Inhibition of aflatoxin B₁ production of Aspergillus flavus isolated from peanut seeds using Lycium halimifolium Mill leaves fractions

Camélia Mosbah¹,²*, Asma Milet¹, Mounira Kara Ali¹, Imen Talhi¹ and Noureddine Kacem Chaouche¹

¹Laboratory of Mycology, Biotechnology and Microbial Activity (LaMyBAM), Brothers Mentouri University, Constantine, Algeria.
²Department of Biology, Faculty of Nature and Life Sciences, Larbi Ben M'hidi University, Oum el Bouaghi, Algeria.

Received 22 November, 2016; Accepted 31 January, 2017

The aim of this study is to explore the impact of Lycium halimifolium Mill leaves extracts on mycotoxin B₁ production in vitro, as well as the inhibitor effect of the aflatoxin B₁ synthesis by Aspergillus flavus strain. The strain was isolated, identified (gi |146746162 | EF409803.1.) and positively tested for the production of Aflatoxin B₁. The antimycotoxinogenesis effect was investigated using L. halimifolium Mill leaves consisting of butanol and ethyl acetate fractions. Indeed, the fractions were tested to stop the secretion of aflatoxin B₁ by A. flavus in submerged culture (YES), simultaneously. The growth of the strain was evaluated as biomass response. The antimycotoxinogenesis activity showed that the two fractions: ethyl acetate and butanol of L. halimifolium Mill leaves were able to inhibit significantly the synthesis of mycotoxin AF₅₁. Thus, the secretion inhibition percentage was calculated in comparison with the control, using HPLC-PDA technique, and estimated at 96.83% for butanolic fraction and 94.99% for ethyl acetate fraction. To verify its correlation with biomass, the growth inhibition percentage was also estimated. The results showed that the dry weight concentration under butanolic and ethyl acetate fractions was 1.6 and 2 g/l, respectively, corresponding to 65.22 and 56.53% of inhibition growth percentage.

Key words: Aflatoxin B₁, antimycotoxinogenesis activity, high performance liquid chromatography coupled with photodiode array detector (HPLC-PDA), natural extract.

INTRODUCTION

Mycotoxins are low molecular weight compounds that are synthetized during secondary metabolism by fungi especially those belonging to the genus Aspergillus, Penicillium and Fusarium (Streit et al., 2012). Aflatoxins are a group of toxic metabolites, called mycotoxins, produced by different species of toxigenic fungi such as

*Corresponding author. E-mail: kamimosbah@yahoo.fr. Tel: 0021364907291. Fax: 00213 31426102.

Author(s) agree that this article remains permanently open access under the terms of the Creative Commons Attribution License 4.0 International License.
Aspergillus flavus, Aspergillus parasiticus, Aspergillus nomius (Huwig et al., 2001). Among the different types of aflatoxins identified, the major groups are aflatoxin B₁ (AFB₁), B₂ (AFB₂), G₁ (AFG₁), G₂ (AFG₂), M₁ (AFM₁) and M₂ (AFM₂). 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 (Giorni et al., 2008). 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 (Trail et al., 1995; Crawford et al., 2008). Aflatoxin B₁ (AFB₁) is the most potent of the known AFs. It was classified within class 1 of human carcinogens especially the hepatocellular carcinoma (Brown, 2009) and considered as potent mutagenic. That caused the damage of cells by two different ways; firstly, AFB₁ (C₁₇H₁₁O₅) is activated to AFB₁-8,9-epoxide and forms adduct primarily at N7 position of guanine. In addition, the AFB₁-8,9-epoxide may interfere with the normal functioning of the nerve cells by forming DNA adducts, protein adducts, oxidative stress factors, mitochondrial directed apoptosis of the nerve cells as well as inhibiting their synthesis of protein, RNA and DNA (McGlynn et al., 2003); secondly, it is a potential immunosuppressive agent which several human studies have reported that aflatoxins cause an increase in circulating alpha tumor necrosis factor suggesting that these mycotoxins are immunotoxic in humans. AFB₁-8,9-epoxide affects also other organs and tissues, such as the lungs, kidney, heart damage and the entire respiratory system (Wagacha and Muthomi, 2008).

Fungi are generally controlled by synthetic fungicides; however, the use of these is increasingly restricted due to the harmful effects of pesticides on human health and the environment (Hermiche et al., 2012). Thus, there are a growing interest on the research of possible use of the plant extracts for control of the pest and diseases in agriculture which is less harmful to one’s health and environment (Logardia, 2012). A large number of compounds, extracts and essential oil from natural sources including medicinal plants such as roots, leaves, seeds and flowers have been reported to inhibit the production of aflatoxin (Paranagama et al., 2003; Benariba et al., 2013; Martins et al., 2014). The negative impacts of aflatoxins on human health led to the investigation of strategies to minimize, inactivate or eliminate the effects of these toxins in contaminated products. In this context, natural sources such as medicinal plants were studied for its therapeutic effect vis-à-vis aflatoxins. Among them, the genus Lycium (Solanaceae family) has been identified as a rich source of antioxidant compounds (Donno et al., 2014). This genus was used as a hedge plant and windbreak; the dry powder of its leaves was used as a protector from eye diseases (Potterat, 2010). In addition, Lycium halimifolium Mill is considered as a healthy food (Amagase and Farnsworth, 2011). The chemical constituents of L. halimifolium Mill are: cyclic peptides, glycoside derived from tryptophan, aldehydes, carotenoids and related compounds (β-carotène, zeaxanthin dipalmitate, β-cryptoxanthin, lutein), phenolic acids and flavonoids (Quercetin-3-O-rutinoside, Kaempferol-3-O-rutinoside, caffeic acid, vanillic acid) and lipopolysaccharides (LP) (glycoproteins formed by a heteroside and conjugated with a polypeptide chain) (Gu et al., 2007; Stephen et al., 2010; Xiaq et al., 2012; Jin et al., 2013). The recent studies indicate that the extracts of some Lycium species possess a range of biological activities, including effects on ageing, neuroprotection, anti-fatigue/endurance, increased metabolism, glucose control in diabetics, glaucoma, anti-oxidant properties, immunomodulation, anti-tumour activity and cytoprotection (Potterat, 2010).

To our knowledge, this study represents the first report of the biological properties of L. halimifolium Mill isolated from the eastern part of Algeria. The main objective of this work is to isolate and identify a fungi strain, test its antimycotoxinogenesis activity in submerged culture (YES) and evaluate its antifungal efficacy as biomass response.

MATERIALS AND METHODS

The plant material

L. halimifolium Mill plant was harvested from the mountain of Djbel Chettaba situated in Constantine (Eastern Algeria) with latitude 36°19’41.02” and longitude 6°27’4”. The samples were collected in spring 2013, corresponding to flowering period. The identification of the plant was established in laboratory of physiology and Ecology, Montouri Brothers University, Constantine, Algeria. The plant leaves were harvested and dried in the shade at room temperature and then were crushed using mortar and pulverized in the mill to obtain powder.

Extraction procedure

For flavonoids extraction, the method of Benhammou et al. (2009) was applied; thus, 20 g of the dry powder of L. halimifolium Mill leaves was extracted in 200 mL of methanol for 24h at room temperature. The extracts were concentrated in a rotary evaporator at 60°C under a reduced pressure. The residue was then boiled with water to dissolve the flavonoids. The aqueous phase containing flavonoids was defatted with petroleum ether and chloroform. The defatted aqueous phase was filtered through whatman paper No 1; firstly, it was extracted with 100 mL of ethyl acetate to obtain ethyl acetate fraction (ACT). The same operation was executed with 100 mL of n-butanol to obtain butanol fraction (BUT). The extracts were evaporated, weighted and preserved for further use in biological activities. The extraction yield was determined by the following formula:

\[
R\% = \frac{\text{mass of fraction residue}}{\text{mass of vegetal powder}} \times 100.
\]
Microbial material

The isolation of the toxigenic fungus was carried out from the local peanuts of the wilaya of El Taref, Algeria. According to Outtara et al. (2011), a sample (50 g) of fifty peanut seed from the crop year 2013 was moistened with sterile distilled water and left at room temperature in Petri plates during 7 days for the development of mycelium. The purification of the interest strain (Aspergillus) was made by successive subcultures on the PDA medium which allows the isolation of pure strain. Fungal isolate was characterized macroscopically and microscopically according to Pitt and Hocking (2009). This characterization was performed in the Laboratory of Mycology, Biotechnology and Microbial Activity (LaMyBAM). The molecular identification of fungal isolate was performed at the Laboratory of Microorganisms and Active Biomolecules (LMBA), Tunisia. The used primers for the PCR amplification were the universal primers ITS1, ITS4 for the ITS gene (Josefa et al., 2004). The purification of the PCR products was achieved using the horizontal agarose gel of ethidium bromide. The amplified genes were sequenced using the same primers sited above and the sequences were corrected by the Bio-edit program and deposited in Genbank data base. The DNA sequences were compared to those previously published in Genbank using the BLASTN program.

Antimycotoxinogenesis activity of L. halimifolium M. leaves fractions

Myco toxinogenesis activity

In order to demonstrate the aflatoxinogenesis of this isolate, a fermentation was established; thus, 50 mL of YES liquid medium consisting of g/L: yeast extracts (20), sucrose (150), MgSO4.7H2O (0.5), ZnSO4.7H2O (0.01), CuSO4.5H2O (0.005) (Pamel et al., 2011) was placed in 250 mL flasks, and autoclaved at 120°C for 15 min. The inoculation was performed by adding 1mL of a spore suspension (10² spores/mL) from interest strain youth culture. The flasks were incubated for 14 days at 25°C with intermittently manual agitation.  

Detection of aflatoxin B1

After the incubation period, the flask content was filtered through Whatman No.1. The mycelia obtained were placed on preweighed Petri plates and dried at 50°C for 6 h and then at 40°C over night. The net dry weight of mycelia was determined. In addition, the filtrate was used to detect the presence of mycotoxins especially, aflatoxin B1. Thus, 50 mL of the filtrate was added to 100 mL of chloroform. The mixture was thoroughly stirred for 10 min and allowed to settle. This operation was repeated successively by adding 50 mL, and then 30 mL of the chloroform to the aqueous phase. The chloroform extract obtained was filtered through Whatman No. 01 and concentrated by evaporation under vacuum using a rotary evaporator until a volume of 2 mL, to constitute the aliquot of mycotoxin crude (AMC) (Multon, 1982). 5 µL of AMC was spotted on silica gel plate (silica gel F254) thin layer chromatography, on the same line where 5 µL of Aflatoxin 1 control (NOVAKITS with 99.7% purity, product code: STD-AFB1, -P1) was deposited. The plate was placed in a chromatography tank and dipped in an eluting solvent mixture consisting of toluene-ethyl acetate- formic acid (5:4:1), respectively. After the migration, the plate was examined under a UV lamp at a wavelength of 365 nm. The presence of aflatoxin B1 was characterized by a blue fluorescence spot with the same Rf as the control. All tests were performed in three replicates (Dutton et al., 1985). The presence of aflatoxin B1 was confirmed using high performance liquid chromatography coupled with photodiode array detector (HPLC-PDA). The HPLC-PDA consisted of Waters e2695 separation Modules equipped with a Photodiode Array Detector (PDA), Gradient Pump (with four pumps), automatic injector, tiny computer with software Empower 3, control and a detector (UV VIS,with a C18 guard column (Machery nagel 250/4.6 nucleod); the column temperature was between 5 to 40°C. To constitute the mobile phase of mycotoxins, a gradient solvent system with solvent A (10 mL/L of Trifluoroacetic acid, TFA) and solvent B (acetonitrile) was used; however, the initial percentage of solvent B was 10%, which was raised to 50% in 30 min, then 90% in 4 min, lowered to 10% in 2min, and held at 10% at a flow rate of 1mL/min. The injection volume was 20 µL for sample extract and the detector was operated at wavelength of 254 nm (Frisvad, 1983).

Anti mycotoxinogenesis activity

To study the effect of two fractions of L. halimifolium Mill leaves, extracted previously (ACT and BUT) as inhibitors of aflatoxin B1-synthesis by interest isolate, the flasks of 250 mL contained 50 mL of YES liquid medium. Three flasks were supplemented with 1mg of ACT fraction; three other flasks were added to 1mg of BUT and the last three flasks were kept without treatment and served as control group. All the flasks were inoculated with 1mL of a spore suspension (10² spores/mL) from an interest youth strain culture. The flasks were incubated for 14 days at 25°C with intermittently manual agitation.

RESULTS AND DISCUSSION

This work is based on the study of antimycotoxinogenesis effect of substances extracted from leaves of L. halimifolium Mill, and the focus of attention is the inhibition of Aflatoxin B1 synthesis by A. flavus.

Determination of extraction yield

Two fractions, namely, ethyl acetate (ACT) and butanol (BUT) were obtained through the applied extraction protocol, and the extraction yield was calculated. It was found that the butanol fraction had the greatest yield with a rate of 28.5%, followed by the ethyl acetate fraction with a rate of 10.08%. The variation of extraction levels may be due to several factors including the interaction with the environment (type of climate, soil, period of harvesting and extraction method).

Characterization and identification of the interest strain

The macroscopic aspect of the colonies' morphology showed that this isolate has very rapid rate of growth (three days), characterized by a powdery texture, with yellow-green to olive green color fronds and may have a white border, denser toward the center. The reverse has cream yellowish color in PDA medium (Figure 1a). Otherwise, the microscopic aspect of the interest strain revealed septed hyphae with rather long conidiophores which have a rather rough texture. The vesicles are spherical to elongate, metulae cover the vesicle from
which the phialides were formed; Conidia are globose pale green with smooth to finely roughened walls (Figure 1b).

These results corroborate those obtained by Botton et al. (1990), attesting that this strain belongs to *A. flavus*. The dominance of the genus *Aspergillus* in the contaminating flora grain has also been reported in several studies (Reddy et al., 2010). However, to confirm this identification, the molecular analysis of sequencing ITS1 and ITS4 region of nuclear ribosomal operon was compared with available sequences in the GenBank. The sequences were similar to each other (99% similarity) (Figure 2). This comparison showed that this isolate is really *A. flavus*. The ITS gene sequences of the strain were submitted to the GenBank database and the accession number established corresponding to gi146746162 | EF409803.1.

**Antimycotoxinogenesis activity of the extracts of *L. halimifolium* Mill leaves**

**Mycotoxinogenesis activity**

After the incubation period (14 days), the filtrate was utilized to confirm the production of aflatoxin B₁ by the *A. flavus* isolated. This filtrate was added to chloroform, cleaned through Whatman No.1, and concentrated by evaporation under vacuum using a rotary evaporator, to constitute the aliquot of mycotoxin crude (AMC) of control, which was spotted on silica gel plate thin layer chromatography. After the visual observation under UV of this plate, a blue fluorescence color of aflatoxin B₁ was revealed with the same Rᵢ value as the control (Figure 3).

The AMC presented many spots with different migration distances, a major spot (Rᵢ = 0, 38) correspond to the same spot of aflatoxin B₁ standard (Rᵢ = 0.38). This result confirms the production of aflatoxins by the interest strain *A. flavus*. Several studies have been reported on the mycotoxin contamination of cereals and peanuts which demonstrated that *A. flavus* produced aflatoxin B₁ (Xianwen et al., 2015; Fakruddin et al., 2015).

The presence of aflatoxin B₁ in the filtrate was confirmed by high performance liquid chromatography HPLC (Figure 4), where the AMC has a retention time (Rt) of 30.13 min (Figure 4b) corresponding to the same standard aflatoxin B₁ with retention time (Rt) of 30.01 min (Figure 4a).

**Antimycotoxinogenesis activity**

In this report, the antimycotoxinogenesis effect of the two
different fractions (ACT and BUT) of *L. halimifolium* Mill leaves was studied. After the fermentation period, the aliquot of Mycotoxin Crude was spotted in the silica gel plate. The chromatographic profile revealed the presence of a fluorescent blue spot which has an Rf equal to 0.38 only in the control (without fractions) and corresponding to the standard AFB1, while the two fractions showed a total absence of aflatoxin B1 (Figure 5).

Our results of TLC demonstrate that the strain *A. flavus* produce aflatoxin B1, which is consistent with the work of Gacem et al. (2013) who used the same fermentation medium and mobile phase, and he has revealed the presence of a blue fluorescent spot corresponding to the aflatoxin B1 in the sample of chloroform extract. Another study used a different mobile phase (chloroform/methanol) demonstrated that *A. flavus* can produce a large amount of aflatoxin B1. Also, Josefa detected Aflatoxins B1 by TLC and he found that *A. flavus* strains produced aflatoxin B1 (Josefa et al., 2004).

Also, the antmycotoxinogenesis effect was also confirmed by high performance liquid chromatography HPLC. A remarkable decrease of aflatoxin B1 synthesis was reported when the growth medium contains the fractions of ethyl acetate and n-butanol (Figure 6); thus, the secretion inhibition percentage was calculated in comparison with the control, using HPLC-PDA technique, and estimated at 96.83% for butanolic fraction (Rt = 30.09 min) (Figure 6a) and 94.99% for ethyl acetate fraction (Rt = 30.18 min) (Figure 6b).

To verify its correlation with biomass, the growth inhibition percentage was also estimated. The results showed that the concentration of dry weight under butanolic and ethyl acetate extracts was 1.6 and 2 g/L consecutively corresponding to 65.22 and 56.53% of inhibition growth percentage. On the other hand, it is very interesting to note that, the production of aflatoxin B2 by the strain in the presence of cited fractions (ethyl acetate and butanol fractions) on growth medium, in comparison with the production of the same toxin by the strain without the presence of fractions, shows a decrease of more than 50% (Figure 6).

However, Neveen et al. (2015) reported that the essential oil of *O. basilicum* has a strong antifungal activity and completely inhibited the growth of *A. flavus* (in the same fermentation medium YES) with a percentage of 70%, while inhibition production of aflatoxin B1 was observed with a retention time equal to 6.94 minutes for the oil tested against a standard retention time to 6.78 min. Another study demonstrated that the methanolic extracts of leaves from *Helianthemum scoparium*, *A. schmittianun* and *Daphne gnidium* inhibited the mycelium growth with 65.33, 83.56% and 100% respectively (Mohammedi and Atik, 2013).

Recent studies have demonstrated the antifungal activity of natural compounds extracted from traditional medicinal plants (Mishra et al., 2012). The inhibition growth of *A. flavus* by secondary metabolites (flavonoids and essential oil) has already been reported. These compounds can interfere with biomembranes causing cell damage and causing leakage of cellular materials and finally the death of microorganisms (Dikbas et al., 2008; Abdel Ghani et al., 2008). Considering the large number of different groups of chemical compounds present in extracts of plants (flavonoids, phenols, saponosid, gallic tannins and catechic tannins and alkaloids), it is interesting to note that the antimicrobial activity is not attributable to a specific mechanism alone but to several targets in the cell.

Plant extracts can completely block the biosynthesis of mycotoxins where fungal growth is not affected (Pusztahelyyi et al., 2015). Several investigators studied the identification and application of natural products for inactivation of AFs and reported that some essential oils and other extracts (several flavonoids, and phenolic compounds) of plants could potentially provide protection against AFB1 (Rasooli et al., 2008; Bluma and Etcheverry, 2008). The natural compounds of plant extracts are identified and have potential candidates against AFB1 and essential oils that are able to reduce DNA binding of aflatoxin and phenolic compounds inhibit the biosynthetic pathway of AFB1 (Alpsoy, 2010). Also the study of Ghorbanian et al. (2008) indicated that inhibition of the aflatoxins synthesis is related to the contact time and the dose of the extract.

This study represents the first report on the evaluation of the antifungal activity and antimycoxygenesis effect of *L. halimifolium* Mill leaves. The results indicate that both fractions; ethyl acetate and n-butanol are able to inhibit in vitro the growth of *A. flavus* and the production...
of aflatoxin B1. These fractions must be subjected to further study to characterize the active compound, define toxicity and evaluate economic feasibility. The study opens up new opportunities for developing the culture of *L. halimifolium* Mill, firstly to reduce the effect of hepatotoxic and carcinogenic aflatoxins B1 and secondly to fight against rust peanuts.

CONFLICT OF INTERESTS
The authors declare that they have no conflict of interests.

ACKNOWLEDGEMENT
This research was supported by the C.N.E.P.R.U.
Figure 5. Profile mycotoxins B$_1$ detection by thin layer chromatography (TLC) of B$_1$ standard, AMC of control, ethyl acetate and n-butanol fractions of L. halimifolium Mill.

Figure 6. HPLC profile of Aflatoxin B$_1$ production by A. flavus: (c) treated with ACT fraction, (d) treated with BUT fraction of L. halimifolium Mill.
research project (code: F00920140051), Ministry of Higher Education and Scientific Research, Algeria. The authors are grateful for its partial financial support.

REFERENCES


