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Management of Aflatoxin B1 Production by *Aspergillus flavus* Isolated from Rice using Methanolic Extract of *Olea europaea* L. Leaves

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Antifungal and antiaflatoxigenic activities of methanolic extract of *Olea europaea* L. Leaves was evaluated against the toxigenic *Aspergillus flavus* isolates from rice. Six rice samples out of seven samples were shown to be contaminated with aflatoxin B1 at concentrations of between 16.3-31.1 µg/Kg. Among 12 isolates of *A. flavus* examined, a total of 7 (58.3%) were capable of producing aflatoxin B1 with ranged from 3.4 to 1.3 µg/ml whereas 5 isolates were not detected aflatoxin B1. Various concentrations (5, 10 and 15% (w/v) of methanolic extract of *Olea europaea* L. Leaves was tested against *Aspergillus flavus*. Our results indicate that inhibition values of the extracts were higher in comparison to the control with different significantly, and this effect gradually increased with concentration. Methanolic extract of *Olea europaea* leaves were inhibited mycelial growth, sporulation and aflatoxin production by *A. flavus* at the three concentrations tested compare to control. %inhibition of aflatoxin production decreased with increasing concentrations. The highest aflatoxin production inhibition rate of the tested fungi was observed at 15%, ranging from 19.0 % to 46.2 at 5%, 41.1 to 76.9 at 10% and 64.7 to 100 at 15%. GC/ MS analysis of methanolic extract of *Olea europaea* leaves lead to separation 27 compounds and phytochemical screening have been described in previous studies.

Key words: Rice, *Aspergillus flavus*, Aflatoxin B1, Methanolic extracts of *Olea europaea*.

Mycotoxins pose a significant risk to the health of humans and animals and food safety (Bhat *et al*., 2010) and constitute a factor for economic losses in food products worldwide (Omidbeygi *et al.,* 2007; Pitt and Hocking, 2009). Aflatoxin is produced mainly by members of Aspergillus section Flavi such as *A. flavus, A. parasiticus, A. pseudotamarii,* and *A. nomius* (Lanier *et al*.,2009; Abdin *et al*., 2010; Reddy *et al*., 2010) *A. flavus* is the most common causal agent of aflatoxin contamination in nature (Klich,2007). Nevertheless, not all isolates of the same species produce toxins (Brase *et al*., 2009). Aflatoxin B1 has been classified as a class one human carcinogen by The International Agency for Research on Cancer (IARC, 1993). The amount of mycotoxin production and microorganism growth will depend on moisture, relative humidity, temperature and spore load (Javaid *et al.,* 2002). Rice (*Oryza sativa* L.) is grown in 114 countries in world (Mexia, 2003) with 722 million tones of total production annually (FAO, 2011) Saudi Arabia imported 1.25 million tones on the average annually (IRRI, 2010). High moisture and temperature conditions before its harvest cause increasing fungi invading lead to reduction in the quality of rice and responsible for causing quantitative and qualitative losses (Uma and Wesely, 2013). Rice is most important principal sources of mycotoxins to human and animals in the world cause is highly cultivated and consumed

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worldwide. *Aspergillus* spp. are common fungal contaminants of rice (Makun *et al*., 2011; *Gautam et al.,* 2012; Uma and Wesely, 2013, Ok *et al*., 2007; Rocha *et al*., 2009; Abd El-Aziz *et al*., 2013; Bakan *et al.,* 2002; Toth and Teren, 2005). Plant extracts have demonstrated antimicrobial effects and antiaflatoxigenic activities as alternative safety method (Beevi *et al.,* 2009; Abd El-Aziz *et al.,* 2013), Therefore, the objective of the present study was to investigate the antiaflatoxigenic activities of *Olea europaea* extract against aflatoxin B1 which produce by *A. flavus* isolated from rice and qualitative analysis of major photochemical present and separation component of *Olea europaea* extract by GC-MS

MATERIALS AND METHODS

Collection of Samples

Seven samples of rice were collected randomly from different markets in Riyadh, Saudi Arabia, during summer 2013.

Extraction of aflatoxin B1 from rice grains:

Ten g of each tested sample was mixed with 100 ml of 4% acetonitrile aqueous solution of potassium chloride (9:1), then shaking for 20 min and filtered through Whatman No.4 filter paper under vacuum condition. 100 ml of n-hexane were added to the filtrate and shaken for 10 min. After separating, the upper phase (n hexane) was discarded. To the lower phase, 50 ml deionized water and 50 ml chloroform was added and this solution was shaken for 10 min. Then, the lower phase was collected and the upper phase was re-extracted twice with 25 ml of chloroform by using the above conditions. Then the chloroform was evaporator in a 40°C water bath at low speed. Methanol at the rate of 2 ml was added and the solution filtered through a 0.45 µl filter. (Zaboli *et al*, 2011).

Fungal isolation and purification

Samples of rice were disinfected using 2% sodium hypochlorite for two minutes, rinsed three times in sterile distilled water then dried by sterile filter paper . Then, three grains were placed randomly onto potato dextrose agar (PDA) and incubated at 25 °C for 7 days. The isolates were purified by the single-spore method. The pure isolates were identified by Regional Center of the Fungi and their Applications, Al-Azhar University, Cairo, Egypt.

Preparation of Methanolic Extracts of *Olea europaea* leaves

The olive leaves were collection from farm of *Botany and Microbiology Department, College of Science, King Saud University* and dried at room temperature and then powdered was extracted successively with methanol Fifty g of the dried *Olea europaea* leaves were soaked in methanol for 48h, then the flask was shaken for 30 min. and finally filtered. The solvent was evaporated under temperature not exceeding 5°C, yielded extracts were used as crude gum weighted and resolved again in the same solvent to give three concentrations (5,10 and 15%) .

Effect of methanolic extracts of *Olea europaea* leaves on aflatoxin B1 produced by *Aspergillus flavus* isolates

Ten ml from different concentrations of solvent *Olea europaea* leaves extract (10, 15 and 20%) were prepared separately and added to 90 ml SMKY liquid medium (sucrose, 20 g; magnesium sulfate, 0.5 g; potassium nitrate, 3 g; yeast extract, 7 g and distilled water, 1000 ml) **,**the flasks were inoculated with discs of 4 mm diameter of the toxigenic *A. flavus* and incubated at 25 °C for 7 days (control was without extract). The filtrates of each flask were treated three times with 50 ml of chloroform in a separating funnel. The chloroform extract was separated and dehydrated with anhydrous sodium sulfate and evaporated till dryness on water bath at 50°C under vacuum. The residues were dissolved in 10 ml methanol **(**Al-Othman *et al*., 2013)

High -Performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC) (model PerkinElmer series 200 UV/VIS) was used for measuring aflatoxin B1. HPLC providing with a C18 column with an internal diameter of 3.9 x 300 mm. and UV detector and fluorescence detection with 365nm excitation and 430nm emission wavelengths. The liquid mobile phase yielded results of methanol: acetic acid: water (20:20:60 v/v/v). The total run time for the separation was approximately 30 min at a flow rate of 1 ml/min (Christian, 1990).

Phytochemical Screening of the *Olea europaea* leaves

Methanolic extract was prepared for phytochemical screening of *Olea europaea* leaves.

The extract was subjected to phytochemical tests for alkaloids (Evans, 2002), glycosides anthraquinones (Siddiq and Ali, 1997), tannins (Iyengar, 1995), phenolics and reducing sugars (Sakthi and Geetha, 2011).

GC/GC-MS analysis of methanolic extract of *Olea europaea* leaves

Methanol extract of *Olea europaea* leaves were analyzed through gas chromatography (model PerkinElmer clarus 500) equipped with a flame ionization detector, and quantitation was carried out by the area normalization method neglecting response factors. The analysis was carried out using a VF-5MS capillary column (30 m x 0.25 mm; 0.25 µm film thickness). The operating conditions were as follow: injection and detector temperature, 250 and 300°C respectively; split ratio, 1: 50; carrier gas, Helium with flow rate (1.0 ml/min). Oven temperature program was 50 to 300°C at the rate of 7°C/min. Mass spectrometer conditions were: ionization potential, 70 eV; mass range from, 40 to 400 m/z; electron multiplier energy, 2000 V. according to the method described by Priyanka *et al.* (2009) The components of plant extracts were identified by comparison of their relative retention times and the mass spectra with those authentic reference compound shown in the literature and by computer matching of their MS spectra with Nist mass spectral library

Statistical analysis

All experiments were repeated three times and treatment means were compared using Least Significant Difference (LSD) at 0.05% analysis according to Daniel (1987).

RESULTS AND DISCUSSION

Determination of aflatoxin B1 from rice grains

Mycotoxin contamination in rice is usually lower as in wheat or corn (Tanaka *et al*., 2007). Data in Table 1. show that , six rice samples out of seven samples were shown to be contaminated with aflatoxin B1 at concentrations of between 16.3-31.1 µg/Kg The highest incidence of the aflatoxin B1 was in samples number 3 (31.1 µg/Kg) while the lowest was in samples number 6 (16.3 µg/Kg). The Aflatoxin B1 contamination was detected in 37 samples of milled rice grains and found that 92% in China according to Liu *et al*., (2006). Prasad *et al.* (1987) tested 56 samples of stored rice and 12 were positive for aflatoxin in India.

Makun *et al*.,(2007) found that aflatoxin B1 was detected in 97% of the samples analyzed at concentrations between 20-1642 ug/kg in Nigeria. Reddy *et al* (2009) found that ranging from 0.1 to 308.0µg/ Kg of rice grains in India. Weidenboerner (2000) recorded that Levels of aflatoxin B1 ranged from 26 to 38 µg/kg in Brasil 8 µg/Kg in Egypt, 28 µg/Kg in Italy and 37 µg/Kg in Thailand. Liu *et al*., (2006) found that 92% of milled rice grains showed positive to aflatoxin B1. Toteja *et al*., (2006) reported the presence of aflatoxin B1 in parboiled rice collected from 11 states in India and found 38.5% of the samples were positive to aflatoxin B1.

However, variation in amount of aflatoxin B1 may be due to moisture content above the

Table 1. Determination of Aflatoxin B1 from rice grains (µg/Kg) by HPLC

Sample No.	Aflatoxin B1(µg/Kg)
1	22.1
2	18.6
3	31.1
4	26.4
5	ND
6	16.3
7	21.2

ND=Not Detected

Table 1. Determination of Aflatoxin B1 from rice grains (µg/Kg) by HPLC

Sample No.	Aflatoxin B1(µg/Kg)
1	ND
2	2.7
3	1.8
4	ND
5	ND
6	2.1
7	3.4
8	1.3
9	ND
10	ND
11	2.6
12	1.9

ND=Not Detected

recommended level for safe storage (14 %) (Taligoola *et al*. 2010; Taligoola *et al*., 2011). Natural occurrence of mycotoxins, about 72% of the samples were found contaminated with mycotoxins according to Gautam *et al*.,(2012) in Himachal Pradesh.

Many authors reported that most of the isolated fungal species belonged to the genus Aspergillus. Aspergillus was the genus most detected at high frequency in rice samples from Nigeria (Makun *et al.,* 2007), Uganda (Taligool *et al.,* 2010, 2011), Nigeria (Makun *et al*. 2007), Thailand (Lapmak *et al.* 2009), Himachal Pradesh (*Gautam et al*., 2012) Tamil Nadu (Uma1 and Wesely, 2013). Korea (Oh *et al*., 2007) and Saudi Arabia (Abd El-Aziz *et al.,* 2013).

Determination of Aflatoxin B1 (µg/ml) were produced by *Aspergillus flavus* by HPLC

The ability of *A. flavus* isolates to produce Aflatoxin B1 is summarized in Table 2. Among 12 isolates of *A. flavus* examined, a total of 7 (58.3%) were capable of producing aflatoxin B1 with ranged from 3.4 to 1.3 µg/ml whereas 5 isolates were not detected aflatoxin B1. Reddy *et al*. (2009) reported that 68% of *A. flavus* isolates produced aflatoxin B1 on Czapeks and PDA agar media and the capacity of aflatoxin B1 production ranged from 0.2–40 µg /g of agar.

Effect of methanolic extracts of *Olea europaea* L .Leaves on growth *and Aspergillus flavus*:

Various concentrations (5, 10 and 15% (w/ v) of methanolic extracts of *Olea europaea* L. Leaves were tested against *Aspergillus flavus*. Results indicate that inhibition values of the extracts were higher in comparison to the control with different significantly, and this effect gradually increased with concentration (Table 3). Methanolic Extracts of *Olea europaea* leaves were inhibited

Table 3. Effect of different concentrations of *Olea europaea* L. leaves on radial mycelial growth of *Aspergillus flavus* isolated from rice

Isolates No.	Control	Radial mycelial growth (cm)					
		5%	%inhibition	10%	%inhibition	15%	%inhibition
2	9.0	3.6	60.0	1.2	86.7	0.8	91.1
3	9.0	5.1	43.3	3.6	60	1.1	87.8
6	9.0	5.3	41.1	2.8	68.9	1.4	84.4
7	9.0	6.0	33.3	4.1	45.4	0.7	92.2
8	9.0	5.6	37.8	2.7	70	1.3	85.6
11	9.0	4.3	52.2	1.9	78.9	0.9	90
12	9.0	5.0	44.4	2.4	73.7	1.5	83.3

LSD at 0.05:Concentration (C)=0.961Isolates (I) =0.735 CXI=0.427

Table 4. Effect of different concentrations of *Olea europaea* L. leaves on sporulation of *Aspergillus flavus* isolated from rice

Isolates No.	Sporulation/ml						
	Control	5%	%inhibition	10%	%inhibition	15%	%inhibition
2	75×10^4	63×10^4	16.0	41×10^4	45.3	16×10^4	78.7
3	64×10^4	50×10^4	21.9	39×10^4	39.1	24×10^4	62.5
6	74×10^4	63×10^4	14.9	84×10^4	35.1	20×10^4	73.0
7	68×10^4	55×10^4	19.1	33×10^4	51.5	13×10^4	80.9
8	70×10^4	61×10^4	21.1	41×10^4	41.4	19×10^4	72.9
11	73×10^4	60×10^4	17.8	36×10^4	50.7	10×10^4	86.3
12	63×10^4	50×10^4	20.6	41×10^4	54.0	14×10^4	77.8

LSD at 0.05:
Concentration (C) = 13×10^4 Isolates (I) = 5×10^4 C×I = 11×10^4

mycelial growth with ranged from 33.3 % to 60.0 at 5%, 45.4 to 86.7 at 10% and 83.3 to 92.2 at 15%.

The results in Table 4. revealed that the gradual concentrations of methanolic extracts of *Olea europaea* L. Leaves lead to significant inhibition in sporulation of *Aspergillus flavus* with ranged from 14.9 % to 21.9 at 5%, 35.1 to 54.0 at 10% and 62.5 to 86.3 at 15%.

Effect of methanolic extracts of *Olea europaea* L .Leaves on aflatoxin B1 produced by *Aspergillus flavus* isolates

Table 5. reveals that methanolic extracts of *Olea europaea* L .Leaves was effective inhibitors on aflatoxin production by *A. flavus* at the three concentrations tested compare to control. %inhibition of aflatoxin production decreased with increasing concentrations. The highest aflatoxin production inhibition rate of the tested fungi was observed at 15%, ranging from 19.0 % to 46.2 at 5%, 41.1 to 76.9 at 10% and 64.7 to 100 at 15%.

Variation in their constituents of the plant extracts lead to variation in ability of plant extracts against the toxigenic *A. flavus* (Cavaleiro *et al.,* 2006). The inhibitory potential depends on the mode of extraction and the concentration of the extracts (Abd El-Aziz *et al*., 2012). The biosynthesis of aflatoxin B is a multistep process and more than 23 enzymatic reactions are required for this pathway (Yu *et al*., 2004). Phenolic compounds present in *O. europaea* play the important role in inhibition fungal growth and aflatoxin production may be these phenolic compounds inhibited steps pathway of aflatoxin production biosynthesis. The antifungal properties of these extracts may be attributed to their distinct phytochemical contents (Rasoolil *et al.,* 2008; Beevi *et al.,* 2009). Al-Rahmah *et al*., (2011) recorded that *O. europaea, Salvadora persica, T. vulgaris* and *Z. spina-christi* have been

Table 5. Effect of *Olea europaea* plant extract on aflatoxin B1 produced by *Aspergillus flavus* at three concentrations after incubation at 28±2°C

Isolates	Radial mycelial growth (cm)					
No.	5%	%inhibition	10%	%inhibition	15%	%inhibition
2	1.6	40.7	1.0	62.9	0.12	95.5
3	1.1	38.9	0.6	66.7	0.31	82.8
6	1.7	19.0	1.0	41.1	0.51	70.0
7	2.1	38.2	1.4	58.8	1.2	64.7
8	0.7	46.2	0.3	76.9	0.0	100
11	2.0	23.1	0.9	65.4	0.4	84.6
12	1.4	26.3	0.5	73.7	0.31	83.6

LSD at 0.05:Concentration (C)=0.961Isolates (I) =0.735 CXI=0.427

studied for antifungal activities against *A. flavus*. Antimicrobial activity of the plant extracts may be cause cross the cell membrane and interacting with the enzymes and proteins of the membrane (Omidbeygi *et al.,* 2007). Olive leaves are recognized for their high flavonoid contents and also for the presence of a potentially bioactive compound oleuropein, a secoiridoid, (El and Karakaya, 2009, Vinha *et al.,* 2005). The leaves of olive tree are rich with biophenols, such as oleuropein, verbascoside, ligstroside, tyrosol and hydroxytyrosol, which have exhibited antimicrobial properties (Caturla *et al.,*2002; Paiva-Martins *et al*., 2003). Winkelhausen *et al*. (2005) re-ported that the phenolic compounds derived from olive hold a good promise as a natural fungicide against common pathogens to crops. Hayes *et al*. (2011) revealed that six major polyphenolic compounds present in olive leaf extract: oleuropein, verbascoside, luteolin-7-O-glucoside, apigenin-7-O-glucoside, hydroxytyrosol and tyrosol. (Corrales *et al*., 2009) showed that *Olea europaea* may present more antimicrobial activity cause the tannin content was highest in *Olea europaea* (37.01 mg CE/ 100g), followed by *Matricaria chamomilla, Melissa officinalis* and *Aloysia triphylla* respectively.

Phytochemical analysis of methanolic Extracts of *Olea europaea* Leaves

Data in table (6) show that anthraquinones, glycosides, phenolics, reducing sugars and tannins were found in methanolic Extracts of olives (*Olea europaea)* while Alkaloids was absent. This result agree with (Mahjoub *et al.,* 2011; Vinha *et al.*, 2012).

Table 6. Qualitative analysis of six major photochemical present in *Olea europaea* L. leaves various extract

Phytochemicals	Qualitative analysis
Alkaloids	-
Anthraquinones	+
Glycosides	+
Phenolics	+
Reducing sugars	+
Tannins	+

(+) present,(–)absent

GC/ MS analysis of methanolic extract of *Olea europaea* leaves

Data in Table 7 shows that main component of methanolic extract of *Olea europaea* leaves by GC-MS according to their retention time (Rt) and percentage of area. Components identified in the present study are listed in Table 7 twenty seven compounds were isolated and characterized by GC-MS. 2-hexenal, the main one was extracted (13.31%) and other compounds present in a relative percentage were β-Pinene (2.21%), Camphene (1.36%), Sabinene (2.01%), β –pinene (3.12%), 1,8-Cineol (6.32%), 4-hydroxy, 3-methoxy cinamic acid (7.30%), Linalool (3.21%) 4-hydroxy cinamic acid (p-coumaric acid) (5.11%)3,4 dimethoxy benzoic acid (veratric acid) (1.14%) Myrcene (1.03%),Ocimene (0.63%) Limonene (1.27%), β-farnesene (0.09%), β-Caryophyllene (0.82%), 3,4-

Table 7. Chemical composition and Relative concentration of *Olea europaea* L. leaves extracts of by GC-Mass

Peak No.	Compounds	Relative concentration
1	α-Pinene	2.21
2	Camphene	1.36
3	Sabinene	2.01
4	β-pinene	3.12
5	1,8-Cineol	6.32
6	4-hydroxy, 3-methoxy sinamic acid (ferulic acid)	7.30
7	Linalool	3.21
8	4-hydroxy sinamic acid (p-coumaric acid)	5.11
9	3,4 dimethoxy benzoic acid (veratric acid)	1.14
10	Myrcene	1.03
11	Ocimene	0.63
12	Limonene	1.27
13	α-farnesene	0.09
14	β-Caryophyllene	0.82
15	3,4-dihydroxy benzoic acid (protocatechuic acid)	1.34
16	Camphor	4.12
17	Menthol	2.22
18	3,4-dihydroxy sinamic acid (caffeic acid)	1.25
19	B-Selinene	0.23
20	2-hexenal	13.31
21	3-methoxy,4-hydroxy benzoic acid (vanilic acid)	8.34
22	2,6-Dimethyl- 1 heptene	1.05
23	4-hydroxy benzoic acid	4.32
24	3-Methyl-4-penten-	0.56
25	γ-Terpinene	4.18
26	Tyrosol	2.14
27	Oleuropein	5.22

dihydroxy benzoic acid (protocatechuic acid) (1.34%), Camphor (4.12%), Menthol (2.22%), 3,4-dihydroxy sinamic acid (caffeic acid) (1.25%), B-Selinene (0.23%), 3-methoxy,4-hydroxy benzoic acid (vanilic acid) (8.34%),6-Dimethyl-1 heptene(1.05%), 4-hydroxy benzoic acid (4.32%),3-Methyl-4-penten (0.56%) β-Terpinene (4.18%) Tyrosol (2.14%) and Oleuropein (5.22%). These results agreement with (Angerosa *et al*., 2000; Flamini *et al*., 2003). According to Kiritsakis (1998) and Salas and Sachez (1999), reported that methods used and conditions applied to obtain olive oil from olive fruit affects its volatile composition. Hence , GC/ MS analysis of methanolic extract of *Olea europaea* leaves show that many phenolic compounds 4-hydroxy, 3-methoxy cinamic acid (ferulic acid), 4-hydroxy cinamic acid (p-coumaric acid), 3,4 dimethoxy benzoic acid (veratric acid), 3,4-dihydroxy benzoic acid (protocatechuic acid), 3,4-dihydroxy cinamic acid (caffeic acid), 3-methoxy,4-hydroxy benzoic acid (vanilic acid), Oleuropein. This finding agreement with (Castilloa *et al*., 2000; Hayes *et al*., 2011).

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