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Article

Screening of lemon balm extracts for anti-aflatoxigenic, antioxidant and other biological activities

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Abstract: Lemon balm extracts by dry plant material of *Melissa officinalis* were tested against their efficacy against for different fungal species *Alternaria alternata, Fusarium oxysporum, Aspergillus flavus* and *Beauveria bassiana*. The aqueous phase of the extract was evaluated for antioxidant, antifungal and also anti-aflatoxigenic activity. A synergism evaluation was also performed concerning, the small quantity of lemon balm polar essential oil compounds extracted into the infusion and the water soluble compounds in the extract. The experiments were conducted in solid cultures and the growth inhibition was demonstrated by measuring mycelial diameter. Additionally, the effect on conidia production was also demonstrated. Lemon balm was also used for in situ test on *Pistachia vera* seed against *Aspergillus flavus* growth and aflatoxin production. Results revealed enhancement of fungal growth by lemon balm extracts however total inhibition of aflatoxins production on *Pistachia vera* seeds was observed, and both actions were tried to attributed to volatile and water soluble compounds identified based on GC/MS, HPLC/DAD and LC/MS, and the observed antioxidant activity. Volatile and water - soluble compounds found to be in absolute synergism in mycelium growth enhancement and the observed anti-aflatoxigenic activity addressed to the high antioxidant activity observed and synergistic action between the other water soluble phenolic compounds identified in the extract. The findings of this study underline the high biological active potential of lemon balm extracts under various screening test since for the first time full phytochemical analysis of lemon balm extracts.

Keywords: *Melissa officinalis* 1, Antioxidant activity 2, Anti-aflatoxigenic activity 3, GC/MS 5, LC/MS , Lemon balm, *in situ*, *Pistachia Vera*

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1. Introduction

Melissa officinalis L., commonly known as lemon balm, is a member of the *Lamiaceae* family that has been used for centuries in form of decoction, infusion or directly in food [1,2], for its outstanding traditional medicinal effects. Lemon balm is nowadays naturalized and cultivated in many countries [3], though originates from the eastern Mediterranean and western Asia region having a long tradition of use as a spice, medicinal plant and herbal tea with mild sedative properties. In addition to the recognized effects in assisting digestion problems, rheumatism or headaches, recently, and apart from some empiric claims of its applicability, other effects have been discovered, namely against melanogenesis, diabetis and anti-Alzheimer, or as also an antitumor, antiproliferative, anticholinesterase, [4,5,6,7]. Modern pharmacological researches demonstrated that lemon balm has several biological activities including antioxidant, hypoglycemic, hypolipidemic, antidepressant, anxiolytic, anti-inflammatory and spasmolytic capabilities [8,9].

These beneficial effects of *M. officinalis* extracts may be primarily attributed to the presence of phenolic compounds as the caffeic acid and other caffeic acid derivatives [1]. The plant contains low amounts of essential oils that include geraniol, citronellal, geranial and neral as citrus aroma compounds and sesquiterpenes, mainly _-caryophyllenene in varying proportions. Phenolic compounds present in the plant are hydroxycinnamic acid derivatives such as rosmarinic acid and flavonoids. Triterpenoids are also reported [10]. The most consumed drinks in the world are teas however, further uses have also been explored, as food ingredient for preservation and stabilization purposes. Antimicrobial properties arousing by these compounds, have gain great attention for applications in the food industry since researchers are trying to prove these multiple antibacterial even antimicotoxigenic properties scientifically [11].

Mycotoxins are secondary metabolites that can be mutagenic, teratogenic or carcinogenic and cause feed refusal in humans or animals are also produced by these fungal agents. Given the adverse effects occasionally reported for synthetic chemical compounds and due to consumer's pressure, the food industry is being forced to seek for alternatives. Researchers have nowadays focused on the discovery of naturally occurring plant-derived antioxidants and antimicrobials [12]. Natural-derived alternatives from plant extracts with proven benefits are being incorporated into food matrices in order to partially or totally replace those synthetic additives. As a consequence, the food industry has invested in solutions based on plants, mushrooms and algae, to be used as natural ingredients, which can act as food additives (e.g. by increasing shelf-life) and, simultaneously, bring health benefits, since they retain the original natural bioactivity of the natural sources.

The production of natural ingredients needs preliminary studies concerning the isolation of these compounds and the establishment of the best extraction methodology and conditions. Although a wide variety of solid–liquid extraction procedures is available to obtain natural ingredients, the use of extended time periods of extraction, the need of large amounts of solvents and the partial loss of natural molecules (such as phenolic compounds) was some of the disadvantages identified by some authors [13]. Recently, it has have demonstrated that extracts rather than dried leaves or essential oils are preferred for use in food cosmetic and pharmaceutical industries, due to limitations arising from the strong smell or taste [14] or their potential toxicity in high doses [15,16].

Their properties regarding the elimination of pathogenic microorganisms, as well as the reduction of lipid oxidation, have been the basis of their application in active food packaging [17]. De-odorized extracts may be the solution. Sequential extraction with solvents of increased polarity can result in de-odorized extracts enriched with antioxidant and antimicrobial constituents [18]. Thus, it is worth investigating natural compounds for developing innovative strategies to counteract biofilm resistance properties to antimicrobials [19]. Up to now, scarce literature exists for the above process in the aforementioned families and the resulting bioactivities. According to our knowledge this is the first report determining *in vitro* and *in situ* the antioxidant and anti-aflatoxigenic activity on aqueous lemon balm extracts along with their biological activities

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2. Materials and Methods

2.1 Plant material and extract preparation

Crude lemon balm extracts were prepared by dry plant material of *M. officinalis* as described in [20]. The crude extract was then extracted three times by petroleum ether in order to further evaluate and analyze the aqueous phase after extraction with petroleum ether (ap–pt) of lemon balm extract. All extracts were then filtered through a Whatman filter No1 Prior to inoculation, an aliquot was further filtrated through a sterile and endotoxin free 0.2 μm polyethersulfone (PES) filter media (Whatman Puradisk 25 mm) to reduce the risk of interference by microorganisms.

RosM is 8.6 mg/mL rosmarinic acid (RA) solution, the common characteristic phenolic compound of *Lamiacae* family, which is an approximate concentration of RA in lemon balm [21] (, in an attempt to interpret the bioactivity of the previous extracts against specific compounds. Prior to application on pistachio seeds, aliquots of the plant extracts were further filtered through a sterile and endotoxin free 0.2 μm PES filter media (Whatman Puradisk 25mm) to reduce the risk of interference by microorganisms. Test solutions were prepared by 5 mL of each plant extract in which 45 mL of double distilled water and 0.01% of the surfactant tween 20 were added.

2.2. Reagents

Analytical standards including ferulic acid (> 98%), caffeic acid (> 98%), p-coumaric acid (> 98%), myricitrin (> 98%), luteolin-7-o-glucoside (> 98%) and kaempherol (> 98%) were of the highest purity and were purchased from Fluka Chemie Ag (Buchs, Switzerland). 2,2-diphenyl-1-picryhydrazyl hydrate (DPPH, 95% purity), Folin-Ciocalteau phenol reagent, gallic acid and rosmarinic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium carbonate (Na2CO3), methanol (HPLC grade) and diethyl ether with butylated hydroxytoluene (BHT) (purity 99.84%) were purchased form Fischer Scientific (Leics, UK). Water for HPLC PLUS was supplied by Carlo Erba (Val-de-Reuii, France). The reagents cyclohexanone (purity 99.0%) and formic acid (purity 98.0%) were obtained from Merck (Darmstadt, Germany) and Panreac (Barcelona, Spain), respectively, whereas standard, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), anhydrous magnesium sulfate (purity 97%) were supplied by Acros Organics (New Jersey, USA). A mixture of n-alkanes (C8–C20) was provided by Supelco (Bellefonte, PA, USA). BrK was supplied by Sigma Aldrich and ELISA test was supplied by (AgraQuant Total AF Test Kit 4-40 ppb, Romer Labs, Singapore).

2.3 Fungal species and physiologicyassays

Alternaria alternata, *Fusarium oxysporum*, *Aspergillus flavus* and *Beauveria bassiana* were grown in potato dextrose agar (PDA) plates under light conditions in order to produce fresh 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. Conidia 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 ^oC. Each treatment was performed in triplicate.

Mycelium diameter was measured at 2, 4 and 7 days for *A. alternata, A. flavus and F.oxysporum*, and 4, 6, 11, 15 and 18 days for *Beauveria bassiana*. Furthermore, % mycelium growth enhancement was calculated as follows:

Mycelium growth enhancement (%) = [(Dsample - Dcontrol) / Dcontrol] x100 ,

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Where, Dcontrol and Dsample are the mycelium diameter values of the control and the test sample at last day of measurement, 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:

Conidia production enhancement (%) = [(Cnsample - Cncontrol) / Cncontrol] χ100

Inhibition on conidia production was calculated as:

Conidia production inhibition (%) = [(Cncontrol - Cnsample) / Cncontrol] χ100

Where Cncontrol and Cnsample are the conidia concentration values of the control and the test sample, respectively.

2.4 Synergism evaluation

During the extraction procedure, a small quantity of lemon balm polar 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 lemon balm extracts. 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 according to [22, 15] 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

2.5 *In situ* evaluation of lemon balm on pistachio seeds infected by *Aspergillus flavus*

Pistachia vera seeds (cv Eginis) were primarily immersed in the plant test solutions that have been described above for 2h and then let dry in a laminar flow cabin for 24h. Pistachio seeds were infected by *Aspergillus flavus* by immersing them in a spore suspension (10^6 conidia/mL) for 30 min. After drying seeds were transferred in sterile petri dishes and were incubated at 28 ^{o}C in the dark for nine days. Each treatment was performed in triplicate. Furthermore, two control series were included, one (TrCt) where the plant extract was replaced by ddH20 as the test solution, and a second control series (Control) where the pistachio seeds were not treated by any test solution and were not inoculated with *A. flavus*, and a treatment of rosmarinic acid solution 8.6 mg/L. Each treatment was performed in triplicate.

2.6 ELISA test

The aflatoxins (AFs) concentration of pistachio samples was determined by the ELISA test (AgraQuant Total AF Test Kit 4-40 ppb, Romer Labs, Singapore). 10 gr ground portion of each pistachio sample was mixed with 50 mL 70% methanol extraction solvent for 3 minutes. Mixture was then filtered and pH was adjusted between 6-8. The filtrates were directly tested with ELISA kit according to the

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manufacturer's guidelines. 100 µL of each extracted filtrate and the AFs standards contained in the kit were mixed with 200 µL of the enzyme conjugated buffer and then were added to antibody strips and left for 15 min at room temperature. Microwells were then emptied and rinsed three times thoroughly before the addition of 100 µL substrated buffer and left for 5 min at room temperature before adding the stop solution buffer. In contaminated samples, AF competes for bidding sites with the enzyme conjugated buffer and in the presence of the substrate develops a blue color inversely proportional to AF concentration. ELISA plates were then read by a dual wavelength microwell reader (Biotek 800 TS). Data on AFs concentrations from the ELISA test were statistically compared by analysis of variance (ANOVA) and Fisher's Least Significant Difference (LSD) using the Statgraphics Plus Software.

2.7 Determination of total phenolic content

Total phenolic content of lemon balm aqueous extract was determined using a modified version of Folin-Ciocalteu assay [23, 24] that consisted in mixing 25 µL of extract (lemon balm aqueous extract), 125 µL of Folin-Ciocalteu reagent and 1500 µL of distilled water and permitted to react for 3 min. Then 375 µL of 20% (w/v) $Na2CO_3$ was added and the volume was made up to 2.5 ml with distilled water. The mixture was incubated at 25°C in the dark for two hours. The absorbance of extract was measured at 725 nm in triplicate on spectrophotometer reader (VMR, V-1200).

Quantification of total phenolic content was performed using a calibration curve of absorbance values derived from standard concentration solutions of gallic acid (0.1-1.0 mg/mL) elaborated in the same manner. Finally, the total phenolic content was expressed as mg of gallic acid per mL of aqueous extract (mg GAE/ mL extract). Each sample was tested in triplicate.

2.8 Determination of antioxidant activity

DPPH assay was used based on previously reported protocols to evaluate the radical scavenging capacity of lemon balm aqueous extract [25, 26]. 3.0 mL DPPH stock solution (100 µM) was mixed with plant extract (30 µL) into well plates. Firstly, was measured the absorbance of DPPH stock solution at 517 nm (Acontrol) and after 1h of incubation in the dark the absorbance of sample (Asample).

The evaluation of DPPH radical-scavenging activity carried out using a spectrophotometer reader (VMR, V-1200) and the calculation as a percentage of DPPH discoloration completed using the formula:

Inhibition (%) = (Acontrol-Asample / Acontrol) x 100,

where Acontrol and Asample are the absorbance values of control and test sample, respectively

Finally, the determination of antioxidant capacity was performed comparing the % inhibition of DPPH value obtained from lemon balm extract with % inhibition values of Trolox using a calibration curve (ranging from 2 to 50µM) and the results were expressed as µmol Trolox equivalents per mL of extract. Each treatment was performed in triplicate.

2.9 Determination of volatile compounds

The analysis of volatile compounds from the aqueous extract of lemon balm was carried out on a Trace Scientific GC Ultra gas chromatograph (Thermo Scientific, Ltd) equipped with a Thermo-5MS column (30 m length, 0.25 mm ID, 0.25 µm film thicknesses) (Thermo Scientific, Ltd) coupled with a Thermo Scientific DSQ II mass detector (Thermo Scientific, Ltd). High purity helium at a flow of 1ml/min was used as the carrier gas. Column temperature was initiated from 60 °C to 250 °C, with a rate 3 °C/ min; each analysis was 63.33 min long. Concentrated samples of 1 mL were injected manually and splitless, into the GC at 220 °C. The MS was set at electron impact mode (70 eV) at the interface temperature of 250 °C (MS transfer line). In the presence of cyclohexanone, as an internal standard, were calculated the relative percentages of the individual volatile components from relative peak areas (%). Identification of the volatile compounds was based on Retention Indices (RI, determined with respect to homologous

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series of n-alkanes C8-C20, under the same conditions of analysis) compared to literature values to support tentative identification and by matching their mass spectra to mass spectra libraries (Adams and x-Calibur).

2.10 Quantification and identification of phenolic compounds

The quantification of water soluble phenolic compounds was performed on a HPLC Agilent model 1100 (Agilent Corporation, MA, USA) system supplied with a Diode Array Detector (DAD). A reverse-phase column Supelco (Discovery HS C18), length 250 mm, internal diameter 4mm with material porosity of 5 μm was used and the column temperature was maintained at 25 °C. Mobile phase consists of two solvents: (A) acidified water, pH=2.5 and (B) acidified methanol and the following gradient program was performed: Initial 75% A, 25% B; 2 min 75% A, 25% B; 40 min 10% A, 90% B; 45 min 10% A, 90% B; 50 min 75% A, 25% B; 60 min 75% A, 25% B. The duration of each analysis was 60min. The flow rate of mobile phase was 0.4 mL/min. The DAD recorded spectra at absorptions of 260, 280 and 330 nm. The sample injection volume was 20 μL. The chromatograms were analyzed using the Agilent Chemstation software.

The LC-MS (ESI) technique was applied for the identification of phenolic compounds in lemon balm infusion. A Shimadzu LC-MS-2010A equipped with an LC-10ADvp binary pump, a DGU-14A degasser, a SIL-10ADvp auto sampler, a SPD-M10Avp Photo Diode Array Detector and a quadrupole mass detector (MSD) with electron spray ion source (MS-ESI). The mass spectrometer was operated in the negative ion mode. The instrumentation and conditions were the same as described for the HPLC method. The peak areas were determined at the characteristic m/z ratio of the compounds. For data acquisition the software Lab Solutions (Shimatzu version 3.40.307) was used.

The phenolic content was quantified based on standard curve of rosmarinic acid. Calibration standards were prepared by diluting a concentrated mixture solution of rosmarinic acid in the concentration range of 0.01 to 300 mg/ mL and the results were expressed as mg rosmarinic acid equivalents per mL of extract. Standard solutions were performed at least in triplicate. The phenolic content was quantified based on standard curve of rosmarinic acid.

Identification of each chromatographic peak was accomplished by comparing retention times, mass spectra, UV, spectra of authentic compounds and published data.

3. Results and discussion

3.1 Effect of crude and ap–pt lemon balm extracts on fungal growth

The biological activity of crude and ap–pt extracts lemon balm, was primarily screened using *in vitro* plate bioassays by measuring mycelium growth and conidia production of *A. alternate, A. flavus, F. oxysprorum and B. bassiana*. As presented in Table 1 lemon balm extracts (crude and ap–pt) found to enhance mycelium growth. Conidia production of was also enhanced noteworthy, up to 349.95% in case of *Alternaria* when treated by ap-pt, and *Asperillus* (234. 98%) when treated by ap-pt and crude extracts respectively. Reduction in conidia development observed only in case of *Aspergillus flavus* when treated by ap-pt extracts and *B. bassiana* for both crude and ap-pt extracts. The differences in biological activity between crude and ap-pt extracts of lemon balm attributed to synergistic action between volatile and water soluble compounds in crude extract. These actions are in detail identified clearly below, by calculating synergism indices as described in section 2.4.

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Table 1. Comparative differences on mycelium development and conidia production due to lemon balm extracts compared to control for all fungal species tested

	Mycelium diameter		Conidia production	
	Crude	ap-te	Crude	ap-te
	%	%	%	%
Alternaria alternata	+ 97.24	+ 37.81	+ 57.02	+ 349.95
Aspergillus flavus	+ 9.82	+ 15.26	+ 234.98	- 45.59
Fusarium oxysporum	+ 17.88	+ 5.59	+ 71.67	+140.63
Beauveria bassiana	+ 0.26	- 1.28	- 40.60	-20.59

Stimulation of mycelium growth and conidia production has been also observed in previous studies [27], and attributed to fungal secondary metabolites produced under stress conditions that are caused by external factors and influence. According to [28], epigenetics is capable to play a very active role in controlling already actively expressed secondary metabolites like aflatoxins.

3.2 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 or antagonism between water soluble and volatile organic compounds as have been previously identified by [15]. Evaluation of the effect of lemon balm crude and ap-te extracts on mycelium growth and conidia production before and after the extraction with petroleum allowed 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 2) volatile and water - soluble compounds are in absolute synergism in mycelium growth enhancement of all fungal species expect *A. flavus*. In case of conidia production where stimulation and reduction noticed both, greatest antagonism found in case of *Fusarium oxysporum*.

These interactions noticed, are may due to the main volatile compounds found in lemon balm as geranial, neral and citronellal β-caryophyllene, nerol acetate [29, 30, 31]. While chemical profile of plant extracts is differentiated according to species, region of cultivation, cultivation practices, harvesting time and post-collective treatment, a full phytochemical analysis of lemon balm extracts including antioxidant activity and total phenolics was conducted in this research in order to make the biological evaluation as more specific and targeted as possible to specific compounds.

Table 2. Synergism indices on fungal mycelium growth (SRm) and conidia production (SRc) of volatile and water soluble compounds of lemon balm extracts

	SRm	Action	SRc	Action
Alternaria alternata	+ 0.39	Synergism	+ 1.96	Antagonism
Aspergillus flavus	+ 1.55	Antagonism	+ 0.40	Synergism
Fusarium oxysporum	+ 0.31	Synergism	+ 6.03	Antagonism
Beauveria bassiana	+ 0.31	Synergism	+ 0.51	Synergism

3.3 Anti-aflatoxigenic activity of lemon balm extracts

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Lemon balm extracts biological activity was also screened against aflatoxins production on *Pistachia vera* seeds infected by *Aspergillus flavus*. Under the same *in situ* protocol was also evaluated the biological action of pure rosmarinic acid, in an equal concentration as in lemon balm extracts. This treatment was applied as rosmarinc acid which is the main characteristic phenolic compound of all *Lamiaceae* species and found in great percentages in lemon balm [21] in order to obtain an idea of rosmarinic acid contribution to the anti-aflatoxigenic activity that was noticed in lemon balm extract. The results of the anti-aflatoxigenic activity of lemon balm crude extract presented in Fig.1, where a total inhibition of aflatoxin production was noticed under the treatment of pistachio seeds by the lemon balm extract. Rosmarinic acid treatment reduced aflatoxins total concentration but not eliminated them to zero as lemon balm extract did, indicating that the great anti-aflatoxigenic activity of lemon balm extracts should be attributed to volatile constituents and possible synergism with the other water soluble compounds determined in the detail phytochemical analysis of lemon balm presented below. Inhibition of aflatoxins due to volatile compounds of plant extracts has also been mentrioned [32 as well as due to water soluble antioxidants of Lamiaceae species [33, 34].

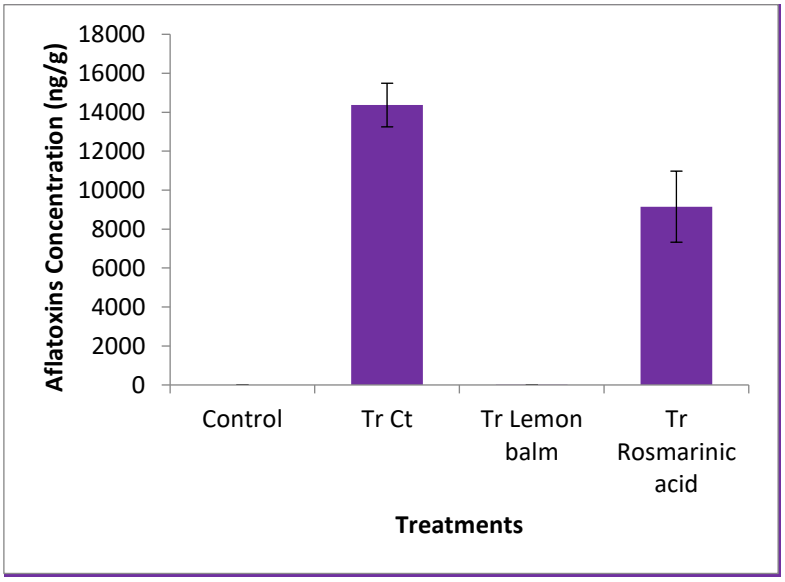

Figure 1. Aflatoxins concentration (ng/g) in *Pistachia vera* seeds infected by *Aspergillus flavus* and treated by lemon balm extract and rosmarinic acid solution. Control: *Pistachia* not inoculated by *Aspergillus flavus*. TrCt: Infected by *Aspergillus flavus* but treated with ddH_2O.

Similar effects on inhibition of aflatoxin production by *Aspergillus flavus* development have been observed in treatment by other natural products like oriental moustard flour, essential oil of Curcuma longa L., and Piper betle var. magahi [35, 36, 37, 38, 39].

The effect of essential oil and extracts of both aqueous and organic solvents of three plant species of the Lamiaceae family (*Thymus daenensis, Satureja khozistanica and Satureja macrosiphonia*) has also been studied in the development and production of aflatoxin by *A. flavus* and *parasiticus*. Based on the results of this study, essential oils and organic solvent extracts mainly prevented the growth of the strain while having little effect on aflatoxin biosynthesis AFB1, as opposed to aqueous extracts which did not reduce both fungal growth as they significantly reduced the production of aflatoxin [32].

3.4 Total phenolic content

The amount of total phenolic content of aqueous extract of lemon balm estimated by Folin-Ciocalteu assay and the value was extrapolated from a standard curve of gallic acid, as previously reported, was 0.176 mg GAE/ mL of extract (Table 3).

Previous study on lemon balm infusion reported 1.003 mg GAE/ mL [40]. Additionally, in other research [41] who studied infusions from various cultured and commercial lemon balm samples reported that TPC ranged from 293.32–959.54 mg GAE/ mL of infusion but the results were expressed differently, thus the values could not compare [42]. Other studies also reported that aqueous extract of lemon balm exhibited the higher phenolic content between other *Lamiaceae* species [15].

3.5 Antioxidant activity of lemon balm extract

The determination of free radical scavenging activity of the lemon balm aqueous extract was quantitatively estimated by measuring the reactivity of DPPH and expressed as μmol Trolox equivalents (TE)/ mL of aqueous extract, as previous described. Lemon balm aqueous extract indicated 39.932 μmol TE/mL (Table 3). This result is higher than the antioxidant values obtained by the same method of other aqueous extracts of lemon balm, specifically by infusions, [40,15] where found 10.970 and 6.340 μmol TE/ mL respectively but this came from the fact of different extraction procedures. Comparison with other published data [42, 43] was not possible due to a different expression of results. Lemon balm in previous studies [15] found to reach the maximum antioxidant activity values compared to other *Lamiaceae* species like oregano, dittany, sage and hyssop and this ranking of antioxidant activity was regardless of the method that antioxidant activity was determined (DPPH or ABTS) and the same observed with the ranking of the total phenolic content.

Table 3: Total phenolic content and antioxidant activity of lemon balm aqueous extract.

Total phenolic content (mg/mL)[a]	Antioxidant activity (μmol/mL)[b]
5.176 ± 0.040	39.932 ± 2.123

[a] Values expressed as gallic acid equivalents / mL of aqueous extract

[c] Values expressed as Trolox equivalents/ mL of aqueous extract

Oxidative stress has found to stimulate aflatoxin production based on previous studies [44, 45], while antioxidant components such as gallic acid have inhibitory effect on their production [46]. In [33] was also analyzed the effect of antioxidant components such as caffeic acid on microarray gene expression by finding that under its effect the expression of most genes involved in the biosynthesis of aflatoxins is restricted. The same results came from [34] regarding antioxidant compounds and aflatoxin production by *A. flavus*.

3.6 Analysis of volatile components of lemon balm extract

Quantification of volatile profile of lemon balm was accomplished by GC-MS. Totally, seven volatile compounds were detected and identified in the studied lemon balm aqueous extract (Table 4). Table 4also presents the retention indices (RIs), which calculated on the basis of homologous n-alkane (C8–C20) under the same conditions and the percentages of the seven detected compounds. The major constituent was geranial (42.63%), followed by high values which obtained from neral (36.63%) and piperitone (20.66 %). The other detected monoterpenes, but with very low values, were neo-isopulegol (0.04%) and the two diastereomers forms carvacrol and thymol at the same percentage (0.01%). The identified two main compounds are in accordance with the previous research [30, 31] that show that the infusion of lemon balm is mainly composed of two the isomers geranial and neral. However, this study reveals that citronellal is also a main compound of lemon balm infusion contrary to piperitone which obtained at the present study [47]. According also to [48] hydroalcoholic extract of lemon balm

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contains neral (Z-citral) and geranial (E-citral) as main compounds, which are the same with identified compounds in the present study, also identified citronellal, derivatives of geranial, trans-caryophyllene and its oxide. The qualitative differences are possibly due to the different climatic regions, specific soil quality and air temperature.

Table 4: Volatile compounds detected in organic extract of lemon balm aqueous extract by GC-MS.

No.	Compounds	RI[a]	%
1	*neo*-isopulegol	1201	0.04
2	neral	1293	36.63
3	carvone	1303	0.01
4	piperitone	1314	20.66
5	geranial	1325	42.63
6	thymol	1359	0.01
7	carvacrol	1362	0.01

[a] Retention indices relative to C9-C24 n-alkanes on a Thermo-5MS column

3.7 Analysis of phenolic compounds of lemon balm aqueous extract

As presented in Table 5 t caffeic acid, ferulic acid, coumaric acid, myricitrin, luteolin-7-o-glucoside, rosmarinic acid and kaempherol were being identified based on their chromatographic behavior (Figure 2) and spectral characteristics using LC-MS (Figure 3).

Table 5: Determination of composition of aqueous extract of lemon balm by HPLC–DAD/LC-MS.

Compounds	RT (min)	mg RA/ mL[a]	%
caffeic acid	19.67	0.836 ± 0.013	7.6
coumaric acid	22.80	0.425 ± 0.004	3.9
ferulic acid	23.24	0.387 ± 0.004	3.6
myricitrin	24.95	0.497 ± 0.002	4.6
luteolin-*7-o*-glucoside	25.921	0.280 ± 0.001	2.6
rosmarinic cid	27.19	1.256 ± 0.004	11.8
kaempherol	36.84	0.023 ± 0.003	0.3

[a]Results are expressed as mean of rosmarinic equivalents ± standard deviation (SD) of three determinations

Moreover, based on the quantification analysis performed with HPLC coupled DAD, by comparison of their retention time with the standards, it appears that the major compound present in lemon balm aqueous extract is rosmarinic acid (1.256 ± 0.004) that represent 11.8 % of the extract. In contrast kaempherol (0.023 ± 0.003) that represent 0.3 % of the extract was the least abundant of the polyphenols determined in lemon balm aqueous extract. Caffeic acid (0.836 ± 0.013) revealed as 7.6 % of the studied extract, followed by myricitrin (0.497 ± 0.002) as 4.6%. Coumaric acid (0.425 ± 0.004) and ferulic acid (0.387 ± 0.004) representing similar percentages 3.9% and 3.6%, respectively. Moreover, luteolin-7-o-glucoside (0.280 ± 0.001) was found as 2.6%.

Rosmarinic was confirmed as being the main component, according to other studies where determine rosmarinic acid content in aqueous extracts [49, 50]. One of the major identified compounds, caffeic acid has already reported to be present in the aqueous extract by other researchers. However,

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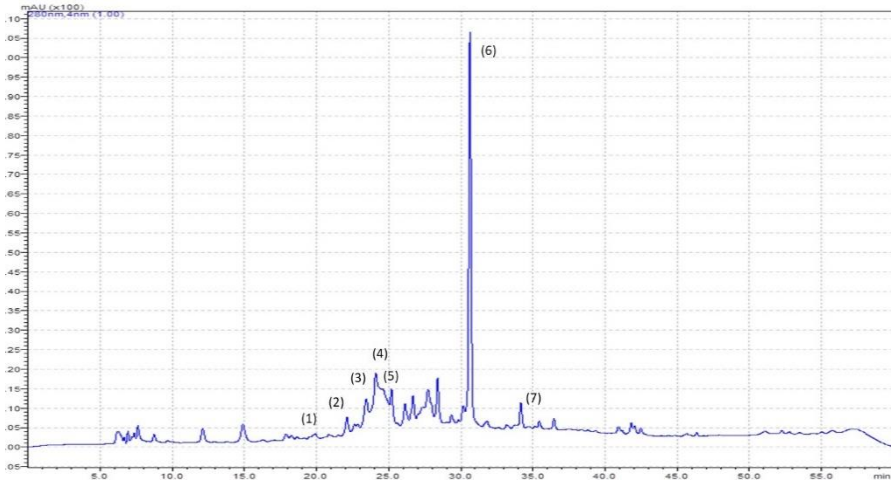
447 the absence of rosmarinic acid at these studies may be due to a variety of reasons ranging from climate
448 and geography to difference in the extraction procedures used (amount of water) [43, 51]. Previous
449 study on lemon balm infusion reported the present of luteolin-7-o-glucoside, caffeic and rosmarinic
450 acids [1]. The presence of ferulic acid, coumaric acid, myricetin and kaempferol in aqueous lemon balm
451 extract detected in this research has not been reported in other studies (Fig 2, 3).
452



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455 **Figure 2.** Peaks of main components identified. (1) caffeic acid, (2) p-coumaric acid, (3) ferulic acid,
456 (4) myricitrin, (5) luteolin7-O-Glucoside, (6) rosmarinic acid, (7) kaempferol
457

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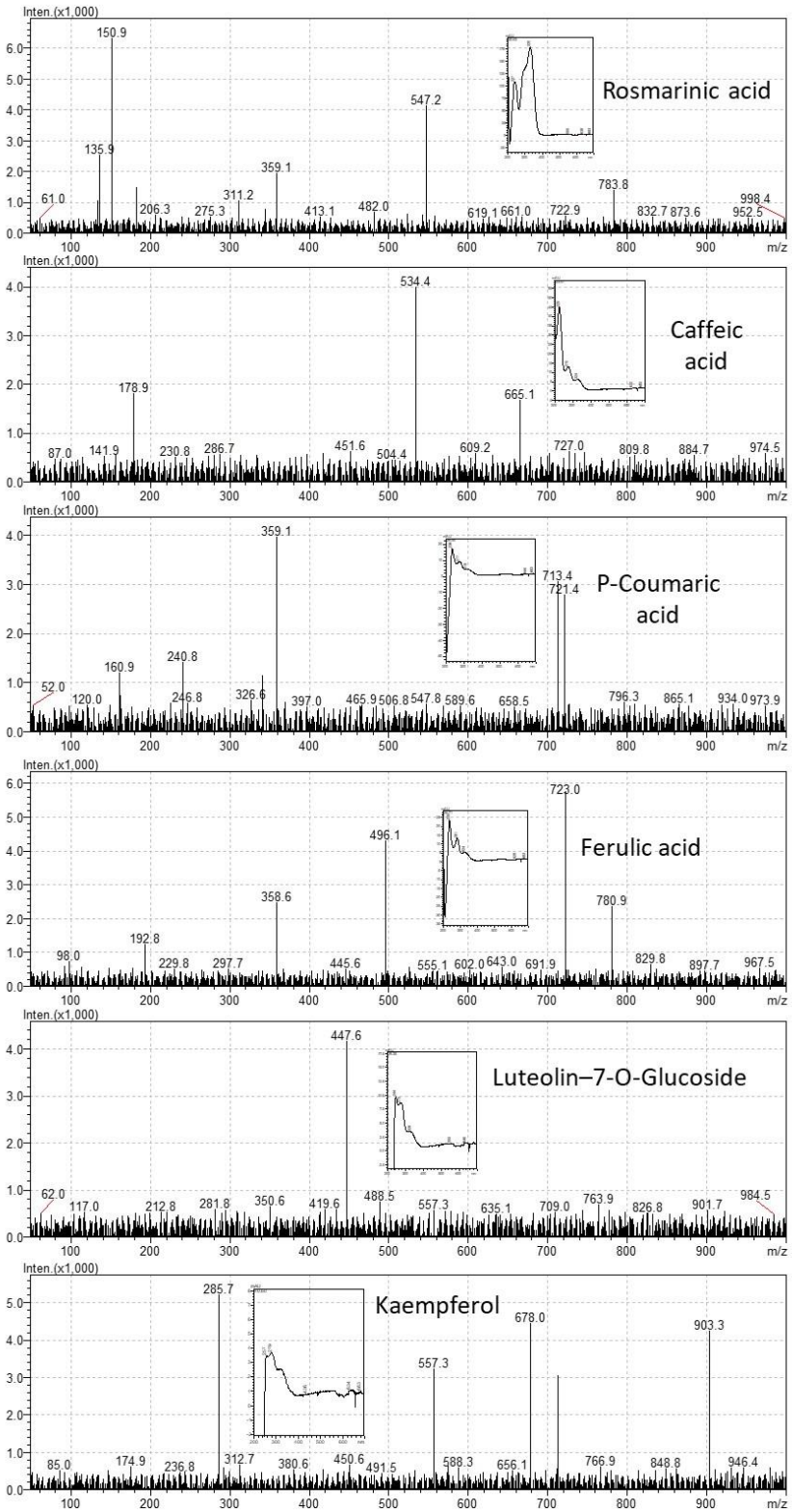

Figure 3. LC/MS Chromatograms of main components

4. Conclusions

Findings of this study underline the high biological active potential of lemon balm extracts under various screening tests. The most interesting action from the point of view of the possible exploitation of beehive from the biotechnology and food technology sector is that the lemon balm extract, although it did not have antimicrobial activity against A. flavus, significantly reduced the aflatoxin production, as quantified by ELISA in situ bioassays with *Pistacia vera* seeds. The anti-aflatoxigenic activity was mainly attributed to its antioxidant activity and antioxidant components which are directly related to the limitation of aflatoxins, as well as the synergism between the water soluble phenolic compounds and the presence of ferulic acid, coumaric acid, myricetin and kaempferol in aqueous lemon balm extract which although detected in this research have not been reported in other studies.

This study addresses new research paths towards the investigation of molecular mechanisms where lemon balm extracts intervene as well as further investigation of biological activities of the specific water soluble and volatile compounds of lemon balm extracts observed.

The results of this study are extremely encouraging for the potential use of lemon balm extracts to treat the presence of aflatoxins in post-harvest foods. The fact, of course, further investigates the effect of a wide range of concentrations of aqueous bee extract on the production of aflatoxins by *A. flavus*, as well as conducting in-situ tests on a wide range of species beyond *Pistachia vera* in order to demonstrate its efficacy as an antiflatoxin agent which can be safely used by food industry.

Authors contributions Efstathia Skotti: Conceptualization, Apply of analytical methods, Writing – Original Draft Preparation; Resources. Review & Editing. Sophia Nefeli Sotiropoulou: Apply of analytical methods, Writing – Original Draft Preparation. Iliada K. Lappa: Writing – Original Draft Preparation; Resources, Review & Editing. Maria Kaiafa: Apply of analytical Methods. Dimitrios I. Tsitsigiannis: Conseptualization, Review and editing. Petros A. Tarantilis: Conseptualization, Review and editing

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