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Evaluation of Antifungal and anti-aflatoxin B_1 efficacy of some crude extracts of *Chamaerops humilis* L. against *Aspergillus flavus* isolated from peanuts (*Arachis hypogea* L.)

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ABSTRACT

The present study strengthens the food preservative potential of *Chamaerops humilis* extracts *viz.* hexane extract, chloroform extract, ethanol extract, chloroform extract, and methanol extract based on their antifungal, antiaflatoxin, and antioxidant efficacy. The isolation of molds from peanuts were carried out by the suspension-dilution technique and inoculation on agar medium. Antifungal determinations were conducted using the agar plate and liquid dilution methods. The effect of *C. humilis* L. extracts on the production of AFB_1 was determined in a SMKY broth. The molds isolated were of the genera *Aspergillus, Penicillium* sp., *Fusarium* sp., and *Alternaria* sp. with *A. flavus* (BBH-6) which is identified as the highest AFB_1 producer. The minimum inhibitory concentration of extracts against the toxigenic strain of *A. flavus* ranged between 3.00 mg/ml and 3.50 mg/ml. The extracts were found more efficacious as they inhibited the dry mycelium weight and aflatoxin production of the aflatoxigenic strain *A. flavus* (BBH-6) at lower concentrations. The extracts showed fungitoxic spectrum against four molds. The IC_{50} value of *C. humilis* L. ranged between 140.4 and 189.46 μg/mg, oxidation of linoleic acid was moderately inhibited by the extracts ranges between 53.54 and 69.33%, while their total phenolic content ranged between 47.80 and 115.71 μg/ml. Based on the findings of present investigation, *C. humilis* L. can be introduced as a proposed solution for formulating plant-based preservative food additives.

1. Introduction

Filamentous fungi have a remarkable ability to grow on varied and relatively simple substrates whose elements they manage to use constituents as nutrients. During their development, they also produce secondary metabolites (Nguefack et al., 2009). Fungal secondary metabolism is very complex and produces many compounds of very different natures. These molecules are synthesized in response to different types of environmental signals and can give their producers competitive advantages. Fungi can be used to produce compounds of pharmaceutical and industrial interest. However, they can also produce a number of toxic metabolites, called mycotoxins, which are dangerous for humans and animals (Frisvad et al., 2005; Prakash et al., 2010, 2011; Reddy et al., 2011). Their presence in food is regulated and these mycotoxins are responsible for losses economic mainly related to the decommissioning of identified contaminated batches. Aflatoxins are among the most studied mycotoxins and the more strictly regulated. This importance is essentially due to their impact health and economics (Upadhyay et al., 2018). In fact, Aflatoxin B_1 (AFB_1) is the most common natural carcinogen known at the present time and it is directly involved in the appearance of liver cancer. L'AFB_1 is classified by the International Agency for Research on Cancer (IARC) in group 1, which includes molecules that are carcinogenic to humans and animals (Liu et Wu, 2010). The health impact of this compound has justified the implementation of consumer protection measures by the establishment of maximum tolerable standards in certain food categories. However, the

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existence of these standards also has important economic repercussions. It therefore seems imperative to develop means of control to prevent the contamination of food by these toxic compounds and/or to limit their harmful effects (Giray et al., 2007; Karami-Osboo et al., 2012).

Pesticides and fungicides have been widely used to prevent development fungal agents. However, because of their own toxicity, their use is of our days subject to certain restrictions. Biological control is also an option possibility of struggle (García-Cela et al., 2012). Likewise, detoxification processes can be put in place to try to reduce the toxicity of these toxins after their synthesis (Reddy et al., 2009; Yoshinari et al., 2007). However, none of these strategies seems, on its own, capable of solving the problem of contamination of raw materials by aflatoxins as evidenced by the many surveys that show levels of sometimes high contamination, especially in regions where the climate is favorable to the development of fungal species that produce these compounds (Cabral et al., 2013; Strosnider et al., 2006).

At present, there is a growing interest in identifying natural compounds able to limit the growth and/or the production of mycotoxins. Thus, the use extracts or essential oils of plants or spices has been able to show their ability to limit food contamination (Kedia & Dubey, 2015; Kedia et al., 2014; Prakash et al., 2012).

Chamaerops humilis L. is a medicinal plant that belongs to the Arecaceae family. It is frequently found in Europe (Italy, Spain, Malta, South of France) and North Africa (Algeria, Tunisia and Morocco). This plant is formerly known through various uses in traditional medicine. The chemical composition of *C. humilis* is rich in several bioactive compounds, including steroids, flavonoids, fatty acids, vitamins, and terpenoids. *C. humilis* extracts showed an inhibitory effect on several bacterial and fungal species, enzymatic inhibition, antidiabetic, antilithic and antioxidant properties (Beghalia et al., 2008; Hasnaoui, Benali, Bouazza, & Benmehdi, 2014; Bouhafsoun et al., 2019).

The present investigation was therefore undertaken by the main objectives; (i) to screen fungi responsible for biodeterioration of peanuts and detect of toxigenic strains of *Aspergillus flavus,* (ii) to determine antifungal and anti-AFB_1 production of *C. humilis* extracts, (iii) to evaluate their total phenolic content (TPC) as well as antioxidant activity, so as to explore the possibility of their recommendation in preserving food items.

2. Materials and methods

2.1. Chemicals and equipment

Chloroform, Methanol, Dimethyl sulfoxide (DMSO) (Sigma Aldrich, France), Isoamyl alcohol, Hexane, Ethanol, Toluene, Silica gel, glass plates, Linoleic acid, Tween 40, Tween-80, Folin-Ciocalteu (Sigma Aldrich, France),Na_2CO_3,Gallic acid (Sigma Aldrich, Germany), Butylated hydroxytoluene (BHT),2,2-diphenyl-1-picrylhydrazil (DPPH) (Sigma Aldrich, France), β-carotene (Sigma Aldrich, France), Potato Dextrose Agar (PDA) medium, Malt Extract Agar (M.E.A), Czapek concentrate, Glycerol Nitrate Agar (G25N), *Aspergillus flavus* and *parasiticus* agar (AFPA), Dichloran Rose Bengale Chloramphenicol (DRBC), SMKY medium. Centrifugation apparatus (Jouan E76), UV lamp (CN-6, VILBER LOURMAY, France), spectrophotometer (6705 UV/Vis, JENWAY) and a light microscope (Motic: BA210, China) were used in the study.

2.2. Sampling

Peanut samples (with envelope, without envelope) were purchased from different markets in the wilaya of Oued Souf, Algeria (33° 22′ 06″ N, 6° 52′ 03″ E. Altitude, 75 m).

Bulk samples were well mixed at 200 g, kept in plastic bags, labeled and then sent to the Microbiology Laboratory of Mohamed Boudiaf M'sila University.

2.3. Moisture content and pH

To calculate moisture content of peanut samples, 50 g of each sample was dried at 100 °C in hot air oven for 24 h and difference with the fresh weight was calculated (Mandeel, 2005). For determination of pH, 1g of each sample was finely ground using a sterilized mixer grinder. A 1:10 (sample: distilled water) (w/v) suspension of each sample was prepared and stirred for 24 h. The pH of the suspension was recorded using an electronic pH meter.

2.4. Mycobiota analysis

The isolation of fungi in the samples were carried out by the suspension-dilution technique and inoculation on agar medium. Ten grams of each sample of crushed peanut were added to 90 ml of distillated water added to tween 80 and homogenized by stirring for 15 min serial dilution (10^{-2}, 10^{-3} and 10^{-4}) were made from the stock solution. One hundred microliters of each dilution were subcultured on the surface of Petri dishes containing the Dichloran Rose Bengale Chloramphenicol (DRBC) medium and incubated at 28 °C for 5–7 days at darkness. Representative isolates from each sample were purified and then subcultured into test tubes containing Potato Dextrose Agar (PDA). After incubation for 7 days at 28 °C, the isolates were stored at 4 °C for subsequent analyzes. The identification of the main fungal genera and species was carried out on Malt Extract Agar (M.E.A), Glycerol Nitrate Agar (G25N), Czapek (CYA) and on *Aspergillus flavus* and *parasiticus* agar (AFPA) (Pitt & Hocking, 2009). The developing fungal colonies were isolated and maintained on PDA at 4 °C.

2.5. Toxigenic strains of A. flavus

A. flavus isolates of peanuts were screened for the production of AFB_1 according to the method describe by Ben Miri et al., (2023). The isolates were cultured separately in 25 ml SMKY (sucrose, 200 g; MgSO4·7H2O, 0.5 g; KNO3, 0.3 g and yeast extract, 7 g; 1 l distilled water) for 10 days at 28 ± 2 °C. After incubation, the mycelia produced in the liquid cultures were removed by filtration and washing on Whatman No.1 filter paper. The weight of the mycelia was determined after incubation at 60 °C for 6 h then at 40 °C overnight. AFB_1 was extracted from the filtrate with 25 ml in a separatory flasks. After stirring, the mixture was left to stand overnight. The chloroform phase was recovered and evaporated to dryness at a temperature of 50 °C in a rotary evaporator. The residue was dissolved in 1 ml of chloroform. Using a microsyringe, 50 µl of the aflatoxin extract were spotted onto the Thin Layer Chromatography (TLC) plate as spots in small portions. AFB_1 standard was spotted at the same time as the samples. The plates were then placed in a chromatography tank whose atmosphere had been previously saturated with Toluene/Iso-amyl alcohol/Methanol (90: 32: 2; v/v/v) (Shukla et al., 2012). After development, the plates were dried in a ventilated hood overnight to remove all traces of the solvent. The chromatograms were then observed under ultra-violet (UV) light to locate the spots exhibiting an absorbance at 365 nm. The fluorescent spots were scraped off, dissolved in 5 ml of methanol; the resulting solution was centrifuged at 3000 g for 5 min. The absorbance of the supernatant was performed in a UV–Visible spectrophotometer at 360 nm. The concentration of AFB_1 was calculated by the formula according to Singh et al. (2010):

$$[AFB_1] \text{ in } \mu g / ml = [(D \times M) / (E \times L)] \times 1000$$

Where, D: absorbance; M: molecular weight of aflatoxin (312 g/mol); E: molar extinction coefficient (21, 800 l/mole/cm); L: optical path length (1 cm cell).

In addition, the inhibition of AFB_1 was calculated as follows (Tian et al., 2012):

$$I\% = (1 - X / Y) \times 100$$

Where: X (μg/ml): concentration of AFB_1 in the treatment; Y (μg/ml): average concentration of AFB_1 in control.

2.6. Plant material and preparation of extracts

The leaves of *C. humilis* L. plant were collected from Hammam Boughrarain Tlemcen region of Algeria, during March and April 2021. The plant was identified with the help of relevant taxonomic literature/flora and its voucher specimens (CH/01) were deposited in the herbarium of the Department of Botany, National School of Agronomy, Algiers.

Collected plant material was dried in the shade and ground in a grinder with 2 mm in diameter mesh. The dried and powdered plant materials (1 g) were extracted successively with 10 ml of hexane, ethanol, chloroform and methanol, by maceration for 24 h at 4 °C. The extracts were filtered using Whatman filter paper (no. 1) and concentrated in vacuo at 45 °C using a Rotary evaporator.

2.7. Plant extracts effects on fungi growth and production of AFB_1

2.7.1. Antifungal assay

The antifungal activity of *C. humilis* L. extracts was tested against the fungal isolates of peanuts by contact assay based on hyphal growth inhibition using PDA medium (Prakash et al., 2012; José Velázquez-Nuñez et al., 2013). *C. humilis* extracts were incorporated into the agar medium at different concentrations (0.50; 1.00; 1.50; 2.00; 2.50; 3.00, 3.50; 4.00 mg/ml). An aliquot (10 μl) of the fungal suspension (1×10^6 spores/ml) were deposited in the center of the culture medium. The control was prepared without extract. Petri dishes were incubated at a temperature of 28 ± 2 °C. Mycelial growth was followed by measuring the diameter along two straight lines perpendicular to the center. The measurements were taken daily for 7 days. The percentage of inhibition (I%) was calculated according to the following formula (Shukla et al., 2012):

$$I\% = (D_{Control} - D_{Test} / D_{Control}) \times 100$$

$D_{Control}$: Diameter of the control growth zone in mm;
D_{Test}: Diameter of the test growth zone in mm.

2.7.2. Determination of minimum inhibitory (MIC) and fungicidal (MCF) concentrations

The minimum inhibitory (MIC) and fungicidal (MCF) concentrations of each extract of *C. humilis* were determined using the liquid dilution method reported by Prakash et al. (2012). Ten (10) μl of the fungal suspension (1×10^6 spores/ml) were inoculated into test tubes containing 10 ml of the SMKY liquid medium at different concentrations. Control tubes containing the SMKY medium were inoculated only with the fungal suspension and incubated at 28 ± 2 °C for 7 days.

2.7.3. Activity of extracts on spore germination

Spores from 7 day cultures of *A. flavus* (BBH-6), previously exposed to *C. humilis* extracts, were harvested with 5 ml of sterile distillated water containing 0.1% (v/v) tween-80 by scraping the mycelial surface gently with L-shaped glass spreader. Control was subjected to the same procedure. Spore suspensions were inoculated into fresh PDA medium in depression slides. The slides containing the spores were incubated at 28 °C for 24 h. For each treatment, 100 spores were examined and the extent of spore germination assessed by looking for germ tube emergence. The number of spores germinated was scored and reported as a percentage of spore germination (Aloui et al., 2014).

2.7.4. Anti-AFB_1 activity of C. humilis extracts

For anti-AFB_1 activity of *C. humilis* extracts, the requisite amount of extracts was dissolved to achieve concentrations from 0.5 to 4.0 mg/ml. The medium was inoculated with ≈1×10^6 spores/ml of seven days of *A. flavus* (BBH-6). The control contained no extract. AFB_1 detection has been conducted by TLC according to the procedures described in the section of toxigenic strains of *A. flavus*.

2.8. Fungitoxic spectrum

The spectrum of fungitoxicity of *C. humilis* extracts was determined by using direct technique against dominant fungi *viz. A. niger*, *P. verrucosum*, *Fusarium* sp., *Alternaria* sp. as described in the section of antifungal assay.

2.9. Total phenolic content estimation

The total phenolic conten (TPC) of *C. humilis* extracts was assessed using the Folin–Ciocalteu reagent, following Dewanto et al. (2002) method. An aliquot of each diluted sample extract (125 μl) of each extract dissolved in 500 μl of distilled water and 125 μl of 10 times diluted Folin-Ciocalteu reagent. After 3 min, 1.25 ml of sodium carbonate solution (7%) was added. After incubation (2 h), the absorbance of each mixture was measured at 760 nm. The total phenolics was calculated from the regression equation of the calibration range, established with the standard gallic acid and expressed in μg of acid equivalents gallic per mg of extract.

2.10. Antioxidant activity

2.10.1. Free radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazil (DPPH) radical scavenging capacity was measured according to Dung et al., (2008) and Nikhat et al. (2009). 2.9 ml of each extract of *C. humilis* L. and Butyl-hydroxytoluene (BHT) at different concentrations was mixed with 100 μl of 0.004% DPPH methanolic solution. The reaction was allowed to stand at room temperature in the dark for 30 min and the absorbance was recorded at 517 nm against a blank (methanol solution). The results were calculated by the following formula: Scavenging effect (%) = $[(A_{blank} - A_{sample})/A_{blank}] \times 100$, where: A_{blank}: Absorbance of the control reaction containing all reagents except extract; A_{sample}: Absorbance of the sample containing a tested dose of extract. The concentration of extract that could scavenge 50% of the DPPH radicals (IC_{50}) was calculated.

2.10.2. β-carotene/linoleic acid assay

The method used was that described by Miraliakbari and Shahidi (2008). A stock solution of β-carotene/linoleic acid emulsion mixture was prepared as follows: 0.5 mg of β-carotene crystals was dissolved in 1 ml of chloroform and 1 ml of this solution was transferred to a flask containing 25 μl of linoleic acid and 200 μl of tween-40. After complete removal of chloroform by evaporation, 100 ml of oxygen-enriched distilled water was added with vigorous stirring. 2.5 ml aliquots of this mixture were transferred to a series of test tubes containing 350 μl of *C. humilis* extract diluted in Dimethyl sulfoxide (DMSO). After vigorous shaking, the tubes were incubated at 50 °C for 2 h. A negative control tube was constructed by replacing the extract with DMSO. The absorbance was finally measured at 470 nm against a blank (emulsion without β-carotene). Antioxidant activities (I%) were calculated using the following formula (Shukla et al., 2012): $I\% = (A_t/A_0) \times 100$, where: A_0: Absorbance of the sample and of the control at t = 0 min; A_t: Absorbance of the sample and of the control at t = 2 h.

2.11. Statistical analysis

All experiments were performed in triplicate except spore germination were repeated six times, the values were represented by the mean ± SD. The results were analyzed by ANOVA test with STATISTICA version 6.1 software (Stat Soft, Inc, France). The level of significance was set at $p < 0.05$.

3. Results and discussion

3.1. Mycological analysis and detection of AFB_1 producing potential

The moisture content of peanut samples with envelope was in the order of 36.5% while that of without envelope was in the order of 41.17%. For both samples, the pH was in the order of 6.13 and 6.26, respectively.

Mold contamination depends on various conditions such as source, harvest period, storage practices and the chemical nature of the substrate (Kedia et al., 2014; Prakash et al., 2010). Moisture content and pH are two other important factors that control the growth and proliferation of molds in stored foodstuffs (Ozkan et al., 2003). In the present study, the moisture content and pH of peanuts were found to be in a range conducive to fungal growth (FAO, 1980).

The analysis of peanut sample with and without envelope revealed a clear dominance of the genus *Aspergillus*. The fungi isolated were species of *A. flavus* (32 and 28%), *A. niger* (24 and 31%), *Penicillium verrucosum* (19%), *Fusarium* sp. (10 and 13%), *Alternaria* sp. (15 and 9%). Bibliography show that *Aspergillus* spp. are widespread in nature (Kedia & Dubey, 2015; Prakash et al., 2012) and especially in hot climate regions such as Algeria (Pitt and Hocking, 2009). *A. flavus* (BBH-6) (774.74 ± 2.18 μg/ml) isolated from peanuts with envelope sample was found to be the most aflatoxogenic and therefore selected as test fungus.

3.2. Antifungal activity

The effects of *C. humilis* L. extracts on the growth of *A. flavus* (BBH-6) during the seven days of incubation are presented in Fig. 1. The growth of mycelium was significantly reduced ($p < 0.05$) in a proportional way to the concentrations of hexane, chloroform, ethanol, and methanol extracts of *C. humilis*, indicating dose-dependent activity.

Hexane and methanol extracts of *C. humilis* inhibited the mycelium growth of *A. flavus* (BBH-6) at 2.50 mg/ml with inhibition percentages of 87.94% and 78.95%, respectively, while, at 3.00 mg/ml of ethanol and chloroform extracts of *C. humilis*, the inhibition percentages were 74.75% and 77.82%, respectively (Fig. 2).

Kumar et al. (2011) reported that methanolic and ethanolic extracts of *Sapium sebiferum* leaves had maximum percentage growth inhibition (26%) against *A. flavus*. Tequida-Meneses et al. (2002) indicated that methanolic extract of *Ambrosia confertiflora* reduced 52.0% of radial growth inhibition for *A. flavus*. Vargas-Arispuro et al. (2005) observed that *A. flavus* showed 86% of inhibition at 0.3 mg/ml of an ethyl acetate extract from leaves of *Larrea tridentata*.

There are three aspects on which most authors agree to attribute their inhibitory function (Cabral et al., 2013; Mishra et al., 2013; Prakash et al., 2015; Tatsadjieu et al., 2010; Tian et al., 2011): i) the presence of OH groups capable of forming hydrogen bonds which have effects on enzymes, modifying a variety of intracellular functions; ii) action on the morphology of microorganisms due to interactions with membrane enzymes, leading to the loss of rigidity and integrity of the cell wall hyphae; and iii) changes in cell membrane permeability, cytoplasmic and cytoplasmic membrane disruption granulation.

Other events that can lead to membrane dysfunction and subsequent disturbances include the following events: dissipation of the two components of the proton motive force; interference with the ATP system in the cell; inhibition of enzymes; and preventing the use of the substrate for energy production (Coutinho de Oliveira et al., 2011; El-Mogy & Alsanius, 2012).

The minimum inhibitory (MIC) and fungicidal (MCF) of the extracts were determined against foodborne fungi by the dilution method in SMKY liquid medium (Table 1). The method provides better conditions for the extracts to be in close contact with the fungal spores and to spread in the medium (Kalemba & Kunicka, 2003). The MIC study is important for determining the minimum concentration to control fungal growth (Prakash et al., 2012). Hexane and methanol extracts at the concentration of 3.00 mg/ml inhibited completely the mycelial growth of *A. flavus* (BBH-6) as well as ethanol and chloroform extracts inhibited *A. flavus* (BBH-6) at 4.00 mg/ml. The MFCs were relatively higher than MICs, showing that the toxicity was fungistatic.

The hexane and methanol extracts of *C. humilis* L. showed significant reduction of the spore germination at 2.50 mg/ml (11.33% and 23.66%, respectively) ($p < 0.05$), while their ethanol and chloroform extracts at 3.00 mg/ml caused 35.66% and 29.50% reduction, respectively ($p < 0.05$) (Fig. 3).

The action of extracts could inhibit spore germination and elongation of the germ tube, suggesting that the effect of the plant compound may be attributed to the perception/transduction of the signals involved in the switch from vegetative to reproductive development (García-Cela et al., 2012).

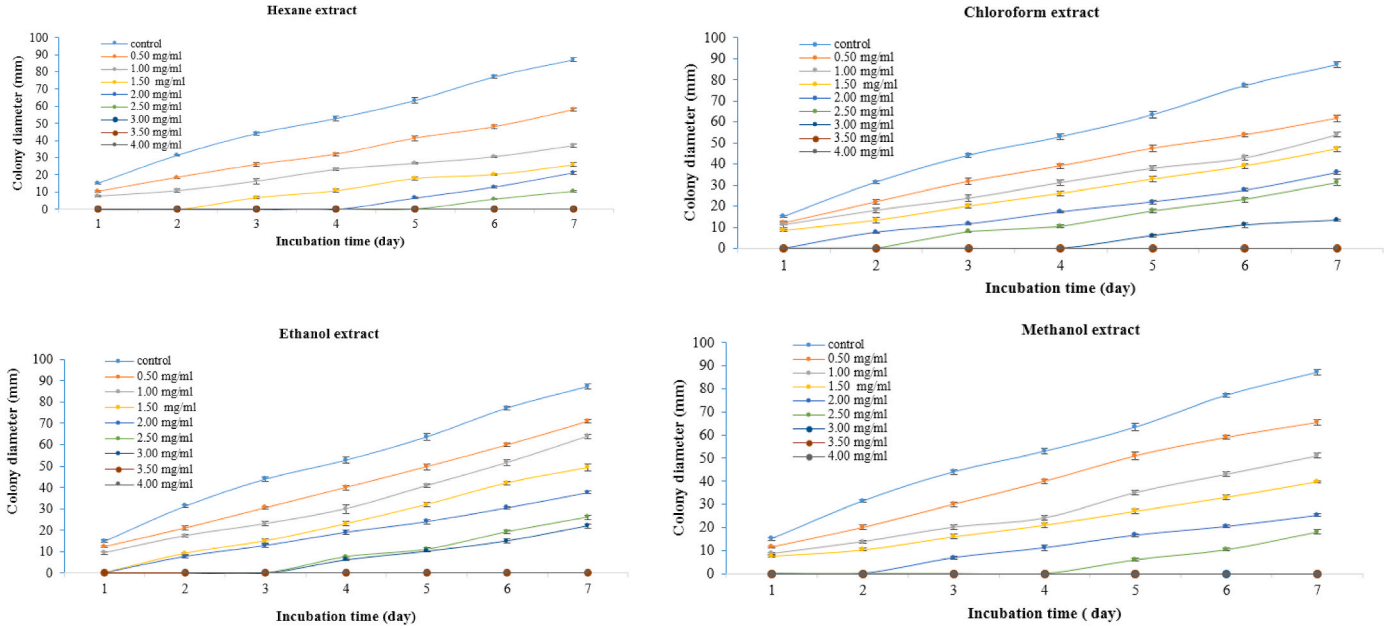

Fig. 1. Effect of extracts of *C. humilis* L. on colony diameter growth of *A. flavus* (BBH-6). Values are means (n = 3) ± SD.

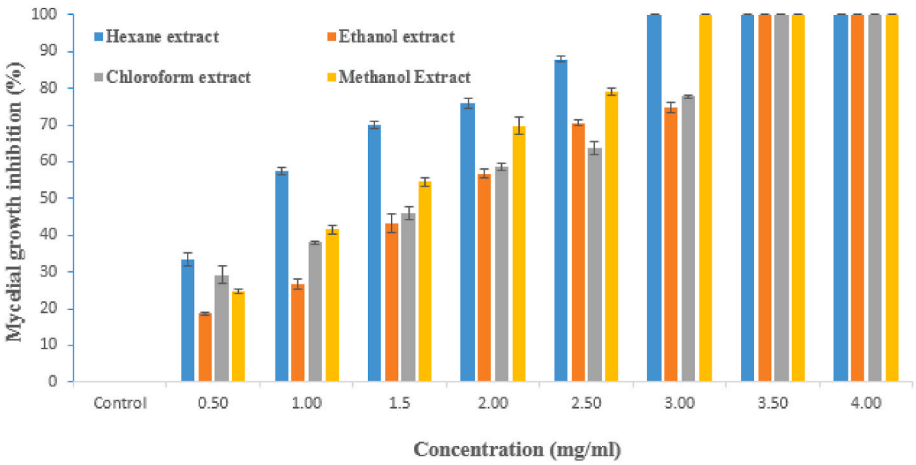

Fig. 2. Percentage inhibition of mycelial growth of *A. flavus* (BBH-6) after 7 days of incubation at different concentrations of *C. humilis* extracts. Values are means (n = 3) ± SD.

Table 1
Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of *C. humilis* extracts.

Extract	MIC (mg/ml)	MFC (mg/ml)
Hexane	3.00 ± 0.00	3.66 ± 0.28
Ethanol	3.50 ± 0.00	> 4.00
Chloroform	3.50 ± 0.00	3.83 ± 0.28
Methanol	3.00 ± 0.00	3.50 ± 0.50

Values are means (n = 3) ± SD.

3.3. Anti-aflatoxin B_1 activity

In parallel with the first test, another complementary test was carried out in order to measure the antifungal potential possessed by the extracts not only on growth but also on AFB_1 production by *A. flavus* (BBH-6), known for its immense potential to produce AFB_1. The production of AFB_1 was measured after an incubation period of 10 days (Table 2).

Results explained a proportional relationship between mycelia dry weight (MDW) and AFB_1 production, which is dose-dependent. Every time, the concentration of *C. humilis* L. extracts were increased in the culture medium, the reduction in MDW was accompanied by a decrease in the secretion of AFB_1 but not identical in percentage. Indeed, the comparison of the reduction rates obtained for the MDW and their equivalent for AFB_1, showed that they were significantly reduced compared to the control ($p < 0.05$).

All *C. humilis* extracts were good inhibitors of the production of AFB_1, the most remarkable activity was observed with *C. humilis* hexane extract. This extract had very good antifungal and antiaflatoxinogenic activity. The percentage reduction of AFB_1 was between 43.47% and 94.88% (*C. humilis* hexane extract), 25.61%–85.82% (*C. humilis* ethanol extract), 43.41%–82.82% (*C. humilis* chloroform extract), and 43.78%–86.75% (*C. humilis* methanol extract), while the percentage reduction of MDW within the range of 29.83%–88.03% (*C. humilis* hexane extract), 13.41%–83.06% (*C. humilis* ethanol extract), 30.31%–85.12% (*C. humilis* chloroform extract), and 22.24%–82.39% (*C. humilis* methanol extract) (Fig. 4).

The results indicated that *C. humilis* L. extracts are a promising alternative for inhibiting AFB_1 production. Various plant extracts exhibited inhibition of AFB_1 production. Velazhahan et al. (2010) reported that seed aqueous extracted by Ajowan showed anti-AFB_1 activity (61%). Plant extracts of *G. cowa* and *G. pendunculata*at 2000−4000 ppb concentration completely inhibited AFB_1 production (100%) (Joseph et al., 2005). Ethanol and ethanol70% extracts of *T. daenensis*, *S. khozistanica* and *S. macrosiphonia* at 2000 mg/l inhibited AFB_1 production (51−87%) (Gorran et al., 2013). The inhibition of AFB_1 by the different concentrations (5, 7.5, 10 and 12.5 g/100 ml) from *H. sabdariffa* calyx ranged between 91.5 and 97.9% (El-Nagerabi et al., 2012).

The search for natural compounds from plant sources, capable of

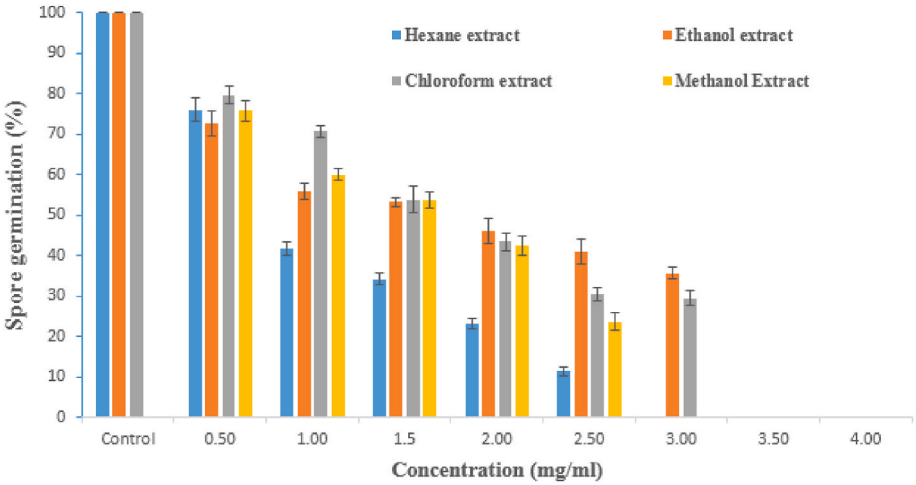

Fig. 3. Effects of the different *C. humilis* extracts on spore germination of *A. flavus* (BBH-6). Values are means (n = 6) ± SD.

Table 2
Effect of *C. humilis* extracts on MDW and AFB_1 production.

Extract	Concentration (mg/ml)	MDW(mg)	AFB_1 (μg/ml)
Hexane	0.50	625.66 ± 4.04	437.94 ± 5.15
	1.00	430 ± 3.00	244.73 ± 5.15
	1.50	280.66 ± 6.02	147.40 ± 4.29
	2.00	205.33 ± 2.51	39.59 ± 2.97
	2.50	106.66 ± 3.78	0.00 ± 0.00
	3.00	0.00 ± 0.00	0.00 ± 0.00
	3.50	0.00 ± 0.00	0.00 ± 0.00
	4.00	0.00 ± 0.00	0.00 ± 0.00
Ethanol	0.50	772 ± 2.00	576.29 ± 3.60
	1.00	673.33 ± 2.08	462.75 ± 5.78
	1.50	542 ± 2.00	345.86 ± 3.60
	2.00	432.33 ± 2.51	167.44 ± 3.78
	2.50	284.66 ± 1.51	109.24 ± 5.02
	3.00	151 ± 2.64	0.00 ± 0.00
	3.50	0.00 ± 0.00	0.00 ± 0.00
	4.00	0.00 ± 0.00	0.00 ± 0.00
Chloroform	0.50	621.33 ± 3.51	438.41 ± 3.59
	1.00	542 ± 2.64	345.86 ± 5.02
	1.50	495 ± 2.00	211,81 ± 2,86
	2.00	394.66 ± 4.72	133.09 ± 5.16
	2.50	292.66 ± 2.51	0.00 ± 0.00
	3.00	132.66 ± 2.51	0.00 ± 0.00
	3.50	0.00 ± 0.00	0.00 ± 0.00
	4.00	0.00 ± 0.00	0.00 ± 0.00
Methanol	0.50	693.33 ± 2.08	435.55 ± 4.37
	1.00	463.66 ± 1.52	265.24 ± 7.05
	1.50	373 ± 3.00	178.89 ± 2.86
	2.00	236.33 ± 6.02	103.04 ± 5.15
	2.50	157 ± 2.00	0.00 ± 0.00
	3.00	0.00 ± 0.00	0.00 ± 0.00
	3.50	0.00 ± 0.00	0.00 ± 0.00
	4.00	0.00 ± 0.00	0.00 ± 0.00
Control	0	891.66 ± 2.08	774.74 ± 2.18

Values are means (n = 3) ± SD.

inhibiting the growth of fungi and the production of aflatoxins is considered a very attractive research area in the prevention of crop contamination and food by aflatoxins. All the extracts demonstrated an action antiaflatoxinogenic. The extracts had two actions, inhibited mycelial growth and the production of AFB_1. Most inhibitors of AF biosynthesis act at one of three levels: modify the physiological environment or other signaling inputs perceived by the fungus, interfering with signal transduction and expression regulatory gene networks upstream of AF biosynthesis, or to block the enzymatic activity. It is possible that natural compounds have the same mechanism as synthetic compounds and exhibit the ideal and possible candidates for reducing AF contamination without incurring ecological and/or health problems. *C. humilis* L. extracts may present an alternative or be associated with synthetic antifungals in the biological control and fight against mycotoxinogenic fungi (Kohiyama et al., 2015; Krishnamsrthy & Shashikala, 2006; Sandoss Kumar et al., 2007).

3.4. Spectrum of toxicity

Results revealed that all extracts had antifungal activity, the intensity of which varied according to the type of extract (solvent used) and the fungal strain tested. *A. niger, P. verrucosum, fusarium sp., Alternaria* sp. The most sensitive fungi were found against the methanol extract (100%) at their MIC. However, remarkable antifungal index (77.26–100.0%) of hexane, ethanol, and chloroform extracts was recorded against fungi (Table 3).

The fungitoxicity was assessed against the isolated foodborne because they are capable of colonizing many products and producing mycotoxins (Scheidegger & Payne, 2003). These fungi must be thoroughly investigated using plant extracts; this suggests that *C. humilis* L. extracts may present an alternative with a broad spectrum of application in the biological control and the fight against mycotoxigenic molds.

3.5. Total phenolic content and Antioxidant activity

Phenols are able to donate H atoms of phenol hydroxyl groups in reaction with peroxyl radicals which can produce stabilized phenoxyl radicals, thereby terminating lipid peroxidation chain reactions. The antioxidant activity of phenols depends on electronic and steric ring effects, substituents and the strength of hydrogen bonding interactions between phenol and solvent (Lucarini et al., 2002).

The TPC of the extracts is given in Table 4. The highest content was recorded for *C. humilis* hexane extract (189.46 μg/mg), followed by *C. humilis* methanol extract (145.93 μg/mg) and *C. humilis* chloroform extract (140.4 μg/mg), while the lowest in the case of *C. humilis* ethanol extract (189.46 μg/mg).

Determining TPC gives an idea of the antioxidant potential of plant extracts, but it cannot be used to predict the antioxidant behavior (Sreenivasulu et al., 2000). Indeed, it is a general method which does not provide information on the nature of the phenolic molecules present in the plant extracts. It will therefore not be possible to establish a relationship between the quantity of phenols and the antioxidant effect (Ben Miri et al., 2018).

The IC_{50} value and % inhibition of oxidation of linoleic acid are presented in Table 4. The lowest IC_{50} value was recorded for *C. humilis* hexane extract (47.80 μg/ml) compared to the control of Butylated hydroxytoluene (BHT) (28.48 μg/ml). Oxidation of linoleic acid was inhibited by the extracts of *C. humilis* ranged between 53.54 and 69.33% compared to the control of BHT (92.00%).

In study performed by Benahmed-Bouhafsoun, Djied, Mouzaz, & Kaid-Harche (2013), methanolic extracts leaves and roots of *C. humilis* showed a remarkable effect on DPPH scavenging free radical (180.71 μg/ml). In another research study done by Bouhafsoun et al. (2019), methanol and water extracts of *C. humilis* leaves and fruits were found to have different levels of antioxidant activity. In the investigation of Gonçalves et al. (2018), the antioxidant activity of the methanolic extracts from grains of *C. humilis* were showed the great ability to scavenge DPPH (81.28 μg/ml). The antioxidant activity of the ethanol and hexane extracts of flowers and grains of *C. humilis* was tested using the DPPH method. The ethanol extract of grains revealed a percentage of inhibition of 84.05% to the effective concentration IC_{50} 2.38 μg/ml, whereas, the antioxidant activity of the other three extracts were significantly lower than that of the ethanol extract of grains (Fekkar, Aiboudi, & Bouyazza, 2016).

The oxidative degradation of lipids (lipid peroxidation) has been studied in the oxidation of foods. Therefore, the presence of antioxidants in extracts will minimize the oxidation of β-carotene by hydrogen peroxide. Deba et al. (2008) reported that polyphenols extracted from plants have antioxidant activity because they have the ability to confer hydrogen atoms to free radicals produced by oxidized linoleic acid, therefore, prevent these radicals free to attack β-carotene. In the same context, Mayachiew and Devahastin (2008), attribute the antioxidant capacity of total phenols to their redox properties, including the scavenging of free radicals and the inhibition of singlet oxygen and their power to give up their hydrogen atoms (Conforti et al., 2006; Kulisic et al., 2004).

3.6. Antioxidant activity and Aflatoxin production

Phenolic compounds play a major role in antioxidant activity. Furthermore, the aflatoxin inhibiting effect of phenolic compounds via the mediation of oxidative stress levels in fungi was also reported by Kim et al. (2008). Kim et al. (2006) demonstrated that growth inhibition of *A. flavus* by phenolic compounds is via targeting of the mitochondrial oxidative stress defense system. Since mitochondria are responsible for supplying acetyl-CoA, a main precursor for aflatoxin biosynthesis, disruption of the mitochondrial respiratory chain may partly explain the inhibitory effects of phenolic antifungal on aflatoxin production. Therefore, *C. humilis* extracts are used as markers to elucidate

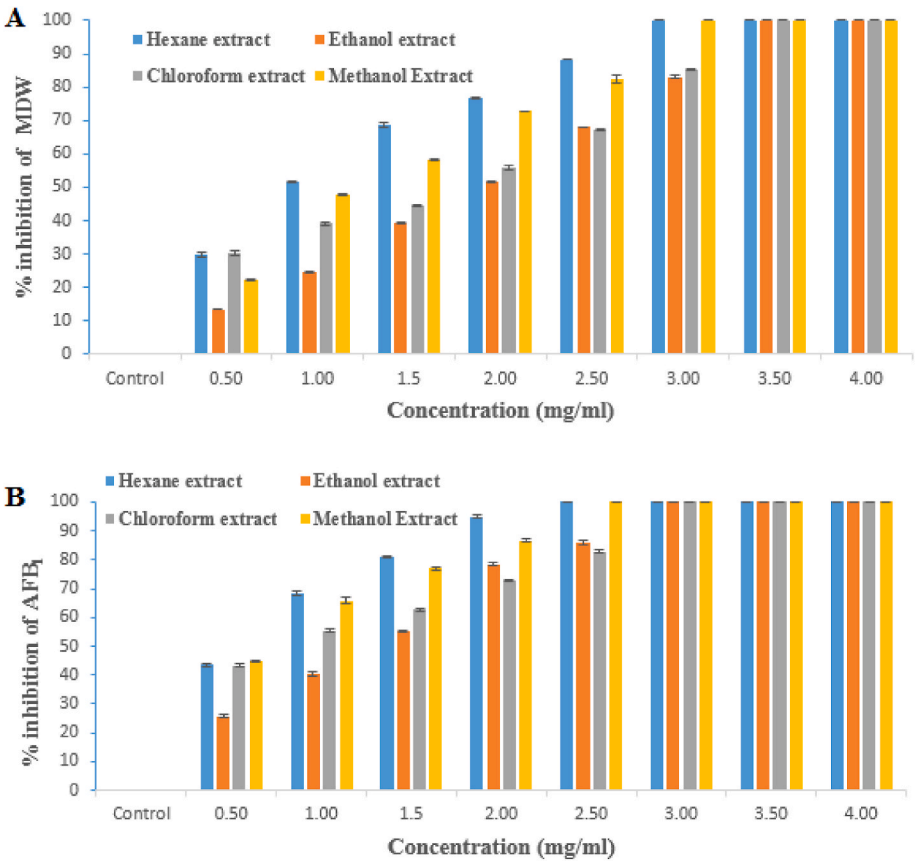

Fig. 4. A and B: Effect of *C. humilis* extracts on mycelial biomass and AFB_1 production by *A. flavus* (BBH-6). Values are means (n = 3) ± SD.

Table 3
Spectrum of toxicity of *C. humilis* extracts (%).

Fungi	Hexane extract	Ethanol extract	Chloroform extract	Methanol extract
A. niger	100.00 ± 0.00	83.14 ± 0.78	85.92 ± 1.00	100.00 ± 0.00
P. verrucosum	77.26 ± 0.27	100.00 ± 0.00	84.15 ± 1.21	100.00 ± 0.00
***Fusarium* sp.**	100.00 ± 0.00	80.75 ± 1.68	100.00 ± 0.00	100.00 ± 0.00
***Alternaria* sp.**	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

Values are means (n = 3) ± SD.

Table 4
Antioxidant activity of *C. humilis* extracts.

Extracts	IC_{50} (μg/ml)	*β*-carotene/linoleic acid inhibition (%)	Total phenolic content(μg/mg)
Hexane	47.80 ± 1.72	69.33 ± 6.09	189.46 ± 7.62
Ethanol	115.71 ± 2.63	66.86 ± 2.16	119.46 ± 3.49
Chloroform	102.92 ± 2.32	53.54 ± 2.58	140.4 ± 2.8
Methanol	86.78 ± 2.04	61.15 ± 1.01	145.93 ± 3.18
BHT	28.48 ± 2.05	92.00 ± 0.96	nd

nd not determined. Values are means (n = 3) ± SD.

antioxidant-based inhibition of aflatoxin biosynthesis.

All extracts of *C. humilis* showed significant antioxidant activity. Thus, the high inhibitory efficacy of AFB_1 production may be due to their antioxidant properties by binding to the inhibition of lipid peroxidation and oxygenation in the process of AFB_1 biosynthesis, due to the anti-aflatoxigenic as well as antioxidant efficacy.

4. Conclusion

In conclusion, to our knowledge, there are no published studies evaluating the inhibitory effects of *C. humilis* L. extracts on *A. flavus* and AFB_1 production. Hexane, ethanol, chloroform and methanol extracts significantly inhibit fungal growth, spore germination and AFB_1 production. The results obtained suggest that *C. humilis* L. extracts are suitable as an antifungal and anti-AFB_1 agent in food protection. However, there are very few data on the composition of such extracts, which limits the formulation of hypotheses on the nature of the active molecules responsible for the anti-aflatoxinogenic effect described in our work. It therefore appears necessary to focus on characterizing the composition of these extracts in order to identify and isolate the active molecules.

CRediT authorship contribution statement

Khaoula Boudjaber: All authors equally contribute to, Conceptualization, Methodology, Formal analysis, Writing, and, Visualization, under, Supervision, of the corresponding authors. **Yamina Ben Miri:** All authors equally contribute to, Conceptualization, Methodology, Formal analysis, Writing, and, Visualization, under, Supervision, of the corresponding authors. **Amina Benabdallah:** All authors equally contribute to, Conceptualization, Methodology, Formal analysis, Writing, and, Visualization, under, Supervision, of the corresponding authors. **Nada**

Bennia: All authors equally contribute to, Conceptualization, Methodology, Formal analysis, Writing, and, Visualization, under, Supervision, of the corresponding authors. **Chaima Hamadi:** All authors equally contribute to, Conceptualization, Methodology, Formal analysis, Writing, and, Visualization, under, Supervision, of the corresponding authors. **Boudjema Soumati:** All authors equally contribute to, Conceptualization, Methodology, Formal analysis, Writing, and, Visualization, under, Supervision, of the corresponding authors. **Djamel Djenane:** All authors equally contribute to, Conceptualization, Methodology, Formal analysis, Writing, and, Visualization, under, Supervision, of the corresponding authors. **Jesus Simal-Gandara:** All authors equally contribute to, Conceptualization, Methodology, Formal analysis, Writing, and, Visualization, under, Supervision, of the corresponding authors.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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