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Saffron (*Crocus sativus* L.) Inhibits Aflatoxin B_1 Production by *Aspergillus parasiticus*

Cryssa Tzanidi, Charalampos Proestos, Panagiota Markaki*
Department of Chemistry, Laboratory of Food Chemistry, National and Kapodistrian University of Athens, Athens, Greece
Email: *markaki@chem.uoa.gr

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ABSTRACT

Aflatoxin B_1 (AFB_1) is a carcinogenic metabolite produced by certain *Aspergillus* species. The aim of the present study was to investigate the effect of saffron stigmas on *A. parasiticus* growth and AFB_1 production in Yeast Extract Sucrose (YES) medium. AFB_1 was extracted from cultures and purified with immunoaffinity columns followed by high performance liquid chromatography (HPLC) coupled to fluorescence detector (FL) analysis. Methods' recovery and limit of detection were 95.3% and 0.02 ng $AFB_1 \cdot ml^{-1}$ of YES respectively. Results indicated that AFB_1 production in samples of YES inoculated with *A. parasiticus* after the addition of saffron dried stigmas (100 $mg \cdot flask^{-1}$) was significantly lower ($p < 0.05$) compared to control samples (inoculated without the addition of saffron stigmas) throughout the entire incubation period (18 days), at the same time as mycelial growth was noticeable. In addition, mycelial growth was observed and AFB_1 was detected, after the 7th day of observation in cultures with saffron alone. Maximum production was observed on the 12th day (0.018 μg $AFB_1 \cdot flask^{-1}$) and on the 9th day (0.051 μg $AFB1 \cdot flask^{-1}$) for samples of YES with the addition of saffron inoculated with *A. parasiticus* and samples with saffron alone (non-inoculated), respectively. In control samples (inoculated without saffron) the maximum production on the same days 12 and 9 was 75.31 μg $AFB_1 \cdot flask^{-1}$ and 64 μg $AFB_1 \cdot flask^{-1}$ respectively. Conclusively when saffron was added to YES inoculated with *A. parasiticus*, AFB_1 production decreased by 99.9% compared to control cultures without saffron addition. This inhibition can be attributed to the antioxidant capacity of saffron constituents.

Keywords: Aflatoxin B_1; *A. parasiticus*; HPLC-FL Analysis; Saffron; Antioxidants

1. Introduction

Aflatoxins are mycotoxins of great significance in foods and feeds and are mainly produced by *Aspergillus* (*A*) *flavus*, *A. parasiticus* and *A. nomius*. Aflatoxin B_1 (AFB_1) is usually found at highest concentrations in contaminated food and feed. Additionally it is regarded to be genotoxic and the most potent liver carcinogen for many animal species as well humans [1,2].

Saffron consists of dried stigmas of the herb *Crocus sativus L.* belonging to the *Iridaceae* family. It is cultivated not only in Mediterranean countries such as Greece, Spain and France but also in India and Iran [3]. There is limited knowledge about the physiology of vegetative development of saffron. It has been shown that the corm size, water availability and cultivation conditions have a significant effect on vegetative development of saffron. The biomass contribution of leaves and mother corm for maintaining the vegetative development of saffron varies throughout the growing season. Photosynthetic rate is consistently very high throughout the year but is reduced in the largest corms [4]. In addition saffron is world's most expensive spice and several studies indicate its potential as anticancer, anti-inflammatory and hypolipidaemic agent [5-9]. Asdaq and Inamdar [10] investigated the potential of saffron and its constituent, crocin, as hypolipidemic and antioxidant agent in rats and reported that saffron was found to be superior to crocin indicating the involvement of other potential constituents of saffron apart from crocin for its synergistic behaviour of quenching free radicals. Moreover according to Kumar *et al*. [11] saffron is known to have antioxidant-like properties. Furthermore, extracts of several spices and herbs have been shown to reduce *A. parasiticus* growth and AFB_1 production [12]. Moreover, essential oils have been considered to inhibit *A. flavus* growth and AFB_1 production [13,14]. Pawar and Thaker [15] investigated 75 essential oils against the fungus *A. niger* growth and saffron was found to have no effect on the fungus. According to Igawa *et al*. [16], in addition to production of inhibitors, the development of plants that can inhibit mycotoxins is a promising approach.

*Corresponding author.

Saffron contains chemical constituents such as crocetin, picrocrocin and safranal which are responsible for color, flavor and aroma respectively. Anthocyanins, flavonoids, vitamins, amino acids, proteins, starch, minerals and other chemical compounds have also been described in saffron [15]. It is important to note that under basic conditions and following the drying process, picrocrocin is converted to safranal [17]. Additionally crocetin ester is degraded upon thermal treatment, although it was affected by external factors [18]. Giaccio [3] reported that crocetin protects against oxidation damage in rats' primary hepatocytes, in particular a suppression of AFB_1-induced hepatotoxic lesions by crocetin. The reduction of toxicity is due to the property of crocetin to stimulate defense mechanisms in the liver cells with the increase of the glutathion-S-transferase and also with the decrease of the alleged AFB_1-DNA.

To our knowledge there is little information in the literature concerning the saffron biological activity as crude product and its effect on AFB_1 production. Hence the aim of the present study was to investigate the effect of saffron dried stigmas on *A. parasiticus* growth and AFB_1 production in Yeast Extract Sucrose medium.

2. Materials and Methods

2.1. Apparatus and Reagents

A laminar flow (Telstar Bio IIA, Spain), an incubator WTB Binder (Tuttlinger, Germany), and a centrifuge Sorvall RC-5B (HS-4) (Norwalk, USA) were used. AFB1 standard was purchased from Sigma-Aldrich Chemical Co, USA. Millipore filters HVLP (0.45 μm) were purchased from Waters (Millipore, USA). The Aflatest immunoaffinity columns were obtained from Vicam, USA. All reagents used were of analytical grade (Sigma Aldrich, USA) while HPLC solvents were of HPLC grade and were purchased from Fisher Scientific, UK. Trifluoroacetic acid was from Fluka, The Netherlands.

2.2. Samples

Samples of saffron were collected from the Athens market during 2010. Saffron purchased in packages was used before the expiration date. The packages were little pots made of glass labeled as "Cooperative de Saffran Kozani". The content of each pot was 1 g of saffron stigmas. All samples were stored in dark and cool place prior to analysis. Just before examination saffron stigmas were transferred in sterile polyethylene bags. Representative sub samples of saffron stigmas (100 mg) were collected aseptically and also used in the present study.

2.3. Media

Aspergillus flavus parasiticus agar (AFPA) was prepared by dissolving 4 g of yeast extract (Oxoid) (Hamshire, UK), 2 g of bacteriological peptone (Oxoid) 0.1 g of ferric ammonium citrate, 0.2 ml of Dichloran 0.2% in ethanol (Fluka Steinheim, The Netherlands), 0.02 g of chloramphenicol (Oxoid) and 3 g of agar (Oxoid) in 200 ml of distilled water, final pH 6.0 - 6.5. Czapek Dox agar (CZA) was prepared by dissolving 0.4 g of sodium nitrite, 0.1 g of potassium chloride, 0.1 g of magnesium sulfate, 0.002 g of ferric sulfate, 0.2 g of dipotassium phosphate, 6 g of sucrose, 3 g of agar, 0.002 g of zinc sulfate and 0.001 g of copper sulfate in 200 ml of distilled water, final pH 6.0 - 6.5 [19].

2.4. Preparation of Spore Inoculum

The aflatoxigenic strain *A.parasiticus* spear (IMI 283883) utilized throughout this study was obtained from the International Mycological Institute (Engham Surrey, UK). An inoculum was obtained by growing the mold on a slant of stock cultures of CZA, which were maintained at 5˚C. Spore inoculum was prepared by growing *A. parasiticus* on CZA for 7 days at 30˚C and spores were harvested aseptically using 10 mL of sterile 0.01% v/v Tween 80 solution. AFB_1 carried over from the initial growth was minimized by centrifuging the spore suspension (1000 g for 10 min) and resuspending the biomass in 10 mL of sterile Tween 80 solution twice. Dilutions (0.1, 0.01, 0.001 and 0.0001) were prepared from the initial spore in sterile tubes containing 10 mL of Tween 80 0.05% v/v suspension [19]. The spore concentration was determined by the spread plate surface count technique, using 0.1 mL of each dilution on four AFPA plates after incubation at 30˚C for 2 days. The population size was estimated by the reverse intense yellow/orange coloration of the colonies. For obtaining an inoculum containing 100 conidia, plates with 10 - 100 colony forming units (cfu) were selected and the desired 100 spore quantity used in the present study was estimated.The quantity of 100 spores $flask^{-1}$ was chose as it was the minimum concentration found in the literature producing a detectable amount of AFB_1 by aspergilli [20].

2.5. Inoculation

For each day of observation, 6 flasks containing 10 mL of YES medium were inoculated with 100 conidia·$flask^{-1}$ of *A. parasiticus*. 100 mg of saffron stigmas with natural microbiota (NM), were added before inoculation into each of the three flasks for each day of observation. Furthermore, 100 mg of saffron with NM were added to three additional flasks of non-inoculated YES, for each day of observation. We should mention that all flasks with YES were sterilized by autoclaving at 121˚C for 15 min. Saffron was added after sterilization and before inoculation. All flasks were incubated under stationary

conditions at 30˚C. Immediately after autoclaving (115˚C, 30 min) for safety reasons [21] the mycelial growth was determined and AFB_1 was assayed on days 0, 3, 7, 9, 12, 15 and 18 of incubation. The experiments were repeated in triplicate.

2.6. AFB_1 Determination

The content of each flask containing YES medium with saffron and flasks control (without saffron addition) was mixed with 30 mL of methanol and shaken well for 10 min. After filtration, an aliquot of 1 ml from each flask was used for AFB_1 analysis. The aliquot of 1 ml from the filtrate was mixed with 10 ml of distilled water. The mixture was transferred onto an Aflatest immunoaffinity column with flow rate 6 ml·min^{-1} and washed twice with 10 ml of distilled water. The column was then allowed once more to dry by passing air through it. AFB_1 was eluted with 2 ml of acetonitrile (flow rate 0.3 ml·min^{-1}) [22]. A derivative of AFB_1 (AFB_{2a}, hemiacetal of AFB_1) was prepared by adding 200 µl of hexane and 200 µl of trifluoroacetic acid to the evaporated solution of AFB_1 eluate, heating at 40˚C in a water bath for 10 min, evaporating to dryness under nitrogen, redissolving in 200 - 500 µl in appropriate volume with water/acetonitrile 9:1, v/v to give concentration of <5 ng·ml^{-1} and analyzing by HPLC (volume injected = 40 µl). AFB_{2a} shows enhanced fluorescence compared to AFB_1 [23]. On the other hand AFB_{2a} is less toxic compared to AFB_1 because of its protein binding properties, since it is not being absorbed from the gastrointestinal tract and therefore it is non toxic to experimental animals. Moreover AFB_{2a} does not interact with nuclear DNA [24].

2.7. Determination of Mycelial Mass in YES Medium

After cooling, mycelia were filtered through filters that were previously dried (24 h at 80˚C) and weighed. The mycelium was washed with distilled water and allowed to dry for 24 h at 80˚C. The dry weight of the mycelium was then measured.

2.8. HPLC-FL Analysis

HPLC was performed using a Hewlett-Packard 1050 (Waldbornn, Germany) liquid chromatograph equipped with a JASCO FP-920 (Jasco Ltd, JAPAN) fluorescence detector and an HP integrator 3395. The HPLC column used was a C_{18} Nova-Pak (250 × 4.6 mm, 4 µm particle size) purchased from Waters (Millipore, USA). For AFB_1 determination isocratic elution was employed and mobile phase consisted of water/acetonitrile/methanol, 20:4:3, v/v/v. Prior to injection samples were filtered through Millipore HVLP (0.45 µm) filters. Detection of the AFB_1 hemiacetal derivative (AFB_{2a}) was carried out at λ_{ex} = 365 nm and λ_{em} = 425 nm. The flow rate was 1 ml·min^{-1}, and the retention time for AFB_{2a} was ~15 min.

3. Results and Discussion

Effect of Saffron on Mycelial Growth and AFB_1 Production

The in-house characterization of the method for determining AFB_1 in YES medium has been reported in detail by Vergopoulou *et al*. [19]. The recovery factor of the method is 95.3% (RSD 9.6%) and the detection limit of the derivatized AFB_1 (AFB_{2a}) was found to be 0.02 ng AFB_1 ml^{-1} of YES. In the present study YES medium was chosen since is an optimum medium for AFB_1 biosynthesis. Gqaleni *et al*. [25] reported that in YES liquid static culture at 30˚C, AFB_1 production by *A. flavus* was higher compared to agar media. In addition, measuring AFB_1 rather than all four AFBs (AFB_1, AFG_1, AFB_2, and AFG2) was followed throughout this study. AFB_1 is the prevalent form and also the most potent of these toxins [26]. *A. parasiticus* was used as most *A. parasiticus* strains are rather stable in aflatoxin production in cultures [27]. **Table 1** shows that the mycelial growth was observed in control cultures inoculated (without saffron addition) and in cultures with saffron, inoculated and non-inoculated with *A. parasiticus*, as well. It is obvious that molds occur along with other microorganisms in saffron natural microbiota. The natural microbiota of saffron stigmas examined, consisted of bacteria (<1000 cfu/gr) and potentially aflatoxigenic molds (authors unpublished results). In that case mycelium growth is likely. Palumbo *et al*. [28] reported that the growth of *A. flavus* was completely inhibited when antagonist bacteria were present in microbiological media. In the present study microbial competition in cultures between saffron natural microbiota and *A. parasiticus* was not proved since the mycelial growth was just slightly reduced compared to cultures control (only inoculated with the fungus, **Table 1**).

The addition of saffron stigmas to cultures inoculated with *A. parasiticus* in this study, inhibited significantly AFB_1 production compared to control cultures (inoculated with *A. parasiticus* without the addition of saffron (**Table 2**, **Figure 1**). AFB_1 production in cultures inoculated with *A. parasiticus* with the addition of saffron was observed up to the 15th day of incubation, while on the 18th day AFB_1 was not detectable. Moreover AFB_1 production was also observed at lower levels after the 7th day of incubation in non- inoculated cultures containing saffron (100 mg·flask^{-1}). AFB_1 in cultures only with saffron was not detectable the days 0 and 3 of incubation, indicating that the saffron utilized throughout this work was not originally contaminated with AFB_1 at detectable levels. Previously Martins *et al*. [29] reported that AFB_1 was

Table 1. *A. parasiticus* growth in YES medium against mycelium growth in YES with saffron addition inoculated and YES with saffron alone.

	Addition of saffron		
	0[a]	100 mg[b]	100 mg + 100 conidia[c]
	Mycelium growth		
Days	g·flask^{-1} (±SD)	g·flask^{-1} (±SD)	g·flask^{-1} (±SD)
0	0.05 (±0.006)	ND[d]	0.06 (±0.01)
3	0.13 (±0.02)	0.08 (±0.02)	0.12 (±0.02)
7	0.18 (±0.003)	0.16 (±0.04)	0.10 (±0.03)
9	0.21 (±0.04)	0.13 (±0.03)	0.11 (±0.02)
12	0.17 (±0.004)	0.13 (±0.02)	0.08 (±0.03)
15	0.17 (±0.01)	0.19 (±0.006)	0.05 (±0.006)
18	0.15 (±0.02)	0.05 (±0.006)	0.04 (±0.006)

[a]Inoculated without the addition of saffron (control); [b]Addition of saffron 100 mg·flask^{-1}; [c]Addition of saffron 100 mg·flask^{-1} inoculated with 100 conidia of *A. parasiticus*; [d]ND: not detected.

Table 2. AFB_1 production (μg/flask) by *A. parasiticus* in YES medium against AFB_1 production in YES with saffron addition inoculated and YES with saffron alone.

	Addition of saffron		
	0[a]	100 mg[b]	100 mg + 100 conidia[c]
	AFB_1 production		
Days	μg·flask^{-1} (±SD)	μg·flask^{-1} (±SD)	μg·flask^{-1} (±SD)
0	0.002 (±0)	ND [d]	0.004 (±0.001)
3	15.79 (±1.85)	ND [d]	0.010 (±0.0006)
7	47.54 (±10.77)	0.008 (±0.002)	0.010 (±0.001)
9	64.00 (±0.75)	0.051 (±0.005)	0.012 (±0.004)
12	75.31 (±2.31)	0.043 (±0.005)	0.018 (±0.003)
15	85.47 (±1.76)	0.020 (±0.001)	0.013 (±0.002)
18	90.42 (±1.61)	0.010 (±0.003)	ND[d]

[a]Inoculated without the addition of saffron (control); [b]Addition of saffron 100 mg/flask; [c]Addition of saffron 100 mg·flask^{-1} inoculated with 100 conidia of *A. parasiticus*; [d]ND: not detected.

found in Portuguese saffron at levels of 1 to 5 μg·kg^{-1}.

Maximum AFB_1 production in cultures with saffron alone and cultures inoculated with 100 conidia of *A. parasiticus* with saffron, was observed on the 9th day (0.051 μg AFB_1·flask^{-1}) and on the 12th day (0.018 μg AFB_1·flask^{-1}) respectively. Maximum AFB_1 production in control samples (inoculated without saffron), on the same days 12 and 9, was 75.31 μg AFB_1·flask^{-1} and 64 μg AFB_1·flask^{-1} respectively. Therefore AFB_1 production was inhibited in inoculated cultures when saffron was

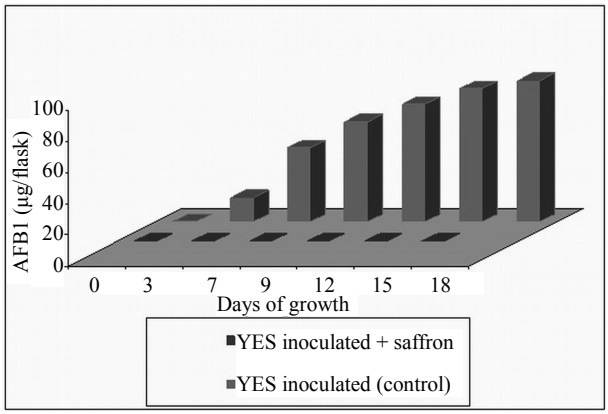

Figure 1. Saffron (100 mg/flask) significantly inhibits AFB_1 production by *A. parasiticus* in YES medium in comparison to AFB_1 production in YES inoculated with *A. parasiticus* without the addition of saffron (control). By the 18th day no AFB_1 was detectable in inoculated cultures with the addition of saffron.

present. Inhibition amounted to 99.9% in cultures with saffron inoculated with *A. parasiticus* for the 15 days of observation. As mentioned previously AFB_1 was not detectable the 18th day (**Table 2**, **Figure 1**). In the present study it was shown that the AFB_1 levels for the samples with saffron only and samples with saffron inoculated with *A. parasiticus*, after reaching their maximum on 9th and 12th day of observation respectively, a decrease was observed. This decrease is due to the aflatoxin degradation. According to Doyle *et al*. [30] as the amount of AFB_1 increases the rate of degradation also increases. This is in agreement with the results of this work (**Table 2**).

One concern with the data on AF inhibitors is that the majority of experiments were conducted in vitro in media that do not approximate conditions on host plant seeds. Screening of inhibitors added to media that incorporate host seed extracts may improve success in identifying efficacious inhibitors [31]. To our knowledge there is no information about the saffron biological ability as crude product. Hence in this work for the first time saffron stigmas were added to YES media without treatment containing their natural microbiota. In the present study 100 mg of saffron flask^{-1} corresponding to 10,000 mg·l^{-1} were utilized, in view of the fact that saffron consists of several compounds both active and inactive. Assimopoulou *et al*. [32] reported previously that methanol extract of saffron exhibited high antioxidant activity at a concentration above 2000 mg·l^{-1}. Furthermore saffron at concentration 100 mg·l^{-1} (corresponding to 1 mg of saffron stigmas·flask^{-1} did not have an effect on the AFB_1 production (authors' unpublished results).

Most inhibitors of AF biosynthesis act at one of three levels: altering the physiological environment or other signaling inputs perceived by the fungus, interfering with

signal transduction and gene expression regulatory networks upstream of AF biosynthesis, or blocking enzymatic activity [31].

Previously, Zaica and Buchanan [33] reported that a large number of compounds were found to inhibit AFB_1 biosynthesis mainly through their effect on fungal growth. In the current study on the 9th and 12th days of maximum production of AFB_1 in samples with saffron alone and samples with saffron inoculated with *A. parasiticus* respectively was 1254 - 1751 and 4183 - 5333 folds lower compared with samples control (inoculated with fungus). Nevertheless the mycelial mass of the same samples at the same days was only 1.3 - 1.6 and 1.9 - 2.1 folds lower, compared to samples inoculated with *A. parasiticus* (control). Therefore AFB_1 decrease was proved and it is assumed that is not correlated to fungus growth. Recently López and Gómez-Gómez [34] reported that infection of plants by pathogens activates a complex system of signal transduction pathways, which results in the activation of defense genes. So the authors isolated and characterized a new chitinase gene from saffron designated Safchi A, which showed chitinase activity in vitro. Chitin is a major component of fungal walls. Upon pathogen attack plants produce chitinases that degrade chitin to chito-oligomers which were shown to elicit strong defense responses in many plant species [35]. The results reported by López and Gómez-Gómez [34] indicated that saffron develops an active defense response to prevent colonization by the fungus. This statement is in agreement with the results presented in the current study since *A. parsiticus* growth was slightly inhibited in the presence of saffron (**Table 1**).

The most interesting approach to the study of the inhibitory effect of saffron on *A. parasiticus* growth and AFB_1 production is that saffron acts as an antioxidant [36]. Oxidative stress has been shown to stimulate aflatoxin biosynthesis in *A. parasiticus* [37,38]. The stimulation and suppression of AF biosynthesis by oxidants and antioxidants, respectively indicates that perturbations in the oxidative state of the fungal cell influence AF biosynthesis. Redox reactions are fundamental to cellular catabolism and anabolism, and antioxidants may hinder vital processes. Inhibition of mitochondrial or peroxisomal b-oxidation by antioxidants may limit the availability of carbon skeletons for polyketide pathways during growth on lipid-rich seeds. Antioxidants could interfere, thereby, reducing the pool of nicotinamide adenine dinucleotide phosphate (reduced form) available for AF biosynthetic reactions [39]. Furthermore a survey of inhibitors of AF biosynthesis has illustrated that many inhibitors have antioxidant activity [31].

In the present study, the significant inhibitory effect of saffron on AFB_1 production should probably be attributed to a synergistic action of the main bioactive constituents crocin and safranal, which is obtained by picrocrocin degradation. Assimopoulou *et al*. [32] reported that saffron methanol extract exhibited high antioxidant activity and established that crocin and safranal showed high scavenging activity which is involved in aging process, anti-inflammatory, anticancer and wound healing activity as well. Additionally Papandreou *et al*. [40] showed the antioxidant properties of saffron stigmas extract and its crocin constituents. It is interesting to report that, in the present study, AFB_1 production in cultures inoculated with *A. parasiticus* with the addition of saffron before autoclaving, was not significantly different compared to control cultures (without saffron) (authors unpublished results). Autoclaving possibly inactivates crucial antioxidants such as safranal (boiling point 70˚C at 1 mm Hg) and crocetin as already mentioned previously.

In the present study the significance was given to the saffron activity as crude material since the antioxidant activity of the principal components has been already shown in the literature as mentioned above. Therefore although saffron consists of many components, both active and inactive, in the present study has been revealed that antioxidant properties dominate. As a result, when saffron stigmas were added to cultures of YES inoculated with *A. parasiticus*, AFB_1 production was down 99.9% compared to control cultures, (without saffron addition) throughout the period of observation. Conclusively taking account saffron's unique properties, it might be utilized under specific conditions for processing agricultural products, to prevent AFB_1 production.

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