

POTENTIAL EFFECT OF THYME AND OREGANO ESSENTIAL OILS TO ANTIMICROBIAL ACTIVITY AND YEAST ENZYMATIC ANTIOXIDATIVE SYSTEM

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ABSTRACT

This *in vitro* study was aimed to assess the effects of *Thymus vulgaris* L. and *Origanum vulgare* L. essential oils (EOs) on cell viability and enzymatic antioxidative system in yeast *Saccharomyces cerevisiae*. Yeasts were cultivated with different concentrations of thyme (*Thymus vulgaris* L.) (0.05 – 1.00 µl/ml) and oregano (*Origanum vulgare* L.) (0.05 – 0.70 µl/ml) EOs for 24 hours. For the present study, gas chromatography-mass spectrometry (GC-MS) analysis was used to quantify chemical composition of EOs. The main components of thyme essential oil were caryophyllene (7.94%), p-cymene (18.55%), and thymol (46.55%). In essential oil of oregano, there was found p-cymene (8.43%) and carvacrol (69.99%) mainly. The antimicrobial activity of selected EOs was investigated by the microbroth dilution method using 96 well microtiter plates. The most potent essential oil able to inhibit growth of *Saccharomyces cerevisiae* was oregano EO with the values of MIC₅₀ 0.468845 µl/ml and MIC₉₀ 0.501845 µl/ml. Also, the activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) were assessed. According to our results, *Thymus vulgaris* L. significantly increased the SOD levels at 0.2, 0.4, 0.5 to 0.9 µl/ml and GPx production at 0.05 to 0.7 µl/ml ($P < 0.001$). In case of *Origanum vulgare* L., significant increase of SOD production at 0.05 - 0.2 µl/ml ($P < 0.001$) was observed. This essential oil also elevated GPx concentration in all experimental groups ($P < 0.001$) significantly. Data from this study suggest that selected EOs have significant effects on enzymatic antioxidants that may affect the first-line cell defense system against oxidative stress.

Keywords: *Saccharomyces cerevisiae*, MICs, *Thymus vulgaris* L., *Origanum vulgare* L., superoxide dismutase, glutathione peroxidase

INTRODUCTION

The antioxidant potential of plant bioactive substances has recently been in the high interest of the scientific and lay public. Plant antioxidants can inhibit the effect of free radicals, thus providing some protection against various diseases (Pratt, 1992; Amarowicz and Pegg, 2019) and are of interest to many scientific studies addressing the potential positive effects on the overall health status (Kiokias *et al.*, 2020; Manuelian *et al.*, 2021), reproduction potential (Zhong and Zhou, 2013; Tvrda *et al.*, 2016; Vizzari *et al.*, 2021) or endocrine system (Golbidi and Laher, 2010; Jambor *et al.*, 2021).

Thymus vulgaris L. is a plant species belonging to the genus *Thyme* (Lamiaceae family). Thyme extract or essential oil has been attributed to many beneficial effects on the human body. These are mainly antioxidative, anti-inflammatory, antitumoral, and antimicrobial effects (Nikolić *et al.*, 2014; Yan *et al.*, 2016; de Oliveira *et al.*, 2017; He *et al.*, 2020; Cisarová *et al.*, 2020). *Thymus vulgaris* L. is an herb found in Europe, Africa, and Asia, where it is also widely used in folk medicine. *Origanum vulgare* L. is an herb which also belongs to Lamiaceae family (genus *Origanum*) that grows in Europe, North Africa, and Asia. Oregano essential oils show, among other features, antioxidative (Spiridon *et al.*, 2011), antimicrobial (De Martino *et al.*, 2009), antifungal (Hlebová *et al.*, 2021) or hepatoprotective (Oniga *et al.*, 2018) activities.

Superoxide dismutase, glutathione peroxidase and catalase are the first line defense antioxidants (Ighodaro and Akinloye, 2018). While catalase is not present in mitochondria of most cells, superoxide dismutase and glutathione peroxidase are systems located in mitochondria (Bai and Cederbaum, 2001).

Yeasts are eukaryotic microorganisms that can be used as excellent cell model for basic research of redox markers. Their undoubted advantages are low cost and confirmed cell response to oxidative stress (Jamieson, 1998; Toledano *et al.*, 2003).

Based on the above, aim of the present study was to evaluate potential antimicrobial and antioxidative activity of thyme and oregano essential oils (EOs). Effect of selected substances to redox markers production was investigated in

yeast. At the same time the GC-MS profiling of thyme and oregano was attempted to understand their chemical composition associated with biologically active substances rendering them biological significance. This study provides a better understanding of underlying enzymatic antioxidant mechanisms of thyme and oregano essential oils.

MATERIAL AND METHODS

Yeast, media, growth conditions and identification (MALDI-TOF MS)

Saccharomyces cerevisiae isolated from commercial baker's yeast was used as the yeast strain in this study. For isolation of yeast strain, Sabourad dextrose agar (Biolife, Italy), 30 ± 1 °C during 24 h was used. Purification of yeast colonies was provided by four-way streak plate method. Isolated species was identified by using MALDI TOF MS (Maldi Biotyper, Bruker Daltonics, Germany) according to Hleba *et al.* (2017).

Chemical analyses of thyme (*Thymus vulgaris* L.) and oregano (*Origanum vulgare* L.) EOs (GC-MS)

The essential oils were obtained from commercial supplier Calendula a.s. (Nová Lubovna, Slovak Republic). EOs were prepared by hydrodistillation and stored in the dark at 4 °C in hermetically sealed bottles, before analyses. The chemical composition of EOs was conducted in an Agilent 7890A GC coupled to an Agilent MSD5975C MS detector (Agilent Technologies, Palo Alto, CA, USA) with an HP-5MS column (30 m × 0.25 mm, 0.25 µm film thickness). Essential oils were diluted in hexane to a concentration of 1 µl/ml. The operating conditions were as follows: one microliter of the sample was injected in split mode 1:12, at an injector temperature of 250 °C and electron ionization energy of 70 eV. Analysis was measured in SCAN mode, mass range was 40–400 m/z. The oven temperature started at 60 °C to a maximum of 231 °C at a rate of 3 °C/min, and then was kept constant for 10 min (Božik *et al.*, 2017). The EOs constituents were identified by

mass spectrometry (MS) and their identity was confirmed by comparing their Kovats retention indices (KI) and their spectra with the retention time of the used authentic standards (Sigma-Aldrich, CZ) (Table 1) or with data available in the National Institute of Standards and Technology Library (NIST, USA), and with literature (Adams, 2007). Only the EOs compounds over 0.1% were included.

Antimicrobial activity of essential oils (cell viability)

The antimicrobial activity of selected EOs was investigated by the microbroth dilution method by using 96 well microtiter plates according to Hleba et al. (2014). EOs were diluted in dimethyl sulfoxide (Sigma-Aldrich, Germany) directly into the Sabouraud broth medium (HiMedia, India). The 96 well microtiter plates were prepared by adding 100 µl of SB medium with tested EOs. For minimum inhibitory concentrations (MICs) determination of EOs, two-fold dilutions of each EO at the concentration of 10 to 0.004883 µl/ml were used. Then a 100 µl of yeast inoculum at the final density of 0.5 McFarland were added to each well to give a final volume 200 µl. After 24 h incubation at 30 °C the inhibition of microbial growth was evaluated by measuring the well absorbance at 450 nm in an absorbance microplate reader Biotek EL808 with shaker (Biotek Instruments, USA). The minimum inhibitory concentrations (MICs) were calculated based on growth control density and were the lowest EO concentrations that completely inhibited the yeast growth. Control sets as the negative control (without yeast inoculum), growth control and purity of EOs were tested simultaneously with the samples. All experiments were carried out in triplicate, independently. The results shown in Table 2 present MIC₅₀ and MIC₉₀ of EOs and were obtained by the probit analysis using the SAS statistical program (confidence level 95%, P<0.05).

Superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity assay

Yeasts were cultivated with different concentrations of thyme (0.05 – 1.00 µl/ml) and oregano (0.05 – 0.70 µl/ml) EOs for 24 hours. Subsequently, the medium was removed from microtiter plates and centrifuged (1 006 g for 10 min). The supernatant was used for SOD and GPx quantification. Analyses were performed using Randox commercial kits (Randox Laboratories, Ltd., United Kingdom) and semi-automated analyzer Randox Rx Monza (Randox laboratories, Ltd., United Kingdom) (Tvrdá et al., 2016; Kovacik et al., 2019). The concentrations of the total protein (TP) were measured using DiaSys (Diagnostic Systems GmbH, Holzheim, Germany) commercial kits and the Randox RX Monza (Randox Laboratories, Ltd., United Kingdom) (Kovacik et al., 2020; Kovacikova et al., 2019). All experiments were carried out in triplicate, independently. The results are expressed as IU/g TP (units per gram of total protein).

Statistical analyses

Probit statistical evaluation was performed using Statgraphics software (STATGRAPHIC Centurion XV, Statgraphics Technologies, Inc., The Plains, Virginia, USA). By using obtained absorbance before and after the analysis, it was possible to express the differences in absorbance between the measurements as a set of binary values. These values were assigned to exact concentrations. Following formula was created for this specific experiment: value 1 (inhibitory effect) was assigned to absorbance values lower than 0.05, while value 0 (no effect or stimulant effect) was assigned to absorbance values higher than 0.05.

Statistical analyses were performed using the GraphPad Prism 6.01 (GraphPad Software Incorporated, San Diego, California, USA). All obtained data were tested for normality using Kolmogorov-Smirnov and Shapiro-Wilk test. Analysis of variance (One-Way ANOVA) followed by Dunnett’s multiple comparison test was used for statistical evaluation. Results were expressed as the mean ± standard deviation (S.D). The level of significance was set at ***(P<0.001), **(P<0.01) and *(P<0.05).

RESULTS

Identification of thyme and oregano EOs chemical compounds

Qualitative and quantitative analyses of EOs are listed in Table 1. The volatile components of tested EOs belong to monoterpenes, monoterpenoid phenols, monoterpenoids, and sesquiterpenes. The main components of thyme (*Thymus vulgaris* L.) essential oil were identified as caryophyllene (7.94%), p-cymene (18.55%), and thymol (46.55%). In essential oil of oregano, there was found p-cymene (8.43%) and carvacrol (69.99%) mainly.

Antimicrobial activity of thyme and oregano EOs

For the determination of minimum inhibitory concentrations (MICs) values, lower concentrations (10–0.004883 µl/ml) of 2 essential oils, were used. The obtained results showed a different antifungal activity of tested EOs with MIC₅₀ and MIC₉₀ values estimated by probit analysis (Table 2).

Table 2 The minimum inhibitory concentrations (MIC₅₀ and MIC₉₀; µl/ml) for essential oils effective against *Saccharomyces cerevisiae* on SB at 30 ± 1 °C after 24 hours of cultivation

Microorganism	Tested essential oils			
	Thyme (<i>Thymus vulgaris</i> L.)		Oregano (<i>Origanum vulgare</i> L.)	
	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀
<i>Saccharomyces cerevisiae</i>	0.937863	1.00129	0.468845	0.501845

Oregano EO was found the most potent essential oil able to inhibit growth of *Saccharomyces cerevisiae*, with the values of MIC₅₀ 0.468845 µl/ml and MIC₉₀ 0.501845 µl/ml.

Table 1 Chemical analysis (%) of used essential oils by GC-MS

RI ^a	Component	Thyme ^c	Oregano
938	^b α-pinene	1.22	0.33
953	^b camphene	1.31	0.29
980	^b β-pinene	0.20	
982	1-octen-3-ol	0.66	
993	β-myrcene	1.36	0.47
997	3-octanol		0.21
1006	^b α-phellandrene	0.1	
1019	^b α-terpinene	1.2	0.91
1029	^b p-cymene	18.55	8.43
1031	^b D-limonene	0.36	
1034	^b eucalyptol	1.47	1.19
1062	^b γ-terpinen	4.27	3.49
1090	^b terpinolene	0.13	0.12
1101	linalool	3.89	3.19
1147	^b camphor	1.98	0.89
1168	^b borneol	1.85	0.87
1179	^b 4-terpineol	1.93	0.77
1192	α-terpineol	0.18	0.41
1238	thymol methyl ether	0.70	
1247	^b (-)-carvone	1.1	0.10
1296	^b thymol	46.55	2.6
1297	menth-1-en-9-ol	1.88	
1306	^b carvacrol		69.99
1360	^b eugenol	0.22	0.15
1376	copaene	0.16	0.14
1420	^b caryophyllene	7.94	1.84
1452	^b α-caryophyllene		0.45
1505	α-bisabolene		0.11
1524	δ-cadinene	0.32	0.44
1574	^b caryophyllene oxide	0.19	0.31
total		99.54	97.70

Legend: ^a – RI: identification based on Kovats retention indices (HP-5MS capillary column) and mass spectra; ^b – Identification confirmed by co-injection of authentic standard; ^c – relative proportion were calculated in % by dividing individual peaks area by total area of all peaks.

Effects of thyme EO on SOD and GPx activity of *Saccharomyces cerevisiae*

Yeast cells were exposed to increasing doses of thyme EO (0.05 - 1.0 µl/ml) during 24 h. As shown in Fig. 1, SOD levels were significantly higher (P<0.001 resp. P<0.01) in experimental groups (from 0.5 to 0.9 µl/ml) in comparison to control.

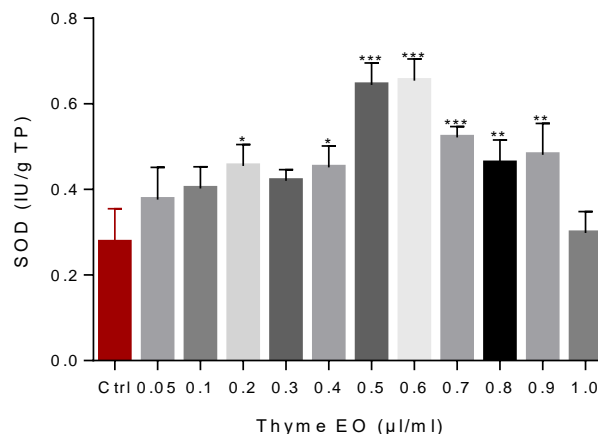


Figure 1 SOD production in yeast *Saccharomyces cerevisiae* cells exposed to different concentrations of experimental essential oil from *Thymus vulgaris* L. in vitro after 24 h cultivation. Ctrl – control group. Each bar represents the mean (±S.D.) of control (untreated) and treated groups.

Slight increases were recorded at 0.2 and 0.4 µl/ml of thyme, also with significant changes ($P<0.05$). At the highest dose of thyme, a statistically significant difference was not observed.

A dose-dependent increase in the GPx production caused by thyme EO is shown in Fig. 2. An increase was recorded in all applied concentrations of thyme except for the highest concentrations (0.8, 0.9 and 1.0 µl/ml). Lower doses of the tested substance (0.05 - 0.7 µl/ml) initiated a successive increase of GPx production ($P<0.001$).

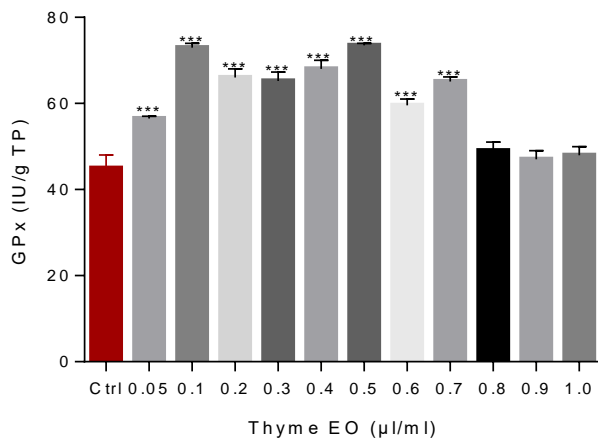


Figure 2 GPx production in yeast *Saccharomyces cerevisiae* cells exposed to different concentrations of experimental essential oil from *Thymus vulgaris* L. *in vitro* after 24 h cultivation. Ctrl – control group. Each bar represents the mean (\pm S.D.) of control (untreated) and treated groups.

Effects of oregano EO on SOD and GPx activity of *Saccharomyces cerevisiae*

Yeast cells were exposed to increasing doses of oregano EO (0.05 – 0.7 µl/ml) during 24 h. A dose-dependent effect in the SOD production caused by oregano is shown in Fig. 3. Lower doses of the tested substance (0.05 - 0.2 µl/ml) initiated an increase of SOD production, the differences were significant ($P<0.001$). Higher doses of the oregano EO (0.5 – 0.7 µl/ml) initiated a successive decrease compared to the control group.

The results shown in Fig. 4 illustrate that experimental doses from 0.05 to 0.7 mg/mL did affect the GPx production significantly ($P<0.001$). GPx production increased in all groups with increasing experimental dose of the test substance.

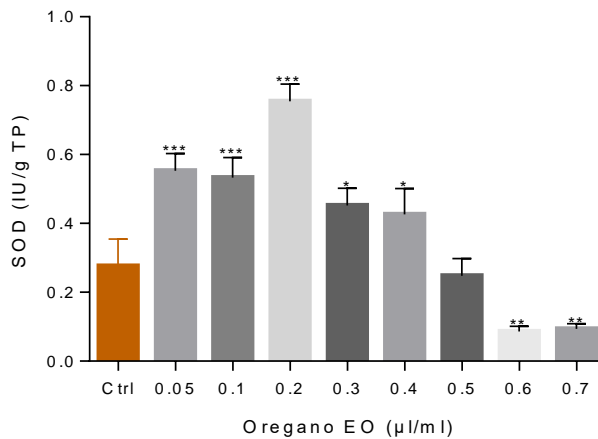


Figure 3 SOD production in yeast *Saccharomyces cerevisiae* cells exposed to different concentrations of experimental essential oil from *Origanum vulgare* L. *in vitro* after 24 h cultivation. Ctrl – control group. Each bar represents the mean (\pm S.D.) of control (untreated) and treated groups.

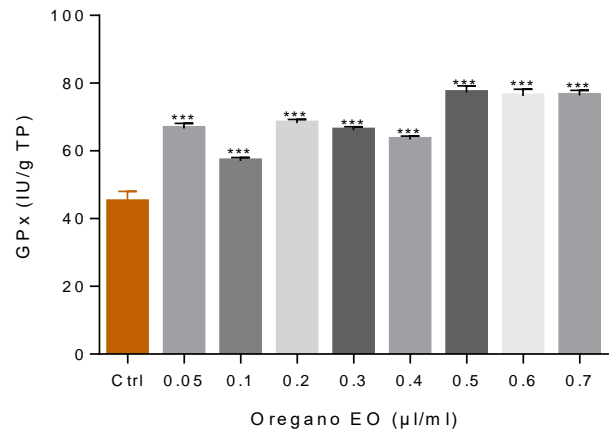


Figure 4 GPx production in yeast *Saccharomyces cerevisiae* cells exposed to different concentrations of experimental essential oil from *Origanum vulgare* L. *in vitro* after 24 h cultivation. Ctrl – control group. Each bar represents the mean (\pm S.D.) of control (untreated) and treated groups.

DISCUSSION

Thyme and oregano EOs chemical compounds

Chemical composition of thyme and oregano EOs, as well as other essential oils, is different and it depends on recovery or extraction methods and procedures (Sefidkon et al., 2006; Lis-Balchin et al., 1998), also on region (Sangun et al., 2007), plant varieties (Ložien et al., 2003) and plant parts (Alsaraf et al., 2020). Compared to other studies, we can confirm that composition of thyme EO is very different and varies from study to study. The main compositions in our thyme EO were thymol (46.55%), p-cymene (18.55%) and caryophyllene (7.94%). Unlike us, authors Alsaraf et al., 2020 acquire thyme EOs by hydro-distillation from leaves and they identified the main compounds as carvacrol (59.29%) and γ -terpinene (29.12%). On the other hand, authors Porte and Godoy, 2008 used hydro-distillation extraction method from leaves and they observed thymol (44.7%), p-cymene (18.6%), γ -terpinene (16.5%) and caryophyllene (0.8%) as the main compounds only. Absolutely different main compounds of thyme EOs obtained using hydro-distillation extraction method from dried areal parts of thyme plants by authors Imelouane et al., 2009 were determined. They detected camphor (38.54%) and camphene (17.19%) and α -pinene (9.35%) as main compounds of thyme EO. In the case of oregano EOs, carvacrol (69.99%) and p-cymene (8.43%) were identified as the main compounds. In contrary, authors Teixeira et al., 2013 identified carvacrol in amount of 14.5%. Except for carvacrol, they detected γ -Terpinene (11.6%), β -fenchyl alcohol (12.8%) and thymol (12.6%) as main compounds. Kulisic et al., 2004 obtained two fractions - hydrocarbons CH fraction and oxygen containing compounds fraction after fractionation of oregano essential oil using column chromatography separation. The main compounds of hydrocarbons CH fraction determined by HPLC were α -terpinene (10.4%), γ -terpinene (31.0%) and p-cymene (22.1%). Oxygen containing compounds fraction contained thymol (47.3%) and carvacrol (46.4%) as main compounds. Obviously, difference between main components of EOs is significant. Therefore, it is very important to determine the composition of all EOs tested in each new study.

Antimicrobial activity of thyme and oregano EOs

Antimicrobial activity of thyme and oregano EOs was tested on *Saccharomyces cerevisiae* by MIC method, where strong antimicrobial activity was observed. Similarly, Konuk et al., 2017 observed high antimicrobial effectivity of thyme and oregano EOs against *S. cerevisiae*. They determined minimal inhibition concentration at the level of 0.2 - 0.3 µl/ml for both EOs. Moreover, they also observed that all tested EOs increased extracellular pH, possibly due to the neutralization of the glucose-induced pH gradient upon impairment of the cell membrane. Bennis et al., 2004 used pure thymol, which is a main compound of thyme EO, for inhibition of *S. cerevisiae* growth. For complete inhibition of *S. cerevisiae* growth, 3 mM of thymol dispersed in 0.2% agar solution was sufficient. Using concentration higher than MIC caused total mortality. Authors confirmed connection between total mortality of *S. cerevisiae* and lysis of yeast cells. Their observing by scanning electron microscopy clearly showed that surface of many cells is deformed significantly and cells in the background showed important damage consisting in apparent cracks. Antimicrobial activity of thyme and oregano EOs was confirmed by many authors (Farag et al., 1989; Özcan et al., 2001; Belletti et al., 2004; Bennis et al., 2004; Chami et al., 2005; Wang et al., 2011; Lv et al., 2011; Cisarová et al., 2020; Hlebová et al., 2021).

Action of thyme and oregano EOs on the antioxidant protection of *Saccharomyces cerevisiae* model cells

In our study, we also investigated the *in vitro* effect of selected EOs on antioxidant enzymes in *S. cerevisiae*. *Thymus vulgaris* L. and *Origanum vulgare* L., are characterized by the presence of numerous bioactive compounds such as monoterpenes, phenolic compounds, sesquiterpenes, thymol, isothymol and other metabolites (Lee et al., 2005; De Martino et al., 2009; Nikolić et al., 2014; Oniga et al., 2018). Bioactive compounds can inhibit the effect of free radicals, thus providing some protection against various diseases (Pratt, 1992; Nour et al., 2017; Amarowicz and Pegg, 2019). SOD, GPx and catalase are the first line defense antioxidant enzymes against oxidative stress (Ighodaro and Akinloye, 2018). We confirmed that some of the analyzed concentrations may affect SOD and GPx production in a dose-dependent manner.

There is a lack of studies on the specific effects of thyme and oregano on yeast. However, there are several experimental studies examining the effect of specific bioactive substances on yeast or other cell models. Comparable consequences have previously been reported by Khan et al. (2015). *Candida Albicans* cells were exposed to two monoterpene phenols (thymol and carvacrol) at concentrations ranging from 5 to 20 µg/ml. The studied phenols increased SOD activity statistically at all tested concentrations. GPx activity decreased significantly after initial increasing with growing concentrations of each compound. Ceker et al. (2012) evaluated the impact of *Origanum vulgare* L. EO on the human lymphocytes stressed by aflatoxin B1 (AFB₁). Cells were cultured together with AFB₁ and experimental concentrations of oregano EO (0.1 to 2.0 µl) for 72 h *in vitro*. Authors monitored, inter alia, the activity of SOD and GPx in the supernatant after essential oil application. Their results showed significant increases in the activities of SOD and GPx compared to control. Höferl et al. (2014) tested antioxidant protection of juniper berry EO on *S. cerevisiae* as the model organism. Activities of SOD, GPx and CAT of yeast were higher (in some cases significantly) compared to controls and yeast treated with dimethylsulfoxid. A similar study was performed using ginger EO, where authors confirmed significant dose dependent increase of antioxidative enzymes and cell viability (Höferl et al., 2015). Several other studies describe the antioxidant potential of thyme and oregano EO or their compounds in different cell (Nikolić et al., 2014; Alsarraf et al., 2020; Avola et al., 2020; Mar et al., 2020) or animal models (El-Nekeety et al., 2011; He et al., 2020; Zhang et al., 2020). Phenolic and terpenoid compounds present in the chemical composition of EO are closely related to their antioxidant function. From the above, the considerable importance of plant essential oils, which deserve future research, is evident.

CONCLUSION

Essential oils can be considered a good source of natural bioactive compounds with antioxidant and antimicrobial effects. The main components of the monitored EOs were caryophyllene, p-cymene, thymol (in *Thymus vulgaris* L.) and p-cymene, carvacrol (in *Origanum vulgare* L.). Data from this study suggest that selected EOs have significant effects on enzymatic antioxidants that may affect the first-line cell defense system against oxidative stress. In our study, we shed a little more understanding the physiological processes taking place in *S. cerevisiae* cells based on the observed RedOX parameters. However, due to a lack of detailed examination of redox markers in yeast as a cell model, further experiments are needed.

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