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Research Article

Potential of *Citrullus colocynthis* L. Schrad. Immature Seed Extracts as Food Preservative against a Fungal Mycotoxigenic Contaminant

Francesca Mussi,[1] **Serena Montalbano**,[1] **Belsem Marzouk**,[2] **Laura Arru**,[3] **Meher Refifa**,[1] **Zohra Marzouk**,[2] **Jamil Kraiem**,[2] **Francesca Degola**,[1] **and Annamaria Buschini**[1,4]

[1]*Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma, Parma, Italy*
[2]*Laboratory of Chemical, Galenic and Pharmacological Development of Drugs, Faculty of Pharmacy, University of Monastir, Monastir, Tunisia*
[3]*Department of Life Sciences, Centre BIOGEST-SITEIA, University of Modena and Reggio Emilia, Reggio Emilia, Italy*
[4]*International Center COMT, University of Parma, Parma, Italy*

Correspondence should be addressed to Serena Montalbano; serena.montalbano@unipr.it

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The world of plant extracts and natural compounds have long been regarded as a promise land for the individuation of healthy alternatives to chemical preservatives, against microbial contamination, in food and feed commodities. A plethora of aromatic and medicinal plant species have been studied from decades to explore their antimicrobial and antioxidant properties, in order to both validate their ethnobotanical use for healing microbial illnesses and assess their suitability as food preservation agents. In fact, after terrestrialization and during the following evolutionary pathway, plants had to develop chemical compounds—constitutive and/or induced—for defence against specific pathogens, therefore becoming a potential source of new natural products usable with antimicrobial purposes. Aside from the most common contaminants that could occur in foodstuff, mycotoxigenic fungal species represent a big concern, mainly in cereals and derived products: aflatoxins in particular are the most dreaded among such toxic and cancerogenic secondary metabolites, and the control of the main producer *Aspergillus flavus* is currently one of the most pursued goals in the field of food safety. As aromatic and medicinal plants have a long history of use in the Mediterranean basin for both food preservation and pest control in crops, the exploitation of native species for the control of mycotoxigenic phytopathogens is almost rationale. The present work provides novel insights into the possible use of *C. colocynthis* seed organic extracts as antimycotoxigenic additives, demonstrating, for some of them, a feasible application as crop and food protectants with specific regard to aflatoxin contamination. Additionally, the evaluation of their cytotoxic potential and nitric oxide production on human cell lines has been reported for the first time.

1. Introduction

In the field of the pathogenic microorganism control, the problem of food-borne outbreaks has represented a true challenge for health regulatory authorities from decades. Indeed, the recent drift toward the use of natural preservatives—and mainly those obtained from plants—in the food chain has increasingly intensified the interest on the most various plant species [1–4]. Being exploited since the ancient times not only for coloring and flavoring, but also for healing purposes, spices and herbs in particular were thought to be the most promising source for the individuation of new preservation agents: in fact, over years, plant-derived extracts, essential oils, and peptides exhibited broad-

spectrum activities, mainly ascribed to relevant antioxidant properties relying on secondary metabolites such as phenylpropanoids, terpenes, flavonoids, and anthocyanins [5–7].

However, despite the demonstrated antimicrobial effect of countless plant extracts, and the advantages that the use of these products as an alternative of chemical additives can bring in terms of chemical residues and microbial resistance, some drawbacks still deter a large use in food industry; for example, the high variability in effectiveness of these compounds (pure or in mixture) against microorganisms in the laboratory system and in real food systems, the costs associated with the processes for their procurement and/or purification, and the concentration required for the achievement of the most effective containment activity are sometimes discouraging. Even if both the European Commission (EC) and the US Food and Drug Administration (FDA) approved few essential oils as food preservatives, the problematic reproducibility of their activity represents a main barrier not overcome yet. Additionally, although some plant compounds enjoy the status of "generally recognized as safe" (GRAS), toxicological assessment of their use in food is scarce or poorly available. Thus, the improvement in cost-effective producing and toxicological information of these compounds is highly desirable, as well as helpful, in the promotion of their use as food biopreservatives. On the other hand, commonly used botanical derivatives have become popular as pesticides in organic farming, as organically produced food raises premium prices; their popularity in agriculture is also due to the perception that they are safe to use on crops for human consumption if compared to chemical treatments [8].

Since the biological properties of plants extracts mainly derive from secondary metabolites, even at different extent, and molecules with antimicrobial function are synthesized in response to biotic stressors [9] that characterize almost less controlled environments, it could be suggested that the highest yield of such compounds should be found preferentially in wild or not completely domesticated species [10]. In this sense, the Mediterranean basin is undoubtedly an exceptional reservoir of genetic resources of both wild and cultivated aromatic and medicinal plants that can be addressed to innovative applications as antimicrobials in several fields related to crop management, from food and feed production to safety and quality. For example, several studies focused on the use of essential oils and crude extracts from Mediterranean species for the control of phytopathogenic fungi affecting the quality and safety of crops have been recently reported [11, 12]. Mycotoxigenic fungal species in particular have been widely considered, because of the big threat that their diffusion on cereal crops and derived commodities poses on human and animal health [13]; among them, *Aspergillus flavus* is probably the most studied.

Citrullus colocynthis L. Schrad. is an annual plant, belonging to the cucurbit family, which grows in arid and semiarid areas. Native to Asia and tropical Africa, it is now widely distributed also in the Arab-Saharan geographic region, and in the desert areas of the Mediterranean basin [14]. Due to its content in glucosides, such as colocynthis, the pulp of the fruit is an effective laxative and an excellent depurative and has been used in the Berber traditional medicine from centuries [15]. Most remarkably, aside from a number of pharmacological properties—ranging from anti-inflammatory, antidiabetic, analgesic, antiepileptic, even to abortive properties—important antimicrobial activities of organic extracts from different parts of the plant have been recognized. For example, aqueous and acetone extracts from roots, stems, leaves, and three maturation stages of fruit and seeds that were screened for their antimicrobial potential against Gram-negative and Gram-positive bacteria and *Candida* spp. demonstrated a broad-spectrum effectiveness [16]. Antimycotic properties have been assessed in plant pathogens also (such as *Alternaria alternata*, *Rhizoctonia solani*, *Fusarium oxysporum*, and *Fusarium solani*) that impact the quality and safety of a number of food feed and commodities [17]. Some aqueous and acetone extracts from *C. colocynthis* plants collected in southern Tunisia (Mednine) showed marked broad-spectrum antiradical properties *in vitro* antioxidant activity tests [18]; but, interestingly, the scavenging potential as assessed in organic extract from leaves, stems, and roots that proved a high effectiveness in inhibiting aflatoxin biosynthesis in *A. flavus* was demonstrated to be uncoupled from the antitoxigenic activity that commonly is thought to rely on the unbalancing of secondary metabolism toward a reduced state [19].

The investigation of traditional medicinal plants and the characterization of their potential as a source of natural preservatives agents are the first step for an optimal utilization of plants extracts as nontoxic-to-humans, sustainable food additives. With this goal, this work seeks to compare the antimycotoxigenic activity of 5 organic extracts from *C. colocynthis* immature seeds, already characterized for their antimicrobial potential on some human pathogens, and to assess their toxicological profile on human cell lines, in order to provide an almost complete information useful for their possible application as cereals derived-products preservatives.

2. Materials and Methods

2.1. C. colocynthis L. Schrad. Immature Seed Organic Extracts. For this work, immature seed organic extracts of *C. colocynthis* collected near Medenine (Tunisia), previously obtained and discussed, were used [20], namely, petroleum ether (PE), chloroform (CHL), ethyl acetate (EA), acetone (AC), and methanol (MET), in ascending polarity, that were obtained through Soxhlet extraction. The CHL extract subfraction F19 [21] was also included.

For biological assays, the crude extracts were redissolved in dimethyl sulfoxide (DMSO) and tested at increasing concentrations.

2.2. Fungal Strains, Media, and Culture Condition. *Aspergillus flavus* strains used in this study were previously isolated from corn fields of the Po Valley and reported [22]. Conidia suspensions were obtained from 10 day YES-agar

[2% (*w*/*v*) yeast extract (Difco, Detroit, MI, USA), 5% (*w*/*v*) sucrose (Sigma, St. Louis, MO, USA), and 2% (*w*/*v*) agar (Difco)] cultures incubated at 28°C; conidia concentration (quantified by OD600) and viability (>90%) were determined according to Degola et al. [23]. Coconut milk-derived medium (CCM) used for microplate assays was obtained as described in Degola et al. [23].

2.3. *Aspergillus flavus* Bioassays

2.3.1. Aflatoxin Production. The effects of seed extracts on aflatoxin biosynthesis were assessed by the microplate fluorescence-based procedure described in Bisceglie et al. [24]. Standard flat-bottom 96-well microplates (Sarstedt, Newton, NC, USA) were used. Suspensions of conidia were diluted to the appropriate concentrations and brought to the final concentration of 5×10^2 conidia/well and inoculated in a final volume of 200 μL/well of CCM medium. Extracts were DMSO resuspended and added to the culture medium. The plates were incubated in the dark under stationary conditions for up to 6 days at 25°C. Total aflatoxin accumulation was monitored by fluorescence emission determination; readings were performed directly from the wells bottom of the culture plate with a microplate reader (TECAN SpectraFluor Plus, Männedorf, Switzerland) using the following parameters: λex = 360 nm, λem = 465 nm, manual gain = 83, lag time = 0 μs, number of flashes = 3, integration time = 200 μs). Inocula were performed in quadruplicate, and experiments were performed in triplicate.

2.3.2. Biomass Production. Mycelium development was assessed in the same microwell cultures used for the toxin biosynthesis evaluation. After aflatoxin measurement, mycelia were manually recovered from the culture wells, slightly paper-dried, and weighted. Inocula were performed in quadruplicate, and experiments were performed in triplicate.

2.4. Human Cell Line, Media, and Culture Condition.

All cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD).

HT29 (ATCC, HTB-3), human colon adenocarcinoma cell line, was maintained in 25 cm^2 flasks with 5 mL of Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% (*v*/*v*) fetal bovine serum (FBS), 1% penicillin (100 U/ml)/streptomycin (100 μg/mL), and 1% L-glutamine (2 mM).

A549 (ATCC CCL-185), human lung carcinoma cell line, was cultured in Roswell Park Memorial Institute (RPMI-1640) medium, supplemented with 10% (*v*/*v*) fetal bovine serum (FBS), 1% penicillin (100 U/mL)/streptomycin (100 μg/mL), and 1% L-glutamine (2 mM).

HFL1(ATCC, CCL-153), human lung normal fibroblast, was cultured in Ham's Nutrient Mixture F-12 with L-glutamine supplemented with 10% (*v*/*v*) fetal bovine serum (FBS) and 1% penicillin (100 U/mL)/streptomycin (100 μg/mL). HFL1 cells were used between passage numbers 5 and 20.

Cell lines were maintained at 37°C in a 5% CO_2 humidified incubator and subcultured twice a week.

2.5. Cytotoxicity on Human Cell Lines. The cytotoxic activities of seed extracts on human cells were measured by MTS assay (Promega, Madison, WI) [25]. Cells were seeded (5×10^4 cell/mL) in 96-well flat-bottom plates. After 24 h of incubation, HT29 cells were treated, in quadruplicate, with increasing concentrations of seed extracts or vehicle control and incubated for 24 h. A volume of 20 μL of MTS solution was added directly to culture wells and after 4 h of incubation the absorbance at 450 nm with a 96-well plate reader (MULTISKAN EX, Thermo Electron Corporation, Vantaa, Finland) was recorded.

2.6. Nitric Oxide Production. The Griess Reagent System (Promega Corporation, Madison, WI, U.S.A) was used to quantify the production of nitric oxide (NO) through the evaluation of nitrite (NO_2^-), one of the NO primary breakdown product, stable and nonvolatile. Briefly, 5×10^3 cells/well were seeded in complete medium (DMEM + 10% FBS), after 24 h of incubation (37°C, 5% CO_2), the medium was discarded, and cells were supplemented with fresh medium without FBS and treated with samples for 24 h. After treatment, Griess reaction was performed as reported in the producer instructions. The absorbance of the reaction mixtures was measured at 570 nm using a microplate reader (TECAN SpectraFluor Plus, Männedorf, Switzerland). The NO concentration was determined by comparison to the nitrite standard reference curve.

2.7. Statistical Analysis. Antifungal and antiaflatoxigenic activity data were analyzed with Past 3.x software (https://past.en.lo4d.com/windows). One-way analysis of variance (ANOVA) was performed. Results of biomass production and AF accumulation were analyzed by Tukey's test; differences were considered significant at $p \leq 0.01$.

Statistical analysis of cytotoxicity assay on human cells was performed by the statistical and graphical function of IBM SPSS Statistics 27 (SPSS Inc., Chicago, IL, USA). The mean values from the repeated experiments were used in ANOVA. If significant F values ($p \leq 0.05$) were obtained, Student's *t*-test (Bonferroni's version) was performed.

3. Results

3.1. Biological Effect of C. colocynthis Immature Seed Organic Extracts on A. flavus. The evaluation of the antifungal potential of *C. colocynthis* immature seed extracts has been conducted on the biomass production in an aflatoxigenic *A. flavus* strain. Increasing concentrations ranging from 10 to 500 mg/mL were tested. As shown in Figure 1, a dose-dependent effect was observed for PE, CHL, EA, and MET extracts; on the contrary, AC extracts did not prove to exert any inhibition on fungal growth. The maximum of antifungal effect (47.5%) was achieved through the treatment with EA extract at the highest concentration (500 μg/mL), followed by PE (42.4%) administrated at the same concentration. Amongst all the organic extracts, both CHL and

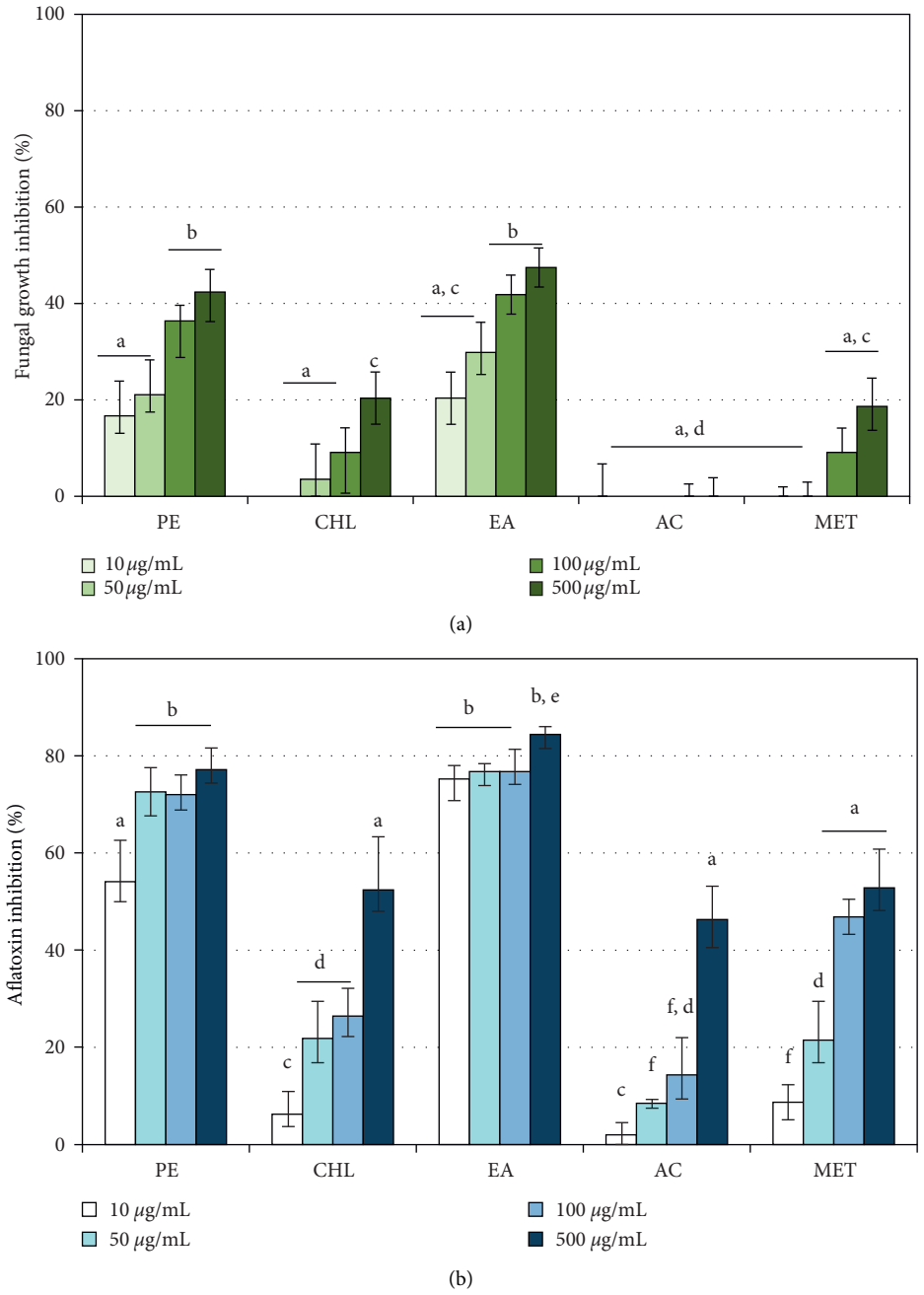


Figure 1: Activity on *A. flavus* growth and aflatoxin biosynthesis. Biomass production (a) and toxin accumulation (b) in CCM cultures after six days of growth with petroleum ether (PE), chloroform (CHL), ethyl-acetate (EA), acetone (AC), methanol (MET) *C. colocynthis* immature seed extracts. Results are expressed as percentage in respect to control (equivalent DMSO amended cultures). Error bars refer to mean values of four replicates S.D. Different letters indicate statistically significant differences at $p \leq 0.01$.

MET were the least effective in lowering the biomass production (and thus the mycelium growth) of *A. flavus*.

The same extracts have been then investigated for their possible effects on aflatoxin biosynthesis (Figure 2). As a general observation, a higher inhibitory potential was globally assessed with respect to the antifungal effect. PE and EA extracts showed the highest antiaflatoxigenic activity, exceeding the 50% inhibition to the PE lowest concentration, and even surpassing about the 70% at the intermediate ones. On the contrary (but according to the effect on the fungal growth), 10, 50, and 100 μg/mL concentration of CHL and AC and 10 and 50 μg/mL concentration of MET extracts resulted in lesser than about 30% aflatoxin containment. For these, the highest concentration was instead quite effective in lowering the toxin accumulation (about 52.4%).

The MET extract was compared, in its biological activity on *A. flavus*, with the derived F19 fraction. In Figure 3, this comparison is reported. As can be observed, F19 behaved as CHL for the efficacy on mycelium inhibition; on the

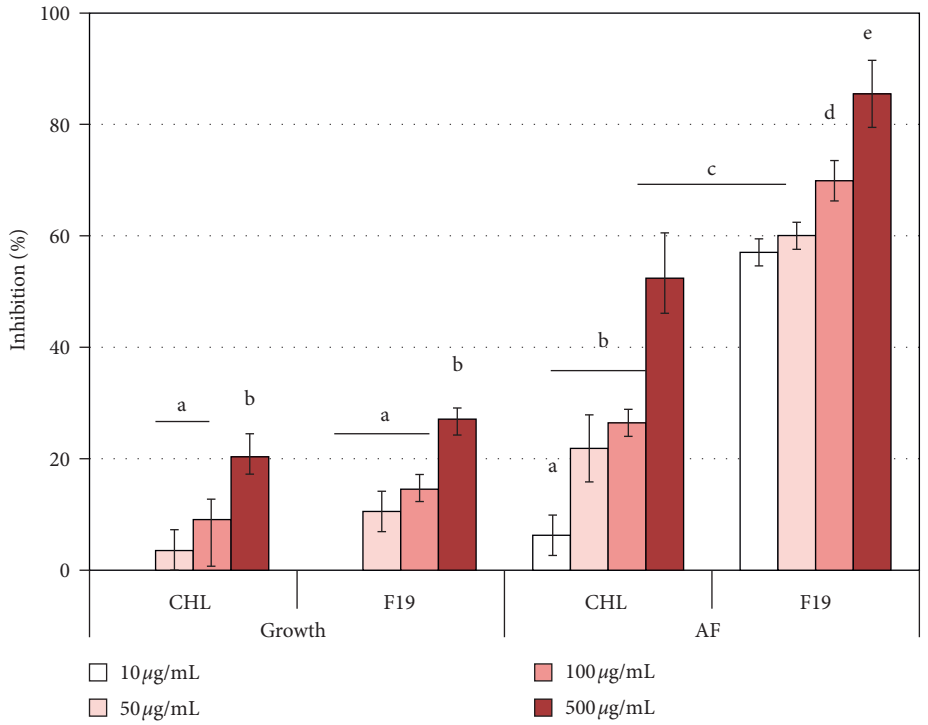
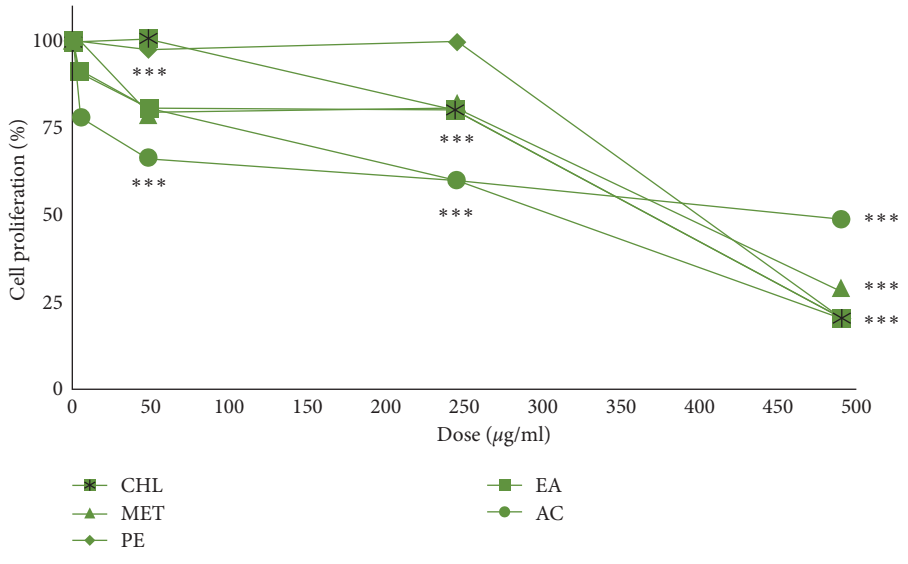


Figure 2: Antifungal and antiaflatoxigenic activity of *C. colocynthis* immature seed chloroform (CHL) extract and its fraction F19. Results are expressed as percentage in respect to control (equivalent DMSO amended cultures). Error bars refer to mean values of four replicates SD. Different letters indicate statistically significant differences at $p \leq 0.01$.



(a)

Figure 3: Continued.

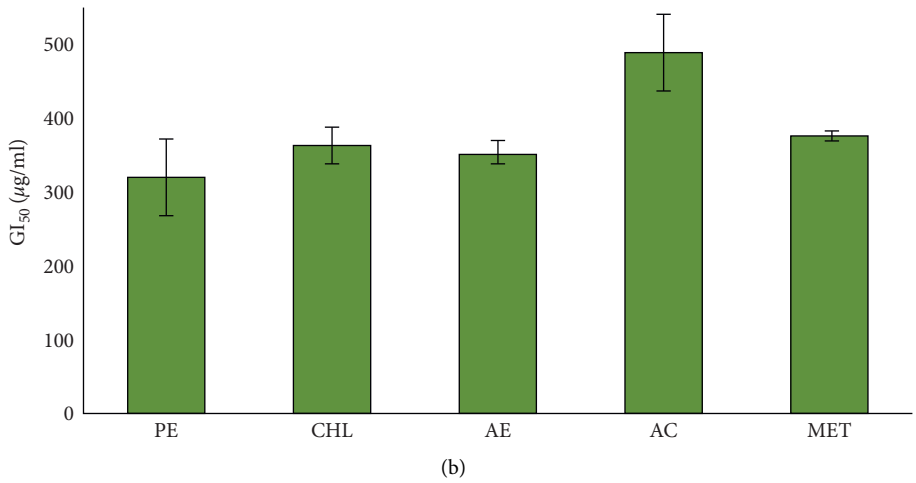


Figure 3: Antiproliferative activity of organic extracts from *C. colocynthis* seed extracts on HT29. (a) Dose-response curve. $^{***}p \leq 0.001$. (b) Concentration able to inhibit of 50% the cell growth (GI_{50}). PE: petroleum ether; CHL: chloroform; EA: ethyl acetate; AC: acetone; MET: methanol.

contrary, it proved to possess a significantly higher potential in aflatoxin containment than CHL.

3.2. Cytotoxicity Assessment on Human Cell Lines. Seed extracts and fraction F19 were evaluated for their cytotoxic activity on human cell lines.

The ability to affect cell proliferation was assessed through the MTS assay, a colorimetric method for determining the number of viable, metabolically active cells in proliferation.

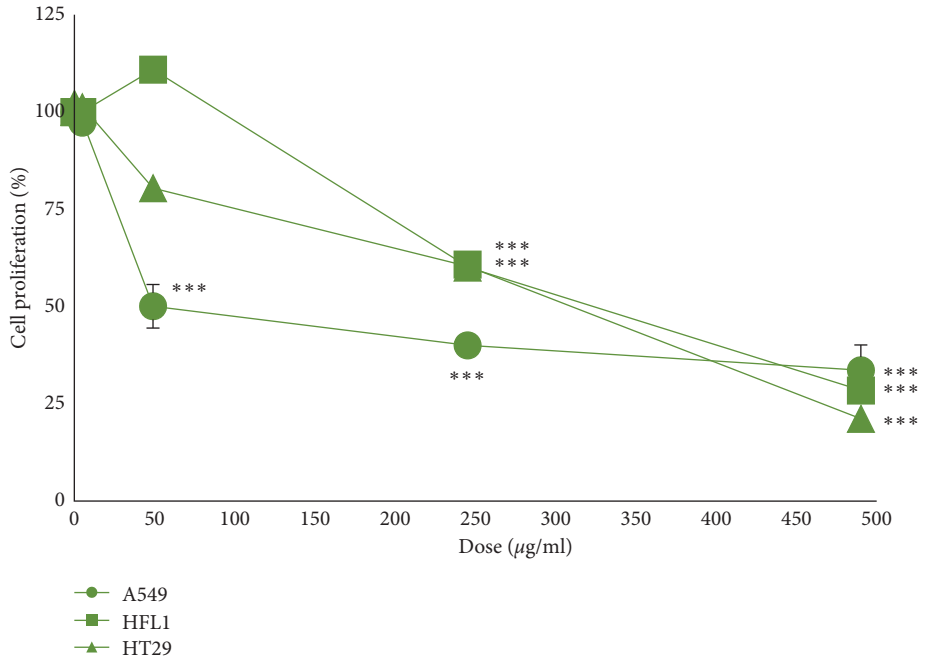
All the seed extracts induced a dose-dependent inhibition on the proliferation of human cell line HT29 (Figure 4(a)). The concentration able to inhibit 50% of the cell growth (GI_{50}) was calculated from the dose-response curve. Based on the GI_{50} values, the inhibitory effects of the seed extracts on HT29 cell proliferation were in decreasing order, as follows: AC (489 μg/mL) > MET (374 μg/mL) > CHL (363 μg/mL) > EA (351 μg/ml) > PE (321 μg/mL) (Figure 4(b)).

The chloroform extract and its F19 fraction, which presented an interesting antiaflatoxigenic activity, were tested on cell lines deriving from different tissues: HT29, A549, and HFL1 (Figure 5(a)). The chloroform extract showed a high, comparable, cytotoxic activity on the three cell lines. F19 induced a dose-dependent cytotoxicity on the cell lines HT29 and HFL1, while the A549 cell line did not show any sensitivity to F19 (Figure 5(b)). Interestingly, F19 was not able to induce an inhibition of 50% of cell proliferation on the different cell lines used. In comparison with the total chloroform extract, the fraction F19 shows a strong reduction in cytotoxic activity.

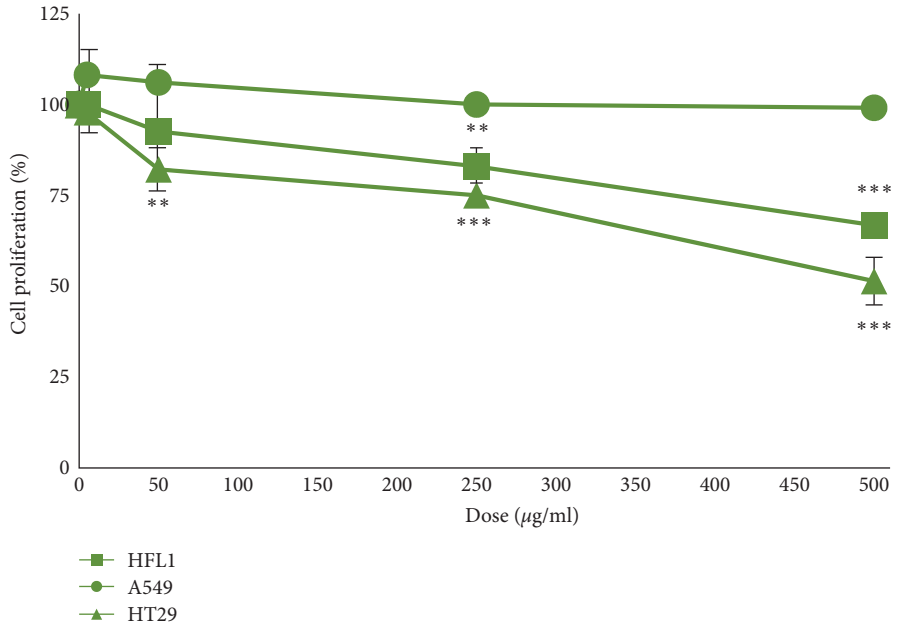
3.3. Evaluation of NO Production. Nitric oxide (NO) has modulating effects on several cellular processes, in particular on inflammation. High levels of NO are produced in response to inflammatory stimuli and mediate proinflammatory and destructive effects. NO production was determined after the treatment of HT29 cells for 24 h with *C. colocynthis* seed chloroform extract and its F19 fraction, using the Griess reaction that evidences nitrite, a stable breakdown product of NO. No significant induction of NO was detected

4. Discussion

Citrullus colocynthis L. Schrad. belongs to the family Cucurbitacea that grow in the arid areas of Mediterranean basin [14] and belongs to the ethnomedicinal arsenal of folk medicine of various Countries, being widely—and long-time—used for many diseases including dermatological, gynaecological, urinary, and pulmonary infections [26, 27]. The most used plant parts addressed to medicinal purposes are mainly fruits and seeds, even if roots and leaves infuses are used for the treatment of urinary infections [28, 29]. However, more recently, organic extracts obtained from leaf, stem, and root material have been successfully applied *in vitro* against some phytopathogenic fungi, and in particular against *A. flavus* and aflatoxin biosynthesis, that is relevant to cereal crops production [19]: in this study, an interesting dependence of the biological activity not only on the plant tissue, but also on the organic solvent utilized for the extraction, was showed. Results obtained with the immature seeds extracts are in accordance with the following: in fact, AC extracts did not exert any inhibitory effect on fungal growth, while others showed a variable extent. On the other hand, aflatoxin containment activity only partially showed a similar behavior, since the organics can be divided for their inhibition rate; indeed, PE and EA from one side, and CHL, AC and MET from the other shared similar antiaflatoxigenic potential. However, the antifungal activity of the same AC extracts determined against some human fungal pathogens [16] proved to be highly dissimilar from results obtained on *A. flavus* biomass production. This observation underlines the necessity, if interested in the specific potential of



(a)



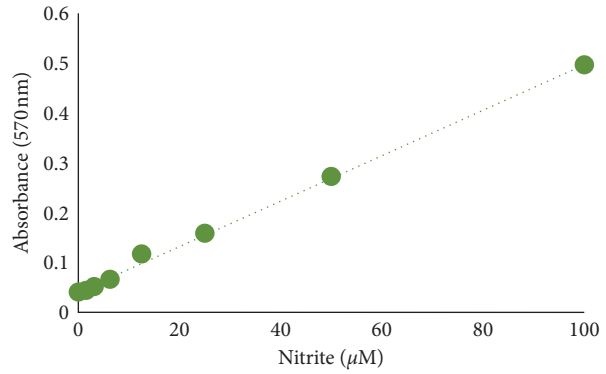
(b)

Figure 4: Antiproliferative activity of *C. colocynthis* immature seed chloroform extract (a) and its fraction F19 (b) on A549, HFL1, and HT29 human cell lines. $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$.

botanicals as crop and/or food protective additives, to directly assess the antifungal activity on the fungal target species, because the inhibitory potential dramatically varies amongst genera—even at the species level.

Despite the demonstrated effectiveness of the most various botanicals for antimicrobial purposes, a deepen and accurate evaluation of their toxicological properties is still required, at least on human cell line models. Hence, we studied the abilities of the different seed extracts in altering human cell proliferation as a parameter of cytotoxicity. Taking in consideration the polarity of the solvent used, the highest activities on HT29 cell line were found with the most polar extracts, such as AC and MET that, on the other hand, were not the most effective as antiaflatoxigenic agents.

When compared to the CHL extract, its F19 subfraction showed not only an increased aflatoxin-containing activity (even at the lower concentration), but also an important



(a)

	DMSO	CHL	F19
NO (μM)	<1	<1	<1

(b)

Figure 5: Nitrite standard curve (a) and NO concentration measured in supernatants from HT29 cells treated with DMSO (control) and *C. colocynthis* immature seed chloroform extract (CHL) and its fraction F19. (b) Evaluation performed after 24 h.

reduction of the cytotoxic effect: in fact, an absence of a GI_{50} value on all the different human cell lines was observed. Furthermore, human cell treatment did not highlight any proinflammatory response, as revealed through NO production. These data are in accordance with a characterization study in which the seed chloroform extract, the F19 subfraction, and the purified compound 11-deoxocucurbitacin-I-2-O-β-d-glucoside (that was thought of as a possible effector present in the F19 subfraction) induced anti-inflammatory effects *in vivo* in rats [20].

5. Conclusions

The present work provides novel insights about the possible use of *C. colocynthis* seed organic extracts as antimycotoxigenic additives, suggesting, for some of them (namely, PE and EA extracts, as well as the F19 subfraction), a possible application in the crop protection and food and feed preservation, with specific regard to aflatoxin contamination. The assessed cytotoxicity on human cells of these extracts, however, seems to not represent a health concern, since their antiaflatoxigenic potential was high starting from the lowest concentration (10 μg/mL), while the relevant GI_{50} values have been assessed to be about 300 μg/mL for PE and EA, and >500 μg/mL for F19. As a final consideration, it should be reminded of that the use of natural compounds/extracts effective in the containment of mycotoxins, whereas not impacting on fungal viability, could represent, in some cases as the crop protection and agricultural purposes, a more ecological strategy, in order to prevent the loss of environmental biodiversity.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Disclosure

F. Degola and A. Buschini must be considered the co-last authors.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

B. Marzouk, F. Degola, and A. Buschini designed the study. F. Mussi, S. Montalbano, M. Refifa, J. Kraiem, and B. Marzouk conducted the experiments. S. Montalbano wrote the original draft. Z. Marzouk, L. Arru, F. Degola, and A. Buschini edited the manuscript. F. Degola and A. Buschini equally contributed as senior.

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