Emirates Journal of Food and Agriculture. 2016. 28(11): 796-804
doi: 10.9755/ejfa.2016-06-618
http://www.ejfa.me/

REGULAR ARTICLE

Biological activity of selected Greek medicinal and aromatic plants extracts on *Alternaria alternata*

Efstathia Skotti[1], Sofia Kountouri[2], Maria Kaiafa[2], Pavlos Bouchagier[3], Dimitrios I. Tsitsigiannis[2], Moschos Polissiou[1], Petros A. Tarantilis[1]*

[1]Laboratory of Chemistry, Department of Food Science and Human Nutrition, School of Food, Biotechnology and Development, Agricultural University of Athens, Lera Odos 75, 11855 Athens, Greece, [2]Laboratory of Phytopathology, Department of Crop Science, School of Agricultural Production Infrastructure and Environment, Agricultural University of Athens, Iera Odos 75, 11855 Athens, Greece, [3]Department of Food Technology, Technological Educational Institute of Ionian Islands, GR 28100 Kefallonia, Greece

ABSTRACT

In this study, the biological activity of selected Greek medicinal and aromatic plants on the phytopathogenic fungus *Alternaria alternata* was investigated. Biological activity was screened on crude aqueous extracts and their aqueous phase (ap-pt) after further extraction with petroleum ether. All the *Lamiaceae* species examined were found to enhance significantly the mycelium growth. *Melissa officinalis* extracts caused the highest stimulation in mycelium growth, (+109.1%) the ap-pt, and (+51.6%) its crude extract. All ap–pt extracts stimulated conidia production, but *Melissa officinalis* caused the highest effect (+349.3%). Crude extracts of *Salvia officinalis* stimulated highly conidial production (+65%) whereas *Hyssopus. officinalis* and *Origanum vulgare* inhibited conidia production at 33.3% and 50.0% respectively. The differences in biological activity between crude and ap–pt extracts for each plant species were attributed through the calculation of correspondent synergism ratios (SR) to the interaction between volatile and water – soluble. Rosmarinic acid, detected as the most abundant phenolic compound in all extracts analyzed and found to correlate strongly (R= 0.84) with the stimulation effect on spore production of *A. alternata*.

Keywords: *Alternaria alternata*; Biological activity; Medicinal and aromatic plants

INTRODUCTION

Natural products attract specific research interest as more safe and effective biopesticide alternatives against phytopathogenic fungi. Biopesticides is the new rapidly growing trend in the European Union that can control pathogens and pests effectively with the minimum environmental impact (Matson et al., 1997). Biopesticides are a form of pesticide based on microorganisms or natural products that may act directly controlling pathogen and pest epidemics or indirectly by enhancing the development of natural enemies (Chandler et al., 2008; Srinivasan, 2012). Bioactive compounds or mixtures have been used for the effective control of fungal pathogens such as *Alternaria alternata* (Guleria et al., 2008). The evaluation of bioactive compounds or mixtures on specific cells is usually conducted through bioassays and molecular tests (Barros et al., 2007; Meca et al., 2010; Muller-Riebau et al., 1995; Skotti et al., 2014; Zambonelli et al., 1996).

Plant extracts, either aqueous or in organic solvents, have shown antimicrobial activity when examined by different screening models (Wang et al., 2011) mainly towards finding successful drug candidates. Biological activity of plant extracts is being investigated through alterations in photosynthetic mechanism or stress status of plants (Bouchagier et al., 2008; Bouchagier and Efthimiadis, 2010), bioassays (Abou-Jawdah et al., 2004; Daferera et al., 2003), tissue or cell culture (Wang et al., 2011), receptor enzyme (Peelman et al., 2006) and biochromatography (He et al., 2008; Kotecha et al., 2007; Kvalheim et al. 2011).

Towards this direction the bioactivity of several plant extracts has been studied (Kumar et al., 2014). The chemical composition of the essential oils, phenolic compounds and flavonoids has been previously studied either for evaluation of aromatic properties or the antioxidant and biological activity (Castilho et al., 2012; Dzamic et al., 2013; Kaliora et al., 2014; Licina et al., 2013; Martins et al., 2014, 2015).

***Corresponding author:**
Petros A. Tarantilis, Laboratory of Chemistry, Department of Food Science and Human Nutrition, School of Food, Biotechnology and Development, Agricultural University of Athens, Lera Odos 75, 11855 Athens, Greece, Tel: +30 210 529 4262, Fax: +30 210 529 4265.
E-mail: ptara@aua.gr

Received: 04 June 2016; **Revised:** 25 October 2016; **Accepted:** 29 October 2016; **Published Online:** 05 November 2016

In case of aqueous plant extracts derived through several extraction techniques, a very small quantity of essential oil compounds comes into the aqueous phase, which either promotes or inhibits partially the bioactivity of water - soluble compounds. Recently, there is an increasing interest in evaluation of potential synergism or antagonism in antimicrobial activity (Al-Bayati, 2008; Endo et al., 2010; Hemaiswarya & Doble, 2009; Moon et al., 2011; Ncube et al., 2012) and antioxidant activity (Romano et al., 2009) of single compounds or mixtures. Evaluation of such synergism in plant extracts between aqueous and volatile compounds has been previously reported by Skotti et al., 2014.

Fungal species have diverse impacts on plants, animals, ecosystems and the environment (Chapin et al., 2000; Desprez-Loustau et al., 2007; Hawksworth, 1991) ranging from nutrient recycling, to biotechnology (Adrio & Demain, 2003; Molitoris, 1995) or emerging animal and plant pathogens. Many fungal pathogens cause serious damage to a large number of crops with significant impacts to agricultural economy (Anderson and Zhu, 2004; Oerke, 2006;). *A.alternata*, commonly grown on vegetation, is one of the most important species among the allergenic fungi (Stierle et al., 1988) and a devastating plant pathogen of several crops worldwide.

The aim of this study is to evaluate the biological activity of *Lamiaceae* medicinal and aromatic plants aqueous extracts against the phytopathogenic fungus *A. alternata,* and to identify the possible interaction of volatile and water – soluble compounds towards this activity.

MATERIALS AND METHODS

Plant material, chemicals, reagents and test organisms

Samples of the *Lamiaceae* family were offered by Aetoloakarnania's Rural Cooperative of Aromatic, Pharmaceutical and Energy Plant Cultivators (Agrotikos Syneterismos Kalliergiton Aromatikon, Farmakeftikon, Energiakon Fyton Aetoloakarnanias, ASKAFEFA), Greece. These samples were, namely, lemon balm (*Melissa officinalis* L.), sage (*Salvia officinalis* L.), oregano (*Origanum vulgare* L.) and hyssop (*H. officinalis* L.). Dittany (*Origanum dictamnus* L.) was supplied from the local market in Crete, Greece. Only leaves of the aforementioned samples were used, with the exception of oregano and hyssop, for which leaves and flowers were used together. The samples were dried at room temperature. Dry plant material was stored at -20°C until used.

Potato dextrose agar (PDA) was supplied by Sigma Aldrich. Potato dextrose broth medium was prepared in the lab using the filtered broth of 200 g of boiled potato small pieces (aprox. 1 cm x 1 cm) in 500 mL of double-distilled water (ddH_2O), adding 20 g of dextrose and the correct amount of ddH_2O to bring the volume to 1 L; the broth was autoclaved for 20 min at 120°C. *Alternaria alternata,* was provided by the laboratory of Phytopathology of the Agricultural University of Athens.

Acetonitrile, and standards of rosmarinic acid, caffeic acid, syringic acid and carnosic acid were supplied by from Sigma-Aldrich Corporation. Standards of luteolin, luteolin -7-*O*- glucoside, kaempferide, apigenin, apigenin-7-*O*-glucoside, chlorogenic acid and kaempferol were supplied by Extrasynthese. Ferulic acid, *p*-coumaric acid and eriodictyol were purchased from Fluka. Petroleum ether was supplied by Merck KGaA (Darmstadt, Germany). Standard stock solutions were stored at -20°C.

Preparation of extracts

The crude extracts of five *Lamiaceae* species (*H. officinalis, M. officinalis, O. dictamnus, O. vulgare,* and *S. officinalis*) at a concentration of 10 g/100mL, were prepared by soaking dry plant material in boiling double distilled water (ddH_2O), mixing thoroughly and then allow to stand for 15 minutes. The herbal extracts were then filtered though a Whatman filter No.1. Prior to inoculation, an aliquot was further filtrated through a sterile and endotoxin free 0.2 μm polyethersulfone (PES) filter media (Whatman Puradisk 25mm) to reduce the risk of interference by micro-organisms.

All the infusions described above were then extracted three times by petroleum ether in order to further analyze the aqueous phase ap–pt extract of this extraction.

High performance liquid chromatography

HPLC analysis was conducted using an Agilent model 1100 (Agilent Corporation, MA, USA) system equipped with a diode array detector. A reverse phase column Supelco (Discovery HS C18), length 250 mm, internal diameter 4.6 mm with material porosity 5 μm was used. The HPLC system is controlled by Agilent Chemstation software. From the absorption spectra of samples and standards solutions, absorptions at 260, 280 and 330 nm were obtained.

Flow rate was adjusted at 0.4 mL/min using the binary gradient of acidified water (pH=2.5) by formic acid, (A) and Acetonitrile (B). The mobile phase consisted of 25% B during the initial 2 min, followed by a gradient increase of the percent of solvent B up to 90% for the next 38 min. Sample injection volume was 20 μL. Components were identified by comparison of their retention times and UV absorption spectra with those of the commercial standards under identical analysis conditions.

Fungal physiological assays

Alternaria alternata, was grown in PDA plates under light conditions in order to produce enough conidia. Fungal mycelium development and spore production studies were carried out on plates containing 19 mL solid PDA medium plus 1 mL of each of the aromatic plants extracts that were added in PDA after autoclaving and cooling down and before medium solidification. The experiments were conducted in 90 mm sterile petri dishes. Conidia of *Alternaria alternata* were collected in 1 mL of ddH_20 (supplemented with 0.01% Tween 80 to facilitate the release of the hydrophobic spores-conidia) using a flat toothpick and then the spore concentration was counted and adjusted to 10^6 conidia/mL using a haemocytometer. 10 μL of the spore suspension (10^4 conidia) was applied to the center of PDA dishes amended with each plant extract, dried completely and then were left to grow in an incubation chamber under light (12h) and dark (12h) conditions at 25°C.

Mycelium diameter was measured at 2, 4 and 7 days. Furthermore, % mycelium growth enhancement was calculated as follows:

$$\text{Mycelium growth enhancement (\%)} = [(D_{sample} - D_{control})]) / D_{control}]\bullet 100,$$

Where, $D_{control}$ and D_{sample} are the Mycelium diameter values of the control and the test sample at day 4, respectively. For conidia concentration, 3 disk cores of 6 mm each in diameter were removed with a cork borer from each plate at 7 days after inoculation, homogenized and vortexed for 1 min in 1 mL sterile water supplemented with 0.01% Tween 80 to facilitate the release of the spores. Spores were counted using a haemocytometer. Furthermore, % Conidia production enhancement was calculated as follows:

$$\text{Conidia productionenhancement (\%)} = [(Cn_{sample} - Cn_{control})] / Cn_{control}]\bullet 100$$

Inhibition on conidia production was calculated as:

$$\text{Conidia production inhibition (\%)} = [(Cn_{control} - Cn_{sample})] / Cn_{control}]\bullet 100$$

Where $Cn_{control}$ and Cn_{sample} are the conidia concentration values of the control and the test sample, respectively.

Synergism evaluation

During the extraction procedure, a small quantity of essential oil compounds is extracted into the infusion. The volatile organic compounds of the essential oil in the extracts may interact with water-soluble compounds and influence the bioactivity of samples to *A. alternata*. In order to evaluate this possible interaction, all crude extracts prepared were further extracted with petroleum ether in order to remove the volatile organic compounds and all the assays were repeated then in triplicate.

The synergism ratios (SR) were calculated as proposed by Hewlett and Placket (1969) and used in several cases of possible synergism evaluation (Anderson and Zhu, 2004 and Otitiloju, 2002; Skotti et al., 2014), as follows:

SRm = (% mycelium growth enhancement ap–pt extract)/ (% growth enhancement of crude extract)

SRc = (% conidia production increase ap–pt extract)/(% conidia production increase of crude extract)

Where, "ap–pt" referred to the aqueous phase after extraction with petroleum ether.

Statistical analysis

The experiments were performed in four replicates. Spore data were statistically compared by analysis of variance (ANOVA) and Fisher's Least Significant Difference (LSD) ranged at $P \leq 0.05$, using the Statgraphics Plus Software.

RESULTS AND DISCUSSION

HPLC analysis

The data of the qualitative analysis of hyssop, lemon balm, dittany, oregano and sage ap–pt extracts were obtained using HPLC coupled with photodiode array. The respective chromatograms with detector responses at 280 nm are presented in Fig 1. The components gallic acid, chlorogenic acid, rutin, *p*-hydrobenzoic acid, luteolin-7-*O*-glucoside, vanillic acid, apigenin-7-*O*-glucoside, ferullic acid, rosmarinic acid, luteolin and apigenin were identified by comparisons to the retention times and UV spectra of authentic standards were analyzed. There were compounds that could not be identified from spectra and retention times of standards were analyzed; however, based on their chromatographic behavior and UV spectra their chemical class, were determined as hydroxycinnamic derivatives or flavonoids.

From the compounds identified, the major and most abundant component present in all extracts was rosmarinic acid. Rosmarinic acid has already been reported to be present in hyssop (Dzamic et al., 2013), lemon balm (Arceusz and Wesolowski, 2013; Barros et al., 2013; Carnat et al., 1998; Dastmalchi et al., 2008), dittany (Liolios et al., 2010; Kaliora et al., 2014; Kouri et al., 2007), oregano (Agiomyrgianaki & Dais 2012; Martins et al. 2014; Shen et al., 2010) and salvia (Cvetkovikj et al., 2013; Kaliora et al., 2014; Martins et al., 2015; Roby et al., 2013; Zimmermann et al., 2011). All plant extracts were analyzed at the same concentration

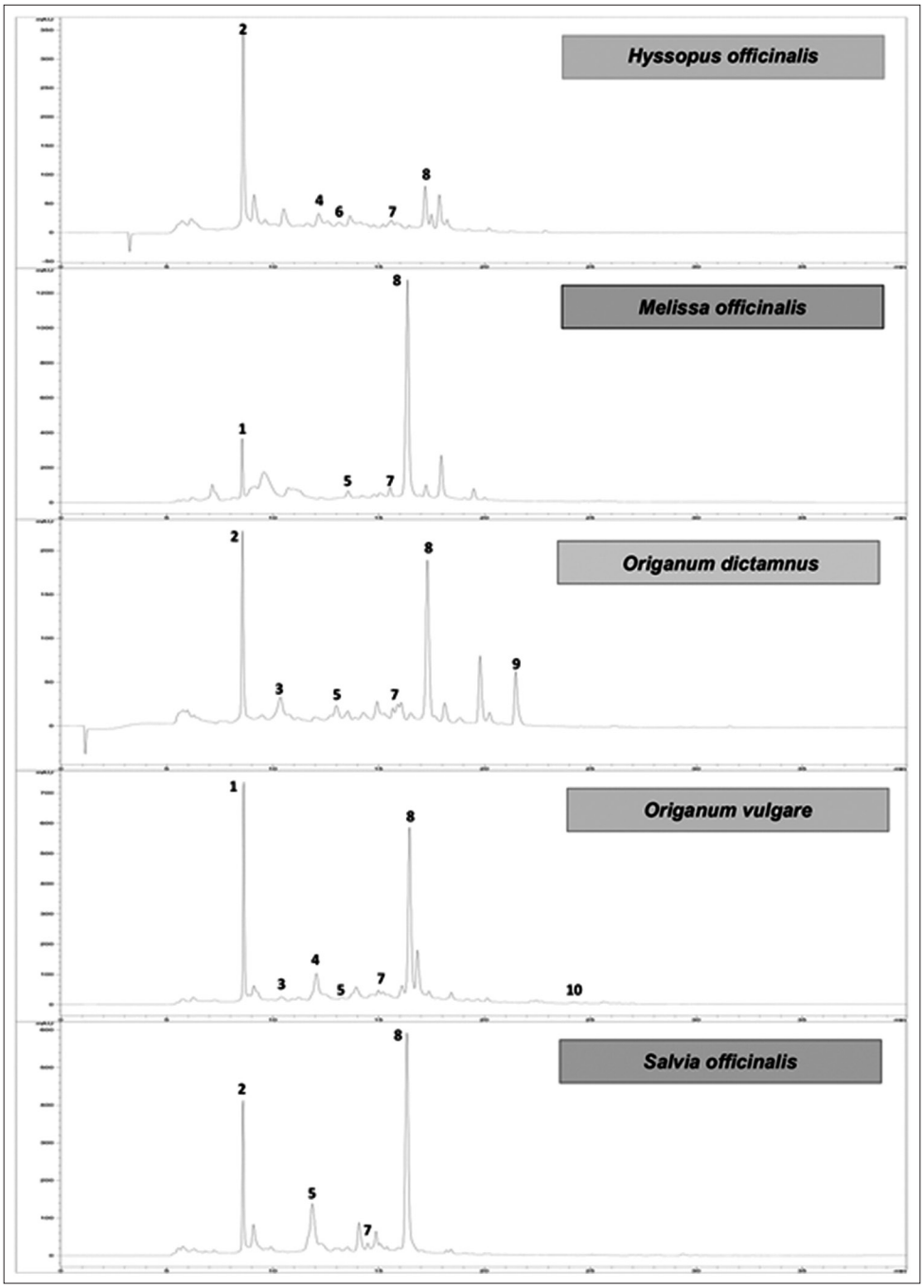

Fig 1. HPLC phenolics and flavonoids profile of *Hyssopus officinalis, Melissa officinalis, Origanum dictamnus, Origanum vulgare* and *Salvia officinalis* infusions at 280nm. Peak identification: (1) Gallic acid, (2) Chlorogenic acid, (3) Rutin, (4) p-hydrobenzoic acid, (5) Luteolin-7-O-glucoside, (6) Vanillic acid, (7) Apigenin-7-O-glucoside, (8) Rosmarinic acid, (9) Luteolin, (10) Apigenin.

of dry plant mass, so their relevant concentration of rosmarinic acid is presented in Table 2 as the area of corresponding peaks in chromatograms. In a number of compositional studies on *Lamiaceae* family plants, luteolin-7-*O*-glucoside has been detected as the main flavonoid compound (Carnat et al., 1998; Martins et al., 2014; 2015). In this study luteolin-7-*O*-glucoside was detected in lemon balm, dittany, oregano and sage. Apigenin-7-*O*-glucoside detected also in all extracts as mentioned also in Cvetkovikj et al., (2013). Gallic acid was detected in lemon balm in accordance to previous studies (Arceusz and Wesolowski, 2013; Pereira et al., 2014) and also in hyssop, oregano and sage. In accordance to previous studies, chlorogenic acid was detected in hyssop (Dzamic et al., 2013; Hatipoglu et al., 2013), lemon balm (Arceusz and Wesolowski, 2013; Pereira et al., 2014), dittany (Kaliora et al., 2014) and sage (Roby et al., 2013; Zimmermann et al., 2011). Ferrulic acid was detected in hyssop (Dzamic et al., 2013), lemon balm (Arceusz and Wesolowski, 2013), dittany (Proestos et al., 2006; 2008) and oregano (Agiomyrgianaki & Dais 2012).

Agipenin was detected in oregano as in Martins et al. (2014). Rutin was detected in oregano and dittany and luteolin in dittany in accordance to Skaltsa and Harvala (1987).

Differences in presence or absence of phenolic or flavonoid compounds in the samples tested, compared to other past compositional analysis of the same plant extracts may be due to many reasons ranging from geography and climate to difference in the specificity of extractions procedures followed (Carnat et al., 1998; Dastmalchi et al., 2008; Zgorka & Glowniak, 2001).

Effect of crude and –pte extracts on *Alternaria alternata*

The biological activity of aromatic plants crude and ap–pt extracts on *A. alternata*, was primarily screened using *in vitro* plate bioassays by measuring mycelium growth (Table 1) and conidia production Fig. 2. All *Lamiaceae* extracts (crude or ap–pt) found to enhance noteworthy mycelium growth Fig. 2. Regarding crude extracts, significant increase in mycelium growth was observed by *M. officinalis* (+109.1%), followed by *O. dictamnus* (+97.0%), *H. officinalis* (+95.5%), *S. officinalis* (+83.3%) and *O. vulgare* (+75.8%). For ap–pt extracts mycelium growth increased by 51.6% for *M. officinalis* and *S. officinalis*, followed by *O. dictamnus* (+48.4%), *H. officinalis* (+45.8%) and *O. vulgare* (+39.9%).

In crude extracts *S. officinalis*, was found to be the more active towards conidia production enhancement (+65.0%), followed by *M. officinalis* (+43.9%) and *O. dictamnus* (+35.4%). *O. vulgare* inhibited strongly conidia production (-50%) followed by *H. officinalis* (-33.3%).

Concerning ap–pt extracts, *M. officinalis* increased significantly conidia production 349.3% (Fig. 3), followed by *H. officinalis* (84.1%), *O. vulgare* (73.9%), *S. officinalis* (68.1%) and *O. dictamnus* (11.6%).

Biological activity of plant extracts seems to be directly related to phenolic constituents and presence of key phenolic compounds responsible for this activity (Hatipoglu et al., 2013). The effect of ap-pt extracts on conidia production of *A. alternata* found to be strongly positive correlated (R = 0.84) with rosmarinic acid in corresponding extracts (Table 2) while this detected as the most abundant phenolic compound in all extracts analyzed. No corresponding correlation was found between rosmarinic acid and conidia production as well as mycelium growth for all the crude extracts. The interaction of volatile and water - soluble compounds may explain differences in correlation with rosmarinic acid between ap–pt and crude plant extracts.

Bioactivity interaction of water soluble and volatile organic compounds

Differences on the biological activity of crude and ap–pt extracts of the same plant may be due to the synergism

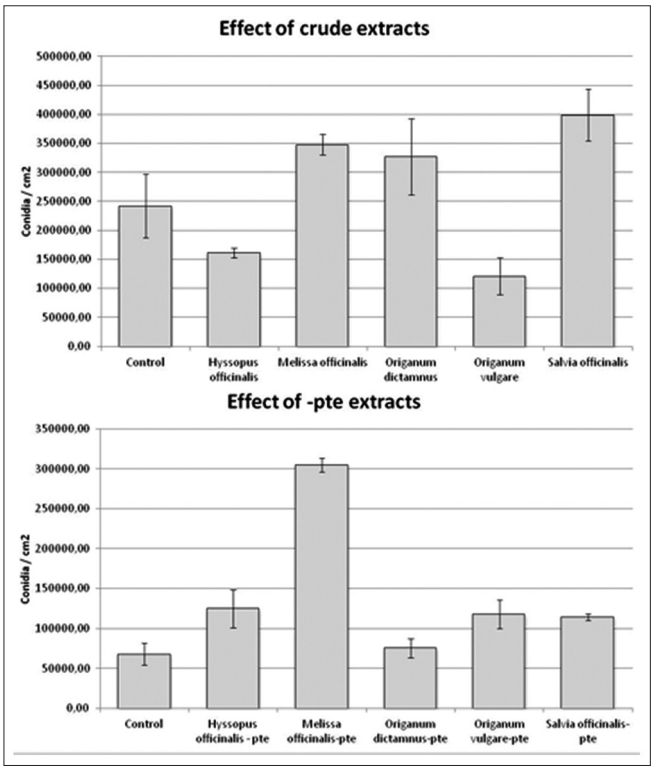

Fig 2. Effect of crude and ap–pt aqueous extracts of *Hyssopus officinalis, Melissa officinalis, Origanum dictamnus, Origanum vulgare* and *Salvia officinalis* on conidia production of *Alternaria alternata*.

Table 1: Effect of aromatic plants crude and ap–pt extracts to mycelium growth of *Alternaria alternata*

Treatment	Mycelium diameter(mm)					
	Crude extracts			ap-pt extracts		
	2 days	4 days	7 days	2 days	4 days	7 days
Control	10.2±0.7^{a}	22.0±0.5^{a}	39.8±0.3^{a}	11.8±0.1^{a}	19.1±0.4^{a}	37.2±1.3^{a}
Hyssopus officinalis	14.8±1.5^{bc}	43.0±0.3^{d}	75.5±1.0^{cd}	14.5±0.3^{b}	27.9±0.4^{bc}	51.2±0.8^{b}
Melissa officinalis	17.8±0.3^{d}	46.0±0.5^{e}	78.5±1.5^{d}	15.6±0.2^{c}	29.0±0.6^{c}	53.9±1.3^{bc}
Origanum dictamnus	16.0±0.3^{cd}	43.3±0.4^{d}	75.2±1.5^{cd}	14.4±0.2^{b}	28.4±0.4^{c}	58.6±1.6^{d}
Origanum vulgare	13.2±0.3^{b}	38.7±0.4^{b}	70.2±1.9^{b}	14.1±0.5^{b}	26.8±0.3^{b}	50.4±0.6^{b}
Salvia officinalis	13.5± 0.6^{b}	40.3±0.2^{c}	72.8±0.9^{bc}	15.0±0.4^{bc}	29.0±0.4^{c}	54.3±1.1^{c}

Equal initial amounts of fungal spores were grown on media agar plates for 7 days. At the end of incubation period fungal cultures were evaluated by measuring mycelium diameter growth and conidia concentration. Results are means of three replicates ± the SE (Standard Error). Superscript letters depict the statistical analysis whereas different letters demonstrate statistically different values for each day according to Tukey's multiple range test at $P \leq 0.05$

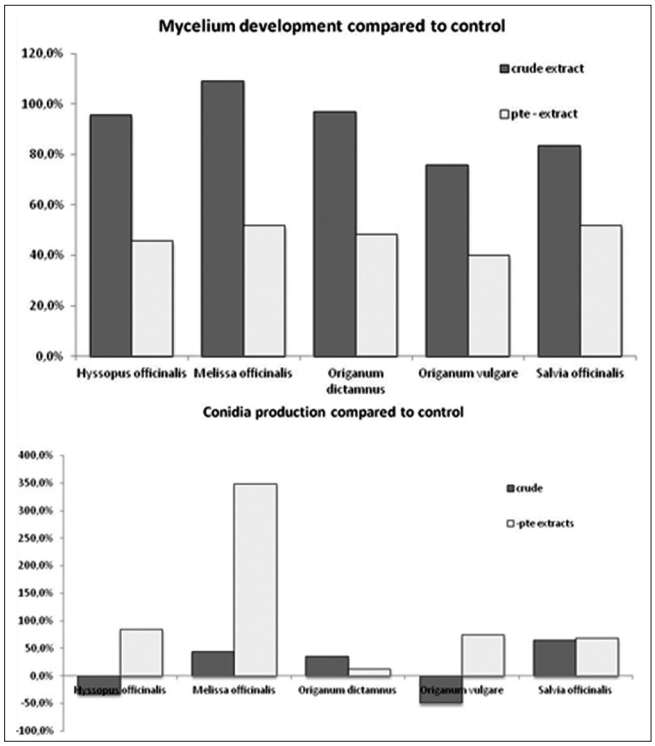

Fig 3. % effect compared to control of crude and ap-pt aromatic plant extracts on mycelium growth and conidia production of *Alternaria alternata*.

or antagonism between water soluble and volatile organic compounds that are present in the crude extract. Evaluation of the effect of samples on *A. alternata* mycelium diameter growth and conidia production before and after the extraction with petroleum ether was allowed through the calculation of synergism ratios (SR) between water soluble and volatile organic compounds of the initial extract. According to the results of SR values calculation (Table 3) volatile and water - soluble compounds are in absolute synergism in mycelium growth enhancement of *A. alternata* where maximum synergism was observed, in lemon balm (SRm = 0.47). In stimulation of conidia production, synergism found only in case of dittany. In all other extracts antagonism was significant but most intense also in case of lemon balm (SRc = 7.96).

For these interactions, responsible seem to be the volatile compounds in the crude extracts where in case of dittany should be carvacrol as has been found the major compound following by γ-terpinene and p-cymene (Liolios et al., 2009; Daferera et al., 2002) or thymol instead of carvacrol if the dittany was cultivated (Daferera et al., 2000); thymol and carvacrol, in case of oregano (Castilho et al., 2012; Kogiannou et al., 2013; Martins et al., 2014) and sage (Kogiannou et al., 2013); geranial, neral and citronellal in lemon balm, the main volatile compounds have found according to Carnat et al., (1998); cis-pinocamphone,

Table 2: Area values of rosmarinic acid in ap–pt extracts

	Concentration g of dry mass / mL	Rosmarinic acid area (AU)
Hyssopus officinalis	0.005	1,593.5 ± 9.5
Melissa officinalis	0.005	23,798.2 ± 598.8
Origanum dictamnus	0.005	3,944.1 ± 107.5
Origanum vulgare	0.005	10,735.2 ± 280.3
Salvia officinalis	0.005	9,409.9 ± 1227.3

Results are means of three replicates ± the SE (Standard Error)

Table 3: SR indexes for mycelium growth (SRm) and conidia production (SRc) of *Alternaria alternata*

Treatment	SRm	Effect	SRc	Effect
Hyssopus officinalis	0.48	synergism	2.52*	Antagonism
Melissa officinalis	0.47	synergism	7.96	Antagonism
Origanum dictamnus	0.50	synergism	0.33	Synergism
Origanum vulgare	0.53	synergism	1.48*	Antagonism
Salvia officinalis	0.62	synergism	1.05	Antagonism

SR<1 indicates synergism; SR>1 indicates antagonism and SR= 1 additive action. *SRc of *Hyssopus officinalis* and *Origanum vulgare* was calculated on % Conidia inhibition; while they inhibited conidia production

pinocarvone, β-pypene, eucalyptol (Hatipoglu et al., 2013) or β-Pipene,1,8-cineole, isopinocamphone and limonene in hyssop (Mazzanti et al., 1998). As volatile compounds found to interact with the aqueous components in all the crude extracts examined further research is necessary on the volatile composition in each extract.

CONCLUSIONS

In this study was observed the biological activity of medicinal and aromatic plants extracts on *A. alternata*. Rosmarinic acid that detected as the most abundant compound in all extracts analyzed, found to correlate with the stimulation effect on conidia production. The bioactive compounds of aromatic plant extracts tested possibly activate molecular mechanisms in fungal cells that lead to stimulation of mycelium growth or conidia production. This is of significant importance as indicates that these extracts may have the potential to be used in biopesticide industry where the bioactive constituents capable to stimulate growth of beneficial microorganisms and especially of fungi, can be used as reinforces of natural enemies in biological control of pests and pathogens. Thus, further investigation is needed on the influence of these extracts in gene signaling pathways that regulate hyphal growth and sporulation of *A. alternata,* and the possible role of rosmarinic acid in this procedure.

ACKNOWLEDGEMENTS

We thank the "Aetoloakarnania's Rural Cooperative of Aromatic, Pharmaceutical and Energy Plant Cultivators (Agrotikos Syneterismos Kalliergiton Aromatikon,

Farmakeftikon, Energiakon Fyton Aetoloakarnanias, ASKAFEFA), for providing the herb samples.

Authors' contribution

E.S. was the main contributor in designing the research, carrying out experiments, data analysis and preparing the manuscript. S.K. and M.K. were involved in carrying out bioassays experiments and collecting data. P.B was involved in data analysis and manuscript writing. D.I.T. contributed in the design and the research for microbiological experiments. M.P. was contributed in research planning. P.A.T. contributing in research planning in every step until manuscript preparation. All seven authors approved of the manuscript for publication and take public responsibility for the content, while declaring no conflict of interest associated with any aspect of this manuscript (financial or other).

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