Antifungal activity of essential oils in the control of food-borne fungi growth and mycotoxin biosynthesis in food

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Debates about the possible negative effects of synthetic preservatives have renewed consumer interest towards natural agents for extension of the product viability and the protection from microbial spoilage. For these reasons, there are current studies on the application of essential oils, extracts, and their components extracted from spices and other aromatic plants, as alternative biopreservatives. These compounds may be useful in limiting or preventing the development of harmful fungi and mycotoxins in food, as additives, as surface protection, or in application for products with modified atmosphere packaging. The subject of this study is the use of essential oils of spices and other aromatic plants in controlling the growth of food-borne fungi and mycotoxin biosynthesis as well as the significance and role of the most frequent food-borne fungi and mycotoxins in food and their biological effects. A special emphasis is given to major antifungal components that constitute oils, antifungal components' mechanism of action at the cellular level, methods for testing antifungal activity, as well as antifungal and antimycotoxigenic effects of essential oil in *in vitro* and *in vivo* condition.

Keywords antifungal and antimycotoxigenic activity; essential oil; food

1. Fungi and mycotoxins in food

Filamentous fungi are widely distributed in environment and are frequent contaminants of food and animal feed. More commonly then other microorganisms, they appear as causers of spoilage under reduced values of water activity (a_w), as are middle (0.75 – 0.90 a_w) and low moist (< 0.75 a_w) food and food with low pH values <4.0 (acidic food). The most common species of fungi isolated from food belong to genera *Aspergillus, Penicillium, Fusarium, Alternaria, Cladosporium, Mucor, Rhizopus, Eurotium* and *Emericella* [1, 2]. Species of genera *Aspergillus, Penicillium* and *Eurotium* are "storage" fungi that can develop at ≤ 0.85 a_w, so they can be isolated from spices, dried fruits and vegetables, pumpkin seeds, sunflower seeds, stored cereals, and similar products. Species of genera *Fusarium* and *Alternaria* are "field" fungi and their development requires higher moisture content in the substrate and lower temperatures. These species are usually found in/on cereal grains and cereal products. Also they are cited as common causers of illness of fruits and vegetables in the field, in addition to species of the genera *Sclerotina, Bortrytis, Monilia, Rhizopus, Mucor,* and *Penicillium.* Fungi are common contaminants of meat and milk products during storage. Species of genera *Penicillium, Aspergillus, Cladosporium, Geotrichum, Mucor, Sporotrichum, Trichoderma* are commonly isolated from these food groups [3].

Growth of fungi in food leads to food spoilage, causing great economic damages. On the other hand, toxin-producing species of genera *Aspergillus* (*A. carbonarius, A. flavus, A. ochraceus, A. oryzae, A. parasiticus, A. versicolor*), *Penicillium* (*P. nordicum*, *P. expansum, P. viridicatum, P. verrucosum*), *Fusarium* (*F. culmorum, F. graminearum, F. oxysporum, F. verticillioides, F. proliferatum*), *Alternaria* (*A. alternata, A. solani, A. brassicae, A. tenuissima, A. tomato*) as well as teleomorphs of the class *Ascomycetes* (*Petromyces alliaceus, Emericella nidulans*, etc.) can biosynthesize toxic secondary metabolites - mycotoxins (aflatoxins, ochratoxin A, sterigmatocystin, patulin, fumonisins, zearalenone, deoxynivalenol, alternariol, alternariol monomethyl ether, tenuazonic acid) [1, 2]. Alimentary intake of mycotoxins in animal and human organisms causes intoxication - mycotoxicosis. Mycotoxicosis are manifested in the form of acute and chronic toxicity (cytotoxicity, hepatotoxicity, neurotoxicity, teratogenicity, mutagenicity, and cancerogenity). At a cellular level, mycotoxins react with nucleic acids and inhibit the biosynthesis of macromolecules DNA and RNA, or act on structures and functions of biological membranes or impair the energy metabolism [4, 5].

Throughout history, there are often encountered data about the mass poisoning of humans and animals that are associated with the consumption of food contaminated with fungi and mycotoxins. One of the first known mycotoxicosis is ergotism caused by ergot alkaloids. Ergotism was responsible for the deaths of thousands of people in medieval Europe. In the 20th and 21st century, the appearance of some mycotoxicosis was described: a disease of horses and pigs in the USA (associated with the intake of rye which was contaminated with *Fusarium graminearum*); stachybotryotoxicosis of horses in USSR and sheep in Slovakia and Hungary; facial eczema of sheep in New Zealand; liver tumours induced by "yellow rice toxins" in Japan after the World War II; alimentary toxic aleukia (ATA) in Siberia in the 1913; Balkan endemic nephropathy in 1952; human aflatoxicosis in Kenya in the 2004, etc. However, mycotoxins and mycotoxicosis have not received much attention until 1960 when the "X" disease of turkeys, ducklings and pheasants caused a great economic loss in England and led to the discovery of the causer − aflatoxins (named after the species which synthesized it, *Aspergillus flavus,* isolated from peanut flour fed to poultry) [1, 4, 5]. Nowadays, more

than 400 types of mycotoxins are known, and with the development of analytic methods their number is steadily increasing. However, only few mycotoxins (aflatoxins, ochratoxin A, *Fusarium* toxins) are well described in toxicology, and aflatoxins are the most studied ones.

Even though rapid technological advances during the last few decades have caused the introduction of new technologies in food production in order to obtain healtly, nutritiously and technologically safe products, the occurrence of fungi and mycotoxins in food products is not negligible. Numerous studies confirmed their presence in almost every type of food product [6, 7] and in feed [8].

Knowing the species of fungi, their properties, and relations to factors that can stimulate or inhibit their growth, is essential for preventing the negative effects they may have as food contaminants.

In order to inhibition fungal growth, and therefore the production of mycotoxins, chemical compounds are usually applied, with or without combination with some of the physical methods. However, today's consumers have high demands for procurement of food that is minimally technologically processed and without synthetic preservatives and additives, because of the possible adverse health effects. Therefore, the food industry is now focused on finding solutions that fully satisfy the criteria of consumers while retaining food safety. For these reasons, there are current studies on the application of essential oils, extracts, oleoresins and their components extracted from spices and other aromatic plants, as alternative biopreservatives [9-18].

2. Essential oils in food

Essential oils (EOs) are liquid products of aromatic plants. Particularly rich in EOs are plants from families *Pinaceae, Zingiberaceae, Umbellifereae, Lamiaceae, Apiaceae, Myrtaceae, Asteraceae, Rutaceae,* and *Laureace*. They are formed in the protoplasm of secretory cells (exogenous or endogenous glands). In plants, they occur freely in the form of droplets in glandular hairs and tubules or secretory organs in underground and overground plant organs. EOs are isolated from the whole plant or some parts (flowers, buds, seeds, leaves, twigs, bark, fruits and roots) by steam distillation, supercritical extraction, fermentation, squeezing under pressure, extraction by easily volatile organic solvents or phytosolts.

Distillation as a method for the production of EOs was first applied in the East (Egypt, India and Persia) more than 2000 years ago, and the first authentic written documents about EOs distillation is attributed to Villanov (C.1235-1311), a Catalan doctor [20, 21].

The first scientific study on the potential application of EOs describes the antimicrobial activity of cinnamon oil against spores of anthrax bacilli, published in 1880. Clove has been used as a preservative against spoilage of meat, syrups, sauces, and sweets. Cinnamon and mustard, in the 1910s, have showed good effects in preserving apples. Since then, other spices and aromatic herbs such as allspice, bay leaves, caraway, coriander, cumin, oregano, rosemary, sage, thyme, basil, onion, and garlic, have been stated to possess significant antimicrobial activity [20-23].
Active components that constitute the EOs are responsible for their antibacterial and antifungal activity.

2.1. Antifungal components of essential oil

So far, over 3000 various components that constitute EOs have been described [24]. Based on the chemical structure, they are divided into five major groups:

- terpenoids (mono-C_{10} and sesquiterpenes-C_{15}),
- aliphatic compounds of lower molecular weight (saturated and unsaturated hydrocarbons, alcohols, aldehydes, esters and lactones),
- volatile aromatic components (benzoic acid derivatives, phenylpropanoids and coumarins),
- nitrogen compounds (indole derivatives and aliphatic amines),
- sulphur compounds (isothiocyanates and organic disulphides)

These compounds build glycosides, saponins, tannins, alkaloids, organic acids, and other compounds that constitute the plant's defence system against microbial infections [23].

The main components (Table 1) make up to 85% of the EOs, while other compounds are present in small amounts or in traces. There are indications that precisely these trace compounds have a key role in the antimicrobial properties, most likely due to the synergistic effects with other ingredients of the oil [20, 21]. However, it is difficult to investigate all synergistic effects and interdependence of all compounds, because of their high numerosity [20].

Table 1 Main antifungal components of EO (adapted from Burt [20], Tajkarimi et al. [21], Ceylan and Fung [23]).

EOs (plant part)	Botanical name	The main components of EO
Anise (seed)	*Pimpinella anisum*	Anethole
Basil (leaf)	*Ocimum basilicum*	D-linalool, methyl chavicol
Caraway (seed)	*Carum carvi*	α-carvone
Cardamom (seed)	*Elettaria cardamomum*	Cineole, α-terpinyl acetate
Celery (seed)	*Apium graveolens*	D-limonene, sedanolide
Cinnamon (bark)	*Cinnamomum zeylanicum*	Cinnamaldehyde
Cassia - Chinese cinnamon (leaves, bark, twigs, stalks)	*Cinnamomum cassia*	Cinnamaldehyde
Clove (flower bud)	*Syzygium aromaticum*	Eugenol
Coriander (leaf)	*Coriandrum sativum*	Linalool, trans-2-decanal
Coriander (seed)	*Coriandrum sativum*	D-linalool
Dill (seed)	*Anethum graveolens*, *A. sowa*	D-carvone
Fennel (seed)	*Foeniculum vulgare*	Anethole
Garlic (clove)	*Allium sativum*	Diallyl thiosulfinate
Ginger (rhizomes)	*Zingiber oficinale*	Zingiberene, cineole, borneol, geraniol, zingerone
Grapefruit (fruit peel)	*Citrus paradisi*	D-limonene, linalool, citral
Nutmeg (flower)	*Myristica fiagrans*	Myristicin, α pinene, sabinene
Mandarin (fruit peel)	*Citrus reticulata*	D-limonene, linalool, citral
Marjoram (leaves)	*Majorana hortensis*	Linalool, methyl chavicol, 4-terpineol
Mint (leaves)	*Mentha piperita, M. spicata*	α-pinene, β-pinene, limonene, cineole, 1-carvone
Mustard (seed)	*Brassica alba* (yellow), *B. juncea* (brown), *B. nigra* (black)	Allyl isothiocyanate
Laurel (leaves)	*Laurus nobilis*	Cineole
Lemon (fruit peel)	*Citrus lemon*	D-limonene, linalool, citral
Onion (bulb)	*Allium cepa*	Trans-S-1-propenyl cysteine sulfoxide
Orange (fruit peel)	*Citrus sinensis*	D-limonene, linalool, citral
Oregano (leaves/flowers)	*Origanum vulgare*	Thymol, carvacrol, γ-terpinene, p-cymene
Pimento (berries/leaves)	*Pimenta dioica*	Eugenol, β-caryophyllene
Paprika (fruit)	*Capsicum annuum*	Carotenoids
Pepper -black (fruit)	*Piper nigrum*	Monoterpene hydrocarbons, piperine
Rosemary (leaves)	*Rosmarinus officinalis*	α- pinene, bornyl acetate, camphor, 1,8-cineol, rosmarinic acid
Sage (leaves)	*Salvia officinalis*	α - and β- thujones, borneol, 1,8-cineole, camphor, α - and β- pinene
Savoury (leaves)	*Satureja hortensis*	Carvacrol, monoterpene hydrocarbon
Tarragon (leaves)	*Artemisia dracunculus*	Methyl chavicol, anethole, γ-terpinene
Thyme (leaves)	*Thymus vulgaris*	Thymol, carvacrol, γ-terpinene, p-cymene
Turmeric (root)	*Curcuma longa*	Turmerone

The chemical structure of the main EO components and their antifungal properties are related. The presence and position of the hydroxyl group in the molecule, the presence of the aromatic nucleus, solubility in fats and spatial orientation affect the antifungal activity [25, 26]. Compounds containing aromatic nucleus and a phenolic OH group are characterized by high antimicrobial activity [27, 28]. Also, presence of the alkyl group in the benzene ring of phenols or guaiacol [29], acetate groups (for example, geranyl-acetate has a stronger antimicrobial activity than geraniol, and bornyl-acetate than borneol) [30], oxygen in monoterpenes and their carbonylated compounds [31] enhance the antifungal activity of the components. Dorman and Deans [30] and Kurita et al. [29] indicate that the type and size of alkyl substituent in the non-phenol ring structure effects the antimicrobial activity. Thus, alkenyl substituent (-CH=CH-) in limonene enhances the antimicrobial activity, in comparison with the alkyl substituent (-CΞC-), as in *p*-cymene, while the compounds with a higher alkyl group have shown stronger antifungal activity. These authors also indicate that β- and *trans*-isomers are much more active in comparison with α- and cis-cis isomers. Phenol components (carvacrol, thymol, eugenol etc.) exhibit the strongest antifungal and antimycotoxigenic activity, followed by alcohols, aldehydes, ketones, ethers and hydrocarbons [32]. Benjilali et al. [33] have examined the antifungal effects of EOs obtained from three chemotypes of wild wormwood, thyme, rosemary and eucalyptus, against 13 *Penicillium* spp., 9 *Aspergillus* spp. and 17 species of other fungi. Wholesomely observed, thyme was the most effective one, followed by wild wormwood,

rosemary, and eucalyptus, which indicates that EOs that have contained mostly phenolic compounds were more effective than oils that have mainly contained ketones. Kurita et al. [29] and Kurita and Koike [32] have examined the antifungal effects of 40 types of aliphatic and aromatic aldehydes, alcohols, phenols, ethers, and carbohydrates, obtained from plant EOs against 7 species of fungi. Cinnamaldehyde showed the strongest antifungal activity out of the aliphatic aldehydes, followed by peril-aldehyde and citral. Aliphatic aldehydes with one or more double bonds conjugated with a carbonyl group had a much stronger activity than those that did not contain a double bond. Antifungal effect of *p*- methylbenzaldehyde was mediocre, while benzaldehyde showed very little effect. Antifungal effects of primary alcohols (such as citronellol, geraniol, perylalcohol and 1-decanol) were also very high. α, β -saturated aliphatic aldehydes (such as citronellal, decanal), secondary alcohols (such as L-menthol, borneol), and tertiary alcohols (such as linalool and terpinol) showed middle antifungal activity, while hydrocarbons showed weak effects.

2.2. Antifungal components' mechanism of action

The possible mechanism of action of EO components on the growth of fungi was reported in several studies. It is generally accepted that the EO components act on the functionality and the structure of the cell membrane [34]. Low concentrations result in changes of the cell structure, inhibiting respiration and changing the permeability of the cell membrane, whereas high concentrations lead to severe membrane damage, loss of homeostasis and cell death [35]. Conner and Beuchat [36] suggest that the antifungal activity is the product of EO components' interaction with enzymes responsible for energy production and the synthesis of structural compounds of the cell. On the other hand, Omidbeygi et al. [12] suggest that the EO components pass through the cell membrane, integrating with enzymes and proteins of membranes, causing loss of macromolecules from the interior of the cell, leading to changes in the cell and ultimately to its death. Cristani et al. [37] indicate out that the antifungal activity of terpene relates to their ability to act not only on the permeability, but also on other functions of cell membranes. These components can pass through the cell membrane and interact with intracellular structures. Daferera et al. [38] state that fungitoxic effect of EOs is a consequence of hydrogen bonds formation between hydroxyl groups of phenolic compounds and active sites of cellular enzymes. According to Sharma and Tripathi [39], the active components cause loss of integrity of the cell wall, and thus the loss of cytoplasmic constituents from the cells of hyphae. Lucini et al. [40] indicated that the inhibition of mycelial growth of fungi is caused by monoterpenes. These components can increase the concentration of lipid peroxides, such as hydroxyl, alkoxyl and alkoperoxy radicals, leading to the destruction of cells. According to Beuchat and Golden [41] and Shenn et al. [42] allicin, the antimicrobial component isolated from garlic, acts as an inhibitor of the enzymes with -SH groups, and affects the synthesis of fatty acids (inhibits acetyl-CoA), lipids, DNA or RNA.

Concerning the effects to mycotoxin biosynthesis, it is considered that active components of EO inhibit one or more steps in the mycotoxin biosynthesis pathway. So, Jayashree and Subranyam [43] indicate that the antiaflatoxigenic activity of eugenol could be linked with the inhibition of lipid peroxidation and oxygenation in the process of aflatoxins B1 biosynthesis. Hua et al [44] indicate out the inhibition of norsolinic acid production (which is one of the precursors in the biosynthesis of aflatoxin) by the acetocyringone, syringaldehyde and sinapinic acid.

2.3. Antifungal activity of essential oils *in vitro*

Antifungal *in vitro* tests are usually performed with the addition of EOs directly to the medium (Agar dilution method) [10, 45-47] or in a volatile atmosphere (Microatmosphere method) [48, 49]. Fewer antifungal tests were performed by disk diffusion (Agar diffusion method) [9, 50, 51], macro and microdilution methods (Liquid broth dilution methods) [52, 53].

Inhibitory parameters that get determined are minimum inhibitory concentration (MIC) or fungistatic concentration and minimum fungicidal concentration (MFC) [54]. MIC is the lowest concentration of EOs without visible fungi growth or turbidity of the broth (that concentration inhibits the growth). MFC is the lowest concentration that demonstrates no fungal growth from subculturing to the agar plate. MIC is not a constant value for an antifungal agent due to its susceptibility to different factors (EO properties, properties of test organisms, the size of the inoculum, content of the nutrient medium, a_w of medium, etc).

When testing the antifungal activity of EOs mixtures, the effect of mixtures is expressed over fractional inhibitory concentration index (FIC_{index}) defined by Davidson and Parish [55] and is calculated by the equation:

$$FIC_{index} = \frac{\text{MFC essential oil A in mixture}}{\text{MFC essential oil A}} + \frac{\text{MFC essential oil B in mixture}}{\text{MFC essential oil B}} \quad (1)$$

in which MFC – is minimal fungicidal concentration (individual concentration or concentration of mixtures of A and B EOs). If the obtained value of $FIC_{index} > 1$, the mixture points to synergic effects (obtained effect of the mixture is higher than the effects of individual EOs concentration). If $FIC_{index} = 1$, the mixture indicates the additive effect (the obtained effect of the mixtures equals the effect of individual EOs concentration). When $FIC_{index} > 2$, the mixture points to antagonistic effects (obtained effect of the mixture is lower than the effects of individual EOs concentration).

When the agar dilatation method is applied, the suspensions of conidial spores in a form of point or mycelial disks of the test fungi are inoculated into the centre of the agar plates that contain the medium with different concentrations of EOs. Growth inhibition was evaluated by a daily measurements of the radial growth of the fungi colonies (in mm). The inhibition percentage was calculated according to the formula:

$$I = \{(C - T)/C\} \times 100 \quad (2)$$

where I - % inhibition, C - fungi control plate colony diameter in mm, T - fungi test plate colony diameter in mm Microatmosphere method differs from the agar dilution method by the placing of a sterile paper disc with added EO, on the bottom of the cover of the inoculated agar plate. The plates are usually sealed with parafilm or a cover with a thread and incubated in an inverted position.

Disk diffusion method was applied for the screening of EOs antifungal activity. Prepared dilution of EO or raw EO in equal amounts are applied to sterile paper disks (usually 6 mm in diameter), which are placed in the centre of the agar medium previously surfacely inoculated (using etaler or a sterile cotton swab) with a conidial suspension of the test fungi in a Petri dish. Antifungal activity is estimated by measuring the diameter of the growth inhibition zone in mm after a certain period of incubation. Clear inhibition zones around the discs indicate the presence of antifungal activity.
In liquid broth dilution method, the effect of different concentrations of EO was determined visually by measuring the optical density (turbidimetrically) or with the count plate method (from no turbidity broths by placing on the agar medium).

Due to the hydrophobic nature of EOs, various organic solvents such as ethanol, methanol, dimethyl sulfoxide (DMSO) were used for dissolving [48, 49, 56, 57].

For the antifungal testing of EOs, the following nutrient substrates were used: Potato Dextrose Agar (PDA), Sabouraud Maltose Agar (SMA), Czapek Doks agar (CDA), Czapek agar (CYA), Yeast Glucose Chloramphenicol Agar (YGCA), Potato Dextrose Broth (PDB), Brain Heart Infusion Broth (BHIB), Malt Peptone Grain (MPG) and Sabouraud Dextrose broth (SDB). Research has shown that fungistatic effect of EOs is stronger on a solid agar substrate than in a liquid one. Moleyar and Narasimham [56] explain this occurrence by the evaporation of the EO components that have accumulated above the substrate. Also, it has been demonstrated that the oils of thyme, cinnamon and clove, containing as main components thymol and eugenol, had the best effect when applied directly into the medium, while the oils of mustard and lime, containing components of lower molecular mass, such as allyl isothiocyanate and citral, were more efficient in the pair method; in both applied methods, oils of orange, sage, and rosemary had a limited effect [49].

EOs of thyme, cinnamon, cloves, oregano, basil, and sage are the most frequent subjects of antifungal tests. EOs of cinnamon, clove, and mustard are considered as the strongest antimicrobial agents, followed by EOs of Jamaican pepper, caraway, coriander, cumin, bay leaves, oregano, rosemary, sage and wild thyme with medium effects and lastly EOs of black and red pepper and ginger with the weakest effect [22, 23].

Most commonly tested are toxigenic species of the genera *Aspergillus* (*A. flavus, A. fumigatus, A. niger, A. ochraceus, A. parasiticus*), *Alternaria* (*A. alternata*), *Penicillium* (*P. chrysogenum*, *P. verrucosum*, *P. expansum*), *Fusarium* (*F. verticillioides, F. proliferatum, F. oxysporum, F. gramenearum, F. culmorum*, etc.) as well as plant pathogens (*Botrytis cinerea, Cladosporium cladosporioides, Sclerotinia sclerotiorum*, etc.) [10, 46, 47, 53, 58].
EOs in *in vitro* studies were applied in concentrations ranging from 0.22 μg/mL to 10000 μg/mL. Concentration depended on the type of EOs, fungi species, test method, and the type of medium.

When applying the agar dilution method (the addition of EOs in the medium) basil EO was fungicidal at concentrations from 0.3 μg/mL to 8000 μg/mL [46, 53, 58]; cinnamon EO from 1000 μg/mL to 20000 μg/mL [58-60]; clove EO from 0.33 μg/mL to 10000 μg/mL [45, 59, 61]; thyme EO from 0.44 μg/mL to 10000 μg/mL [45, 58, 61-64]; mandarin EO from 2.0 μg/mL to 9400 μg/mL [34, 65]; dill EO from 1000 μg/mL to 10000 μg/mL [58, 61, 66]; oregano EO from 0.22 μg/mL to 4000 μg/mL [62, 67-72]; sage EO from 625 μg/mL to 20000 μg/mL [61, 62]; eugenol 0.2 μg/mL to 2000 μg/mL [47, 73, 74].

Research by Edris and Farrag [75] indicates that the main components linalool, eugenol (isolated from the basil EO), menthol and menthone (isolated from the mint EO), and their synergistic combination showed stronger antifungal activity than the basil EO and mint EO on *Penicillium expansum*, *Botrytis cinerea* and *Monilia fructigena.* Kumar et al. [47] suggest that eugenol at a concentration of 0.2 μL/mL acts fungicidally to *Alternaria alternata, Aspergillus candidus, A. fumigatus, A. niger, A. paradoxus, A. terreus, A. versicolor, Cladosporium cladosporioides*, *Culvularia lunata*, *Fusarium nivale, F. oxysporum* and *Penicillium* spp., while the basil EO achieves fungicidal effect on the tested fungi at a concentration of 0.3 μL/mL.

2.4. Antimycotoxigenic activity of essential oils *in vitro*

Antimycotoxigenic investigation of EOs are most commonly performed on the grains of wheat, corn and rice, and other applied media include: broth or agar with yeast extract and sucrose (YES or SMKY), broth of palm seeds (PKB), Maize Meal Extract Agar (MMEA), Peanut Meal Extract Agar (PMEA), and Potato Dextrose Broth (PDB). Aflatoxins B1 and G1 are the most common subjects of research. EOs for antimycotoxigenic tests were applied in concentrations of 0.2

μL/mL to 5000 μg/mL (Table 2). The EO components (geranial, eugenol, neral) were tested at concentrations of 0.1 to 0.8 μL/mL.

EOs of anise, cinnamon, basil, oregano, cassia, coriander, clove, bay leave, wild carrot, lupines, thyme, and spearmint, had effect on the reduction of aflatoxins B1 biosynthesis by the aflatoxigenic fungi (*A. flavus*, *A. parasiticus,* and *A. nomius*) cultured on wheat grains, or in PKB or SMKY broth [47, 58, 76-82]. EOs of onion and garlic have inhibited the biosynthesis of sterigmatocystin by *A. versicolor* in the YES broth [17]. Good results in inhibiting the synthesis of ochratoxin A were achieved by EOs of anise, cinnamon, wild thyme, spearmint, basil, coriander, peppermint, oregano, and sage [58, 83] on YES broth and wheat grains. EOs of anise, basil, cinnamon, clove, lemon, lemon-grass, wild thyme, oregano, and spearmint had an inhibitory effect on the production of *Fusarium* toxins (deoxynivalenol, fumonisin B1, and zearalenone) by *F. graminearum, F. proliferatum, F. verticillioides* on the grains of corn and wheat [46, 58, 59, 68, 84].

Table 2 Applied concentrations of EO in preventing the formation of mycotoxins.

Mycotoxins	EOs/*components	Applied concentrations	Nutrient medium	Literature
Aflatoxin G1	Anise, cinnamon, thyme, spearmint	2%	Wheat grains	[58]
	Basil, cassia, coriander, bay laurel	5%	PKB	[82]
Aflatoxin B1	Anise	2% 150 mg/kg 1000 μg/g	Wheat grains MMEA, corn grains	[58] [79-81]
	Basil	5%, 0.2 μL/mL	PKB, SMKY	[47, 82]
	Cinnamon	250 μg/mL, 2%	SMKY, wheat grains	[58, 85]
	*Geranial	0.6 μL/mL	SMKY	[86]
	Cassia, coriander, bay laurel	5%	PKB	[82]
	Clove	250 μg/mL 500 μg/g, 1000 μg/g, 5 g/kg	SMKY MMEA, corn grains, rice grains	[85] [79-81, 87]
	Cumin	1000 μg/g	PDB YES	[88-90]
	Turmeric	5 g/kg	Rice grains	[87]
	Lippia rugosa	1000 mg/L	SMKY	[91]
	Lippia alba	0.8 μL/mL	SMKY	[86]
	*Eugenol	0.1 μL/mL	SMKY	[47]
	Eucalyptus	50 μL/mL	YES	[92]
	Thyme	500 μg/g, 1000 μg/g, 250 mg/kg 2%	MMEA, corn grains, YES, wheat grains	[58, 76, 77, 79-81]
	Nana	700 mg/kg	MMEA	[81]
	*Neral	0.8 μL/mL	SMKY	[86]
	Oregano	7000 mg/kg 0.6 μL/mL	MMEA, SMKY	[79, 93, 94]
	Rosemary	450 mg/kg	YES	[78]
	Spearmint	2%	Wheat grains	[58]
Sterigmatocystin	Onion, garlic	5 μL/100 mL 10 μL/100 mL	YES	[17]
Ochratoxin A	Anise, cinnamon, thyme, spearmint	2%	Wheat grains	[58]
	Basil, coriander, mint, oregano, sage	1000 mg/kg	YES	[83]

Fumonisin B1	Anise, cinnamon, thyme, spearmint	2%	Wheat grains	[58]
	Basil	100 mg/kg	Corn grains	[46]
	Cinnamon, lemon- grass, clove, oregano, shallot	1000 µg/g	Corn grains	[68]
Zearalenone	Cinnamon, lemon- grass, clove, oregano, shallot	500 µg/g, 1000 µg/g	Corn grains	[59, 84]
Deoxynivalenol	Cinnamon, lemon- grass, clove, oregano, shallot	500 µg/g, 1000 µg/g	Corn grains	[59, 84]

3.2. Application of EOs in the preservation of food from mycological and mycotoxicological contamination *in vivo*

In vivo tests were carried out on cereals [58, 85], cherry tomato [13, 64, 95], oranges [96], apples [74], in the preservation of black rye bread, biscuits [49, 97] and ketchup [12].

For *in vivo* studies, higher concentrations were required compared to *in vitro* studies [12, 49, 58, 64, 74, 82, 96]. EOs of thyme, clove and savoury at concentrations of 350 and 500 ppm showed stronger inhibitory effects (87.5 to 100%) towards *A. flavus* in liquid medium (SDB) than ketchup (48-87% inhibition at 500 ppm) [12]. Cassia EO has completely inhibited the growth of *A. alternata* on PDA supplemented with 300 ppm, while the concentration of 500 ppm reduced the infection of cherry tomato by 34.2% (artificially contaminated fruits) and 19.1% (naturally contaminated fruits) [64]. The minimum inhibitory concentrations of cinnamon EO, which was added to PDA medium, was 0.64% (v/v) for *Rhizopus nigricans*, 0.16% (v/v) for *Aspergillus flavus* and *Penicillium expansum*, while antifungal protection of orange fruit needed 2% (v/v) of this oil [96] (Table 3). These differences in *in vivo* and *in vitro* studies arise from the complex composition of food, pH, a_w, storage temperature, the presence of O_2 [98]. The high content of fat and / or proteins protects microorganisms from the effects of EO. It is assumed that EO dissolves in the lipid phase of food, so that a relatively small portion remains free to act on the microorganisms in the aqueous phase. On the other hand, reduced a_w of food compared to laboratory broth hinders the progress of EO towards microorganisms. It was also found that the physical structure of food limits the antimicrobial effect of EO. So, lower MIC was achieved in broth than in the gel matrix, due to the difficulty of diffusion of EO in the gel matrix [99]. Some authors are of the opinion that larger and richer availability of nutrients in food compared to laboratory media allows a microorganism to rapidly repair damaged cells, and thus increase the resistance to EO [100].

Literature data suggest that EOs may find practical applications in post-harvest prevention of fungi contamination of stored grains [58, 101-103]. According to these data, basil EO significantly reduces the contamination of seeds with *F. verticillioides* [101]. EO of *Ocimum gratissimum* showed high efficacy against *F. verticillioides* at a concentration of 200 ppm, since it reduces infection of the seed with this fungi by 95-100% [102]. *In vivo* studies have shown that oil of *O. basilicum* at a concentration of 4.8 µL/g significantly reduces the incidence of *F. verticillioides* and fumonisin biosynthesis in artificially inoculated maize grain as compared to the control [103]. Mixture of eugenol oil (2 mg/mL) and soy lecithin (50 mg/ml) reduces the appearance of *P. expansum* (7%), *P. vagabonds* (6%), *B. cinerea* (4%) and *M. fructigena* (2%) in apple fruits during six months of storage at 2°C [74]. The cinnamon EO at a concentration of 2% and 3% completely suppresses the growth of *Rhizopus nigricans, A. flavus* and *P. expansum* on fruits of mandarin and jojoba during three days of storage at 25°C [96]. Caraway EO was applied in *in vivo* condition at a concentration of 500 ppb for tomato fruit protection from contamination by *A. alternata* and *P. digitatum* [104]. During the ten-day storage at +13°C, the percentage of infected fruits was reduced by 72% (*A. alternata*) and 47% (*P. digitatum*). Thyme EO at a concentration of 500 ppm had positive effects in protection of ketchup from *A. flavus* [12]. Nielsen and Rios [48] suggest the possible application of AITC (isolated from mustard EO) in the preservation of rye and "hot dogs" bread from fungi contamination using active atmosphere packaging. EOs of oregano, mustard, thyme, cinnamon, sage, rosemary and lemon-grass added to the atmosphere packaging of rye bread at a concentration of 135 and 270 µL/L have delayed or inhibited the growth of *P. roqueforti, P. corylophilum, A. flavus* and *E. repens* [49].

Table 3 Antifungal and antimycotoxigenic protection of food using EOs *in vivo.*

Fungi/mycotoxins	EOs/*components	Applied concentrations	Food	Literature
Alternaria alternata	Cassia	100 mg/kg	Cherry tomato	[64]
Aspergillus flavus	Anise, green mint	2%	Wheat grains	[58]
	*AITC (allyl isothiocyanate)	2 μL/gas phase	Rye bread, "hot dog" bread	[48]
	Cinnamon	2%	Wheat grains, orange fruits	[58]
	Cinnamon, clove, bay laurel, thyme, rosemary, sage,	270 μL/L gas phase	Black rye bread	[49]
	Thyme	2%	Wheat grains	[58]
	Lemon grass, orange, mustard	135 μL/Lgas phase	Black rye bread	[58]
	Thyme, clove, savory	300 mg/kg, 500 mg/kg	Tomato ketchup	[12]
A. ochraceus, A. parasiticus	Anise, cinnamon, thyme, green mint	2%	Wheat grains	[58]
Botrytis cinerea, Colletotrichum coccodes	Cinnamon	500 mg/kg	Cherry tomato, pepper	[13]
Fusarium verticillioides	Anise, cinnamon, thyme, green mint	2%	Wheat grains	[58]
Eurotium repens	Cinnamon, clove, bay laurel, rosemary, sage	270 μL/L gas phase, 50 μL/gas phase	Black rye bread, biscuits analogue	[49, 97]
	Thyme, lemon grass, orange, mustard	135 μL/L gas phase, 50 μL/gas phase	Black rye bread, biscuits analogue	[49, 97]
Penicillium commune	*AITC	2 μL/gas phase	Rye bread, "hot dog" bread	[48]
P. corylophilum	Cinnamon, cloves, bay laurel, thyme, orange, rosemary, sage	270 μL/L gas phase	Black rye bread	[49]
	Lemon-grass, mustard	135 μL/L gas phase	Black rye bread	[49]
	*AITC	2 μL/L gas phase	Rye bread, hot dog bread	[48]
P. expansum	Cinnamon	2.0% (v/v)	Orange peels	[96]
P. roqueforti	Cinnamon, clove, lemon-grass, bay laurel, thyme, orange, rosemary, sage	270 μL/L gas phase	Black rye bread	[49]
	Mustard	135 μL/L gas phase	Black rye bread	[49]
	*AITC	2 μL/L gas phase	Rye bread, "hot dog" bread	[49]
Rhizopus nigricans	Cinnamon	2.0% (v/v)	Orange fruits	[96]
Sclerotina sclerotiorum	Dill, oregano	3.2 μg/mL	Tomato fruits	[95]
Monilia fructigena, Phlyctema vagabonds, B. cinerea, P. expansum	*Eugenol	2 mg/mL	Apple fruits (″Golden Delicious")	[74]
Aflatoxin B_1	Cinnamon, clove	1000 mg/kg	Corn grains	[85]
Aflatoxin B1, G1	Basil	100 mg/g	Melons, peanuts, sorghum, corn	[82]

Research show that EOs in antifungal and antimycotoxigenic protection of food can be applied as surface protection (by smearing or applying in the form of biofilm) [74, 96], as an addition to the modified atmosphere packaging, [48, 49], or as an addition to food [12]. However, a small number of EOs are used as commercial preservatives in the food industry. One such product is "DMC Base Natural" by the manufacturer DOMCA SA (Alhendín, Granada, Spain), and it consists of 50% of the EOs of rosemary, sage, citrus and 50% glycerol [20]. Similar products are also "Biotecta™ 60", and "Biotecta™ 60 PLUS" by the manufacturer Bavaria Corp (Apopka, FL, USA), made up of plant extracts and classified as safe (GRAS).

It is undeniable that EOs have the potential to control the development of fungi which contaminate food, as well as the production of mycotoxin by the toxin-producing species. However, the general ascertainment of researches who deal with antifungal testing of EOs is that the methods of determining the antifiungal activity should be standardized. It is necessary to complete the antifungal and antimycotoxigenic research of combined effects of EOs *in vivo* and *in vitro,* since stronger antifungal effects can be achieved through the application of lower concentrations of combined EOs in food or in active packaging. Science and practice should provide the answers to which of the EOs and which combinations are to have strong antifungal effect in food, while not being harmful to human health, having a suitable effect on the sensory quality of the product, and being economically profitable.

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