African Journal of Microbiology Research Vol. 6(12), pp. 3079-3084, 30 March, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR12.569
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Antifungal activity of lemon balm and sage essential oils on the growth of ochratoxigenic *Penicillium verrucosum*

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Accepted 20 March, 2012

In this study, the chemical compositions of essential oils (EOs) from lemon balm (*Melissa officinalis*) and sage (*Salvia officinalis*) were determined using gas chromatography/mass spectrometry (GC/MS) method, indicating that 1,2-propanediol (31.26%) and *α*-thujone (24.92%) were the major components in lemon balm and sage, respectively. In addition, antifungal effects of these EOs against ochratoxigenic *Penicillium verrucosum* Dierckx (D-99756) isolated from Kashar Cheese were investigated. In order to test their antifungal effects, the EOs dilutions were prepared in methanol at seven different concentrations (500, 250, 125, 62, 31, 15.5 and 7.75 µl/ml; the values corresponding to 0.5, 0.25, 0.125, 0.062, 0.031, 0.015 and 0.00775% based on agar medium). Their antifungal activities were determined with respect to minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC). The MIC values of lemon balm and sage EOs against *P. verrucosum* were determined to be the 125 and 62 µl/ml, respectively; and their MFC values were found to be 250 and 125 µl/ml, respectively, indicating that sage exhibited two fold fungistatic and fungicidal effect against ochratoxigenic *P. verrucosum* than did lemon balm. The results of this study suggest that lemon balm and sage EOs can be considered as natural antifungal agents that can be used to inactivate ochratoxigenic *P. verrucosum* with respect to food safety applications.

Key words: *Penicillium verrucosum*, lemon balm, sage, fungicidal and fungistatic effect.

INTRODUCTION

Penicillium verrucosum is predominantly responsible for occurrence of ochratoxin A (OTA) mainly in cereals and cereal products, especially in northern Europe where cooler damp harvesting conditions exist (Lund and Frisvad, 2003; Magan and Olsen, 2004). Being a potent nephrotoxic mycotoxin, OTA is linked to kidney problems in both livestock and human populations. It is a well-known genotoxic mycotoxin, which has also carcinogenic, teratogenic and immunotoxic properties. Although cereal and cereal products are held responsible for most of the worldwide human intake (Cholmakov-Bodechtel et al., 2000), natural occurrence of OTA has also been reported in a variety of common foods and beverages such as chocolate, coffee, fried fruits, grape juice, pork, poultry, beer and wine (Clark and Snedeker, 2006).

Occurrence of OTA has generally been linked to inefficiently dried grains, which can result in pockets of growth by *P. verrucosum* in storage (Magan et al., 2003, 2004). It has also been shown that sub-optimal levels of fungistats that are one of the aliphatic acid-based treatments may stimulate growth and mycotoxin production by *P. verrucosum* strains (Arroyo et al., 2005). Furthermore, OTA has been suggested to be phenotypically produced

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under some stress factors such as preservative concentration and interactions with water presence and temperature (Schmidt-Heydt et al., 2007).

There have been several efforts to control growth of these mycotoxigenic spoilage fungi. In this respect, several chemical preservatives such as calcium propionate and potassium sorbate have been extensively used to prevent growth of the fungi. On the other hand, use of such preservatives has been suppressed by consumers who demand use of natural ones (Schmidt-Heydt et al., 2007). For this reason, there have been many efforts so far exerted to prevent or control the fungal growth in different food products using several antifungal chemical additives. On the other hand, there has been considerable interest in use of extracts and essential oils (EOs) obtained from species and plant materials whose main components possess a wide range of antimicrobial effects (Guynot et al., 2003; Suhr and Nielsen, 2003; Rasooli and Abyaneh, 2004; Klaric Segvec et al., 2006; Pawar and Thaker, 2006; Gutierrez et al., 2008; Viuda-Martos et al., 2008).

In other words, researches on the potential antimicrobial effects of essential oils have been remarkably increased, for instance, on their antifungal effects to control and inhibit growth food pathogens as well as food native microflora (Ponce et al., 2003). In this respect, lemon balm (*Melissa officinalis*) and sage (*Salvia officinalis*) have been reported to have antifungal activity (Velluti et al., 2003; Pinto et al., 2007) due to the presence of bioactive substances such as essential oil and phenolic compounds. On the other hand, the use of essential oils in foods should meet demands of consumers for mildly processed or natural food products (Nychas, 1995); for this reason, their practical application is limited due to concerns with respect to sensorial characteristics resulting from their undesirable flavor agents. Also, their effectiveness is moderated due to interaction with food ingredients and structure (Juven et al., 1994; Skandamis and Nychas, 2000; Skandamis et al., 2000), leading a difficult task to compromise between effective doses of such flavoring agents and sensory acceptability (Skandamis and Nychas, 2001). Therefore, it is necessary to determine the minimal inhibitory and lethal concentrations of the EOs that will be able to be used in food technology (Moreira et al., 2005). Due to the above reasons, the objective of present study was to determine chemical composition of lemon balm and sage essential oils and evaluate antifungal activity namely, minimum inhibition concentration (MIC) and minimum fungicidal concentration (MFC) of their EOs on the growth of *P. verrucosum* in culture medium.

MATERIALS AND METHODS

Solvent (methanol), peptone, Tween-80, basal medium (Yeast Extract Sucrose (YES) agar and broth), sodium chloride (NaCl) and Malt Extract Agar were obtained from Merck, Germany. Lemon balm (*Melissa officinalis*) and sage (*Salvia officinalis*) EOs were obtained from a local market (Fetih Baharat, Samsun-Turkey).

Characterization of essential oils using GC-MS analysis

Gas chromatography/mass spectrometry (GC/MS) system was used to characterize the chemical composition of the lemon balm and sage EOs as outlined (Sagdic et al., 2009). GC-MS/Quadropole detector analysis was conducted to determine the volatile oil composition using a Shimadzu QP 5050 system, equipped with an FFAP capillary column (50 m × 0.32 mm (i.d.), film thickness: 0.25 µm). Detector and injector temperature were set at 230℃. The temperature program for FFAP column was from 120℃ (1 min) to 230℃ at a rate of 6℃/min and then held at 200℃ for 35 min. Helium was used as a carrier gas at a flow of 14 psi (Split 1:10), and injection volume of each sample was 1 µl. The components were identified by comparison of their mass spectra with those of Wiley and Nist, Tutore Libraries. The ionization energy was set at 70 eV.

Preparation of the essential oil dilutions

The tested concentrations of the EOs dilutions ranged from 7.75 to 500 µl ml^{-1} (500, 250, 125, 62, 31, 15.5 and 7.75 µl/ml; the values corresponding to 0.5, 0.25, 0.125, 0.062, 0.031, 0.0156 and 0.00781% based on agar medium) and were prepared in methanol as a diluent (Rasooli and Abyaneh, 2004). As a control, absolute methanol not including the EOs was used in the antifungal assays.

Fungal strain tested and preparation of spore suspension

P. verrucosum Dierckx (D-99756), the fungal strain isolated from Kashar Cheese was obtained from mould culture collection of Food Institute, Marmara Research Center (Kocaeli-Turkey), The Scientific and Technological Research Council of Turkey (TUBITAK). The strain from frozen stocks was cultivated on Malt Extract Agar (MEA, 2% malt, 0.1% peptone, 1.5% agar) slants at 25℃ in the dark for 7-10 days until they yielded good sporulation (Cabanas et al., 2009). Spores were collected from the slant surface with a sterile loop needle by adding 10 ml of sterile saline solution (0.85%) containing Tween 80 (0.1% v/v). The spore suspension was adjusted to approximately 10^6 spores ml^{-1} in 5 ml of YES broth tubes using haemocytometer (Elias et al., 2006; Cabanas et al., 2009; Nguefack et al., 2009). In order to confirm the initial spore counts, the pour plate method was used and negative (methanol) and positive (essential oils) controls were prepared. The test organism was enumerated on growing broth media by serial dilution method (Ozkan et al., 2010). The viability and inoculums size of the strain were checked using quantitative colony counts. The plates were incubated for 72 h at 25℃ in the dark (Tavares et al. , 2008).

Antifungal assays to determine MIC (fungistatic) and MFC (fungicidal) values

A macrodilution broth method was used to determine the MIC and MFC)(Lass-Florl et al., 2003; Pinto et al., 2007; Rasooli et al., 2008; Tavares et al., 2008). To determine MIC values, YES broth was used, while YES agar was used to determine if the inhibition was reversible or permanent (Bragulat et al., 2008). In this respect, fifty micro liters from seven EOs dilutions at various concentrations (500, 250, 125, 62, 31, 15.5 and 7.75 µl ml^{-1}) were added in 5 ml sterile YES broth tubes containing 10^6 spores ml^{-1} and the resultant solutions were mixed gently. The tubes were incubated at 25℃ for

Table 1. Essential oil compositions of lemon balm and sage.

Compounds	Lemon Balm		Sage	
	RT [a]	%	RT	%
1,8-Cineole	- [b]	-	5.53	11.53
1-propanol 2-hydroxypropoxy	21.40	14.36	-	-
1,2-propanediol	15.35	31.26	-	-
2-propanol 1,1 oxybis	20.41	23.30	-	-
2-α-Pinene	-	-	3.60	1.70
3-cyclohexene-1-methanol	17.04	3.74	-	-
Benzene 1,1-oxybis	23.25	6.20	-	-
Borneol	-	-	17.01	2.85
Camphene	-	-	3.06	7.87
Camphor	-	-	12.66	15.30
Cyclohexene	2.81	3,34	-	-
Dihyromycrenol	11.89	10.99	-	-
Dipropylene glycol	22.93	3.48	-	-
d-Limonene	-	-	5.28	5.90
Linalool	-	-	13.74	1.51
trans-Caryophyllene	-	-	14.26	5.07
α -Humulene	-	-	15.90	4.08
α -Myrcene	-	-	4.66	2.24
α-Pinene	-	-	2.56	6.36
α-Thujone	-	-	10.56	24.92
γ-Terpinene	-	-	6.22	1.45
Δ-3-Carene	-	-	4.30	1.17

[a] Retention time (as minutes), [b] not detected.

Table 2. MIC[a] and MFC[b] values of lemon balm and sage essential oils against *P. verrucosum*.

Plant species	MIC (µl ml^{-1})[c]	MFC (µl ml^{-1})
Lemon balm	125	250
Sage	62	125

[a]MIC: minimum inhibition concentration, [b]MFC: minimum fungicidal concentration, [c] The values corresponding to 0.25, 0.125 and 0.062 % based on agar medium.

7-10 days on a shaking incubator in order to homogeneously disperse the oils thoroughly in the broth within tubes. The fungal growth was checked by the turbidity. MIC was determined based on the highest dilution level (the lowest concentration) at which no visible growth was observed considering the EO-free controls. Regarding determination of the MFC test, it was conducted to determine if the inhibition was reversible or permanent. In this respect, the oil dilutions indicating inhibitory effect were further tested for lethal (fungicidal) effects, as well. One millilitre of inoculums from the tubes was subcultured on YES agar plates and then, incubated at 25°C for 48 h. The EO concentrati ons at which typical *P. verrucosum* colonies could not grow on the plates were regarded as fungicidal effect. In other words, the MFC was defined as the lowest EO concentration at which 99% of the inoculums were killed. All the experiments were performed in triplicate with two replications.

RESULTS AND DISCUSSION

Chemical composition of EOs

The main compounds of the tested essential oils and their percentages are presented in Table 1, which indicates that different components of lemon balm and sage EOs, respectively were characterized. As can be seen, major components of lemon balm EOs were 1,2-propanediol (31.26%) and 2-propanol 1,1 oxybis (23.30%), while those of sage EOs were α-thujone (24.92%) and camphor (15.30 %). In literature, the EO composition of these plants varies from one to another, which can be expected since the composition of the extracts obtained from these plants is known to vary significantly because of difference between species and chemotypes (Tantaoui-Elaraki et al., 1993), geographical origins (Perry et al., 1999), seasons (Senatore, 1996), extraction procedure, time of harvest and the plant part colected (Hammer et al., 1999; Scaneberg and Khan, 2002).

Fungistatic and fungicidal effects of the essential oils

The ochratoxigenic *P. verrucosum* grew in the positive

control tubes, whereas mycelial formations were well observed in all of the negative control tubes. The fungistatic (MIC) and fungicidal (MFC) effects on the strain were observed when pure forms of lemon balm and sage EOs were applied. The fungistatic (MIC) and fungicidal (MFC) effects of the EOs are presented in Table 2. As can be seen, the MIC value (125 µL ml^{-1}) of lemon balm EO was higher than that (62 µL ml^{-1}) of sage EO, revealing the fact that the inhibitory effect of sage EO against *P. verrucosum* was twofold higher than that of the lemon balm. Similar trend was also seen in the MFC values; namely, the irreversible inhibition of *P. verrucosum* caused by lemon balm and sage EOs was defined as MFC values. The fungicidal effect was observed when 125 µL ml^{-1} concentration of sage EO was applied on the fungal strain, while 125 µL ml^{-1} concentration of lemon balm was applied. These results clearly indicated that sage (*Salvia officinalis*) EO exhibited more fungistatic and fungicidal activity than did lemon balm EO (*Melissa officinalis*).

The observed fungistatic and fungicidal effects of the EOs were thought to result from the major volatile compounds in these EOs; namely, *2*-propanediol, *2*-propanol 1,1 oxybis in lemon balm EO and *α*-thujone and camphor in sage EO. Accordingly, it was reported that the antimicrobial effect of essential oils was mainly due to the most abundant components (Farag et al., 1989). A limited number of studies have appeared to examine the antifungal activity of the tested oils against *P. verrucosum*. Tzortzakis and Economakis (2007) found that the inhibition of fungal sporulation could be completely retarded at 500 ppm and that up to 70% inhibition was achieved at 25 ppm lemon grass oil concentration. This concentration level was lower than our findings (Table 2). On the other hand, consistent results were also reported. The composition of *M. officinalis* oil (lemon grass) has been published by several authors (Venturini et al., 2002; Tzortzakis and Economakis, 2007; Figueirinha et al., 2008; Fisher and Phillips, 2008; Iordache et al., 2009; Viuda-Martos et al., 2008; Nguefeck et al., 2009) who reported that methylmristate, methylpalmitate, loliolide and citral were the major compounds that exhibited *in vitro* antifungal activity such as *P. expansum* (Venturini et al., 2002), *P. verrucosum* (Viuda-Martos et al., 2008; Nguefack et al., 2009), *P. chrysogenum* (Viuda-Martos et al., 2008), *P. digitatum* (Fisher and Phillips, 2008) and the reduction of fungal spore up to 70% (Tzortzakis and Economakis, 2007).

Regarding the sage EO, the composition and a broad antifungal spectrum of the EOs of the *Salvia* species (Perry et al., 1999; Salgueiro et al., 2003; Pina-Vaz et al., 2004; Pinto et al., 2007; Ozkan et al., 2010) have also been studied. Ozkan et al. (2010) determined total phenolics, total flavonols, total flavanols, antioxidant and antimicrobial activities and chemical composition of Turkish endemic *Salvia pisidica* extract and essential oil. The major compounds of the oil were camphor (23.8%), sabinol (19.2%), α-thujone (14.2%) responsible for bacteriostatic and bacteriocidal effects. On the other hand, the antifungal effect of sage (*Salvia officinalis*) has been demonstrated to be insufficient against *P. verrucosum* isolated from various food stuffs. As far as the effects of another EOs against *P. verrucosum* were concerned, thyme oil was reported to show strong inhibition activity against *P. verrucosum* at 200-300 µg g^{-1} concentration (Aldred et al., 2008). On the other hand, the mycelium growth of *P. corylophilum* could be prevented and delayed in culture media by adding 50 µl pure EO of thyme oil (Guynot et al., 2003). Given these reports, it can be seen that the EO dose levels at which fungistatic and fungicidal effects were observed in this study were less or higher than those observed in the other results. Differences between the results could be attributed to the fact that there were differences between the geographical origins, extraction procedures, harvest seasons and the plant part collected.

In spite of the fact that a considerable number of studies have been conducted reporting the antifungal effects of essential oils, the exact mechanism actions of their main constituents against fungi have not been still reported. However, several assumptions were reported. In this respect, the antimicrobial properties of essential oils were attributed to their phenolic compounds; thus, presence of an aromatic nucleus and OH group, which causes degeneration in cell wall, cell membrane integrity and cellular organelles such as mitochondria (Shelef, 1983; Farag et al., 1989; Ultee et al., 2002; Rasooli and Mirmostafa, 2003; Rasooli and Owlia 2005). In addition, the phenolic components of essential oils were reported to denaturize the enzymes that control spore germination (Nychas, 1995). Zambonelli et al. (1996) attributed the antifungal effects of EO to degeneration of fungal hyphae.

Conclusions

The present work evaluated the potential antifungal activity of lemon balm (*M. officinalis*) and sage (*S. officialis*) EOs against ochratoxigenic *P. verrucosum* isolated from Kashar cheese. Given the fact that these EOs can negatively affect the organoleptic properties of the food such as kashar cheese because of having strong aromatic flavor, they should be added in lower quantities. Therefore, in this study, MIC and MFC values of the essential oils were calculated so that their minimal effective concentrations could be found. The results revealed that both EOs could show both fungistatic and fungicidal effects against the fungal strain; however, the effect of sage EO was more pronounced. The observed fungicidal effects also indicated that mycotoxin production might be prevented if the strain is treated with sage EO with specified concentration. Briefly, it can be concluded that these oils can be potentially used as natural

antimicrobial agents instead of chemical-based antimicrobials against ochratoxigenic *P. verrucosum* in food safety applications.

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