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CHEMICAL COMPOSITION AND ANTIOXIDANT PROPERTIES OF JUNIPER BERRY (*JUNIPERUS COMMUNIS* L.) ESSENTIAL OIL

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Abstract

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The chemical composition of essential oils of juniper berries (*Juniperus communis* L.) were analyzed using GC/FID and GC/MS. Antioxidant properties were defined by 7 different *in vitro* models. The antioxidant activity attributable to electron transfer made juniper berry essential oil a strong antioxidant. IC_{50} for hydroxyl radical (OH) scavenging and for chelating capacity were 0.0235 ig.$(cm^3)^{-1}$ and 0.024**6** ig.$(cm^3)^{-1}$ respectively. The essential oil exhibited hydrogen peroxide scavenging activity and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^+$) radical cation scavenging activity – the activity of 10 mg of juniper berry oil is equivalent to 4.77 mM Trolox. The antioxidant activity of the oil attributable to hydrogen atom transfer was lower. IC_{50} for 2,2-Diphenyl-1-picrylhydrazyl radical scavenging (DPPH) was found to be 944 ig.$(cm^3)^{-1}$. Lipid peroxidation inhibition by the essential oil in both stages, i.e. hydroperoxide formation and malondialdehyde formation, was less efficient than the inhibition by BHT. Through *in vivo* analyses with *Saccharomyces cerevisiae* yeast, the essential oil effect on the levels of the antioxidant enzymes SOD, catalase, and glutathione peroxidase was established.

Key words: Juniperus communis L, GC/FID, GC/MS, antioxidant, *Saccharomyces cerevisiae*, antioxidant enzymes

Abbreviations: DPPH: 2,2-Diphenyl-1-picryl hydrazyl; OH: hydroxyl radical; ABTS: 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid); BHT: Butylated hydroxy-toluene; SOD: Superoxide dismutase; CAT: Catalase; GPx: Glutathione peroxidase; IC_{50}: Inhibitory concentration at 50%; ROS: reactive oxygen species

Introduction

Reactive oxygen species (ROS) such as H_2O_2, superoxide radical, and hydroxyl radical, are produced in the cells (organisms) during breathing and cellular metabolism. At lower concentrations, they participate in cellular physiological reactions (Schopfer et al., 2001). Their overproduction, however, largely determines cell survival. The ROS inactivation and removal depends on the non-enzymatic and enzymatic protective mechanisms. Research on ROS-induced damage has shown that antioxidant production is genetically controlled in the cells (Kim et al., 2010). The focus on antioxidants naturally contained in essential oils is directly related to their application aimed at the prevention of oxidative damage to biological systems by ROS. Low-molecular antioxidants can enhance organism stability under oxidative stress (Martorell et al., 2011).

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For centuries, juniper berries have been used in folk medicine for the treatment of opportunistic infections, as a spice for meat, as flavor in the preparation of gin and raki (Darwin 2000; Foster et al., 1999). The IFRA recommendations contain no restrictions regarding the use of this oil.

The antioxidant activity of essential oils from different juniper berry species has been established *in vitro* (Emami et al., 2007). Anti-radical activity depends on the oil components (their chemical nature and concentration) (Misharina and Samusenko, 2008; Misharina et al., 2009; Wei and Shibamoto, 2007; Ruberto and Baratta, 2009). Regardless of the differences in the composition of juniper berry essential oils, they are dominated by terpene hydrocarbons. In many cases, the essential oil antioxidant activity cannot be attributed to the dominant compounds á- and β-pinene. These monoterpene hydrocarbons in juniper berry essential oil do not contribute to a significant inhibition of malondialdehyde formation (Wei and Shibamoto, 2007). The carriers of antioxidant properties in relation to lipid peroxidation in both its stages are α- and γ-terpinenes and, to a significantly lesser extent, their sesquiterpene analogues. This has been established both for juniper essential oils (Misharina and Samusenko, 2008; Misharina et al., 2009; Wei and Shibamoto, 2007) and for pure terpene hydrocarbons: terpinolene, α-terpinene and γ-terpinene (Ruberto and Baratta, 2009). Myrcene, α- and β-pinene only inhibit lipid peroxidation in the second stage; sabinene, limonene, α-pinene, and myrcene demonstrate anti-radical activity in relation to DPPH radical (Bua-in and Paisooksantivatana, 2009; Roberto et al., 2010).

The scavenging effect of hydroxyl radicals and the protection of deoxyribose against degradation is mainly due to β-pinene and limonene (Emami et al., 2007); the superoxide radical neutralization is determined by germacrene-D (Karioti et al., 2004)**.** The tenmembered *ring system* and the three double bonds acting as electron-rich centers in germacrene-D determine its anti-radical activity.

A number of studies have shown that the monoterpene components also contained in juniper essential oil enhance, through their antioxidant activity, the oxidative stress resistance of living organisms. Their antiradical activity affects the levels of the most important enzymes responsible for the neutralization of ROS: SODs, catalases, peroxidases, and glutathione transferase (van Lieshout et al., 1998; Sepici-Dincel et al., 2007; Roberto et al., 2010). The *S. cerevisiae* yeast is widely used for the better understanding of the cellular protection against ROS. Its enzymatic anti-ROS antioxidant protection has been well studied (Jakubowski et al., 2000; Manfredini et al., 2004; Longo et al., 1996; Tsuzi et al., 2004; Inoue et al., 1999). In this aspect, it was interesting to study the possibility of increasing the antioxidant protection of yeast cells using juniper berry essential oil and oxidant detoxification *in vivo*. The antioxidant properties of the essential oil both *in vitro* and *in vivo* are important for the overall evaluation of its action.

The aim of this study was firstly, to investigate the chemical composition of the essential oils of juniper berries, and secondly, to assess *in vitro* the antioxidant activity of juniper berry essential oil and prove *in vivo* its preventive effect upon the oxidative damage in *S. cerevisiae* due to its action on the antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase.

Materials and Methods

Materials. Essential oil samples for chemical investigation were obtained from Kurt Kitzing GmbH, Wallerstein, Germany. The following references were used: essential oil of juniper berries 2x rect. 801116 lot 16785. All samples were subjected to GC analysis, undiluted, with a 0.5 µl plunger-in-needle syringe at a very high split rate.

Gaschromatography/massspectrometry analyses of essential oil. GC/FID and GC/MS analyses were carried out simultaneously using a Finnigan ThermoQuest TraceGC with a dual split/splitless injector, an FID detector and a Finnigan Automass quadrupole mass spectrometer. One inlet was connected to a 50 m x 0.25 mm x 1.0 µm SE-54 (5% Diphenyl, 1% vinyl-, 95% dimethyl-polysiloxane) fused silica column (CS Chromatographie Service, Germany), the other injector was coupled to a 60 m x 0.25 mm x 0.25 µm Carbowax 20M (polyethylene glycol) column (J &W Scientific, USA). The two columns were connected at the outlet with a quartz Y connector and the combined effluents of the columns were split simultaneously to the FID and MS detectors with a short (ca. 50 cm) 0.1 mm ID fused silica restrictor column as a GC/MS interface. The carrier gas was helium 5.0 with a constant flow rate of 1.5 cm^3/min, injector temperature was 230°C, FID detector temperature 250°C, GC/MS interface heating 250°C, ion source at 150°C, EI mode at 70 eV, scan range 40 – 300 amu. The following temperature program was used: 46°C for 1 min to 100°C at a rate of 5°C/min.; 100°C to 230°C at 2°C/min; 230°C for 13.2 min. Identification was achieved using Finnigan XCalibur 1.2 software with MS correlations through the NIST (NIST/EPA/NIH Mass Spectral Library (2008), Adams essential oils (Adams, 2007) MassFinder (König et al., 2007) and our own library. Retention indices of reference compounds and from literature data (Davies, 1990; ESO 2000, update 2006) were used to confirm peak data. Quantification was achieved through peak area calculations of the FID chromatogram.

Antioxidant activity *in vitro*

Scavenging effect on 2,2-diphenyl-1-picryl hydrazyl radical (DPPH). The radical scavenging ability was determined according to the method of Mensor et al. (2001). One cm^3 of 0.3 mM alcohol solution of DPPH was added to 2.5 cm^3 of the samples with different juniper berry oil concentrations. The samples were kept at room temperature in the dark and after 30 min the optic density was measured at 518 nm. The optic density of the samples, the control and the blank was measured in comparison with ethanol. One synthetic antioxidant, Butylated hydroxy-*toluene* (BHT) was used as positive control.

Detection of hydroxyl radicals by deoxyribose assay. The assay was performed as described by Halliwell et al. (1987) with minor changes. All solutions were freshly prepared. 1.0 cm^3 of the reaction mixture contained 28 mM 2-deoxy-D-ribose (dissolved in KH_2PO_4 K_2HPO_4 buffer pH 7.4), 0.5 cm^3 solution of various concentrations of the juniper berry oil, 200 μM $FeCl_3$ and 1.04 mM EDTA (1:1 v/v), 10 mM H_2O_2 and 1.0 mM ascorbic acid. After an incubation period of 1 h at 37 ^{0}C, the extent of deoxyribose degradation was measured by the thiobarbituric acid (TBA) reaction. 1.0 cm^3 of TBA (10 g.(dm3)$^{-1}$ in 50 mM NaOH) and 1.0 cm^3 of trichloroacetic acid (TCA) were added to the reaction mixture and the tubes were heated at 100 ^{0}C for 20 min. After cooling, the absorbance was read at 532 nm against a blank (containing only buffer and deoxyribose). The percentage inhibition was calculated by the formula $I(\%)=100 - (Abs_{sample}/Abs_{control})x100$.

The IC_{50} value represented the concentration of the compounds that caused 50 % inhibition of radical formation. Quercetin was used as a positive control.

Total antioxidant capacity. The total antioxidant capacity was determined using an Antioxidant Assay Kit (SIGMA, product code CSO 790).

The antioxidant assay principle is the formation of a ferryl myoglobin radical from metmyoglobin and hydrogen peroxide, which oxidizes the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) to produce a radical cation, $ABTS^+$, a soluble chromogen that is green in color and can be determined spectrophotometrically at 405 nm. Antioxidants suppress the production of the radical cation in a concentration dependent manner and the color intensity decreases proportionally. Trolox, a water-soluble vitamin E analog, serves as a standard or control antioxidant.

Evaluation of antioxidant activity in linoleic acid model system. Linoleic acid emulsions were prepared by mixing 0.285 g of linoleic acid, 0.289 g of Tween 20 as emulsifier and 50 cm^3 phosphate buffer (pH 7.2). The mixture was homogenized for 5 min according to Yen et al. (2003). The antioxidant was added at the final concentrations of 0.2 g.$(dm^3)^{-1}$ of oil, BHT 0.1 g.$(dm^3)^{-1}$ was used as control. The mixture was incubated in an oven at 37^{0}C for 19 d. The course of oxidation was monitored by measuring the conjugated diene formation (CD) and thiobarbituric acid reactive substances (TBARS).

The antioxidant activity at the end of the assay time was expressed as reduction percentage of peroxidation (RP %) for each indicator. The control containing no antioxidant was 0%.

RP % = □(peroxidation indicator value without antioxidant) – (peroxidation indicator value with antioxidant)/ peroxidation indicator value without antioxidant)□x100. A higher percentage indicates a higher antioxidant activity.

Determination of conjugated diene formation. Aliquots of 0.02 cm^3 were taken at different intervals during incubation. After incubation, 2 cm^3 of methanol in deionised water [600 $cm^3.(dm^3)^{-1}$] were added, and the absorbance of the mixture was measured at 233 nm (Zainol et al., 2003).

Determination of thiobarbituric acid reactive substances. A modified thiobarbituric acid reactive substances (TBARS) method was used to measure the antioxidant activity of oil in terms of inhibition on lipid peroxidation. 0.1 cm^3 of sample was taken from the emulsion every day, and the following were sequentially added: the TBA-TCA solution [20 mM TBA in 150 g.$(dm^3)^{-1}$ TCA]. The mixture was heated in a 100^{0}C water bath for 15 min and cooled at room temperature. After 2 cm^3 of chloroform were added, the mixture was mixed and centrifuged at 2000 rpm for 15 min. The chloroform layer was separated and the absorbance of the supernatant was measured at 532 nm against a blank containing TBA-TCA solution (Romero et al., 2004).

Hydrogen peroxide scavenging activity of juniper berry oil. Juniper berry oil in different concentrations [40, 60, 80, 100, 120, 160 and 200 μg.$(cm^3)^{-1}$, dissolved in 0.01, 0.015, 0.02, 0.025, 0.03, 0.04 and 0.05 cm^3 DMSO respectively] were added to 1 cm^3 20 mM H_2O_2 in phosphate buffer (0.1 M, pH 7.3). The initial and the final absorbance of the samples were measured at 240 nm. The final absorbance of the samples was measured after an incubation period of 1h at 25^{0}C and the difference in absorbance was calculated ΔA. Hydrogen peroxide concentration was determined according to the formula:

$$\text{Concentration} = \frac{\Delta A}{\varepsilon} \text{ mM},$$

where ΔA – the difference in absorbance at the end and at the beginning of reaction; ε - molar absorptivity of H_2O_2 [ε = 43.6, 1.$(M.cm)^{-1}$].

Samples containing 20 mM H_2O_2 and the same DMSO volumes were prepared in a similar way.

Antioxidant activity in vivo (assays for antioxidant enzymes in yeast cells treated with juniper berry essential oil)

Model organism. For the *in vivo* analyses, *S. cerevisiae* yeast from the collection of the Biotechnology Department at

the University of Food Technologies, Plovdiv, were used. The strain was cultivated aerobically in a liquid medium [g.$(dm^3)^{-1}$: yeast extract -10, Bacto-peptone - 10, glucose – 20] for 48 h at 30^0C. The cells were centrifuged (3000 min-1), washed with phosphate buffer (50 mM, pH 7) and centrifuged again. Then they were resuspended in potassium-phosphate buffer, pH 7 until final $OD_{600\ nm}$ of 0.256. Samples containing 1 cm^3 suspension and juniper berry oil in different concentrations (40, 60, 80, 100, 120, 160 and 200 μg.$(cm^3)^{-1}$ added in 0.01, 0.015, 0.02, 0.025, 0.03, 0.04 and 0.05 cm^3 DMSO respectively) were prepared. Yeast suspension controls were also prepared using the same DMSO volumes. The samples were incubated for 1 h in the dark with periodic shaking. Then the yeast cells were centrifuged at 4000 min^{-1} for oil and DMSO removal, washed twice with phosphate buffer (50 mM, pH 7), centrifuged and resuspended in phosphate buffer to 1 cm^3. This whole cell suspension was used for the evaluation of the enzymes superoxide dismutase, catalase, and glutathione peroxidase. The resultant enzyme activities were compared to those of *S. cerevisiae* cells not treated with oil.

For evaluation of the protein content in yeast cells, the suspension was subjected to heat treatment for 20 min at 60^0C. The resultant cell lysate was centrifuged at 4000 min^{-1} and the protein in the supernatant was determined according to the Lowry method.

Superoxide dismutase (SOD) activity. SOD (EC 1.15.1.1) activity was assayed by the nitroblue tetrazolium (NBT) test (Beauchamp and Fridovich, 1971). NBT was reduced to blue formazan by O^-_2, which has a strong absorbance at 560 nm. The presence of SOD inhibited the reaction. The assay mixture consisted of sodium carbonate buffer (pH 10.2) containing xanthin, NBT, EDTA and 0.025 cm^3 of yeast suspension. The reaction was initiated by the addition of 0.05 cm^3 of xanthine oxidase [0.1 mg.$(cm^3)^{-1}$] and the mixture was incubated for 30 min at room temperature. The reaction was stopped by adding 6 mM copper (II) chloride and the mixture was centrifuged at 1500 rpm for 10 min. The absorbance of blue formazan in the supernatants was measured at 560 nm. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction. Activity was expressed as units per mg protein.

Catalase (CAT) activity. CAT (EC 1.11.1.6) activity was measured according to the method of Carrillo et al. (1991). The decomposition of H_2O_2 [30 g.$(dm^3)^{-1}$] was monitored by a decrease in absorbance at 240 nm. The assay mixture contained 0.025 cm^3 of yeast suspension in 50 mM phosphate buffer (pH 7.0) at a final volume of 1.0 cm^3. The samples were incubated for 2 min at 37^0C and the absorbance of the samples was monitored for 3 min. One unit of CAT was defined as the enzyme amount causing decomposition of 1 μmol H_2O_2 in 1 min. Activity was expressed as units per mg protein and calculated according to the formula:

$$CAT = \frac{\Delta A}{\varepsilon.3.mg}, U.(mg)^{-1}$$

where: ΔA – the difference in absorbance at the beginning and at the end of the reaction; ε - molar absorptivity of H_2O_2 [ε = 0.0436, 1.(imol.cm)$^{-1}$]; 3 – reaction time, min; mg – protein content.

Glutathione peroxidase (GPx) activity. GPx (EC 1.11.1.9) was assayed by the method of Paglia and Valentine (1967). The reaction mixture contained 0.1 M phosphate buffer (pH 7.0), EDTA, glutathione (GSH), NaN_3, 1 unit of glutathione reductase, 1.5 mM NADPH and 0.025 cm^3 of yeast suspension. After incubation for 10 min at 37^0C, H_2O_2 was added to each sample at a final concentration of 20 mM. The GPX activity was measured as the rate of NADPH oxidation at 340 nm. One unit of GPX was defined as the enzyme amount causing oxidation of 1 μmol NADPH in 1 min. Activity was expressed as units per mg protein and calculated according to the formula:

$$GPx = \frac{\Delta A}{\varepsilon.3.mg}, U.(mg)^{-1}$$

where**:** ΔA - the difference in absorbance at the beginning and at the end of the reaction; ε - molar absorptivity of NADPH [ε = 6.3, 1.(imol.cm)$^{-1}$]; 3 – reaction time, min; mg – protein content.

Assays for antioxidant enzymes in yeast cells subjected to oxidative stress with hydrogen peroxide. For these analyses, 1mM hydrogen peroxide (final concentration) was added to 1 cm^3 of yeast suspension ($OD_{600\ nm}$ 0.256), which was then incubated for 1 hour in the dark with periodic shaking. The yeast cells were centrifuged at 4000 min^{-1}, washed twice with phosphate buffer (50 mM, pH 7), centrifuged and resuspended in phosphate buffer to 1 cm^3. This whole cell suspension was used for determination of the antioxidant enzymes as described in the preceding section.

Statistical analysis. The experimental data analysis included approximation through fourth order polynomial dependences. For all cases, the plural correlation coefficient R^2 was determined. The concentration level corresponding to 50 % of inhibition was calculated according to the approximated dependence for which R^2 was the maximum. The mathematical analysis of the data was carried out with MATHLAB software.

Results are expressed as means ± SD (n =3).

All values of the enzyme activities are presented as mean ± SD (n =3). The statistical differences between the activities of the treated and untreated yeast, between oil-treated yeast and yeast-treated with DMSO were analyzed by Student's t-test. Difference showing a *p*-value of = 0.05 were considered statistically significant.

Results and Discussion

Chemical composition of juniper berry oil

Juniper berries (*Juniperus communis* L.) were analyzed using GC/FID and GC/MS (Table 1**).** The composition of juniper berry essential oil was dominated by monoterpenes (α-pinene: 35.4%, myrcene: 15.3%, sabinene: 7.6%, limonene: 7.3%), sesquiterpene (â-caryophyllene: 4.2%, germacrene D: 1.8%, δ-cadinene: 1.5%). The major oxygenated terpenoids were terpinen-4-ol: 2.4%, α- and γ-terpinenes: 0.5 and 1.8%.

Table 1
Chemical composition of juniper berries oil

Compound	RI †	RI ¥	Area§ [%]
Tricyclene	929	1000	0.1
α-Thujene	932	-	0.7
α-Pinene	943	1018	35.4
α-Fenchene	954	1045	0.1
Camphene	956	1053	0.5
Thuja-2,4(10)-diene	960	-	0.1
Sabinene	981	1110	7.6
β-Pinene	986	1097	3.3
p-Mentha-2,8-diene	990	1120	0.3
Myrcene	997	1150	15.3
p-Mentha-1(7),8-diene	1010	1156	0.7
δ-*3*-Carene	1016	1135	0.1
α-Terpinene	1022	1165	0.5
p-Cymene	1030	1251	2.1
Limonene	1035	1187	7.3
(*E*)-Ocimene	1049	-	0.1
γ-Terpinene	1063	1230	1.8
Terpinolene	1095	1267	1.2
Linalool	1104	-	0.2
(*E*)-Pinocarveol	1148	-	0.1
Terpinen-4-ol	1187	-	2.4
α-Terpineol	1199	-	0.2
Fenchyl acetate	1227	-	0.1
Bornyl acetate	1293	-	0.2
Terpinyl acetate	1355	-	0.1
α-Cubebene	1359	1443	0.5
α-Copaene	1387	1478	0.5
β-Elemene	1402	1569	0.6
β-Caryophyllene	1433	1579	4.2
Thujopsene	1443	1599	0.3
(*E*)-β-Farnesene	1462	-	0.3
α-Humulene	1466	1645	1.2
Germacrene D	1493	1685	1.8
δ-Cadinene	1533	1730	1.5

By way of comparison, Estonian juniper berry oil (*Juniperus communis* L.) is dominated by α-pinene: 47.9%, β-pinene: 1.2%, germacrene D: 3.7%, myrcene: 3.4%, limonene: 1.2%, α- and γ-terpinenes in trace amounts (Orav et al., 2010). The oil from *Juniperus communis* subsp. *Hemisphaerica* is dominated by sabinene: 25.1% and α-pinene: 13.6% (Emami et al., 2007).

Regardless of the domination of monoterpene compounds in the oils, there are differences in their quantitative composition due to a number of factors: geographical location, degree of ripeness and age, production method, etc. These differences underlie the individual biological properties of juniper berry essential oils.

Antioxidant activity in vitro

2,2-diphenyl-1-picrylhydrazyl radical scavenging. DPPH assay was one of the *in vitro* tests used in this study to determine the ability of juniper berry oil components to act as hydrogen atom donors. 2,2- Diphenyl-1-picrylhydrazyl (DPPH) assay is usually regarded as a reaction of hydrogen atom transfer, but on the basis of the kinetic data, an electron transfer mechanism can also be considered for this assay (Foti et al., 2004; Huang et al., 2005).

Juniper berry essential oil was a DPPH radical reducer with IC_{50} value of 944 ig.$(cm^3)^{-1}$ (R^2=0.995) (Table 2). The antiradical activity of BHT (a control in the study) was much stronger than that of the oil having IC_{50} value of 4.414 ig.$(cm^3)^{-1}$ (R^2=0.999), i.e. 213.8 times as strong as that of essential oil.

Limonene in 10-50 ig.$(cm^3)^{-1}$ concentrations causes DPPH inhibition from 16% to 25% (Roberto et al., 2010). Emami et al. (2007) established that γ-terpinene (17.74%) showed antiradical activity in relation to DPPH radicals, β-pinene had extremely low activity (0.96%), and α-pinene had no activity.

Deoxyribose degradation assay. The results of the deoxyribose degradation inhibition also showed other action mechanisms of the antioxidants in juniper berry essential oil (Table 2). During incubation of Fe^{3+}-EDTA with H_2O_2 and ascorbic acid at pH 7.4, hydroxyl radicals were formed, which was indicated by the 2-deoxy-D-ribose degradation by them. 2-deoxy-D-ribose degrades to fragments which yield a pink colour when heated with thiobarbituric acid at low pH (Halliwell et al., 1987). The juniper berry oil added to the reaction mixture removed the hydroxyl radicals from the sugar and protected it against degradation (Table 2, assays with EDTA). The effect of the inhibition of hydroxyl radical by juniper berry oil was expressed by IC_{50} 0.0235 μg.$(cm^3)^{-1}$ (R^2=0.998), which was considerably higher than that of quercetin having IC_{50} 6.15 μg.$(cm^3)^{-1}$ (R^2=0.996). In the absence of EDTA in the reaction mixture, some of the Fe^{3+} ions were able to form a complex with deoxyribose and participate in the formation of hydroxyl radicals. Only the molecules, which can

chelate Fe (III), form a more stable complex with iron (III) than ETDA, and inactivate them, can inhibit deoxyribose degradation. This action mechanism of juniper berry oil was proved in our studies (Table 2, assays without EDTA). Juniper berry oil showed significant chelating capacity with IC_{50} 0.0246 μg.$(cm^3)^{-1}$ (R^2=0.930), fully comparable to hydroxyl radical scavengers. The chelating capacity of the oil was many times stronger than that of quercetin with IC_{50} 6.2 μg.$(cm^3)^{-1}$ (R^2=0.999)

In the deoxyribose degradation assay, Emami et al. (2007) established the strongest effect for pure compounds β-pinene and limonene. The *Juniperus oblonga* berry oil demonstrated the strongest anti-radical effect, which, as the authors believe, may be attributed to the large amounts of β-pinene (20.8%) in oil.

Establishing the chelation of Fe (III) by juniper berry essential oil is important for our future studies using the *S. cerevisiae* model organism. The main source of OH radical production was the Fenton reaction, which occurred between Fe^{2+} and H_2O_2. Srinivasan et al. (2000) showed that in yeast (wild-type and *sod m*utants), unlike in *E. coli* and mammals cells, most, if not all, EPR-detectable iron (free iron) was present in the Fe(III) state. On the other hand, excess superoxide could generate iron reduction by the Haber–Weiss reaction and, in turn, the ferrous ion could take part in the Fenton reaction.

ABTS radical cation scavenging activity. The full antioxidant capacity of juniper berry essential oil was also characterized by neutralization of the radical cation of *2,2'*-azino-bis(3-ethylbenzo thiazoline-6-sulphonic acid) - $ABTS^{+}$**.** Trolox was used as a reference antioxidant. The juniper berry essential oil in 10 mg concentration had antioxidant activity equivalent to 4.77 mM Trolox (Table 2).

Antioxidant activity determination in linoleic acid emulsion. An important mechanism of antioxidant activity is the inhibition of linoleic acid oxidation. Polyunsaturated fatty acids such as linoleic acid are easily oxidized by atmospheric oxygen. This auto-oxidation leads to chain reactions with formation of conjugated double bonds and by-products such as aldehydes, ketones and alcohols. The unoxidized linoleic acid molecules have two unconjugated double bonds and no absorbance at 233 nm. During the oxidation of lipid molecules, conjugated double bonds are formed, whereby lipid peroxides and hydroperoxides are produced, their absorbance at 233 nm increasing in relation to their concentration.

Linoleic acid peroxidation caused by the formation of conjugated double bonds showed two absorbance maximums: on the 4th and the 7th day of incubation (control in this study) (Figure 1a). In the samples containing juniper berry oil in 0.2 g.$(dm^3)^{-1}$ concentration, 100% inhibition was observed at the first peak, and 18.82% at the second peak of peroxide production.

Table 2
Antioxidant activities of juniper berry oil

	DPPH test IC_{50} [μg.$(cm^3)^{-1}$]		OH• radical scavenging activity. Assays with EDTA IC_{50} [μg.$(cm^3)^{-1}$]		Fe^{3+} chelating activity. Assays without EDTA IC_{50} [μg.$(cm^3)^{-1}$]	ABTS test
Juniper berry oil	944 (R^2=0.995)	Juniper berry oil	0.0235 (R^2=0.998)	Juniper berry oil	0.0246 (R^2=0.930)	10 mg juniper berry oil had total antioxidant capacity correspondent to 4.77 mM Trolox
BHT (standard)	4.414 (R^2=0.999)	Quercetin (standard)	6.15 (R^2=0.996)	Quercetin (standard)	6.2 (R^2=0.999)	

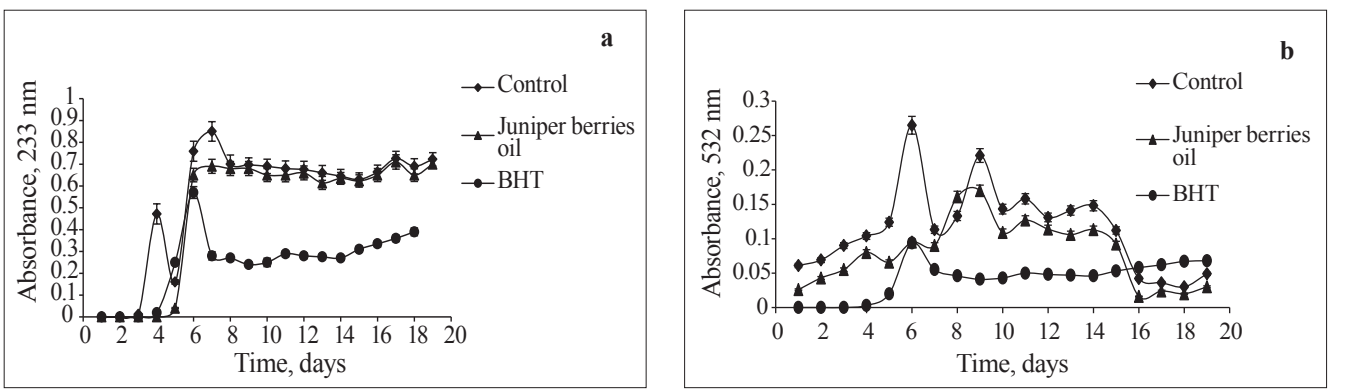

Fig. 1. Effect of juniper berries oil on conjugated dienes (a) and TBARS (b) in a linoleic acid/water emulsion system

The use of thiobarbituric acid as reagent showed the presence of malonaldehyde: a secondary product of the linoleic acid peroxidation. Malonaldehyde yielded a pink colour with thiobarbituric acid, with absorption maximum at 532 nm. The control in this study showed 4 peaks in the formation of lipid peroxidation by-products: on the 6th, 9th, 11th and 14th d of the study (Figure 1b).

The first two peaks were the most significant ones, followed by a period of attenuation in the formation of lipid peroxidation by-products. The results also showed that the formation of by-products of lipid peroxidation as a process began after the initial formation of peroxides and hydroperoxides in the reaction medium. The addition of juniper berry oil to the reaction emulsion reduced significantly the formation of lipid peroxidation by-products. On the 6th day of the process, 64.15% inhibition of lipid peroxidation was achieved; on the 9th day, it was 23.07%. Juniper berry oil inhibited largely the second of the two lipid peroxidation mechanisms, i.e. conjugated double bond formation and production of by-products of linoleic acid. Juniper berry oil was less efficient than BHA in both processes of lipid peroxidation inhibition.

Ruberto et al. (2009) proved that α- and γ-terpinene and terpinolene had the highest antioxidant activity in both lipid peroxidation stages, the activity of α- and γ-terpinene being comparable to that of α-tocopherol. α-Pinene, sabinene and limonene exhibited weak activity only at the stage of by-product formation.

The lower degree of lipid peroxidation inhibition by the juniper berry essential oil studied was determined by its composition. The oil was dominated by α-pinene (35.4%) and myrcene (15.3%), with considerably lower concentration of terpinolene (1.2%), α-terpinene (0.5%) and γ-terpinene (1.8%), carriers of higher antioxidant activity.

Hydrogen peroxide scavenging activity of juniper berry oil. At essential oil concentrations from 40 to 200 μg.(cm^3)$^{-1}$, a decrease in H_2O_2 concentration was observed within the whole range studied (Figure 2). The initial concentration of 20x10^{-3} M H_2O_2 decreased to 5.8 x10^{-3} M (3.44 times as low as the initial concentration) in the presence of 200 μg.(cm^3)$^{-1}$ essential oil after 1 h of action. Thus, the oil imitated the action of catalase, the substrate for its action being H_2O_2. Under enzyme action, however, the enzyme remained unchanged whereas the juniper oil action in relation to hydrogen peroxide was probably due to the oxidation of some of the oil components. These results were in conformity with the research of Misharina et al. (2009), Rudbäck et al. (2012), which proved that cyclic monoterpene hydrocarbons α- and γ-terpinenes (contained in juniper berry essential oil) were oxidized to the aromatic hydrocarbon p-cymene.

The DMSO solvent used in increasing volumes had a weak effect on H_2O_2, the largest input volume of 0.05 cm^3 reducing its concentration by 13.65%. Gülçin et al. (2010) also reported scavenging activity of clove oil on H_2O_2.

The hydrogen peroxide scavenging property of essential oil is of great biological significance. Hydrogen peroxide is not a free radical but can generate the exceptionally strong hydroxyl radicals. Furthermore, H_2O_2 easily diffuses through mitochondrial membranes and can oxidize a number of compounds (MacDonald-Wicks et al., 2006; Clarkson and Thompson, 2000).

The 7 tests used for *in vitro* evaluation of juniper berry essential oil demonstrated its different action mechanisms. Its hydrogen atom (electron) donating capacity was proved by the DPPH assays and lipid peroxidation inhibition in both its stages. The investigated oil also had an electron yielding capacity – a mechanism underlying both OH and $ABTS^+$ scavenging and OH formation (chelating capacity). The antioxidant activity which was due to electron transfer made juniper berry essential oil a strong antioxidant. IC_{50} for OH and for chelating capacity were 0.0235 μg.(cm^3)$^{-1}$ and 0.0246 μg.(cm^3)$^{-1}$ respectively, i.e. 261.7 and 252.03 times as low as those for quercetin. The antioxidant activity of the oil attributable to hydrogen atom transfer was lower. IC_{50} of 944 μg.(cm^3)$^{-1}$ for DPPH was 200 times as high as that for BHT. Lipid peroxidation inhibition by the essential oil was much less efficient than inhibition by BHT. Lipid peroxidation (established through malondialdehyde formation) was inhibited by the essential oil by 64.15% (6 d) and 23.7% (9 d) in relation to 64.15% (6 d) and 81.44% (9 d) inhibition by BHT (Figure 1b).

A number of researchers believe that the data on the antioxidant activity of essential oils or their components obtained according to different methods are practically incomparable. This is due both to the difference in the protocols used and to the different composition of the essential oils studied.

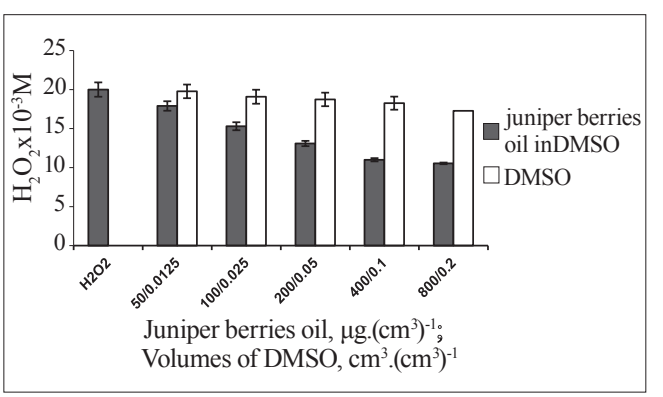

Fig. 2. Hydrogen peroxide scavenging activity of juniper berries oil

Antioxidant activity in vivo. *Action of the essential oil on the antioxidant protection of Saccharomyces cerevisiae model organism.*

The *in vitro* tests used showed that juniper berry essential oil exhibited antioxidant activity. In this aspect, the essential oil effect on whole cells of wild *S. cerevisiae* strain was studied. It was evaluated *in vivo* on the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Figure 3 a,b,c). Molecular oxygen is assimilated as a substrate by the living cells and participates in different reactions catalysed by the enzymes oxygenase, oxidase and hydroxylase. All these enzymes function interrelatedly and the study of exogenous antioxidants upon certain enzymes in whole cells would provide an evaluation that would be as close as possible to the metabolic processes occurring in the cells. Oxygen assimilation is at the expense of partially reduced oxygen species, including the production of free radicals: superoxide radical ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$). With a view to studying the effect of juniper berry essential oil (exogenous antioxidant) on the antioxidant enzymes in the cells, their enzyme levels were established before and after treatment with different essential oil concentrations. The *S. cerevisiae* cell suspension was treated with the essential oil for 1 h. During that period, the cells were also subjected to starvation.

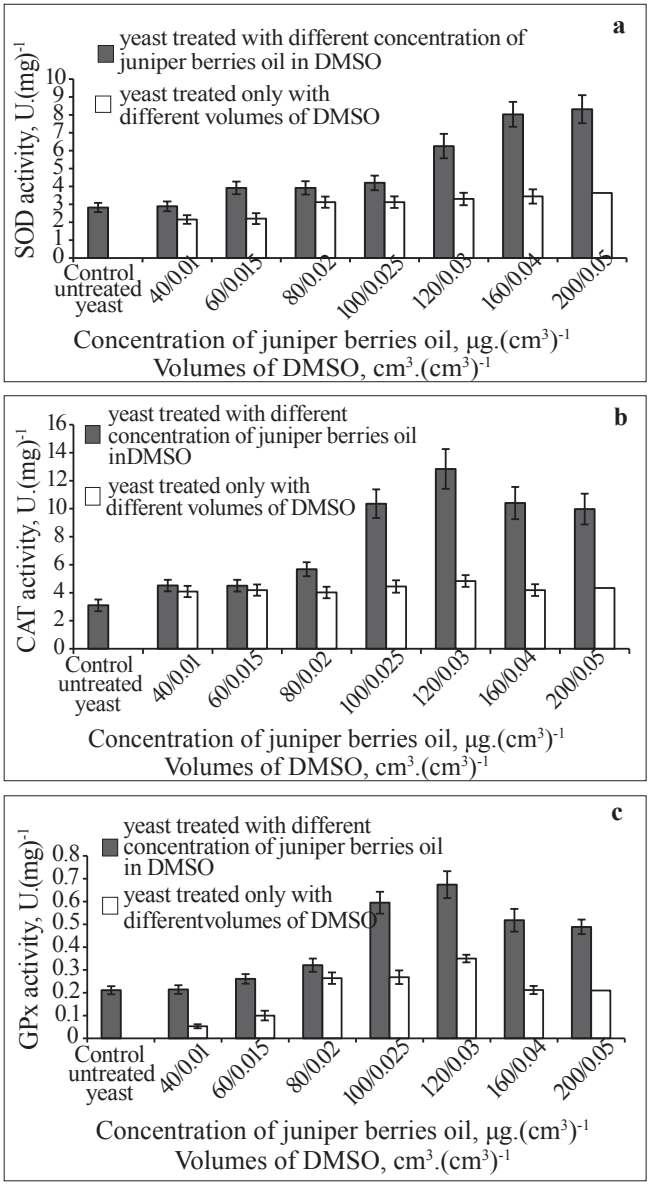

Fig. 3. Effect of juniper berries oil on superoxide dismutase (a), catalase (b) and glutatione peroxidase (c) activity in *S. cerevisiae* cells

For SOD evaluation in the whole yeast cells, the xanthine-xanthine oxygenase system generating a superoxide radical was used. The addition of whole cells only in the presence of xanthine caused xanthine oxidation under the effect of their own xanthine oxidase. Therefore, for the evaluation of SOD as control, the xanthine-xanthine-oxidase system and heat-inactivated cells (for elimination of the action of their own xanthine oxygenase) were used.

The evaluation of CAT using whole cells was facilitated by the fact that the substrate for the action of this enzyme, i.e. H_2O_2, was non-ionized and easily diffused through the hydrophobic membranes of the mitochondrial biological membranes (Clarkson and Thompson, 2000; MacDonald-Wicks et al., 2006**).**

Superoxide dismutase (SOD) participated in the dismutation of superoxide radicals in hydrogen peroxide and molecular oxygen. CAT and GPx metabolized H_2O_2: catalase only decomposed H_2O_2, and yeast glutathione peroxidase acted both on H_2O_2 and organic hydroperoxides.

Yeast cells treated with juniper berry essential oil exhibited direct dependence of the 3 enzymatic activities on the essential oil concentration (Figure 3 a,b,c). Under the effect of low essential oil concentrations from 40 to 100 μg.(cm^3)$^{-1}$, the SOD activity increased slightly. Oil concentrations above 100 to 200 μg.(cm^3)$^{-1}$ caused a greater increase in SOD activity. The enzyme level was the highest, reaching 8.32 U.(mg)$^{-1}$, at 200 μg.(cm^3)$^{-1}$ concentration of juniper berry essential oil. This activity was 2.95 times as high as SOD of untreated yeast (control: 2.82 U.(mg)$^{-1}$).

Within the whole range studied, the change in SOD activity only in the presence of DMSO followed the change in enzymatic activity in the presence of the essential oil. As absolute values, however, these activities were lower than those obtained in the presence of juniper berry essential oil. The

SOD activity in the presence of the solvent was the highest at a volume of 0.05 cm^3: 3.64 $U.(mg)^{-1}$, which was 2.28 times as low as the activity registered at 200 $\mu g.(cm^3)^{-1}$ essential oil.

CAT in cells treated with different essential oil concentrations showed a more complex dependence on SOD. Within the 40 to 120 $\mu g.(cm^3)^{-1}$ range of essential oil, enzyme activity increased to 12.84 $U.(mg)^{-1}$, i.e. 4.14 times as high as the control (3.10 $U.(mg)^{-1}$). Oil concentrations above 120 $\mu g.(cm^3)^{-1}$ led to a decrease in the enzymatic activity in relation to the one obtained at 100 $\mu g.(cm^3)^{-1}$. Nevertheless, these enzyme levels were higher than the control: 10.41 and 9.98 $U.(mg)^{-1}$ respectively at 160 $\mu g.(cm^3)^{-1}$ and 200 $\mu g.(cm^3)^{-1}$ essential oil.

The change in GPx activity in relation to the essential oil concentration was analogous to catalase activity. The activity increased in the 40 – 120 $\mu g.(cm^3)^{-1}$ range to 0.674 $U.(mg)^{-1}$, which was 3.19 times as high as the control (0.211 $U.(mg)^{-1}$). Concentrations of 160 $\mu g.(cm^3)^{-1}$ and 200 $\mu g.(cm^3)^{-1}$ caused a decrease in the enzyme level, which was 0.518 $U.(mg)^{-1}$and 0.489 $U.(mg)^{-1}$ respectively. Although the enzyme levels were reduced at these concentrations, they remained higher than the activity of untreated yeast cells.

The change in CAT and GPx activity in the presence of DMSO alone was again analogous to the enzyme activities obtained in the presence of essential oil, but they were considerably lower as absolute values. The highest CAT (4.84 $U.(mg)^{-1}$) and GPx (0.350 $U.(mg)^{-1}$) activity values were 2.6 times and 1.92 times as low as the enzyme levels at 120 $\mu g.(cm^3)^{-1}$ of essential oil added with 0.03 cm^3 DMSO.

Activities of SOD, CAT and GPx of yeast treated with juniper berries oil compared with the controls (untreated yeast) were statistically significant (p-value of = 0.05). Those activities compared with the activities of the yeast, treated with DMSO, showed p-value of = 0.05 and are considered statistically significant too.

We compared the effect of juniper berry essential oil on the 3 antioxidant enzymes in the yeast cells to the effect of 1 mM H_2O_2 on these enzymes (Figure 4). The yeast cells responded to the oxidative stress induced by 1 mM H_2O_2 for a period of 1 h with an increase in the CAT (5.28 $U.(mg)^{-1}$) and GPx (0.284 $U.(mg)^{-1}$) activities by 1.70 and 1.34 times respectively, and in the SOD (2.94 $U.(mg)^{-1}$) activity by 1.04 times compared to untreated cells. The increase in the CAT activity in yeast cells treated with hydrogen peroxide can be explained by the inducible transcription of the CTT1 gene encoding catalase in the *S. cerevisiae* cytoplasm (Schüller et al., 1994; *Jamieson et al., 1994).* It has also been proved that under oxidative stress, GPx genes of *S. cerevisiae* also encode phospholipid hydroperoxide glutathione peroxidases and that these enzymes protect yeast against phospholipid hydroperoxides (Inoue et al., 1999; Avery and Avery, 2001).

Under starvation conditions and treatment with juniper essential oil (up to 120 $\mu g.(cm^3)^{-1}$), yeast cells exhibited higher antioxidant capacity than the antioxidant protection of cells subjected to oxidative stress by 1 mM H_2O_2. The enhanced antioxidant protection was indirect evidence of the change in the endogenous levels of H_2O_2 and other organic peroxides in this microorganism. Higher enzyme activities in the yeast cells meant better ability of the cells to degrade hydrogen peroxide, organic hydroperoxides and phospholipid hydroperoxides. Thus, it would follow that the level of these peroxides within the cells would be lower at higher enzyme activity. Roberto et al. (2010) proved that a lower H_2O_2 endogenous level corresponded to a higher CAT and GPx activity in lymphocyte cells treated with the monoterpene compound limonene. We proved *in vitro* that juniper oil could degrade hydrogen peroxide similarly to catalase action (Figure 2). Monoterpene compounds are known to be able to penetrate cells (Misharina and Samusenko, 2008) and therefore neutralize endogenous H_2O_2. The significant increase in the CAT and GPx activity in yeast cells in the presence of juniper essential oil and under starvation stress conditions may be due to inducible gene transcription and probably to a larger extent to the catalase-like action of the oil components.

SOD is the first protection line against oxidative stress in living organisms (Schauss et al., 2006). The scavenging of superoxide radicals, which are precursors of highly reactive species such as hydroxyl radicals, is particularly important for organism adaptation under oxidative stress. Considering the fact that the expression of the genes encoding Cu/Zn SOD and Mn SOD in *S. cerevisiae* is not inducible by oxidative stress (Jamieson et al., 1994), as well as the studies of Karioti et al. (2004) on the scavenging activity of germacrene-D in relation to the superoxide radical, we can assume that the increasing SOD activity resulted from the action of juniper berry essential oil.

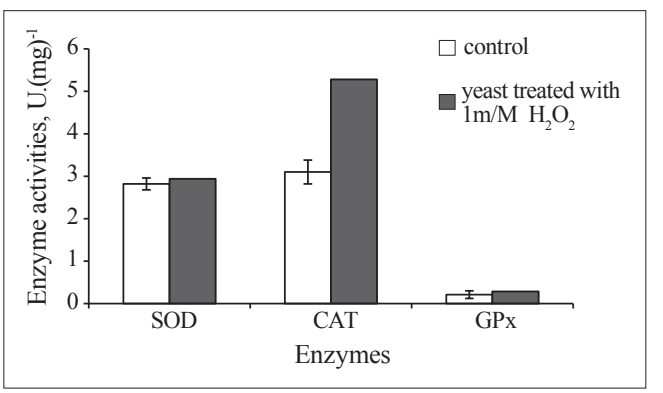

Fig. 4. Effect of 1 mM H_2O_2 on enzyme activities in *S. cerevisiae* cells

The studies showed that the 120 μg.(cm^3)$^{-1}$ concentration of essential oil proved to be a threshold concentration for all three enzymes. With SOD, it marked the beginning of a more significant increase in activity; with CAT and GPx the enzyme levels increased up to this concentration, and then started to decrease. Juniper berry essential oil increasingly neutralized H_2O_2 *in vitro* within the whole 40 to 200 μg.(cm^3)$^{-1}$ range, resembling catalase action (Figure 2). The changes in the CAT and GPx activities, having H_2O_2 as their substrate, however, increased only to 120 μg.(cm^3)$^{-1}$ of essential oil (Figure 3 b,c). If the increasing activity of these enzymes enables cells to neutralize the reactive oxygen species, their decreasing activity should be considered as a decreasing ability of cells to neutralize them. This was proved by the studies of Roberto et al. (2010) on lymphocyte cells. Their treatment with high limonene concentrations was related to an increased endogenous H_2O_2 level and reduced CAT and GPx activities. Juniper berry oil concentrations exceeding 120 μg.(cm^3)$^{-1}$ probably induced damage to *S. cerevisiae*. Antimicrobial action has been established for juniper berry essential oil (Wanner et al., 2010). Parveen et al. (2004) reported damages induced to *S. cerevisiae*. The authors found that the *S. cerevisiae* participating in ergosterol biosynthesis and assimilation, lipid metabolism, cell wall structure and function, and cellular transport were affected by á-terpinene treatment.

Conclusion

In vitro antioxidant research of juniper essential oil proved the existence of several mechanisms, which enabled radical scavenging, H_2O_2, the prevention of radical formation (chelating capacity) and protection against lipid peroxidation. *In vivo* studies confirmed these effects of the oil, which created the possibility of blocking the oxidation processes in yeast cells, and enhance their adaptivity to ROS. The biological effects of juniper berry essential oil *in vivo* were directly dependent on the concentrations applied.

It is well known that ROS contribute to organism aging and the etiopathogenesis of various diseases. The proved ability of juniper berry essential oil to enhance adaptivity to ROS *in vivo* adds new details to the essential oil properties. These properties determine its potential for food additive production, an efficient way of people's health and quality of life improvement. Furthermore, it expands the areas of application to perfumery, cosmetics, pharmacy and medicine**.**

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