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Fungitoxic effect of natural extracts on mycelial growth, spore germination and aflatoxin B1 production of *Aspergillus flavus*

Zohra Mohammedi*, Fawzia Atik

Natural Products Laboratory, Department of molecular and cellular biology, faculty of sciences, university of Abou Bakr Belkaid, BP 119 Tlemcen 13000, Algeria

***Corresponding author: mdi3zhr@gmail.com**

Abstract

The methanolic extracts of leaves from *Tamarix aphylla*, *Tamarix pauciovulata*, *T. hirsuta*, *D. gnidium*, *C. procera*, aerial parts from *H. scoparium*, *A. schmittianum*, *P. argentea* and *M. canescens* were investigated for their antifungal and antiaflatoxigenic activities against the aflatoxigenic fungus *A. flavus*. The results of antifungal activity evaluated by two methods indicate that three plant species (*H. scoparium*, *A. schmittianun* and *D. gnidium*) among the nine investigated showed significant ($p<0.05$) mycelium growth inhibition which is of 65.33%, 83.56% and 100% respectively. Moreover five plant species showed significant ($p<0.05$) spore germination inhibition. These are extracts of *C. procera* (81.53%), *T. pauciovulata* (83.81%), *T. aphylla* (92.33%), *M. canescens* (97.73%) and *D. gnidium* (100%). Extracts from *D. gnidium* exhibited complete inhibitory effect on mycelium growth and spore germination, while *H. scoparium* exhibited complete inhibitory effect only on spore germination. In addition, all extracts from different plant species were found efficacious in checking aflatoxin B1 production and extract of *D. gnidium* was the more potent inhibitor of aflatoxin B1 biosythesis by the fungitoxic strain *A. flavus* at the concentration of 10µg/ml. The findings obtained reveals that the natural extract of *D. gnidium* is an important source of potentially useful molecules for the development of new antifungal agents and the possible exploitation of *D. gnidium* extract to protect the spoilage grains and food stuffs against the aflatoxigenic fungus *A. flavus*.

Keywords: aflatoxins, *Aspergillus flavus*, food protection, medicinal herbs, mycotoxins, natural extracts.
Abbreviation: AF-Aflatoxin, AFB1-Aflatoxin B1, PDA-Potato Dextrose Agar, SD-Standard Deviation, SMKY- Sucrose/ Magnesium sulphate/ potassium (Kalium) nitrate/ Yeast extract, TLC-Thin Layer Chromatography.

Introduction

Mycotoxins are a group of structurally diverse secondary metabolites produced by various fungal species. These toxic compounds can contaminate foodstuffs, crops or human foods. The ingestion of these contaminated materials may be pathogenic in animals and humans as they may lead to serious health problems, such as liver, kidney or nervous system damage, immunosuppression and carcinogenesis (Bennett and Klich, 2003). They comprise a group of several hundreds of chemically different toxic compounds (Huwig, 2001). Aflatoxins (AFs) are a group of mycotoxins produced by the spoilage of fungi *Aspergillus*, particularly *Aspergillus flavus* and *Aspergillus parasiticus* (Blesa and al., 2003). They are most commonly known for causing acute or chronic liver disease (Chao and al., 1991), but they are also considered immunosuppressive, hepatotoxic, mutagenic, teratogenic, and carcinogenic (Patten, 1981). The four major aflatoxins are B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2), which are each distinguished by the colour of their fluorescence under ultraviolet light (B, blue; G, green). The International Agency for Research on Cancer (IARC) has clarified AFB1, AFB2, AFG1 and AFG2 in the group I as human carcinogen (IARC, 2002). *A. flavus* generally produces only AFB1 and AFB2 (Santacroce and al., 2008) and AFB1 is the most toxic form (Speijers and Speijers, 2004), its mechanism of genotoxicity results from liver cytochrome P-450 epoxidation of AFB1 to AFB1 *exo*-8,9-epoxide (AFBO). This epoxide reacts with DNA at the guanyl N7 atom after intercalation, forming a genotoxic DNA adduct (Essigmann and al., 1977; Johnson and Guengerich, 1997). Different strategies have been established for preventing AFs contamination of susceptible plants and crops (Cleveland and Bhatnagar, 1992). The application of chemicals compounds has led to a number of environmental and health problems due to their residual toxicity, carcinogenicity, hormonal imbalance and spermatotoxicity (Pandey, 2003; Kumar and al., 2007). There is a need to design new and environmentally safe methods of reducing infection by aflatoxigenic *aspergilli* and to inhibit aflatoxin biosynthesis. Plants are considered as sources of useful metabolites (Razzaghi-Abyaneh and al., 2008). Plants contain a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, and flavonoids, reported to have in vitro antifungal properties (Arif and al., 2009). The objectives of this study were to evaluate the fungitoxic effect of various plant extracts against *Aspergillus flavus* and to test the selected plants for their ability to inhibit aflatoxin B1 production.

Results

The results of antifungal screening of methanolic extracts of nine plant species are presented in table 1 and 2. In this study, all extracts showed inhibition activity against the fungus *A. flavus*. The mycelial growth inhibitory effect of CME was in

the following order: *D. gnidium*, *A. schmittianum*, *H. scoparium*, *M. canescens*, *T. aphylla*, *T. pauciovulata*, *T. hirsuta* and *P. argentea*. Extracts from *D. gnidium*, *A. schmittianum* and *H. scoparium* shown good antifungal activity on mycelium growth, more than 50%. CME of *D. gnidium* led to complete inhibition (100%) of mycelium growth, while only about 85.56% and 65.33% inhibition was achieved by the same concentration of CME from *A. schmittianum* and *H. scoparium* respectively. Results presented in table 2 show the anti-germination activity of CME against the spores of fungitoxic strain *A. flavus*. The CME of all plants with the exception of CME of *P. argentea* have inhibitory effect on spore germination and CME of *D. gnidium* and *H. scoparium* have resulted in complete inhibition of germination. CME of these two species have shown the highest antifungal activity by growth inhibitory test, but the CME of *H. scoparium* has not caused complete inhibitory in the first test, then the CME of *D. gnidium* was the most effective against growth and spores germination of the toxigenic strain *A. flavus*. However, our results also indicate that CME of *M. canescens*, *C. procera*, *T. aphylla* and *T. pauciovulata* were more effective on spore germination than mycelial growth. At the concentration of 5µg/ml, CME of these four plants exhibited higher inhibitory activity, more than 80% on spore germination, while moderate activity less than 50% were observed in growth inhibitory test. CME of *M. canescens* exhibited 97.73%, *C.procera* 81.53%, *T. aphylla* 92.33% and *T. pauciovulata* 83.81% inhibitory effect on spore germination. In addition, CME of *A. schmittiamun* showed only 21.3% inhibitory effect on spore germination and it was more active on mycelium growth than spore germination. Furthermore, data in table 2 clearly indicate that CME of *P. argentea* did not inhibit spore germination of filamentous fungus *A. flavus* and statistically this result (0.57%) was not significant ($p>0.05$) and then it was similar to control. The CME of all plant species on aflatoxin B1production in SMKY liquid medium by the aflatoxigenic strain *A. flavus*, measured by TLC-Spectrophotometric method have shown a great inhibitory effect, which is dose-dependent. It's noted that the increase in concentration of CME in medium provoke enhance inhibitory activity on AFB1 biosynthetic. The same observation was shown for biomass production by the fungus *A. flavus*, but not similar in percentage. The results presented in table 3 indicate that biomass and aflatoxin contents were significantly ($p<0.05$) affected by the extracts from different source of plant species. At low concentration, only CME of *T. pauciovulata* and *H. scoparium* showed not significant ($p>0.05$) inhibitory effect on AFB1 production and consequently CME of these two species have not antiaflatoxigenic activity at the concentration of 2.5µg/ml, but the effect on AFB1 production appear at the concentration of 5µg/ml and enlarge with improve concentration. In general, at 10µg/ml all CME have a very high antiaflatoxigenic potential, which is more than 50%. *D. gnidium* shown the best activity, its CME of the leaves exhibited 93.50% reduction of AFB1 production, followed by the crude extracts of *M. canescens* (89.75%), *H. scoparium* (85.51%), *T. pauciovulata* (85.49%) and *A. schmittianum* (82.24%).

Discussion

Plants contain several phytochemicals compounds, which are known to play important role in defense against bacteria, fungi, herbivores, insects and viruses (Duke and Bogenschutz-Godwi, 1999). Various reports on medicinal plants extracts have shown inhibitory effects against phytopathogenic fungi in vitro (Senhaji and al., 2005; Pak and al.,2006; Oyedeji and al., 2011). Our results of the mycelium growth inhibition assay suggested that the crude methanolic extracts from *D. gnidium*, *A. schmittianum* and *H. scoparium* were the most active against the fungus *A. flavus*. The CME of *D. gnidium* and *H. scoparium* were also more active on spore germination inhibition with the crude methanolic extracts from *C. procera*, *M. canescens*, *T. aphylla* and *T. pauciovulata*. These plants are an important part of traditional medicine in North Africa and are extensively used in Algeria. It should be noted that polyphenolic, flavonoids, phenolic acids, tannins, quinines, coumarins, terpenoids and alkaloids possess antifungal proprieties (Ferheen and al., 2005; Omidbeygi and al., 2007; Oyedeji and al., 2011). The mechanisms thought to be responsible for toxicity against fungi may involve various targets: interference with the synthesis of cellular walls, alteration of cell permeability, interference with the transport of electron, the nutrient absorption, the adenosine triphosphatase and other metabolic processes of the cell, deactivation of various cellular enzymes and denaturation of cellular proteins (Marjorie, 1996; Cowan, 1999; Feng and Zheng, 2007; Al-Amiery and al., 2012). *D. gnidium* contains coumarins, flavonoids and diterpenoids (Kupchan and al., 1976; Deiana and al., 2003). Its Crude methanolic extract promoted total growth inhibition of the mycelium and prevents spore germination, while crude methanolic extract from *H. scoparium* provoked only complete inhibition in the second antifungal test (spore germination inhibition). A number of studies have been reported that the presence and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity. The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Arif and al., 2009). Most foods are prone to biodeterioration by *A. flavus* and other fungi during post-harvest processing, transport, and storage. Mould infections alter the chemical and nutritional characteristics of grains and seeds and, more importantly, contaminate the remaining food with mycotoxins (Shukla and al., 2008). Aflatoxins are the most economically mycotoxins for which their toxicity for human has been well established (Eaton and Groopman, 1994). Regarding the results of aflatoxigenic test, it can be concluded that the most methanolic extracts from all plant species can reduce production of AFB1, but this efficacy is limited and the strong inhibition was found with the extract from *D. gnidium*. This result explains that the bioactive compounds in extract of *D. gnidium* applied three potent inhibitory actions, on mycelium (vegetative form), on spore and on AFB1 production. Various reports in literature definite the prominent role of *D. gnidium* as antimicrobial and antimycotic and several research have demonstrate that secondary metabolites from plant species such as flavonoids and coumarins have inhibitory effect on growth and on aflatoxin production (Buchanan and Lewis, 1984; Deiana and al., 2003; Gonçalez and al., 2001; Greene-McDowelle and al., 1999; Norton, 1999; Pak and al., 2006; Paster and al., 1988; Weidenborner and al., 1989). Anti-mycelial growth, anti-germination and anti-aflatoxin activities of *D. gnidium* were due to synergic effect of whole phytochemical compounds in it methanolic extract in particulary flavonoids and coumarins. These bioactive compounds act simultaneously or differently and take the same or different

Table 1. Toxicological effect of plant extracts (5µg/ml) on mycelial growth of the fungus strain *A. flavus*

species	Diameter of colony (mm) ± SD	Percentage of inhibition (%)
Tamarix aphylla	45 ± 1.00***	40
Tamarix pauciovulata	52.50 ± 3.53**	30
Thymeleae hirsuta	53.33 ± 2.58**	28.89
Haloxylon scoparium	26 ± 3.60***	65.33
Paronychia argentea	56.50 ± 3.10*	24.67
Morettia canescens	41.67 ± 2.88***	44.44
Calotropis procera	39.50 ± 1.08***	47.33
Arthrophytum schmittianum	12.33 ± 2.12***	83.56
Daphne gnidium	0***	100
Control	75 ± 1.41	0

n=3 ; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$

Table 2. Effect of extracts on the germination of spores at the concentration of 5µg/ml

species	Percentage of germination	Inhibition of spore germination (%) (mean ± SD)
Tamarix aphylla	7.67	92.33 ± 0.700
Tamarix pauciovulata	16.19	83.81 ± 0.245
Thymelaea hirsuta	68.75	31.25 ± 0.905
Haloxylon scoparium	0	100
Paronychia argentea	99.43	0.57 ± 1.125^{ns}
Morettia canescens	2.27	97.73 ± 0.240
Calotropis procera	18.47	81.53 ± 1.000
Arthrophytum schmittianum	78.69	21.31 ± 1.560
Daphne gnidium	0	100
control	100	0

n=3 ; ns: not significant ($p > 0.05$)

ways, acting together or separately on one or several defined targets, associate allelopathic mechanism and inhibition of enzymes involved in growth and in the biosynthetic pathway of aflatoxins and leads to an effective antifungal and antiaflatoxigenic activity. This present finding indicates the possible exploitation of CME of *D. gnidium* in treatment of food commodities against fungal infestations and aflatoxin production.

Materials and methods

Plant materials

Plant species: *Calotropis procera* (Ait.) (leaves), *Paronychia argentea* (Lam.) (aerial part), *Morettia canescens* (Boiss.) (aerial part), *Haloxylon scoparium* (Pomel) (aerial part), *Thymelaea hirsuta* (Endl.) (leaves), *Tamarix aphylla* (L.) Karst. (leaves), *Tamarix pauciovulata* J. Gay (leaves), *Daphne gnidium* (L.) (leaves) and *Arthrophytum schmittianum* (Pomel) M. et W. (aerial part) were collected from arid and sub arid area from Algeria (table 4). The collected material was dried in darkness at room temperature. Then it was powdered and stored in experimental plastic bag.

Extraction procedure

To prepare the crude extract of each species, the powdered material was macerated in methanol (70%) at room temperature for 48hours. The mixture was filtrated through whatman n°1 filter paper to separate the residue plant material from the liquid. Then to remove the solvent, the filtrate was evaporated under low pressure and temperature with a rotary evaporator (Laborota 4000). The dry residue was dissolved in sterilized distilled water, frozen and lyophilized to get dry lyophilized power, called crude methanolic extract (CME).

Fungal strain

The aflatoxigenic strain *Aspergillus flavus MNHN 994294* (National Museum of Natural History) used in this study was obtained from Natural Products Laboratory, Department of molecular and cellular Biology, University of Abou Bakr Belkaid, Tlemcen (Algeria). The strain was maintained on potato dextrose agar (PDA) medium at 4°C.

Antifungal activity

Growth inhibitory assay

Inhibition of mycelial growth was assayed using the food poison method described by Nene and Thapliyal (1979). The autoclaved medium was maintained in a water batch at 45°C. For each sample, the CME was added to molten PDA to obtain final concentration of 5µg/ml. The homogeneous mixture was poured in sterilized Petri dishes. A 6mm mycelium ager disc was taken from seven days old culture of toxigenic fungus and placed in the center of each Petri plate. Three replicate plates were used per treatment. Plates containing mycelium disc without CME were used as negative controls. All plates were incubated at 28 ± 2 °C for 4 days. Fungal growth was measured as colony diameter and toxicity of CME against *A. flavus* was measured in terms of percent mycelia inhibition by the formula:

Growth Inhibition (%) = $[(Dc - Dt)/Dc] * 100$

Where,

Dc: Diameter of colony in the control (mm)

Dt: Diameter of colony in the treatment (mm)

Spore germination inhibition assay

Fungal spores were collected using sterile distilled water containing 0.1% Tween 80, from PDA plates after 7 days of

Table 3. Effect of natural extracts on *A. flavus* growth and aflatoxin productivity in SMKY liquid medium.

Species	Concentration (µg/ml)	Biomass (g)	Biomass reduction (%)	Amount of AFB1 (µg/g)	Inhibition of Aflatoxin B1 production (%)
Tamarix aphylla	2.5	0.1396±0.00028	49.42	66.195±0.07425	51.46
	5	0.1367±0.00071	50.47	63.775±0.54801	53.24
	7.5	0.1341±0.00078	51.41	61.356±0.17183	55.01
	10	0.1334±0.00028	51.67	39.775±0.24395	70.84
Tamarix pauciovulata	2.5	0.1457±0.0029	47.21	130.594±1.12713^{ns}	04.24
	5	0.139 ±0.00042	49.49	43.264±1.22754	68.28
	7.5	0.1351±0.00092	51.05	31.744±0.88813	76.72
	10	0.1302±0.00191	52.83	19.784±0.15274	85.49
Thymelaea hirsuta	2.5	0.1368±0.00226	50.44	106.943±4.90944	21.58
	5	0.1311±0.00311	52.50	61.725±2.31577	54.74
	7.5	0.1089±0.00785	60.54	54.938±3.09571	59.72
	10	0.1014±0.00099	63.26	40.431±1.64049	70.35
Haloxylon scoparium	2.5	0.1398±0.0053	49.35	131.470±1.73241^{ns}	3.60
	5	0.1198±0.00339	56.59	110.750±5.48008	18.79
	7.5	0.1141±0.0029	58.66	40.441±2.43315	70.35
	10	0.1014±0.00778	63.26	19.768±2.26981	85.51
Paronychia argentea	2.5	0.1409±0.00042	48.95	120.560±3.83252	11.60
	5	0.1408±0.00057	48.99	86.002±4.24405	36.94
	7.5	0.1398±0.00601	49.35	51.329±2.97621	62.37
	10	0.0528±0.01393	80.87	36.562±2.90267	73.19
Morettia canescens	2.5	0.1385±0.00057	49.82	67.774±1.29118	50.30
	5	0.1370±0.00141	50.36	41.151±1.34562	69.83
	7.5	0.1322±0.00156	52.10	36.275±2.19132	73.40
	10	0.1284±0.00332	53.48	13.976±1.39654	89.75
Calotropis procera	2.5	0.1441±0.00198	47.79	87.521±1.13915	35.82
	5	0.1357±0.00219	50.83	83.953±2.04566	38.44
	7.5	0.1312±0.00085	52.68	61.022±2.46215	55.25
	10	0.1306±0.02164	52.46	56.997±4.24123	58.21
Arthrophytum schmittianum	2.5	0.1418±0.00085	48.62	111.537±5.33866	18.21
	5	0.1415±0.00106	48.73	62.720±3.71938	54.01
	7.5	0.1316±0.00113	52.32	29.727±2.79166	78.20
	10	0.1286±0.00431	53.41	24.220±1.73595	82.24
Daphne gnidium	2.5	0.1259±0.0029	54.39	86.378±3.87707	36.66
	5	0.1235±0.00106	55.25	37.929±1.31522	72.19
	7.5	0.1154±0.00382	58.19	34.315±1.94313	74.84
	10	0.0159±0.00156	94.24	8.866±0.97123	93.50
control	0	0.2760±0.01435	0	136.375±5.46099	0

n=3 ; ns: not significant ($p > 0.05$)

Table 4. area of collect and botanical information of plants used.

Scientific name	Family	Plant organ	Area of collect	Use
Morettia canescens	Brassicaceae	Aerial part	*Adrar*	For animals
Calotropis procera	Asclepiadaceae	leaves	*Adrar*	Medicinal, for animals
Tamarix aphylla	Tamaricaceae	leaves	*Adrar*	Medicinal, for animals
Tamarix pauciovulata	Tamaricaceae	leaves	*Adrar*	Medicinal, for animals
Thymelaea hirsuta	Thymelaeaceae	leaves	*Mascara*	Medicinal
Paronychia argentea	Caryophyllaceae	Aerial part	*Mechria*	Medicinal
Haloxylon scoparium	Chenopodiaceae	Aerial part	*Ghardaïa*	Medicinal
Arthrophytum schmittianum	Amaranthaceae	Aerial part	*Tebessa*	Medicinal
Daphne gnidium	Thymelaeaceae	leaves	*Tlemcen*	Medicinal

growth at 28°C. Final concentrations of spores were adjusted to 1.45×10^6 spores/ml. for assay; autoclaved PDA medium (20ml) was mixed with 5µg/ml of CME and 1.45×10^6 spores and then poured into Petri plates (10cm diameter). After solidification, plates were incubated at 28 ± 2 °C for 4 days. The percent germination reduction of spores was determined using the following formula:

Inhibition of spore germination (%) = $[(Nc - Nt)/Nc] * 100$

Where,

Nc: Number of fungal colony in control

Nt: Number of fungal colony in treatment

Anti-aflatoxigenic Activity

The efficacity of CME of each sample to reduce aflatoxins production was evaluated by the method of Diener and Davis (1969). A fungal disc of 6mm diameter from seven days old culture of *A. flavus* on PDA medium was ascetically inoculated on 25ml of the SMKY liquid medium in conical flask supplemented with a required amount of CME. The control sets comprised the medium without the CME. The flasks were incubated at 28 ± 2 °C for 10 days. After incubation, content of each flask was filtered through

cheesecloth and Whatman filter paper n°1. The fungal mats were dried in oven at 110°C for 12 hours and mycelium weight was determined (biomass). The filtrate was extracted with chloroform (1:1, v/v) in a separating funnel. The chloroformic fraction was evaporated to dryness using a rotary evaporator and the residue was dissolved in 1ml of chloroform. The amount of aflatoxin B1 (AFB1) produced in the treatment and control sets was determined by the TLC technique following Nabney and Nesbitt (1965). 50 µl of the chloroform extract spotted on TLC plate along with the standard of aflatoxin, was run in toluene: isoamyl alcohol: methanol (90:32:2 v/v). The developed plates were air dried and observed under long wave of UV light 365nm. Initial identification of different components of aflatoxins was made on visual basis by comparing the color and intensity of fluorescence of the sample and standard spots. For the quantitative estimation, spot of AFB1 on TLC plate were scrapped out and dissolved in chloroforme (1ml). The amount of aflatoxin B1 present in the sample was estimated by a spectrophotometer Shimadzu UV mini1240 at 363nm and calculated according to the following formula:

AFB1 content ($\mu g.ml^{-1}$) = [(D x M)/ (E x L)] * 1000

Where,

D: absorbance, M: molecular weight of aflatoxin (312), E: molar extinction coefficient of aflatoxin B1 (21,800), and L: path length (1cm)

Statistical analysis

All analysis was performed in triplicate. The data were recorded as means ± standard deviation (SD). One-way analysis of variance (ANOVA) and Tukey multiple comparisons were carried out to test for any significant differences between means. Differences between means at 5% ($p<0.05$) were considered significant.

Conclusion

Our data showed that extracts from the extracts of *Daphne gnidium* have broad antifungal and antiaflatoxigenic efficacy against *Aspergillus flavus* and are potentially useful ingredient in various preparations for food and crops preservation.

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