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Inhibition of *Aspergillus flavus* growth and aflatoxin B1 production by olive mill wastewater

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The study aimed to investigate the effect of crude olive mill wastewater (OMW) and phenolic extract from olive mill wastewater (PE) on *Aspergillus flavus* growth and aflatoxin B1(AFB1) production in SMKY broth medium and in maize powder. The SMKY medium and maize powder were supplemented with various concentrations of OMW and PE, inoculated with *A. flavus* and incubated at 28 °C for 7 days. The mycelia biomass (dry mycelium weight) and fungal colony diameter were determined in SMKY and maize powder, respectively. Aflatoxin B1 synthesis was quantified by direct competitive ELISA method and chemical compositions of PE were analyzed by HPLC. In SMKY medium, results showed that mycelia biomass was reduced to 50 % and 46.2 % with concentrations of 250 mg/L and 4 g/kg of the PE and OMW, respectively. Complete inhibition was obtained with 500 mg/L and 10 g/L of the PE and OMW, respectively. In maize powder, PE and OMW reduced the mycelial growth by more than 50 % with 800 mg/L and 16 g/kg, and complete inhibition was obtained at 1000 mg/kg and 20 g/kg, respectively. Aflatoxin B1 was totally inhibited in SMKY medium to witch 400 mg/L and 8 g/L of PE and OMW, respectively, had been added. Results obtained in the present study indicate the possibility of exploiting PE and crude OMW in the control of aflatoxigenic *A. flavus* in stored foods products**.**

Keywords: *Aspergillus flavus*, aflatoxin B1, maize, olive mill wastewater, phenolic extract, direct competitive ELISA method.

INTRODUCTION

Fungi are significant destroyers of foodstuffs and grains during storage (Bryden, 2012). More than 25 % of the world's cereals are contaminated with mycotoxins (Richard, 2007). Of all the mycotoxins, aflatoxins (AFs), produced by *Aspergillus flavus*, *A. parasiticus*, and *A. nomius*, are of greatest concern in terms of incidence in food and feed and toxicity to humans and animals especially in countries with warm climate. Aflatoxin B1 (AFB1) is the most frequent and toxic, and is a powerful hepatotoxic, teratogenic and mutagenic compound. It was classified within class 1 of human carcinogens (IARC, 2012). Because of their deleterious effect on human and animal health, and their importance in the international food trade, food contaminated with these toxigenic fungi has received worldwide attention. Numerous studies have been conducted to determine the effects of various food additives, preservatives, and chemical, and environmental condition to inhibit growth and mycotoxin production. Effective and efficient control of toxigenic fungi can be achieved by the use of synthetic chemical fungicides but the application of high concentrations of these synthetic chemicals in an attempt to control postharvest deterioration of food commodities increases the

risk of toxic residues remaining in the products (Kumar and Kalita, 2017). Effective strategies and tools are required to address the prevention, control, and suppression of aflatoxigenic fungi and AFs in food and feed.

Recently, there has been increasing interest in strategies for controlling phytopathogens with natural substances released by plants, namely allelochemicals (allelopathic compounds), rather than with chemical compounds (Khan et al., 2016). Thus, there are reasons to develop alternatives to conventional pesticides, alternatives that are of low environmental risk and present a lower risk of the development of pesticide resistance in the pathogen; a characteristic that will enhance the durability of agriculture and the environment. On the other hand, massive quantities of agro-industrial by-products are produced worldwide every day. A great proportion of these by-products could be used in beneficial ways as fertilizers or bio pesticides (Welzenbach et al., 2009). An example of agro-industrial by-product with high potential is olive mill wastewaters which are generated during olive oil extraction by the traditional milling and press processes (Zbakh and El-Abbassi, 2012). The antimicrobial and antifungal activity of the phenolic compounds they contain has also been reported (Lagrouh et al., 2017).

Maize is second only to wheat among world cereal crops for the quantity produced and is often invaded by *A. flavus* before harvest (Tola and Kebede, 2016). In order to evaluate *A. flavus* growth and AFB1 production, maize was experimentally contaminated with *A. flavus* and treated with phenolic extract from olive mill wastewater (PE) and with crude olive mill wastewater (OMW) from an olive tree of the *chemlal* variety, which is largely cultivated in Kabylia (Algeria).

MATERIALS AND METHODS

Sampling

Olive mill wastewater (OMW) came from discontinuous three phase olive processing in the village in Tizi-Ouzou, Kabylia region, the most important producer of olive oil from *chemlal* variety olive trees in Algeria. This sample was taken at the middle of the olive harvest season, fractioned, and kept at -40 °C. To study the antifungal and anti-aflatoxigenic effects of crude OMW, samples were frozen and lyophilised for 24 h, and dehydrated for 6 h to obtain a powder.

Ten kg of maize grains (*Zea mays* L.) imported from Argentina and intended for human consumption were investigated in this study. A sample was collected in June 2015 from the market in Tizi-Ouzou, Algeria. The sample was fractioned (200 g) and stored at 4 °C until the analysis. The maize grains were checked for sterility and absence of AFB1.

Reagents and diagnostic kit

Ethyl acetate, hexane, methanol, and HPLC-grade solvents were purchased from Merck (Germany). All solvents were of analytical grade and used without further purification. Folin-Ciocalteu reagent was purchased from Sigma-Aldrich (France). Gallic acid, hydroxytyrosol, tyrosol, *p*-coumaric acid, protocatechuic acid, oleuropein, vanillic acid and caffeic acid standards were purchased from Sigma–Aldrich (France). Hydrochlorid acid and sodium carbonate were from the ICS Science group (France). The *ELISA* kit was a *Celer AFLA B1* (MA220/MA221; *Tecna R & D•Diagnostics-Biotechnolgy*, Italy). All other solvents and reagents were of analytical grade and were purchased from Merck, Germany.

Culture of fungus and medium

A toxigenic strain of *A. flavus* (NRRL 3251^T) was used for aflatoxin estimation. The strain, procured from the Biology of Microbial Systems Laboratory (LBSM, ENS Kouba), was stored in 20 % glycerol at -40 °C.

Physicochemical characteristics of OMW

The electrical conductivity (EC) and the pH of OMW were directly measured in the sample using a pH-meter. The OMW dry matter determined before and after drying the sample at 105 °C. Protein concentration was measured by Bradford method (1976) and the total lipid content was determined by the method of Knight et al.(1972). Total sugar content was measured according to the method of Dubois et al.(1956) and reducing sugars were measured by the DNS method (Miller, 1959). Analyses were carried out in triplicate, and the results are given as mean values ± SD.

Extraction of Phenolic compounds from OMW

Liquid–liquid extraction with ethyl acetate was carried out according to the method of De Marco et al.(2007). After acidification at pH 2 with HCl (4 N) and the lipid fraction was removed with hexane. The mixture (solvent-OMW) (10 mL, v/v) was shaken vigorously and centrifuged for 15 min

at 4000 *g*. The phases were separated, and the extraction was repeated three times in succession. All the runs were performed at ambient temperature (25 °C). The ethyl acetate was evaporated in a rotary evaporator at 40 °C. The dry residue was dissolved in 3 mL of methanol for the quantification and characterization of phenolic compounds. For the assessment of the antifungal and anti-aflatoxigenic effects of PE, the sample was lyophilised after extraction and dehydrated for 5 h to obtain a powder.

HPLC separation and identification of phenolic compounds

The analysis was performed on a HPLC-UV system (Ultimate 3000, Thermo Fisher Scientific®) and UV detection was operating at 280 nm. The column used for polyphenol analysis was a reversed phase Hypurity, C18, 250 x 4.6 mm, 5 µm. The solvent system used a gradient of solvent A (water: trifluoro acetic acid) (90:10, v/v) and solvent B (acetonitrile: methanol) (80:20, v/v).

The flow rate was 1 mL/min and the injection volume was 20 µL. The phenolic compounds were identified on the basis of their retention time in comparison with phenolic standards. The total phenolic compounds content of OMW extracts was determined using Folin–Ciocalteau reagent (Singleton and Rossi, 1965). The absorption at a wavelength of 750 nm was determined with a spectrophotometer. The data are expressed as gallic acid equivalents (g gallic acid/100g of OMW).

Effect of PE from OMW and crude OMW on mycelial growth

In SMKY broth medium

An aliquot of 25 mL of SMKY broth medium (200 g of sucrose; 0.5 g $MgSO_4$, 7H2O; 0.3 g KNO_3 and 7 g yeast extract distilled water, 1000 mL; pH 5.6) was taken in a 100 mL conical flask. Amounts of PE (50, 125, 250, 400 and 500 mg/L) and OMW (2, 4, 6, 8, and 10 g/L) were added to medium. The flasks were aseptically inoculated with a 5.0 mm disc of 7 days old culture of aflatoxigenic *A. flavus* in PDA medium. The control sets comprise the medium without PE or OMW. The flasks were incubated for 7 days at 28 °C. Each control and treated condition was tested in triplicate. After incubation, the content of each flask was filtered through Whatman filter paper n°1. The mycelial dry weight (biomass) was measured after filtration and drying at 105 °C.

In maize powder

The initial humidity of the maize grains was 11.5 %. The grains were ground to powder in order to obtain a radial mycelial growth. The powder was autoclaved at 115 °C for 30 min. For all experiments, maize powder was hydrated to 30 % of humidity by the addition of sterile distilled water (Hesseltine, 1976).This moisture content was chosen because it allowed *A. flavus* to synthesise significant quantities of AFB1. Concentrations of PE (250, 500, 800 and 1000 mg/kg) and OMW (8, 12, 16 and 20 g/kg) were added to the sterile distilled water. The hydrated maize powder (20 g for each Petri dish) was inoculated with 100 µL of 10^6 spores/mL of *A. flavus* (NRRL 3251^T). The Petri dishes were incubated at 28 °C for 7 days. The effect of PE and OMW was determined by measuring the fungal colony diameter using a centimeter scale, after 7 days of incubation.

Effect of PE from OMW and crude OMW on production of AFB1

In SMKY broth medium

The SMKY filtrate was extracted with 20 mL methanol in conical tubes. After incubation for 3 h at room temperature, methanolic extracts of each tube were centrifuged at 4000 *g* for 20 min (Nabney and Nesbitt, 1965).The extract was evaporated to dryness in a water bath at 50°C under vacuum. The residues were dissolved in 2 mL of methanol and were filtered through a 0,45 µm micro filter (Millex-HP Syringe Filter Unit, Millipore Corporation, USA) then analyzed by Thin Layer Chromatography (TLC). Quantification of AFB1 was performed by direct competitive ELISA assay using a polyclonal antibody specific to AFB1 with a sensitivity of detection of 1 ppb.

In maize powder

AFB1 was extracted according to the *Celer AFLA B1* test kit directions. Briefly, the content of each contaminated plate (20 g) was mixed with 20 ml of methanol/water (70/30) and centrifuged at 4000 *g* for 20 min. The analysis methods were as described above (*In SMKY broth medium*).

Detection of aflatoxin B1 by TLC

The detection of AFB1 production by *A. flavus* was carried out on silica gel (60 plates 20 x 20 cm, Merck). The spotted plates were developed in chloroform: acetone (90:10, v/v) solvent system. Twenty-five microliters of each treatment and control was spotted on TLC plates and run for 45 min in a TLC tank. Plates were air dried and

observed under UV light (365 nm) for the presence and intensity of fluorescent spots (AOAC, 2005).

Direct competitive ELISA determination of aflatoxin B1

After the extraction and filtration, the sample was ready for determination of AFB1 following the *Celer AFLA B1* test kit directions. Briefly standard solutions used for making the calibration curve contained AFB1: 0, 1, 5, 20 and 40 ppb. The assay was performed in plastic microwells that had been coated with anti-aflatoxin antibody. In the premixing wells, the enzyme-labelled aflatoxin and the standard solutions, or the samples were mixed and then transferred into the anti-aflatoxin microtiter plate. Fifty microlitres of the standard/sample was dropped into wells and 100 µL of (AFB1-HRP) was added to each premixing well and 100 µL of the content of each premixing well was transferred immediately into the corresponding anti-aflatoxin B1 antibody coated microwell and incubated for 10 min at room temperature (25 °C). After incubation, the wells were emptied by inverting the micro plate and tapping it vigorously against the absorbent paper. It was washed 3 times with PBS- Tween (0.01 M). One hundred microliters of substrate (tetramethylbenzidine, TMB) was added to the wells and left in the dark for 5 min. The enzyme converted the colourless chromogen into a blue product. Addition of the stop reagent (50 µL of 2 M sulfuric acid) led to a colour change from blue to yellow. The intensity of the colour was inversely proportional to the concentration of AFB1 in the sample or standard, which was measured at 450 nm by micro ELISA Reader (Biotek EL 311). The curve of optical density of the standards was plotted, and the optical densities of the samples were compared with the curve to calculate the concentration of AFB1.

STATISTICAL ANALYSIS

All the measurements were replicated three times for each treatment. Data were expressed as means ± SD and were statistically analyzed by one-way analysis of variance (ANOVA) followed by the Tukey post-test. Values of $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Olive mill wastewater characterization

The physicochemical characteristics of the OMW sample used in these experiments are given in (Table. 1).

Table 1: Physicochemical characteristics of olive mill wastewater used in these experiments

Parameters	Value
pH	4.8 ± 0.00*
Dry matter (%)	11.85 ± 1.50
Lipids (g/ 100g)	1.11 ± 0.08
Total phenols (g/100g)	5.29 ± 1.67
Total sugars (g/100g)	11.39 ± 2.12
Reducing sugars (g/100g)	3.69 ± 0.90
Total proteins (g/100g)	1.04 ± 0.06

* Each value is the mean of three represented measurements ± SD

The OMW was slightly acidic, having a pH of value at 4.8 ± 0.00, and contain high levels of total sugar (11.39 ± 2.12 g/100g of OMW) and phenolic compounds (5.29 ± 1.67 g/100g of OMW). In contrast, the total protein concentration was extremely low (1.04 ± 0.06 g/100g of OMW). The composition of OMW has been widely discussed in the literature. OMW is an acidic effluent, having pH values from 3 to 5 and generally composed of water (83 to 94%), organic matter (4 to 16%) and mineral salts (0.4 to 2.5%) (Eroğlu et al. 2004). Oils (1 to 14%), polysaccharides (13 to 53%) (Hamdi, 1992; Aggoun et al., 2016), proteins (8 to 16 %), organic acids (3 to 10 %), polyalcohols (3 to 10 %) and polyphenols (2 to 15 %) are listed as its main organic contents. Mineral salts of OMW are mainly carbonates (21 %), phosphates (14 %), potassium (47 %) and sodium (7 %) (Dermeche et al., 2013). Both the quality and quantity of OMW are highly variable and are affect by several factors such as: type of production process, type of olives, use of pesticides and fertilisers, area under cultivation, climatic conditions, and harvesting time (i.e. stage of olive maturity) (Eroğlu et al., 2009 ; El-Abbassi et al., 2017).

HPLC identification of phenolic compounds

The total phenolic content of PE from OMW measured by Folin Ciocalteu assay and calculated from the calibration curve (R^2 = 0.998), was 5.290 ± 1.670 (g Gallic acid equivalent /100 g of OMW). Of the eight quantified phenolic compounds quantified, hydroxytyrosol was the most abundant with a proportion of (54.23% ± 0.22) (Fig. 1 and Table. 2).

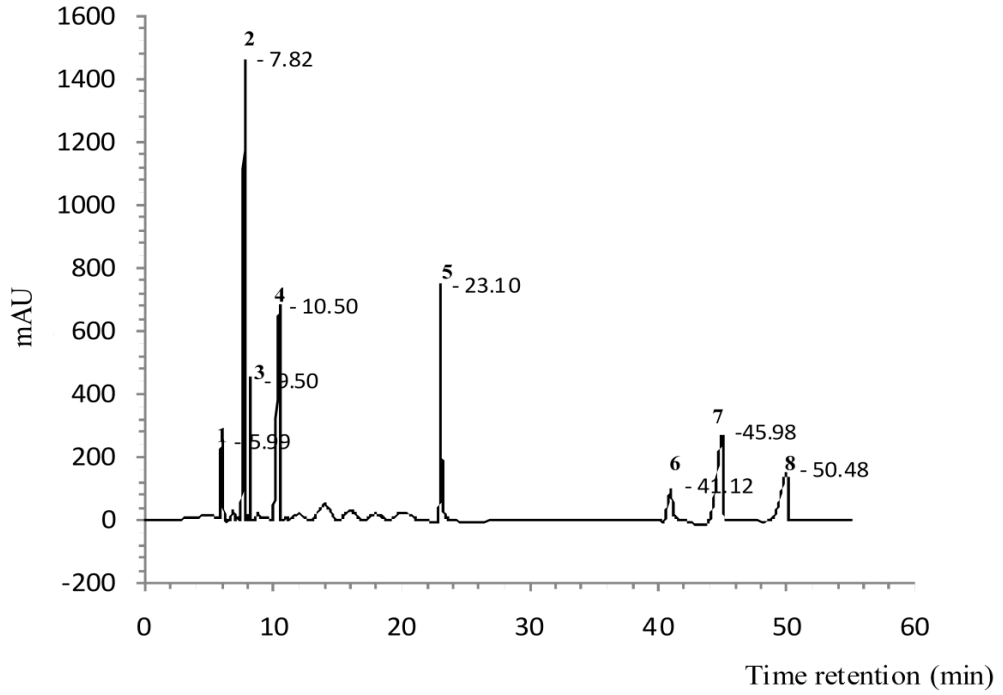

Figure. 1. HPLC chromatogram of the phenolic profile of OMW: (1) gallic acid, (2) hydroxytyrosol, (3) tyrosol, (4) *p*-coumaric acid, (5) protocatechuic acid, (6) oleuropein, (7) vanillic acid, (8) caffeic acid. Peak quantification was carried out at 280 nm. The main phenolic compounds were established by comparison with relative retention times of pure compounds.

Table 2: Retention time, concentration and relative abundance (%) of identified phenolic compounds of olive mill wastewater.

Phenolic compounds	Retention time (min)	Concentration (g of GAE*/100 g of OMW)	Proportion (%)
Gallic acid	5.99	0.337 ± 0.040	6.37 ± 0.22
Hydroxytyrosol	7.82	2.869 ± 0.012	54.23 ± 0.22
Tyrosol	9.50	0.506 ± 0.010	9.56 ± 0.12
***p*-coumaric acid**	10.50	0.253 ± 0.050	4.78 ± 0.39
Protocatechuic acid	23.10	0.759 ± 0.044	14.35 ± 0.57
Oleuropein	41.12	0.046 ± 0.017	0.87 ± 0.36
Vanillic acid	45.98	0.084 ± 0.021	1.58 ± 0.10
Caffeic acid	50.48	0.064 ± 0.007	1.21 ± 0.12
Total phenols	/	5.290 ± 1.670	100

* GAE: gallic acid equivalent. Each value is the mean of three represented measurements ± SD.

Polyphenols are found in OMW at concentrations ranging from 0.63 to 5.45 g/100 g of OMW (Dermeche et al. 2013), depending on the processing system used for olive oil production. Phenolic compounds generally comprise a great number of organic substances that have the common characteristic of possessing an aromatic ring with one or more substitute hydroxyl group and a functional chain. The widespread classes of hydrophilic phenolic compounds identified and quantified in OMW include phenolic acids, phenolic alcohols, flavonoids, secoiridoids, and lignans. Up to now, more than 50 phenolic compounds have been identified in OMW (Caporaso et al. 2017). A group of polyphenols found in OMW is derived from cinnamic acid such as *p*-coumaric acid, caffeic acid, and ferulic acid. Another group of phenolic compounds present in OMW derives from benzoic acid: benzoic acid, p-hydroxybenzoic acid, protocatechuic acid, vanillic acid, veratric acid, gallic acid, syringic acid and β-3,4-dihydroxyphenyl ethanol derivatives, such as tyrosol, hydroxytyrosol and 3,4-dihydroxyphenylglycol. In addition, OMW contains flavonoids, which are mainly represented by apigenin, luteolin, quercetin, and rutin together with secoiridoids (oleuropein). Of all olive polyphenols, hydroxytyrosol is considered as the major phenolic compound in OMW (El-Abbassi et al. 2017) and could make up as much as 70 % of the monocyclic phenolic compounds present in OMW. Hydroxytyrosol is probably generated from the hydrolysis of oleuropein by an esterase during the milling process. It has been an important focus of research since its discovery (Ragazzi and Veronese, 1973) and has been associated with a spectrum of interesting bioactivities (El-Abbassi et al. 2017).

Effect of PE from OMW and crude OMW on mycelia growth of *A. flavus*

A corresponding decrease in fungal mycelial growth in SMKY medium and maize powder with increasing concentration of PE and crude OMW was observed in the present study, after 7 days of incubation at 28 °C. The mycelia growth (biomass) was significantly reduced with increasing concentration of PE and OMW both in SMKY medium (Table. 3) and in maize powder (Table. 4). In SMKY medium, results showed that mycelia biomass inhibition by OMW was 30.8% with 2 g/L of OMW; 61.5% with 6 g/L ($P < 0.001$) and complete inhibition with 10 g/L. The PE reduced 50% the fungal growth by 50 % and complete inhibition was obtained with 500 mg/L. In maize powder, PE and OMW reduced the mycelial growth by more than 50% when present at 800 mg/L and 16 g/kg, and complete inhibition was obtained at 1000 mg/kg and 20 g/kg, respectively.

Aflatoxin B1 inhibition detected by Thin Layer Chromatography

Aflatoxin B1 was inhibited by increasing concentrations of PE and crude OMW both in SMKY medium and maize powder. The AFB1 inhibition was demonstrated by the reduction of fluorescence intensity at 365 nm (Fig. 2). In SMKY medium, blue fluorescence intensity of AFB1 was clearly reduced with 4 and 6 g/L of OMW. The AFB1 was not detected at 8 and 10 mg/L. In presence of PE, the blue fluorescence intensity of AFB1 was reduced by half with 125 mg/L and at 400 mg/L and 500 mg/L AFB1 was not detected (Fig. 2a and Fig. 2b). The same results were observed in maize powder AFB1 blue fluorescence decreased with increasing concentration of OMW and PE (Fig. 2c and Fig. 2d).

Aflatoxin B1 analysis by Direct competitive ELISA

The effect of PE and OMW on AFB1 production, in SMKY medium and maize powder, was analyzed with direct competitive ELISA. The synthesis of AFB1 was inhibited by up to 90 % in SMKY medium and maize powder in the presence of PE at 250 mg/L and 500 mg/kg , respectively as shown in (Table. 5). PE at 500 mg/L and 1000 mg/kg was completely inhibited AFB1 synthesis in SMKY and maize powder, respectively ($P < 0.001$). The synthesis of AFB1 was inhibited up to 50 % in SMKY medium and maize powder in the presence of 6 g/L and 12 g/kg of OMW, respectively (Table. 6). OMW at 8 g/L, 10 g/L and 20 g/kg completely inhibited AFB1 synthesis in SMKY medium and maize powder, respectively ($P < 0.001$).

Crop protection technologies, which include herbicides, insecticides, fungicides and biotechnology products, help to control thousands of weed species, harmful insects and numerous plant diseases that affect crops. Many researchers have tried to take advantage from the phytotoxic and antimicrobial properties of OMW by using it in agriculture as bio pesticide for crop protection (Yangui et al., 2010; Larif et al., 2013; Esmail et al., 2015).

Table 3: Concentration effect of phenolic extract and crude olive mill wastewater on mycelia biomass (g) ± SD of *A. flavus* in SMKY medium, after 7 days of incubation at 28°C.

Treatment	Concentration	Mycelial Biomass (g) ± SD	Reduction percentage (%)
Phenolic Extract (PE) (mg/L)	0	0.26 ± 0.05*	0
	50	0.22 ± 0.01	15.4
	125	0.20 ± 0.01	23
	250	0.13 ± 0.00	50
	400	0.08 ± 0.00	69.2
	500	0.00 ± 0.00	100
Olive Mill Wastewater (OMW) (g/L)	0	0.26 ± 0.05*	0
	2	0.18 ± 0.03	30.8
	4	0.14 ± 0.02	46.2
	6	0.10 ± 0.01	61.5
	8	0.06 ± 0.01	76.9
	10	0.00± 0.00	100

*Each value is the mean of three represented measurements ± SD, Significantly different from control, $P < 0.05$.

Table 4 : Effect of phenolic extract and crude olive mill wastewater on mycelial growth of *A. flavus* in maize powder after 7 days of incubation.

Treatment	Concentration	Diameter (mm)	Inhibition (%)
Concentration of PE (mg/kg)	0	79 ± 0.88*	/
	250	79 ± 0.44	0
	500	65 ± 0.04	17.7
	800	38 ± 0.71	51
	1000	00 ± 00	100
Concentration of OMW (g/kg)	0	79 ± 0.88*	/
	8	80 ± 0.04	0
	12	67 ± 0.44	15.2
	16	39 ± 0.04	50.6
	20	00 ± 00	100

*Each value is the mean of three represented measurements ± SD, Significantly different from control, $P < 0.05$.

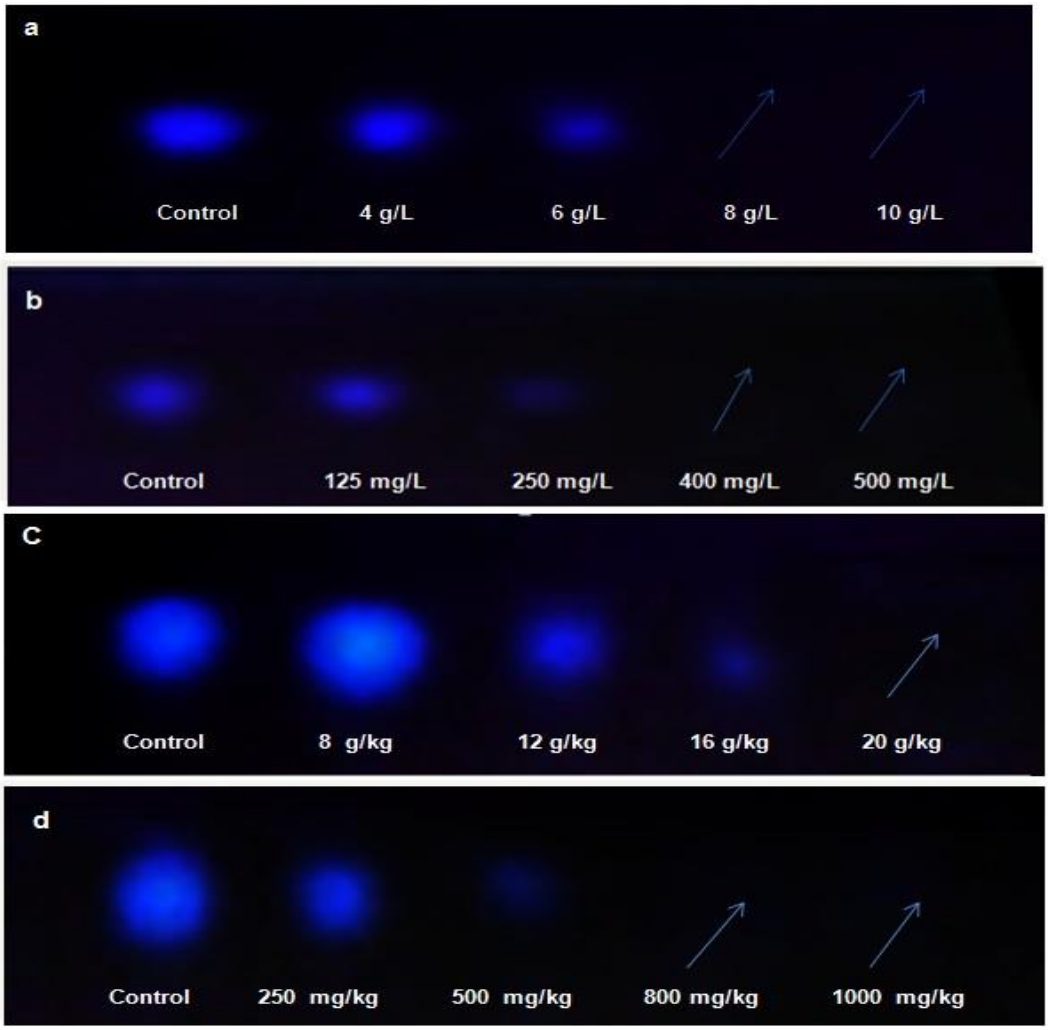

Figure. 2. TLC Chromatograms showing AFB1 inhibition by crude olive mill wastewater (OMW) and phenolic extract by OMW (PE) in SMKY medium (a, b) respectively and in maize powder (c, d) respectively after 7 days of incubation.

Table 5 : Percentage of AFB1 inhibition of *A. flavus* produced by different concentrations of phenolic extract in SMKY medium and maize powder by direct competitive ELISA after 7 days of incubation.

Treatment	Concentration	Amount of AFB1 (ppb)	Inhibition of AFB1 (%)
PE in SMKY medium (mg/L)	0	130.62 ± 0.46*	/
	125	90.56 ± 0.83	30.68
	250	8.00 ± 0.24	93.87
	400	0.00 ± 0.00	100
	500	0.00 ± 0.00	100
PE in maize powder (mg/kg)	0	360.01 ± 0.12*	/
	250	140.00 ± 0.45	61.11
	500	6.00 ± 0.40	98.33
	800	0.00 ± 0.00	100
	1000	0.00 ± 0.00	100

**Each value is the mean of three represented measurements ± SD. LOQ estimated for the method is 1 ppb for maize.

Table 6: Percentage of AF B1 inhibition of *A. flavus* produced by different concentrations of crude olive mill wastewater in SMKY medium and sterile maize powder by direct competitive ELISA after 7 days of incubation.

Treatment	Concentration	Amount of AFB1 (ppb)	Inhibition of AFB1 (%)
OMW in SMKY medium (g/L)	0	130.68 ± 0.46*	/
	4	110.10 ± 0.13	15.73
	6	60 ± 0.23	54.07
	8	0.00 ± 0.00	100
	10	0.00 ± 0.00	100
OMW in maize powder (g/kg)	0	360.01 ± 0.12*	/
	8	520 ± 1.13^{ns}	0
	12	130.26 ± 0.70	63.82
	16	6.75 ± 0.81	98.13
	20	0.00 ± 0.00	100

ns: not significant ($P > 0.05$)
*Each value is the mean of three represented measurements ± SD. LOQ estimated for the method is 1 ppb for maize.

The present work is the first investigation showing the protective effects of PE from OMW and of crude OMW against aflatoxigenic strain *A.flavus*. Significant reduction in mycelial growth and AFB1 synthesis was observed. Various essential oils have frequently been reported as inhibitors of aflatoxin production and mycelia growth of *Aspergillus* species in food commodities (Passone et al., 2013; Pandey et al., 2016). However, there is no significant literature available on the bio efficacy of PE and OMW on *A.flavus* growth and AFB1 production.

The biosynthesis of aflatoxins is strongly dependent on chemical and physical growth conditions, that is, substrate composition, temperature, pH, water activity and the particular combination of different parameters that can be completely inhibited or activated by the biosynthesis of these aflatoxins (Hahlbrock and Scheel, 1989). Moreover, the biosynthesis of aflatoxin is established by the conversion of acetate to norsolorinic acid (NOR) by synthases (FAS) and a polyketide synthase (NR-PKS, PksA) which are involved in the synthesis of the polyketide from a hexanoyl (the initial substrate for aflatoxin formation) (Crawford et al., 2008). Phenolics are secondary metabolites synthesized via the phenylpropanoid biosynthetic pathway. These compounds are building blocks for cell wall structures, serving as a defence against pathogens (Hahlbrock and Scheel, 1989). These compounds not only inhibited AFB1 biosynthesis but also reduced production of norsolinic acid (*NOR*) the first stable aflatoxin precursor (Ferreira et al., 2013). It is clear that phenolic compounds inhibited one or more early rather than late steps in the AFB biosynthesis pathway (Ferreira et al., 2013). Hitokoto et al., (1980), reported cases in which the formation of aflatoxins was prevented while the fungal colony growth was apparently normal, justifying the need to study both the potential to inhibit fungal growth and the potential to inhibit toxin production. The mechanism by which the latter inhibition occurs is not very clear. Aflatoxins are synthesized extra-mitochondrially from acetylcoenzyme A during a period of rapid glucose utilization (Hsieh and Mateles, 1970). According to Tatsadjieu et al*.,* (2009), the essential oils of *L. rugosa* from Cameroon may restrict carbohydrate catabolism in *A. flavus* by acting on some key enzymes and this may result in the decrease of its ability to synthesise AFB1.

Also, the number and position of substitutions of the phenolic acids and the saturated side-chain length influenced the antimicrobial potential of the phenolic acids against the various microorganisms, but in different ways (Nazzaro et al., 2013). According to Farag et al*.,* (1989) and Nychas (1995), the presence of phenolic OH

groups able to form hydrogen bonds with the active sites of target enzymes increased antimicrobial activity.

Mekki et al., (2009), reported that the phytotoxic and antimicrobial properties of OMW are associated with its phenolic compound composition. Some studies have demonstrated interesting bactericidal and fungicidal activities of OMW and especially of its phenolic monomers, such as hydroxytyrosol and tyrosol (Yangui et al., 2009). Hydroxytyrosol-rich OMW extracts have been shown to exhibit a powerful fungicidal activity against phytopathogens with a minimal inhibition concentration ranging between 7.18 and 57.4 mg/L (Yangui et al., 2010). *Verticillium wilt*, caused by *Verticillium dahliae Kleb*, is one of the most destructive plant diseases worldwide and it is a challenge for producers to find effective means of controlling it. Some researchers have suggested the incorporation of OMW into the soil as an eco-friendly alternative to fumigants for crop protection against *V. dahlia*. OMW curative control activity was demonstrated through a pot experiment on a tomato crop under field conditions (Yangui et al. 2011). This might be attributed to the direct effect of fungicidal compounds such hydroxytyrosol or an induction of defence response mechanisms in the plant. The experimental treatment of The soil inoculated with *Rhizoctonia solani* (6 g/kg d.w. of soil) by OMW obtained from a centrifugal olive mill in the area of Messinia, in South Greece, can also inhibit the growth of *Rhizoctonia solani* if applied at high doses (400 ml/kg d.w. of soil) (Kotsou et al. 2004), but no antifungal activity of OMW extracts by Mission and Frantoio olive fruit (Australia) was found against *Candida albicans* and *Aspergillus niger* at 5 mg/disc by disc diffusion method (Obied et al., 2007). However, filtered OMW application on fruits and vegetables infested with can inhibit the sporulation of *Botrytis* and *Penicillium spp* and also as the mycelium growth of the soil-borne pathogens *Fusarium oxysporum f. sp.* and *lycopersici* on tomato plants; in this experiment twenty one tomato seedlings were incubated in *Fusarium oxysporum f. sp.* and *lycopersici conidia* suspension (10^6 conidia/ ml) for 10 min and 21 were incubated in the conidial suspension treated with olive OMW (5 ml/100 ml in total solution) (Vagelas et al. 2009).

CONCLUSION

The results show that OMW and phenolic extract from OMW inhibits growth and the production of AFB1 *in vitro* (SMKY medium) and in maize powder. The use of natural compounds like phenolic compounds with lower toxicity than that of synthetic products can be a good alternative for aflatoxin control and may be a possibility for protection against the production of AFB1 in corn. In addition, the therapeutic use of phenolic compounds may also provide a solution to the rapid development of fungal resistance to various prevalent antifungal therapeutics that is currently observed. These results, obtained justify future researches emphasizing the antifungal properties of the by-products of olive oil and their possible use as viable alternatives to control microbial growth in stored food commodities.

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CONFLICT OF INTEREST

The authors declare no financial or other conflicts of interest.

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Add acknowledgment here

AUTHOR CONTRIBUTIONS

Designed research: N. Oularbi-Senani, F. Moulti-Mati and A. Riba.
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