



Phenolic Content of Dill Seed Extracts as Antifungal Agent against *Aspergillus spp.*

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ABSTRACT

This study was performed to examine the effect of phenolic compounds and antioxidant content of four dill seed extracts. These extracts were prepared by hydro-distillation, ethanol, methanol and acetone. They were examined as antifungal agent against eight *Aspergillus spp.* Results revealed that hydro-distillation extract (HE) and ethanol extract (EE), derived from dill seed, had higher total phenolic content (45.67 and 39.71 GAE/g, respectively), as well as higher contents of butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and octyl methylcinnamate (OMC) (4.98, 1.50, 39.65 and 4.37, 1.10, 33.26 mg/ml, respectively) as compared to the methanol and acetone extracts. HE recorded a high diameter of inhibition zone (DIZ) with *A. flavus* IFO 6343, *A. niger* DSM 371 and *A. parasciticus* NRRL 2999, with MIC = 15 mg/l. While EE was more effective on *A. niger* CAIM 147, *A. niger* NRRL 337 and *A. oryzae* NRRL 9362, with MIC = 15 mg/l as compared with methanol and acetone extracts under the same conditions. Antifungal potential of HE and EE were found to be highly effective on enumeration of fungal strains used in this study. Obtained results confirmed strong relationship between the total phenolic content of dill seed extracts and its amounts of BHA as an antifungal agent. It is recommended to use dill HE (light yellow color) with foods highly expected to be infected with *A. flavus* IFO 6343, *A. niger* DSM 371 and *A. parasciticus* NRRL 2999, at 15 and 20 mg/l, while EE (pale yellow color) is recommended to prevent foods to be susceptible to *A. niger* CAIM 147, *A. niger* NRRL 337 and *A. oryzae* NRRL 9362 at 15 and 20 mg/l.

Keywords: Dill seed extracts, hydro-distillation, Ethanol, methanol, acetone, *Aspergillus spp.*, phenolic compounds, antioxidants.

1. Introduction

Dill plant (*Anethum graveolens*) is an important member of the *Umbelliferae* family, which is native to southwest Asia, southeast Europe and also the Mediterranean region (Tian *et al.*, 2012 and Nada *et al.*, 2018). It grows in many countries such as Iran, India, Russia and Egypt (Noah *et al.*, 2017). It is known in Egypt as Shapt (Dahiya and Purkayastha, 2012) and has been used as an aromatic, medicinal and culinary herb since early decades (Hojjati, 2017). Compounds from aromatic plants have been recognized as a natural source for antibacterial and antifungal properties since early time in several countries. They are rich source of essential oils (EO) as it contains several biologically active ingredients used as antimicrobial agents (Hussein *et al.*, 2015 and Nguyen *et al.*, 2020) as well as flavoring agents in food and beverages (Najaran *et al.*, 2016). Essential oil containing bioactive phenolic and antioxidant compounds are often gained by hydro-distillation or extracted by some organic solvents from the whole plant or different parts i.e. leaves, stems or even their seeds (Tongnuanchan and Benjakul, 2014). Moreover, these extracts might have antimicrobial effect (Delaquis *et al.*, 2002 and Arora and Kaur 2007). Different experimental techniques have been used to explain the antimicrobial

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characteristics and the chemical composition of these essential oils (Delaquis *et al.*, 2002, Al-Ma'adhedi, 2012, Taota *et al.*, 2016 and Nada *et al.*, 2018).

Essential oils of dill seeds have more than 40 constituents, which could be identified as essential volatiles and when tested as an antimicrobial agent, it showed a high activity against *Aspergillus niger*, *Saccaromyces cerevisiae* and *Candida albicans* (Jirovetz *et al.*, 2003 and Nehdia *et al.*, 2020). Dill seed oil consists of α -phellandrene, eugenol, anethole, flavonoids, coumarine, triterpenes and phenolic acid (Jana and Shekhawat, 2010 and Said-Al Ahl and Omer, 2016). EO chemical composition differs according to the plant part, developing stage and also the extraction method (Singh *et al.*, 2006 and Al Ma'adhedi 2012). Volatile oil obtained from dill seed hydro-distillation and acetone extraction was found to have an inhibition effect on the fungal mycelial growth as, inhibition zone increased with the volatile oil dose, where, it completely inhibited *Fusarium graminearum* growth, followed by *F. citrinum*, *Aspergillus flavus* and *A. terreus* (Singh *et al.*, 2006 and Prakash *et al.*, 2012).

Aspergillus is a filamentous fungus, with more than 185 identified species worldwide. More than 20 species of which were reported to cause harmful infection in humans, animals and plants (Cleveland *et al.*, 2009). *A. flavus* and *A. parasiticus* are the most harmful species due to their direct infection and toxification to human kind (Tian *et al.*, 2012 and Noah *et al.*, 2017). They are mainly concerned with food poisoning as they play a main role in aflatoxins production which are involved in food contamination and toxification (Kumar *et al.*, 2017). They also affect plant metabolism by altering with vital cellular enzymes, pigmentation and decrease germination ability (Alpsoy, 2010). Polyphenols concentration and microbial enzymes activity are related, as polyphenols can interfere with cell membrane specific sites to interact with microbial membrane proteins, enzymes and lipids, and alter their cell permeability to permit protons, ions and macromolecules loss (Taotao *et al.*, 2016). This cellular components loss cause deformation in cell structure and consequently led to inhibition of the cellular growth (Bajpai *et al.*, 2013). Phenolic antioxidants i.e. butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butyl-hydroquinone (TBHQ) are classified as phenols that have antimicrobial activity, against gram positive and negative bacteria as well as molds and yeasts (Suh *et al.*, 2005). It was reported that, those phenolic antioxidants belong to some compounds that retard oxidation such as BHA, BHT, tert-butylhydroquinone (TBHQ) and propyl gallate (PG) (Shahidi and Ambigaipalan, 2015). Some scientists had observed that these compounds had an excellent effect against a wide range of microorganisms (Razavi-Rohani and Griffiths, 1999 and Arroyo, 2003) such as viruses, protozoa, bacteria, yeast and molds growth (Swamy *et al.*, 2016). From the previous four phenolic antioxidants, both BHA and Polypropylene (PP) showed the highest antimicrobial activity *in vitro* (Arroyo, 2003).

The objective of this study was to determine the phenolic and antioxidant compounds content of four dill seed extracts, as well as the antifungal evaluation of these extracts against eight *Aspergillus* strains.

2. Materials and Methods

2.1. Plant Material Source:

Dill (*Anethum graveolens* L.) seeds -GoldKrone- were purchased from Enza Zaden, Netherlands. Seeds were selected carefully to remove bad ones, washed well with running water for 30 minutes, dried and then grinded. Extraction was done by hydro-distillation and by three other solvents (ethanol, methanol and acetone).

2.2. Dill Seed Extraction Method:

Hydro-distillation was performed using Clevenger type apparatus for three hours as recommended by Asadipour *et al.* (2003), while extraction by solvents, were done using ethanol, methanol and acetone (El-Gomhouria Co., for Trading pharmaceutical, chemicals and medical appliance, Alexandria governorate, Egypt) according to Gomaa *et al.* (2016). Fifteen grams of grinded dill seeds were added to 300ml of each solvent individually, for 12 hours under shaking at room temperature then filtrated. After then, solvents were evaporated using rotary evaporator (Yamato Scientific Ltd., Japan; using pump, JEIO TECH, VE-11, Korea) at 40°C until dryness. Extracts were stored in dark sealed glass bottles at 4°C until used (Gomaa and Abdel Rahman, 2013).

2.3. Total Phenolic Content:

Total phenolic content were determined by The Folin Ciocalteu reagent (FCR), also called the gallic acid equivalence method (GAE), as mentioned by Singleton and Rossi (1965). Ultraviolet-visible (UV-Vis) spectrophotometry (Thermo electron corporation- Korea) was used. The total phenolic content was expressed as milligram of Gallic acid equivalent per gram of dill seeds dry weight (mg GAE/g).

2.4. Phenolic and Antioxidants Compounds Extraction:

Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and octyl methylcinnamate (OMC); were determined as described by Akkbi *et al.* (2013); 0.5g of extract sample were added to 25 ml n-hexane; followed by 25ml of acetonitrile, followed by refluxing for 30mins at 70°C with stirring. After that, crude extract was transferred to a separator funnel to separate n-hexane from acetonitrile. The later was concentrated using a vacuum rotary evaporator at 45°C, then the residue was re-dissolved in 10 ml of acetonitrile, filtered and diluted to 25ml with acetonitrile. Stock solution was stored in dark place at 4°C until use. Acetonitrile, n-hexane and acetic acid. D-gallic acid standards of BHA, BHT and OMC were supplied from Sigma-Aldrich (St. Louis, USA). Quantitative and qualitative analysis of each BHA, BHT and OMC were performed by Shimadzer HPLC (model: LC-20AT equipped with four pumps and Shimadzu SPD-20 AV UV/Vis) detector using 50µl of each samples. HPLC analysis condition was modified from Saad *et al.* (2007) using 280 nm as maximum wave length with mobile phase; acetonitrile and (water: acetic acid 99:1/v/v) and flow rate of 0.8 ml/min. Average concentration of phenolic antioxidants BHA, BHT and OMC were expressed by milligram per gram (mg/g).

2.5. *Aspergillus* Strains:

Eight culture strains of *Aspergillus spp.*, were used in the present study, illustrated as follows:

Code	Strain name	Source	Origin
1	<i>Afla I</i> <i>A. flavus</i> ATCC 16875	The American Type Culture Collection (ATCC).	USA
2	<i>Afla II</i> <i>A. flavus</i> IFO 6343	Institute for Fermentation, Osaka (IFO).	Japan
3	<i>Aniger I</i> <i>A. niger</i> CAIM 157	Cairo Mircen Culture Collection Microbiology Resource Center– Ain Shams University (CAIM).	Egypt
4	<i>Aniger II</i> <i>A. niger</i> DSM 731	German Collection of Microorganisms and Cell Cultures GmbH, Deutsche Sammlung von Mikroorganismen GmbH (DSM)	Germany
5	<i>Aniger III</i> <i>A. niger</i> NRRL 337	Northern Regional Research Laboratory (NRRL).	USA
6	<i>Aoryz.</i> <i>A. oryzae</i> NRRL 9362	Northern Regional Research Laboratory (NRRL).	USA
7	<i>Aparast.</i> <i>A. parasiticus</i> NRRL 2999	Northern Regional Research Laboratory (NRRL).	USA
8	<i>Aphoen.</i> <i>A. phoenicis</i> CAIM 149	Cairo Mircen Culture Collection Microbiology Resource Center– Ain Shams University (CAIM).	Egypt

Aspergillus strains were cultured in Potato dextrose agar (PDA) and incubated at 32 °C for 7 days until complete sporulation as mentioned by Difco Manual (1984).

2.6. Essential oil (EO) Effect against *Aspergillus spp.*:

Diluted spores' suspensions of the eight fungal strains were prepared by adding 10ml of 0.1% sterilized peptone water. Spores enumeration was made by cell count indirect technique (De Moss and Bard, 1957). Disc-diffusion method was used for diameter of inhibition zone (DIZ mm) determination with different dill seed extracts as suggested by Efstirion *et al.* (2012). One ml of each fungal suspension was added to 10 ml of PDA medium (45-50°C) and poured into petri dishes. After media solidification, sterilized filter paper discs (4mm in diameter) were put on the surface of inoculated media, then injected with 10 mg/l of dill extract to be tested, while 10% dimethyl sulfoxide (DMSO) was used as a negative control (Abd-Alla *et al.*, 2013 and Gomaa *et al.*, 2016). Clotrimazole (1% w/w) was used as a positive control as recommended by Villanueva *et al.* (2008) and Madugula *et al.* (2017).

Plates were incubated for 48 hours at 28 °C. The antifungal activity was measured as diameter of inhibition zone (DIZ) in mm around filter paper disc. This experiment was repeated in triplets.

The most effective extracts were selected to find the minimum inhibitory concentration (MIC) using serial dilutions (5, 10, 15, 20, 30 mg/l). *Aspergillus* strains under this study were added to sterilized PDA media along with 1 ml of each *Aspergillus* strain spore suspension (in 0.1% peptone water) and incubated for 48 hours at 28 °C. Then, MIC values were determined as the lowest antifungal concentration that inhibited visible growth of tested *Aspergillus* strains. Each test was performed in triplets.

2.7. Statistical analysis:

Data were performed using Costat software (version 6.400) as one-way randomized complete design (RCD). Comparisons among the means of different treatments were done using the least significant differences (L.S.D) test procedure at $p \leq 0.05$ level of probability, as illustrated by Snedecor and Cochran (1980).

3. Results and Discussion

The present study was conducted to examine the antifungal effect of dill seed EO extracted by hydro-distillation, ethanol, methanol and acetone against eight *Aspergillus* strains.

Dill seed extract obtained by hydro-distillation, was light yellow color. While the color of ethanol, methanol and acetone extracts were pale-yellow. Color of the extract is very important factor and essential when adding these extracts to foods for the purpose of preservation. These results came in agreement with Dahiya and Purkayasthat (2012), who stated that volatile essential oil of dill seeds, has light yellow color. Also, Singh *et al.* (2006) and Al-Ma'adhedi (2012) stated that, dill seed oil has a pale yellow color. They also reported that, dill seed oil amount varies depending on the extraction method.

Essential oils yield obtained from dill seeds by different extraction methods are presented in Table (1). Data showed that, hydro-distillation extract yield was 2.2%, while ethanol extract (EE) yield was 2.5% with no significant difference between them. Methanol extract (ME) and acetone extract (AE) yields were 1.3 and 1.8% respectively. Total phenolic content (mg GAE/g) showed in Table (1) significantly gave the highest content by HE (45.67 mg GAE/g). EE possessed 39.71 mg GAE/g followed by ME and AE (27.40 and 25.50 mg GAE/g, respectively).

Table 1: Total phenolic yield and content of different dill seed extracts.

Extract	Total phenolic yield (%)	Total phenolic content (mg GAE/g)
HE	2.20 ± 0.30 ^a	45.67 ± 0.19 ^a
EE	2.53 ± 0.21 ^a	39.71 ± 0.29 ^b
ME	1.30 ± 0.10 ^c	27.40 ± 0.30 ^c
AE	1.80 ± 0.17 ^b	25.50 ± 0.26 ^d

Mean ± standard deviation of triplicate determined in dry weight basis.

HE: Hydro-distillation extract, EE: Ethanol extract, ME: Methanol extract, AE: Acetone extract.

Means within a column followed by the same letter are not significantly different according to LSD test at $p \leq 0.05$.

Concerning antioxidant phenolic compounds shown in Table (2), HE significantly had higher content of BHA, BHT and OMC (4.98, 1.50, 39.65 mg/g, respectively) as compared with other tested solvents. EE came in the second place in this respect as it recorded 4.37, 1.10 and 33.26 mg/g, regarding BHA, BHT and OMC contents, while ME and AE recorded (3.09, 0.71, 32.80) and (2.89, 0.75, 20.42) mg/g, respectively. Generally, ME and AE significantly possessed lower values for the phenolic compounds as compared with HE and EE extracts. These results came in agreement with Singh *et al.* (2006) who found that dill seed extraction by hydro-distillation in a Clevenger type apparatus for 6 hours was 2.6%, while the yield was 1.8% when extracted by acetone at Soxhlet apparatus for 5 hours. Also this was supported by Stanojevic *et al.* (2015) who mentioned that hydro-distillation of dill seeds yielded 2.8 ml/100g dry weight.

Table (3) shows the antifungal assay conducted by the disc diffusion method for measuring the diameter of inhibition zone (DIZ) of *Aspergillus spp.* using 10mg/l of the extracted oils. Results obtained from this study showed that dill seed extracts were effective on all tested *Aspergillus* strains. HE significantly had the highest inhibitory effect on *Afla II*, *Aniger II* and *Aparast* as they recorded DIZ of 18.33, 20.67 and 19.67 mm, respectively; with a significant difference from the commercial positive control (Clotrimazole). Also, data showed that EE extract significantly gave the highest DIZ against *Aniger I*, *Aniger III* and *Aoryz* strains (20.33, 20.67 and 20.00 mm, respectively). As for *Afla I* and *Aphoen* strains, Clotrimazole (positive control) significantly gave the highest DIZ (18.00 and 15.67 mm, respectively) followed by HE with DIZ 11.00 and 10.00 mm for the previous mentioned strains, respectively. Clotrimazole, generally had a higher significant DIZ than ME and AE at all *Aspergillus spp.* under study.

Table 2: Average concentration (mg/g) of phenolic compounds in different dill seed extracts.

Extract	BHA	BHT	OMC
HE	4.98 ± 0.49 ^a	1.50 ± 0.20 ^a	39.65 ± 0.05 ^a
EE	4.37 ± 0.28 ^b	1.10 ± 0.10 ^b	33.26 ± 0.17 ^b
ME	3.09 ± 0.05 ^c	0.71 ± 0.09 ^c	32.80 ± 0.78 ^c
AE	2.89 ± 0.28 ^c	0.75 ± 0.22 ^{bc}	20.42 ± 0.18 ^d

Mean ± standard deviation of triplicate determined in dry weight basis.

HE: Hydro-distillation extract, EE: Ethanol extract, ME: Methanol extract, AE: Acetone extract.

Means within a column followed by the same letter are not significantly different according to LSD test at p<0.05.

Table 3: Diameter of Inhibition zone (DIZ)* of extracted EO of dill seeds (10mg/L) against tested *Aspergillus* strains growth.

HE	EE	ME	AE	DMSO	Clotrimazole
<i>Afla I (A. flavus ATCC 16875)</i>					
11.00 ^b	8.33 ^c	8.00 ^c	8.33 ^c	0.00 ^d	18.00 ^a
<i>Afla II (A. flavus IFO 6343)</i>					
18.33 ^a	12.00 ^c	11.00 ^{cd}	9.67 ^d	0.00 ^e	15.00 ^b
<i>Aniger I (A. niger CAIM 147)</i>					
12.33 ^c	20.33 ^a	10.00 ^d	11.33 ^{cd}	0.00 ^e	16.67 ^b
<i>Aniger II (A. niger DSM 731)</i>					
20.67 ^a	11.33 ^c	10.67 ^c	11.33 ^c	0.00 ^d	18.00 ^b
<i>Aniger III (A. niger NRRL 337)</i>					
11.33 ^c	20.67 ^a	7.67 ^d	6.00 ^e	0.00 ^f	16.33 ^b
<i>Aoryz (A. oryzae NRRL 9362)</i>					
12.00 ^c	20.00 ^a	8.00 ^c	10.00 ^d	0.00 ^f	15.67 ^b
<i>Aparast (A. parasiticus NRRL 2999)</i>					
19.67 ^a	12.00 ^c	7.67 ^d	7.66 ^d	0.00 ^e	15.67 ^b
<i>Aphoen. (A. phoenicis CAIM 149)</i>					
10.00 ^b	8.67 ^c	7.67 ^c	5.00 ^d	0.00 ^d	15.67 ^a

*Mean of Diameter of inhibition zone (DIZ) in mm, including disc paper diameter of 4 mm.

HE: Hydro-distillation extract, EE: Ethanol extract, ME: Methanol extract, AE: Acetone extract.

DMSO: Dimethyl sulfoxide, Clotrimazole: commercial positive control.

Means within the same row followed by the same letter are not significantly different according to LSD test at p<0.05.

This was supported by El-Gayyar *et al* (2001) who found that DIZ for *A. niger* was 12 mm with 24 mg of dill seed extract by hydro-distillation. Also, Singh *et al* (2006) found that DIZ of EE were 100% and 82.5% against both of *A. niger* and *A. flavus*, respectively, while AE gave 42.3 and 56.5% effectiveness, respectively. These results came in agreement with Nada *et al.* (2018) who observed that HE gave DIZ results against, *A. flavus*, *A. niger*, *Penicillium expansum* and *P. islandicum*, by 70, 60, 34 and 33 mm respectively. Consequently, dill seed extracts can be considered a potential source of antifungal growth, as its EO has the ability to inhibit *Fusarium* species through damaging cytoplasm and cellular constituents as well as distortion of mycelia (Taotao *et al.*, 2016 and Mani-López *et al.*, 2021).

According to the previous results illustrated in Table (3), HE was the most effective against *Afla II*, *Aniger II* and *Aparast.*. Also, EE affected *Aniger I*, *Aniger III* and *Aoryz*. Accordingly those 2 extracts were selected to be tested against the above mentioned *Aspergillus* strains with serial dilutions.

Results of HE serial dilutions in Table (4) showed that, total count of *Afla II*, *Aniger II* and *Aparast.* were inversely proportional to the extract concentration. Viable count of *A. nigerII* was reduced by 1/3 initial inoculum count (322×10^4 CFU/ml) when using 5 mg/l of the extract, while at 10 mg/l, the total viable count recorded 5×10^4 CFU/ml and no growth was recorded at higher concentrations. On the other hand using (15mg/l) extract decreased the total count of *Afla II* and *Aparast.* from initial inoculum (250×10^4) to (11×10^4 CFU/ml) and from (266×10^4) to (18×10^4) CFU/ml, respectively. And at 20 mg/l, HE inhibited fungal growth completely. Accordingly to the previous data revealed in Table (4), growth of *Aniger II* was totally inhibited at 15 mg/l of HE while *Afla II* and *Aparast.* growth was totally inhibited at 20 mg/l concentration.

Table 4: Fungal count* of MIC** values using HE (mg/l) against the three *Aspergillus* selected strains.

<i>Aspergillus</i> strains	0	5	10	15	20	30
<i>Afla II</i>	250×10^4	192×10^4	87×10^4	11×10^4	0	0
<i>Aniger II</i>	322×10^4	110×10^4	5×10^4	0	0	0
<i>Aparast.</i>	266×10^4	180×10^4	90×10^4	18×10^4	0	0

* Fungal count: CFU/ml

**MIC: the minimum inhibitory concentration

Concerning EE serial dilutions illustrated in Table (5), results indicated 15 mg/l stopped totally *AnigerI* growth. *Aoryz.* count decreased from 273×10^4 CFU/ml at 5 mg/l to 12×10^4 CFU/ml at 15 mg/l of EE, while at 20 mg/l no growth was noticed. Same results were obtained *Aniger III* at 15 mg/l as total viable count of initial inoculum was reduced from 392×10^4 CFU/ml to 4×10^4 CFU/ml. But no growth was observed at 20 mg/l by the same dill seed oil. Accordingly, MIC of EE was 15 mg/l for *Aniger I*, while it was 20 mg/l for both *Aniger III* and *Aoryz*.

Table 5: Fungal count* of MIC** values using EE (mg/l) against the three *Aspergillus* selected strains.

<i>Aspergillus</i> strains	0	5	10	15	20	30
<i>Aniger I</i>	415×10^4	362×10^4	17×10^4	0	0	0
<i>AnigerIII</i>	392×10^4	214×10^4	19×10^4	4×10^4	0	0
<i>Aoryz.</i>	390×10^4	273×10^4	52×10^4	12×10^4	0	0

* Fungal count: CFU/ml

**MIC: the minimum inhibitory concentration

Results were supported by Tian *et al.* (2012) who observed that dill seed extracts have ability to disrupt permeability barrier of plasma membrane of fungi and can cause morphological change in the cell. Again, Ghadimipour and Eveghad (2015) proved that, the BHA has an important role as antifungal agent in culture media. Taotao *et al.* (2016) stated the BHA can cause cytoplasm and cellular constituents leaking and destroy the fungal mycelia. Fung *et al.* (1977) stated that BHA had an inhibitory effect on growth and toxicity of six *Aspergillus flavus* of non-toxigenic strains, while BHT at the same concentration had no visible inhibitory effect. Also as a result Raccach (1984) reported that inhibition mechanism of bacteria, yeasts and molds growth by phenolic antioxidants has been found to affect the cellular membrane function and composition as well as the synthesis of DNA, RNA, protein, lipid, and the function of the mitochondria, these effects were also supported by Ilhami *et al.* (2004) who confirmed that the inhibitory powers of BHA are due to disruption of cytoplasmic membrane of microbial cell, also phenolic compounds may function as a membrane perturbed which is inserted between lipid layer of the microbial cell, causing disruption of ordered state of the alkyl chains.

Conclusion

It is recommended to use dill HE (light yellow color) with foods highly expected to be infected with to *A. flavus* IFO 6343, *A. niger* DSM 731 and *A. parasiticus* NRRL 2999 at 15 and 20 mg/l, while dill EE (pale yellow color) is recommended to be used with foods to prevent food from contamination with *A. niger* CAIM 147, *A. niger* NRRL 337 and *A. oryzae* NRRL 9362 at 15 and 20 mg/l.

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