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Survival of some food-borne fungi in the presence of oregano essential oil

KEYWORDS:

- *Oregano essential oil*
- *Antifungal activity*
- *Surviving curves*

ABSTRACT: *Alimentary diseases, usually caused by microorganisms, are growing public health problem worldwide. Successful control of food-borne pathogens requires the use of multiple preservation techniques, including application of diverse additives. The modern trends in nutrition suggest substitution of synthetic food additives with natural ones. Plant essential oils are reported to be an interesting source of antimicrobial compounds. This study evaluates the antimicrobial potential of *Oreganum heracleoticum* L. essential oil against six commonly found food-borne fungi by screening their survival in the presence of this oil in growth media. Results showed that oregano essential oil possesses strong in vitro antifungal activity against tested fungi, so the concentration of 0.31 µL/mL was needed for total growth inhibition of yeast species, 0.62 µL/mL for *Aspergillus* species and 1.25 µL/mL for *Penicillium* species. When applied in adequate concentration, oregano essential oil has a great potential to be used as natural antifungal preservative in foods.*

INTRODUCTION

Food-borne diseases are growing public health problem worldwide. Successful control of food-borne pathogens requires the use of multiple preservation techniques in the manufacturing and storage of food products. Increasingly negative consumer perception of synthetic preservatives put the food industry under pressure to replace them with natural ones. Plant essential oils could be an interesting alternative for synthetic preservatives, with a few advantages: they are characterized by a broad-spectrum activity, including antifungal, antibacterial and antiviral activities; they are considered as safe; they are from natural resources which have been used as food for a long time; they have functional properties; they have acceptable sensory properties, etc. (1, 2, 3).

Essential oils (EOs) extracted from plant material have been used for centuries. Their initial application was in medicine, then in 19th century their use as aroma and flavour became their major employment, and nowadays they represent an interesting source of natural antimicrobials for food preservation (4). Potential application of EOs in food preservation requires detailed knowledge about their properties, such as the minimum inhibitory concentrations, mode of action, range of target organisms, etc. Numerous studies have been published on the antimicrobial activities of plant EOs against food-borne pathogens (5, 6, 7, 8, 9, 10, 11, etc).

Essential oil of *Oreganum heracleoticum* L. is widely used in the food industry, mainly to enhance the food aroma and taste, but oregano has been shown to exhibit antifungal activity as well (12, 13). They possess broad-spectrum activity against fungi (14, 15, 16, 17, 18, 19), do not exhibit adverse effects on human health, and are proven not to cause either significant or marginal toxic effects at cellular level. Also, the concentrations at which they exhibit

antimicrobial activity do not reach the possible genotoxic level (4, 20). One limitation on the use of oregano essential oil or its constituents in foods is its herbal aroma, because when using in higher concentrations it may cause an increase of flavour intensity in the food products, which sometimes negatively affects the consumer acceptability.

In the present study, antifungal potential of oregano essential oil was assessed against 6 fungal species that are reported to often cause food spoilage and poisoning. The aim was to determine the concentrations of the oil that may *in vitro* inhibit tested fungi and construct surviving curves for each species, in order to estimate the possible behavior of tested fungi in the presence of this antimicrobial agent. The obtained surviving curves would be needed for potential application of oregano EO as food preservative.

MATERIALS AND METHODS

Isolation and identification of fungal species

Fungal species used in this investigation were all isolated during the process of dry fermented sausage manufacturing, except *Saccharomyces cerevisiae*, which is ATTC strain (2601). *Aspergillus flavus*, *Aspergillus ochraceus* and *Rhodotorula glutinis* were isolated from raw material for sausage production, such as spices and meat butter, while *Penicillium aurantiogriseum* and *Penicillium brevicompactum* were isolated from air in producing facility. Isolation and identification of these species were described previously (21, 22).

Isolation of oregano essential oil

Oreganum heracleoticum L. essential oil (EO) was obtained from plant material collected and identified by the Institute of Medicinal Plant Research Dr. Josif Pančić (Belgrade, Serbia) by hydro-distillation process. Procedure of essential oil isolation and identification of oil components was described previously (12).

Screening

Broth microdilution method was used for screening of antifungal activity, in order to estimate the range of oil concentration active against specific fungal species. Concentrations of 7-day culture suspensions were in range 10^5 - 10^6 cfu/ml, and concentrations of oregano EO applied on fungal suspension were: 5 μ L/mL, 2.5 μ L/mL, 1.25 μ L/mL, 0.62 μ L/mL, 0.31 μ L/mL, 0.15 μ L/mL. Each well of the microtiter plate was poured with 160 μ L of Sabouraud maltose broth (SMB), 20 μ L of oregano oil dilution and 20 μ L of spore suspension. The well for growth control contained 180 μ L of SMB and 20 μ L of spore suspension and well for sterility control contained 180 μ L of SMB and 20 μ L of oil. Inoculums were grown for 48 h at 25 °C, and then they were subcultured into Petri dishes (10 μ L), poured with Sabouraud maltose agar (SMA) and incubated for 7 days at 25 °C. After the incubation period all Petri dishes were inspected on the presence of mould growth and the first EO concentration that totally inhibit the fungal growth was detected.

Broth macrodilution method

Seven-day mould cultures and 24-hours yeast cultures grown on SMA were used for preparation of the spore suspension. The spores were harvested with sterile loop in 50 mL of physiological saline solution containing 0.5mL/100mL Tween 80, and aseptically transferred into sterile Erlenmeyer flasks. The final concentrations of spore suspensions were in range 10^5 - 10^6 cfu/ml. Concentrations of *Origanum heracleoticum* L. EO used for each species were chosen based on the results of screening procedure (Table 2), so the last concentration that totally inhibited the growth and the first concentration that allowed some growth were used. Proper amount of pure oil was diluted in propylene-glycol, to obtain desired concentration in final inoculated mixture.

Total of three Erlenmeyer flasks for each species were poured with 100 mL of inoculum mixture. Content of all mixtures and final concentration of EO used for specific species are given in Table 1.

Table 1. Content of mixtures and final concentration of EO

Species	V [mL]			EO concentration [$\mu\text{L}/\text{mL}$]		
	SMB	SS [*]	EO	Control ^{**}	Test-1	Test-2
<i>Penicillium aurantiogriseum</i>	80	10	10	0	0,62	1,25
<i>Penicillium brevicompactum</i>	80	10	10	0	0,62	1,25
<i>Aspergillus flavus</i>	80	10	10	0	0,31	0,62
<i>Aspergillus ochraceus</i>	80	10	10	0	0,31	0,62
<i>Saccharomyces cerevisiae</i>	80	10	10	0	0,15	0,31
<i>Rhodotorula glutinis</i>	80	10	10	0	0,15	0,31

Table 1 (legend):

* - spore suspension;

** - in control flasks the pure solvent propylene-glycol was used, so the oil concentration is 0 $\mu\text{L}/\text{mL}$

Inoculated flasks were incubated at 25 °C. After 12, 24, 48, 72 and 96 hours the amount of 1 ml was taken from the each flask, series of ten-fold dilutions were prepared, and 100 μL from 10^{-2} and 10^{-4} dilutions were transferred into Petri plates and poured with 15 mL of SMA. Furthermore, pH of the each mixture was measured immediately after inoculation and after 96 h of incubation. Biomass of the control and test samples was measured after incubation period of 96 hours. The method uses a dried and measured filter paper to retain the biomass. After filtering the sample, the filter is dried to a constant weight at 103–105 °C. The percentage of biomass reduction was calculated following the equation:

$$\% \text{ reduction} = (A-B)/A \cdot 100,$$

Where A is the dry matter of control sample and B is the dry matter of test sample.

All experiments were conducted in triplicates.

RESULTS AND DISCUSSION

Results of the screening procedure are given in Table 2.

Table 2. Results of screening of the oregano EO antifungal activity

Species	EO concentration [$\mu\text{L}/\text{mL}$]							
	5	2.5	1.25	0.62	0.31	0.15	GC	SC
<i>Penicillium aurantiogriseum</i>	-	-	-	+	+	+	+	-
<i>Penicillium brevicompactum</i>	-	-	-	+	+	+	+	-
<i>Aspergillus flavus</i>	-	-	-	-	+	+	+	-
<i>Aspergillus ochraceus</i>	-	-	-	-	+	+	+	-
<i>Saccharomyces cerevisiae</i>	-	-	-	-	-	+	+	-
<i>Rhodotorula glutinis</i>	-	-	-	-	-	+	+	-

Table 2 (legend):

GC - Growth control

SC - Sterility control

'+' - Visible growth observed

'-' - No visible growth observed

Besides the information about proper oil concentration to be used in experiments, screening procedure gave us the valuable information about different genera susceptibility on oregano EO. As the Table 2 shows, oregano EO had the strongest effect on the yeast species, where the concentration of 0.31 $\mu\text{L}/\text{mL}$ was enough for total growth inhibition. *Aspergillus* species were a bit less susceptible, and the concentration of 0.62 $\mu\text{L}/\text{mL}$ was needed for total inhibition, while the *Penicillium* species showed the highest resistance of the tested species, but they were also totally inhibited with the 1.25 $\mu\text{L}/\text{mL}$ oregano EO solution.

Results for all species except the *Saccharomyces cerevisiae* are given on Figs 1-5. Logarithmic scale was used for surviving curve presentation, standard deviation for three measurements are presented as error bar.

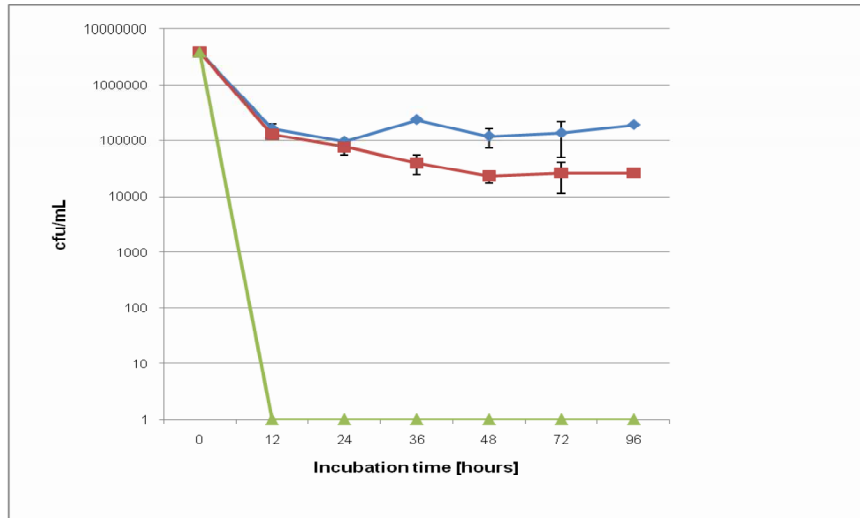


Figure 1 – Surviving of *Aspergillus flavus* in the presence of oregano EO (blue:CONTROL; red:TEST-1; green:TEST-2)

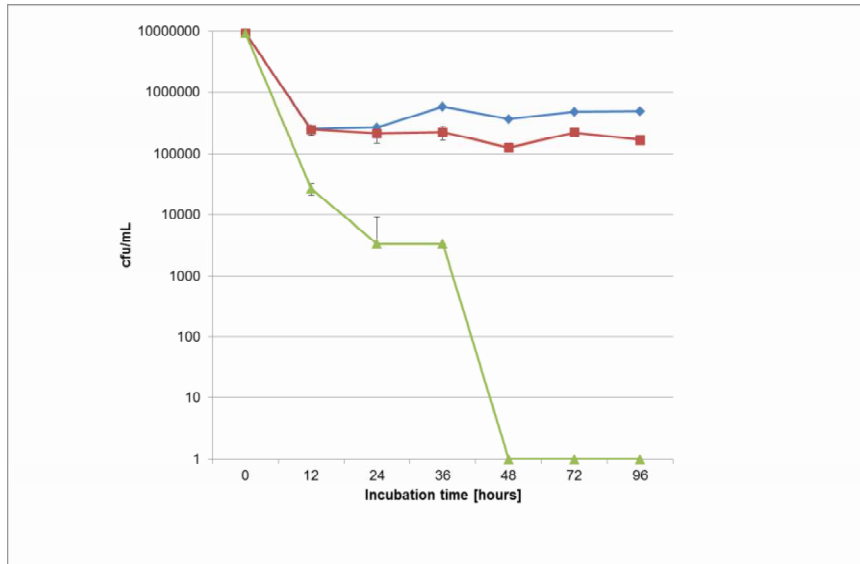


Figure 2 - Surviving of *Aspergillus ochraceus* in the presence of oregano EO (blue:CONTROL; red:TEST-1; green:TEST-2)

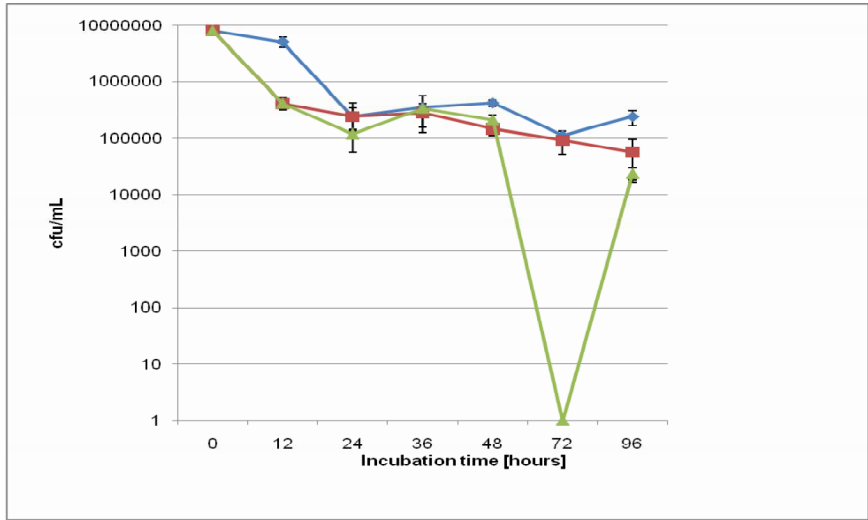


Figure 3 - Surviving of *Penicillium aurantiogriseum* in the presence of oregano EO (blue:CONTROL; red:TEST-1; green:TEST-2)

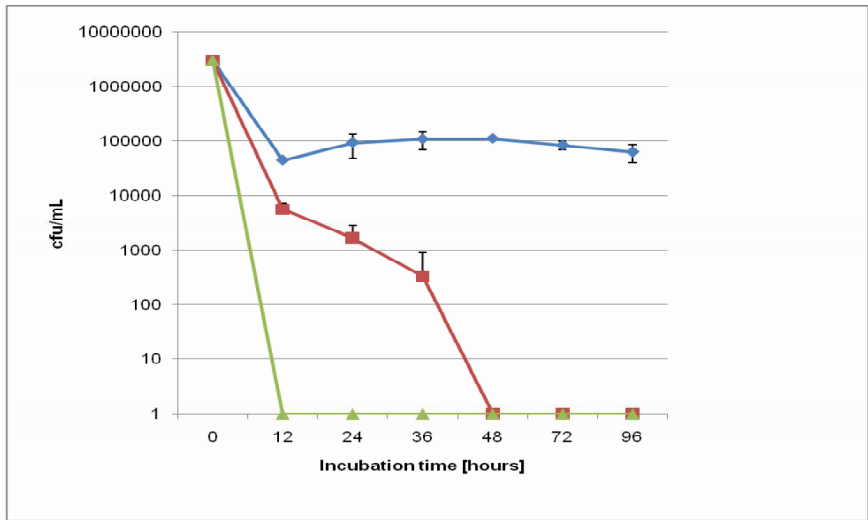


Figure 4 - Surviving of *Penicillium brevicompactum* in the presence of oregano EO (blue:CONTROL; red:TEST-1; green:TEST-2)

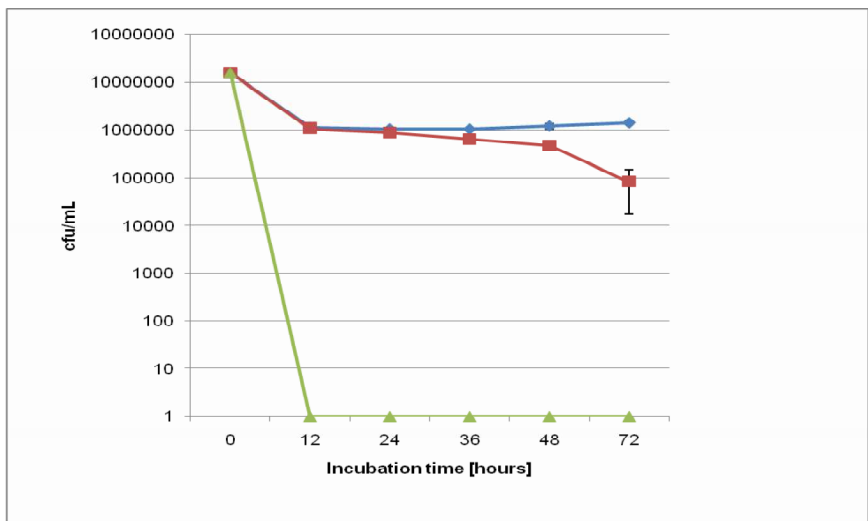


Figure 5 - Surviving of *Rhodotorula glutinis* in the presence of oregano EO (blue:CONTROL; red:TEST-1; green:TEST-2)

For *Saccharomyces cerevisiae* results are presented in table (Table 3).

Table 3. Surviving of *Saccharomyces cerevisiae*

Incubation time [h]	Control [cfu/mL]	Test-1 [cfu/mL]	Test-2 [cfu/mL]
0	1.4×10^7	1.4×10^7	2.5×10^4
12	$1.4 \cdot 10^6$	9.7×10^5	0
24	$\gg 3 \cdot 10^7$	1.2×10^6	0
36	$\gg 3 \cdot 10^7$	1.8×10^6	0
48	$\gg 3 \cdot 10^7$	$\gg 3 \cdot 10^7$	0
72	$\gg 3 \cdot 10^7$	$\gg 3 \cdot 10^7$	0

As it can be noticed from the Table 3, in *Saccharomyces cerevisiae* control sample (after 24 hours) and in Test-1 sample (after 48 hours), it was difficult even to estimate the exact number of colonies, because this number exceeded 300 cfu per Petri plate of the dilution used in the experiments. So, the table presentation of the results was much more convenient than graphic presentation. As results indicated, higher concentration used in the experiments (Test-2 concentration for each species) needed different time to kill all vital cells presented in the mixture. Cells of *A. flavus*, *P. brevicompactum*, *R. glutinis* and *S. cerevisiae* was totally inhibited within 12 hours, so sampling after 12 h of incubation gave no growth in Petri plates. *A. ochraceus* was totally inhibited within 48 hours, while *P. aurantiogriseum* was the most resistant species, and it was totally inhibited within 72 hours. Very important fact obtained in this investigation is that this concentration actually killed all vital cells, within some period of time. Taking into account that periods of time needed for total inhibition are applicable in food industry, that this concentrations are sensorial acceptable (7, 23, 24) and that the inhibition was total even the concentrations of fungal spores were high above concentrations that could be possibly found in food - the conclusion to be made is that oregano essential oil has a great potential as natural antifungal additive.

Test-1 concentration gave diverse results. The number of living cells in the mixtures decreased over time, but total inhibition was observed only for *P. brevicompactum*. For *A. flavus*, *A. ochraceus*, *P. aurantiogriseum* and *R. glutinis*, the number of living cells decreased to some level, and then it remained quite unchanged until the last sampling, after 96 hours. It could be presumed that after some time this number would start increasing, because the germination and sporulation of tested species may be just delayed buy antifungal agent, so the semi-damaged cells may recover their ability to reproduce (25, 26). This is exactly what happened with *S. cerevisiae*, where the number of vital cells decreased within the first 12 hours of incubation, after which it started to increase, slowly at the beginning, and then rapidly, so after 48 h this number highly exceeded initial concentration of spore suspension. So, the minimal fungicidal concentration of oregano essential oil for each of the tested species are definitively found between those two concentrations (Test-1 and Test-2), obtained from screening procedure. Further researches could be based on finding even lower concentration of Test-2, that shows the same efficacy as the concentration used in this research.

According to the GC analysis (12), the EO of oregano contained carvacrol as a principal component (69.00%), followed by p-cymene (10.50%) and thymol (7.94%). We suggest that carvacrol and thymol, as the most prominent volatile components of EO contribute most to its antifungal activity (27). Thymol and carvacrol are the registered flavourings and are considered to present no risk to the health of consumer. Besides, The United States Food and Drug Administration (FDA) also classifies these substances as Generally Recognized As Safe (GRAS). The crude essential oil of oregano has been also classified as GRAS by FDA (28).

Results of biomass investigations are presented on Figs 6 and 7. Fig. 6 shows absolute values of filtrated biomass dry matter, expressed in grams. These results show decreasing trend of biomass between control, test-1 and test-2 samples. Error bars are given for standard deviation of 3 measurements.

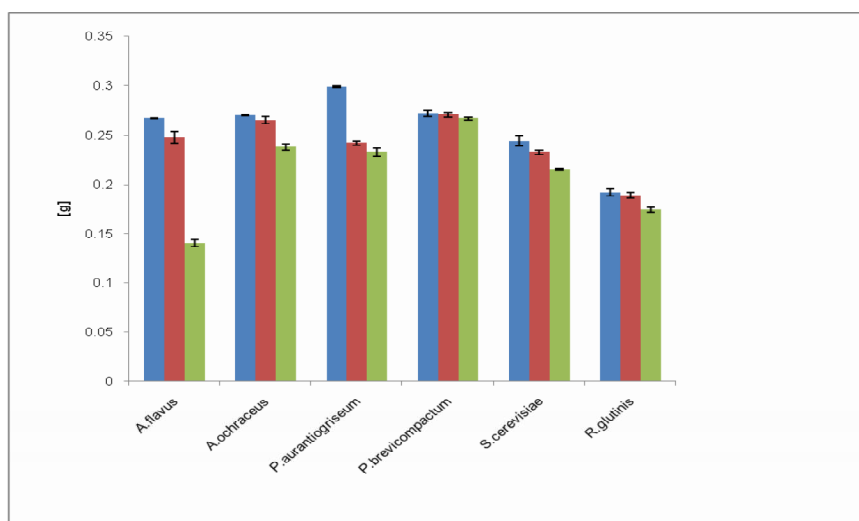


Figure 6 – Absolute biomass reduction [g] (blue: CONTROL; red: TEST-1; green: TEST-2)

Relative biomass reduction was used to emphasize the antifungal activity of oregano essential oil. Results express the relative reduction in biomass of Test-1 and Test-2 sample comparing to the control sample, and are presented in Fig. 7.

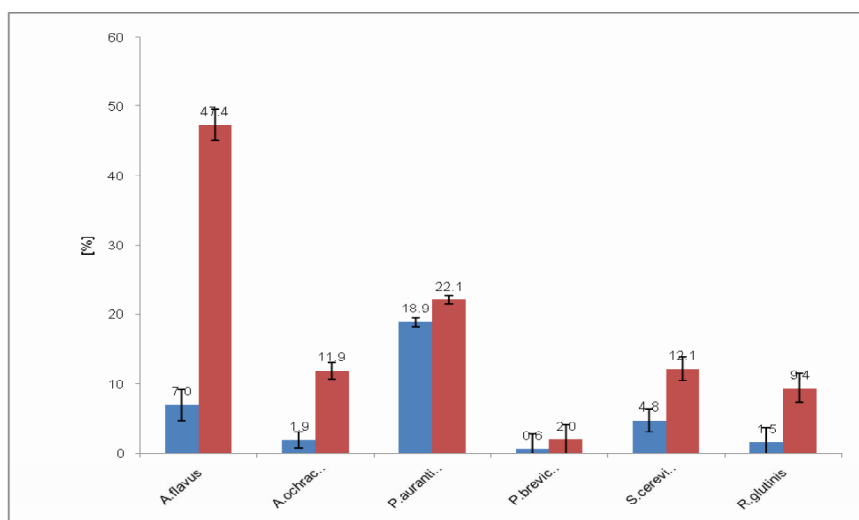


Figure 7 - Relative biomass reduction [%] (blue: TEST-1/CONTROL; red: TEST-2/CONTROL)

For all species, Test-2 concentration gave higher percentage of biomass reduction, but relative biomass reduction values vary between species a lot. The highest reduction was obtained for *A.flavus* (Test-2 sample – 47%). For *P.brevicompatum*, the results of the antifungal activity of the inhibition of biomass gave no results (relative reduction was below 1%), but this not exclude the antifungal activity. From our macrodilution method results it can be observed that EO have antifungal activity against this mould species, and some literature data also confirm that antifungal activity and biomass reduction are not necessary in correlation (29). There is not yet a clear explanation for the perceived difference in the two ways of evaluation of the antifungal activity, but it is sure that this difference does not exclude the antifungal activity.

All samples were also submitted to the pH measuring immediately after inoculation and after incubation. Results are presented in Table 4.

Table 4. pH measuring results

Species	Control		Test-1		Test-2	
	pH ₁	pH ₂	pH ₁	pH ₂	pH ₁	pH ₂
<i>Aspergillus flavus</i>	5.92±0.01	5.54±0.19	5.95±0.03	5.54±0.01	5.93±0.02	5.86±0.11
<i>Aspergillus ochraceus</i>	5.74±0.03	5.62±0.02	5.73±0.01	5.68±0.05	5.71±0.03	5.65±0.01
<i>Penicillium aurantiogriseum</i>	5.53±0.02	5.45±0.05	5.65±0.02	5.52±0.01	5.7±0.01	5.84±0.02
<i>Penicillium brevicompactum</i>	5.56±0.03	5.53±0.01	5.53±0.02	5.48±0.02	5.43±0.01	5.45±0.03
<i>Saccharomyces cerevisiae</i>	5.6±0.09	4.9±0.04	5.62±0.06	5.56±0.15	5.64±0.03	4.78±0.04
<i>Rhodotorula glutinis</i>	5.68±0.1	5.49±0.04	5.7±0.13	5.65±0.14	5.71±0.02	5.61±0.02

Table 4 (legend):

- Standard deviation for 3 measurements

Some literature data suggest that medium composition, especially pH value, may significantly affects *in vitro* susceptibility of microorganism to some antimicrobial agent (30, 31). In order to evaluate possible effect of pH on rate of some antimicrobial agent efficacy, growth curves for each species at various pHs should be constructed. Results in literature are very diverse, depending on microorganism, antimicrobial agent, pH range, other conditions in medium, etc. Interesting to be noticed, some significant differences in susceptibility or growth pattern were recorded usually when pH varied for at least couple of units. In this investigation, pH slightly varied (0.02-0.86) as a result of fungal cells metabolism, so it is unlikely that this change of pH had some significant effect on fungal susceptibility to oregano EO.

CONCLUSION

The intensive use of antibiotics has often resulted in the development of resistant strains. Because of this drug resistance, the search for new antibiotics continues unabated. In this context plants continue to be a rich source of therapeutic drugs as the active principles of many drugs are found in plants or a produced as secondary metabolites. Results obtained in this research suggest that oregano essential oil, although originally added to change or improve taste, has been attractive choice for substituting synthetic preservatives in food, because of its potential antimicrobial activity. However, there are several limitations for application of oregano essential oil as food preservative. Firstly, there are regulatory limitations on the accepted daily intake of essential oils in general, and sometimes high concentrations are needed to achieve sufficient antimicrobial activity. Secondly, in many food products some essential oil components are impaired by interactions with food matrix components, such as fat, starch and proteins. Furthermore, the potential antimicrobial activity depends on pH, temperature and the level of antimicrobial contamination. And finally, the intense aroma of EOs limits their application to spicy foods where the acceptable sensory threshold is relatively high. For the potential application of EO as food preservative, all of the named limitations have to be considered and evaluated, using food model media and some *in vivo* methods. Therefore, further investigations are needed to more fully evaluate the possible application of oregano EO as food preservative.

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