



## Research Article

# Inhibiting *Aspergillus flavus* Growth and Aflatoxin Decrement in Aquatic Feed with *Satureja hortensis* Essential Oil

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**Abstract** | *Aspergillus flavus* from inner part of pistachio was placed on the surface of PDA media culture to produce the spores. Then, different doses (400, 500 and 600 ppm) of *savory* (*Satureja hortensis*) essential oil (specifying its component by GC/MS analysis) were added to media to determine its effects as fungal inhibitor. Also, fish feed samples (300 g each) combined with above-mentioned doses of *savory* essential oil were inoculated with 2 ml spore suspension to evaluate fungal inhibition of *savory* oil at feed by HPLC method. The extent of the inhibition of fungal growth and aflatoxin (AF) production was dependent on the concentration of essential oil applied. The results indicate that Carvacrol (41.239%) and thymol (20.718%) are the most ingredients of *savory* oil and use of 500 and 600 ppm *savory* essential oil in aquatic feeds decrease AF contamination produced by *Aspergillus flavus* and it could be an appropriate alternative to chemical anti-fungal compounds.

**Keywords** | Aflatoxin, Carvacrol, Fish feed, *Savory*, Thymol

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## INTRODUCTION

Aflatoxins (AFs) are important fungal toxic compounds which are produced by an expanding list of closely related fungi (Eaton and Groopman, 1994). AFB<sub>1</sub> is the most important toxic compound among all known AFs that causes serious risk to human and animal health (Diaz et al., 2009; Sepahdari et al., 2010). Despite the interesting data that have now been established about AFB<sub>1</sub> in animals, the presence and impact of AFB<sub>1</sub> in farmed aquatic species are still underestimated. Very little has been documented on the toxicity of AFB<sub>1</sub> for cultured aquatic invertebrates fed by artificially contaminated diets (Alinezhad et al., 2011). The history of AFs in fish returns to 1960 when a prevalence of liver tumors was reported in rainbow trout hatcheries in USA due to feed with AF contaminated cottonseed meal as a raw ingredient in trout meal (Ashley and

Halver, 1963). AF contamination of feed and feed ingredients used in aquaculture has been reported for a wide range of substrates including rice, maize, peanuts, cottonseed, fish meal and dried fish (Ashley and Halver, 1963; Ashley, 1970). AFs demonstrate a fundamental impact on the fish farming production, causing anemia, hemorrhage, liver damage, weight loss, increased susceptibility to secondary infectious diseases, increased mortality and a gradual decline of reared fish stock quality, thus representing a significant problem in aquaculture systems (Santacroce et al., 2008). Also, this toxin affects some hydrothermal fishes such as channel catfish and tilapia due to more use of plant compounds in comparison to animal compounds (Gowda et al., 2004) that plant compounds are more sensitive to AFs. Improper methods of feed processing and storage are among the most important factors favouring the growth of AF-producing moulds and they are major elements that

can be controlled by the fish producer (Fraga et al., 2007).

During the last decade, development of antibiotic resistance as well as unpleasant side effects of some antibiotic drugs has terminated to the investigation for new antimicrobial agents. Also, a lot of people are interested in usage of organic productions (Rasooli et al., 2008). Therefore, many researchers have experimented the antimicrobial activity and other biological effects of different plants and their essential oils.

*Satureja hortensis* (Savory) belongs to the *Lamiaceae* family and is a medical herb which grows in more than 30 hectares of Iran. It has been traditionally used as diuretic, carminative, anti-diarrheal and intensive disinfectant. Some biological effects of *savory* such as antiviral, anti-inflammatory, antimicrobial and antifungal have been confirmed (Razzaghi-Abyaneh et al., 2008). About 2-5 ml essential oil is obtained from every 100 g *savory* which the aim of the recent study was therefore to evaluate the antifungal effect of essential oil of *savory* and its impression on toxin prevention in aquatic diet.

## MATERIAL AND METHODS

The protocol of the experiment was reviewed and approved by the Animal Science Research Department of Agriculture and Natural Resource Education and Research Center of Kerman, Iran.

### ESSENTIAL OIL AND GC/MS ANALYSIS

The essential oils from aerial parts of *savory* were prepared from Barij Essence Pharmaceutical Company (Kashan, Iran) for this experimental trial. The samples were stored at 4°C until used. The oil analysis was done using GC and GC/MS. The GC device was Shinadzu-9A system equipped with F.I.D detector and Chromatepac data-processor, capillary column of DB-5 (30 m×0.25 mm, film thickness 0.25 µm). The oven temperature program was raised up to 100-220°C at a rate of 2°C/min. The injector temperature was 230°C. Helium was used as the carrier gas at a flow rate 22.7 cm/s and ionization energy in mass spectrophotometer was 70 electron volts. GC/MS analysis was done on a Varin-3400 GC system connected to Saturn 2 mass spectrometer with the same-above-mentioned capillary column, ion trap detector, carrier gas helium with flow rate 50 cm/s, ionization energy in mass spectrometer 70 electron volts, with temperature program 60-240°C at a rate of 3°C/min and injector temperatures 250°C. The compounds of the oil were identified by comparison of their retention indices (RI), mass spectra fragmentation with those on the stored Wiley 7 n.1 mass computer library, and NIST (National Institute of Standards and Technology) (Mahboubi and Kazempour, 2011).

**MICROBIAL STRAINS AND PURIFICATION OF PATHOGEN**  
Pathogen fungi of *Aspergillus flavus* PTCC5006IR6 from inner part of Rafsanjan pistatue was obtained from Persian Type Culture Collection (Tehran, Iran) as a lyophilized fungus with high toxin production. At first, fungus was placed on the surface of PDA (potato dextrose agar) media culture and petri dishes were incubated for 7-10 days in 28±2°C to produce the spores. Isolates were purified by single spore methods. The media culture was used after the fourth stage of processing and preparation (passage) of pure cultures.

### SAMPLE PREPARATION

At first, different doses of *savory* essential oil (400, 500 and 600 ppm) were added to media and flasks were inoculated with discs of 1 cm diameter of the toxigenic *Aspergillus flavus*. Prepared petri dishes were used to determine its effects as fungal inhibitor. Secondly, fish feed samples (300 g each) were weighted in containers. Prepared containers were autoclaved in 121°C for 15 minutes to be sterile. Then, different doses of *savory* essential oil (400, 500 and 600 ppm) in 3 ml of distilled water (to supply moisture) were prepared and added to feed samples. Three replicates were performed for each concentration and control was carried out with 3 ml distilled water. The samples were inoculated with 2 ml spore suspension in sterile conditions under microbial hood. Dishes were incubated at 28±2°C and shook every 24 hours to assimilate oxygen, fungal infection and *savory* essential oil in the whole of media culture. Light, temperature and oxygen conditions were similar for all samples.

### DETERMINATION OF HPLC

The first, second and third samplings were done After 20, 40 and 60 days of the inoculation, respectively. Analysis of compounds was performed on Aflatoxins by High-Performance Liquid Chromatography (HPLC) model (model PerkinElmer series 200 UV/VIS) with a C18 column with an internal diameter of 300 mm × 3.9 mm, 4 micron. The HPLC was equipped with an UV detector and fluorescence with 365 nm excitation and 430 emission wavelengths. The mobile phase consists of methanol:acetic acid:water (20:20:60 v/v/v). The total run time for the separation was approximately 25 min at a flow rate of 1 ml/min (Christian, 1990; Abd-El-Aziz et al., 2015).

### STATISTICAL ANALYSIS

All of the data were analyzed using SPSS 10.0 statistical software and significant differences between mean values were determined with Duncan's multiple range test ( $P < 0.05$ ), followed by one-way ANOVA.

## RESULTS

### INGREDIENTS OF *Savory* ESSENTIAL OIL

Table 1 shows the ingredients of *savory* oil. Carvacrol

(41.239%), thymol (20.718%),  $\gamma$ -terpinene (16.498%) and P-cymene (10.082%) were the main components of savory oil.  $\alpha$ -terpinene (4.498%),  $\beta$ -pinene (0.588%),  $\alpha$ -pinene (1.480%) and myrcene (1.788%) were the other minor components of oil.

**Table 1: Ingredients of savory oil**

Name of Compound	%	Name of Compound	%
Alpha – thujene	1.008	Limonene	0.755
Alpha- pinene	1.480	Gamma-terpinene	16.498
Beta- pinene	0.588	Thymol	20.718
Myrcene	1.788	Carvacrol	41.239
Alpha- phellandrene	0.287	e- caryophyllene	0.538
Alpha-terpinene	4.498	p- cymene	10.082
Beta-bisabolene	0.527		

**DETECTION OF AFB<sub>1</sub>, G<sub>1</sub> AND TOTAL B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub> PRODUCED BY *Aspergillus flavus* IN CONFRONTING WITH Savory OIL**

Data in Table 2 shows the amount of AFs faced to different doses of savory oil in different days of sampling. Obviously, since day 40 onwards, 500 and 600 ppm savory oil decreased all kinds of AFB<sub>1</sub> and total B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub>. Results of AFG<sub>1</sub> indicate that 500 and 600 ppm savory essential oil in all days declined this kind of fungal toxin.

**DETECTION OF AFB<sub>1</sub>, G<sub>1</sub> AND TOTAL B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub> PRODUCED BY *Aspergillus flavus* IN CONFRONTING WITH Savory OIL AT AQUATIC FEED SAMPLES**

Effect of different doses of savory essential oil on AFB<sub>1</sub>

and G<sub>1</sub> inhibit at aquatic feed samples during different days are shown in Table 3. Amount of AFB<sub>1</sub> reduced linearly during time in all feed samples including control but noticeably, all doses of savory essential oils (400, 500 and 600 ppm) could completely inhibit fungi growth in feed samples at day 60 of experimental period. On the other hand, although savory essential oil decreased amount of AFG<sub>1</sub> at aquatic feed samples at day 20 but its effect did not continue until days 40 and 60 which are apparent in Table 3.

**DISCUSSION**

Data show that 500 and 600 ppm savory essential oil appeared effective on the growth inhibit of fungi compared to control. There is an upstanding relationship between the inhibitory effect of the essential oils and their doses (Abd-El-Aziz et al., 2015, Al-Gahtani et al., 2013; Rasooli et al., 2008). The results indicate that the test toxigenic fungus is sensitive to the savory essential oil and particularly sensitive to doses upper than 500 ppm. This result is confirmed by some other researchers (Atanda et al., 2007; Eweis et al., 2012).

Chemical composition of savory oil exhibited the presence of thymol,  $\gamma$ -terpinene, p-cymene and carvacrol as the main component. In the present study, carvacrol was the main component of savory oil. It is a monoterpenoid phenol. It has a pungent and warm odour characteristic. Thymol was identified as the second most common compound in savory oil and it is part of a naturally occurring class of compounds known as biocides, with strong antimicrobial attributes when used alone or with other biocides such as carvacrol.

**Table 2: Effect of different doses of savory oil on amount of AFs**

Treat-ments	AFB <sub>1</sub>			AFG <sub>1</sub>			Total AFB <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>		
	Day 20	Day 40	Day 60	Day 20	Day 40	Day 60	Day 20	Day 40	Day 60
Control	0.53±0.012	0.57 <sup>a</sup> ±0.028	1.13 <sup>a</sup> ±0.035	1.99 <sup>a</sup> ±0.040	1.21 <sup>a</sup> ±0.041	1.49 <sup>a</sup> ±0.038	1.89±0.201	1.78 <sup>a</sup> ±0.084	2.62 <sup>a</sup> ±0.328
400 ppm	0.53±0.014	0.58 <sup>a</sup> ±0.017	0 <sup>b</sup> ±0	2.10 <sup>a</sup> ±0.051	1.11 <sup>a</sup> ±0.038	0.48 <sup>ab</sup> ±0.020	1.93±0.196	0.82 <sup>b</sup> ±0.073	0.82 <sup>b</sup> ±0.182
500 ppm	0.56±0.022	0.22 <sup>b</sup> ±0.021	0 <sup>b</sup> ±0	1.19 <sup>bc</sup> ±0.032	0.64 <sup>b</sup> ±0.021	0.24 <sup>b</sup> ±0.015	1.75±0.141	0.86 <sup>b</sup> ±0.195	0.52 <sup>b</sup> ±0.020
600 ppm	0.56±0.018	0.15 <sup>b</sup> ±0.019	0 <sup>b</sup> ±0	0.87 <sup>c</sup> ±0.021	0.44 <sup>b</sup> ±0.017	0.15 <sup>b</sup> ±0.012	1.46±0.093	0.84 <sup>b</sup> ±0.068	0.15 <sup>c</sup> ±0.064
P value	0.226	0.000	0.000	0.025	0.030	0.009	0.527	0.016	0.021

<sup>a,b,c</sup>, Means within a column with different subscripts differ (P<0.05)

**Table 3: Effect of different doses of savory oil on amount of AFs at aquatic feed samples**

Treat-ments	AFB <sub>1</sub>			AFG <sub>1</sub>			Total AFB <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>		
	Day 20	Day 40	Day 60	Day 20	Day 40	Day 60	Day 20	Day 40	Day 60
Control	0.58±0.051	0.34 <sup>a</sup> ±0.063	0.24 <sup>a</sup> ±0.059	1.20 <sup>a</sup> ±0.037	0.62±0.029	0.72±0.021	1.58±0.121	1.84 <sup>a</sup> ±0.115	1.76 <sup>a</sup> ±0.103
400 ppm	0.53±0.038	0.40 <sup>a</sup> ±0.051	0 <sup>b</sup> ±0	1.25 <sup>a</sup> ±0.042	0.45±0.033	0.50±0.037	1.43±0.320	0.93 <sup>b</sup> ±0.103	0.87 <sup>b</sup> ±0.992
500 ppm	0.57±0.049	0.22 <sup>b</sup> ±0.071	0 <sup>b</sup> ±0	1.20 <sup>a</sup> ±0.039	0.59±0.027	0.75±0.022	1.69±0.211	0.81 <sup>b</sup> ±0.093	0.93 <sup>b</sup> ±0.121
600 ppm	0.58±0.057	0.15 <sup>b</sup> ±0.068	0 <sup>b</sup> ±0	0.90 <sup>b</sup> ±0.051	0.68±0.035	0.71±0.030	1.80±0.187	0.98 <sup>b</sup> ±0.098	0.61 <sup>b</sup> ±0.143
P value	0.826	0.014	0.005	0.024	0.308	0.481	0.299	0.037	0.041

<sup>a,b</sup>, Means within a column with different subscripts differ (P<0.05)

$\gamma$ -terpinene was the third component of this oil that is natural and has been isolated from a variety of plant sources. The fourth most abundant compound was p-cymene which is a naturally aromatic compound classified as monoterpene hydrocarbon consisting of a benzene ring substituted with a methyl and isopropyl groups (Kedia et al., 2014; Mahboubi and Kazempour, 2011).

Antimicrobial activities of this essential oil is apparently attributable to high phenolic compounds such as thymol and carvacrol (Mahboubi and Ghazian bidgoli, 2010; Gowda et al., 2004; Boyraz and Ozcan, 2006; Gulluce et al., 2003), or p-cymene. The antimicrobial effect of thymol and carvacrol is due to damage in membrane integrity with change in pH hemostasis also equilibrium of inorganic ions, p-cymene does not have antimicrobial activity but it increases the antimicrobial activity of thymol or carvacrol (Delgado et al., 2004; Ultee et al., 2002). P-cymene is hydrophobic compound with ability to dissolve cytoplasmic membrane of bacterial cells (Mahboubi and Kazempour, 2011). Also, based on Dikbas et al. (2008) report, AFs inhibit effects of carvacrol is more than thymol but all major and/or minor components of *savory* essential oil play an important role on its antimicrobial activities. It is found that the whole essential oils have a greater antibacterial activity than the mixed major component (Mourey and Canillac, 2002) so the minor components of essential oil play a critical role for activity of oil.

Regional differences in AFs contamination of herbal foods may be attributable to climatic conditions and to agricultural practices. Humidity (Nawar, 2008), pre-harvest conditions of temperature and humidity in the field and improper post-harvest handling and storage (Nakai et al., 2008; Khodavaisy et al., 2012) play a fundamental role in the development and spread of fungal contaminations.

It has been reported that *savory* essential oil decreases mycelium weight of *Aspergillus flavus* (Dikbas et al., 2008), inhibits growth of AFB<sub>1</sub> and G<sub>1</sub> produced by *Aspergillus parasiticus* (Razzaghi et al., 2008), prevents toxigenic fungi in plants and foods (Maskouki and Mortazavi, 2004) and has intensive antimicrobial activity (Mahboubi and Kazempour, 2011).

Results from current experiment indicates that use of more than 500 ppm *savory* essential oil in aquatic feeds decrease AFs contamination produced by *Aspergillus flavus* and it might be an appropriate alternative to chemical anti-fungi compounds.

## AUTHORS' CONTRIBUTION

Both authors contributed equally in this article.

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