extracts, biological activity may sometimes be attributed to a single molecule, but two or more metabolites may contribute to the effect through synergistic or antagonistic mechanisms. Determination of which metabolites are effective and how they contribute to the activity has some challenges [Williamson, 2001].

Nowadays, metabolite-activity correlation studies are a beneficial approach for determining the effective metabolites and contribution levels [Gonulalan et al., 2020].

Recently, the increase in the prevalence of *Candida* infections has made them a substantial public health problem. Candidemia is shown as the fourth most common hospital-acquired disease in the United States due to its high mortality rates [Bibi et al., 2021]. Especially in hospitalized and immunocompromised patients, candidiasis is a major cause of fungal infections. It is especially common in individuals suffering from diabetes, cancer, AIDS, and transplant patients as well as those with other severe diseases [Ng'uni et al., 2022]. In order for antifungal medication to be effective, new solutions are required as the

epidemiology of these fungal infections is continually changing [Meirelles et al., 2017].

Candida tropicalis often ranks third or fourth as a cause of candidemia but is a significant contributor to invasive candidiasis with a 30-day mortality rate as high as 40–50%. However, it is one of the leading causes of candidemia in parts of the Asia Pacific and Latin America. Although *C. tropicalis* is known to be susceptible to many antifungal drugs, resistance to the azoles has been increasing reported [Kighley et al., 2022]. However, recent multicenter studies in Turkey have shown an increase in the prevalence of azole-resistant and azole-tolerant isolates, especially in blood isolates [Arastehfar et al., 2020].

The need for novel, alternative treatments is urgent due to the widespread use of antifungals and the rise in infections brought on by emerging species. Natural medicines have been utilized for centuries in Turkey and other parts of the world. Checkerboard method can be used for synergistic antifungal activity between extracts and FLZ [Tullio et al., 2019].

Alzheimer's can be short-defined as an irreversible neurodegenerative disease that causes severe memory loss, unusual behavior, personality changes, and cognitive dysfunction [Rao et al., 2012]. There is no cure for Alzheimer's and the drugs available are inadequate. Acetylcholinesterase inhibitors are effective and used in the treatment of Alzheimer's [Björkholm and Monteggia, 2016].

The genus *Salvia*, which belongs to Lamiaceae family, is important in terms of its richness of species, its traditional use and, its preference as a cultivated plant. Some *Salvia* species are reported to be effective in the treatment of conditions such as coronary heart disease, hepatitis, dysmenorrhea, and insomnia [Lu and Foo, 2002].

Monoterpenes, diterpenes, triterpenes, and flavonoids are the major ingredients of *Salvia* species, which are abundant in essential oils [Amaro- Luis et al., 1998].

Aim of this study is to elicit both concerned activities and metabolite profile of *Salvia* species known for their anti-fungal and anti-acetylcholinesterase (AChE) activities [Rus et al., 2015; Kobus-Cisowska et al., 2019].

2. Material and Methods

2.1. Plant Materials

Aerial parts of *S. cryptantha* Montbret & Aucher ex Benth. (HUEF20028), *S. tchihatcheffii* (Fisch. & C.A.Mey.) Boiss. (HUEF20035), *S. virgata* Jacq. (HUEF20036) were harvested during the flowering season from Ankara Beytepe region. Only *S. officinalis* L. (TBC-S-001) was purchased from the Selçuk University.

2.2. Extraction of Plant Materials

Each of the 5 g of powdered crude drug materials was extracted with 50 mL of methanol over a 30-minute period in a reflux cooler at 40 °C. The residue was then extracted for 15 minutes with 30 mL of methanol and then filtered once more. The filtrates were combined, dried with an evaporator, and then lyophilized. Quantity of extracts were obtained as 856.2, 939.7, 820.3, 690.1 mg for *S. cryptantha*, *S. tchihatcheffii*, *S. officinalis*, and *S. virgata* respectively.

2.3. Sample Preparation

2.3.1. Sample Preparation for Activity Assays

Stock solutions were prepared in methanol as 4000 ppm and diluted to final concentrations, which were 200, 100, 50, 25, and 12.5 µg/mL for extracts and 20, 10, 5, 2.5, and 1.25 µg/mL for galanthamine. The concentration ranges applied in the MIC assay were 0.5-256 µg/mL for FLZ and 2-1024 µg/mL for each *Salvia*

species. For the checkerboard assay, the ranges for FLZ and *Salvia* species extracts were 0.25 to 128 g/mL and 2 to 256 g/mL, respectively.

2.3.2. Sample Preparation for GC-MS

10 mL of methanol was used to dissolve 1 mg of lyophilized extract. Myristic acid was added as an internal standard and centrifugated. 200 mL of this solution was separated and vacuum-dried. 20 µL of 20 mg/mL methoxyamine in pyridine was used for methoxyamination (30 °C for 90 min). 80 µL of MSTFA (N-Methyl-N-(trimethylsilyl) trifluoroacetamide with 1% TMCS (trimethylchlorosilane) was used for derivatization (37 °C for 30 min). And then solutions are placed in vials with 200 µL silanized inserts for GC-MS analysis.

2.3.3 Sample Preparation for LC-QTOF-MS

1000 µg/mL plant extracts were diluted from the stock solution and 10 µg/1000 mL phenylalanine was added as an internal standard. Lyophilized plant materials were dissolved in 10 mL methanol. The samples were transferred into LC-QTOF-MS vials.

2.4. Antifungal and Checkerboard Assays

2.4.1. Strain, Culture Condition and Chemicals

The reference strain *Candida tropicalis* ATCC 750 was sub-cultured on Sabouraud Dextrose Agar (SDA, Merck) and incubated for 24 h at 37 °C. Periodically at -80°C, strains were replenished from frozen stocks. Experiments were conducted in RPMI 1640 medium supplemented with l-glutamine but lacking sodium bicarbonate (Sigma) and buffered with 0.165 M morpholinepropanesulfonic acid (MOPS) [Alkhalifa et al., 2022].

The stock solution of FLZ powder that was purchased from Abcr was prepared in dimethyl sulfoxide (DMSO) (1024 µg/mL). The stock solutions of the extracts were

prepared in DMSO at a concentration of 2048 µg/mL. Stock solutions were kept at -20 °C until use. All experiments were conducted in duplicate. It was previously done to control using DMSO 2%.

2.4.2. Antifungal Activity

C. tropicalis were assayed for susceptibility to four *Salvia* sp. and to FLZ by a broth microdilution method (MIC), in accordance with the guidelines of CLSI M27-A3 document [CLSI, 2008]. The concentration ranges used were 0.5–256 µg/mL for FLC, 2–1024 µg/mL for each extract. Plates were incubated at 37 °C for 24 h. The MIC was established as the lowest concentration of FLC and *Salvia* extract at which no visual turbidity was observed in *C. tropicalis* ATCC 750.

2.4.3. Checkerboard Microdilution Assay

The most used technique for evaluating antibacterial combinations in vitro is the checkerboard approach. Synergic interactions between FLZ and the extracts were determined by the checkerboard microtiter assay. Checkerboard synergy testing was performed by the microdilution method as previously described [Canturk, 2018]. The final concentrations for FLZ and the extract varied from 0.25 to 128 µg/mL and 2 to 256 µg/mL, respectively, from the initial concentration of fungal suspension in RPMI 1640 medium of 10³ CFU/mL. Microtiter plates were incubated at 37°C for 24 h. The fractional inhibitory concentration index (FICI) was figured out using the first non-turbid well in each column and row at 96 well U-bottom microplate. $FICI \leq 0.5$ is considered synergistic; >0.5 to <4 indifference, and ≥ 4 antagonistic.

2.5. Anti-AchE Assay

The *in vitro* modified Ellman's spectrophotometric assay was used to determine the anti-AChE activities of the samples [Ellman et al., 1961]. Briefly,

potassium phosphate buffer (pH:7.5), 5,5'-dithio-bis-(2-nitrobenzoic acid) (1.25 mM), acetylcholinesterase enzyme (electric eel, Type VI-S) and the different concentrations of test samples were incubated in a 96 well plate for 10 min. The enzymatic reaction was initiated by adding the substrate acetylthiocholine iodide (7.5 mM). The changes in the absorbance were read at 412 nm for 4 min. Galantamine was used as the reference compound.

2.6. Metabolomics Analysis

2.6.1. LC-QTOF-MS Analysis

Conditions of the LC-QTOF-MS analysis system have been previously described in Gonulalan et al., [2020].

Metabolites' auto MS-MS data were recorded above the 200-count threshold between 100 and 1700 m/z. The plant metabolites were fragmented by MS/MS using a collision energy of 20 eV.

The recorded raw MS data was processed using MS-Dial 3.96 for deconvolution, peak identification, and alignment [Tsugawa et al., 2015]. The minimum amplitude for peak detection was set at 2000 amplitude. Tolerances for MS1 and MS2 were switched to 0.01 and 0.025 Da.

The RIKEN tandem mass spectral database (ReSpect), a plant-specific ms/ms-based database, was used to predict the molecular formula and structure. A positive mode was used for the identification of metabolites. The identification score cut off value was set to 60%, and the mass tolerances for the MS1 and MS2 were modified to 0.01 and 0.05 Da. Microsoft Excel was used for correlation analysis.

2.6.2. GC-MS Analysis

Metabolomic analysis based on GC-MS was carried out as previously described [Nemutlu et al., 2015]. Microsoft Excel was used for correlation analysis.

3. Results and Discussion

3.1. Antifungal Activity

Salvia species are well-known herbal therapeutic medicines that have been utilized for centuries. Because of their antioxidant, antibacterial, antifungal, and anti-leishmanial characteristics, they are popular medicines both inside and outside of European nations [Nikmehr et al., 2014]. Due to the increase in yeast resistance, there is a need to search for new strategies in the treatment of candidiasis. In this study, a potential pharmacological strategy in anti-candidal therapy was investigated, specifically the combination of FLZ which is one of the conventional antifungals with *S. cryptantha*, *S. tchihatcheffii*, *S. officinalis* and *S. virgata* extracts of the Lamiaceae family.

In our study, we initially determined the minimum inhibitory concentrations of the FLZ and the *Salvia* species extracts in a liquid culture medium. **Table 1** show antifungal effects of FLZ and *Salvia* species extracts against to *C. tropicalis* ATCC 750. MIC results were evaluated as susceptible to *C. tropicalis* ATCC 750 in terms of CLSI standards.

Candida tropicalis strains is frequently corporate with candidiasis in neutropenic patients. Antifungal therapy can be challenging, and the yeast cellular wall's thickness has been described as a shield to polyene antifungal drugs [Pozzatti et al., 2008]. All *Salvia* species had modest

antifungal activity in this research. The best results were determined as *S. virgata* extract with 32 µg/mL against *C. tropicalis* ATCC 750 strain. Recently, the antifungal activity of *Salvia officinalis* has been studied by Martins et al., [2015] and the MICs found for the methanol extract of *Salvia officinalis* were higher than those found by us.

3.2. Checkerboard Microdilution Assay

Antifungal drug resistance problems approaches propose several possible ways of preventing and overcoming drug resistance. The efficacy antifungal drugs can be developed by using combination therapy [Samber et al., 2015]. The effect of antifungals in combination with Lamiaceae species extracts may contribute to the reduction of pathogen resistance to drugs thus making the treatment more effective [Mirghani, 2022]. This is the first time that antifungal activity of the aforementioned *Salvia* species methanol extracts in combination with FLZ has been studied.

Checkerboard microdilution assay was performed to study the synergistic activity between the antibiotic FLZ and the methanol extracts of all *Salvia* species on *C. tropicalis* ATCC 750. **Table 2** provides a summary of the findings of the checkerboard analysis. Combinations of FLZ and all of the *Salvia* species extracts which used in this study showed synergetic effects against *C. tropicalis* ATCC 750.

Table 1. Determination of MICs of *Salvia* species methanol extracts and FLZ against *C. tropicalis* ATCC 750.

	MIC (µg/mL)	CLSI M27-A3 Breakpoints		
		S	SSD	R
<i>S. cryptantha</i>	64	-*	-	-
<i>S. tchihatcheffii</i>	64	-	-	-
<i>S. officinalis</i>	128	-	-	-
<i>S. virgata</i>	32	-	-	-
FLZ	2	≤ 2.0	4	≥ 8.0

*(-) breakpoints not provided by CLSI documents M27-A3.

Table 2. Interaction of FLZ with *Salvia* sp. methanol extracts against common *C. tropicalis* evaluated by checkerboard and interpretation by fractional inhibitory concentration index.

Strain	Checkerboard MICs of Combination			
	FLZ ($\mu\text{g/mL}$)	<i>Salvia</i> sp. extracts ($\mu\text{g/mL}$)	FICI	Outcome
<i>C. tropicalis</i> ATCC 750	1	2 ^a	0.28	Synergy
	2	2 ^b	0.15	Synergy
	1	4 ^c	0.10	Synergy
	1	16 ^d	0.5	Synergy

a S. cryptantha Montbret & Aucher ex Benth, *b S. tchihatcheffii* (Fisch. & C.A.Mey.) Boiss. *c S. officinalis* L. *d S. virgata* Jacq.

The combinations of studied *Salvia* species extract and FLZ gave very pronounced synergistic effects regarding *C. tropicalis* ATCC 750 with FICI values between 0.10 and 0.5.

There is a lack of studies demonstrating the synergistic effect of *Salvia* species, synergy studies with different components have been discussed. In a study conducted by Nóbrega et al. (2019). *C. tropicalis* ATCC 13803 strain sensitive to the carvacrol/FLZ combination resulted in additive effects (FICI=1.25). Another study using thymol and carvacrol in combination with FLZ showed synergistic effects against the *C. tropicalis* ATCC 750 strain [Ahmad et al., 2013]. The findings of our research provide evidence of a synergistic effect in terms of the results compared to those obtained from other studies. The results presented here thus support the design of clinical studies to assess the effectiveness of this combination therapy. Natural medicines with intrinsic antimicrobial activity or substances that promote the action of

extensively used antibiotic/antifungal treatments may be employed as new ways to treating multi-resistant pathogens and preventing the interactions of these microorganisms with synthetic drugs. Natural substances can also be used with conventional antimicrobials to increase the antimicrobial effectiveness of both [Ermenlieva et al., 2022]. However, the cases such as overdose or side effects that may occur in the use of these *Salvia* species, which are widely used for traditional treatment, with antifungals should be investigated in further studies.

3.3. Anti-AChE Assay

The anti-AChE activity of four *Salvia* species and galanthamine are shown in **Table 3** as the percentages of inhibition. Among these four species, the most active one was determined to be *S. officinalis*. None of the *Salvia* species has significant anti-AChE activity.

Table 3. Anti-acetylcholinesterase (AChE) activity results of *Salvia* species and galanthamine.

	200 $\mu\text{g/mL}$	100 $\mu\text{g/mL}$	50 $\mu\text{g/mL}$	25 $\mu\text{g/mL}$	12.5 $\mu\text{g/mL}$
<i>S. cryptantha</i>	32.72	17.93	11.27	3.87	-
<i>S. tchihatcheffii</i>	5.90	1.47	-	-	-
<i>S. officinalis</i>	43.96	23.88	12.1	1.19	0.86
<i>S. virgata</i>	12.10	4.68	1.21	0.96	0.33
	20 $\mu\text{g/mL}$	10 $\mu\text{g/mL}$	5 $\mu\text{g/mL}$	2.5 $\mu\text{g/mL}$	1.25 $\mu\text{g/mL}$
Galanthamine	80.56	69.53	57.50	37.93	24.48

3.4. Metabolomics Analysis

Metabolomics analyses (Phytomics in plants) is a new and effective holistic approach to clarifying the metabolic profile of plant extracts, as well as providing some unique opportunities to identify active metabolic compound(s) when combined with correlation analysis [Waris et al., 2022].

After the GC-MS chromatograms were deconvolved and aligned, 1703 mass spectral features were found, of which 295 were analyzed using retention index libraries. Major compound groups and quantities of the metabolites are listed in **Table 4**.

Table 4. GC/MS Based Metabolomic Profiling (Major metabolites).

Super Class	Class / Sub Class / Parent	Quantity of Metabolites
Organic acids and derivatives	Amino acids, peptides, and analogues	52
	Hydroxy acids and derivatives	7
	Dicarboxylic acids and derivatives	6
	Keto acids and derivatives	6
Organic oxygen compounds	Carbohydrates and carbohydrate conjugates	56
Lipids and lipid-like molecules	Fatty Acyls	33
	Prenol lipids	10
Benzeneoids	Benzene and substituted derivatives	19
	Phenols	11
Organoheterocyclic compounds	Azoles, Indoles and derivatives, Pyridines and derivatives, etc..	27
Phenylpropanoids and polyketides	Cinnamic acids and derivatives, Flavonoids, Phenylpropanoic acids, etc..	24

69354 peaks were discovered in the LC-qTOF-MS data, 346 of which were recognized by the MS/MS spectrum. **Table 5** lists the major compound groups and quantity of metabolites.

Table 5. LC-QTOF-MS Based Metabolomic Profiling (Major metabolites).

Types of Metabolites	Quantity of Metabolites
Flavonoid-7- <i>O</i> -glycosides	15
Flavonoid-3- <i>O</i> -glycosides	10
Anthocyanidin-3- <i>O</i> -glycosides	10
Anthocyanidin-5- <i>O</i> -glycosides	5
Flavonols	5
Methoxyphenols	5
Hydroxycinnamic acids	4
Hydroxybenzoic acid derivatives	3
7-hydroxycoumarins	2

3.5. Correlation Analysis

Correlation analysis can be used to find active ingredients of plant materials. For this purpose, total compound groups are usually selected [Muflihah et al., 2021]. In our study different and more specific compound groups are correlated with the activity. Also the number of the correlated metabolites and the correlation coefficients has determined.

The LC-QTOF-MS analysis's correlation studies revealed that 30 metabolites positively correlated ($r \geq 0.85$) with the anti-AchE activity, while 22 metabolites were negatively correlated ($r \leq -0.85$).

The GC-MS analysis's correlation studies revealed that 43 metabolites positively correlated ($r \geq 0.85$) with the Anti-AchE activity, while 9 metabolites were negatively correlated ($r \leq -0.85$).

The LC-QTOF-MS analysis's correlation studies revealed that 14 metabolites had a positive correlation ($r \geq 0.85$) with the synergistic effects with FLZ, while 66 metabolites displayed a strongly negative correlation ($r \leq -0.85$).

The GC-MS analysis's correlation studies revealed that 4 metabolites had a positive correlation ($r \geq 0.85$) with the synergistic effects with FLZ, while 51 metabolites displayed a strongly negative correlation ($r \leq -0.85$).

4. Conclusion

The present study provides new information regarding the anticandidal potential of *S. cryptantha*, *S. tchihatcheffii*, *S. officinalis*, *S. virgata* methanol extracts and their synergistic effects with FLZ. The results indicate that all *Salvia* species studied possess anticandidal activity as well as combined high anticandidal effect and high synergistic interaction with the FLZ. We selected a relatively small number of samples because we regarded it as a form of a pilot study. Additional studies are necessary to

determine the mechanism of these synergistic antifungal associations.

According to our results, FLZ and *Salvia* species extracts combination may be a potential therapeutic option for the treatment of *C. tropicalis* related infections.

According to the anti-AChE activity results, four *Salvia* species has no significant anti-AChE activity. Only *S. officinalis* showed moderate effect. Further studies are needed to clarify metabolite/metabolites as determined by the correlation analyses may have higher activity or not.

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Author Contribution

EMG contributed Anti-Acetylcholinesterase activities and correlation analysis, considered and designed the research, drafted the manuscript, and approved the final version of the manuscript. CK performed anti-Acetylcholinesterase activities and also contributed to the draft of the manuscript. NBB contributed the antifungal and synergic activities by using the broth microdilution method, and checkerboard microdilution assay, respectively, and also contributed to the draft of the manuscript. GBY performed the antifungal and synergic activities by using the broth microdilution method and checkerboard microdilution assay, respectively, and also contributed to the draft of the manuscript.

Conflicts of Interest

The authors have no conflicts of interest to declare and disclose any financial field.

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