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CARACTERISATION OF ORIGANUM VULGARE EXTRACTS

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ABSTRACT. *Origanum vulgare* L., commonly known as oregano, is a woody, rhizomatous perennial plant. Oregano extracts are a complex mixture of different compounds, in which major constituents are terpenes (mono- and sesquiterpenes). The flavor of oregano oil is generally due to the presence of thymol and carvacrol. Carvacrol has multiple properties: antioxidant, antimicrobial, antitumor, antimutagenic, antigenotoxic, analgesic, antispasmodic, anti-inflammatory, angiogenic, antiparasitic, antiplatelet, insecticidal, antihepatotoxic and neuroprotective. The oregano extract was characterized by analyzing, the antioxidant activity, the total polyphenols content (obtaining 2.4 - 3 mg gallic acid/g), the content of phenolic acids and flavonols by HPLC chromatography, the content of carvacrol (21, 29% in essential oil and 19.78% in ethanolic extract) and thymol (2.59% in essential oil and 2.67% in alcoholic extract) by GC-MS chromatography. Oregano extracts were tested for antimicrobial activity against *Staphylococcus aureus*. The test results demonstrate the significant antimicrobial activity of these preparations.

Keywords: *Oregano vulgare, antioxidant activity, polyphenols, HPLC, GC-MS*

INTRODUCTION

Phytotherapy is the science that studies the use of plant products for therapeutic purposes. Herbs and products extracted from them are used to treat various infectious diseases in traditional medicine because of their

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properties. It is also important to note that, according to the World Health Organisation, about 80% of the world's population currently uses herbs and medicinal plants as traditional remedies. However, although these products are natural, they are slower acting in the acute phase of the disease compared to synthetic

Oreganum vulgare L has beneficial properties due to the presence of essential oils, which contain volatile aromatic compounds that function as secondary metabolites of the plant, while the primary metabolites are responsible for the survival of the plant. Essential oils provide the plant with protection from hazards, predators or other threats, promoting healthy reproduction and helping to attract pollinators. [1]

Origanum vulgare L., commonly known as oregano, is a woody, rhizomatous perennial plant, with stems up to 90 cm or more, usually branched at the top, pubescent, hirsute or velvety, rarely glabrous. The name oregano can be translated as 'mountain delight' and comes from the Greek '*oros*' meaning mountain and '*ganos*', joy. Oregano belongs to the *Lamiaceae* family of the order *Lamiales*. The genus *Origanum* includes more than 70 species, 49 taxa (species and subspecies) and natural hybrids found throughout most of the Mediterranean area, including northern Africa, but also in temperate Asia and Central America. Wild oregano species can grow at different altitudes, from coastal to mountainous areas, on different soils and are able to withstand quite low temperatures.

An average of 1-2% essential oil is extracted from a plant, but the amount depends on the species grown. In *Origanum vulgare L* oil was found terpenes (limonene, α-pinene, γ-terpinene, p-cymen, α-myrcene), alcohols (thymol, carvacrol, eugenol, linalool, 2-phenyl-ethanol, 3-hexen-1-ol, mimoquinone, thymoquinone, octen-3-ol, 1,8-cineole, cis-p-ment-2-en-1-ol, terpinen-4-ol, α-terpineol, spatulenol and benzyl alcohol). [2]

The essential oil content, as well as the ratio of carvacrol and thymol to the total amount of oil, is quite variable depending on the species of oregano, the soil and terrain of origin, the time of harvesting, and the method of distillation.

While thymol is antibacterial, anti-inflammatory, antiprotozoal it also has antioxidant, cytotoxic, and pesticidal activities and is a good allelopathic agent. Carvacrol has multiple properties: antioxidant, antimicrobial, antitumor, antimutagenic, antigenotoxic, analgesic, antispasmodic, anti-inflammatory, angiogenic, antiparasitic, antiplatelet, insecticidal, antihepatotoxic and neuroprotective. [3]

GC-MS chromatographic analysis identified the following components in oregano essential oil: 27% monoterpene hydrocarbons (α-pinen, camphen, β-pinen, myrcen, α-phellandren, α-terpinen, p-cymen, limonen, γ-terpinen),

66% oxygenated monoterpenes (1,8-cineole, linalool, α-thujone, camphor, borneol, terpinen-4-ol, α-terpineol, geranial, thymol, carvacrol), 6% sesquiterpene hydrocarbons (α-copaen, β-caryophyllene, α-humulene), 0.5% oxygenated sesquiterpenes (caryophyllene oxide) and 0.5% aromatic compounds (phenylpropanoids: 1-octen-3-ol), listed according to the Kovat index retention (RI), C8-C32 n-alkane, calculated by gas chromatograph on an apolar HP-5MS column. [4]

The oregano plant is also very rich in minerals such as Ca (1043 mg/kg), Fe (159 mg/kg), K (19625 mg/kg), Cr (7.43 mg/kg), and Ba (79.8 mg/kg), which are important structural components of tissues and could influence water and acid-base balance. [5]

In vitro studies, the antioxidant activity of the oregano plant was found to be 12 times higher than in oranges, 30 times higher than in potatoes and 42 times higher than in apples. The beneficial health effects of extracts (ethanolic, methanolic and in acetone) of oregano are attributed to its high antioxidant activity, determined by carvacrol and thymol. Several studies demonstrate that a high content of polyphenols in oregano leads to a major antioxidant potential, which is determined by organic acids with biological effects mostly related to remarkable antioxidant, antimutagenic, anticarcinogenic and anti-inflammatory activities. Polyphenolic acids have a high capacity to scavenge reactive oxygen radicals. These polyphenols are able to inhibit inflammatory processes and tumor propagation by deactivating pro-oxidative enzymes, described below. In some *in vivo* animal studies, *Origanum* plants have been shown to be a dietary supplement *and* have been used in animal diets for weight gain, infection control and immune enhancement. [6,7,8]

Various studies using *Origanum* plants have shown good results, showing that its inclusion in the diet increases the weight of animals; helps fight parasite infections and increases the animals' innate immune response. These important effects on farm animals are due to the presence in the essential oils of the polyphenols: carvacrol, linalool, borneol, α-terpinene, γ-terpinene, α-pinen and β-pinen.[9,10,11]

RESULTS AND DISCUSSIONS

Extraction methods are based on the distribution of the components of a mixture in a polar solvent. Maceration was carried out using polar solvents with different polarities: methanol, ethanol, acetone.

The Folin-Ciocalteau test is the most common procedure used to determine the total polyphenol content of different extracts. The amount of polyphenols with antioxidant properties is expressed in gallic acid equivalents

(mg/ml). In the concentration range of 0.05 - 2.0 mg/ml (gallic acid) a strong dependence of concentration on absorbance is obtained. The line obtained has the equation y = 4.3916x + 0.1236, the correlation coefficient R^2 = 0.9975 showing a good correlation of the results.

The extracts obtained by maceration and essential oils of DoTERRA and Purarom were analyzed and the results are presented in table 1.

It is observed that in the case of extractions in methanol and acetone the amount of polyphenols extracted is higher than in ethanolic solutions and in essential oils 0.61 mg/ml.

The health benefits of oregano extracts are explained by the high antioxidant activity due to the polyphenols they contain. In order to determine antioxidant activity, the analysis is based on the measurement of free radical neutralizing capacity. The unpaired electron of the nitrogen atom in DPPH is reduced by accepting a hydrogen atom from the antioxidants at the corresponding hydrazine.

Table 1. Amount of polyphenols and absorbances of samples analysed

No.	Type of extract used	Absorbance (nm)	Polyphenol content in the test solution (mg gallic acid/ml)
1	Purarom essential oregano oil	1.1935	0.48±0.02
2	doTERRA® Oregano essential oil	1.2095	0.49±0.03
A	Methanolic extract	1.4713	0.61±0.018
B	Acetone extract	1.4731	0.61±0.02
C	Ethanolic extract	1.2003	0.49±0.017

The total polyphenol content of extracts ranged from 0,48 mg GA/ml to 0,61 mg GA/ml. These results were like previous oregano powder findings [xx]. Ethanolic extract of oregano powder is rich in rosmarinic acid quercetin coumarinic acid, caffeic acid [12] demonstrated by HPLC analysis of the extracts.

DPPH is a stable paramagnetic radical. The unpaired electron from the nitrogen atom is delocalized, belonging to the whole molecule. The solution is dark purple, showing an absorption maximum at a wavelength of 517 nm. The antioxidant activity of the three extracts and the essential oils of oregano was determined. Table 2 shows the results of the determinations.

From the analysis of the experimental results obtained, it is observed that doTERRA oregano essential oil shows the highest free radical neutralizing activity. In the case of extraction by the maceration method, the ethanolic

sample shows the highest free radical neutralizing activity. This is due to the higher solubility of compounds with antioxidant activity in ethyl alcohol. Licina [13] reported the DPPH radical scavenging activity of different extracts of *O. vulgare* L. in terms of IC50 values and found that ethanol extract was the most active with an IC50 value of 34.5 μ g/ml, while acetone extract was the least active with an IC50 value of 86 μ g/ml.

Table 2. Antioxidant activity results of analyzed samples

No	Type of extract used	Absorbance (nm) after 60 minutes	Antioxidant activity (%) neutralizing free radicals
1	Purarom essential oil of oregano	0.0884	89.89%
2	doTERRA® Oregano essential oil	0.0363	95.84%
A	Methanolic extract	0.7067	19.17%
B	Acetone extract	0.1283	85.32%
C	Ethanolic extract	0.0840	90.39%

By HPLC chromatographic analysis the polyphenol content was determined. Initially, the calibration curves of some polyphenols that were determined in extracts were determined. The calibration curves obtained with analytical standards are shown in Table 3.

Table 3. Results obtained by HPLC chromatographic analysis

Compound	Retention time (min.)	Equation of the calibration line	R^2
Gallic acid	2.2	Y=28572x + 457.97	0.9943
Caffeic acid	4.6	Y=42330x =157.55	0.9960
Kaempherol	15.1	Y=207.88x - 4.5454	0.9966
Quercetin	22.1	Y=19277x-1233.2	0.9998
Umbelliferone	6.4	Y=1739.9x + 2751.8	0.9988

After HPLC chromatographic analysis of the extracts, the polyphenol content was determined and quantification was carried out using calibration curves for each compound. In Table 4, the results of the experimental determinations obtained from the analysis of oregano extracts/essential oils by the chromatographic method are presented.

Table 4. Amount of polyphenols in the analyzed samples

Sample	Gallic acid	Caffeic acid	Umbelliferone	Kaempferol	Quercetin
Methanolic extract (mg/ml)	0.5	0.8	0.06	0.9	0.16
Ethanol extract (mg/ml)	0.4	0.2	0.5	0.21	1.1
Acetone extract (mg/ml)	29	1.1	0.4	-	5.5
Purarom essential oil (mg/ml)	0.09	0.072	0.015	0.42	0.16
doTerra oil (mg/ml)	0.015	0.08	0.025	Cannot quantify	

From the experimental results obtained it can be seen that gallic acid, caffeic acid, and umbelliferone were identified in all samples. Along with these compounds, the flavonols kaempferol and quercetin were identified. In the extract made with an aqueous acetone solution, the highest amounts of these compounds were obtained. The analyses of volatile oil were carried out by GC-MS chromatography. The results are shown in Table 5.

Table 5. Results obtained by GC-MS chromatographic analysis

Compound	Rt (min)	Earth Oil	Ethanolic extract
		Conc (%)	Conc(%).
Izobutilacetat		1.37	1.24
α-Phellandren	5.949	0.25	0.28
3,6,6-trimethyl-2-norpinene	6.078	1.54	1.62
β-Mircen	6.972	3.53	2.97
(+)-2-Carene	7.377	2.74	2.58
A-Cimene	7.513	5.52	5.73
D-Limonen	7.574	1.12	0.98
2-Thujen	8.040	6.24	6.61
Beta-cis-Terpincol	8.180	0.41	0.49
Linalool	8.663	10.18	11.12
Endo-Borneol	9.734	1.48	1.55
Carvona	11.098	0.42	0.39
Thymol	11.438	2.59	2.67
Carvacrol	11.620	21.29	19.78
Cariophilen	13.356	1.94	2.11
β-Bisabolen	14.353	3.15	3.67

The results show a high concentration of carvacrol and thymol, compounds that have antibacterial activity, 21.29%, and 2.59% respectively in the essential oil and 18.78% and 2.67% in the hydroalcoholic extract.

In studies carried out on oregano extracts, it was determined a carvacrol content of 17-27%, depending on the country of origin. [14] The results obtained are comparable to others from the literature. [15] Antimicrobial activity was tested using the *Staphylococcus aureus* reference strain. In parallel, four antibiotics were tested: azithromycin, erythromycin, levofloxacin, and tetracycline. Different volumes of essential oil and oregano extract were tested. If in the case of antibiotics the diameters obtained were between 25 mm (azithromycin) and 32 mm (erythromycin) in the case of oregano preparations the diameter was 32 mm even at 0.2 µl extract respectively oil essence. The test results demonstrate the significant antimicrobial activity of oregano extracts. In the literature, a correlation between carvacrol content and antimicrobial activity is shown [16]

In addition, in agreement with literature data [17] terpenoids show analgesic, antitumoral, and antinociceptive activity. These data explain the free radical neutralizing activity and antimicrobial activity of oregano extracts.

Considering the obtained experimental results, oregano extracts are potential candidates for antimicrobial agents. At the same time, the combination of marked antioxidant properties with antibacterial action makes extracts of oregano a good candidate to be used in medicine, pharmaceutical, and cosmetic industry, as an antimicrobial, analgesic, antioxidant, and skin whitening agent. Being a widely used spice and having above mentioned biological activities, oregano could also be used as a food preservative.

CONCLUSIONS

Oregano is a widely used medicinal herb due to its special phenolic compounds and medicinal properties. In this study, we characterized the extracts of *Oregano vulgare*. In this study, we characterized the extracts of oregano... and essential oils. In ethanol: water extract, obtained by maceration, a polyphenol content of 0,48 mg GA/ml was determined. The ethanolic extract is rich in quercetin, and caffeic acid, which also explains the neutralization activity of free radicals at 90.3% in 60 minutes. The GC-MS analysis determined the content of 19.78% carvacrol and 2.67% thymol in the ethanolic extract, respectively. These compounds have antioxidant and antibacterial activity.

Oregano is a common spice and can be important in the prevention and treatment of many diseases, including bacterial infections. As a rich source of phenolic compounds and other secondary metabolites with

antioxidant and antimicrobial activity, oregano extracts could be used as nutritional supplements for the prevention of degenerative diseases. The potential of these extracts should be further evaluated for their application in modern medicine, the food industry, and cosmetics due to their antioxidant and antibacterial properties.

EXPERIMENTAL SECTION

Materials

All chemicals (methanol, acetic acid, sodium carbonate) used for assays were of analytical grade. 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, vallinic acid, synaptic acid, quercetin, catechin, caffeic acid, umbelliferone, kaempferol were procured from Sigma-Aldrich Company. Folin- Ciocalteau reagent were procured from Merck Co.

The oregano plants used were grown from selected seeds, under organic conditions. When the plant reached maturity, the leaves were harvested, washed in order to remove impurities, then dried in the air current. The dry plant was further processed.

Equipment

A UV-visible spectrophotometer from Analitic-Jena was used for measuring the absorbance.

Determination of phenols by HPLC was performed on a HPLC ACME 3000 Younglin Instrument, equipped with SP 930 D module and UV730D detector module.

Thermo GC-MS (Model Trace 1310 ISQ7000) equipped with an HP-5MS capillary column (30 m length x 0.32 mm internal diameter x 0.25µm film thickness). GC-MS spectroscopic detection, an electron ionization system with an ionization energy of 70eV was used.

Extract sample preparation

Dried plants were ground into a fine powder and then subjected to phytochemical extraction. We performed extraction by maceration, using several solvents: methanol: water 1:1, ethanol: water 1:1, and acetone: water 1:1. Plant powder (1 gram) was macerated with 10 ml solvent at room temperature for 2 days with shaking from time to time. The suspension

obtained was filtered. The extract is filtered by a Millipore filter with a 0,45 µm and diluted with the same solvent at 10 ml The solution was stored at 4° C in the refrigerator until analyzed.

1. Determination of Total Phenolic Content

The total phenols content of each extract was evaluated using the Folin- Ciocalteu reagent described by [39] with slight modifications. It is a colorimetric method based on the transfer of electrons, in an alkaline medium, from phenolic compounds to form a blue chromophore consisting of a phospho- and phosphomolybdenum complex in which the maximum absorption is proportional to the total concentration of polyphenolic compounds. The reduced forms of Folin-Ciocalteau reagent are detectable with a spectrophotometer in the 690 to 750 nm range. The reaction temperature was used to reduce the time to reach maximum color, t = 37°C. Gallic acid was used as the standard reference compound according to the method described by the International Organization for Standardization ISO 14502-1. A calibration curve is initially constructed using a compound with significant antioxidant properties as a reference.

In a test tube, was introduced 8 ml distilled water, 1 ml standard working solution or analysis solution and 1 ml Foline-Ciocateau reagent (diluted 1:10). It was shaken and left to rest 1 minute, then 2 ml Na_2 CO_3 20% solution was added and was maintained at 40° C for 1 hour. The absorbance of the resulting blue color was measured at 765 nm with a UV-VIS spectrophotometer.

Quantification was done on the basis of the standard curve of gallic acid concentration range between 0.05-0.25 mg/ml (y= 4.3916x+0.1236; R^2 =0.9975). The amount of total phenolic compounds was expressed as mg gallic acid equivalent (GAE)/ml extract.

2. DPPH scavenging assay

The hydrogen donating or the radical-scavenging ability of the extraction solution was measured using the stable radical DPPH-. DPPH radical scavenging assay of each sample was carried out by using a DPPH method with modification [40].

DPPH solution was prepared by dissolving 2 mg DPPH in 50 ml methanol (about 3 mM). 0.2 ml of each extracted sample was mixed with 2.8 ml DPPH solution. The mixture was incubated at room temperature in the dark. After 60 minutes, the absorbance was determined at 517 nm and gallic acid

was used as a positive control. The DPPH radical scavenging ability of each extracted sample was calculated using the following equation:

$$\text{DPPH radical scavenging rate (\%)} = (A_{control}-A_{sample}) \times 100 / A_{control}$$

where $A_{control}$ is the absorbance of DPPH solution (without extract sample, A_{sample} is the absorbance of the extract sample.

Analysis of phenols by reverse-phase high-performance liquid chromatography(HPLC)

Determination of phenols was performed on a HPLC, under the following chromatographic conditions: isocratic conditions, mobile phase methanol: water: acetic acid 300:700:2, flow 1 ml/min. Chromatographic separation of the constituents of the sample was obtained using a reverse-phase YMC-Pack ODS AQ column, 150 cm long and with an internal diameter of 4.6 mm. A volume of 0.2 µl of each sample was injected into the chromatograph at room temperature.

GC-MS analysis

The GC-MS analysis of various organic crude extract leaves of *Origanum vulgare L*. was performed using a Thermo GC-MS GC-MS spectroscopic detection, and an electron ionization system with an ionization energy of 70eV was used. Helium was used as a carrier gas at 30 cm s^{-1} and the injection volume was 1 µl. The mass transfer line and injector temperature were set at 220^{o} C and respectively 290^{o} C. The oven temperature was programmed at 45^{o} C for 1 min, raised to 250^{o} C at 5 C/min, and maintained at 250^{o} C for 5 min. Diluted samples of 1 µl were injected in the split mode with a split ratio of 120:1. The relative percentage of the chemical constituents in the dried extracts was expressed as the percentage by peak area normalization.

Determination of antimicrobial activity

To test the antimicrobial activity of the extract, agar-type Mueller-Hinton plates with a layer thickness of 90 mm were used and the test method was the diffusion method. Testing of the antimicrobial activity of the extract was performed on Gram-positive bacteria (*Staphylococcus aureus*) compared to the different antibiotics. The oregano extract was applied at different concentrations and after 24 H at 37^{o} C, the diameter of the inhibition zone was compared to that produced by the antibiotics.

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