

Mycotoxins Associated with Maize Wastes Treated with Comprised Capsule of *Spirulina platensis* Biomass

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Spirulina platensis has roles in biotechnological product advancement due to its safety for humans and animals. Analysis of *S. platensis* extract by high-performance liquid chromatography reflected the presence of 14 phenolic and flavonoid compounds with different concentrations such as ellagic acid (333 µg/g), gallic acid (294 µg/g), methyl gallate (147 µg/g), naringenin (144 µg/g), and chlorogenic acid (142 µg/g). The isolated *Aspergillus flavus* from silage of maize was tested to evaluate the effect of *S. platensis* extract on aflatoxins B1, B2, G1, and G2 production. The treated silage with *S. platensis* extract showed the presence of 37±0.33 ppb, 0.59±0.16 ppb, 0.26±0.22 ppb, and 0.21±0.18 ppb compared to untreated silage that showed 3.14±0.15 ppb, 0.81±0.08 ppb, 0.46±0.05 ppb, and 0.26±0.23 ppb aflatoxins of B1, B2, G1, and G2. These results were shown on the 10th day of incubation. On the 15th day of incubation, the treated silage showed less mycotoxins than un-treated silage. At different incubation periods, glucosamine was estimated as a growth development biomarker. The content of glucosamine was inhibited as a result of the effect of *S. platensis* extract on fungus growth with 53.71%, 49.19%, 47.84%, 38.47%, and 35.72% inhibition on the 3rd, 6th, 9th, 12th, and 15th day.

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INTRODUCTION

Maintaining high quality grains and grain products necessitates monitoring for mycotoxins and mycotoxigenic fungi. In addition, accurate fungal identification is crucial for informing researchers about which mycotoxins may be present (Magnoli *et al.* 2007; Hamed *et al.* 2016; Abdelghany *et al.* 2017). Mycotoxins of potential food safety concern can be produced by toxic fungi while grains are stored. They should be carefully monitored and controlled (Penagos-Tabares *et al.* 2022). Xerophilic fungi, such as *Aspergillus restrictus* and *Aspergillus glaucus*, grow in grains during storage at low water activity (a_w 0.75). They are the most prevalent fungal species at the start of storage and have little impact on grain quality (Anke *et al.* 1980). However, the development of *A. flavus*, *Aspergillus ochraceus*, and *Penicillium sp.* growth can spoil grain and its products that have been stored. They can yield toxic or cancer-causing metabolites that endanger both people's and animals' health (Nesci *et al.* 2003; Amézqueta *et al.* 2012). Mycotoxins are a

threat to the health of livestock for animals, the aquaculture industry, and humans due to its intermittent occurrence in their feeds. This problem affects animal, fish, and human feeds globally (El-Taher *et al.* 2012). Fungi, as well as its spores, develop on grain and food products in different ways. They often develop from the surface of plant fragments throughout pre-harvest or harvest transport, storage, and handling (Klich 2002).

Spirulina is formally called Arthrospira, with symbiotic and filamentous characters belonging to blue-green alga (Cyanobacteria). *Spirulina* is a genus with a distinctive capacity for photosynthetic activity (Komárek *et al.* 2009). Three *Spirulina* species, *S. platensis*, *Spirulina maxima*, and *Spirulina fusiformis* are being thoroughly researched because they are edible. They also have high nutritional and potential therapeutic values. The most popular and widely accessible *Spirulina* is the *S. platensis*. The majority of published research and public health decisions reference this species (Karkos *et al.* 2008). *S. platensis* contains high amounts of proteins (70% of the dry weight), 5 to 8% of lipid, 14% of carbohydrates besides glycolipids, sulpholipids, and minerals such as zinc, calcium, magnesium, iron, selenium, and manganese (Cohen 1997).

Several medicinal and environmental applications such as anti-fungal, anti-oxidant, anti-biofilm, wastewater treatment, bioenergy, and degradation of toxic dyes such as malachite green and crystal violet have been associated to algae and its products (Renganathan *et al.* 2014; Abdelghany *et al.* 2018; Salem *et al.* 2019; Salem *et al.* 2021; Harini and Rajkumar 2022). One promising application is the detoxification or degradation of toxic substances such as mycotoxins was documented by *S. platensis* as mentioned recently (Yadavalli *et al.* 2023).

Ramamurthy and Raveendran (2009) documented the activity of *S. platensis* extract against the growth of *Candida albicans*, *Trichoderma viride*, *Aspergillus niger* and *Penicillium javanicum*. In other report, *A. fumigatus*, *Fusarium solani*, *F. oxysporum*, *Mucor vulgaris*, and *Penicillium expansum* were inhibited using *S. platensis* (Oltu and Rudic 2016). The inhibitory action was observed by Al-Ghanayem (2017) on *F. oxysporum* after *Aspergillus flavus* and *Aspergillus niger*. It was observed by using *S. platensis* extract. Bajpai (2016) demonstrated that the polyphenols and polysaccharides of *S. platensis* represent antifungal compounds that inhibit the fungal growth or destroy the fungal ultrastructures. Furthermore, Al-Ghanayem (2017) stated that the antifungal activity of the alga occurs because of the presence of its intracellular and extracellular metabolites. Fayyad *et al.* (2020) presented that *S. platensis* extract exhibited inhibitory activity towards *Candida albicans*, *C. glabrata*, *C. fameta*, *C. lusitana*, *Aspergillus niger*, *A. flavus*, *Mucor sp.*, and *Botrytis sp.* Bencheikh *et al.* (2022) recently enhanced the production *S. platensis*, investigating its ability to produce antifungal compounds against *Fusarium culmorum*, *Alternaria solani*, and *Cladosporium sp.* *F. culmorum* was the most sensitive to the extract, with 90% inhibition. Fungal growth and mycotoxins production were influenced by *Spirulina sp.* extract. According to Pagnussatt *et al.* (2014), *Fusarium graminearum* growth and mycotoxin secretion were inhibited using phenolic extract of *Spirulina sp.* Extract of *Spirulina sp.* containing phenolic acids with higher content of gallic acid inhibited the growth of *F. graminearum*. It reduced the concentrations to 68% of trichothecene, nivalenol, and deoxynivalenol (Pagnussatt *et al.* 2014), with reductions of glucosamine levels (40%) and amylase activity (62%). The objective of this investigation was to evaluate the phenolic and flavonoid contents of *Spirulina* and their activity against mycotoxins production.

EXPERIMENTAL

Source of *Spirulina platensis*

Blue green capsule and powder of *Spirulina platensis* was obtained from the National Research Center in Egypt. Distilled water was used to prepare the fresh suspension of *S. platensis*.

Methanolic Extract of *S. platensis* Preparation

Methanolic extract of freeze-dried *S. platensis* (50 g) was prepared through a stirring process with 200 mL of methanol at 40 °C for 1 day. The extract was filtrated and then concentrated *via* rotary evaporator (Laborota 4000, Heidolph, Milan, Italy). The obtained extract was kept at 4 °C for further utilization.

Phenolic and Flavonoid Content of *S. platensis* Extract Analysis by HPLC

Five µL of the prepared *S. platensis* extract were subjected for analysis *via* injection in an HPLC device (Agilent 1260 series, Agilent Technologies, Santa Clara, CA, USA). Separations were performed using an Eclipse C18 column (4.6 mm x 250 mm i.d., 5 µm). Water represents the mobile phase (A), with trifluoroacetic acid (0.05%) in acetonitrile (B) at a flow rate of 0.9 mL/min. Mobile phases were programmed sequentially with a linear gradient as follows: 0 to 5 min (80% A), 5 to 8 min (60% A), 8 to 12 min (60% A), 12 to 15 min (82% A), 16 min (82% A), and 16 to 20 min (82% A). A multi-wavelength detector (UV) was monitored at 280 nm. Column temperature was preserved at 40 °C. The identified phenolic or flavonoid in the extract was detected quantitatively based on the introduced standards constituents (Al-Rajhi 2022).

Isolation of Mycotoxin Producing Fungus from Maize Silage (*Zea mays* L.)

Ensiled maize plants were used for animal feed in the form of silage. Under a determined condition of storage, the silage becomes spoiled with some fungi. Small parts of silage contained with fungi were placed on the surface of potato dextrose agar (PDA) medium in petri dishes. It was incubated at 30 °C for 6 days. The developed fungal colonies were re-cultivated and purified. According to identification keys including morphological and microscopical description, the fungal isolates were identified (Raper and Fennell 1973; Domsch *et al.* 1980).

Mycotoxins Production Conditions

Glass conical flasks with a capacity 250 mL contained sterile 100 mL of tap water amended with 2% maize silage. The influence of *S. platensis* extract on the mycotoxins production was carried out *via* its addition at 2% w/v to the flasks containing the growth medium. The producer fungus to aflatoxins (*A. flavus*) was inoculated and incubated up to 15 days at 30 °C. The productivity of aflatoxins was estimated on the 10th and 15th day of the control and treated samples. Glucosamine was estimated on the 3rd, 6th, 9th, 12th, and 15th day of the incubation period.

Mycotoxins Assay

A microtitre plate enzyme-linked immunosorbent assay (ELISA) reader (automated Chem-well) with test kit of Aflatoxin B1, B2, G1, and G2 were utilized for ELISA analyses, which was designated by company (r-biopharm) manufacturer (Enzyme Immunoassay for the quantifiable investigation of toxins) (Leszczynska *et al.* 2001). The

fungus broth (10 mL) was mixed with 20 mL of methanol (70%), then vortexed by a magnetic stirrer for 10 min. The extract was filtrated with Whatman no. 1 filter paper. This was then followed by the dilution of 5 mL of the filtrate with distilled water (15 mL) and Tween 20 (0.25 mL). It was mixed by a magnetic stirrer for 2 min. Standard solutions of 50 μ L toxins at different concentrations including 5 ppb, 10 ppb, 15 ppb, 20 ppb, and 25 ppb were inoculated in the wells of a micro-titer plate. In addition, 50 μ L of the prepared extracts were injected in the micro-titer plate. After the incubation of plates in the dark for 30 min at 25 °C, all liquid was detached from the wells. The well was washed using 250 μ L of PBS-Tween-Buffer (pH 7.2). Then, 50 μ L of enzyme substrate and 50 μ L of tetramethyl-benzidine as a chromogen were added to each well for 30 min at 25 °C. Stop reagent (100 μ L of 1M H₂SO₄) was added to the reaction mixture, and the absorbance at 450 nm was measured in ELISA reader.

Glucosamine Estimation

The content of glucosamine in the fungal biomass was detected *via* the addition of 5 mL of 6 M HCl to fungal dry biomass (0.2 g). It was autoclaved for 10 min at 121 °C. After autoclaving, the reaction mixture was cooled to room temperature (25 °C). This was followed by filtration and collected in a volumetric flask (5 mL capacity). In a 25 mL volumetric flask, 1 mL of the reaction mixture was mixed with a drop solution of phenolphthalein dissolved in ethanol (0.5% w/v). With NaOH solution (3 M), it was neutralized. KHSO₄ 1% (w/v) was added until reverse titration occurred, and the pink color disappeared. Distilled water was added to complete the flask volume. One mL of solution was transferred from the flask, followed by mixing with acetyl acetone (1 mL) in 50 mL of 0.5 M Na₂CO₃ in a test tube. The test tube was heated for 20 min in boiling water, followed by cooling and an addition of ethanol (6 mL) and Ehrlich's reagent (1 mL). In an oven, the tubes were incubated for 10 min at 65 °C. The absorbance was measured at 530 nm to determine the content of glucosamine. The quantity was calculated from the standard curve ranging from 1 to 30 μ g/mL (Scotti *et al.* 2001).

Statistical Evaluation

The experiments were performed in three replicates to calculate the mean of the results and the standard deviation (SD). The variance was designed by the SPSS ver. 22.0 software (version 14, IBM, Armonk, NY, USA).

RESULTS AND DISCUSSION

In the present study, *S. platensis* was cultivated and propagated in a suitable broth medium, then it was available in the form of capsules (Fig. 1). HPLC analysis of *S. platensis* detected 14 compounds at different retention times. The extract was enriched with certain phenolic and flavonoid compounds including ellagic acid, gallic acid, methyl gallate, naringenin, and chlorogenic acid with different concentrations of 333, 294, 147, 144, and 142 μ g/g, respectively. Cinnamic acid, syringic acid, and apigenin were detected in low levels of 2.78, 5.87, and 6.43 μ g/g, respectively. Other compounds were recognized in moderate concentrations such as caffeic acid, catechin, and ferulic acid. However, there was a lack of pyro catechol, rutin, coumaric acid, vanillin, and hesperetin in the extract.

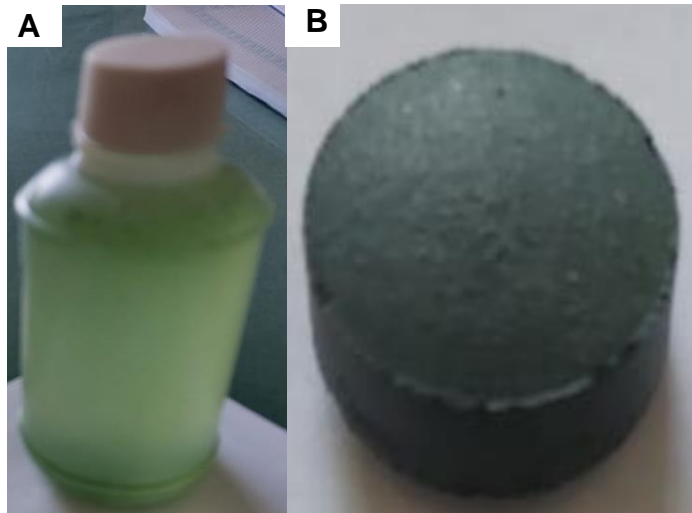


Fig. 1. Available form *S. platensis* in broth (A) and capsule form (B)

Table 1. Flavonoid and Phenolic Compounds Detected in *S. platensis* Extract

Compound	Retention Time	Area	Area (%)	Concentration ($\mu\text{g/g}$)
Gallic acid	3.375	65.12	2.3462	293.72
Chlorogenic acid	4.167	19.82	0.7142	141.60
Catechin	4.456	2.82	0.1015	36.23
Methyl gallate	5.594	51.63	1.8603	147.41
Caffeic acid	5.891	18.68	0.6730	75.84
Syringic acid	6.313	1.61	0.0580	5.87
Pyro catechol	6.808	0.00	0.0000	0.00
Rutin	8.032	0.00	0.0000	0.00
Ellagic acid	8.554	31.08	1.1196	332.67
Coumaric acid	9.209	0.00	0.0000	0.00
Vanillin	9.808	0.00	0.0000	0.00
Ferulic acid	10.227	16.96	0.6111	60.02
Naringenin	10.709	23.45	0.8449	144.48
Daidzein	12.530	2.82	0.1016	9.03
Quercetin	12.769	15.97	0.5753	108.69
Cinnamic acid	14.045	2.89	0.1042	2.78
Apigenin	14.387	1.64	0.0593	6.43
Kaempferol	15.046	5.68	0.2047	21.35
Hesperetin	15.636	0.00	0.0000	0.00

From previous analysis, different phenolic compounds were detected in *Spirulina* sp., such as gallic, caffeic, salicylic, and trans-cinnamic acids (Pagnussatt *et al.* 2014). Other studies identified these four compounds besides chlorogenic acid. This difference may occur due to varying conditions of cultivation. The phenolic acid (ellagic acid) in the *S. platensis* extract in current findings reflected inhibitory action toward aflatoxin B1 production by 82% (Pierzynowska and Grzesiuk 1998). The enhancement of fermentation quality, maintenance of protein, and a decrease in the abundance of dangerous

microorganisms in stylo silage have been observed after the addition of ellagic acid (Zou *et al.* 2021). Exogenous chlorogenic acid exhibited the inhibition of mycotoxin synthesis by *Fusarium spp.* This result was mainly dependent on the concentration of phenolic acid (Ferruz *et al.* 2016a). Ferruz *et al.* (2016b) tested several phenolic acids such as chlorogenic, caffeic, *p*-coumaric, and ferulic acids against T-2 and HT-2 mycotoxin production by *Fusarium sporotrichioides* and *Fusarium langsethiae*. It was determined that *F. langsethiae* and *F. sporotrichioides* biomass were not affected by these acids, while T-2 toxin was reduced to 23.1% and 26.5% using chlorogenic and ferulic acids, respectively.

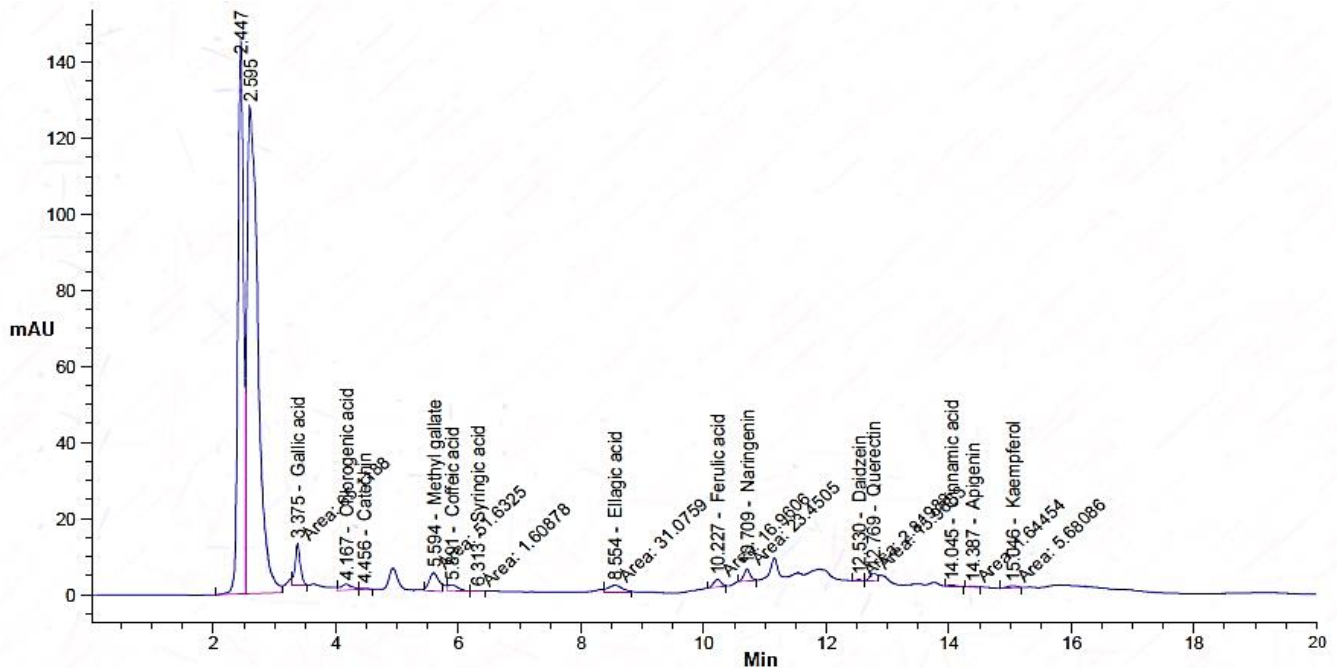


Fig. 2. HPLC chromatograph of *S. platensis* extract

The identification approaches documented the occurrence of three filamentous fungi on maize silage: *Aspergillus flavus*, *A. niger*, and *Fusarium sp.* Bakri (2021) demonstrated the presence of *Aspergillus sp.*, *Fusarium sp.*, *Penicillium*, *Mucor sp.*, and *Rhizopus sp.* on maize silage. The presence of various fungi, particularly mycotoxigenic on maize silage, were considered unattractive characteristics. *A. flavus* was used to study the efficacy of *S. platensis* extract on the aflatoxins production. In a recent review study, several biological activities such as anti-oxidants, anti-biofilm, antifungal, and detoxification of mycotoxins were associated with algae and its products (Yadavalli *et al.* 2023); the data suggests that *S. platensis* is detoxicant agent for mycotoxins. The level of mycotoxins production was affected by the period of silage incubation and treatment by *S. platensis* extract. From Table 1, the mean of detected aflatoxin B1, B2, G1, and G2 was 3.14 ± 0.15 ppb, 0.81 ± 0.08 ppb, 0.46 ± 0.05 ppb, and 0.26 ± 0.23 ppb. On the 10th day, it became 2.37 ± 0.33 ppb, 0.59 ± 0.16 ppb, 0.26 ± 0.22 ppb, and 0.21 ± 0.18 ppb when the silage was treated with *S. platensis* extract. The treated silage showed less content of aflatoxins B1, B2, G1, and G2 compared to untreated silage on the 15th day. It was expected that the aflatoxins in the treated silage on the 15th day would not exceed the aflatoxins on the 10th day. However, the results were opposite from this. It was found that the inhibition

percentage of aflatoxins B2 and G2 production was higher on the 15th day (30.61% and 77.77%) than the 10th day (27.16% and 19.23%). The appearance of aflatoxins on the 15th day indicated that the producer fungus growth was not completely inhibited by the *S. platensis* extract. The current outcomes were in agreement with other studies. For instance, Christ-Ribeiro *et al.* (2019) showed that the phenolic compounds of *Spirulina* sp. were effective against fungal growth with 20.2% inhibition as well as ochratoxin A production with 29% inhibition. In previous studies, methanolic extract of *S. platensis* was applied to suppress the growth and aflatoxin synthesis of *A. flavus* and *A. niger* (Pugazhendhi *et al.* 2015). Synthesis of several *F. graminearum* mycotoxins including nivalenol, deoxynivalenol, and trichothecene were inhibited by *Spirulina* sp. extract enriched with gallic acid (Pagnussatt *et al.* 2014). The benefit of the application of algal macromolecules is its ability to bind mycotoxins without dissociating in the uncontaminated feed. Mycotoxins then go through the digestive system of animals (Yadavalli *et al.* 2023).

Table 2. Effect of *S. platensis* Extract on the Aflatoxins Production at 10th Day and 15th Day

Treatment and Test Time	Aflatoxin B1 ppb	Aflatoxin B2 ppb	Aflatoxin G1 ppb	Aflatoxin G2 ppb
Control at 10 th day	3.06	0.73	0.41	0.00
	3.05	0.81	0.46	0.37
	3.31	0.88	0.50	0.41
	3.14±0.15*	0.81±0.08*	0.46±0.05*	0.26±0.23*
Control at 15 th day	3.70	0.98	0.56	0.45
	3.83	1.02	0.58	0.47
	3.51	0.93	0.53	0.43
	3.68±0.16*	0.98±0.05*	0.56±0.03*	0.45±0.02*
Treated at 10 th day	1.99	0.41	0.00	0.00
	2.53	0.67	0.38	0.31
	2.60	0.69	0.39	0.32
	2.37±0.33*	0.59±0.16*	0.26±0.22*	0.21±0.18*
Treated 15 th day	2.77	0.66	0.37	0.00
	2.92	0.67	0.00	0.31
	2.99	0.71	0.40	0.00
	2.89±0.11*	0.68±0.03*	0.26±0.22*	0.10±0.18*

*, Average of triplicate values of detected mycotoxins and standard deviations

Glucosamine as a biomarker of treated and untreated (control) *A. flavus* growth by *S. platensis* extract was estimated at different incubation periods (Fig. 3). *S. platensis* extract was effective on the glucosamine content of the fungus biomass at all periods of the incubation compared to the untreated fungus with inhibition of 53.7%, 49.2%, 47.8%, 38.5%, and 35.7% on days 3, 6, 9, 12, and 15. Souza *et al.* (2011) mentioned that the estimation of glucosamine is the best to examine the influence of *S. platensis* contents on the development of fungal biomass due to its creation. It was linear along the period in the untreated fungus and is affected by phenolic compounds that reflected antifungal activity *via* inhibiting the production of glucosamine up to 56%. Similar findings were recorded in previous reports using *Aspergillus oryzae* (Nagel *et al.* 2001) and *Rhizopus oligosporus* (Nopharatana *et al.* 2003)

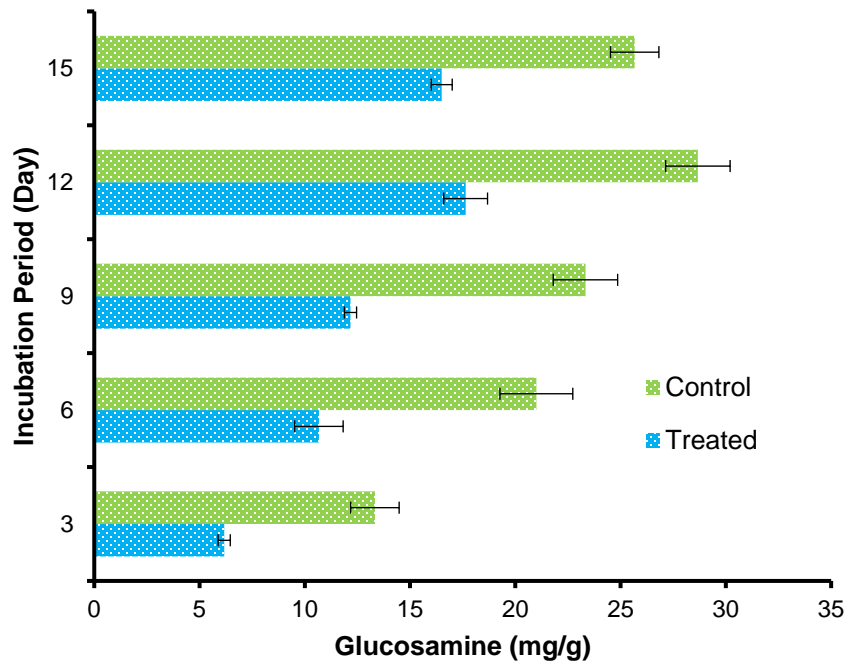


Fig. 3. Fungal biomass glucosamine at different incubation periods

CONCLUSIONS

1. Overall, outcomes indicated that *S. platensis* contains several flavonoid and phenolic acids.
2. *S. platensis* extract decreases the production of mycotoxins.
3. Based on the result of glucosamine detection, *S. platensis* extract had a repressive effect on *A. flavus* growth.

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Conflicts of Interest: The authors declare no conflict of interest

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