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# *Ocimum Basilicum* : A Candidate Plant Against Aflatoxins Production with Antioxidant Activity

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## Abstract

The leaves of *Ocimum basilicum* have been traditionally used as a food spice, in perfumery and medical industries. The crude plant extract of leaves was tested as antifungal using *Aspergillus parasitiucus* and *Aspergillus flavus* as fungal strains., moderate growth inhibition was observed even at the lowest concentration (1mg/mL) of *Ocimum Basilicum* extract applied. The anti-aflatoxigenic potentials of the leaves of *Ocimum Basilicum* were investigated for its effects on aflatoxins (B1, B2, G1, and G2) production in maize flour. Four maize flour samples inoculated with *Aspergillus flavus* and *Aspergillus parasitiucus* were treated with plant leaves powder and stored for the period of six months at 28°C with 16% moisture level. At the end of each month, aflatoxin (AFB1, AFB2, AFG1, and AFG2) concentrations were determined using high performance liquid chromatography HPLC., and as a result; the contents of aflatoxins have been reduced in tested samples. In addition, the present work included the free radical scavenging activity of the *Ocimum Basilicum* extract using DPPH assay. The methanolic extract of Ocimum Basilicum (IC50 value = 6.20 mg/mL) showed stronger radical scavenging activity than that of synthetic antioxidant BHT (IC50 = 14.31mg/mL), It is noted that, the lower the IC50 value, the higher the antioxidant activity.

## 1. Introduction

Bad harvesting practices, improper drying, handling, packaging, storage and transport conditions contribute to fungal growth and increase the risk of mycotoxin production [1]. Aflatoxins are a group of naturally occurring mycotoxins that are produced by two closely related fungi: *Aspergillus flavus* and *Aspergillus parasitiucus* They are highly toxic, carcinogenic and mutagenic in humans and animals [2]**.** Aflatoxins (AFs) is one of the most important naturally occurring mycotoxins in agricultural products. AFs are coumarinsdifurano-composed from two furans and a coumarin ring [3,4]. These fungi can grow under favorable temperature and humidity conditions on different improperly stored commodities and food stuffs like maize, rice, peanuts, chili peppers, vinegar, cotton seed, corn, tree nuts, wheat, and in spices [5]. Aflatoxins enter into the food stuff when impure food is processed where they have been transferred in both animals and human food. Aflatoxins are reported to be genotoxic, carcinogenic, teratogenic, and hepatotoxic in nature., For example, its toxicity is 416 times more than melamine, 68

times more than arsenic and ten times of in potassium cyanide [6-8]. Among 18 different types of AFs identified, major members are Aflatoxins B1, B2, G1, G2, M1, and M2 (AFM1 and AFM2 are the hydroxylation products of AFB1 and AFB2, have been identified in milk and dairy products). Aflatoxin B1 (AFB1) is the most abundantly produced and the most toxic followed by G1, B2, and G2. AFB1 is classified by the International Agency of Research on Cancer as Group 1 carcinogen [9-11]. *Aspergillus flavus and Aspergillus parasitiucus* are the most prevalent microbes responsible for contamination of food, feed and other agricultural commodities, and have been associated with several acute and chronic aflatoxin outbreaks in the past [12,13].

Medicinal plants and their products have been an important role over the world for humans since prehistoric times. *Ocimum Basilicum* has previously been reported as antifungal, antibacterial, antiradical, antimycotoxigenic and antioxidant activities [14,15]. *Ocimum Basilicum* as medicinal plant was investigated for inhibition of AFTs in stored corn white. The aim of this research was to evaluate the potential of *Ocimum Basilicum* to inhibit AFTs produced by *Aspergillus flavus* and *Aspergillus parasitiucus* during storage, and also its antioxidant activity. The treatment efficiency was evaluated on the basis of AFT inhibition and nutritional value of the stored maize flour samples over the period of six months of storage. The free radical scavenging activity of the *Ocimum Basilicum* extract had been studied using DPPH, and the antioxidant activity of test sample was expressed as an IC50 value.

## 2. Methodology

### *1-2- Chemicals and reagents*

Acetonitrile and methanol (HPLC-gradient grade) were obtained from Sigma-Aldrich (Germany)., Standard AFB1, AFB2, AFG1, and AFG2 were obtained from Supelco (Spin)., Immunoaffinity columns for AFs were purchased from Alfa Test (Germany)., Dimethyl Sulfoxide., Sabouraud 4% dextrose agar (Avonchem., UK), terbinafin from local markets., Whitman No. 4 filter paper (Whatman International, Maidstone, UK)., maize grains samples were purchased from local markets., DPPH (Sigma-Aldrich, Germany).

### *2-2-Apparatus*

HPLC method was performed on a Shimadzu (Kyoto, Japan) liquid chromatography system, equipped with a model LC-20 AT pump and CTO-20A oven. The detector was a fluorescence detector (Shimadzu RF-10 AXL, Kyoto, Japan) programmed to monitor at 365 nm for excitation and 435 nm for emission. $AF_S$ were completely separated using a stainless steel column of dimension ($4.6\times250$ $mm^2$) packed with symmetry C18 and 4µm particle size (Merck, Germany). Memmert oven (Germany)., Finally, Uv-Vis spectrophotometers (phylo., Italy)., Shaker apparatus., and a rotary evaporator system from ( Stuart., UK) were used.

### *3-2-Plant collection and extraction*

Leaves of *Ocimum Basilicum,* Fig. 1., were collected from plantation in Damascus (Syria). Plant material was cleaned, washed gently in running water. Leaves were excised and shade dried on a white paper for two weeks, then a commercial blender with dried leaves were grounded into fine powder, and the grounded samples were stored in dark in polythene bags at $4^oC$ for further work. Antifungal extracts were prepared from the leaves by dissolving 25 g powder in 100 ml methanol (80%) and shaken for 48 h at room temperature. The mixture was filtered to separate the extract from residues. The extracts were concentrated by using rotary evaporator at 45°C and stored in a refrigerator at 4°C.

**Figure 1.** *Ocimum Basilicum* L. plant

### *4-2-Antifungal activity*

Antifungal activity of crude plant extract of leaves was screened by agar tube dilution method. *Aspergillus parasitiucus* and *Aspergillus flavus* were used as fungal strains. Test sample was prepared by dissolving 24mg plant extract in 6ml of dimethyl sulfoxide DMSO. Fungus media was prepared by dissolving 6.5g of sabouraud dextrose agar in 100ml of sterilized distilled water and pH was adjusted to 5.6. Agar was poured in screwed capped test tubes and the autoclaved. Agar containing test tubes were allowed to cool up to 50°C, added test sample in each tube and allowed to solidify in slanting position. After solidification, each slant was inoculated by 4mm diameter piece of fungal strain. Positive control was adjusted by adding terbinafin at concentration(5 µg/mL) and negative control slants were without test sample. These test tubes were incubated at 28°C for seven days. Readings were measured in mm and growth inhibition was calculated with reference to negative control. Antifungal assay was performed at different concentrations of extracts (2, 4, 8 mg/ml) to find the optimum effective concentration of extract against *Aspergillus parasitiucus* and *Aspergillus flavus*. Percentage inhibition was calculated by following formula, Eqn. 1 [16]:

$$\text{Percent inhibition} = \frac{\text{Growth in sample(cm)} - \text{Growth in negative control (cm)}}{\text{Growth in sample(cm)}} \times 100 \quad \textbf{Eqn. 1}$$

### *5-2-Maize flour samples treatment*

Toxin free maize flour samples (6 kg) were obtained by grinding toxin free maize grains. The sample was dried in an oven at 60°C and divided into 20 parts (200g). Each was autoclaved, then, moistened (25%) with sterilized distilled water. The maize flour samples were inoculated by 4ml of *Aspergillus flavus* suspension (1×107 spores/ml) and also another maize flour samples were inoculated by 4 ml of *Aspergillus parasitiucus* suspension. Plant leaves powder at three different concentrations (5, 10 and 15%w/w) was added separately in 200g of inoculated maize flour samples. Non-treated maize flour samples were considered as control. The treated and control samples were stored at 25% moisture and 28°C for six months. At the end of each month, maize flour samples were drawn and AFTs were determined by HPLC.

### *6-2-Aflatoxin extraction and analysis*

Tested maize flour samples (25g) were carefully weighed and mixed with 5 g of sodium chloride and 125 ml of extracting agent ACN/$H_2O$ (70:30 v/v) into a blender jar. After blending and mixing for 2min at high speed, the extract was filtered through Whitman No.4 filter paper, 15ml was removed and

30ml of water was added. It was mixed thoroughly and the extract was filtered through whatman No. 4 filter paper. Finally, 15ml of the reconstituted extract were passed through a immunoaffinity column at a flow rate of 2ml/min. The column was washed with two aliquots of 10ml ultrapure water at a flow rate of 5ml/min, and the aflatoxin were slowly released from the antibody using 1ml of methanol and eluted with 1ml ultrapure water in vials. Vital was fully mixed in a vortex and made suitable for the high-performance liquid chromatography (HPLC) [17]. The chromatographic experiments for determine AFs were carried out using HPLC-FLD as follows: Mobile phase: Water, acetonitrile, and methanol (60:20:20, v:v:v), Column oven temperature: 40°C, Injection volume: 50µL, Flow rate: 1.3 mL/min, Detection fluorescence: excitation at 365 nm, emission at 435 nm [18]. The AFTs inhibition was calculated using the relation shown below in the Eqn. 2, where X is the concentration of AFTs in treated samples and Y is the concentration of AFTs in control.

$$\text{Inhibition}(\%) = \frac{X-Y}{Y} \times 100 \quad \textbf{Eqn. 2}$$

### *7-2-DPPH radical scavenging assay*

The method of Adel F. Ahmed *et al*. [19] was utilized to test the DPPH radical scavenging activity. DPPH was dissolved in methanol to give a 200µM solution; 10µL of the extract sample in methanol (or methanol itself as blank control) was added to 175µL of the methanol DPPH solution. Different concentrations were tested (20, 10, 5, 2.5 and1.25 mg/mL ). After mixing, the lowering in absorbance was determined at 515nm after 20min. The actual decrease in absorption induced by the test compound was calculated by subtracting that of the control. The antioxidant activity of test sample was expressed as an IC50 value, i.e. the concentration in mg/ml that inhibits by 50% and was calculated from the concentration-effect linear regression curve. BHT was used for positive control. The DPPH radical scavenging activity of each sample was calculated as the percentage inhibition., Eqn. 3.

$$\%\ \text{Inhibition of DPPH radical activity} = [(A^0 - A^1)/A^0] \times 100\% \qquad \textbf{Eqn. 3}$$

Where: $A^0$ is the absorbance of the DPPH itself; $A^1$is the absorbance of sample and the positive control.

## 3.Results and Discussion

### *1-3-Antifungal activity of Ocimum Basilicum*

Extract of *Ocimum Basilicum* was studies against *Aspergillus flavus* and *Aspergillus parasitiucus.*, and the antifungal activities are given in **Table 1**. Moderate growth inhibition was observed even at the lowest concentration (1mg/mL) of *Ocimum Basilicum* extract applied. At 8mg/mL concentration complete inhibition was observed for *Aspergillus flavus* and *Aspergillus parasitiucus* fungal.

**Table 1:** Antifungal activities of *Ocimum Basilicum* extract (%)

| Fungal | *Ocimum Basilicum* extract | | | | Terbinafin (control) (5 µg/mL) |
|---|---|---|---|---|---|
| | 1mg/mL | 2 mg/mL | 4mg/mL | 8 mg/mL | |
| *Aspergillus flavus* | 35 | 55 | 78 | 100 | 61 |
| *Aspergillus parasitiucus* | 38 | 60 | 84 | 100 | 66 |

### *2-3-Aflatoxin inhibition*

In this research, the validated methods were used and the internal and external quality control experiments were performed. Regarding internal quality control, the accuracy and precision of the methods were verified. For this purpose, AFB1, AFB2, AFG1 and AFG2 recoveries were recorded by analyzing a blank sample, spiked at 4 ng/g for AFS. The recovery rate values for aflatoxins were close to each other and about 78-84% and the mean coefficient of variation were 4.2%. The AFTs level was corrected, according to the recovery value. LOD and LOQ for AFB1 were 0.06 µg/kg and 0.100 µg/kg, respectively, LOD and LOQ for AFB2 were 0.05 and 0.10µg/kg, respectively, LOD and LOQ for AFG1 were 0.08 and 0.13 µg/kg, LOD and LOQ for AFG2 were 0.09 and 0.14µg/kg, respectively. The linearity in the working standard solutions at three determinations of six concentration levels was reliable, 0.9996 for AFB1 and 0.9992 for AFB2, 0.9987 for AFG1 and 0.09985 for AFG2.

It has been reported that *Ocimum Basilicum* possesses antifungal, antibacterial, antiradical, antimycotoxigenic and antioxidant properties [14]. *Ocimum Basilicum* plant leaves can be used as an antiaflatoxigenic agent. Aflatoxins cause deterioration of food on large scale. To ensure human and economy, antifungal agents offer an alternative to prohibit from fungal contamination in food stuff and its extract having a lot of bioactive compounds to control aflatoxin production [18].

Maize flour samples (which were inoculated with *Aspergillus flavus and Aspergillus parasitiucus* fungal) were treated with different concentrations of *Ocimum Basilicum* plant leaves powder were stored for a period of six months and the AFTs) AFB1, AFB2, AFG1 and AFG2) concentrations were determined at the end of each month. The obtained results are shown in **Table 2**.

Maize flour treated with plant powder showed that *Ocimum Basilicum* plants have promising activity to inhibit of AFTs production by *Aspergillus flavus* and *Aspergillus parasitiucus*. prior reports also showed that spices, plant extracts, essential oils have ability to play a role as antifungal against toxigenic microorganisms [14, 20,21]. *Ocimum Basilicum* plant in this study prevented *Aspergillus flavus* and *Aspergillus parasitiucus* growth efficiently and as a result, the contents of aflatoxins were also reduced. Results of the present study are in line with Dahham *et al*. [22] who announced that the methanolic extracts of *P. granatum* have an effective antifungal activity.

In the present study four mycotoxins (B1, B2, G1 and G2) are present in aflatoxins. These are produced by two closely linked fungi *Aspergillus flavus* and *Aspergillus parasitiucus*. Toxigenic strains of *Aspergillus flavus* produce only two types of aflatoxins, B1 and B2. Strains of *Aspergillus parasitiucus* produce aflatoxins of four types (B1, B2, G1 and G2) [23]. In the present study, and all *Ocimum Basilicum* extracts showed a good result against *Aspergillus parasitiucus* than *Aspergillus flavus*.

### *3-3-DPPH radical scavenging assay of extract of Ocimum Basilicum*

Free radical scavenging activity of the *Ocimum Basilicum* extract was measured by DPPH test. Free radical scavenging capacity increased with increasing extract concentrations. The IC50 value was calculated to determine the concentration of the sample required to inhibit 50% of radical. The methanolic extract of *Ocimum Basilicum* (IC50 value = 6.20 mg/mL) showed stronger radical scavenging activity than that of synthetic antioxidant BHT (IC50= 14.31mg/mL). It is noted that, the lower the IC50 value, the higher the antioxidant activity, this is consistent with the reference studies [24,25].

Survey literature indicated that phytochemical screening of aqueous extract and elemental analysis of *O. basilicum* showed the presence of saponins, tannins and cardiac glycosides. The phenolic compounds

known to be reported the most in basil are phenolic acids and flavonol-glycosides. Phenolic acid type in the form of caffeic acid derivatives has been also detected [26-30]. This richness in aromatic compounds play a major role in various biological activities.

**Table 2:** Aflatoxin content* (ppb) in maize flour treated with Ocimum Basilicum leaves at incubation period of 1-6 month.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Month 1** | ***Ocimum Basilicum* (g/100gm)** | ***Aspergillus flavus*** | | ***Aspergillus parasitiucus*** | | | |
| | | **B1** | **B2** | **B1** | **B2** | **G1** | **G2** |
| | 00 | 10.71±0.02 | 3.56±0.03 | 2.57±0.06 | 2.23±0.08 | 1.14±0.03 | 1.81±0.07 |
| | 05 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 |
| | 10 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 |
| | 15 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 |
| **Month 2** | *Ocimum Basilicum* (g/100gm) | *Aspergillus flavus* | | *Aspergillus parasitiucus* | | | |
| | | B1 | B2 | B2 | G1 | G2 | B2 |
| | 00 | 18.78±0.07 | 6.73±0.05 | 4.41±0.06 | 6.86±0.05 | 2.39±0.09 | 3.61±0.05 |
| | 05 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 |
| | 10 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 |
| | 15 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 |
| **Month 3** | *Ocimum Basilicum* (g/100gm) | *Aspergillus flavus* | | *Aspergillus parasitiucus* | | | |
| | | B1 | B2 | B2 | G1 | G2 | B2 |
| | 00 | 26.71±0.06 | 10.84±0.05 | 6.97±0.04 | 10.73±0.03 | 3.45±0.03 | 5.35±0.04 |
| | 05 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 |
| | 10 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 |
| | 15 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 |
| **Month 4** | *Ocimum Basilicum* (g/100gm) | *Aspergillus flavus* | | *Aspergillus parasitiucus* | | | |
| | | B1 | B2 | B2 | G1 | G2 | B2 |
| | 00 | 35.13±0.05 | 18.23±0.07 | 10.66±0.05 | 17.52±0.03 | 5.93±0.24 | 9.43±0.05 |
| | 05 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 |
| | 10 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 |
| | 15 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 |
| **Month 5** | *Ocimum Basilicum* (g/100gm) | *Aspergillus flavus* | | *Aspergillus parasitiucus* | | | |
| | | B1 | B2 | B2 | G1 | G2 | B2 |
| | 00 | 45.81±0.09 | 26.52±0.04 | 20.41±0.64 | 25.13±0.07 | 9.47±0.04 | 18.41±0.64 |
| | 05 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 |
| | 10 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 |
| | 15 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 | 0.00±0 |
| **Month 6** | *Ocimum Basilicum* (g/100gm) | *Aspergillus flavus* | | *Aspergillus parasitiucus* | | | |
| | | B1 | B2 | B2 | G1 | G2 | B2 |
| | 00 | 57.51±0.08 | 34.82±0.05 | 28.31±0.64 | 35.93±0.09 | 16.87±0.05 | 25.31±0.04 |
| | 05 | 2.14±0.05 | 1.09±0.03 | 1.04±0 .05 | 1.05±0.04 | 0.34±0.02 | 0.94±0 .05 |
| | 10 | 1.31±0.05 | 0.48±0.01 | 0.55±0.06 | 0.74±0.03 | 0.19±0.03 | 0.67±0.04 |
| | 15 | 0.00±0 | 0.00±0 | 0.15±0.02 | 0.44±0.03 | 0.00±0 | 0.19±0.05 |

* The data obtained in this study were expressed as mean ± SD. All tested samples were statistically analyzed.

## Conclusion

Methanolic extract of *Ocimum Basilicum* leaves has shown a good reactivity as antioxidants and as antifungal. The methanolic extract of *Ocimum Basilicum* showed stronger radical scavenging activity than that of synthetic antioxidant BHT by using DPPH. The anti-aflatoxigenic potentials of *Ocimum Basilicum* extract has shown a good result against producing aflatoxins by two fungi *Aspergillus flavus* and *Aspergillus parasitiucus* in maize flour samples. Thus, it is recommended to deepen studies on the use of important medicinal plants that have a good effect against the growth of fungi to limit the spread and growth of fungi producing aflatoxins, which in turn constitute a carcinogenic factor.

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