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Research Article

Evaluation of essential oil and hydrolate from a new hyssop variety (*Hyssopus officinalis* L.)

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Abstract

The main objective of this study was to evaluate the quality of essential oil (EO) and hydrolate (HY) obtained from a new Romanian variety of hyssop (*Hyssopus officinalis* L., Lamiaceae family), namely 'Cătălin'. The chemical composition and the concentration of the compounds was established by gas chromatography coupled to mass spectrometry (GC/MS). The main constituents identified in hyssop EO and HY were *cis*-pinocamphone (34.63% and 67.00%), *trans*-pinocamphone (11.72% and 14.58%), thujenol (1.39% and 6.05%). The evaluation of the antioxidant capacity was performed by three methods (DPPH, ABTS and FRAP), EO proving a higher oxidizing activity compared to HY one. The antimicrobial activity of the essential oil was evaluated *in vitro,* in order to detect its ability to inhibit G^- phytopathogenic bacteria (*Pseudomonas syringae*) and plant pathogenic fungi (*Fusarium oxysporum*). Eugenol, linalool and estragole standards were used as reference volatile compounds. Regarding *Pseudomonas syringae* (LMG5090) bacterium, assays showed that hyssop oil does not inhibit its growth. Estragole and eugenol showed pronounced antibacterial activity in all tested concentrations, both in the first 24 hours of incubation and after 3 days. Linalool instead has bacteriostatic activity only at high concentrations (50% and 100%), an inhibitory activity that is maintained only in the first 24 hours of incubation. The results obtained against *Fusarium oxysporum* reveal that the EO tested has no fungicidal activity but only fungistatic, and it is able to delay mycelial growth and the degree of inhibition depending on the concentration used.

Keywords: antimicrobial activity; antioxidant activity; chemical composition; hyssop
Abbreviation: essential oil (EO); hydrolate (HY)

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Introduction

Hyssop (*Hyssopus officinalis* L., fam. Lamiaceae) is one of the important medicinal plants which is cultivated in Romania, a xerophytic type plant, well adapted to drought (Ciocârlan, 2009). Although it has a bitter taste, hyssop is used as a food flavouring and has traditionally been used for medicinal purposes, in antispasmodic, antifungal and cough treatments (Fathiazad and Hamedeyazdan, 2011; Vlase *et al.*, 2014).

The main constituents of hyssop include several polyphenolic compounds, primarily flavonoids: apigenin, quercetin, diosmin, luteolin and their glycosides, followed by other phenolic compounds: chlorogenic, protocathetic, ferulic acids, syringic, p-hydroxybenzoic and caffeic acids (Fathiazad and Hamedeyazdan, 2011). In addition to the essential oil, the hyssop plant also contains phenolic acids, tannins, diterpene lactones (marubina) and triterpenoid compounds such as ursolic and oleanolic acid (Venditti *et al.*, 2015). The essential oil is the most important and most frequently researched product of hyssop (Benedec *et al.*, 2003; Vlase *et al.*, 2014). Data from the literature on wild and cultivated plants indicate that the plant produces between 0.3-1% essential oil with several main compounds, terpenoids of *iso*-pinocamphone type dominant compound, along with β-pinene, 1,8-cineole, pinocarvone, linalool, sabine and methyl eugenol (Hristova *et al.*, 2015).

Studies conducted by Chalchat *et al.* (2001) showed that the main compounds of hyssop essential oil are mainly *cis-* and *trans-* pinocamphone and pinocarvone, along with smaller amounts of germacrene-D, bicyclo-germacrene, elemol and spathulenol. Also, the presence of aliphatic fatty acids: palmitic acid (15.60%), stearic acid (10.73%), linolenic acid (63.98%), arachidic acid (2.64%) and eicosadienoic acid (0.68%), was determined in hyssop oil (Benedec *et al.*, 2003). The content and quality of the essential oil may vary within a single species from one growing season to another and may be affected by the variety used, the extraction method, the climatic conditions of that year and the agrotechnics applied (Ghalem and Mohamed, 2009; Xu *et al.*, 2011). The harvesting stage also has a significant effect on the quantity and quality of the essential oil. This is one of the main reasons why it is important to choose the right harvesting period to obtain a superior essential oil, both quantitatively and qualitatively.

There is a relationship between production yields, ontogenetic variations and different parts of plants. All these parameters together, significantly affect the essential oil and its composition in the semi-arid climatic conditions of Turkey. It was confirmed that the pre, complete and post-flowering ontogenetic stages of hyssop contained over 1.0% essential oil, but also a high content of *iso*-pinocamphone and β-pinene, present in different parts of the plants. Therefore, hyssop could be profitably cultivated for commercial production of *iso*-pinocamphone and β-pinene under semi-arid conditions.

The extracts and essential oil obtained from hyssop showed moderate antioxidant and antimicrobial activity against the activity of G^+ and G^- bacteria, along with antiviral, antifungal and insecticidal properties '*in vitro*' studies (Judžentienė, 2016).

The present study aimed to characterize the chemical composition, quantitative dosage of three compounds (linalool, estragole, eugenol), antioxidant and antimicrobial activity of the EO and HY obtained from a new hyssop variety ('Cătălin'), of Romanian origin. Although the three compounds (linalool, estragole and eugenol) are not compounds with a high level in EO and HY of 'Cătălin' variety, they were nevertheless chosen as standards because they have antimicrobial activity for other strains of bacteria and fungi.

Materials and Methods

Biological material

The plant material was represented by the first Romanian variety of hyssop (*Hyssopus officinalis* L., fam. Lamiaceae), namely 'Cătălin', which is in the process of homologation. The variety comes from Line 1 selected

from the local population 'De Ciorani', which is part of the Germplasm Resources Collection of the Vegetable Research-Development Station Buzau, Romania. It is a perennial, semi-early variety that can be grown in the open field, with good resistance to drought and frost. Hyssop crop was established in 2019 (Băneasa area, Bucharest: 44°30'2" N and 26°4'20" E, altitude 90 m), agricultural year characterized by lack of rainfall and temperatures of over 35 °C during the vegetation period of the plants. During the establishment, the cultivation technology of this species was followed in terms of land preparation, seedling planting, crop maintenance works, harvesting. The harvesting was carried out in a mechanized way in June and September, and the total production obtained was 1,500 kg ha^{-1} green plant mass.

Isolation of EO and HY

To obtain the EO and HY, the plants were harvested at the time of flowering, and hydrodistillation was used as the extraction method, using shoots and inflorescences. The hydrodistillation was performed with the help of an Aura Distillateur installation. It consists of: 130 l stainless steel main tank, a stainless-steel spiral condensation vessel with the role of transforming water and oil vapour into a liquid mixture, an electric steam generator (15 kW) and a glass tank (Florentine flask) with a capacity of 60 l, which has the role of separating EO from HY. The hydrodistillation was done in series of 10 kg of green plant, and the distillation time was on average 2.5 hours/series. The same procedure was used for the HY as in the case of EO of hyssop.

Prior to use, EO and HY were stored in dark bottles, in the refrigerator (t=4 °C). The amount of oil obtained from vegetable material was calculated as:

$$\text{Oil (\%v/w wet base)} = \text{Observed volume of oil (mL)}/\text{Weight of sample (g)} \times 100 \quad (1)$$

EO and HY analysis

Their chemical composition, the concentration of the compounds was determined by gas chromatography coupled to mass spectrometry (GC/MS), using an Agilent Technologies gas chromatograph type 7890 A GC system; MS Agilent Technologies type 5975 C Mass Selective detector; Column macrogol 20,000; carrier gas - helium for R chromatography with a flow rate of 1.5 mL/minute; temperature regime – 250 °C (10 degrees/min) up to 280 °C (const. 5.5 min); Injector temperature 220 °C; detector temperature 235 °C; mobile phase - helium 1 ml/min; split injector; split ratio - 1:100; automatic injection system for the sample to be analysed; volume used for the analysis -1 mL essential oil. Prior to injection, EO was dissolved 100 times in hexane R, and 15 ml of undiluted HY was extracted into 10 ml of hexane R, then dried over anhydrous sodium sulfate R. Standards used: linalool (97% purity), estragole (98% purity) and eugenol (99% purity), purchased from Sigma-Aldrich.

Using the retention times and spectra in the chromatograms of the reference solutions, their compounds were located in the chromatograms obtained with each test solution. The individual constituents were identified by their identical retention indices, referring to compounds known from literature data (Adams, 1995). Identification was based on standard mass library used by National Institute of Standards and Technology and Wiley libraries to detect the possibilities of EO and HY components.

Analysis of EO and HY antioxidant activity

The use of the three methods provides an overview of the antioxidant activity of EO and HY of hyssop, each method is based on different principles, and the information complements each other.

The scavenger activity of the DPPH radical (2,2-Diphenyl-1-picrylhydrazyl) is based on the ability of antioxidants to reduce the DPPH radical. The percentage of DPPH remaining in the solution is calculated according to the formula:

$$\%DPPH = [(A_{control\ sample} - A_{sample})/A_{control\ sample}] \times 100 \quad (2)$$

$A_{control\ sample}$ = absorbance of the control sample

A_{sample} = absorbance of the sample

The amount of sample required to reduce DPPH absorbance by 50% is called IC_{50}. All samples were worked in triplicate; to determine IC_{50}, 5 concentrations were tested for each sample.

The scavenger activity of the ABTS radical (2,2`-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) - the method is known as TEAC (Trolox Equivalent Antioxidant Capacity) because the expression of the antioxidant capacity of the extracts can be done as Trolox equivalents. The antioxidant capacity was expressed in mM Trolox using 3 calibration curves of the Trolox standard.

Ferric ion Reducing Antioxidant Power (FRAP) - is based on the ability of antioxidants to reduce the yellow-coloured tripyridyltriazine - Fe^{3+} (Fe(III)-TPTZ) complex to the blue-coloured tripyridyltriazine - Fe^{2+} (Fe(II)-TPTZ) complex , by the action of electron release by antioxidants. The samples were worked in triplicate, and the FRAP values of each sample were expressed in mM Trolox g^{-1} for EO and $L^{-1}h$ for HY respectively.

Microbial strains and growth conditions

The G^- bacterial strain, *Pseudomonas syringae* (LMG5090), used in the experiments, was provided by the Research and Development Institute for Plant Protection, Bucharest, Romania. *Pseudomonas syringae* is a phytopathogenic bacterium with an extremely varied host range. Due to this aspect, the species is used as a model organism in numerous studies (Xiu *et al.*, 2018), to understand the pathogenicity mechanisms encountered in bacteria. Over 50 pathological varieties have been identified in this bacterial species, and these pathovars can infect almost all plants of economic interest. Bacterial inoculum was obtained from fresh cultures, prepared in Luria Bertani (LB) broth at 28 °C, under orbital shacking at 150 rpm.

The phytopathogenic strain of the fungus *Fusarium oxysporum* (ZUM 2407) was provided by the USAMV Bucharest, Faculty of Biotechnology, Romania. It comprises more than 120 special forms and resistance breeds, and most of them are pathogenic to plants of agricultural and horticultural interest, with a very wide range of host plants. Sometimes it can also develop saprophytically, on plant debris and in the soil, or as an asymptomatic endophyte, harmless to the host (*http://eol.org/*). The fungal inoculum was prepared as mycelia plugs, 6 mm in diameter, collected from 14-days old cultures obtained on Potato-Dextrose-Agar.

Essential oil emulsion

For antimicrobial assays the EO were tested undiluted (100% conc.) and in 75%, 50% and 25% concentration. Emulsions were prepared in 10% DMSO supplemented with 0.5% Tween 80 (Prabuseenivasan *et al.*, 2006). This solvent reveals no influence on the microbial growth.

Antibacterial assay

The antibacterial potential of EO was tested and analysed in vitro conditions. Non-ventilated, sterile, polypropylene Petri dishes, 90 mm in diameter, were used in this study. Each plate was filled with 20 ml of LB agar and plated with fresh bacterial suspension (10^8 CFU/ml). EO was placed equidistantly spotted (10 µl/spot) four times on each plate. Four replicate plates were prepared for each concentration of the tested oils. Positive controls, without EO, were also prepared for phytopathogenic bacterium. All plates were sealed with parafilm, incubated at 28 °C. Biometric measurements were taken after one to 24 hours and 3 days after inoculation. Similarly, the three commercial compounds (estragole, eugenol and linalool), used undiluted (100%) or in the proportion of 1/2 (50%) and 1/4 (25%), were tested. Antibacterial activity was estimated based on the clear areas where the pathogen could not colonize the growth substrate.

Antifungal assay

The antifungal assay was performed in similar conditions as the previous test. However, to sustain the fungal growth, Potato-Glucose-Agar (PDA) medium was used. The plates were inoculated in the centre with mycelia plugs, 8 mm in diameter. Four sterile paper disks, 5 mm in diameter, were placed equidistantly at 2 cm

distance from the fungal inoculum. Each disk was filled with 10 µl of EO emulsion. Four concentrations were tested, one concentration/plate, each plate in four replicates. Positive controls, without EO, were also prepared for each plant pathogenic fungi. Plates were sealed with parafilm, incubated at 26 °C to 28 °C and analysed on a daily basis for the first 10 days after inoculation. Subsequently, after 10 days of maintenance at room temperature, the plates were visually analysed to confirm the antifungal activity. Biometric analyses were made to the fungal growth, in order to evaluate the antifungal potential of the EO. Fungal inhibition efficacy was calculated according to the formula proposed by Lahlali and Hirji (2010):

$$E\%=[(Rc-Rt)/Rc] \times 100 \quad (3)$$

R_c = the radius of the fungal colony in control plates,

R_T = the fungal radius in the test plates.

Statistical analysis

The results of the antioxidant activity for EO and HY were analyzed and calculated using Microsoft Excel software. Data were analyzed by unidirectional analysis of variance (ANOVA) and T-Test ($P < 0.05$). Values of $P < 0.05$ were considered statistically significant.

The statistical analysis performed for the interpolation of the experimental data on the antimicrobial activity of hyssop essential oil were performed with the Matchcad 2000 programs (Reference manual, Math Soft, 2000) and Excel from the MS Office 2007 package.

Results and Discussion

EO and HY chemical composition by GS/MS

EO_s regardless of the plant they come from, are complex mixtures of volatile, lipophilic and odoriferous substances in the secondary metabolism of plants. They are composed of monoterpenes, sesquiterpenes and their oxygenated derivatives (alcohols, aldehydes, esters, ketones, phenols and oxides). Bicyclic monoterpene such as pinocamphone (*trans*-pinocamphone and *iso*-pinocamphone), *cis*-pinocamphone are the main characteristic compounds for oils obtained from *Hyssopus officinalis* (Hussein *et al.*, 2015). When hyssop oil is used industrially, for quality control it is recommended to identify plant samples at the intraspecific level and to carry out a rigorous chemical analysis. The regulation used to certify the quality of hyssop oil in international trade reports, mentions the presence of 13 compounds as standard, among which pinocamphone, *iso*-pinocamphone and pinene are the most abundant (40-90%) (ISO 9841:2013). Pinocamphone, due to its camphorated smell, slightly spicy, with moderate to weak intensity, is widely used in perfumery, but also in the composition of liqueurs.

Hydro distillation of aerial parts of *H. officinalis* yield oil about 0.27% (v/w). The EO and HY were analysed by GC/MS for determination of their components and results are given in Tables 1 as a relative peak area of each constituent. The chemical composition of EO and HY obtained from *H. officinalis*, 'Cătălin' variety is shown in Table 1, and the EO chromatogram in Figure 1. Twenty-eight volatile compounds were identified in hyssop EO, which represented 99.65% of total compounds. The majority of the compounds were: *cis*-pinocamphone (34.63%), *trans*-pinocamphone (11.72%), β-pinene (10.46%), germacren-D (7.27%) and terpinene (7.19%). Minor contributions to the total chemical compounds also had elemene (6.20%), β-caryophyllene (2.63%), α-thujene (2.05%), thujenol (1.39%).

For HY obtained by hydro distillation (time: 2.5 hours) from 'Cătălin' variety, the results obtained from the analyses made by means of GC/MS are also presented in Table 1. Seventeen compounds representing 96.15% of the total separated compounds were identified. C*is*-pinocamphone (67.00%), *trans*-pinocamphone (14.58%) and thujenol (6.05%), were the majority compounds identified. Minor contributions to the total chemical compounds also had pinenol (2.87%), linalool (1.29%), α-therpineol (1.05%).

Table 1. Chemical composition of the EO and HY isolated from aerial part of the hyssop (*Hyssopus officinalis* L., fam. Lamiaceae), 'Cătălin' variety

Compound name	EO of hyssop 'Cătălin' variety		HY of hyssop 'Cătălin' variety	
	RT	Area %	RT	Area %
α-Pinene	5.45	0.66	nd	nd
α-Phellandrene	5.69	0.32	nd	nd
β-Pinene	9.16	10.46	nd	nd
α-Thujene	10.26	2.05	nd	nd
Myrcene	13.20	1.78	nd	nd
D-Limonene	14.45	1.03	nd	nd
1,8-Cineole	14.70	nd	14.70	0.36
Terpinene	14.80	7.19	nd	nd
β-Ocimene	17.02	1.02	nd	nd
Carenol	21.15	1.86	nd	nd
Octenol	23.45	nd	23.45	0.33
Hexanol	24.49	nd	24.49	0.18
trans-Pinocamphone	*25.00*	*11.72*	*25.00*	*14.58*
Aristolene	25.31	0.71	nd	nd
Ethylenhexanol	25.41	nd	25.41	0.15
cis-Pinocamphone	*25.78*	*34.63*	*25.78*	*67.00*
Linalol	26.03	0.74	26.03	1.29
Pinenol	26.38	1.74	26.38	2.87
β-Caryophyllene	27.04	2.63	nd	nd
Terpinen-4-ol	27.30	nd	27.30	0.76
Myrtenol	27.91	nd	27.91	0.13
Longifolene	28.22	2.02	nd	nd
Iso-Pinocarveol	28.54	nd	28.54	0.23
α-Caryophyllene	28.81	0.44	nd	nd
Estragole	28.99	0.22	nd	nd
Myrtenil acetate	29.35	0.81	nd	nd
α-Terpineol	29.60	0.20	29.60	1.05
Germacrene-D	29.76	7.27	nd	nd
β-Elemene	30.33	6.20	nd	nd
Cadinene	30.90	0.37	nd	nd
Thujenol	31.72	1.39	31.72	6.05
Pinonediol	35.18	nd	35.18	0.26
Isoeugenol	36.39	nd	36.39	0.27
Guaiol	37.63	1.13	nd	nd
Pholedrine	37.94	nd	37.94	0.17
Spathulenol	38.45	0,38	nd	nd
Eugenol	39.32	0.42	39.32	0.47
Ɉ Eudesmol	40.39	0.26	nd	nd
Total of major compounds	28 compounds identified 99.65%		17 compounds identified 96.15%	
Monoterpene hydrocarbons	24.51		-	
Monoterpenes oxigenated	55.33		94.62	
Sesquiterpenes hydrocarbons	17.62		0.17	
Sesquiterpenes oxigenated	2.19		0.70	
Others	-		0.66	

RT-Retention time; Area- the values were expressed as [area percentage]; nd–not detected.

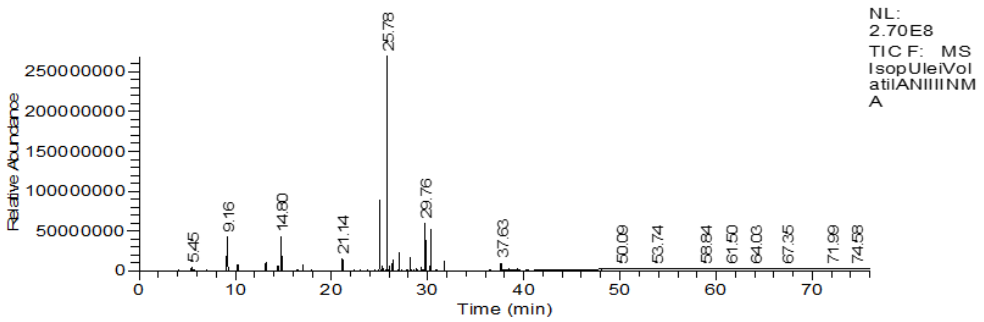


Figure 1. Chromatogram EO of hyssop (*Hyssopus officinalis* L.), 'Cătălin' variety

Subsequent dosing of the 3 compounds of interest, using high purity standards, showed that linalool was present in EO (1.29%), estragole (0.22%), while eugenol could not be dosed because the compound was not identified in the chromatogram.

The classification by class of the identified compounds, showed that for EO, monoterpenes oxigenated have priority by 55.33% followed by monoterpenes hydrocarbons by 24,51%, sesquiterpenes hydrocarbons 17.62% and sesquiterpenes oxigenated 2.19%. For HY monoterpenes oxigenated have priority with 94.62%.

The literature (Fathiazad and Hamedeyazdan, 2011; Ogunwande *et al.*, 2011) mentions the characteristic compounds of hyssop oil: *iso*-pinocamphones (syn. *cis*-(3)-pinanone), pinocamphone (syn. *trans*-(3)-pinanone) and their β-pinene precursor. Other compounds mentioned are pinocarvone, sabinene, germacrene-D, germacrene D-4-ol, α-, β-phellandrene, 4-carvomentenol, thymol, carvacrol, elemol, limonene, linalool, 1,8-cineole, α-terpinene, myrtenol, myrtenyl acetate, (methyl) eugenol, etc.

Khan *et al.* (2012), in the studies performed on EO obtained from hyssop growing in Kashmir, showed that in notable quantities are: mirene (2.24%), terpinene (1.03%), (Z)-sabinene hydrate (2.02 %), linalool (1.04%), terpinen-4-ol (2.55%), germacrene-D (1.61%) and elemol (3.43%). The main constituents of the oil previously reported were: pinocamphone, *iso*-pinocamphone, β-pinene, 1,8-cineole, myrtenol and pinocarvone. The same study also notes that essential oil yield is affected by the harvesting stage, so that the young plants in the pre-flowering stage have a lower yield in obtaining the essential oil compared to the older plants, which are in full bloom and post-flowering stage. It has also been observed that pinocamphone (the main compound in hyssop oil) gradually increases quantitatively until after the post-flowering stage. The other important compound, β-pinene, quantitatively in the plant first increased and then decreased, after the full flowering stage.

Although the three compounds (linalool, estragole and eugenol) in EO and HY of hyssop do not have a high level, the literature cites the antimicrobial activity (Pereira *et al.*, 2018), but on other phytopathogenic strains. Herman *et al.* (2016) showed that Linalool has the potential to significantly increase the antimicrobial efficacy of essential oils. In the study by Tan and Nishida (2012), it is mentioned that methyl-eugenol (eugenol precursor) has high antimicrobial activity against some bacteria G^+ (*B. subtilis* and *B. aureus*), G^- (*E. coli* and *Salmonella*), fungi (*A. niger* and *A. oryzae*).

Linalool (3,7-dimethyl-1,6-octadien-3-ol) is an acyclic tertiary monoterpene alcohol and one of the major floral fragrances that comes from nature. It is widely used as a fragrant ingredient, being a component of the top notes of perfumes (Venditti *et al.*, 2015).

Eugenol is a natural compound found in a variety of sources such as spices (nutmeg, allspice), medicinal plants (basil, tarragon), fruits (bananas, oranges) and is a basic compound found in essential oils from citronella plants (Southwell *et al.*, 2011; Venditti *et al.*, 2015). In the analysed samples, this compound was identified only in hyssop hydrolate, but in small quantities (0.47%).

Studies on hyssop from Bulgaria showed that the main constituents of EO were: 1,8-cineole (39.6-48.2%), *iso*-pinocamphone (16.3-28.0%) and pinene (9.4-11.4%), while methyl eugenol was absent. A similar

composition was found in a hyssop population growing in eastern Serbia, with 1,8-cineole (36.4%), pinene (19.6%) and *iso*-pinocamphone (15.3%) being the important compounds (Dzamic *et al.*, 2013).

The results of the study (Venditti *et al.*, 2015) conducted on 2 subspecies of hyssop from Italy (subsp. *officinalis* and subsp. *aristatus*) showed the presence of monoterpenic ketones such as pinocamphone, *iso*-pinocamphone and phenylpropanoids (methyl eugenol) in the EO.

The study on the EO obtained from the aerial part of hyssop plants grown in Egypt (Hussein *et al.,* 2015), identified the presence of 33 compounds that accounted for 99.99% of total oil. The major compounds were *cis*-pinocamphone (26.85%), β-pinene (20.43%) and *trans*-pinocamphone (15.97%). Studies conducted in Poland by Zawiślak in 2013, reported that the main compounds of EO obtained from hyssop were: *cis*-pinocamphone (33.52-37.13%), *trans*-pinocamphone (23.43-28.67%), β-pinene (7.89-8.12 %), elemol (5.86-8.95%), germacrene-D (3.23-4.65%), E-caryophyllene, (2.67%), etc.

Monoterpenes (*iso*-pinocamphone, *trans*-pinocamphone), the main representatives in this oil, are relatively rarely detected in larger quantities in the essential oils of other species. In number, at the most, 44 compounds were detected in hyssop oil (Hüsnü Can Baser and Buchbauer, 2020). In addition to the compounds mentioned, there are also other monoterpenes such as β-pinene, pinocarvone, myrtenol, etc., but they are present in minimal concentrations.

In this study it was observed that by distillation, compounds belonging to the classes of monoterpene hydrocarbons and sesquiterpene hydrocarbons pass completely into hyssop EO and the HY contains only oxygenated terpenes which have not fully distilled. Monoterpenes such as myrcene, thujene, terpinene are found in low concentrations in EO, so the chemical composition differs. Comparing the results obtained with those existing in the literature, the composition of our sample is similar, but it shows quantitative differences between compounds, here the main compound is *cis*-pinocamphone, *trans*-pinocamphone (Chalchat *et al.*, 2001) followed by β-pinene and germacrene-D. This variation of the chemical composition of EO obtained from hyssop, variety 'Cătălin' exists and can be attributed to environmental conditions, location and harvesting period.

Antioxidant activity of the EO and HY

One molecule of antioxidants can prevent oxidation or delay the production of other molecules. Oxidation covers chemical reactions in which electrons are transferred from one substance to another. Free radicals are created by oxidation reactions that cause chain reactions that damage cells. Antioxidants limit these chain actions by temporarily eliminating free radicals and decreasing other oxidation reactions. To become a stable diamagnetic molecule, DPPH is a stable free radical and accepts an electron or a hydrogen radical (El-Beltagi *et al.*, 2019).

The antioxidant capacity of the EO and HY obtained from 'Cătălin' variety was determined by three methods (Tamokou *et al.*, 2013; Vlase *et al.*, 2014). Three methods were used to evaluate the antioxidant activity for hyssop EO and HY, to find out which of them is the most sensitive, so as to best reflect their antioxidant capacity.

The results regarding the antioxidant activity obtained by the three methods (DPPH, ABTS, FRAP), for both EO and HY obtained from the hyssop variety 'Cătălin', are presented comparatively in Table 2.

The antioxidant activity of the EO obtained from 'Cătălin' variety was characterized by low values of IC_{50}, 3.23±0.05g l^{-1}, 0.13±0.00g^{-1} and 6.03±0.81mMTrolox g^{-1} (DPPH, ABTS and FRAP tests, respectively), which demonstrates high antioxidant activity, identified by all three methods (Table 2).

Regarding hyssop HY, the IC_{50} values obtained were very high in the DPPH test (1741.39±5.22 g l^{-1}), which corresponds to a very low antioxidant activity. It should be noted that, by the ABTS method, the antioxidant capacity is relatively equal for both EO (0.13±0.00 g^{-1}) and HY (0.16±0.00 g^{-1}) of hyssop. It can be stated that the HY obtained from the new variety of hyssop 'Cătălin', has antioxidant activity but significantly

lower than that of EO. The results obtained by the ABTS method are in reasonable agreement for both the EO and the HY obtained from hyssop.

Various papers have reported high antioxidant activities of EOs obtained from medicinal and aromatic plants due to the richness of their chemical composition (Mutlu-Ingok *et al.*, 2020; Ovidi *et al.*, 2021). According to the results of this study, hyssop extract showed a high antioxidant activity, but lower compared to extracts of rosemary, sage and thyme (Babovic *et al.*, 2010). In general, the antioxidant properties of EOs depend on their components, especially phenolic compounds. *Cis*-pinocamphone, important compund identified in hyssop EO, whose antioxidant activity has not yet been reported in the literature. The activities of EOs, such as antioxidants, depend on their structural characteristics, but also on many other factors, such as concentration, temperature, light, substrate type and physical state of the system, as well as microcomponents that act as a pro-oxidizing or synergistic (Yanishlieva-Maslarova, 2001). For slow-reacting compounds, the influence has been attributed to the complex reaction mechanism. In our study, most likely, the constituents of *H. officinalis* essential oil are involved in one or more side reactions, which led to a slower reduction of DPPH solutions.

Table 2. Antioxidant capacity of EO and HY obtained from a new variety of hyssop, 'Cătălin'

Den.no.	Sample description hyssop, 'Cătălin' variety	DPPH[a] IC_{50} g l^{-1}	ABTS[a] MTrolox g^{-1}	FRAP mMTrolox g^{-1}
1.	Essential oil	3.23±0.05	0.13±0.00	6.03±0.81
2.	Hydrolate	1741.39±5.22	0.16±0.00	0.02±0.00

[a]Values are expressed as mean ± SD (n=3)

Antimicrobial activity of the EO

The antimicrobial activity of the EO depends on the chemical composition and the amount of unique, individual compounds. These compounds can be naturally active in the plant or can be activated by specific enzymes, when the plant is subjected to a certain biotic or abiotic stress (Saranraj and Durga Devi, 2017). An important feature of EOs (of their compounds) is hydrophobicity, which allows them to mix with lipids in cell membranes and mitochondria, making them more permeable. The mechanisms of action of EOs include cell wall degradation, cytoplasmic membrane damage, cytoplasm coagulation, membrane protein damage, increased membrane permeability allowing the leakage of ions and cellular components (Nazzaro *et al.*, 2013). Studies have shown that *H. officinalis* has moderate antimicrobial activity *in vitro* against G^+ and G^- bacteria and antioxidant activity along with antifungal, antiviral and insecticidal activities. (Fathiazad *et al.*, 2011). The efficiency of an antimicrobial compound depends on the type, genus, species and strain of the target microorganism, in addition to environmental factors: pH, temperature, atmospheric composition and initial microbial load of the substrate (Saranraj and Durga Devi, 2017).

Antibacterial activity

The determinations performed 24 hours after the beginning of the experiment and after 3 days, intended to assess the antibacterial potential of hyssop EO obtained from 'Cătălin' variety. The results showed that it did not inhibit the growth of the bacterium *Pseudomonas syringae* LMG5090, in any of the 4 concentrations tested (100%, 75%, 50% and 25%). However, in some repetitions, a slight decrease in the bacterial growth density was observed on the mark left by the oil spot. The obtained results confirm the tests performed by Venditti *et al.* (2015), with EO obtained from *H. officinalis* subsp. *aristatus*, in which no activity was detected against two G^- bacteria (*Enterococcus faecalis* and *Pseudomonas aeruginosa*). The results confirmed previous data obtained with the essential oil of *H. officinalis* var. *decumbens*, to which the antimicrobial activity was attributed due to the high content of linalool. This can be explained by the fact that the cell wall of G^- bacteria was more resistant to the activity of the EO and its compounds, which did not allow hydrophobic molecules to penetrate

as easily as in the case of G^+ bacteria. Thus, the EO is less able to affect the cell growth of G^- bacteria (Nazzaro *et al.*, 2013).

In vitro tests performed to determine the antibacterial potential of linalool against *Pseudomonas syringae* LMG5090 showed that this compound in a concentration of 50% and 100% reduces the bacterial density in the areas of dissemination of test compound spot. However, at a concentration of 25% or after 3 days of incubation in the presence of 50% and 100% concentrations, this compound did not disturb the bacterial growth of the tested strains (Figure 2; Table 3). *In vitro* tests performed to determine the antibacterial potential of estragole against *Pseudomonas syringae* LMG5090 showed that this compound inhibits bacterial growth when applied in different concentrations (25%, 50% and 100%). Antibacterial activity can be observed in the first 24 hours of incubation, but it has also been maintained after 3 days of incubation (Figure 3; Table3). *In vitro* tests performed to determine the antibacterial potential of eugenol against *Pseudomonas syringae* LMG5090 showed that this compound inhibits bacterial growth when applied in concentrations of 25%, 50% and 100%. The clear antibacterial activity can be observed in the first 24 hours, but it has also been maintained after 3 days of incubation (Figure 4, Table 3), similar aspects being observed as in the case of estragole.

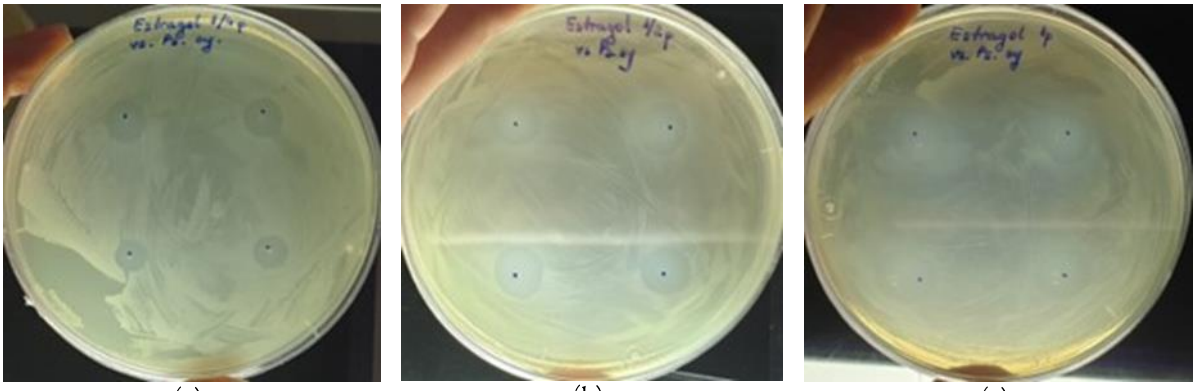
Table 3. The action of compunds linalool, estragole, eugenol on the bacterium *Pseudomonas syringae* LMG5090

Sample	Conc. %	Bacterial growth inhibition zone diameter (cm)	
		After 24 h	After 3 days
Control	100	0	0
	50	0	0
	25	0	0
Linalool	100	10,00	0
	50	8,00	0
	25	0	0
Estragole	100	15,00	15,00
	50	11,00	11,00
	25	8,50	8,50
Eugenol	100	15,00	14,50
	50	11,50	11,50
	25	9,00	9,00

In conclusion, estragole and eugenol show pronounced antibacterial activity in all tested concentrations (25%, 50%, 100%), both in the first 24 hours of incubation and after 3 days. Linalool, on the other hand, has higher bacteriostatic activity only at high concentrations (50% and 100%), inhibitory activity that is maintained only in the first 24 hours of incubation.

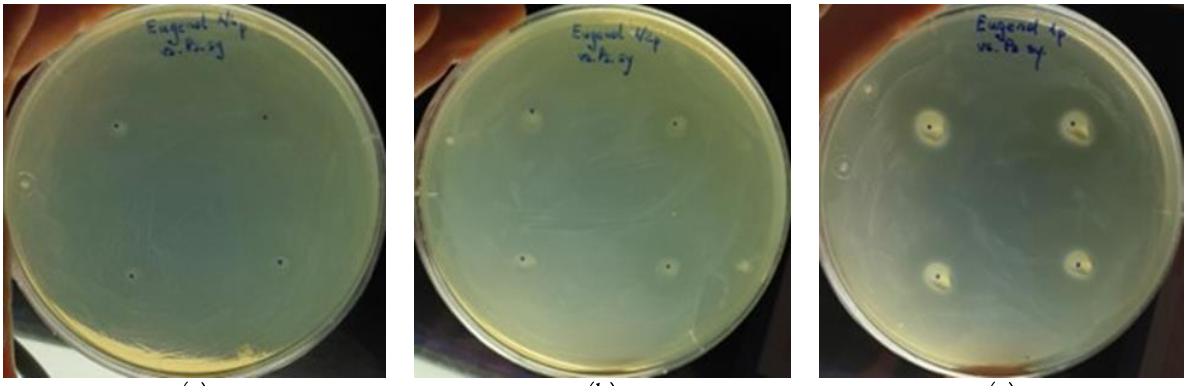
(a) (b) (c)

Figure 2. Culture of *Pseudomonas syringae* LMG5090 grown in the presence of Linalool, in different concentrations: 25% (a); 50% (b); 100% (c)



(a) (b) (c)

Figure 3. Culture of *Pseudomonas syringae* LMG5090 grown in the presence of Estragole, in different concentrations: 25% (a); 50% (b); 100% (c)



(a) (b) (c)

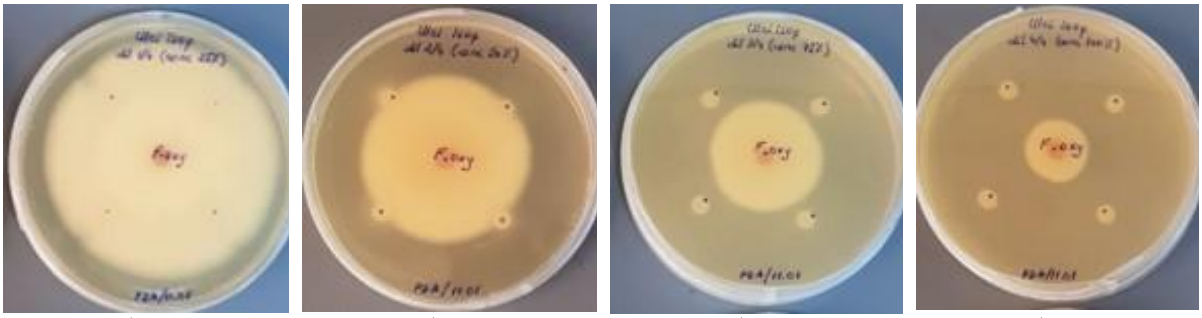
Figure 4. *Culture* of *Pseudomonas syringae* LMG5090 grown in the presence of Eugenol, in different concentrations: 25% (a); 50% (b); 100% (c)

Antifungal activity

EO obtained from hyssop variety 'Cătălin' by hydro distillation, was tested on the phytopathogenic fungus *Fusarium oxysporum* which produces fungal vascular wilt that causes the wilting of plants, or the blackening and rot of their roots and collar. Other symptoms, with less negative implications, are the partial yellowing of the basal leaves, on half of the leaf blade; twisting of rosette leaves, especially because of the asymmetrical development of the leaf blade, or uncharacteristic embossing of the leaves (Gilardi *et al.*, 2016; Velarde-Félix *et al.*, 2018). In the present study, to inhibit the growth of the pathogen *Fusarium oxysporum* ZUM 2407, the EO was used undiluted (100%) or diluted in proportions of 3/4 (75%), 1/2 (50%) and 1/4 (25%). The samples were incubated at 27 °C and analysed in the first 10 days after inoculation. *In vitro* tests against *Fusarium oxysporum* show that the solvent used to prepare EO dilutions does not influence the growth of the pathogen. The study showed that *in vitro*, with undiluted use of hyssop EO, it has better inhibitory activity compared to the control sample. The inhibitory activity of the oil was maintained at the tested concentration of 75% (Figure 5c). After 10 days of incubation at 27 °C, *F. oxysporum* grown in the presence of undiluted hyssop EO (100%), had inhibited mycelial growth of 82.4% compared to untreated control sample, and at a concentration of 75%, its inhibition effectiveness was 62.4%. As the concentration of hyssop essential oil decreases, the inhibitory efficacy also decreases, so that at a concentration of 50% it has a low inhibitory

efficacy (43.6%), following that at a concentration of 25%, after 10 days of incubation, the inhibition of mycelial growth shall be insignificant compared to the untreated control sample. Optical microscopy analysis of EO inhibited mycelial growth of *F. oxysporum* did not show noticeable changes in morphology, but only a weaker ramification, in the control culture the mycelium being denser, an aspect observed even with the naked eye (Figure 5). The literature mentions a series of results obtained from tests performed with hyssop oil. Thus, two essential oils of *H. officinalis* grown in two different localities in Italy and grown at 1000 m above sea level showed a very high antifungal activity against different strains of phytopathogenic fungi (Fraternale *et al.*, 2004). The study conducted by Ghfir *et al.* (1997) on growing mycelial hyphae, fractionated and isolated from the cell walls of *Aspergillus fumigatus* grown in the absence or presence of hyssop EO, showed that its presence causes a decrease in the level of uronic acid, protein and neutral sugars, while the levels of phosphorus, lipids and amino acids were increased. Neutral sugars were mainly galactose, mannose and glucose, while the amino acids were galactosamine and glucosamine, identified by HPLC analysis. The presence of hyssop oil induced changes in the content of galactosamine and galactose in the growing medium (Tahir *et al.*, 2018).

In conclusion, it should be mentioned that the hyssop EO tested has no fungicidal activity but only fungistatic, being able to delay mycelial growth at least 10 days according to Figure 5, the degree of inhibition depending on the concentration of essential oil used and its composition.



(a) (b) (c) (d)

Figure 5. Inhibitory effect (after 10 days) of EO hyssop, 'Cătălin' variety on the *Fusarium oxysporum* ZUM 2407, in different concentrations: 25% (a); 50% (b); 75% (c); 100% (d)

Statistics on antimicrobial activity

The expression of the optimal polynomial function represents the measure of the efficacy of the hyssop oil obtained from the 'Cătălin' variety, on the phytopathogenic fung *Fusarium oxysporum*. The statistical technique of interpolation of experimental data was used to obtain functions with two variables. For the essential oil obtained from hyssop cv. 'Cătălin', the polynomial function of fourth degree efficacy has the expression:

$$E_{UI}(t,c) = -423.363 + 151.583t + 18.477c - 1.852tc - 30.53t^2 - 0.337c^2 + \\ + 0.169t^2c + 0.012tc^2 + +2.68t^3 + 0.003087c^3 - 0.0004284t^2c^2 - 0.006222t^3c - \\ - 0.00002207tc^3 - 0.085t^4 - 0.00001125c^4 \quad (4)$$

Function (4) is a function with 2 variables, time and concentration of essential oil used in exercises as a treatment for the control of phytopathogenic fungus.

An overall estimator is the mean value over time of the Efficacy (function (5)) and which is given by the formula:

$$\overline{E}_{UBG}(c) = \frac{1}{T - T_0}\int_{T_0}^{T} E_{UBG}(t,c)dt \quad (5)$$

where $T_0 < T$ are the times that delimit the interval for testing the efficacy of the treatment. This average value depends on the concentration used and apparently contains the parameters of the integral limits to be

included in the experimental time interval. An average value of the Efficacy function can be defined for the range of experimental concentrations used, which will depend on the time:

$$\hat{E}_{UBG}(t) = \frac{1}{C - C_0} \int_{C_0}^{C} E_{UBG}(t, c)dc \quad (6)$$

wherein C_0 <C are the times that delimit the interval for testing the efficacy of the treatment. Function (6) is the average value of concentrations, efficacy for hyssop oil and time dependent. The two mean values (5) and (6) are different variables and efficacy functions. and a variant of treatment in their terms is difficult to formulate.

In order to eliminate this last obstacle, in order to obtain a numerical estimator of the treatments efficacy, we mediate over the function concentration range (5) or over the experimental time interval, function (6). An average estimator is obtained over the experimental interval, cartesian product, [T_0, T] x [C_0, C]:

$$\hat{\hat{E}}_{UBG}(c) = \frac{1}{(T - T_0)(C - C_0)} \int_{C_0}^{C} \int_{T_0}^{T} E_{UBG}(t, c)dtdc \quad (7)$$

The values of the global average Efficacy estimator for hyssop EO, 'Cătălin' variety (from the experimental data obtained), were estimated at 74.023% on the phytopathogenic fungus *Fusarium oxysporum*.

The efficacy of hyssop EO on the phytopathogenic bacterium *Pseudomonas syringae* was calculated using the following formula:

$$E = \frac{S_i}{S} \quad (8)$$

este where S = the area of the disk, and the string of areas S_i, is:

$$S_i = \pi\phi_i \quad (9)$$

where ϕ_i is the average diameter measured in experiments for all substances analyzed and the order i = 1...16. The classification of the efficacy of the standards and of the hyssop oil tested according to concentrations is shown in Figure 6.

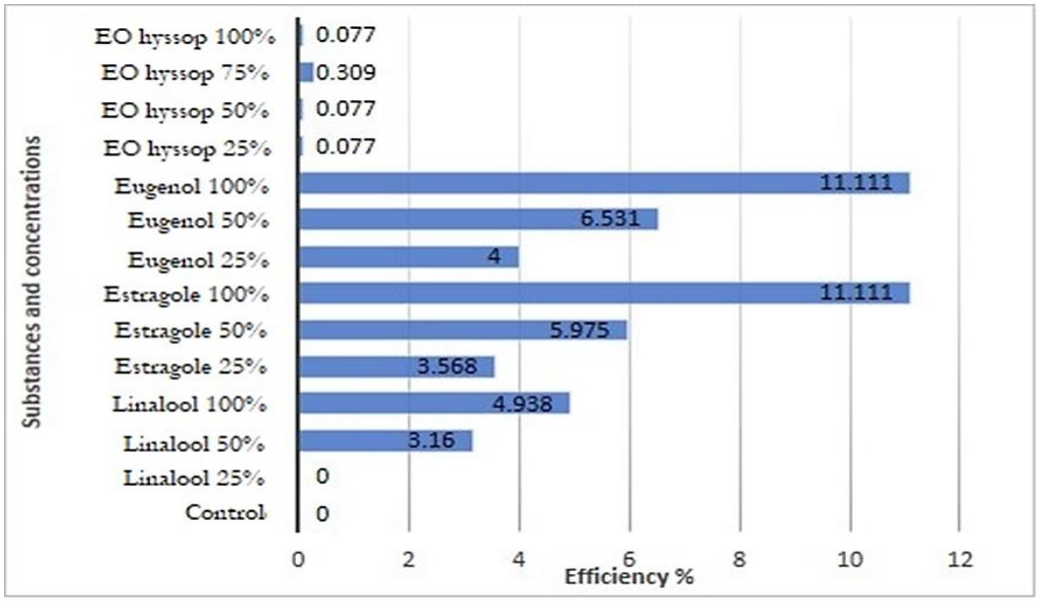


Figure 6. Efficacy of hyssop EO and compounds (linalool, estragole, eugenol) on phytopathogenic bacteria *Pseudomonas syringae* LMG5090

The graph shows that the most efficacy treatment against this bacterium, is the one with high purity substances (eugenol and estragole) c = 100%, followed by linalool, but the efficacy rate is halved at the same concentration.

Conclusions

The originality of this study is given by the fact that the 'Cătălin' variety is the first Romanian hyssop variety, for which the evaluation of EO and HY was made. The results obtained regarding the chemical composition showed that *cis*-pinocamphone and *trans*-pinocamphone, followed by thujenol and pinenol, were the major compounds identified in both EO and HY. In terms of antioxidant activity, of the three methods used, DPPH is the best method for assessing the antioxidant capacity of hyssop. This is high in the case of EO, being also present in HY, but significantly lower than in EO. Regarding antimicrobial activity, *in vitro* tests showed that EO did not inhibit the growth of the bacterium *Pseudomonas syringae* (LMG5090), and those for the phytopathogenic fungus *Fusarium oxysporum* showed rather the fungistatic activity of EO. For estragole and eugenol, antibacterial activity was observed at all concentrations used, and linalool had bacteriostatic activity only at high concentrations (50% and 100%).

Further research will focus on testing EO on other strains of bacteria and phytopathogenic fungi, as well as on testing the allelopathic activity of both (EO and HY).

Authors' Contributions

Conceptualisation - S.(T)C., and I.R.F.; Investigation – P.C., G.F., and S.O.-A.; Data curation – V.N.V., and M.A.; Writing-original draft preparation – S.(T). C., and M.A.; Writing-review and editing – I.R.F., and B.F.; Funding acquisition – V.N.V., S.(T)C.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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