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Inhibitory effects of Ajowan (Trachyspermum ammi) ethanolic extract on *A. ochraceus* growth and ochratoxin production

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Abstract: Ajowan is an aromatic seed spice that has a medicinal value. In this paper Ajowan Ethanolic Extract (AEE), which was prepared from Ajowan seeds, was assessed for antibacterial and antifungal activity against selected pathogenic bacteria and fungi by agar well diffusion assay. AEE exhibited considerable inhibitory effects against all the organisms tested. Emphasis of the study was on the affect of AEE on the mycelial growth and spore germination of toxigenic fungi *A. ochraceus*. Cultures were incubated on yeast extract-sucrose (YES) broth, at concentrations of 50, 150, 250 ppm at 25 °C. At 250 ppm, AEE completely inhibited germination of spores, the fungal growth and ochratoxin A (OTA) production, showing the immense antitoxigenic potential of AEE. Also, the application of AEE in food samples resulted in considerable inhibition of the growth of *A. ochraceus* in foods such as maize and poultry feed at 125 mg/g and no detectable amount of OTA was found at a high moisture level of 20%, even after 7 days.

Key words: Ajowan ethanolic extract, ochratoxin A, antibacterial, antifungal, inhibition, fungal growth

Ajowan (Trachyspermum ammi) etanol ekstraktının *A. ochraceus* bitkisinin büyüme ve okratoksin üretimi üzerine inhibitör etkisi

Özet: Ajowan tedavi edici özelliği olan aromatik bir baharat tohumudur. Ajowan tohumlarından hazırlanan Ajowan etanol ekstraktının (AEE) agar kuyucuk difüzyon yöntemi kullanılarak seçilmiş patojen bakteriler ve mantarlara karşı antibakteriyel ve antifungal özellikleri tayin edilmiştir. AEE tüm denenen organizmalara karşı önemli bir inhibitör etki göstermiştir. Çalışmanın önemi, toksik etkili mantar *A. ochraceus*'un misel büyümesi ve spor çimlenmesi üzerine AEE' nin etkisiydi. Kültürler maya ekstrakt-sükroz (YES) sıvı besiyerinde 50, 150, 250 ppm konsantrasyonlarında 25 °C' de inkübe edilmiştir. 250 ppm' de, AEE spor çimlenmesi, fungal büyüme ve AEE' nin çok büyük antitoksijenik potansiyel gösterdiği okratoksin A (OTA) üretimi tamamıyle inhibe edilmiştir. Gıda örneklerinde AEE uygulaması da, 125 mg/g' da mısır ve kümes hayvan yemleri gibi yiyeceklerde A. ochraceus' un büyümesi büyük bir oranda engellenmiştir ve 7 gün sonunda % 20' nin üzerinde en yüksek nem oranında hiçbir OTA miktarı belirlenememiştir.

Anahtar sözcükler: Ajowan etanol ekstraktı, okratoksin A, antifungal, inhibisyon, fungal büyüme

Introduction

Food conservation for nutrition and superior shelf life can be obtained by controlling the growth of food borne pathogenic microorganisms and food spoilage. This could be achieved by suppressing one or more factors that are essential for microbial survival (1). Suppression might be possible by adding suitable chemical substances and by controlling physical factors for the growth of microbes (2,3). These methods either kill organisms or could make survival unviable. Consumers in general prefer to have food free from preservatives or added at low levels (4). Moreover, there has been a demand for food with long shelf life and without any risk of food contaminants. This warrants the use of natural preservatives as alternatives to chemical ones leading to increasing interest in testing natural compounds as antimicrobials for food preservation (5,6). Accordingly, natural plant products with antimicrobial properties have obtained recognition for its possible applications in food in terms of preventing bacterial and fungal growth (7). Several plant extracts have been studied for their antimicrobial properties. Especially, studies on spices s and herbs with antifungal and antitoxigenic oils have been the subject of many investigations (8-10). Ajowan is one of the aromatic seed spices, which is generally used for medicinal purposes as a digestive stimulant or to treat liver disorders. Thymol, the major phenolic compound present in Ajowan, has been reported to be a germicide, antispasmodic, and antifungal agent (11). In the present study, the author investigates the antimicrobial activity of Ajowan Ethanolic Extract (AEE) and affects of AEE on growth and Ochratoxin A (OTA) production by *A. ochraceus* CFR 221.

Material and methods

Microorganisms

The following strains were obtained from cultures maintained at CFTRI, Mysore; bacterial: *Bacillus cereus* F 4810, *Bacillus subtilis*, *Staphylococcus aureus* FRZ 722, *Streptococcus* sp., *Listeria monocytogenes* Scott-A, *Escherichia coli* MTCC 118, *Pseudomonas aeruginosa,* and *Yersinia enterocolitica* MTCC 859; fungal: *Aspergillus flavus*, *Aspergillus niger, Aspergillus orzyae, Fusarium* sp. GF-1019, *Penicillium* sp., *and Aspergillus ochraceus* CFR 221. Bacterial cultures were maintained on nutrient agar slants and fungal isolates on potato dextrose agar and stored at 4 ^{o}C.

Raw materials and chemicals

Ajowan seeds, maize, and poultry feed were procured from a local market. All chemicals used were of analytical grade and purchased from typical chemical companies.

AEE

Ajowan seeds (250 g) were loaded into glass columns and eluted with 50% aqueous ethanol (600 ml).The material was desolventised using rotovac at 30^{o}C. AEE (20% yield), which resulted in an antimicrobial substance that was used for the study.

Antibacterial activity

The antibacterial activity was carried out using agar well diffusion method. Bacterial strains (18-24 h old) grown in nutrient broth and incubated at 37 ^{o}C (10^{8}-10^{9} cfu/mL) were used in this study. Culture broth (0.1 mL) was spread on nutrient agar plate by the spread plate method. Wells were bored (8 mm diameter) in the agar plates, filled with 50 µL (2.5 mg) of AEE extract and were incubated at 37 ^{o}C for 24 h. At the end of the incubation period, the susceptibility of the test organisms was determined by measuring the radius of the zone of inhibition around the well. The results given are an average of duplicated experiments.

Preparation of spore suspension

All the fungal cultures were grown on PDA slants at 25 ^{o}C until the spores ramified the slant (7-10 days). Spores were suspended by adding sterilized Tween 80 solution (0.01% v/v) in distilled water and inoculated with approximately 10^{6} viable spores per mL.

Antifungal activity of AEE

The antifungal activity of the AEE was studied by agar well assay against various fungi (*A.flavus, A. ochraceus, A. niger, A. orzyae, Fusarium moniliforme, Penicillium* sp.) (12). AEE concentrations of 2.5 mg were used on test organisms. Each test was carried out in duplicates and fungal toxicity was measured in terms of percentage of mycelial inhibition calculated according to the following formula:

percentage of mycelial inhibition = [(dc - dt) / dc] × 100

dc and dt are the average diameter of mycelial colony of control and tested sets, respectively.

Effects of AEE on fungal growth

The antifungal activity of the AEE was tested at different growth periods. The spore suspension from *A. ochraceus* CFR 221 was inoculated on PDA by spread plate technique. AEE (12.5 mg) was added into the fungus-seeded plate in agar wells on days 1, 2, 3, 4 and 5, and the treated plates were incubated at room temperature (28 ± 2 °C) for 7 days, and observed for zone of inhibition.

Effects of AEE on fungal growth and toxin production in culture medium

Yeast Extract-Sucrose (YES) was used as a basal medium for growth and OTA production in stationary conditions (13*). A. ochraceus* spores suspension prepared in Tween 80 was inoculated into sterile YES broth. AEE was incorporated into YES medium at zero hour to give concentrations of 50, 150, 250 ppm. The cultures were incubated at 30 °C for 7 days. The fungal biomass was determined as dry weight (drying at 95 °C to constant weight). OTA was extracted from acidified culture broth (pH 4.0 with 1N HCl) using chloroform (25 × 3 mL). OTA production was monitored using Shimadzu LC-6A liquid chromatograph HPLC system equipped with a C-18 column. The mobile phase comprised acetonitrile, acetic acid, and water (49.5:1:49.5) at a flow rate of 1 mL/min. The detection was done at 460 nm and OTA had standard retention time of 8.03 min.

Effects of AEE in food substrate

The antifungal preparation was tested for inhibition of growth of *A. ochraceus* in maize and poultry feed. Maize and poultry feed were ground to grits and 2 g of each substrates were dispersed into Erlenmeyer flasks. The autoclaved maize and the poultry feed samples were inoculated with spore suspension of *A. ochraceus* CFR 221 (10^6 spores) and treated with AEE at concentration of 25, 75, and 125 mg/g. The final moisture of the samples was adjusted to 20% with sterile distilled water. The treated samples were incubated at 28 ± 2 °C for 7 days. The samples were analyzed for fungal biomass and OTA content. The maize and poultry feed sample without AEE served as a control sample.

Statistical analysis

The antimicrobial activity evaluated by the agar well diffusion method was expressed as mean ± standard deviation of the diameter of the growth inhibition zones (mm). The correlation coefficient was calculated between the content of mycelial biomass and ochratoxin using the Origin 6.0 software (Northampton, MA, USA).

Results and discussion

Antibacterial activity of AEE

The antibacterial activity of AEE was observed against food borne pathogens at 2.5 mg dosage. The extract was found to be highly effective for *B. cereus* with 48 mm zone of inhibition followed by *S. aureus*, *B. subtilis,* and *L. monocytogenes*. On the other hand, lesser inhibition was observed in *Streptococcus, Y. enterocolitica, E. coli*, and *P. aeruoginosa* (Figure 1). However, for all tested bacteria the data were found to be significant. Most of the gram-positive bacteria, such as *B. cereus, B. subtilis, S. aureus, and L. monocytogenes*, showed good inhibition action when compared to gram-negative bacteria (e.g. *E. coli and P. aeruginosa*). Generally, gram-negative bacteria have been reported to be more resistant than gram-positive samples to the antimicrobial effect of essential oils given the differences in the lipopolysaccharide constitution of their cell walls (14). Several hypotheses have been put forward which involve hydrophobic and hydrogen bonding of phenolic compounds to

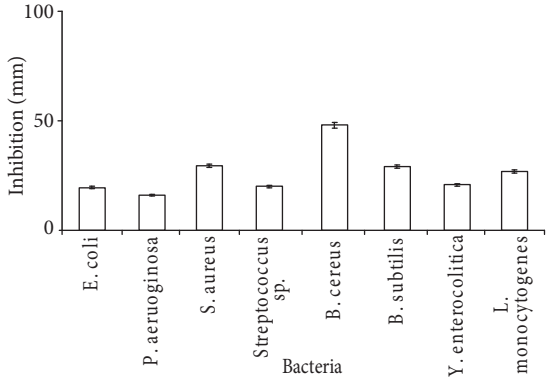

Figure 1. Antibacterial activities of Ajowan ethanolic extract (AEE).

membrane proteins, followed by partition into the lipid bilayer, perturbation of membrane permeability, membrane disruption, destruction of electron transport systems, and cell wall perturbation (15-19). The AEE activity can be explained due to the effect of the major chemical constituent, i.e. thymol. The high activity of the phenolic component may be further explained in terms of the alkyl substitution into phenol nucleus, which is known to enhance the antimicrobial activity of phenols. Phenolic compounds, such as thymol and carvacol, are known to be either bactericidal or bacteriostatic agents depending on the concentration used (20). In the present study, AEE has exhibited a broad spectrum of antibacterial activity.

Antifungal activity of AEE

The AEE was found active *against Aspergillus flavus, Aspergillus ochraceus, Aspergillus niger, Aspergillus orzyae, Fusarium moniliforme, and Penicillium* sp. by agar well assay method. High incidence was observed in the development of mycelial and sporulation control of these fungi. Growth of toxigenic fungi, *A. ochraceus* and *A. flavus,* was inhibited by 69% and 38%, respectively. The results are given in the Figure 2. It has been reported that antifungal activity occurs in Cymbopogan, Ajowan, and Dill oils against *Colletotrichum lindemuthianum* (21). The major and minor components present in the extract, i.e. phenolic and non-phenolic alcohols, are considered to be mostly antifungal agents (22,23). The studies on the essential oil of ajowan have been reported to inhibit some of the dermatophytes and are fungistatic towards *A. niger* and *T. violaceam* (24).

Effects of AEE on fungal growth

AEE was found to be both fungicidal and fungistatic. The AEE was active in inhibiting the growth of *A. ochraceus* when it was applied at the time of inoculation (0 day) before germination of spores (Figure 3). Sporulation was also prevented when AEE was added on day 1 and 2 of incubation. However, treatment of the inoculated plate with AEE in the Agar wells on day 3, 4, and 5 was not effective as mycelial growth was observed in these cases. Thymol and carvacol have been reported to cause disruptive action on cytoplasmic membrane. Likewise, thymol, the phenolic compound present in the AEE, appears to impair fungal enzyme system sensitizing the membrane permeability; thus, making vital intracellular constituents unavailable for the growth of organisms. The AEE was most effective when it was applied before germination of spores. Reproduction occurs predominantly by the production of asexual spores, which are an important source of fungal infestation in food and responsible for their rapid proliferation. Hence, prevention of germination of spores by the application of AEE may be used as an effective strategy for preventing fungal infestation in foods.

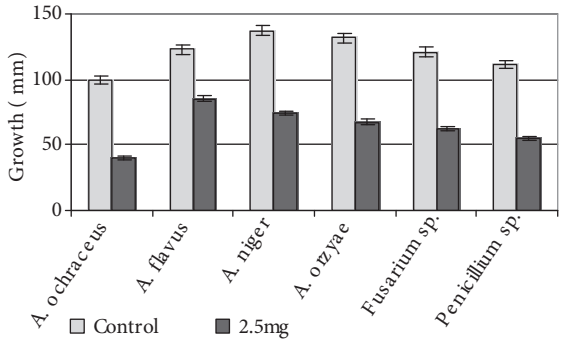

Figure 2. Antifungal activity of Ajowan ethanolic extract (AEE) with pathogenic fungi.

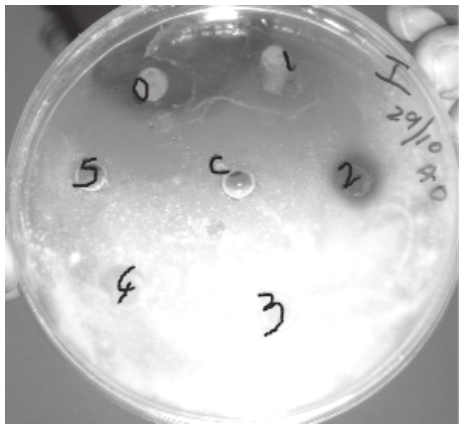

Figure 3. Effect of AEE on growth of ochratoxigenic fungi C-control with out AEE, 0-addition of AEE during inoculation, 1 to 5-addition of AEE 1 to 5 days after inoculation.

Effect of AEE on fungal growth and OTA production in cultures

The effects of AEE on the fungal biomass and OTA production in liquid culture medium are shown in the Table. Doses depended on inhibition of fungal biomass and OTA production. Growth and OTA production decreased progressively with increase in concentration of AEE. It was observed that at a dose 250 ppm of AEE, complete inhibition of fungal growth and OTA production could be possible. The inhibitory activity of AEE was significant in *A. ochraceus* in pure culture growing in YES broth. However, the inhibition towards fungal growth and OTA production was not linear. It is reported that the inhibitory effect of spice oils was mainly due to the most abundant component present in the spice extract (25). At a particular concentration, the potency of a fungitoxic compound can depend on the inoculum density of the test fungus apparently due to detoxifying enzymes produced by the organism (24). The inhibitory reaction of natural products on moulds involves cytoplasm granulation, cytoplasmic membrane, rupture and inactivation, or inhibition of intracellular and extracellular enzymes. These biological events could take place separately or concurrently culminating in inhibition of mycelium germination (26).

Application of AEE in Food Systems Figure 4 shows fungal biomass and OTA production in tested and control samples of maize and poultry feeds. The inhibition of fungal growth was apparent in treated samples at 125 mg/g, where complete inhibition of fungal biomass and 100% inhibition of ochratoxin were observed. The inhibitory effect of AEE was mainly due to thymol and carvacol. Thymol has demonstrated to have a high microbiocidal and antiaflatoxigenic effects

Table. Determination of effective concentration of AEE on YES medium.

Treatment (ppm)	Fungal biomass Dry wt (mg)	Inhibition (%)	OTA (ppm)	Inhibition (%)
Control	800.0 ± 0.02	0	0.23 ±0.4	0
50	726.4 ± 0.03	71.5 ± 0.08	0.17 ±0.1	73.9± 0.03
150	118.5 ± 0.01	85.4 ± 0.1	0.02 ±0.08	86.9 ± 0.1
250	No growth	100.0 ±0.08	0	100 ± 0.06

Incubation period – 7 days

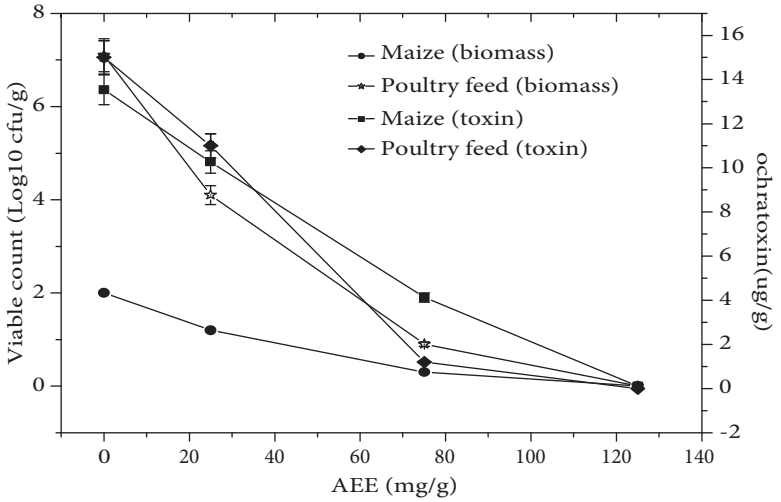

Figure 4. Effect of AEE on inhibition of *Aspergillus ochraceus* CFR 221 growth and OTA production in maize and poultry feed.

due to the presence of a phenolic -OH group (25). The aqueous extract of ajowan seeds was found to contain aflatoxin inactivation factor. An approximately 80% reduction in total aflatoxin content over the control was observed. Moreover, it was observed that toxin decontamination in spiked corn samples could be achieved by using the aflatoxin inactivation factor (27,28).

Conclusion

In summary, it can be concluded that the AEE possesses significant antibacterial and antifungal activity. AEE was antitoxigenic against *A. ochraceus* fungi producing OTA. The inhibitory effects were exhibited in broth culture as well as in solid food substrate. AEE inhibited 100% of biomass and OTA production in maize and poultry feed compared to control. This study underscores the potent antimicrobial effects of AEE with special reference to prevention of mycotoxin contamination in many foods indicating that AEE can find application as an alternative to synthetic antifungal products.

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