

RESEARCH ARTICLE

Rapid evaluation of *Salvia pachystachya* essential oil against three *Colletotrichum* species causing anthracnose on strawberries

Nurhayat Tabanca^{1*}, Betül Demirci², David E. Wedge³, Zeki Aytaç⁴, K. Hüsnü Can Başer⁵

¹ USDA-ARS, Subtropical Horticulture Research Station, 13601 Old Cutler Rd., Miami, FL 33158 USA

² Department of Pharmacognosy, Faculty of Pharmacy, Anadolu University, 26470, Eskisehir, TURKEY

³ USDA-ARS, Natural Products Utilization Research Unit, University of Mississippi, University, MS 38677 USA

⁴ Department of Biology, Faculty of Science, Gazi University, 06500 Ankara, TURKEY

⁵ Department of Pharmacognosy, Faculty of Pharmacy, Near East University, Nicosia, N. Cyprus, Mersin 10, TURKEY

*Corresponding author. Email: Nurhayat.Tabanca@ars.usda.gov

Abstract

The objective of this study was to evaluate the antifungal activity of *Salvia pachystachya* Trautv. (Lamiaceae) essential oil against plant pathogens that cause anthracnose, *Colletotrichum acutatum* J. H. Simmonds, *C. fragariae* A.N. Brooks and *C. gloeosporioides* (Penz.) Penz. & Sacc., and to determine its chemical composition. The essential oil was analyzed by gas chromatography-mass spectrometry (GC-MS) and major components were identified as α -pinene (18.9%), (Z)- β -ocimene (11.8%) and 1,8-cineole (8.0%). The oil showed weak antifungal activity against three *Colletotrichum* species using direct-bioautography assay.

Keywords: Antifungal, bioautography, GC-MS, sage, monoterpenoids, *Salvia*, Turkey

Introduction

In recent years, consumption of strawberries has increased due to their potential human health benefits (Henning et al., 2010; Giampieri et al., 2012). Based on 2017 statistics, (Retrieved from www.statista.com/statistics/194235/top-10-strawberry-producing-us-states), California and Florida are the two top strawberry producing states in the United States, with California production totaling over 29.22 million cwt (hundredweight), followed by Florida at 2.41 million cwt. Consumer interest is for large, attractive, blemish-free strawberries, sweeter and more flavorful, yet produced with less agrochemical input.

However, anthracnose of strawberry (*Fragaria x ananassa* Duch.), caused by *Colletotrichum* species, is a highly damaging disease in strawberry nursery and fruit production fields in the United States (Wedge et al., 2007; Smith, 2008). The pathogens *C. acutatum* J. H. Simmonds, *C. fragariae* A.N. Brooks and *C. gloeosporioides* (Penz.) Penz. & Sacc. can occur alone or in combination and spores can infect flowers, fruit, leaves, petioles, stolons, and crowns on the strawberries (Smith, 2008). Anthracnose fruit rot appears as dark brown to black spots at any stage of symptoms on both immature and mature fruit (Figure 1) (Retrieved from <https://content.ces.ncsu.edu/anthracnose-fruit-rot-of-strawberry>). Increasing incidence of resistance to fungicides by plant pathogens and undesirable side effects to humans and the environment has focused attention towards natural products that can be used as Integrated pest management alternatives to commonly be used single-site fungicides.

Figure 1. Anthracnose Fruit Rot showing a range of symptoms on green and ripe fruit, including newly formed lesions, fruit nearly covered by lesions and spores, and a mummified fruit that has desiccated.



(Photo credit: Frank J. Louws, NC State Extension Publications)

The mission of the U.S. Department of Agriculture-Agricultural Research Service (USDA-ARS) at the Natural Products Utilization Research Unit (NPURU) at the Oxford, Mississippi is to develop natural products for uses in agriculture in order to produce more environmental and toxicologically benign disease and pest management tools. This broad mission includes exploration of plant-derived chemicals to control important plant pathogenic fungi. Therefore, we selected unique plants that are often used in traditional medicine or aromatic plants that previously were not studied as potential sources for new agrochemical fungicides. Plant essential oils and extracts have been of great interest to replace synthetic fungicides with natural ones. *Salvia pachystachya* Trautv. (Lamiaceae) collected from eastern Turkey was chosen for this study and its essential oil was evaluated for antifungal activity against three *Colletotrichum* species. To the best of our knowledge, the antifungal activity of *S. pachystachya* essential oil against these plant pathogens has not been studied previously for potential anthracnose control.

Salvia L. species (sage) are one of the most important aromatic plants in the Mediterranean region and Turkey is one of the most important sage-producing countries in the world (Engels, 2011). The genus *Salvia* is represented by 107 taxa, including 58 taxa (54%) that are endemic to Turkey (Celep & Dirmenci 2017). Sage species have been traditionally used as tea under the common name “adaçayı” in Turkey and are commonly used as remedies for colds and sore throats (Tabanca et al., 2017). *Salvia pachystachya* is of Irano-Turanian origin which is widespread in Transcaucasia and N.W. Iran and mainly distributed in eastern and northeastern portions of Anatolia (Retrieved from www.bizimbitkiler.org.tr). The plant is known as “Yedişalba” in Turkey (Retrieved from www.bizimbitkiler.org.tr) and morphologically differs from other species with dwarf mat-forming subshrubs, with 2-4 pairs of leaflets, leaflets obovate and terminal segments lanceolate; petiole long ciliate; verticillasters 2-6 flowered; bracts ovate to acuminate; calyx campanulate and often purplish, eglandular pilose to villose with sessile glands to densely capitate-glandular; corolla violet-blue to lilac (rarely white). *Salvia pachystachya* grows on rocky limestone and igneous slopes, screes, subalpine pasture, at an altitude of 1200- 3200 m, flowering time is May to July.

Materials and Methods

Plant material

Aerial parts of *S. pachystachya* were collected from Ağrı: Doğubeyazıt-Çaldıran, 16 km, 2150 m, steppe, collected on June 08, 2001. The specimen (Figure 2) was identified by Prof. Dr. Zeki Aytaç (Gazi University, Ankara, Turkey) using Flora of Turkey (Hedge, 1982). A voucher specimen has been deposited at the GAZI Herbarium in Ankara, Turkey (Karaveliogullari 3056 et al.).

Isolation of the essential oil

The air-dried plant materials (flowers, leaves, and stems) were subjected to hydrodistillation for 3 hours using a Clevenger-type apparatus in 2001. The oil yield was calculated as 0.15%, v/w on dry weight basis and oil was stored at 4°C in the dark until analyzed.

Figure 2. Herbarium specimen of the *Salvia pachystachya* (Photo courtesy of Zeki Aytaç)



Gas chromatography–mass spectrometry (GC-MS) analysis conditions

GC-MS analysis of *S. pachystachya* was subsequently performed with a Hewlett-Packard GCD system. Innovax FSC column (60 m x 0.25 mm, 0.25 µm film thickness) was used with helium as carrier gas. The oven temperature was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/min, then kept constant

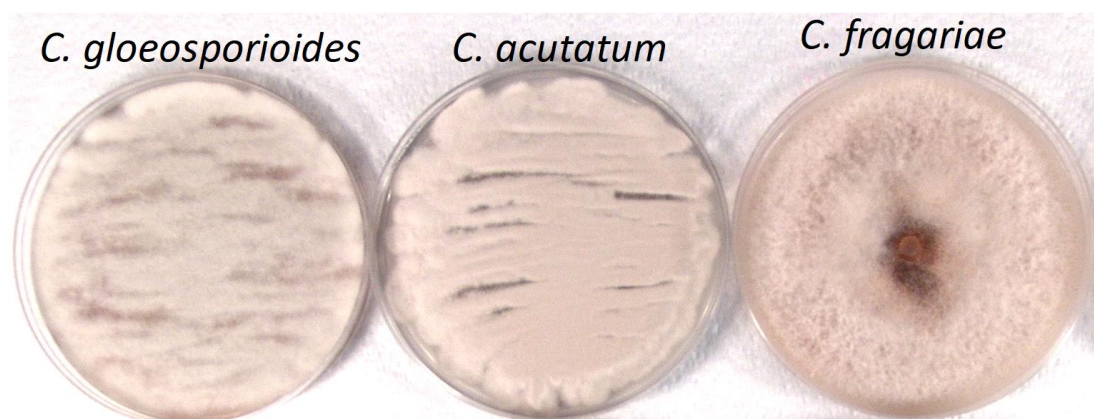
at 220°C for 10 min and programmed to 240 °C at a rate of 1°C/min. Split flow was adjusted at 50 mL/min. The injector temperature was at 250°C. Mass spectra were recorded at 70 eV. Mass range was from m/z 35 to 425. Relative percentages of the characterized components were as cited in Table I.

Identification of the essential oil components was carried out by comparison of their relative retention times with those of authenticated samples or by comparison of their relative retention index (RRI) to series of *n*-alkanes. Computer matching against commercial libraries, Wiley GC/MS Library, MassFinder 3 Library, (McLafferty & Stauffer, 1989; Koenig, Joulain, & Hochmuth, 2004) and in-house “Baser Library of Essential Oil Constituents” built up by chemical standards and known components of essential oils, as well as MS literature data (Joulain & Koenig, 1998; ESO 2000, 1999).

Direct bioautography for antifungal activity

Isolates of *Colletotrichum acutatum*, *C. fragariae*, *C. gloeosporioides* were obtained from Barbara J. Smith, USDA, ARS, Small Fruit Research Station, Poplarville, MS, USA. Fungal cultures were grown on potato dextrose agar (PDA, Difco, Detroit, MI) in 9-cm petri dishes (Figure 3) and incubated in a growth chamber at 25 ± 2 °C with a 12-h photoperiod under 60 ± 5 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light. Conidial concentrations were determined photometrically and suspensions were adjusted, based on a standard curve, with sterile distilled water to a stock concentration of 1.0×10^6 conidia/mL (Wedge and Kuhajek, 1998; Wang et al., 2008). Conidia were harvested from two-week-old cultures by flooding plates with 5 mL of sterile distilled water and dislodging conidia from acervuli using an L-shaped glass rod. Conidial suspensions were filtered through sterile Miracloth (Calbiochem-Novabiochem Corp., La Jolla, CA, USA) to remove mycelia. Subsequently, the conidia of *Colletotrichum* suspensions were adjusted to 3.0×10^5 conidia/mL with liquid potato-dextrose broth (PDB, Difco, Detroit, MI, USA) and 0.1% Tween-80. To detect biological activity directly on the Thin-layer chromatography (TLC) silica gel plate, TLC plates (10 x 10 cm, 250 μm , Silica Gel GF Uniplate, Analtech, Inc., Newark, DE, USA) were inoculated with a spore suspension. Inoculated plates were placed in a 30 x 13 x 7.5 cm moisture chamber (398-C, Pioneer Plastics, Inc. Dixon, KY, USA) and incubated in a growth chamber at 24 ± 1 °C and 12-h photoperiod under 60 ± 5 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light. For manual reading, inhibition of fungal growth diameters was measured with a ruler and recorded in mm. Technical grade fungicide captan (Chem Service, Inc. West Chester, PA, USA) was used as controls at 2 mM in 2 μL of 95% ethanol. The bioassay was performed in 2001.

Figure 3. Fungal cultures of three *Colletotrichum* test species in potato-dextrose agar (PDA) (Photo courtesy of Nurhayat Tabanca & David E. Wedge)



Results and Discussion

The chemical composition of *S. pachystachya* essential oil was analysed by GC-MS and the analysis showed that forty-nine components accounted for 93.4% of the total oil. The main components consisted of α -pinene (18.9%), (Z)- β -ocimene (11.8%) and 1,8-cineole (8.0%) (Table 1). Chemical composition of *S. pachystachya* essential oil from Turkey (Temel et al., 2016; Hatipoglu et al., 2016) and Iran has been previously reported (Shakeri et al. 2018). *Salvia pachystachya* essential oil obtained from Erzincan (Turkey) was found to be rich in α -pinene (24.0%), α -pinene (12.2%), spathulenol (10.4%), viridiflorol (7.7%) and 1,8-cineole (6.5%) (Temel et al., 2016). Volatile components of *S. pachystachya* collected from another source of Turkey (Agri) was analysed by a thermal desorption (TD)-GC-MS technique and borneol (10.70%), β -pinene (10.10%), cedryl propyl ether (7.14%), α -pinene (6.27%) and spathulenol (6.26%) were detected as the main components (Hatipoglu et al., 2016). *Salvia pachystachya* essential oil obtained from Iran exhibited a different chemical profile. It contained camphor (31.0%), 1,8-cineole (13.5%), camphene (11.7%), α -pinene (8.0%), β -bourbonene (5.1%) and spathulenol (4.3%) (Shakeri et al. 2018). This variation could be due to differences in genotype, ecological conditions and/or extraction methods.

Table 1. The composition of the essential oil of *Salvia pachystachya*

RRI*	Compound	%**
1032	α-Pinene	18.9
1035	α -Thujene	1.6
1118	β -Pinene	0.8
1132	Sabinene	2.2
1174	Myrcene	1.8
1203	Limonene	4.3
1213	1,8-Cineole	8.0
1246	(Z)-β-Ocimene	11.8
1255	γ -Terpinene	0.2
1266	(E)- β -Ocimene	0.7
1280	<i>p</i> -Cymene	1.2
1290	Terpinolene	<0.1
1382	<i>cis</i> -Alloocimene	0.4
1406	α -Fenchone	0.4
1429	Perillene	0.1
1497	α -Copaene	2.2
1535	β -Bourbonene	1.1
1553	Linalool	1.8
1562	Octanol	0.3
1586	Pinocarvone	0.1
1589	β -Ylangene	0.3
1591	Bornyl acetate	0.5
1600	β -Elemene	0.9
1612	β -Caryophyllene	3.2
1641	Methyl benzoate	0.1
1648	Myrtenal	0.1
1661	Alloaromadendrene	2.1

1687	α -Humulene	4.3
1706	α -Terpineol	2.4
1726	Germacrene D	5.7
1751	Carvone	0.3
1773	δ -Cadinene	0.5
1776	γ -Cadinene	0.3
1804	Myrtenol	0.2
1830	2,6-Dimethyl-3(E),5(E),7-octatriene-2-ol	0.1
1864	<i>p</i> -Cymen-8-ol	0.2
1866	Methyl hydrocinnamate	0.3
1988	2-Phenylethyl-2-methylbutyrate	0.2
2008	Caryophyllene oxide	1.8
2071	Humulene epoxide-II	1.4
2122	Hedycaryol	4.4
2144	Spathulenol	0.6
2148	(Z)-3-Hexen-1-yl benzoate	0.2
2185	γ -Eudesmol	1.3
2200	α -Guaiol	0.3
2204	Eremoligenol	0.3
2239	Carvacrol	0.1
2250	α -Eudesmol	1.5
2257	β -Eudesmol	1.9
Total		93.4

*RRI Relative retention indices calculated against *n*-alkanes, **% calculated from TIC data

To discover natural fungicides, *S. pachystachya* essential oil was dissolved in *n*-hexane and applied directly on the TLC plates without separation. TLC plates were then subjected to the direct bioautography bioassay against *C. acutatum*, *C. fragariae* and *C. gloeosporioides*. *Salvia pachystachya* essential oil demonstrated clear zones of inhibition between $2.6 \text{ mm} \pm 1.41 \text{ mm}$ and $3.3 \pm 1.73 \text{ mm}$ at the 80 and 160 μg applications, respectively. The diameter of the inhibition zone did not increase significantly with increasing concentrations of *S. pachystachya* essential oil. The positive control captan is a well-known multisite inhibitor fungicide and it showed clear inhibitory zones of $16.63 \pm 2.03 \text{ mm}$ at 1.2 μg concentration. The weak antifungal result observed with *S. pachystachya* essential oil might be due to its monoterpene rich composition. Based on our previous studies, we found that essential oils rich in monoterpene hydrocarbons did not show inhibitory effect on the growth of *Colletotrichum* species (Tabanca et al., 2006, 2014). In this study, *S. pachystachya* essential oil contained twelve monoterpene hydrocarbons (43.9%), twelve oxygenated monoterpenes (14.2%), ten sesquiterpene hydrocarbons (20.6%), oxygenated sesquiterpenes (13.5%) and six other components (1.2%) (Table 1). We previously observed higher activity in the essential oils rich in oxygenated monoterpenes [Tabanca et al. 2007a; Altintas et al. 2013] or phenylpropanoids [Tabanca et al. 2005 & 2007b]. It can be concluded that the chemical profile of essential oil plays an important role in antifungal activity. Essential oils rich in monoterpenoids maybe eliminated by employing them antifungal assays against these three *Colletotrichum* species.

Direct bioautography is a rapid screening assay to determine antifungal activity in plant extracts such as essential oils. Antifungal compounds can be directly seen on the TLC plates where clear zones inhibit fungal

growth. Chemical composition of the essential oils plays a critical role in the antifungal activity, and essential oils rich in α -pinene, (Z)- β -ocimene and 1,8-cineole are not likely to exhibit significant antifungal activity against *Colletotrichum* species.

ACKNOWLEDGMENT

The authors thank Ms. Linda Robertson and Ms. Ramona Pace for performing antifungal assays. We thank Dr. Paul E. Kendra and Dr. Alina Puig (USDA-ARS, SHRS, Miami, FL, USA), and the journal editor/referees for critical reviews of an earlier version of this manuscript. The mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, Agricultural Research Service and does not imply its approval to the exclusion of other products that may also be suitable. The authors declare that they have no conflict of interest.

REFERENCES

- Altintas, A., Tabanca, N., Tyihak, E., Ott, P. G., Moricz, M., Mincsovcics, E., Wedge, D. E. (2013). Characterization of Volatile Constituents from *Origanum onites* and Their Antifungal and Antibacterial Activity. *Journal of AOAC International*, 96, 1200-1208.
- Celep, F., & Dirmenci, T. (2017). Systematic and Biogeographic overview of Lamiaceae in Turkey. *Natural Volatiles & Essential Oils*, 4(4), 14-27.
- Hedge, I. C. (1982). *Salvia* L., in Davis, P. H., Edmonson J. R., Mill, R. R., Kit Tan (eds.) *Flora of Turkey and the East Aegean Islands*, vol. 7, pp. 400-462, Edinburgh Univ. press, Edinburgh.
- Engels, G. (2011). Sage, *Salvia officinalis*, *HerbalGram*, *The Journal of the American Botanical Council*, 89, 1-4.
- ESO 2000, (1999). *The Complete Database of Essential Oils*. Boelens Aroma Chemical Information Service: The Netherlands.
- Giampieri, F., Tulipani, S., Alvarez-Suarez, J. M., Quiles, J. L., Mezzetti, B., Battino, M. (2012). The Strawberry: Composition, Nutritional Quality, and Impact on Human Health. *Nutrition*, 28, 9-19.
- Hatipoglu, S. D., Zorlu, N., Dirmenci, T., Goren, A. C., Ozturk, T., Topcu, G. (2016). Determination of Volatile Organic Compounds in Forty-Five *Salvia* Species by Thermal Desorption-GC-MS Technique. *Records of Natural Products*, 10, 659-700.
- Henning, S. M., Seeram, N. P., Zhang, Y., Li, L., Gao, K., Lee, R.P., Wang, D.C., Zerlin, A., Karp, H., Thames G., Kotlerman, J., Li, Z., Heber, D. (2010). Strawberry Consumption is Associated with Increased Antioxidant Capacity in Serum. *Journal of Medicinal Food*, 13, 16-22.
- <https://www.statista.com/statistics/194235/top-10-strawberry-producing-us-states/> (accessed on December 7, 2019).
- <https://www.bizimbitkiler.org.tr> (accessed on December 7, 2019).
- <https://content.ces.ncsu.edu/anthracnose-fruit-rot-of-strawberry> (accessed on February 14, 2019).
- Joulain, D., & Koenig, W. A. (1998). *The Atlas of Spectra Data of Sesquiterpene Hydrocarbons*. E.B.-Verlag: Hamburg.
- Koenig, W. A., Joulain, D., & Hochmuth, D. H. (2004). Terpenoids and related constituents of essential oils. MassFinder 3. Hamburg, Germany: Hochmuth Scientific Consulting.
- McLafferty, F. W., & Stauffer, D. B. (1989). *The Wiley/NBS Registry of Mass Spectral Data*; J. Wiley and Sons: New York.
- Shakeri, A., Sharifi, M. J., Bazzaz, B. S. F., Emami, A., Soheili, V., Sahebkar, A., Javad, A. (2018). Bioautography Detection of Antimicrobial Compounds from the Essential Oil of *Salvia pachystachys*. *Current Bioactive Compounds*, 14, 80-85.
- Smith, B. J. (2008). Epidemiology and pathology of strawberry anthracnose: A North American Perspective. *Hortscience* 43, 69-73.

- Tabanca, N., Bedir, E., Ferreira, D., Slade, D., Wedge, D. E., Jacop, M. R., Khan, S. I., Kirimer, N., Baser, K. H. C., Khan, I. A. (2005). Bioactive Constituents from Turkish *Pimpinella* Species. *Chemistry & Biodiversity*, 2, 221-232.
- Tabanca, N., Demirci, B., Baser, K. H. C., Aytac, Z., Ekici, M., Khan, S. I., Jacob, M. R., Wedge, D. E. (2006). Chemical Composition and Antifungal Activity of *Salvia macrochlamys* and *Salvia recognita* Essential Oils. *Journal of Agricultural and Food Chemistry*, 54, 6593-6597.
- Tabanca, N., Demirci, B., Crockett, S. L., Baser, K. H. C., Wedge, D. E. (2007a). Chemical Composition and Antifungal Activity of *Arnica longifolia*, *Aster hesperius*, and *Chrysothamnus nauseosus* Essential Oils. *Journal of Agricultural and Food Chemistry*, 55, 8430-8435.
- Tabanca, N., Demirci, B., Baser, K. H. C., Mincsovcics, E., Khan, S. I., Jacob, M. R., Wedge, D. E. (2007b). Characterization of Volatile Constituents of *Scaligeria tripartita* and Studies on the Antifungal Activity against Phytopathogenic Fungi. *Journal of Chromatography B*, 850, 221-229.
- Tabanca, N., Gao, Z., Demirci, B., Techen, N., Wedge, D. E., Ali, A., Sampson, B. J., Werle, C., Bernier, U. R., Khan, I. A., Baser, K. H. C. (2014). Molecular and Phytochemical Investigation of *Angelica dahurica* and *Angelica pubescentis* Essential Oils and Their Biological Activity against *Aedes aegypti*, *Stephanitis pyrioides*, and *Colletotrichum* Species. *Journal of Agricultural and Food Chemistry*, 62, 8848-8857.
- Tabanca, N., Demirci, B., Aytac, Z., Baser, K. H. C. (2017). The chemical composition of *Salvia verticillata* L. subsp. *verticillata* from Turkey. *Natural Volatiles & Essential Oils*, 4, 18-28.
- Temel, H. E., Demirci, B., Demirci, F., Celep, F., Kahraman, A., Dogan, M., Baser, K. H. C. (2016). Chemical characterization and anticholinesterase effects of essential oils derived from *Salvia* species. *Journal of Essential Oil Research*, 28, 322-331.
- Wang, X., Wedge, D. E., Tabanca, N., Johnson, R. D., Cutler, S. J., Pace, P. F., Smith, B. J., Zhou, L. (2008). Development of a miniaturized 24-well strawberry leaf disk bioassay for evaluating natural fungicides. *Natural Product Communications*, 3, 1079-1084.
- Wedge, D. E., Kuhajek, J. M. (1998). A microbioassay for fungicide discovery. *Saas Bulletin Biochemistry and Biotechnology*, 11, 1-7.
- Wedge, D. E., Smith, B. J., Quebedeaux, J. P., Constantin, R. J. (2007). Fungicide management strategies for control of strawberry fruit rot diseases in Louisiana and Mississippi. *Crop Protection*, 26, 1449-1458.

Received: 08.03.2019

Accepted: 16.04.2019