Antifungal activity of oleoresins used in meat industry on some toxigenic *Aspergillus* spp.

**Abstract:** Different spice oleoresins are widely used in meat industry. They contribute to the specific aroma and flavor of the end products, but they have also been reported to have strong antimicrobial activity. These properties open a plenty of possibilities to be used for defining the specific sensory profile of the product but also as natural food preservatives. This paper focuses on the antifungal activity of four oleoresins against different foodborne toxigenic *Aspergillus* species. Oleoresins used in the experiments were cayenne pepper, black pepper, garlic and rosemary oleoresins, and they were tested against the following *Aspergillus* species: *A. clavatus, A. flavus, A. fumigatus, A. niger, A. ochraceus* and *A. versicolor*. Antifungal activity was tested using microtitre-plate-based assay incorporating resazurin as an indicator of cell growth and broth microdilution-method.

**Key words:** Oleoresins, antifungal activity, *Aspergillus* spp.

**Introduction**

Nowadays, different meat products are commonly produced and consumed worldwide. Small-scale production of traditional meat products, which have been well recognized by consumers, still occurs, but large quantities are also produced at butchers’ shops and in meat processing companies (Papaggian et al., 2007). Meat products are protected against microbial spoilage by different preservation methods in combination with controlled atmosphere or vacuum packaging or by cold storage throughout distribution chain (Sørensen et al., 2008). However, molds periodically cause problems in this kind of food products, especially in the traditional ones (Škrinjar et al., 2012).

In recent decades, the question of mold occurrence and toxicity has attracted attention, especially in the fields of agriculture and food industry (Mížaková et al., 2002, Škrinjar et al., 2012). Microscopic filamentous fungi often contaminate vegetable and animal products, which become the source of diseases in man and slaughter animals. The reason for an increasing interest is the ability of molds to produce secondary metabolites – mycotoxins,
that have unfavorable effects, such as carcinogenesis, mutagenicity, teratogenicity, etc. (Frisvad and Thrane, 2002).

The difficulties of controlling these undesirable fungi as well as the growing interest of the consumers in natural products have been forcing the industry to find new alternatives for food preservation. One such possibility is the use of biopreservatives as antifungal additives (Čabarčapetaal., 2011). Numerous naturally occurring compounds found in plants, herbs, and spices have been shown to possess antimicrobial functions and serve as a source of antimicrobial agents against foodborne pathogens (Škrinjar and Nem et, 2009). Some biopreservatives that can be used in food processing are plant extracts, such as oleoresins. Spice oleoresins constitute the true essence of spices in their most concentrated form, containing both volatile essential oil and non-volatile resinous fraction (Ponceet al., 2010). Oleoresins are applied in food, cosmetics and pharmaceutical industry as flavoring agents and antimicrobials (Rajama et al., 2012). They are the most convenient substitutes for raw spices in foods since they are free of microorganisms and may be standardized to desired flavor (Ponce et al., 2008). Antimicrobial potency of oleoresins is generally lower in food systems than in vitro, depending on the food composition, processing steps and storage temperature which could strongly influence the effectiveness of these antimicrobial agents (Burt, 2004). Accordingly, larger amounts of oleoresins are required in food systems, which could seriously interfere with the food’s finally sensory profile. Therefore, the applied concentrations have to be well tested and optimized, considering their impact on sensory properties in final product and antimicrobial effectiveness.

Among a number of oleoresins, few of them are of special interest for meat industry due to their flavor and aroma and the greatest potential for use in industrial applications. Those are oleoresins of garlic, rosemary, cayenne pepper, sweet pepper, black pepper, oregano, etc. (Busatta et al., 2008).

The aim of the presented study was to evaluate the antifungal properties of some oleoresins commonly used in meat industry against foodborne *Aspergillus* species.

**MATERIALS AND METHODS**

**Oleoresins.** Four different oleoresins were tested: garlic, rosemary, black pepper and cayenne pepper. They were obtained from Milex Ltd., Rumenka, Serbia. They were used in meat industry to enhance flavor, aroma and to substitute some conventional spices. They were added in small amounts to the additives that are normally used in meat industry, so their final concentration in the final product was relatively small.

Rosemary oleoresin was extracted from dried leaf of *Rosmarinus officinalis* L. It is viscous homogenic greenish liquid that contains approximately 11% of carnosic acid which was thought to have antimicrobial activity (Wecskesser et al., 2007). Black pepper oleoresin was extracted from dried fruit *Piper nigrum* L. It is dark green viscous liquid with approximately 40% of piperine. Also this
oleoresin has a share of ethereal oil in amount of 20%. Garlic oleoresin contains around 10% of ethereal oil. It was extracted from cloves of *Allium sativum* L. Cayenne pepper oleoresin is also used as colorant in food industry. It is red-brownish liquid that was extracted from dried fruit from *Capsicum annum* L. or *Cappsisicum frutescense* L. It is also a potential antimicrobial agent since it contains capsaicin (cca 6%) (Singh and Chittenden, 2008).

**Microorganisms.** The antifungal activity of oleoresins was evaluated on six different species from genus *Aspergillus*: *A. clavatus*, *A. flavus*, *A. fumigatus*, *A. niger*, *A. ochraceus* and *A. versicolor*. Test cultures belonged to the culture collection from the Laboratory for Food Microbiology, Faculty of Technology in Novi Sad. The cultures were maintained on Sabouraud maltose agar (SMA, Himedia) slants and were stored at temperature of +4 °C.

**Antifungal assay.** Evaluation of the antifungal activity of the tested oleoresins against selected mold isolates was done by determining the minimal inhibitory concentrations (MIC). The MIC is defined as the lowest concentration, recorded in mg/l, of an antifungal agent that inhibits the growth of a fungus (Rodriguez-Tudela et al., 2008). For MIC determination in vitro, two different methods were used: a) microtitre plate-based antifungal assay incorporating resazurin as an indicator of cell growth and b) broth microdilution method.

a) Microtitre plate-based antifungal assay incorporating resazurin as an indicator of cell growth; Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide, Himedia) allows the detection of microbial growth in extremely small volumes of solution in microtitre plates without the use of a spectrophotometer (Sarker et al., 2007). A stock solution was prepared by adding 0.01% of the resazurin sodium salt powder in sterile distilled water. It was filter-sterilized and kept at 4°C (Hussain et al., 2011).

The suspension of the fungal isolates was prepared from the 7-day-old cultures. Spores were taken by adding 10 ml of sodium chloride solution containing 0.5% Tween 80 onto slant, scraped with sterile loop and aseptically transferred into sterile test tubes. Determination of total mold count per 1ml of suspension was performed using the standard Koch’s method. Final concentration of spore suspension was approximately 1×10⁸ cells/ml. One ml of suspension was inoculated in each sterile Petri dish, poured with about 15 mL of SMA in triplicate. Petri dishes were left to incubate for 7 days at 25°C.

Oleoresins were diluted in propylene (PG) to the test concentrations of 250, 125, 62.5, 31.25, 15.62 and 7.8 µl/ml.

Twenty microliters aliquots of all tested oleoresin solutions were added to 96-well microtitre plates in the abovementioned concentrations. After that, aliquots of 160 µl of Sabouraud maltose broth (SMB, Himedia) were added into each microplate. At the end, 20 µl of standardized fungal spore suspensions (approximately 10⁵ cfu/ml) were inoculated into each microplate. The test was performed in a total volume of 200 µl with final oleoresin concentrations of 25-0.78 µl/ml. Growth control contained 180 µl of SMB and 20 µl of standard spore suspension, while negative control had 180 µl of SMB with 20 µl of diluted oleoresin sample. The inoculated microtitre plates were incu-
bated at 25°C for two days. After the end of the incubation period, 10 µl of resazurin solution was added into each well and left to re-incubate overnight. A change of color from blue (oxidized) to pink (reduced) indicated the growth of molds. The MIC was defined as the lowest concentration of each substance that prevented this change in color.

b) Broth microdilution assay. The preparation of microtitre plates for broth microdilution method was the same as method under a). The difference was in the last step, where instead of adding resazurin solution in each well, test samples were sub-plated on SMA, taking 10 µl from each well into Petri dishes and then pouring SMA. Petri dishes were left to incubate for 7 days at the temperature of 25°C. The minimal fungicidal concentration (MFC) is defined as the lowest concentration of the oleoresins, at which 99.9% of the inoculated microorganisms were killed.

Reduction degree (RD [%]) is calculated in order to express antifungal efficiency of all oleoresins on selected fungal strains.

$$\text{RD} [\%] = \left(1 - \frac{S}{GC}\right) \cdot 100\%$$

where S is the number of colony forming units per Petri dish (CFU/P.d.) of every probe and GC is CFU/P.d. of growth control.

RESULTS AND DISCUSSION

a) Microtitre plate-based antifungal assay incorporating resazurin as an indicator of cell growth

Microtitre plate-based antifungal assay incorporating resazurin as an indicator of cell growth was applied to all oleoresins in order to investigate their potential antifungal activity. Due to the intensive color of cayenne paprika, black pepper and rosemary oleoresin, this method was not reliable. Therefore, it can be concluded that only antifungal activity of garlic oleoresin was evaluated according to this method.

Table 1 shows the results of antifungal activity of garlic oleoresin on all tested Aspergillus species. The first six test concentrations of garlic oleoresins showed very strong antifungal effect on all fungal species used in this experiment. The absence of color change was observed in all wells, except in those that presenting the growth control. Considering these facts, it was concluded that garlic oleoresin was diluted to the final concentration of 0.015 µl/ml in order to find MIC. For A. flavus and A. ochraceus, MIC was between 0.78 and 0.50 µl/ml. Minimal inhibitory concentration of garlic oleoresin for A. niger was 0.062 µl/ml, while for A. versicolor, A. clavatus and A. fumigates, new series of dilution did not determine MIC, so new series of dilution were required and prepared to the final concentration of 0.0002 µl/ml. New series of dilutions gave the following results: A. clavatus had MIC between 0.015 and 7·10⁻³ µl/ml. Garlic oleoresin had the strongest antifungal effect on A. versicolor and A. fumigatus, 7·10⁻³ µl/ml. These concentrations are significantly lower than those found in literature (B n k e b l l i a, 2004).
Tab. 1. – Inhibitory effect of garlic oleoresin on some fungal species from genus *Aspergillus*

<table>
<thead>
<tr>
<th>Fungal-sppces</th>
<th>Final concentration of garlic oleoresin in microtiter plate wells [10⁻¹ µl/ml]</th>
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<td></td>
<td>NC</td>
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<tr>
<td>A.niger</td>
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<td>A.versicolor</td>
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<td>A.clavatus</td>
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<td>A.flavus</td>
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<td>A.fumigatus</td>
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<td>A.ochraceus</td>
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GC – Growth control, NC- negative control

**b) Broth microdilution assay**

Figure 1 shows the results of activities of black pepper oleoresin against some food-borne fungi from genus *Aspergillus*. Results represent mean value of viable count of molds per Petri dish with standard deviation.
Apart from black pepper oleoresin antibacterial activity (Karshe and Lakshmi, 2010), it has also expressed antifungal activity by inhibiting the growth of *A. fumigatus*, *A. niger* and *A. versicolor* at concentrations of 25 µl/ml. In the case of *A. versicolor*, lower concentrations (12.5 and 6.25 µl/ml) were also efficient (Figure 2). *A. ochraceus* showed the highest resistance to black pepper oleoresin (Figure 1).

![Graphs showing reduction degree (%) of tested oleoresins in some species from genus Aspergillus](image)

Fig. 2. – Reduction degree [%] of tested oleoresins in some species from genus *Aspergillus*

Figure 3 shows the results of rosemary oleoresin activities against the tested species from genus *Aspergillus*. Rosemary oleoresin, at concentration of 25 µl/ml, inhibited growth (MFC) of *A. flavus*, *A. fumigatus*, *A. ochraceus* and *A. versicolor*, and it had strong antifungal effect on *A. clavatus* (RD=90.91%) (Figure 2). In the case of *A. niger*, rosemary oleoresin did not show MFC, only the inhibition in the range of 62.5-91.2%. Figure 2 shows other values of reduction degree.
In comparison to the other two oleoresins, cayenne pepper oleoresin demonstrated low inhibitory effect on molds growth (Figure 4). It had a significant value of reduction degree for *A. clavatus* and *A. versicolor* species only at concentration of 25 µl/ml (Figure 2).
CONCLUSION

Garlic oleoresin was reported to have very high antifungal activity using resazurin as a growth indicator, hence the following concentrations of 0.78 µl/ml (*A. ochraceus* and *A. flavus*), 0.062 µl/ml (*A. niger*), 0.015 µl/ml (*A. clavatus*) and 7×10⁻³µl/ml (*A. fumigatus* and *A. versicolor*). Black pepper oleoresin had significant antifungal activity at concentrations of 25-12.5 µl/ml, while cayenne pepper oleoresin showed low antifungal activity, demonstrating growth inhibition at concentration of 25 µl/ml only in the cases of *A. clavatus* and *A. versicolor* species.
REFERENCES


АНТИФУНГАЛНА АКТИВНОСТ ОЛЕОРЕЗИНА КОЈИ СЕ КОРИСТЕ У МЕСНОЈ ИНДУСТРИЈИ НА НЕКЕ ТОКСИГЕНЕ ВРСТЕ РОДА ASPERGILLUS SPP.

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Резиме

Различите врсте олеорезина имају широку примену у месној индустрији. Они доприносе специфичној ароми и укусу крајњих производа, али такође је показано да поседују и изузетну антимикробну активност. Ове особине омогућавају широк спектар разноврсних комбинација у циљу формирања новог сензорног профила производа, али такође могу да послуже као природни прехрамбени конзерванси. У овом научном раду је испитивана антифунгална активност 4 различита олеорезина на неколико токсигених врста из рода Aspergillus. Тестирани су олеорезини љуте паприке, црног бибера, белог лука и рузмарина. Као тест микороргананизми коришћене су следеће врсте рода Aspergillus: A. clavatus, A. flavus, A. fumigatus, A. niger, A. ochraceus и A. versicolor. Антифунгална активност је испитивана бујон-микродилуционом методом са додатком ресазурина као индикатора ћелијског раста, као и класичном бујон-микродилуционом методом.

КЉУЧНЕ РЕЧИ: Олеорезини, антифунгална активност, Aspergillus spp.

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