Effect of Plant Essential Oils and Gamma Irradiation on Growth and Aflatoxin Production by *Aspergillus Flavus* Isolated from Wheat Grains

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**ABSTRACT**

The antifungal potential of essential oils of *Thyme (Thymus vulgaris L.*) and camphor (*Eucalyptus rostrata L.*) was determined on *Aspergillus flavus* link isolated from wheat grains on Potato dextrose agar (PDA). They inhibited completely mycelial growth of the fungus at 1000 and 2000 ppm, and prevented aflatoxin production at sublethal dose 500 and 1000 ppm respectively. Gamma radiation was used to control mycelial growth of *Aspergillus flavus* Link and inhibiting aflatoxin production. A dose level of 3.5 KGY gamma radiation prevented the fungal growth and aflatoxin production by *A. flavus* link, where a dose of 2.5 KGY (the sublethal dose) prevented about 85% of aflatoxin production.

**Key Words:** Essential oil, Camphor, Thyme, A. Flavus, Gamma Radiation, Aflatoxin

**INTRODUCTION**

Recent records suggest that 25% of the world's crops were threatened by infection with more than 300 fungal genus, which known to produce metabolites as mycotoxins in their seeds and grains, specially under unsuitable storage conditions (Galvano et al.\(^1\)). Moulds are usually caused by one or more fungus, depending on locality and entires as well as on the environmental factors. In particular *Aspergillus* spp. and *Fusarium* spp. are the most prevalent through storage (El-Naggar\(^2\)).

*Aspergillus flavus* produce aflatoxins in food and feedstuffs. Aflatoxins are known as potent hepatocarcinogens in animals and humans (Galvano et al.\(^3\)).

Numerous studies have documented the antifungal (Cairns and Magan\(^4\)) and antibacterial (Canillac and Mourey\(^5\)) activity of plant essential oils.

Mady\(^6\) found that powdered cardamom proved to have much more antifungal activities against the mycotoxigenic and other tested fungi compared with powdered black mustard. The growth of all tested fungi were inhibited at different levels of cardamom concentrations.

Dimitra *et al.*\(^7\) tested oils of oregano, thyme, dictamnus, marjoram, lavender, rosemary, sage and pennyroyal for their effectiveness against *Botrytis cinerea, Fusarium solani* and *Clavibacter michiganensis* on artificial growth media. Their growth was completely inhibited by the tested essential oils at relatively low concentrations (85-300 μg/ml).

Gowda *et al.*\(^8\) found that *Eugenia caryophyllus* L. oil at 0.5-1% completely inhibited aflatoxin production of *A. parasiticus*, while a moderate reduction in toxin production occurred with 0.2-1% of *Curcuma longa* L. (63-84%), 0.1-1% of *Allium cepa* (64-76%) and 0.2-1% *A. sativum* (71-84%) essential oils in feeds.

Helal *et al.*\(^9\) reported that 1.5 μl/ml of *Cymbopogon citrates* L. essential oil completely inhibited mycelial growth of *Aspergillus flavus* applied by fumigation and the sublethal doses of 1.0 μl/ml inhibited about 65% of fungal growth after five days of incubation and delayed conidiation as
compared with the control. Also, sublethal dose completely inhibited aflatoxin B\textsubscript{1} production from \textit{A. flavus}.

Irradiation has been used to preserve foods to be free of pathogenic microorganisms and it is considered an important tool for control of food contaminating microorganisms. Gamma radiation is used to evaluate the quality of stored foods and reduces economic losses and helping in acceptance of products exported by developing countries (Loaharanu\textsuperscript{(10)}).

Various methods of preservation such as fumigation, fungicide application and heat treatment had been applied to eliminate mould contamination, but none of these methods offered complete control of pathogenic and toxigenic fungal species, meanwhile, it has been proved by several authors that gamma irradiation is a promising method applied for the decontamination and preservation of plant crops and different food and feed stuffs considering the disadvantages caused by alternative methods (Gharib and Aziz\textsuperscript{(11)} and Aziz et al.\textsuperscript{(12)}).

Hassanein\textsuperscript{(13)} found that exposure of \textit{A. flavus} to low doses of gamma irradiation (0.0 to 2 KGY) increased both fungal growth and aflatoxin production followed by complete inhibition at irradiation dose of 3.0 KGY.

Chiu et al.\textsuperscript{(14)} reported that after 4 weeks incubation of the irradiated (0.0 to 15 KGY) inoculated peanut seeds in a humidified conditions, aflatoxin produced by surviving \textit{Aspergillus parasiticus} decreased when the irradiation dose increased.

Aquino\textsuperscript{(15)} demonstrated that mycotoxins are secondary metabolites produced by different fungi genera as \textit{Aspergillus}, \textit{Fusarium} and \textit{Penicillium}. Among the mycotoxins aflatoxin B\textsubscript{1} is the most commonly found in food and is considered to be the most toxic compound, gamma irradiation can be used to preserve foods.

Recently Ribeiro, et al.\textsuperscript{(16)} found an opposite results when observing the effect of gamma radiation (2 KGY) on \textit{Aspergillus flavus} and \textit{Aspergillus ochraceus}, the level of mycotoxins produced by irradiated strains were two times greater than those produced by control strains.

**MATERIALS AND METHODS**

**Experimtinal Fungus:**

\textit{Aspergillus flavus} link was isolated from wheat grains, it was locally identified according to Raper and Fennell\textsuperscript{(17)} and Moubasher\textsuperscript{(18)}.

**Essential Oil:**

Camphore (\textit{Eucalyptus rostrate} L.) and Thyme (\textit{Thymus vulgaris} L.) were kindly obtained from sekum company, Hikstep region, Cairo, Egypt.

**Culture Media:**

Media used were prepared according to Gams et al.\textsuperscript{(19)} The fungus was isolated and maintained on potato – dextrose agar (PDA) medium and identified on Czapek’s and malt agar media. PDA media consists of: Potato extract, 230 mL; glucose, 20 g; water, 770 mL. Potato extract was prepared by adding 100 g potatoes (pleeded and sliced in a mincer) to 300 mL tap water; it was left overnight at 4\textdegree C, and filtered through cloth.

**Gamma Irradiation Treatment:**

Gamma irradiation process was carried out in Co\textsuperscript{60} Gamma Cell at the National Center for Radiation Research and Technology (NCRRT), Nasr City, Cairo, Egypt. The dose rate was 0.9KGY/hr at the time of the experiment.
Effect of Different Concentrations of Essential Oil on Linear Growth of *Aspergillus Flavus*:

Different concentrations (100, 500, 1000 and 2000 ppm) of Camphore (*Eucalyptus rostrata*) and Thyme (*Thymus vulgaris*) were individually added to 250 mL Erlenmeyer flasks each contains sterilized PDA medium (1% Tween 80 was used as emulsifying agent). The plates from each concentration were prepared. The plates were inoculated singly with disks (3 mm diam.) of fungal growth taken from 7 days old culture of *Aspergillus flavus*. Three replicates were prepared from each concentration of both treatments and linear growth of the fungus was measured when the control plates reached full growth at 27±2°C.

**Effect of Gamma Radiation on Growth and Aflatoxins Production by *Aspergillus Flavus***:

The effect of increasing doses of gamma radiation on growth and aflatoxins production determined as follows: One mL spore suspension (10^6-10^7 spore/mL) was added to 9 mL sterile saline solution in test tubes. Then the tubes were exposed to different doses of gamma radiation (0.0, 0.5, 1.5, 2.5 and 3.5 KGy). After irradiation, one mL spore suspension of each dose was inoculated into 50 mL of sterilized Czapek's yeast extract broth medium in 250 mL conical flask. Triplicate flasks were made for each dose. The flasks were shaken for 30 min and incubated at 28°C for 10 days. After the incubation period cultures were autoclaved for 1 min. to kill spores and mycelia and to facilitate the extraction of aflatoxins. Then the mycelial mat was separated dried at 60°C overnight and weighed to determine the mycelial dry mass as a measure of fungal growth.

The extract was successfully filtered through Whatman No.1 filter paper. The chloroform layer was separated in a separating funnel over anhydrous sodium sulfate and transferred into 400 mL beaker then purification, mycotoxins were quantified by direct comparison of the sample extracts with appropriate dilutions of standard mycotoxins solutions. TLC and high-pressure liquid chromatography were used for qualitative and quantitative estimation of mycotoxins (Grabarkiewicz-Szczesna et al. (20)).

**Determination of D_{10} Values**:  
Radiation D_{10}-values is the dose which reduce the initial microbial population by 90% determined as follows: 1 mL of the fungal spore suspension was added to 9 mL of sterile saline solution in test tubes. The tubes were exposed to various doses of gamma radiation (0.0, 0.5, 1.5, 2.5 and 3.5 KGy). Three replicate tubes were used after irradiation serial dilutions were made from each replicate and the count of fungal survivors was enumerated on malt extract agar medium using pour plate technique (Koburger and Marth (21)). All plates were incubated at 28°C for 4-5 days. The colonies were counted in each plate and D_{10}-value was calculated according to WHO (22).

**Experimental Design and Statistical Analysis**:

All treatments in this study were arranged in complete randomized design. The obtained data were subjected to analysis of variance using the general linear module procedure of SAS (23). Appropriate treatment means were separated using Duncan's multiple range test (Duncan (24)).

**RESULTS**

**Effect of Camphore and Thyme Essential Oil on Mycelial Growth and Aflatoxin Production by *Aspergillus Flavus***:

Data in Table (1) indicate that the inhibition activity of Camphore and Thyme oils were more evident when their concentrations increased. The data also show that Thyme oil was more effective than Camphore on mycelial growth. Moreover, Thyme oil inhibited mycelial growth at 1000 ppm and Camphore oil inhibited mycelial growth at 2000 ppm. Also, Thyme oil at sublethal concentration 500 ppm prevented aflatoxin production and Camphore oil at 1000ppm sublethal concentration prevented aflatoxin production.
Table (1): Effect of Camphore and Thyme essential oil on linear growth and aflatoxin production by Aspergillus flavus.

<table>
<thead>
<tr>
<th>Essential oil treatment</th>
<th>Concentration (ppm)</th>
<th>Linear growth (cm)</th>
<th>Aflatoxin (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Camphore</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>7.60±0.16 A</td>
<td>3.5±0.11 A</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>6.10±0.14 B</td>
<td>2.4±0.41 B</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>4.50±0.21 C</td>
<td>1.5±0.13 C</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>3.30±0.10 d</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Thyme</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>7.30±0.17 A</td>
<td>2.8±0.21 A</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>5.20±0.13 B</td>
<td>1.4±0.11 B</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>2.60±0.12 C</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

Means with the same letter are not significantly.

Effect of Incremental Irradiation Doses on the Viable Count (cfu/mL) of Aspergillus Flavus:

Data in Table (2) and Fig. (1) show that as the radiation dose leads to increasing the counts of fungal colony which decreased and at 3.5 KGY no colony appeared (lethal dose).

Table (2): Effect of incremental irradiation doses on the viable count (cfu/ml) of Aspergillus flavus.

<table>
<thead>
<tr>
<th>Irradiation dose (KGY)</th>
<th>Counts (mL)</th>
<th>Log counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>7.0 x 10^6</td>
<td>6.84 A</td>
</tr>
<tr>
<td>0.5</td>
<td>3.5 x 10^5</td>
<td>5.54 B</td>
</tr>
<tr>
<td>1.5</td>
<td>2.6 x 10^4</td>
<td>4.41 C</td>
</tr>
<tr>
<td>2.5</td>
<td>1 x 10^2</td>
<td>2.0 d</td>
</tr>
<tr>
<td>3.5</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Means with the same letter are not significantly.

Fig (1) the relation between irradiation dose (KGY) and log counts of Aspergillus flavus colony and from the histogram D_{10}-value calculated 0.48 KGY.

![Graph](image1.png)

Fig. (1): Effect of incremental irradiation doses on the viable count (cfu/mL) of Aspergillus flavus.

126
Effect of Different Gamma Radiation Doses on Mycelium Dry Weight and Aflatoxin Production by Aspergillus Flavus:

Data in Table (3) demonstrated that as gamma radiation doses increased the mycelium dry weight decreased and sublethal dose of gamma radiation 2.5 KGy and lethal dose of gamma radiation is 3.5 KGy (No fungal growth). Also, the table reported that as gamma radiation doses increased the amount of aflatoxin production by Aspergillus flavus decreased.

Table (3): Effect of different gamma radiation doses on mycelium dry weight and aflatoxin production by Aspergillus flavus.

<table>
<thead>
<tr>
<th>Irradiation dose (KGy)</th>
<th>Mycellium dry.wt (gm)</th>
<th>Aflatoxins (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.19±0.14 A</td>
<td>5.04±0.11 A</td>
</tr>
<tr>
<td>0.5</td>
<td>0.13±0.12 B</td>
<td>4.70±0.21 B</td>
</tr>
<tr>
<td>1.5</td>
<td>0.10±0.11 C</td>
<td>3.92±0.35 C</td>
</tr>
<tr>
<td>2.5</td>
<td>0.07±0.10 d</td>
<td>0.76±0.29 d</td>
</tr>
<tr>
<td>3.5</td>
<td>0±0.0</td>
<td>0±0.0</td>
</tr>
</tbody>
</table>

Means with the same letter are not significant.

DISCUSSION

Fungi are significant destroyers of foodstuffs during storage, rendering these foods unfit for human consumption by retarding their nutritive value and sometimes by producing mycotoxins (Mishra and Dubey (25)).

Aflatoxins are a family of toxic and carcinogenic substances which are considered secondary metabolites. The principle aflatoxins producers are, A. parasiticus and A. flavus (Rasooli and Abyaneh (26)). Due to their ubiquity, the persistence in the atmosphere and dispensability of these moulds, many types of foodstuffs can be contaminated by aflatoxins (Salmeron et al. (27)).

Essential oils are used as antimicrobial agents and they have two main characters. The first is their natural origin which means more safety to the people and the environment. The second is that they are considered of low risk for resistance strains development by pathogenic microorganisms (Mahmoud (28)).

The obtained results revealed that campphore and thyme inhibited the growth of A. flavus and thyme is more effective than camphor, the two essential oils prevented the fungal growth at 2000 and 1000 ppm, respectively. These results are in agreement with Zambonelli et al. (29) who observed that fungal growth inhibition was associated with the degeneration of fungal hyphae after treatment with Thymus vulgaris L., Lavandula and Mentha piperita essential oils. Similar results were obtained in case of A. niger treated with Cymbopogon nardus oil (De Billerbeck et al. (30)) on Saccharomyces cerevisiae when treated with Cymbopogon citratus L. essential oil (Helal et al. (31)) and on A. niger when treated with C. citratus L. essential oil (Helal et al. (32)).

Gamma irradiation has been known as a successful method for food preservation through lowering or completely elimination of microorganisms present which caused food spoilage and deterioration A. flavus is known to be a common aflatoxigenic fungi. Growth of this fungus and subsequent aflatoxin production represents a serious public health concern to the human beings. Therefore, the effect of different gamma radiation doses (0.0, 0.5, 1.5,2.5 and 3.5 KGy) on the growth and aflatoxin production by A. flavus was investigated. The results show that as the gamma irradiation dose increased both mycelial dry weight and aflatoxin production decreased.

Gamma irradiation dose of 2.5 KGy was very effective and greatly reduced both mycelial dry weight and aflatoxin production, while 3.5 KGy completely inhibit the growth of A. flavus and subsequently aflatoxin production. These results are in agreement with the results obtained by EL-
The presence of water has an important role in the destruction of aflatoxin by gamma radiation, since radiolysis leads to the formation of highly reactive free radicals. These radicals can readily attack aflatoxin at the terminal giving products of lower biological activity (Diehl (36)).

The mutagenic activity of aflatoxin in an aqueous solution (5 gL⁻¹ water) was reduced by 34%-44%, 74% and 100% after exposure to gamma rays at 2.5, 5, 10 and 20 KGY, respectively. Rustom (37) and Aquino et al. (38) studied the influence of water activity (a W) on the aflatoxin reduction in maize sample, since, the reduction of aflatoxin B₁ and aflatoxin B₂ in samples irradiated with 5 KGY was 46% and 94% respectively. Also, they found that higher gamma energy resulted in formation of highly reactive free radicals from broken molecules of water, in the same work, it was demonstrated that high dose of 10 KGY resulted in no detectable levels of aflatoxin.

Microorganisms differ greatly in their resistance to gamma radiation and radiation resistance of the same microorganism differ within their species and even within their strains, although the range of resistance among strains of a single species is usually weak enough to be ignored for practical purposes (Stegeman (39)).

D₁₀-values for individual microorganisms are very important and useful in calculating lethal and sub-lethal doses to know the relative resistance of microorganisms to gamma radiation which may be beneficial in various applications such as inactivation of fungal infection and ensure the hygienic quality of foods. El-Hadi (33) reported that D₁₀-value of A. flavus was 0.44 KGY. Hammad et al. (40) found that D₁₀-value for A. fumigatus spores ranged from 0.5 KGY to 0.6 KGY. Shahin (35) indicated that D₁₀-values of six tested fungi were 0.86, 0.71, 0.61, 0.53, 0.53 and 0.47 KGY for Eurotium amstelodami, Aspergillus fumigatus, Emericella quadrilineata, A. flavus, A. flavus forming Sclerotia and Paecilomyces variotii, respectively.

REFERENCES


