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Original Article

EFFICACY OF EXTRACTS OF SOME PLANTS IN AVOIDING FUNGAL DISEASES OF STORED CEREALS

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ABSTRACT

Objective: Plant metabolites and plant-based pesticides appear to be one of the better alternatives to synthetic pesticides. The aim of the current study is to evaluate the antifungal properties of the aqueous, ethanolic, methanolic and chloroform extracts of the leaves and stems of five wild plants (*Astrachantha echinus*; *Seriphidium herba-album*; *Peganum harmala*; *Diplotaxis acris* and *Tamarix aphylla*) collected from Tabuk deserts of KSA, against seed-borne mycoflora isolated from stored corn, wheat and barley grains.

Methods: *In vitro* antifungal activities of the 5 extracts were tested using the poisoned food technique. Separation of the different fractions from selected extracts was carried out using Thin Layer Chromatography (TLC). High Performance Liquid Chromatography (HPLC) was used to characterize the separated fractions and estimate the *in vivo* reduction in mycotoxins levels due to plants extracts.

Results: Most of these extracts significantly suppressed radial growth of the tested fungi *in vitro* with varying levels and/or caused their complete inhibition (100%, $P \leq 0.01$). Results of separation of selected extracts using TLC, and then their characterization by HPLC analysis revealed that these promising extracts were rich in p-Coumaric acid, Catechin, Caffeic acid, Cinnamic acid, Ferulic acid, Vanillic acid and Gallic acid in concentrations ranging from 97.0 µg/ml (Vanillic acid) to 471.1 µg/ml (Gallic acid). In addition, methanolic, ethanolic extracts of *Astrachantha echinus* and *Seriphidium herba-album* plants showed potent *in vivo* antimycotoxigenic activity ($P \leq 0.01$) against toxigenic isolates of *Apergillus flavus* and *Fusarium verticillioides* in comparison with the synthetic fungicide.

Conclusion: These novel methanolic, ethanolic extracts of *Astrachantha echinus* and *Seriphidium herba-album* plants could be commercially used as worldwide biodegradable and ecofriendly phytofungicides to prevent fungal biodeterioration of cereals; prevent mycotoxins production and/or decreasing them to an acceptable level; displace the use of synthetic fungicides and high cost refrigeration of cereals during storage.

Keywords: Grains, Storage fungi, Plants extracts, Phenols, Mycotoxins, Phytofungicides.

INTRODUCTION

Cereal grains such as corn; wheat and barley are the main sources of human diet; however, they are prone to great hazards and diseases by mold fungi especially during storage. Presence of molds in stored grains may lead to various forms of deterioration; decreased nutritive value and mycotoxins production [1, 2]. Aflatoxins (AFs) are one of the most hazardous mycotoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus* [3]. Aflatoxin B1 (AFB1) is frequently found in many feeds and foods such as cereals, and its unanticipated consumption poses alarming risks to public health worldwide [4, 5]. The International Agency for Research on Cancer has classified AFB1 as a group 1 carcinogen [6]. Based on the chemical structure and highly lipophilic properties of AFB1, it is found that AFB1 easily passes through the cells plasma membrane and converts into more or less toxic hydroxylated metabolites like AFB1 epoxide, aflatoxicol, and so on causing potent oxidative stress in cells [7-9]. In addition, fumonisin B1 mycotoxin produced by *Fusarium verticillioides* is the most common contaminant of corn during pre and post-harvest conditions [10, 11].

Seed treatment is the safest and the cheapest way of control of seed-borne fungal diseases, mycotoxins production and preventing biodeterioration of cereal grains [12, 13]. To manage biodeterioration causing fungi, the regular practice in agriculture is to use large quantities of chemical fertilizers; chemical growth regulators and chemical pesticides [14]. However, when they are concentrated on the grains, could induce chemical poisoning; environmental toxicity and development of resistance by fungi to these chemical agents [15]. Use of natural products like botanical amendments or botanical extracts for the management of fungal diseases in plants is considered as a substitute method to synthetic fungicides, as they are biodegradable and have less negative impacts on the human health and environmental hazards. This may be used for formulating new, safer and ecofriendly fungicides.

In vitro evaluation of antifungal potency of plants extracts against phytopathogenic fungi in general and biodeterioration causing fungi in particular is the first step towards developing plant based phytofungicides [16]. Different crude extracts of plants materials rich in polyphenolics and alkaloids are becoming important in food industries because of their antifungal and antiaflatoxigenic activities. Hence, such plants extracts could potentially be used to control mycotoxigenic fungi in foods and feeds, and for avoiding the use of synthetic chemicals [17]. The aims of the present study are to a)-develop novel plants extracts with high antifungal and *in vivo* antimycotoxigenic efficacy that could be used as ecofriendly phytofungicides; b)-protect cereal grains during storage from fungal diseases and mycotoxins; c)-prevent their biodeterioration; d)-increase their shelf life; g)-displace the use of the deleterious synthetic fungicides and high cost refrigeration of cereals during storage.

MATERIALS AND METHODS

Collection and identification of desert plants

Five plants were collected from different locations of Tabuk deserts, Saudi Arabia such as El-Zeta Mountain; Al Lawz Mountain; Al Harrah road and El-Madinah road. These plants were botanically identified [18, 19].

Aqueous and solvents extraction of the plant's secondary metabolites

The aqueous and solvents (methanol, ethanol and chloroform) extraction from the 5 desert plants were carried out [20].

Collection of samples of stored cereals

Four samples of stored corn, wheat and barley grains (200 g each) were collected from different storage markets of Tabuk, Saudi Arabia. They were preserved at 5 °C for 24h until isolation of their mycoflora.

Isolation and mycological identification of the storage seed-borne mycoflora

Forty grains of each cereal were surface sterilized with 2% (v/v) sodium-hypochlorite solution (Sigma, USA) for 3 min and then rinsed twice with sterile distilled water. The sterilized grains were plated on Potato dextrose agar medium (PDA) (2 grains per plate) and subjected to agar plating method, to isolate their frequently occurring seed-borne mycoflora [21]. The plates were incubated under alternating periods of 12 h darkness and 12 h of light at 25±2 °C for 7 d. The percent incidence of these seed-borne fungi obtained from all grains was determined using this formula: Percent Incidence (PI) = number of seeds on which the fungus is encountered in each sample/total number of seeds tested in each samples x 100 [22]. All the isolates were identified mycologically up to the species level using different fungal keys [23-25].

Examination of the *in vitro* antifungal potential of all plant's extracts against the isolated mycoflora and determination of their minimum inhibitory concentrations (MIC)

One gram of each solvent extracts was dissolved in 1l of Dimethyl sulfoxide (DMSO)(Sigma, USA), PDA with different concentration of each of the solvents extracts were prepared to have a final concentrations of: 0.8; 1.6; 3.2 and 4.8 µg/ml. PDA medium amended with the same concentrations of these respective solvents served as negative control. Mycelial discs (5 mm) from the margins of seven d old cultures of each fungal species were placed in the center of medium. The plates were incubated at 28±2 °C for seven d and three replicates were maintained for each treatment. The percent inhibition of mycelial growth was determined by the formulae PI = C-T/C×100, where C= Diameter of control colony, T= Diameter of treated colony [26]. The same procedure was followed for the synthetic fungicide Topsin-m (70% of active principle) (Cerexagri-Nisso LLC), preparing concentrations of 25; 50 and 100 µg/ml standard recommended dosage. The experiment was repeated three times for confirmation of results. Minimum inhibitory concentrations (MIC) of all extracts were determined [27]. The MIC was taken as the lowest concentration of the extract that prevented growth of the test fungi.

Separation of different fractions from *Astrachantha echinus* and *Seriphidium herba-album* extracts using thin layer chromatography (TLC)

The methanol, ethanol extracts of the selected *Astrachantha echinus* and *Seriphidium herba-album* extracts was separated into different fractions using TLC [26]. These separated fractions were characterized qualitatively and quantitavely by High performance liquid chromatography (HPLC) [28].

***In vitro* antifungal activities of the separated phenolic components against the seed borne mycoflora**

Each of the phenolic separated components of the selected extracts was dissolved in 1 ml of (DMSO) to prepare a final concentration of 4.8 µg/ml of medium, and their antifungal potential was detected [26].

Estimation of *in vivo* antimycotoxigenic activities of plants extracts against selected toxigenic isolates of *Aspergillus flavus* and *Fusarium verticillioides*

The *in vivo* reduction of aflatoxin (AFB1) and fumonisin (FB1) mycotoxins production levels in corn seeds co-inoculated separately with *Aspergillus flavus*, *Fusarium verticillioides* (selected toxigenic isolates), with the extracts of *Astrachantha echinus* and *Seriphidium herba-album* plants, respectively, were estimated[29]. Fifty g of commercial corn was soaked with distilled water (15 ml) in a separate Erlenmayer flasks (300 ml) overnight before sterilization run for 30 min at 121 °C. Each flask was inoculated separately with *A. flavus* and *F. verticillioides* conidial suspensions (2 ml of 1× 106 CFUs/ml) in combination with each of methanolic and ethanolic extracts of both plants (5 ml) at three different concentrations (1.6, 3.2 and 4.8 µg/ml). Other sets of flasks were inoculated with the toxigenic fungi and the synthetic Topsin-m (70% a. p.) (Cerexagri-Nisso LLC) (5 ml of 4.8 µg/ml). In the positive control flasks, each toxigenic fungus was inoculated separately. All cultures were incubated at 25 °C and shaken each d to support a uniform growth of mycelium. Six replicates were made for each set of flasks. After 21 d incubation, corn seeds colonized by fungi were dried in a thin layer at room temperature and mycotoxins contents were analyzed. The process of extraction and estimation of aflatoxin level (AFB1) and fumonisin level (FB1) were carried out using HPLC [30-32].

Data analysis

All treatments were replicated three times (unless otherwise specified), data were reported as mean±SD (standard deviation) and were subjected to analysis of variance (ANOVA) using Statistical Software (version 6.0; Stat Soft Inc., Tulsa, OK, USA).

RESULTS

Identification of the desert plants

The collected five desert plants were belonging to 5 different families identified as; *Astrachantha echinus* (DC.) Podlech (family: Leguminosae); *Seriphidium herba-album* (Asso) Soják (family: Asteraceae); *Peganum harmala* L. (family: Zygophyllaceae); *Diplotaxis acris* (Forssk.) Boiss (family: Brassicaceae) and *Tamarix aphylla* (L.) Karst (family: Tamaricaceae).

Isolation and identification of the seed borne mycoflora

Isolation of the seed borne mycoflora on PDA medium led to the recovery of 8 fungal species which were identified mycologically, and their percent incidence from all grains was calculated. They were identified as, *Fusarium verticillioides* Sheldon (92%); *Fusarium solani* (Martius) Appel and Wollenweber (88%); *Aspergillus niger* Van Tighem (90%); *Aspergillus flavus* Link (80%); *Aspergillus versicolor* Teraboschi (75%); *Alternaria alternata* (Fries) Keissler (78%); *Macrophomina phaseolina* (Tassi) Goidanich (88%); *Epicoccum nigrum* Link (75%).

Examination of the *in-vitro* antifungal potential of the plant's extracts against the isolated mycoflora and determination of their minimum inhibitory concentrations (MIC)

All plants extracts showed high antifungal potency against the selected fungi (with different degrees) at almost all concentrations tested compared with the negative and positive control. Their antifungal activities increased with increasing their corresponding concentrations.

Astrachantha echinus

As it is clear in (tables 1a, b), all extracts especially methanolic and ethanolic ones caused significant suppression of fungal radial growth ranging from 35% ($P \leq 0.01$) to complete inhibition (100%, $P \leq 0.01$) at all the tested concentrations.

Seriphidium herba-album

Chloroform extract completely prevented mycelial growth of *Epicoccum nigrum*; *A. versicolor* (100%, $P \leq 0.01$) at concentrations of 1.6, 3.2, 4.8 µg/ml and caused the same inhibition of *Macrophomina phaseolina* at concentrations of 3.2, 4.8 µg/ml. Methanol extracts of this plant recorded inhibition of *Macrophomina phaseolina*; *Epicoccum nigrum* at all tested concentrations (100%, $P \leq 0.01$).

Ethyl alcohol extract showed high inhibitory activity (100%, $P \leq 0.01$) of *Epicoccum nigrum* at all used concentrations. The aqueous extract completely prevented growth (100%, $P \leq 0.01$) of *Macrophomina phaseolina* at 3.2, 4.8µg/ml and *Epicoccum nigrum*, *A. versicolor* at 4.8 µg/ml.

Peganum harmala

Alternaria alternata and *Epicoccum nigrum* were highly sensitive to the methanol and ethanol extracts as their growth was completely inhibited (100%, $P \leq 0.01$) at 4.8 µg/ml. The remaining mycoflora showed moderate to low sensitivity ranging from 81%-46% ($P \leq 0.01$).

Table 1(a): *In vitro* antifungal activity of aqueous and ethyl alcohol extracts of *Astrachantha echinus* plant against seed borne mycoflora of cereals

Mycoflora	Aqueous extract				MIC	Ethyl alcohol extract				MIC
	Concentration (µg/ml)					Concentrations (µg/ml)				
	0.8	1.6	3.2	4.8	µg/ml	0.8	1.6	3.2	4.8	µg/ml
	Percentage of inhibition (%)					Percentage of inhibition (%)				
Fusarium verticillioides	54.4^{a}±0.64	56.3^{b}±0.37	65.5^{c}±0.64	75.5^{d}±0.64	0.1	54.4^{a}±0.64	54.4^{b}±0.64	56.67^{c}±0.64	73.33^{d}±0.64	0.2
Fusarium solani	64.8^{a}±1.33	64.0^{b}±0.74	65.5^{c}±0.64	77.4^{d}±0.74	0.1	48.8^{a}±0.64	50.7^{b}±0.74	52.22^{c}±1.11	73.70^{d}±0.74	0.4
Aspergillus niger	63.3^{a}±1.09	65.8^{b}±0.17	68.8^{c}±0.73	100^{d}±0	0.1	18.3^{a}±1.72	38.6b±0.70	41.59^{c}±0.20	61.90^{d}±2.01	0.1
Aspergillus flavus	66.5^{a}±4.30	66.4^{b}±3.56	68.5^{c}±0.47	100^{d}±0	0.1	45.5^{a}±2.31	48.1^{b}±0.37	51.48^{c}±0.98	100.0^{d}±0	0.1
Aspergillus versicolor	67.5^{a}±0.17	71.8^{b}±1.30	75.5^{c}±0.14	100^{d}±0	0.1	55.3^{a}±0.68	67.0^{b}±0.46	76.61^{c}±1.21	100.0^{d}±0	0.1
Alternaria alternata	47.7^{a}±0.75	51.0^{b}±0.91	71.3^{c}±1.45	82.9^{d}±0.39	0.2	35.1^{a}±0.98	49.7^{b}±1.04	67.79^{c}±0.86	59.83^{d}±0.72	0.4
Macrophomina phaseolina	64.2^{a}±1.31	65.1^{b}±0.16	72.7^{c}±0.40	100^{d}±0	0.1	60.7^{a}±1.16	70.9^{b}±2.83	79.02^{c}±3.10	81.25^{d}±1.54	0.2
Epicoccum nigrum	75.0^{a}±0.4	76.0^{b}±1.5	79.9^{c}±0.43	100^{d}±0	0.1	65.2^{a}±0.66	68.4^{b}±0.65	100.0^{c}±0	100.0^{d}±0	0.1

Results show the percentages of inhibition of radial growth of the isolated mycoflora of cereals by the aqueous and ethanol extracts of *Astrachantha echinus* plant which was tested *in vitro* using the poisoned food technique. The MIC of the extracts was also estimated using the same technique. Values are the mean of three replicates, ± standard deviation. The value followed by different superscript letters (a, b, c, ….) indicates that means are not significantly different at P ≤ 0.01 when subjected to analysis of variance (ANOVA). Pattern of percentage inhibition increase is not uniform for all the microorganisms. Where; MIC: minimum inhibitory concentration.

Table 1(b): *In vitro* antifungal activity of methyl alcohol and chloroform extracts of *Astrachantha echinus* plant against seed borne mycoflora of cereals

Mycoflora	Methyl alcohol extract				MIC	Chloroform extract				MIC
	Concentration (µg/ml)					Concentrations (µg/ml)				
	0.8	1.6	3.2	4.8	µg/ml	0.8	1.6	3.2	4.8	µg/ml
	Percentage of inhibition (%)					Percentage of inhibition (%)				
Fusarium verticillioides	66.3^{a}±0.3	66.6^{b}±0.5	67.0^{c}±0.3	78.5^{d}±0.3	0.1	63.3^{a}±1.92	63.7^{b}±1.33	65.1^{c}±0.37	80.7^{d}±0.74	0.1
Fusarium solani	74.8^{a}±0.7	75.1^{b}±0.3	76.3^{c}±0.37	75.1^{d}±0.7	0.1	74.4^{a}±0.6	75.1^{b}±0.37	75.9^{c}±0.98	78.8^{d}±0.6	0.1
Aspergillus niger	60.8^{a}±1.4	62.8^{b}±0.1	63.8^{c}±0.60	66.8±0.9	0.1	62.8^{a}±3.99	72.7^{b}±0.57	80.7^{c}±0.77	100^{d}±0	0.1
Aspergillus flavus	68.9^{a}±2.15	70.9^{b}±0.5	71.3^{c}±1.09	100^{d}±0	0.1	57.9^{a}±1.76	62.6^{b}±2.37	68.39^{c}±2.13	100^{d}±0	0.1
Aspergillus versicolor	65.4^{a}±1.3	72.3^{b}±0.9	78.7^{c}±0.74	100^{d}±0	0.1	50.5^{a}±0.26	69.6^{b}±0.97	79.27^{c}±0.55	100^{d}±0	0.1
Alternaria alternata	51.0^{a}±1.1	65.2^{b}±0.8	69.4^{c}±0.48	100^{d}±0	0.2	58.5^{a}±1.71	60.2^{b}±0.67	67.3^{c}±1.01	100^{d}±0	0.1
Macrophomina phaseolina	74.1^{a}±1.1	74.5^{b}±0.6	75.4^{c}±0.56	100^{d}±0	0.1	70.9^{a}±0.32	73.2^{b}±0.12	75.0^{c}±0.51	100^{d}±0	0.1
Epicoccum nigrum	61.9^{a}±0.6	72.8^{b}±2.1	78.8^{c}±0.8	100^{d}±0	0.1	65.7^{a}±0.96	71.7^{b}±2.10	78.2^{c}±1.47	100^{d}±0	0.1

Results show the percentages of inhibition of radial growth of the isolated mycoflora of cereals by the methanolic and chloroform extracts of *Astrachantha echinus* plant which was tested *in vitro* using the poisoned food technique. The MIC of the extracts was also estimated using the same technique. Values are the mean of three replicates, ± standard deviation. The value followed by different superscript letters (a, b, c….) indicates that means are not significantly different at P ≤ 0.01 when subjected to analysis of variance (ANOVA). Pattern of percentage inhibition increase is uniform for all the microorganisms. Where; MIC: minimum inhibitory concentration.

Diplotaxis acris

Epicoccum nigrum was less resistant to the methanol and chloroform extracts as they completely prevented its radial growth (100%, P ≤ 0.01) at a concentration of 4.8 µg/ml and suppressed to 83%, 81% (P ≤ 0.01) by the aqueous and ethanol extracts; respectively.

Tamarix aphylla

A. versicolor and *Epicoccum nigrum* were very sensitive to the chloroform extract causing inhibition (100%, P ≤ 0.01) at the concentration of 4.8 µg/ml. The synthetic fungicide Topsin-m (70% a. p.) exhibited various capabilities to suppress the seed borne mycoflora on the solid medium (table 2). The inhibitory activities of most extracts at the concentration of 0.8 µg/ml were equivalent to the same level of activity of the chemical fungicide at 100 µg/ml.

All extracts of *Astrachantha echinus* and *Seriphidium herba-album* plants especially methanolic, ethanolic ones showed the most effective antifungal potency against the seed borne mycoflora; hence were selected for further research.

Table 2: *In vitro* antifungal activity of Topsin-m (70% a. p.) fungicide against seed borne mycoflora of cereals

Mycoflora	Concentrations of fungicide (µg/ml)		
	25	50	100
	Percentages of growth inhibition (%)		
Fusarium verticillioides	82.59^{a}±0.37	100.0^{b}±0	100.0^{c}±0
Fusarium solani	54.81^{a}±0.37	64.81^{b}±0.37	76.67^{c}±0.64
Aspergillus niger	28.65^{a}±3.61	38.01^{b}±3.68	51.84^{c}±2.36
Aspergillus flavus	29.26^{a}±1.48	38.15^{b}±0.37	61.48^{c}±0.37
Aspergillus versicolor	37.31^{a}±0.75	51.76^{b}±1.63	64.19^{c}±0.55
Alternaria alternata	43.09^{a}±0.66	45.19^{b}±0.19	51.88^{c}±0.62
Macrophomina phaseolina	74.01^{a}±1.61	81.38^{b}±0.45	100.0^{c}±0.97
Epicoccum nigrum	25.58^{a}±0.94	39.58^{b}±1.21	54.56^{c}±1.16

Results show the percentages of inhibition of radial growth of the isolated mycoflora of cereals by the synthetic fungicide (Topsin–m) which was tested *in vitro* using the poisoned food technique as positive control treatments. Values are the mean of three replicates, ± standard deviation. The value followed by different superscript letters (a, b, c….) indicates that means are not significantly different at P ≤ 0.01 when subjected to analysis of variance (ANOVA). Pattern of percentage inhibition increase is uniform for all the microorganisms. Where; (70% a. p.): 70% active principle of Topsin-m fungicide.

Studying the MIC of all extracts compared with Topsin-m (70% a. p.) showed that the MIC of *Astrachantha echinus* plant extracts were very minute varying between 0.1-0.8 µg/ml, which were less than the MIC of the synthetic fungicide (25 µg/ml) as it is clear in(tables 1a, b).

Separation of different phenolic components from the selected extracts using TLC and their characterization using HPLC

Separation of the phenolic components of the selected extracts by TLC led to the recovery of 7separate bands with different R_f. After characterization of these bands using HPLC, we found that the highest conc. of phenols was that of Gallic acid about 471.1 µg/ml, which were detected in the methanolic extract of *Astrachantha echinus* plant. P-Coumaric acid and Gallic acid levels were high in the methanolic, ethanolic extract of the same plant (448.1, 466 µg/ml; respectively). Moderate levels of Gallic acid and p-Coumaric acid were observed in the methanolic extract of *Seriphidium herba-album* plant (349, 183.2 µg/ml; respectively); Ferulic acid level was good enough also in the ethanolic extract of this plant (327 µg/ml) (table 3). The remaining phenolic components such as Catechin, Caffeic acid, Vanillic acid and Cinnamic acid were also characterized with different concentrations.

Table 3: Qualitative and quantitative characterization of separated phenolic components using HPLC in methanol, ethanol extracts of *Astrachantha echinus* and *Seriphidium herba-album* plants

Phenolic compounds	*Astrachantha echinus* extract		*Seriphidium herba-album* extract	
	Conc. of phenols (µg/ml)		Conc. of phenols (µg/ml)	
	Methanol	Ethanol	Methanol	Ethanol
Catechin	101.4±0.01	170.7±0.74	189.1±0.01	182.2±0.01
Gallic acid	471.1±0.01	466±0.13	349±0.21	192.4±0.41
Caffeic acid	126.8±0.36	100.8±0.99	173±0.01	116.9±1.20
Vanillic acid	190.05±0.23	97.0±0.01	156.4±1.08	126±0.62
p-Coumaric acid	448.1±0.41	110.8±1.01	183.2±1.43	132.4±0.93
Ferulic acid	195.8±0.01	121.6±0.57	155±0.88	327±0.01
Cinnamic acid	189.5±0.91	98.1±0.01	129.5±0.23	109±1.07

Results show the characterization of the phenolics separated from the extracts of *Astrachantha echinus* and *Seriphidium herba-album* plants using HPLC. Values are the means of three replicates, ± standard deviation. Where; Conc.: Concentration

***In vitro* antifungal activities of the separated phenolic components against the seed borne mycoflora**

All the separated phenolic components of the selected extracts showed potent antifungal potency against seed borne mycoflora at4.8 µg/ml, compared with that of the synthetic fungicides (tables 4a-d). Ferulic acid and Gallic acid in methanol extract of *Astrachantha echinus* plant showed high antifungal potency against *F. verticillioides*, *A. versicolor* and *Alternaria alternata* (89, 86 and 88 %, respectively).

In ethanol extract of the same plant, *F. verticillioides*, *A. flavus* and *Alternaria alternata* were most sensitive to Ferulic acid and Gallic acid showing inhibition percentages of (89, 89 and 88%, respectively). Gallic acid and Ferulic acid phenolic components in the methanol and ethanol extracts of *Seriphidiumherba album* showed potentially high percentages of inhibition of radial growth of *F. verticilliodes*, *A. flavus* and *Macrophomina phaseolina* ranging from 86-89 %.

The synthetic fungicide (Topsin-m) showed good inhibitory action against the radial growth of all the tested mycoflora ranging from 89-93 %.

The antifungal activities of the Gallic acid, p-Coumaric acid and Ferulic acid phenolic components were much more than that of the remaining 4 ones.

Table 4 (a): Antifungal activity of phenolic components recorded in methanol extract of *Astrachantha echinus* at 4.8 µg/ml against seed borne mycoflora

Mycoflora	% of inhibition of mycoflora							
	Phenolic components							Topsin-m
	Catechin	Gallic acid	Caffeic acid	Vanillic acid	p-Coumaric acid	Ferulic acid	Cinnamic acid	
Fusarium verticillioides	77^{a}±0.1	82^{b}±0.23	68^{a}±0.41	61^{ab}±0.21	71^{ab}±0.82	89^{a}±0.1	78^{a}±0.1	91
Fusarium solani	65^{a}±0.23	74^{a}±0.32	68^{a}±0.21	67^{a}±0.1	74^{b}±0.21	80^{a}±0.23	71^{b}±0.41	89
Aspergillus niger	68^{a}±0.23	78^{a}±0.32	69^{b}±0.1	67^{a}±0.41	73^{a}±0.1	79^{b}±0.1	70^{b}±0.82	89
Aspergillus flavus	87^{a}±0.41	80^{ab}±0.23	80^{ab}±0.63	62^{ab}±0.63	79_{b}±0.23	80^{b}±0.41	74^{b}±0.32	93
Aspergillus niger	78^{b}±0.1	86^{b}±0.82	78^{ab}±0.32	70^{ab}±0.23	79^{b}±0.1	78^{b}±0.21	74^{ab}±0.1	91
Alternaria alternata	76^{a}±0.9	86^{b}±0.1	78^{b}±0.27	66^{a}±0.40	71^{b}±0.41	79^{b}±0.21	78^{ab}±0.82	89
Macrophomina phaseolina	79^{b}±0.71	84^{a}±0.21	77^{a}±0.21	70^{ab}±0.1	79^{ab}±0.21	77^{ab}±0.1	70^{b}±0.21	92
Epicoccum nigrum	79^{ab}±0.41	81^{ab}±0.71	71^{a}±0.11	71^{a}±0.1	73^{b}±0.1	80^{a}±0.23	68^{b}±0.21	92

Results show the percentages of inhibition of radial growth of the isolated mycoflora of cereals by the separated phenolic components from the methanol extract of *Astrachantha echinus* plant, which was tested *in vitro* using the poisoned food technique. Values are the mean of three replicates,± standard deviation. The value followed by different superscript letters (a, b, c, ….) indicates that means are not significantly different at $P \leq 0.01$ when subjected to analysis of variance (ANOVA). Pattern of percentage inhibition increase is not uniform for all the microorganisms.

Table 4 (b): Antifungal activity of phenolic compounds recorded in ethanol extract of *Astrachantha echinus*at 4.8 µg/ml against seed borne mycoflora

Mycoflora	% of inhibition of mycoflora						
	Phenolic components						
	Catechin	Gallic acid	Caffeic acid	Vanillic acid	p-Coumaric acid	Ferulic acid	Cinnamic acid
Fusarium verticillioides	71^{a}±0.23	83^{b}±0.11	76^{ab}±0.1	69^{b}±0.1	74^{b}±0.24	89^{a}±0.23	78^{a}±0.1
Fusarium solani	78^{a}±0.1	83^{a}±0.31	77^{a}±0.24	73^{b}±0.23	68^{ab}±0.1	87^{a}±0.31	79^{a}±0.11

Aspergillus niger	70^{a}±0.24	81^{a}±0.17	78^{a}±0.17	71^{ab}±0.11	68^{a}±0.23	85^{b}±0.1	71^{ab}±0.17
Aspergillus flavus	76^{a}±0.31	82^{ab}±0.23	73^{b}±0.16	70^{b}±0.1	69^{a}±0.23	89^{a}±0.24	79^{a}±0.11
Aspergillus versicolor	79^{b}±0.17	87^{a}±0.24	76^{ab}±0.31	67^{b}±0.11	74^{ab}±0.24	86^{a}±0.11	71^{a}±0.23
Alternaria alternata	73^{a}±0.23	88^{ab}±0.1	72^{b}±0.23	69^{b}±0.23	73^{b}±0.17	82a±0.16	75^{b}±0.31
Macrophomina phaseolina	77^{a}±0.11	84^{b}±0.1	77^{a}±0.16	70^{b}±0.1	71^{b}±0.1	84^{ab}±0.24	73^{a}±0.1
Epicoccum nigrum	78^{a}±0.24	80^{b}±0.24	73^{a}±0.23	72^{a}±0.31	69^{ab}±0.31	81^{b}±0.23	73^{a}±0.16

Results show the percentages of inhibition of radial growth of the isolated mycoflora of cereals by the separated phenolic components from the ethanol extract of *Astrachantha echinus* plant, which was tested *in vitro* using the poisoned food technique. Values are the mean of three replicates, ± standard deviation. The value followed by different superscript letters (a, b, c, ….) indicates that means are not significantly different at P ≤ 0.01 when subjected to analysis of variance (ANOVA). Pattern of percentage inhibition increase is not uniform for all the microorganisms.

Table 4 (c): Antifungal activity of phenolic compounds recorded in methanol extract of *Seriphidium herba-album*at 4.8 µg/ml against seed borne mycoflora

Mycoflora	**% of inhibition of mycoflora**						
	Phenolic components						
	Catechin	**Gallic acid**	**Caffeic acid**	**Vanillic acid**	**p-Coumaric acid**	**Ferulic acid**	**Cinnamic acid**
Fusarium verticillioides	71^{a}±0.11	88^{a}±0.11	78^{a}±1.04	69^{a}±0.23	74^{a}±0.11	88^{a}±0.16	78^{b}±0.23
Fusarium solani	78^{a}±0.17	87^{b}±0.23	79^{ab}±0.31	73^{a}±0.16	68^{b}±0.16	83^{ab}±1.04	79^{b}±0.25
Aspergillus niger	70^{b}±0.23	87^{b}±0.16	78^{ab}±0.17	71^{b}±0.31	68^{b}±0.23	83^{a}±0.31	71^{b}±0.23
Aspergillus flavus	76^{b}±0.16	88^{b}±0.31	73^{b}±0.23	70^{b}±0.11	69^{a}±0.17	87^{b}±0.11	79^{ab}±0.17
Aspergillus versicolor	79^{a}±0.23	85^{ab}±0.23	78^{a}±0.23	67^{b}±0.23	74^{a}±0.17	85^{b}±0.23	71^{b}±1.04
Alternaria alternata	73^{ab}±0.17	80^{a}±0.11	72^{a}±0.16	69^{ab}±0.17	73^{a}±0.16	85^{b}±0.17	75^{a}±0.31
Macrophomina phaseolina	77^{a}±0.31	86^{a}±1.04	77^{b}±0.31	70^{b}±0.11	71^{a}±0.17	83^{b}±0.31	73^{b}±0.16
Epicoccum nigrum	78^{ab}±0.11	81^{a}±0.31	73^{a}±1.04	72^{a}±0.16	69^{a}±0.23	85^{b}±0.17	73^{b}±0.16

Results show the percentages of inhibition of radial growth of the isolated mycoflora of cereals by the separated phenolic components from the methanol extract *Seriphidium herba-album* plant, which was tested *in vitro* using the poisoned food technique. Values are the mean of three replicates, ± standard deviation. The value followed by different superscript letters (a, b, c….) indicates that means are not significantly different at P ≤ 0.01 when subjected to analysis of variance (ANOVA). Pattern of percentage inhibition increase is not uniform for all the microorganisms.

Table 4 (d): Antifungal activity of phenolic compounds recorded in ethanol extract of *Seriphidium herba-album*at 4.8 µg/ml against seed borne mycoflora

Mycoflora	**% of inhibition of mycoflora**						
	Phenolic components						
	Catechin	**Gallic acid**	**Caffeic acid**	**Vanillic acid**	**p-Coumaric acid**	**Ferulic acid**	**Cinnamic acid**
Fusarium verticillioides	73^{a}±0.11	87^{b}±1.04	78^{a}±0.25	69^{a}±0.11	74^{a}±1.03	89^{b}±0.16	78^{a}±0.34
Fusarium solani	78^{b}±1.03	87^{a}±1.06	79^{ab}±1.04	73^{a}±0.16	68^{a}±0.34	86^{a}±1.04	79^{a}±1.03
Aspergillus niger	70^{b}±0.16	81^{b}±0.16	78^{ab}±0.34	71^{ab}±0.24	68^{a}±0.34	79^{a}±1.04	71^{a}±0.16
Aspergillus flavus	76^{b}±0.25	88^{b}±0.11	73^{b}±0.11	70^{a}±0.34	69^{ab}±0.16	89b±1.03	79^{ab}±0.25
Aspergillus versicolor	79^{a}±0.31	78^{b}±1.03	78^{b}±0.16	67^{b}±1.04	74^{b}±1.03	85^{b}±1.03	71^{a}±1.03
Alternaria alternata	73^{a}±1.04	79^{ab}±0.25	72^{a}±0.11	69^{b}±1.04	73^{a}±0.11	84^{ab}±0.34	75^{a}±1.03
Macrophomina phaseolina	77^{a}±0.31	76^{b}±0.16	77^{b}±1.03	70b±1.03	71^{ab}±0.25	81^{ab}±1.06	73^{b}±0.16
Epicoccum nigrum	78^{ab}±1.04	82^{b}±1.04	73^{a}±1.03	72^{b}±1.03	69^{a}±0.11	83^{b}±0.11	73^{b}±0.11

Results show the percentages of inhibition of radial growth of the isolated mycoflora of cereals by the separated phenolic components from the ethanol extract *Seriphidium herba-album* plant, which was tested *in vitro* using the poisoned food technique. Values are the mean of three replicates, ± standard deviation. The value followed by different superscript letters (a, b, c, ….) indicates that means are not significantly different at P ≤ 0.01 when subjected to analysis of variance (ANOVA). Pattern of percentage inhibition increase is not uniform for all the microorganisms.

Table 5 (a): *In vivo* estimation of aflatoxin (AFB1) and fumonisin (FB1) mycotoxins levels in corn seeds co-inoculated separately with *Aspergillus flavus*, *Fusarium verticillioides* isolates and methanol, ethanol extracts of *Astrachantha echinus* plant

Mycotoxins	***Astrachantha echinus* plant**						**Fungicide (µg/ml)**	**Positive control**
	Methanolic extract (µg/ml)			**Ethanolic extract (µg/ml)**				
	1.6	**3.2**	**4.8**	**1.6**	**3.2**	**4.8**	**4.8**	
AFB1 (ng/g of corn)	3^{a}±2.03	2.3^{a}±1.87	1.97^{a}±1.23	3.3^{a}±2.2	2.9^{a}±0.78	2.4^{a}±0.01	2.1^{a}±0.5	9.2^{a}±0.02
FB1(µg/kg of corn)	231^{b}±2.1	211^{b}±0.89	179^{b}±1.11	254^{b}±0.01	209^{b}±0.46	189^{b}±1.06	182^{b}±2.0	456^{b}±1.37

Results show the decrease in aflatoxin B1 and fumonisin B1 mycotoxins levels which was estimated *in vivo* on corn grains co-inoculated separately with the methanol, ethanol extracts of *Astrachantha echinus* plant together with the mycotoxigenic isolates of *A. flavus* and *F. verticillioides*, using HPLC. Values are the mean of six replicates, ± standard deviation. The value followed by different superscript letters (a, b, c, ….) indicates that means are not significantly different at P ≤ 0.01 when subjected to analysis of variance (ANOVA). Where; AFB1: aflatoxin B1, FB1: fumonisin B1, Fungicide: Topsin-m (70% a. p.), Positive Control: corn inoculated separately with mycotoxigenic isolates of *A. flavus* and *F. verticillioides* only.

***In vivo* antimycotoxigenic activities of *Astrachantha echinus* and *Seriphidium herba-album* plants extracts**

Methanolic, ethanolic extracts of *Astrachantha echinus* and *Seriphidium herba-album* plants showed high *in vivo* antimyco toxigenic activity against toxigenic *A. flavus* and *F. verticillioides*, as they significantly reduced the aflatoxin (AFB1) and fumonisin (FB1) levels at all tested concentrations, respectively, compared with the positive control and fungicide treated corn grains (tables 5a, b). The methanolic extract of *Astrachanthaechinus* was very effective at concentration of 4.8 µg/ml, it reduced the AFB1 level in corn grains to (1.97 ng/g of corn) even below that of the synthetic fungicide (2.1

ng/g of corn), and that of FB1 to (1.79 µg/kg of corn) compared with the fungicide (182 µg/kg of corn). The AFB1, FB1 production levels were recorded to decrease with increasing the corresponding concentrations of these two plants extracts.

Table 5 (b): *In vivo* estimation of aflatoxin (AFB1) and fumonisin (FB1) mycotoxins levels in corn seeds co-inoculated separately with *Aspergillus flavus*, *Fusarium verticillioides* isolates and methanolic, ethanolic extracts of *Seriphidium herba-album* plant

Mycotoxins	*Seriphidium herba-album* plant						Fungicide (µg/ml)	Positive Control
	Methanolic extract (µg/ml)			Ethanolic extract (µg/ml)				
	1.6	3.2	4.8	1.6	3.2	4.8	4.8	
AFB1 (ng/g of corn)	3.3^{a}±2.11	2.66^{a}±1.78	2.2^{a}±2.88	3.22^{a}±0.97	2.89^{a}±1.46	2.4^{a}±0.20	2.1^{a}±0.53	9.2^{a}±0.02
FB1 (µg/kg of corn)	251^{b}±0.21	201^{b}±2.97	184^{b}±3.11	253^{b}±1.89	221^{b}±2.34	187^{b}±1.67	182^{b}±2.0	456^{b}±1.37

Results show the decrease in aflatoxin B1 and fumonisin B1 mycotoxins levels which was estimated *in vivo* on corn grains co-inoculated separately with the methanol, ethanol extracts of *Seriphidium herba-album* plant together with the mycotoxigenic isolates of *A. flavus* and *F. verticillioides*, using HPLC. Values are the mean of six replicates, ± standard deviation. The value followed by different superscript letters (a, b, c….) indicates that means are not significantly different at P ≤ 0.01 when subjected to analysis of variance (ANOVA). Where; AFB1: aflatoxin B1, FB1: fumonisin B1, Fungicide: Topsin-m (70 % a. p.), Positive Control: corn inoculated separately with mycotoxigenic isolates of *A. flavus* and *F. verticillioides* only.

DISCUSSION

Isolation of the seed borne mycoflora from stored cereal grains led to the recovery of several fungi with high percent incidences, well known of causing cereal diseases such as *F. solani*, *Alternaria alternata* and *Macrophomina phaseolina* (causing root and foot rot, black point, charcoal rot, respectively) and/or producing mycotoxins such as *F. verticillioides, A. niger* and *A. flavus* known of producing Fumonisins, Ochratoxins and Aflatoxins, respectively, that can adversely affect human and animal health if they enter the food chain.

In accordance [33-35]. Extracts of *Peganum harmala*, *Diplotaxis acris* and *Tamarixaphylla* showed less *in vitro* antifungal potency against the mycoflora compared with those of *Astrachantha echinus* and *Seriphidium herba-album,* this may be attributed to their low level of active antifungal components such as polyphenols and flavonoids thus the mycoflora were less sensitive to them, and/or due to their low solubility in water and in the other extracting solvents. The tested mycoflora had different levels of sensitivity to all extracts ranging from 2% to complete inhibition.

A. versicolor, *Alternaria alternata*, *Macrophomina phaseolina* and *Epicoccum nigrum* were highly sensitive to all extracts of the five plants especially to those of methanolic, ethanolic and chloroform solvents. However, *F. verticillioides*, *F. solani*, *A. flavus* and *A. niger* showed moderate resistance to these extracts. The basis of these varying degrees of sensitivity might be due to the intrinsic tolerance of these microorganisms, in addition to the nature and combinations of phytocompounds present in these crude extracts [36].

In previous studies, various secondary metabolites have been isolated from *Seriphidium herba-album* extracts such as the sesquiterpene; flavonoids; essential oils, *Peganum harmala* extract showed beta-carboline alkaloids such as harmalol; harmaline and harmine. In addition, methanolic extracts of *Tamarixaphylla* showed presence of tannin; phenolic compounds; anthraquinones; saponins; alkaloids; flavonoids and terpenoid s [37-41]. These compounds are associated with defense mechanisms of plants by their repellent or attractive properties [42]. The antimicrobial components of these plant extracts cross the fungal cell membrane interacting with its enzymes and proteins, so producing a flux of protons towards the cell exterior which induces change in the cell and ultimately their death [43, 44].

Extracts of *Astrachantha echinus* and *Seriphidium herba-album* plants recorded high promising *in vitro* antifungal potency at all tested concentrations as they suppressed and/or completely inhibited (100%) the radial growth of most of the mycoflora especially at 4.8 µg/ml, lower concentrations (0.8, 1.6 and 3.2 µg/ml) showed moderate activities, difference between replicates were not significant(P ≤ 0.01) . Most of the current plant extracts were more effective than the reference synthetic fungicide Topsin-m (70% a. p.), as they suppressed and/or completely inhibited radial growth of all mycoflora on PDA medium at low concentrations of 0.8-4.8 µg/ml, however, a huge concentration reaching to 25 µg/ml of this effective fungicide was required to attain the same effect, means were not significant (P ≤ 0.01).

The MIC of the developed extracts was very low ranging from 0.1-0.8 µg/ml compared with that of the synthetic fungicide (25µg/ml), these results demonstrates their high and effective *in vitro* antifungal potency. Results of the current study showed that, methanol and ethanol solvents based extracts had better antifungal potency than aqueous and chloroform ones. Separation of the selected methanolic, ethanolic extracts of *Astrachantha echinus* and *Seriphidium herba-album* using TLC and then characterization of the obtained bands using HPLC led to the recovery of 7 phenolic components with different concentrations. All the separated phenolic components showed high *in vitro* antifungal potency (68-89%) against the tested mycoflora at 4.8 µg/ml, especially those of Gallic acid, p-Coumaric acid and Ferulic acid.

In addition, these selected extracts of *Astrachantha echinus* and *Seriphidium herba-album* plants showed effective *in vivo* anti-aflatoxigenic (AFB1) and anti-fumonisin (FB 1) activities in inoculated corn grains compared with the toxigenic isolates of *A. flavus* and *F. verticillioides*, respectively, and with the synthetic fungicide (means of the 6 replicates were not significant, P ≤ 0.01). Methanolic extract of *Astrachantha echinus* was more efficient in terms of *in vivo* decrease of mycotoxins levels in inoculated corn (AFB1= 1.97 ng/g and FB1=1.97 µg/kg) compared with the synthetic fungicide (2.1 ng/g, 182 µg/kg, respectively) at concentration of 4.8µg/ml. These observed results indicated that the efficient *in vitro* antifungal and *in vivo* antimycotoxigenic activities of these two selected plants extracts were due to the vital role of high concentrations of different phenols detected in their methanolic and ethanolic extracts, especially those of Gallic acid, p-Coumaric acid and Ferulic acid, similar results were obtained in previous studies [45-47].

Whether these phenolic principles acted singly or synergistically they might prevent and/or decreased the growth of the toxigenic fungi by disturbing the ir respiratory chains, denature enzymes and proteins within the fungal cells; in addition, they might inhibit the biosynthetic pathways of both aflatoxins and fumonisins in these mycotoxigenic fungi. In accordance, high antifungal and anti-aflatoxigenic activities due to polyphenolic compounds such as Gallic acid were recorded in methanolic extracts of *Zingiber officinalis* and *Punica granatum* plants [48], and in methanolic extract of *D. gnidium* [49]. Presence and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity [50]. In accordance, Gallic acid, p-Coumaric acid and Ferulic acid detected in the current extracts are characterized by high hydroxylation.

For the best of our knowledge, this is the first record of potent broad spectrum *in vitro* antifungal and *in vivo* antimycotoxigenic activities of *Astrachantha echinus* plant extracts. The significances of the current work are that the novel and promising methanolic, ethanolic extracts of *Astrachantha echinus* and *Seriphidium herba-album* plants could be formulated in the future, to be used as worldwide economic and ecofriendly phytofungicides to prevent fungal biodeterioration of cereals; preventing and/or decreasing their mycotoxins contents to an acceptable level; increasing their shelf

life, displacing the use of health hazard chemical fungicides and refrigeration of cereals in store.

CONCLUSION

Finally, we concluded that the methanol, ethanol extracts of *Astrachantha echinus* and *Seriphidium herba-album* plants could be used as efficient, ecofriendly and biodegradable phyto fungicides, to prevent biodeterioration; mycotoxins production and/or decreasing them to an acceptable level in cereals, displace the use of health hazard chemical fungicides and refrigeration of cereals during storage.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest

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