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Full Length Research Paper

Antifungal and antiaflatoxigenic activities of some plant extracts

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The efficiency of five methanolic plant extracts obtained from *Euclayptus globolus, Olea europaea*, *Salvadora persica, Thymus vulgaris* and *Ziziphus spina-christi* were investigated for their inhibitory effect on the growth and aflatoxin B_1 production of the toxigenic *Aspergillus flavus* strain isolated from wheat seeds. Extract of *T. vulgaris* showed the highest antifungal and antiaflatoxigenic activities as aflatoxin B_1 production was absolutely arrested at 5 mg/ml, while the mycelial growth of *A. flavus* was completely inhibited at 15 mg/ml. *E. globolus* and *O. europaea* extracts showed a moderate antifungal activities and exhibited a significant antiaflatoxigenic efficiency as they inhibited aflatoxin B_1 production at 15 mg/ml. On the other hand, *S. persica* showed weak antifungal activities while no effect was detected with *Z. spina-christi* extract. The analysis of plant extracts by GC/MS revealed that *T. vulgaris* extract was mainly composed by thymol (38.73%), carvacrol (19.31%) and ρ-cimene (10.13%), *O. europaea* extract was composed by anthracenedione (20.63%), terbutaline (13.96%) and propiolic acid (12.02%) while eucalyptol (30.62%), globulol (18.94%) and silane (9.13%) were the principal components of *E. globolus* extract. These extracts of *T. vulgaris*, *E. globolus* and *O. europaea* which are effective and environmentally safe are promising for protecting wheat seeds against aflatoxigenic strain of *A. flavus*.

Key words: Aflatoxins, wheat, antiaflatoxigenic, methanolic extracts, *Euclayptus globolus, Olea europaea, Salvadora persica, Thymus vulgaris.*

INTRODUCTION

Aflatoxins are carcinogenic and immunosuppressive metabolites produced by *Aspergilllus flavus* and *Aspergilllus parasiticus* when these fungi infect crops before and after harvest thereby contaminating food and feed and threatening both human and animal health (Jonsyn-Ellis, 2001; Omidbeygi et al., 2007). These toxins have been incriminated as the cause of high mortality in livestock and some cases of death in human being (Mori et al., 1998; Egal et al., 2005). Among all classes of aflatoxins, aflatoxin B_1 is known to be the most significant in terms of animal and human health risk (Coulombe, 1993). Thus, foods contaminated with these toxigenic fungi and presence of aflatoxins is a major concern which has received world wide attention due to their deleterious effect on human and animal health as well as their importance in international food trade (Mishra and Das, 2003; Soubra et al., 2009).

Currently, there is a strong debate about the safety aspects of fungicides in use since they are considered responsible for many carcinogenic and teratogenic attributes as well as residual toxicity (Prabhu and Urs, 1998). For these reasons, the use of numerous plant extracts, spices and their constituents may provide an alternative way to prevent fungal growth and mycotoxins formation (Holley and Patel, 2005; Lee et al., 2001, 2007). Many spices and plant extracts have proven to be efficient antifungal against toxigenic moulds (Rasooli and Owlia, 2005). Bluma and Etcheverry (2008) demonstrated antifungal and antiaflatoxigenic properties

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Table 1. The ethnobotanical data, plant parts employed and extract percentage yield of plant species.

Plant species	Family	Common name	Plant part used[@]	Extract yield (%)
Euclayptus globolus	Myrtaceae	Eucalyptus	L	8.93
Olea europaea	Oleaceae	Olive	L	13.94
Salvadora persica	Salvadoraceae	Arak	B	10.72
Thymus vulgaris	Lamiaceae	Thyme	L	11.05
Ziziphus spina-christi	Rhamnaceae	Seder	L	9.43

[@]Plant parts used: L, leaves; B, bark.

of some essential oils including *Thymus vulgaris*, *Cymbopogon citrates, Ocimum gratissimum* and *Pimpinella anisum* against *A. flavus*. They reported that *T. vulgaris* completely inhibited AFB_1 at 3000 $\mu g g^{-1}$. On the other hand, Kumar et al. (2008) and Razzaghi-Abyaneh et al. (2009) reported that *Euclayptus citriodora*, *Euclayptus camaldulensis* and *T. vulgaris* significantly inhibited mycelial growth and aflatoxins production by the toxigenic fungi. In addition, many species and herbs such as basil, cinnamon, marigold, arak and spearmint (Ahmed et al., 2008; Soliman and Badeaa, 2002), thyme (Rasooli et al., 2006; Pinto et al., 2006), cassia and sweat basil (Atanda et al., 2007) have been reported to inhibit toxigenic and food borne moulds. In the present study, the effect of five kinds of plant extracts including *Euclayptus globolus, Olea europaea*, *Salvadora persica, Thymus vulgaris* and *Ziziphus spina-christi* on growth and aflatoxin B_1 production of *A. flavus* are evaluated.

MATERIALS AND METHODS

Fungal isolation

Wheat seeds were disinfected using 2% sodium hypochlorite for three minutes, rinsed three times in sterile distilled water and dried between layers of sterile filter paper (Whatmann, No. 1). Ten groups of wheat seeds, each with five seeds were plated out on 15 ml of potato dextrose agar medium (PDA) (5 seeds / plate) and incubated at 25°C for 5 days. Subculturing was repeated several times for mycelial tips to obtain pure cultures which were preserved on PDA slant till identified.

Fungal strain

The toxigenic isolate *A. flavus* was isolated from seeds of *Triticum* spp. and identified on the basis of colony and morphological characteristics (Raper and Fennell, 1977). The toxigenicity of the isolates was determined using coconut milk agar medium (Pallavi et al., 1997) and selected for detailed study. The culture of toxigenic *A. flavus* strain was maintained on potato dextrose agar slant in the laboratory of Botany and Microbiology Dept. Collage of Science.

Preparation of plant extraction

Plant materials of five plant species belonging to five botanical families (Table 1) included in this study were collected, washed with tap water, disinfected by immersion in 2% sodium hypochlorite solution for 30 min, rinsed with sterile distilled water to eliminate residual hypochlorite and dried in shade. The shade-dried material of each plant species was grounded into a powdered material using a blender to pass 100 mm sieve and the mince was sealed in polyethylene bags until extraction. For preparation of methanolic extracts, 50 g of dry powder plant material from each plant species was soaked in methanol (10 ml of methanol /g of plant material) with stirring for 48 h then filtered through double layers of muslin, centrifuged at 9000 rpm for 10 min and finally filtered again through Whatman filter paper No. (41) to remove leaf debris and obtain a clear filtrate. The filtrates were evaporated and dried under reduced pressure and temperature below 40°C.

Antifungal screening test of plant extracts

Antifungal activity was evaluated on the toxigenic *A. flavus* strain using Czapek dox broth medium (sucrose, 30 g; sodium nitrate, 3 g; dipotassium phosphate, 0.5 g; magnesium sulfate, 0.5 g; potassium chloride, 0.5 g; ferrous sulfate, 0.01 g; distilled water, 1000 ml; pH, 6.5). The plant extract residues were re-dissolved in 5 ml of Czapek broth, sterilized in disposable Millipore filter (0.22 µm pores) and mixed with 45 ml of sterile Czapek broth in 150 ml Erlenmyer flasks to obtain final concentration of 10 mg/ ml of each plant extract. The control set was kept in parallel to the treatment sets without plant extracts. The flasks were inoculated with discs of 6 mm diameter of the toxigenic *A. flavus* isolate and incubated at 25 ± 2°C for 7 days. After incubation, content of each flask was filtered (Whatman No. 1) and biomass of the filtered mycelium was determined after drying at 70°C for 4 days till their weights remains constant. The percentage of mycelial inhibition was calculated (Table 2) using the following formula.

Percentage of mycelial inhibition = [C – T / C] x 100

(Where, C and T are the mycelial dry weight (mg) in control and treatment respectively).

Antifungal and aflatoxins inhibition assay

The most effective plant extracts including *T. vulgaris*, *E. globolus, O. europaea* and *S. persica* were used to determine minimal inhibitory concentration (MIC), minimal fungicidal concentration (MFC) and their anti-aflatoxigenic efficacy using yeast extract sucrose broth (YES) medium supplemented with 0.8% sodium chloride (Ezzat and Sarhan, 1991). Different concentrations of each plant extract (5, 10, 15, 20 and 25 mg/ml) were prepared separately by dissolving their requisite amount in 10 ml of YES broth, sterilized through Millipore filter and mixed with 40 ml of sterile YES broth in 150 ml Erlenmeyer flasks. Three replicates were performed for each concentration and the control set was kept parallel to the treatment without plant extract. The flasks were inoculated with discs of 6 mm diameter of the toxigenic *A. flavus* and incubated at 25 ± 2°C for 7 days. After incubation, content of each flask was

Table 2. Antifungal screening of some plant extracts (10 mg/ml) against toxigenic *A. flavus* isolate.

Plant species	Mycelial dry weight (gm)	Percentage of mycelial growth inhibition
Euclayptus globolus	0.226* ± 0.008	65.23
Olea europaea	0.255* ± 0.001	60.77
Salvadora persica	0.384* ± 0.005	40.92
Thymus vulgaris	0.121* ± 0.002	81.39
Ziziphus spina-christi	0.726* ± 0.014	00.00
Control	0.650* ± 0.005	00.00

Values in the same column followed by asterisk (*) are significantly different at (P = 0.05), data are means (n = 3) ± standard error of three replicates.

filtered (Whatman, No. 1) and biomass of filtered mycelium was dried at 70°C for 4 days till their weights remains constant. Mycelial dry weights of treatments and control was determined then MIC and MFC were calculated for each plant extract.

For aflatoxins extractions, the filtrates of each flask was treated three times with 50 ml of chloroform in a separating funnel. The chloroform extract was separated and dehydrated with anhydrous sodium sulfate and evaporated till dryness on water bath at 50°C under vacuum. The residues were dissolved in 1 ml chloroform and 50 µl of chloroform extract spotted on thin layer chromatography (TLC) plates (20 x 20 cm^2) coated with 0.25 mm thick silica gel (Alugram, Germany). The plates were developed with benzene-methanol- acetic acid (95: 5: 5 v/v/v) solvent system described by (AOAC, 1995) and aflatoxin B_1 was detected by visual examination of TLC plates under UV lamp at 365 nm and comparison of the fluorescent band with that of the standard aflatoxin. The presence of aflatoxin $B_1$was confirmed chemically by spraying trifluoroacetic acid (Bankole and Joda, 2004). Quantification of aflatoxin B_1 was done by photodensitometry (Bio-metera-Germany) comparing the area and density of the spot samples with aflatoxin B_1 standard (Supelco - USA). Aflatoxin B_1 content was expressed in terms of μgl^{-1} and aflatoxin inhibition was calculated as follow; percentage of inhibition = [Y – X / Y] x 100, where X" is the concentration of aflatoxin in treated sample and Y" is the concentration of aflatoxin in control.

GC/GC-MS analysis of the effective plant extracts

The effective plant extracts of *E. globolus*, *O. europaea* and *T. vulgaris* were analyzed through gas chromatography and mass spectroscopy (GC-MS) Varian model, 450 equipped with a flame ionization detector and quantization was carried out by the area normalization method neglecting response factors. The analysis was carried out using a VF-5MS capillary column (30 m x 0.25 mm; 0.25 µm film thickness). The operating conditions were as follow: injection and detector temperature, 250 and 300°C respectively; split ratio, 1 : 50; carrier gas, Helium with flow rate (1.0 ml/min). Oven temperature program was 50 to 300°C at the rate of 7°C/min. Mass spectrometer conditions were: ionization potential, 70 eV; mass range from m/z, 40 to 400 amu; electron multiplier energy, 2000 V. The components of plant extracts were identified by comparison of their relative retention times and the mass spectra with those authentic reference compound shown in the literature (Adams, 2007) and by computer matching of their MS spectra with Wiley and Nist 8 mass spectral library.

Statistical analysis

All measurements were replicated three times for each treatment and the data were reported as mean ± SE (standard error). The data were also analyzed statistically using One-way analysis of variance (ANOVA) and differences among the means were determined for significance at P ≤ 0.05 using Duncan's multiple range test (by SPSS, 16.1 Chicago, USA).

RESULTS

The ethnobotanical data (botanical and local name) of the plants used in this study and the extract percentage yield of the selected plant species are demonstrated in Table 1. A total number of 5 plants from five different families were studied regard to their antifungal and antiaflatoxigenic activities against the toxigenic *A. flavus* isolate. Among the five methanolic plant extracts screened for their antifungal activity, *T. vulgaris* showed the highest antifungal activity at 10 mg/ml while the extract of *E. globolus* and *O. europaea* showed a moderate antifungal activity against the toxigenic *A. flavus* isolate. On the other hand, the aqueous extracts of *S. persica* showed a weak antifungal activity and no effect was detected with *Z. spina-christi* (Table 2). Hence, the methanolic extracts of *T. vulgaris*, *E. globolus*, *O. europaea* and *S. persica* were selected for further investigation.

The MIC and MFC were employed by poisoned food technique to assess fungistatic and fungicidal properties of the effective plant extract. As illustrated in Figure 1, the inhibitory plant extracts show various capabilities to suppress *A. flavus* grown on broth medium. Although, the inhibitory effect of the plant extracts increased in proportion to their concentrations and reached to a maximum in the final concentration of 20 mg/ml, the extract of *T. vulgaris* showed absolute growth inhibition at concentration 15 mg/ml followed by *E. globolus* and *O. europaea* which inhibited mycelial growth with (96.46%) and (91.68%) at 20 mg/ml respectively while *S. persica* extract need more than 25 mg/ml to suppress fungal mycelial growth. This inhibition was reported to be significant for all plant extracts at the level of 0.05 (ANOVA). *T. vulgaris* were strongly active at MIC of 10 mg/ml and at MFC of 15 mg/ml while *E. globolus* and *O. europaea* showed fungistatic activity against the toxigenic

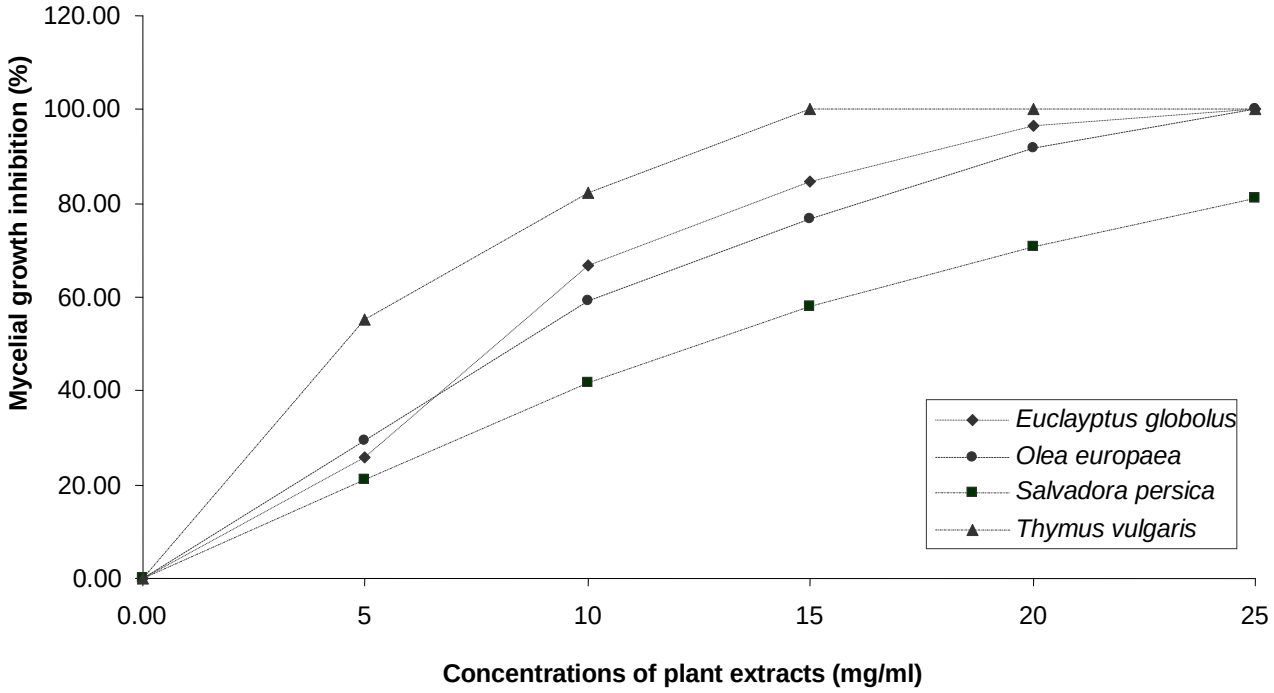

Figure 1. Percentage of mycelial growth inhibition of the toxigenic *A. flavus* isolate by various concentrations of plant extracts.

A. flavus with MIC of 15 mg/ml and MFC of 20 mg/ml. None of the tested plant extracts were able to suppress fungal growth at concentration of 5 mg/ml as shown in the Figure 1. In addition, extract of *T. vulgaris* was found efficient to arrest the aflatoxin B_1 produced by *A. flavus* and completely inhibited at 5 mg/ml (Table 3).

A direct correlation was found between fungal growth and AFB_1 production that is a significant decrease in mycelial biomass resulted low AFB_1 production and vice versa. As a fungitoxicant, *T. vulgaris* extract was evaluated to be more efficacious fungitoxicant than the other plant extracts. The extract of *T. vulgaris* completely inhibited AFB_1 production by reduction mycelial growth with (55.64%) at concentration 5 mg/ml while the extracts of *E. globolus* and *O. europaea* inhibited AFB_1 production by (82.60%) and (70.84%) with biomass reduction of (66.62%) and (59.2%) at a higher concentration 10 mg/ml. Although, *O. europaea* and *E. globolus* showed a moderate antifungal activities, they exhibited a significant antiaflatoxienic efficiency as they completely inhibited aflatoxin B_1 production at 15 mg/ml

The main constituents of the effective plant extracts that is *E. globolus, O. europaea* and *T. vulgaris* identified by GC-MS spectrometer analysis are summarized in Table 4 according to their retention indices (RI) and percentage composition. Euclayptol (cineol) (30.62%), globulol (18.94%), silane (9.13%) and valencene (7.78%) were the principal components of *E. globolus* extract. *O. europaea* extract was composed by anthracenedione (20.63%), terbutaline (13.96%) and propiolic acid (12.02%) while *T. vulgaris* extract was mainly composed by thymol (38.73%), carvacrol (19.31%) and ρ-cimene (10.13%), respectively.

DISCUSSION

Aflatoxins are known to be potent hepatocarcinogens in animals and human. Therefore, the presence of toxigenic fungi and mycotoxins in foods and grains stored for long periods of time presents a potential hazard to human and animal health. In the present study, a toxigenic strain of *A. flavus* isolated from wheat seeds was chosen because of its toxigenic productivity and its strong affinity to colonize wheat seeds and various food commodities render them unfit for human consumption (Bandyopadhyay et al., 2007). Adverse effects of chemical pesticides on environment and human health are burning issues and there is a need to search for a new fungicides with improved performance as well as eco-friendly in nature. Hence, some extracts of ethnomedicinally important higher plant species were tested for their antifungal activity. Such plant products would be biodegradable and safe to human health (Mohanlall and Odhav, 2006). In continuing of the studies on antifungal and aflatoxins inhibitors, a number of 5 plant species from five different families were evaluated for their antifungal effects against aflatoxin producing *A. flavus*. The methanolic extracts from *T. vulgaris* was effectively suppressed fungal growth followed by *E. globolus* and *O. europaea*. These results are in accordance with that of Kumar et al. (2008) who reported

Table 3. Antiaflatoxigenic efficacy of some plant extracts.

Plant extracts	Concentration (mg/ml)	Biomass (g)	R_f	Aflatoxin B_1 content ($\mu g l^{-1}$)	% Inhibition of aflatoxin B_1
Eucalyptus globolus	0.00	0.707* ± 0.020	1.96	384.60	---
	5.00	0.523* ± 0.012	1.98	162.30	57.80
	10.0	0.236* ± 0.008	1.95	66.80	82.60
	15.0	0.109* ± 0.001	---	00.00	100.0
	20.0	0.025* ± 0.001	---	00.00	100.0
	25.0	0.000 ± 0.000	---	00.00	100.0
Olea europea	0.00	0.745* ± 0.022	1.97	386.20	---
	5.00	0.527* ± 0.014	1.94	225.4	41.67
	10.0	0.304* ± 0.002	1.96	112.6	70.84
	15.0	0.173* ± 0.002	---	0.000	100.0
	20.0	0.062* ± 0.001	---	0.000	100.0
	25.0	0.000 ± 0.000	---	0.000	100.0
Salvadora perisca	0.00	0.693* ± 0.028	1.98	384.60	---
	5.00	0.548* ± 0.003	1.95	252.80	34.27
	10.0	0.405* ± 0.002	1.96	168.40	56.21
	15.0	0.292* ± 0.004	1.98	117.80	69.37
	20.0	0.202* ± 0.005	1.97	72.60	81.12
	25.0	0.131* ± 0.002	---	0.00	100.0
Thymus vulgaris	0.00	0.701* ± 0.009	1.98	381.60	---
	5.00	0.311* ± 0.002	---	00.00	100.0
	10.0	0.124* ± 0.001	---	00.00	100.0
	15.0	0.000 ± 0.000	---	00.00	100.0
	20.0	0.000 ± 0.000	---	00.00	100.0
	25.0	0.000 ± 0.000	---	00.00	100.0

Values in the same column followed by asterisk (*) are significantly different at (P = 0.05), Data are means (n = 3) ± standard error of three replicates.

that *T. vulgaris* essential oil is absolutely inhibited mycelial growth of *A. flavus* at 0.7 µl/ml. On the contrary, no inhibitory effect was detected with *Z. spina-christi* even at higher concentration more than 25 mg/ml. A variation in fungitoxicity of the concerned plant extracts against the toxigenic *A. flavus* may be due to considerable variation in their constituents (Cavaleiro et al., 2006). The study of MIC of the fungitoxicants is necessary to find out their minimum amount to check the pest population and it would be helpful in saving unnecessary wastage of the pesticides. The MIC of *T. vulgaris* extract was comparatively lower than some earlier reported extracts that is *E. globolus* and *O. europaea* (Kumar et al., 2008).

In addition, *T. vulgaris* extract showed marked efficacy in arresting aflatoxin B_1 production by the toxigenic *A. flavus* isolate. The mycelial growth and AFB_1 production were recorded to decrease on increasing the concentrations of the plant extracts. It is evident from Table 3 that the extract of *T. vulgaris* was found to be more potential as antifungal and antitoxicant than *E. globolus* extract. It was found that at 5 mg/ml of *T. vulgaris* extract, mycelial growth was inhibited to (55.64%) and AFB_1 production was completely checked while mycelial growth and aflatoxin B_1 production were inhibited to (26.03%) and (57.8%) with *E. globolus* extract at the same concentration. Hence, *T. vulgaris* extract is more efficient as aflatoxin-inhibitor. Generally, in order to reduce the aflatoxins production, mycelial growth must be below the threshold limit so that aflatoxins could not be produced. Compositional analysis of *T. vulgaris* extract showed that phenolic compounds as thymol, carvacrol and ρ-cimene present in *T. vulgaris* extract as well as other phenolic compounds present in *E. globolus* play the vital role in growth and aflatoxin inhibition. These phenolic compounds inhibited one or more early rather than late steps in the AFB_1 biosynthesis pathway. Some researcher attributed the inhibitory effect of these plant extracts to hydrophobicity characters of these plant extracts and their components. This enables them to

Table 4. Phytochemical composition of the most effective plant extracts and their relative contents (%).

Compound	Plant extracts					
	Eucalyptus globolus		*Olea europaea*		*Thymus vulgaris*	
	RI	%	RI	%	RI	%
α-Phellandrene	10.570	1.52	----	----	----	----
Eucalyptol (Cineol)	12.174	30.62	----	----	----	----
Isopinocarveol	15.295	2.26	----	----	----	----
Cis-carveol	16.274	2.04	----	----	----	----
4-Terpineol	16.331	1.15	----	----	----	----
α-Terpinol	16.699	3.43	----	----	----	----
Silane	19.077	9.13	----	----	----	----
Valencene	22.420	7.78	----	----	----	----
Aromadendrene	22.888	3.59	----	----	----	----
Epiglobulol	24.980	3.12	----	----	----	----
Globulol	25.469	18.94	----	----	----	----
Isolonaifolol acetate	25.647	5.28	----	----	----	----
β-Eudesmol	26.209	4.30	----	----	----	----
β-Guaiene	26.684	1.23	----	----	----	----
δ-Selinene	26.760	1.46	----	----	----	----
3-Hexadecyne	29.833	4.13	----	----	----	----
2,3 Dihydroxypropanal	----	----	5.458	3.73	----	----
1,3 Dihydroxypropanone	----	----	7.511	2.29	----	----
Octamethylcyclotetrasiloxane	----	----	10.182	3.47	----	----
Decamethylcyclopentasiloxane	----	----	14.035	5.38	----	----
Methyl coumalate	----	----	17.529	2.63	----	----
9,10-Anthracenedione	----	----	17.612	20.63	----	----
4-Vinylguaiacol	----	----	18.033	2.59	----	----
4-Carbomethoxybenzaldehyde	----	----	19.161	13.56	----	----
Tyrosol	----	----	20.164	3.20	----	----
Terbutaline	----	----	23.517	13.96	----	----
α-Lumicolchicine	----	----	23.521	3.15	----	----
Phenylthioethanol	----	----	24.089	7.59	----	----
Propiolic acid	----	----	25.419	12.02	----	----
Octasiloxane	----	----	28.288	5.82	----	----
α-Thujane	----	----	----	----	8.135	2.31
α-Terpinolene	----	----	----	----	8.631	5.94
β-Olimene	----	----	----	----	9.742	4.73
Bornyl acetate	----	----	----	----	11.020	6.12
Piperitinene oxide	----	----	----	----	11.631	2.13
α-Gurjunene	----	----	----	----	12.473	1.72
ρ-Cimene	----	----	----	----	13.027	10.13
Borneol	----	----	----	----	15.321	3.27
Carvacol	----	----	----	----	21.173	0.90
Thymol	----	----	----	----	22.430	38.73
Carvacrol	----	----	----	----	23.251	19.31
Bisabolene	----	----	----	----	26.651	3.71

RI: Retention time.

partition in the lipids of the fungal cell wall membrane and mitochondria, disturbing their structure and rendering them more permeable. Leaking of ions and other cell contents can then occur (Burt, 2004). Other researchers have suggested that antimicrobial components of the essential oils present in the plant extracts cross the cell

membrane interacting with the enzymes and proteins of the membrane, so producing a flux of protons towards the cell exterior which induces change in the cell and ultimately their death “that is alter metabolic pathway” (Omidbeygi et al., 2007). The results of the present study revealed that some plant extracts have been emerged as safe alternatives to replace chemical fungicides and preservatives and can be used as eco-friendly fungicides. On the basis of present findings, extracts of *T. vulgaris*, *E. globolus* and *O. europaea* can be recommended as potentially effective and environmentally safer alternative fungicides to protect the spoilage of wheat seeds from the toxigenic *A. flavus* and they should find a practical application as eco-friendly fungicides.

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