Anti-Fungal and Anti-Aflatoxigenic Properties of Organs of *Cannabis sativa* L.: Relation to Phenolic Content and Antioxidant Capacities

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Abstract

Aflatoxin B1 is a carcinogenic mycotoxin that frequently contaminates crops worldwide. Current research indicates that the use of natural extracts to combat mycotoxin contamination may represent an eco-friendly, sustainable strategy to ensure food safety. Although *Cannabis sativa* L. has long been known for its psychoactive cannabinoids, it is also rich in many other bioactive molecules. This study examines extracts from various organs of *Cannabis sativa* L. to determine their ability to limit aflatoxin production and growth of *Aspergillus flavus*. The results indicate that flower extract is most effective for limiting the synthesis of aflatoxin B1, leading to an almost-complete inhibition of toxin production at a concentration of 0.225 milligrams dry matter per gram of culture medium. Since flower extract is rich in phenolic compounds, its total antioxidant ability and radical-scavenging capacity are determined. Compared with other anti-aflatoxigenic extracts, the anti-oxidative potential of *Cannabis sativa* L. flower extract appears moderate, suggesting that its anti-mycotoxin effect may be related to other bioactive compounds.

Introduction

Mycotoxins are toxic secondary metabolites produced by various fungal species as they develop on crops. They can contaminate cereals and many other agricultural products, either in the pre-harvest period or during storage, producing numerous carcinogenic, immuno-toxic, repro-toxic, and nephro-toxic effects on both humans and animals (Pleadin et al. 2019).

Of the hundreds of known mycotoxins, aflatoxin B1 (AFB1) is one of the most problematic for worldwide food safety. AFB1 belongs to group I of carcinogenic compounds for humans, as defined by the International Agency for Research on Cancer (IARC, 1993), since responsible for the appearance of hepatocellular carcinoma in Human. In addition, the hepatitis B virus produces a synergistic effect with AFB1 (Hamid et al. 2013), and it is responsible for impairing growth in children as well as for immunosuppression in different species (Meissonnier et al. 2008; Khlangwiset et al. 2011).

AFB1 is synthesized by *Aspergillus* section *Flavi* species, *Aspergillus flavus*, with *A. parasiticus* being the most frequent species isolated from foods and feeds (Frisvad et al. 2019). These are thermo-preferable species, which is why AFB1 is a major contaminant in hot climates. An estimated 4.5 billion people in the world are regularly exposed to this carcinogenic agent through their diet (Hamid et al. 2013). Moreover, ongoing climate change is directly affecting the distribution of mycotoxins, leading to their recent appearance in regions that were heretofore considered mycotoxin-free. Thus, AFB1 is now an emerging contaminant in European crops (Bailly et al. 2018).

To protect consumers from toxic compounds, many countries have set up strict regulations to minimize the presence of AFB1 in foods. For example, in Europe, AFB1 tolerance ranges from 2 to 5 µg/kg, depending on the foods (European Union 2006). Once present, AFB1 is stable and is not degraded by food processing.

To date, one of the most common ways to limit mycotoxin contamination is to apply pesticides during plant growth to limit fungal development and to implement good storage practices (especially proper drying) to avoid fungal development after harvest (Da Luz et al. 2017; Ayofemi et al. 2020). However, the use of pesticides is increasingly questioned due to the toxicity of these molecules and to the increased resistance developed by the target organisms (Lucas et al. 2015). Good storage practices can also be complicated to maintain, especially in regions with wet climates where grain may become moist during storage.

Significant research is now ongoing to find sustainable strategies to limit mycotoxin contamination of foods. One such strategy is the use of plant extracts, which is of major interest. Plants produce many bioactive compounds that help them resist fungal attacks and that could be used to combat mycotoxin biosynthesis (Loi et al. 2020).

Cannabis sativa L. is receiving increasing attention in many areas as a plant that produces potent bioactive molecules. This herbaceous annual plant belongs to the *Cannabaceae* family and has been cultivated by humans for a thousand years for multiple purposes: the stem for fiber production, the seeds for food, and the buds for medicine, owing to the presence of various psychoactive molecules (Russo 2007). These active compounds are derived from the cannabinoid acid produced by the plant, which can be converted into psychoactive cannabinoids, for example, by heat or ultraviolet irradiation (Flores-Sanchez et al., 2008).

Cannabis sativa L. comprises many other potential bioactive molecules; a total of 545 compounds have been identified from this plant, including at least 120 terpenoïdes, 26 flavonoids, and 11 steroids (Jin et al. 2020). Phenolic compounds (phenylpropanoids) belonging to the family of flavonoids generally play the role of antioxidant in plants and shield against oxidative stress. They also contribute to the vibrant color of many fruits and vegetables. Many different flavonoids have been identified in *Cannabis*, including apigenin, luteolin, quercetin, kaempferol, cannflavin A, cannflavin B (only found in the *Cannabis* species), *β*-sitosterol, vitexin, isovitexin, kaempferol, and orientin (Elsohly et al. 2017).

Other than cannabinoids, *Cannabis sativa* L. is a potential source of many natural bioactive compounds with antioxidant and antimicrobial activities that could find numerous applications (Pellati et al. 2018). Moreover, law 178 of May 28, 2020 has legalized the cultivation of *Cannabis sativa* L. for medical and industrial purposes in Lebanon (Lebanese Republic 2020). The cannabis industry is expected to generate by-products (leave, stem, and flower remains) that may represent a source of beneficial compounds, including polyphenols (Fiorini et al. 2019), that could be valorized in a myriad of ways depending on their properties (anti-oxidative, antimicrobial, etc.).

Since these compounds are secondary metabolites, climatic conditions (and thus their geographical origin) and time of harvest can significantly affect their content in *Cannabis sativa* L. (Calzolari et al. 2017), and the distribution of these molecules can vary over the plant. For example, the content of most cannabinoids increases with plant height, both in the flowers and in the inflorescence leaves (Marzorati et al. 2016).

The present study thus evaluates the antifungal and anti-aflatoxigenic properties of different organs of the Lebanese *Cannabis sativa* L. plant to determine their total phenolic content and antioxidant potential.

Material And Methods

Plant samples

Cannabis sativa L. plants were collected from the village “Yammoune” (34° 07’ 46.52”N, 36° 01’ 51.49”E), which is northwest of the city of Baalbek, within the main region of cannabis cultivation in Lebanon. Plants were collected, sun-dried, and preserved hermetically in a sealed plastic bag. Hashish was used for comparison. It was prepared locally in Yammoune village, using traditional methods without the addition of any other ingredients than cannabis trichomes. Samples were then transported to the Faculty of Sciences, Saint Joseph University. Collection and transportation were authorized by agreement number 873 between the legal authorities, the Lebanese Agronomic Research Institute (LARI), and the Ethics Council of the Saint Joseph University.

Chemicals

All solvents and chemicals were provided by Sigma-Aldrich (Darmstadt, Germany) and used as received. All solvents were HPLC grade.

Preparation of plant extracts

Three different organs were manually collected from plants of *Cannabis sativa* L.: stems, leaves and flowers. The organs (25 g each) were ground separately with an electrical grinder. Then, 9 g of powder were extracted with 90 mL of acetone:water (1:1, v:v) mixture in 150 ml sterile glass Erlenmeyer flasks. This solvent mixture was found to be the most effective for polyphenol extraction in different plants (data not shown). The flasks were held at 50°C in a water bath for 2 hours, following which extracts were filtered through sterile gauze followed by a second filtration through a Whatman 1PS phase separator (GE Healthcare Life Sciences, Vélizy-Villacoublay, France). The acetone was evaporated with the help of a rotary evaporator at 60°C, following which 90 mL of distilled water were added. Extracts were filtrated again through 22 µm nylon syringe filters (Sigma-Aldrich, Darmstadt, Germany). The final extracts were stored at + 4°C until use.

Determination of total phenolic compounds

The Folin-Ciocalteu technique was used to evaluate the total phenolic content of the extracts, as described by Singleton et al. (1999) and Ainsworth and Gillespie (2007). A volume of 0.8 mL of sodium carbonate (75 mg/mL) was added to 0.2 mL of each extract, and the solution was mixed with 1 mL of the 1/10 diluted Folin-Ciocalteu reagent. After 2 hours, the absorbance at 750 nm was acquired by using a UV-Vis spectrophotometer (Biochrom Ltd, Cambridge, England), and the total phenolic content was expressed in mg of gallic acid equivalents per gram of dry matter (mg GAE/g DM) based on a gallic acid calibration curve.

Total antioxidant activity

To measure the antioxidant activity of the extract during incubation with *A. flavus*, increasing concentrations of extracts (0.03125, 0.05625, 0.1125, 0.225, 0.45, 0.9, 1.8, 3.6 and 7.2 mg DM/mL) were used for analysis. A phosphomolybdenum reduction assay was used to determine the total antioxidant capacity of extracts, as described by Abi-Khattar et al. (2019). A reagent solution consisting of 28 mM of sodium phosphate, 4 mM of ammonium molybdate, and 0.6 M of sulfuric acid was prepared. Next, 1 mL of this solution was mixed with 100 µL of the extract at different concentrations. The mixtures were incubated for 90 min at 95°C, following which the absorbance was measured at 685 nm. The antioxidant activity of extracts was expressed in µg of ascorbic acid equivalent per milliliter based on an ascorbic acid calibration curve.

Radical-scavenging ability

Anti-radical activity was measured by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) reduction (Abi-Khattar et al. 2019) using the same concentrations of plant extract than for antioxidant activity analysis. A volume of 1.45 mL of DPPH (0.06 mM) (Sigma-Aldrich, St-Quentin Fallavier, France) was added to 50 µL of the extract at various concentrations (Sigma-Aldrich, St-Quentin Fallavier, France). After 30 min of incubation at room temperature in the dark, the absorbance was measured at 515 nm. The radical-scavenging ability percentage (RSA %) was calculated by using

RSA % = [(absorbance of control − absorbance of sample)/absorbance of control]×100.

Effects of Cannabis sativa L. extracts on fungal growth and AFB1 production

This study used the *A. flavus* strain NRRL 62477 (El Mahgubi et al. 2013) maintained on malt extract agar medium (Biokar Diagnostics, Allone, France) at 27°C. For experiments, 10 µL of a calibrated spore suspension (10^5 spores/mL) prepared in a tween 80 solution (0.05%) were inoculated centrally. To test how extracts from different *Cannabis sativa* L. organs affected the fungal development and AFB1 production, different volumes of extract were added to the medium prior to fungal inoculation, corresponding to increasing dry matter (DM) content: 50 µl (4.5 mg DM); 100 µl (9 mg DM); 200 µl (18 mg DM); 400 µl (36 mg DM); 800 µl (72 mg DM); 1600 µl (144 mg DM). Controls were incubated after adding the same volume of distilled water. Petri dishes were incubated for seven days at 27°C in the dark. The impact on fungal growth was quantified by measuring the colony diameter at the end of the experiment.

AFB1 determination

At the end of the incubation period, culture media were mixed with 30 mL of chloroform and agitated for 2 hours on a horizontal shaking table at 150 rpm and 25°C. Samples were filtered through a Whatman 1PS phase separator (GE Healthcare Life Sciences, Vélizy-Villacoublay, France). Two milliliters of filtrate were then evaporated at 50°C until dry and then dissolved in 2 mL methanol. All samples were then filtered through 0.45 µm syringe filters (Thermo Scientific Fisher, Villebon-Sur-Yvette, France). The AFB1 production was analyzed by using reverse-phase High-Performance Liquid Chromatography (HPLC; Waters Alliance, Milford, MA, USA) coupled with a fluorescence detector. Separation was done on a C18 column (5 µm particle size; 250 mm × 4.6 mm; Supleco, Bellefonte, PA, USA) fitted with a HS C18, Supelguard Discovery precolumn (5 µm particle size, 20 mm × 4 mm; Supelco, Bellefonte, PA, USA) at a

constant temperature of 40°C. The mobile phase consisted of water:methanol:nitric acid 4 M (55:45:0.35 v:v:v) in which 119 mg/L KBr was added extemporaneously (Miklos et al., 2020). The flow rate was 0.8 mL/min. Each run lasted 35 min, the injection volume was 20 µL, and the excitation and emission wavelengths were 360 and 430 nm, respectively. Production levels of AFB1 were calculated based on a standard calibration curve. The limit of detection and limit of quantification of the method were 3.4 and 15 ng/L, respectively. The IC50 of the different extracts corresponded to concentrations that inhibited AFB1 production by 50% compared with untreated control cultures. The IC50 was calculated by using Compusyn software (Compusyn 229 inc, Paramus, NJ, USA; https://www.combosyn.com).

Statistical analysis

The results are expressed as mean values ± standard deviation of triplicates. STATGRAPHICS® Centurion XV (Statgraphics 18, The Plains, VA, USA) was used for analysis of variance and least significant difference tests.

Results And Discussion

Phenolic content of Cannabis sativa L. extracts

The total phenolic content of each part of the plant was determined by using the Folin-Ciocalteu method. As Table 1 shows, the phenolic contents of stems and leaves are comparable. It is approximately 6 times more important in flower extract. This later is also more than 3 times more concentrated in polyphenols than cannabis resin (hashish). This content of total phenolic compounds is consistent with that reported recently by Izzo et al. (2020), who measured phenolic contents ranging from 26 to 33 mg GAE/g in inflorescence methanolic extracts of four different varieties of *Cannabis sativa* L.

Table 1
Phenolic content, antifungal activity, and anti-aflatoxigenic activity for various amounts of extracts from different organs of *Cannabis sativa* L.

	Extract			
	Stems	**Leaves**	**Flowers**	**Hashish**
Total phenolic content (mg GAE/g DM)	5.97 ± 0.99	4.94 ± 1.09	32.49 ± 1.00	8.83 ± 0.76
Concentration of *C. sativa* L. extract*	Fungal development (% of control)			
0	100%	100%	100%	100%
0.225	100%^{ns}	100%^{ns}	83%**	97%^{ns}
0.45	98%^{ns}	98%^{ns}	81%**	96%^{ns}
0.9	103%^{ns}	97%^{ns}	77%**	97%*
1.8	99%^{ns}	101%^{ns}	74%**	94%**
3.6	97%^{ns}	98%^{ns}	70%**	91%**
7.2	95%*	100%^{ns}	64%**	84%**
Concentration of *C. sativa* L. extract*	AFB1 production (% of control)			
0	100%	100%	100%	100%
0.225	115%**	102%^{ns}	7%**	87%**
0.45	101%	95%**	3%**	72%**
0.9	98%**	89%**	nd	61%**
1.8	92%**	81%**	nd	47%**
3.6	87%**	72%**	nd	35%**
7.2	83%**	64%**	nd	13%**

** milligram of Cannabis dry matter per gram of culture medium*

*GAE: gallic acid equivalent; DM: dry matter; nd = not detectable; ns = not significant; * p < 0.05; ** p < 0.01*

The phenolic content measured in *Cannabis sativa* L. extracts is moderate compared with that of other plants or extracts. It is slightly higher than that reported for various aqueous extracts of medicinal plants such as *Rosa canina*, *Salvia officinalis* L. or *Achillea filipendulina* Lam, which have phenolic contents of 4.09, 5, and 4.12 mg GAE/g DM (Sytar et al. 2018), respectively, and is much less than that measured in methanol-water extracts of nettle leaf (> 150 mg GAE/mg DM) (Otles et al. 2012) or Saint John's Wort (> 250 mg GAE/mg DM) (Oztürk et al., 2009). Nevertheless, the phenolic content of stems and leaves is comparable to that reported for dates (Oztürk et al. 2009). The concentration in *Cannabis sativa* L. flowers is equivalent to that reported in grape seeds, which are considered as potent sources of phenolic compounds (Oztürk et al. 2009).

Impact of Cannabis sativa L. extracts on growth of Aspergillus flavus and production of AFB1

To evaluate the impact of plant extracts on both fungal development and AFB1 production, different amounts of each plant extract were incubated with *Aspergillus flavus* NRRL62477. As Table 1 shows, incubation with extract from leaves and stems leaves fungal development essentially unchanged, with the exception that the highest concentration of stem extract leads to a slight (5%) reduction in growth. In contrast, incubation with flower extract leads to a dose-dependent inhibition of fungal development that becomes significant at 0.225 mg of DM per mL of culture medium and reaches 36% reduction in fungal growth with 7.2 mg DM/mL. Hashish leads to a reduction of only 16% in fungal growth at the highest concentration tested.

In parallel, AFB1 production was measured, and Table 1 summarizes the results. All extracts lead to a dose-dependent reduction of AFB1 synthesis, albeit with significant differences in the magnitude of reduction. Incubation with the highest concentration of stem extract leads to a reduction of only 17% of AFB1 production. Leaf extract more strongly affects mycotoxin production, reducing it by 36% compared with the control culture. However, flower extract has, by far, the most important effect on AFB1. Incubation with only 0.225 mg of DM/ml leads to a reduction of AFB1 production by 93% (Table 1). This motivated us to investigate the effect of lower concentrations, and the results are presented in Fig. 1.

Interestingly, flower extract rapidly inhibits AFB1 production, producing an IC50 of about 0.056 mg DM/mL. In comparison, hashish also leads to a dose-dependent reduction of AFB1 production, but its IC50 is around 1.8 mg DM/mL. Compared with the reduction previously reported for other plant extracts, the *Cannabis sativa* L. flower extract appears to be a very potent inhibitor of AFB1 synthesis. The IC50 reported for cumin, coriander, and hyssop range from 0.3 to 0.625 mg DM/mL. For these plant extracts, total inhibition of AFB1 production requires concentrations as high as 1.25, 1.5, and 15 mg DM/mL for cumin and coriander essential oils and hyssop aqueous extract, respectively (Khoury et al. 2017; Ben Miri et al. 2018). These results are over tenfold greater than those obtained from *Cannabis sativa* L. flower extract.

Relation between anti-aflatoxigenic activity and anti-oxidative potential

To investigate the relationship between the anti-aflatoxigenic property of *Cannabis sativa* L. flower extract and its anti-oxidative ability, the antioxidant potential was measured by using the phosphomolybdenum

method and a DPPH scavenging assay (see results in Fig. 2). The total antioxidant capacity determined by the phosphomolybdenum method increases with the quantity of extract and reaches 3.72 mg equivalent ascorbic acid/mL. The radical-scavenging ability is also dose dependent, with the reaction saturating at 3.6 mg DM/mL, where the IC50 value is 1.068 mg DM/mL.

Both the total antioxidant capacity and the radical-scavenging ability of *Cannabis sativa* L. flower extract are much lower than those reported for other plant extracts at concentrations that inhibit AFB1 production, such as *Cuminum cyminum* or *Coriandrum sativum* essential oils (Ben Miri et al. 2018). They are both also much lower than that reported for eugenol, another well-known inhibitor of AFB1 synthesis (Gulcin et al. 2011; Caceres et al. 2016). These results suggest that, rather than total antioxidant ability, the nature of the phenolic compound present in the extract may be responsible for the anti-AFB1 effect of cannabis flower extract.

Izzo et al. (2020) recently reported that polyphenols present in methanolic extracts of *Cannabis sativa* L. flowers might contain compounds such as caffeic and ferulic acid. These phenolic acids reportedly modulate the production of certain mycotoxins, mostly produced by the *Fusarium* species. Ferruz et al. (2016) reported that both phenolic acids mildly reduce the production of T-2 and HT-2 toxins, although this effect was detected at concentrations far exceeding those encountered in cannabis extracts. In contrast, low doses of ferulic acid also reportedly increase the production of fumonisins in *Fusarium verticillioides* (Ferrochio et al., 2013), and coumaric acid, another component of *Cannabis sativa* L. flower extracts, increases the production of both T-2 and HT-2 toxins (Ferruz et al. 2016).

Quercetin is also present in cannabis flower methanolic extracts at concentrations of 10–28 mg/kg (Izzo et al., 2020). This molecule reportedly inhibits aflatoxin production in *Aspergillus flavus* (Li et al., 2019), although this inhibition was accompanied by a strong limitation of fungal development that is not detected in the present work. Cannflavin A and B may also be found in *Cannabis sativa* L. extracts and at greater concentrations in flowers than in other parts of the plant (Izzo et al., 2020; Pollastro et al. 2018). These methylated isoprenoid flavones are *Cannabis*-specific and offer a potent anti-inflammatory activity (Rea et al., 2019). However, Cannflavin A and B are not part of the cannabinoid family, which may explain why hashish, which is rich in cannabinoids, is less active than flower extract. This suggests that the anti-AFB1 effect is not related to cannabinoids. It would thus be of interest to test these molecules for their anti-mycotoxin effect. Of course, given that the extracts are quite complex, the effect detected may also be due to other compounds. The identification of the anti-AFB1 molecules present in *Cannabis sativa* L. flower extract could help uncover new mechanisms of AFB1 regulation that would subsequently lead to new targets to trigger and thereby block the biosynthesis of this carcinogenic agent, thus reducing its contamination of food and feed.

Conclusion

We report herein the anti-mycotoxin property of *Cannabis sativa* L. extracts, which could open the way to new uses for this plant and a better valorization of its by-products. Its mild impact on fungal growth may

help to improve food safety without affecting biodiversity. Although the anti-aflatoxin potential is correlated with the phenolic content of these extracts, it does not seem to be causally related to their global anti-oxidative capacity.

Declarations

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Author contributions: Anthony Al Khoury and Rheid Sleiman conducted the experiments, Pamela Hindieh participated in the characterization of the extracts, Ali Atoui and Richard Maroun helped interpret the results, and Jean-Denis Bailly and André Khoury supervised the study. All authors participated in writing the manuscript.

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Figures

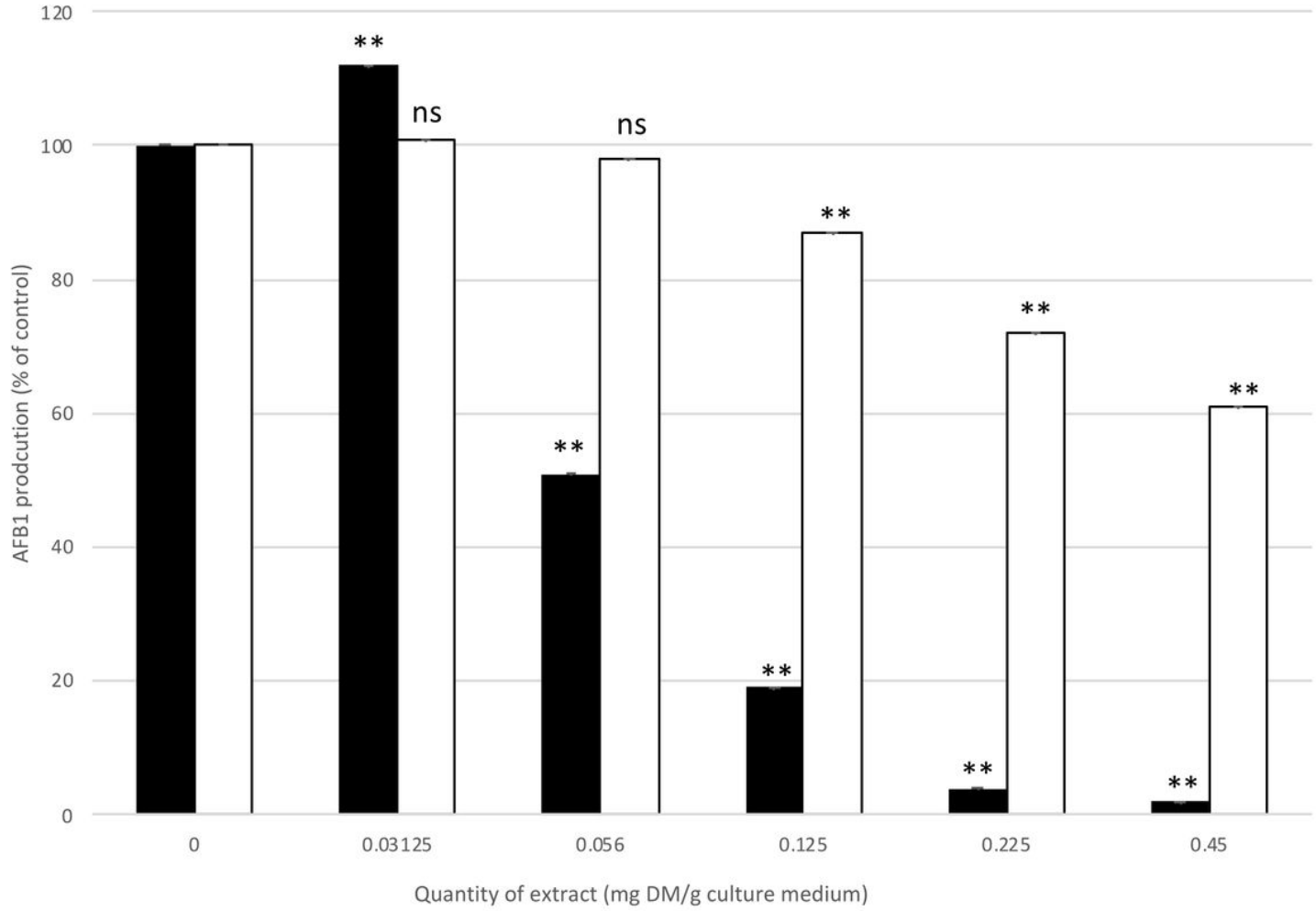

Figure 1

AFB1 as a function of Cannabis sativa L. flower extracts (black bars) compared with the effect of hashish extract (white bars). Results are expressed as percent of control production and are the mean values of three separate experiments ± the standard deviation. ns = not significant; ** means $p < 0.01$.

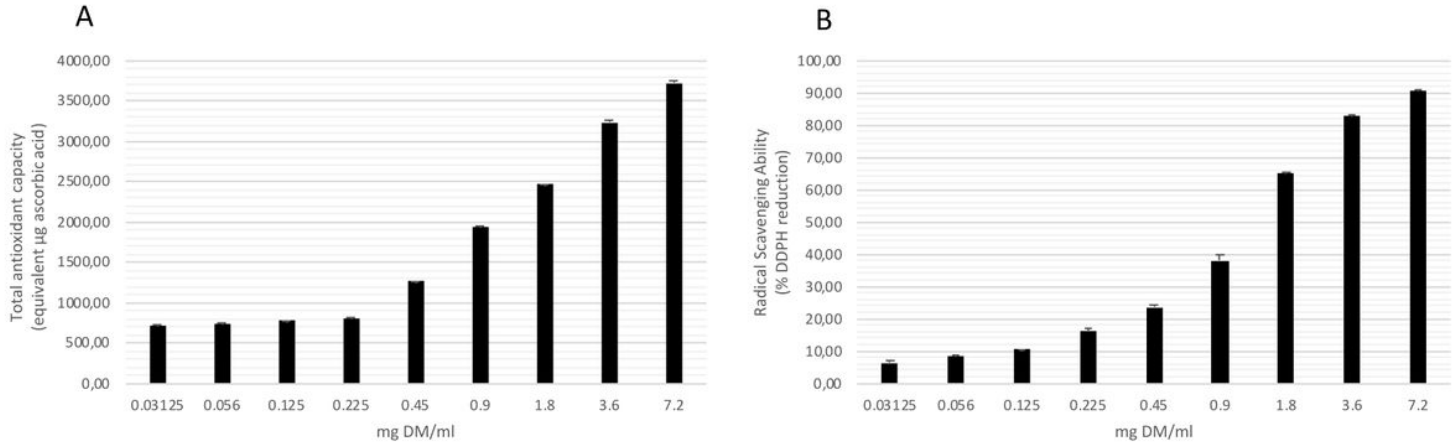

Figure 2

(A) Total antioxidant capacity and (B) radical-scavenging activity of increasing amounts of Cannabis sativa L. flower extract (mg dry matter per mL) as determined by phosphomolybdenum and DDPH assays, respectively. Results correspond to the mean ± the standard deviation of three separate measurements and are expressed as (A) equivalent µg/mL ascorbic acid and (b) percent reduction of DDPH.