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Inhibitory Effects of *Anchusaitalica* and *Calotropisprocera* Extracts on *Aspergillusochraceus* and *Fusariumgraminearum* Growth under *in Vitro* Conditions

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Abstract: **This study was conducted to identify the fungi producing mycotoxins, infecting corn seeds and evaluate the activity of alcoholic and water extracts from Borage *Anchusaitalica* and French jasmine *Calotropisprocera* toward *Aspergillusochraceus* and *Fusariumgramimarum* in culture media. Results showed that several genus of fungi including, *Aspergillus*, *Fusarium*, *Penicillium*, *Cladosporium*, *Cylindrocarpon*, *Mucor* and *Rhizopus* were found associated with corn seeds. It was found that 9 of 18 isolates of *A. ochraceus* produced ochratoxinA (ochre A) and 14 of 30 isolates of *F. graminearum* produced Deoxynivalenol (Don) toxin. High inhibition of both of *A. ochraceus* and *F. graminearum* on PDA containing Borage and French jasmine extracts was observed. Inhibition of 100 % was obtained with alcoholic Borage extract, while 70.5 and 75.5 % were obtained with alcoholic French jasmine extract at 1 % in the culture media for *A. ochraceus* and *F. graminearum* respectively. Inhibition of 78.90 % and 84 %, 93.00 % and 98.00 % at 2 % of cold and hot water of borage extract were obtained for the two fungi respectively. Lesser effects of French jasmine water extract, 74.00 % and 75.00 %, 78.6 % and 83.0 % at 2 % of cold and hot water extracts toward the two fungi were obtained respectively. Higher inhibition of fungal growth was obtained with French jasmine latex, 100 % at 0.5 % of latex for the two fungi was obtained respectively.**

Keywords: ***Anchusaitalica, Calotropisprocera, Aspergillusoc- hraceus, Fusariumgraminearum***

1. INTRODUCTION

The corn *Zea mays* L. corp is considered as the third of the most important cereal crops in the world and Iraq that constitute the main part of poultry and animal diet (1). It has been reported that corn seeds infected with many fungi producing mycotoxin in the field and under storage conditions (2, 21). It was found that of the fungi associated with corn seeds, *Aspergillusochraceus* producing ochratoxin A and *Fusariumgraminearum* producing Deoxynivalenol (DON) toxins were among the most important that colonized corn seeds and contaminated them with mycotoxins (13, 32). The mycotoxins are secondary metabolites produced by many pathogenic fungi on cereals particularly corn seeds under certain conditions in the fields and under storage. The seeds contaminated with mycotoxin constitute very serious problems for human, directly through consuming food made from these seeds, or indirectly through consuming meet from poultry previously taking diet containing contaminated corn seeds (5, 7).

As a result of some previous studies indicated that small grain cereals including corn were highly contaminated with mycotoxins which are harmful to both human and animals, this research was conducted to identify the most important fungi producing mycotoxins associated with corn seeds in storage and evaluate the activity of Borage anf French jasmine extracts to inhibit the fungal growth in culture media.

2. MATERIALS AND METHODS

Fungi isolates

Aspergillusochraceus and *Fusariumgraminearum* isolates were isolated from corn seeds collected from storages at different areas in Iraq. The seeds were surface sterilized with sodium hypochlorite 2 % for 3 min., rinsed thoroughly with sterile distilled water, dried on filter paper and cultivated on potato succrose agar (PSA) (200 g potato, 10 g sucrose, 20 g agar in 1 liter water) in petriplates of 9 cm diameter (5 seeds/plate). The plates were maintained at 25 ± 2 ˚c for 5 days and the growing fungi were purified and identified. *A. ochraceus* isolates were purified on Subouroud Dextrose Agar (SDA) (65 g/L distilled water) and on potato dextrose agar (PDA) (39 g/L distilled water) in petriplates of 9 cm diameter. The plates were maintained at 25 ± 2 ˚c for 5–7 days.

Fusarium isolates were purified by single spore technique through serial dilutions in sterile distilled water on PDA in Petriplates. The plates were maintained at 25 ± 2 ˚c for 24 hours. The growing colonies were conserved in sterile soil and the species were identified based on morphological charecters according to Booth (8), Pitt and Hoching (24).

Ability of isolates to produce mycotoxin

A. ochraceus

A. ochraceus isolates were grown on yeast extract sucrose broth (YESB) (20 g yest extract, 200 g sucrose in 1 l distilled water) in 250 ml flasks. Each flask containing 100 ml YESB was inoculated with 0.5 cm diameter disk of fungal growth from PDA culture. The flasks were maintained at 26 ± 2 ˚c for14 days (11).

Ochra A extraction

The suspension of each isolate was filtered through Whatman filter paper No. 4. Fifty ml of the filtrate were transferred into 250 ml separating funnel containing 100 ml of chloroforme. The funnel was well agitated and let to

settle for 15 min. The lower layer was passed through Na_2SO_4 to eliminate water and the filtrate was dried in waterbath at 50 ˚c. The dried material was dissolved in 5 mlchloroforme, redried and conserved in small vials under freezing.

Ochra A detection

The dried samples were dissolved separately in 1 ml of chloroforme and ochra A was detected by thin layer chromatography on plates of 20X20X2.5 cm using standard ochra A (sigma chemical co.) as control. The plate was activated at 110 ˚c for 2 hours and charged with 10 µl of each sample at 2 cm from the lower side spacing of 1.5 cm. The chromatography was performed in a mixture of Toluene:Ethylacetate:Formic acid (60V:30V:10V), and the spots were detected by UV viewing cabinate at 360 nm (6).

F. graminearum

F. graminearum isolates were grown on rice seeds. One hundred twenty five ml distilled water were added to 100 g of rice seeds in petriplate of 20 cm diameter and 5 cm depth. The plate was autoclaved twice at 121 ˚c and 1.5 Kg/cm^2 for 20 min. during 24 hours. The sterilized seeds were inoculated with 2 disks of 0.5 cm diameter of fungal growth, and homogenized. The plate was maintained at 25 ± 2 ˚c for 14 days, then transferred to 13-16 ˚c for additional 14 days to induce the production of DON. The contaminated seeds were dried and ground in electrical grinder (2).

DON extraction

Fifty gm of rice seeds powder were added to 200 ml of Acetonitril:water mixture (84V:16v) in 500 ml flask. The flask was agitated for 30 min. and the extract was filtered through Whatman No.2 filter paper. One hundred twenty five ml of the filtrate were added to 50 ml of Hexane in separating funnel, subjected to agitation for 20 sec. and let to settle. Fifteen gm of ammonium sulfate were added to the lower layer and passed through filter paper (Whatman No.2). The filtrate was passed through 10 gm of anhydrous Na_2SO_4 and the filtrate dried and conserved in dark vial.

DON detection

The same procedure of Ochra A detection was followed for DON detection except that the mixture of chromatography used was, Chloroforme:Acetone:Isopropanol (8V:1V:1V). At the end of chromatography the plate was sprayed with 20 % ammonium chloride in methanol (31). The plate was maintained at 120 ˚c for 7 days and the spots were detected by UV light.

Activity of plant powders against *A. ohraceus* and *F. graminearum* on culture media

Leaves and flowers of Borage were obtained from local market, while leaves of French jasmine were collected from shrubs naturaly grown at Agric. College/University of Baghdad. The plant parts were air dried, ground by grinder (Willy mill model No.3, Arther Thomas co.), and passed through fine sieve. The powder was added into PDA medium before solidification (50 ˚c) at 10, 30, 50 g/l and poured in petriplates of 9 cm diameter. The center of each plate was inoculated with 0.5 cm diameter desk of fungal growth. The plates were maintained at 25 ± 1 ˚c and the diameters of growth colonies were calculated. The percentage of inhibition was calculated according to the following equation:

% of inhibition = Colony diameter in control – colony diameter in treatment / Colony diameter in control X 100

Activity of plant part extracts against A.ochraceusand F. graminearum in culture media

Fifty gm of plant parts powder were added to 200 ml of cold water in 500 ml flask. The flask was subjected to agitation for 24 hours and the extract was passed through Whatman No.1 filter paper in Buchner funnel with vacuum. The filtrate was concentrated in rotary evaporator to obtain densed liquid. In other trial the same procedure was followed and the flask containing the extract was maintained at 100 ˚c for 15 min. in water bath (28).

Alcoholic extract

Fifty gm of plant parts powder were added to 100 ml of ethyl alcohol 95 % in 500 ml flask. The flask with the mixture was agitated in electric shaker for 24 hours and the extract passed through Whatman No.1 filter paper in Buchner funnel with vacuum. The filtrate was concentrated in rotary evaporator at 45-50 ˚c (16). The concentrated extracts were added into the culture media at 0.2, 0.5, 1 % of alcoholic extract, 0.5, 1, 2 % of cold and hot water extracts. Also, French jasmine latex was used at 0.1, 0.3, 0.5 % with culture media. The medium containing the extracts was poured in petriplates. Each plate was inoculated in the center with 0.5 cm diameter disk of fungal growth, maintained at 25 ± 1 ˚c and the percentage of inhibition was calculated as before.

3. RESULTS

Identification of fungi isolated from corn seeds

Several species of fungi belong to the genus *Aspergillus*, *Fusarium*, *Penicillium*, *Cladosporium*, *Cylindrocarpon*, *Mucor* and *Rhizopus*, were found associated with corn seeds. *Aspergillus* species, *A. flavus*, *A. niger* and *A. ochraceus*, were the mor prevailing ate 32 %, followed by *Fusarium* species, *F. moniliforme*, *F. graminearum*, at 25 %. The other species were found at percentages ranged between 1.7-23 %. Similar results concerning the fungi associated with corn seeds were previously reported (21). *A. flavus* and *F. moniliforme* were submitted to many studies in our department, therefore *A. ochraceus* and *F. graminearum* were selected for this study.

Ability of A. ochraceus and F. graminearum to produce mycotoxins

The analysis of fungal cultures filtrate on thin layer chromatography (TLC) plates showed that 9 of 18 isolates of *A. ochraceus* were able to produce ochratoxin A (ochre A), and 14 of 30 isolates of *F. graminearum* produced DON toxin fig (1). The more active isolate of *A. ochraceus* (OTA 18 MR) and the more active one of *f. graminearum*(F1. DON. Fg) in producing mycotoxins, as shown by High Performance Liquid Chromatography (HPLC), fig(2), were adopted for the next experiments.

These results were found in accordance with many studies concerning the ability of these fungi to produce mycotoxin (15, 13).

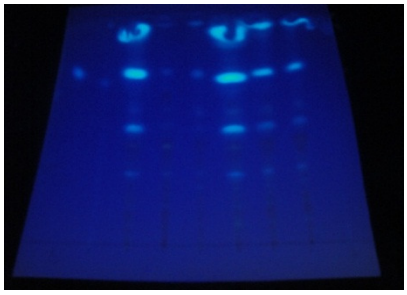


Fig 1(a) Ochratoxin

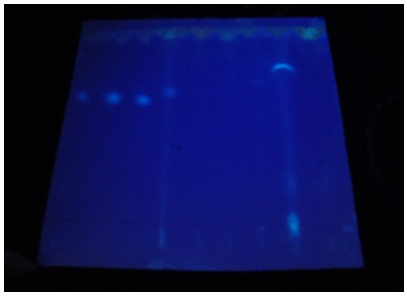


Fig 1(b) DON

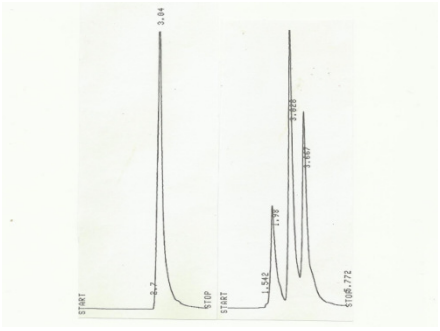


Fig 1 (c) Ochratoxin

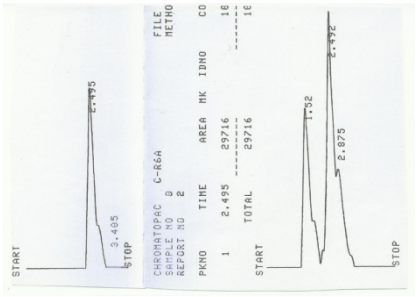


Fig 1 (d) DON

Fig 1. Antagonistic effect of plant powders against *A. ochraceus* and *F. graminearum* on PDA

It has been found that both of Borage *Anchusaitalica* and French jasmine powders exhibited significant reduction on the radial growth of *A. ochraceus* and *F. graminearum* on culture media compared with control. The inhibition percentages were found to be 32.22 and 37.70 %, 51.10 and 56.50 %, 65.90 and 68.88 % with *A. ochraceus* and *F. graminearum* at 1, 3, 5% of Borage powder respectively. Similar results were obtained using French jasmine powder that cause inhibition percentages, 21.10 an 19.40 %, 48.88 an 48.75 %, 55.50 and 57.55 % with two fungi at 1, 3, 5 % respectively, table (1) and fig (1). Several previous studies indicated the ability of many medicinal plant extracts to inhibit the growth of fungi producing mycotoxins (14, 30, 22).

Fig.2.(a)

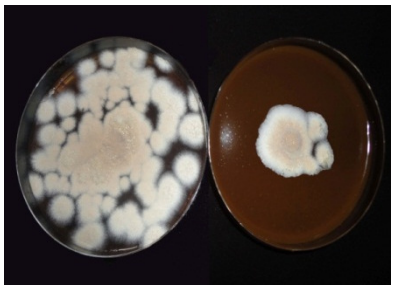


Fig.2.(b)

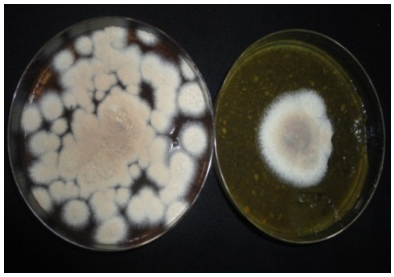


Fig.2.(c)

Fig.2.(d)

Fig.2. Antagonistic effect of plant powders against A. ochraceus and *F. graminearum* on PDA.

Table 1.Antagonistic effect of Borage and French jasmine powders against *A. ochraceus* and *F. graminearum* on PDA

Powders' concentrations	Inhibition percentages	
	A. ochraceus	*F. graminearum*

Borage powder 1%	32.22	37.70
Borage powder 3%	51.10	56.50
Borage powder 5%	65.90	68.88
French jasmine 1%	21.10	19.42
French jasmine 3%	48.88	48.75
French jasmine 5%	55.50	57.55
LSD P = 0.05	8.49	9.16

Activity of alcoholic and water plant extracts against A. ochraceus and F. graminearum in culture media

Alcoholic extracts of Borage and French jasmine induced significant reduction in *A. ochraceus* and *F. graminarum* radial growth in culture media. The inhibition percentages were 67.75 and 72.80 %, 74.50 and 75.60 %, 100 and 100 % at 0.2, 0.5, 1 % of alcoholic Borage extract for the two fungi respectively. Lesser activity of alcoholic French jasmine extract against the two fungi was observed, 31.20 and 35.80 %, 61.50 and 67.70 %, 70.50 and 75.50 % at 0.2, 0.5, 1 % respectively. Higher inhibition of fungal growth values were obtained with French jasmine latex in the culture media. Inhibition percentages of 55.50 and 74.50 %, 100.00 and 100.00 %, 100.00 and 100.00 % at 0.1, 0.3, 0.5 % were registered for the two fungi respectively, table (2), fig (2).

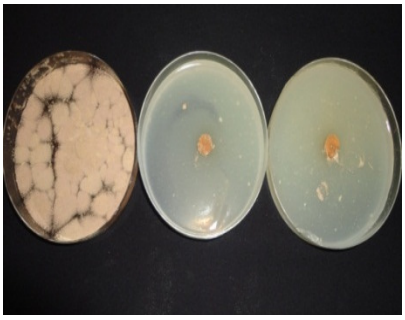


Fig.3(a)

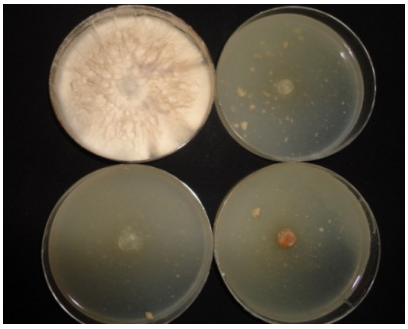


Fig.3(b)

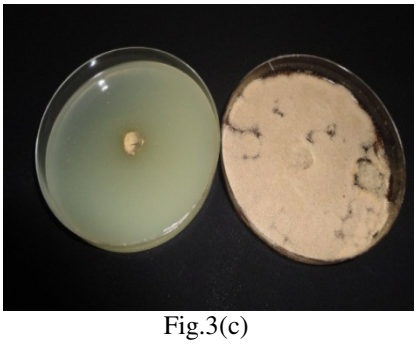


Fig.3(c)

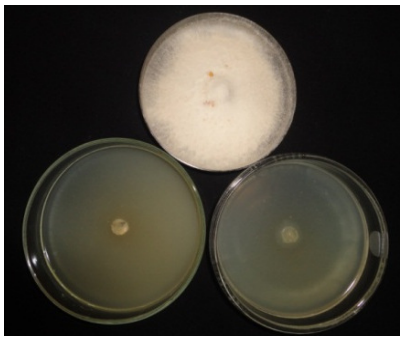


Fig.3(d)

Fig.3. Activity of alcoholic and water plant extracts against A. ochraceus and *F. graminearum* in culture media

Table (2):Activity of alcoholic plant extracts and latex against *A. ochraceus* and *F. graminearum* in culture media

Alcoholic extracts	Concentration	Inhibition percentages	
		A. ochraceus	*F. graminearum*
Borage extract	0.2	67.75	72.80
	0.5	74.50	75.60
	1	100	100
French jasmine extract	0.2	31.20	35.80
	0.5	61.50	67.70
	1	70.5	75.5
French jasmine latex	0.1	55.50	74.50
	0.3	100	100
	0.5	100	100
LSD P = 0.05	----	10.63	8.02

Cold and hot water extracts of Borage and French jasmine showed high efficiency in reducing A. ochraceus and F. graminearum radial growth in culture media.

Inhibition percentages of 56.10 and 59.70 %, 71.30 and 72.60 %, 78.90 and 84.50 % at 0.5, 1, 2 % of cold water Borage extract in the media were achieved for the two fungi respectively. While 62.10 and 71.40 %, 73.50 and 75.00 %, 93.00 and 98.00 % were obtained with 0.5, 1, 2 % of hot water Borage extract for the two fungi respectively.

Lesser effects were registered using French jasmine water extracts, 55.60 and 57.50 %, 58.80 and 61.70 %, 74.00 and 75.00 % with cold water extracts, 50.50 and 51.50 %, 63.80 and 66.70 %, 78.50 and 83.30 % with hot water extracts at 0.5, 1, 2 % in the culture media for the two fungi respectively, table (3), fig (3).

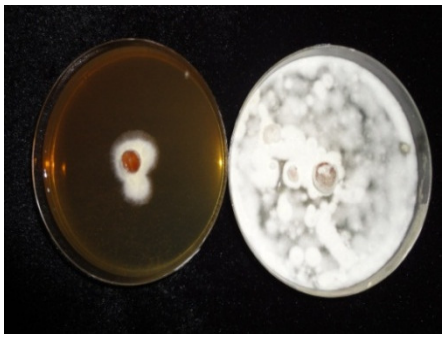


Fig.4(a)

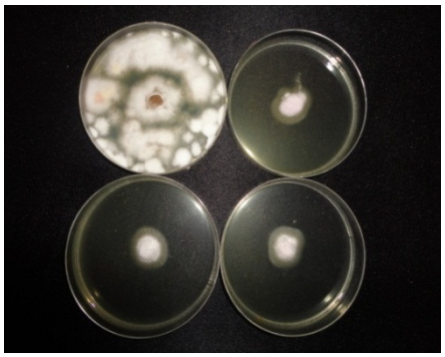
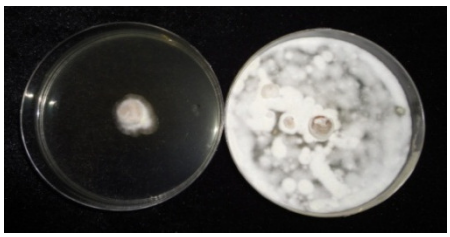


Fig.4(b)



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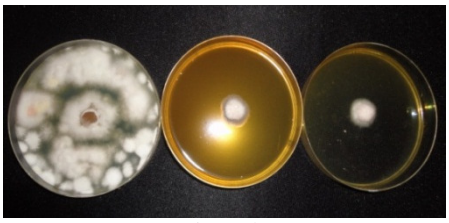

Fig.4(d)

Fig.4(e)

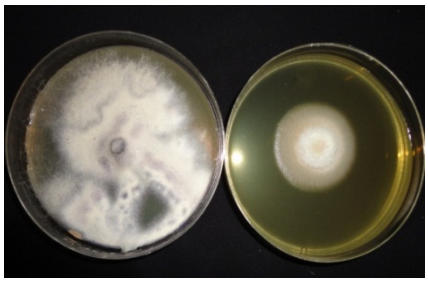

Fig.4(f)

Fig.4. Cold and hot water extracts of Borage and French jasmine showed high efficiency in reducing A. ochraceus and *F. graminearum* radial growth in culture media.

Several previous studies reported the efficiency of Borage and French jasmine water and alcoholic extracts against fungi and bacteria (31, 4, 23, 26, 18).

Table 3.Activity of watery plant extracts against *A. ochraceus* and *F. graminearum* in culture media

Water extracts	Concentrations	Inhibition percentages	
		A. ochraceus	*F. graminearum*
Cold water extract of Borage	0.5	56.10	59.70
	1	71.30	72.60
	2	78.90	84.50
Cold water extract of French jasmine	0.5	55.00	57.50
	1	58.80	61.70
	2	74.00	75.00
Hot water extract of Borage	0.5	62.10	71.40
	1	73.50	75.00
	2	93.90	98.00
Hot water extract of French jasmine	0.5	50.50	51.50
	1	63.80	66.70
	2	78.50	83.30
LSD P = 0.05	----	9.59	10.81

4. DISCUSSION

The results of this study demonstrated that corn seeds were internally infected by many species of fungi under storage conditions. The infection of seed may started in the field (preharvest infection) and continue to develop under poor grain storage practice. Of these fungi, *Aspergillusochraceus* and *fusariumgraminearum* were found among the more important and representing a serious problem to human and animals through producing mycotoxins. Similar results concerning the growth of fungi from surface sterilized corn seeds were previously reported (23, 10) reported that maize kernels invaded by molds including *Aspergillus* spp. before storage deteriorated more rapidly under conditions favorable to the fungi growth. The source of seed infection by *A. ochraceus* may came from the fungal colonies on maize residues in the soil or other hosts in the field. It has been reported that in cultivated fields *Aspergillus* spp. may actively colonize the residues of maize and other crops (35, 27), and these residues ecame more important since they may be colonized by insects vectoring the moulds to ripening maize cars (20).

A. ochraceus is a weak pathogen requiring breaks in the pericarp as entering points to inter the grain. These entering points may be caused by insects, birds, previous infection by other fungi, or climatic stress such as warm temperature.It was reported that high temperature stress results in poor kernel filling and kernel abortion which provides entry points for kernel rotting fungi such as *Aspergillus* (25). Smarts et al (29) reported that exposure of corn plants to high temperature during grain development leads to cracks in the testa that allow internal colonization grains by *Aspergillusflavus*. High temperature during grain maturation found to be associated with high level of mycotoxin in the field (33). *A. ochraceus* inoculum may be arrived and colonize maize grains as air born spores or insect-transmitted contaminating the silks and grow into the developing ear. Several previous studies reported that moths of corn ear worm, *Heliothiszea*, and European corn borer, *Ostrinianubitalis* have been implicated as vectors of *A. flavus* (19, 22). Doupnik (12) reported that infection by the ear rot fungus

Helminthosporiummaydis predisposed the kenels to *A. flavus* infection and toxin production.

The infection of corn seeds by *F. graminearum* may happened through airborne spores deposited on silk and the fungal hyphae grow into the developing grains and this associated with production of DON toxin. Some previous studies reported that infection of wheat seeds by *F. graminearum* is initiated by deposition of spores on flowering spikelets (9). High activity of Borage and French jasmine alcoholic and water extracts as well as French jasmine latex against *A. ochraceus* and *F. graminearum* growth on culture media was observed. The activity of Borage extract may be due to its content of tanines (Mucilag, Pyrrolizidine, alkaloids, Lycopsamine, supenedeneviridiflorate). Borage extract was widely used to inhibit the growth of several pathogenic fungi (23).

The activity of French jasmine may came from its content of trypsin, calotropin, uscharin, and calotroxin. It was reported that French jasmine extract has completely inhibited the sporulation of *Aspergillus*spp. (5), and the growth of *F. semitectum* on culture media (17). French jasmine latex was effectively inhibited the growth of many pathogenic species of yeast and fungi infecting human (26).

The inhibition activity of Borage and French jasmine extracts toward *A. ochraceus* and *F. graminearum* may be promising to manage these fungi and reduce the mycotoxins that produced in corn seeds in storage.

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