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Original Research Article

Antifungal Activity of *Syzygium aromaticum* (Dianthus) against toxigenic *Rhizopus stolonifera* and its immunomodulatory effects in aflatoxin-fed mice

Gehan Ahmed Othman[1]*, Dalia Abd El Moneim Ahmed[1], Huda A Alghamdi[1], Asmaa Mahmoud Radwan[2]

[1]Biology Department, College of Science, Girls Branch, King Khalid University, Abha. Kingdom of Saudi Arabia, [2]Plant and Microbiology Department, Faculty of Science, Girls branch, Al Azhar University, Cairo, Egypt

For correspondence: **Email: Othman_gehan@yahoo.com*

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Abstract

Purpose: *To evaluate the antifungal potency of Syzygium aromaticum (Dianthus) seed extract against the growth and aflatoxin production of Rhizopus stolonifer, and its immunomodulatory effect.*
Methods: *Disc diffusion method was used for assay of antifungal effect of aqueous extract of Dianthus. Lymphoid cell counts, total and differential peritoneal exudate cell counts (PEC), phagocytic activity of PEC, and plaque-forming activities were determined. In addition, E-rosette-forming cells (RFC), T-cell mitogenesis cells and liver functions were measured.*
Results: *The aqueous extract of Dianthus (50 %) exhibited high inhibition zone against most isolates of R. stolonifera. It produced significant increases in the number of splenocytes, as well as in the absolute number and relative proportion of macrophages ($p < 0.05$). The extract also produced a gradual increase in the scavenging activity of PEC, and significant reduction in serum ALT, relative to control.*
Conclusion: *These results suggest that Dianthus modifies biological responses by enhancement of the immune system, activation of phagocytosis, boosting of immune response, and prevention of liver damage.*

Keywords: *Aflatoxin, Syzygium aromaticum, Rhizopus stolonifer, Lymphocytes, T-cells, B-cells, Macrophages*

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INTRODUCTION

It is now well established that fungi and their mycotoxins cause extensive pollution of human food and animal feeds, and so constitute serious health problems. Thus, it is important to address the safety problems associated with mycotoxins. Numerous reports exist in the literature on the adverse effects of fungi and their mycotoxins on the health and safety of consumers [1]. In addition to the unlimited economic losses resulting from the presence of moulds in food, mycotoxins constitute a serious public health problem [2]. Mycotoxins (especially aflatoxins) can be extremely harmful to animals through their hepatotoxic, nephrotoxic, immunotoxic,

carcinogenic and genotoxic properties [3].

Aflatoxins B1, B2, G1 and G2 are considered the most dangerous types of mycotoxins. Aflatoxin B1 is responsible for aflatoxicosis in different species of animals. It is a poisonous metabolite synthesized by *Aspergillus flavus* and *Aspergillus parasiticus,* and it exerts carcinogenic effects on the liver and kidney [4]. Studies have shown that aflatoxins exert deleterious effects on the reproductive systems of a wide spectrum of domestic animals [5].

Exposure to AFB1 caused abnormal levels of kidney oxidative stress biomarkers in rats, an indication of cellular damage [6]. A study has reported that aqueous extract of *Azadirachta indicia* bark ameliorated aflatoxin-induced damage to blood cells and hepatotoxicity in albino mice [7]. The antioxidant components of aqueous extract of rosemary reduced immune responses and oxidative disorders due to oxidants produced during experimental aflatoxinogenesis [8].

Dianthus caryophyllus contains triterpenes, alkaloids, coumarins and cyanogenic glycosides [9]. The major color-determining pigments of carnation flower are cyanidin, pelargonidin and isosalipurposide [10]. The present study was carried out to determine the antifungal potency of *Dianthus* seed extract against the growth and aflatoxin production of *Rhizopus stolonifer,* and its immunomodulatory effect on aflatoxin-fed mice.

EXPERIMENTAL

Materials

Syzygium aromaticum seeds were obtained from a local authorized market in Saudi Arabia. Petri dishes (100 x 15 mm), sterile blank paper disk (0.64 cm in diameter), cell/counting chamber, potato dextrose agar and microplates were used. Male Swiss albino mice (8 - 10 weeks old and weighing about 20 g each) were used in this study. They were maintained in a quiet room at 28 ^{o}C in the laboratory animal unit of College of Science, Helwan University, Cairo, Egypt. All the animal experiments were conducted in accordance with international guidelines [11].

Preparation of *Syzygium aromaticum* seeds extract

Syzygium aromatic was ground to fine powder and the extract was prepared by percolating the powder for 48 h, filtration and concentration using rotary evaporating [12].

Agar diffusion assay

Agar diffusion assay was carried out by the adaptation of the procedure outlined in a previous study [13].

Experimental design and treatment regime

Doses of 0.30, 0.40 and 0.50 g of *Dianthus* extract/100 g body weight were orally administered to aflatoxin- fed mice daily for two successive weeks. Mice in the control group were orally administered 0.2 ml of distilled water in place of extract.

Harvesting of peritoneal exudate cells (PEC)

Normal and treated groups were intraperitoneally injected with 2 ml of starch suspension, after which they were sacrificed and the peritoneal exudate cells (PEC) were obtained as described earlier [14].

Determination of lymphoid cell counts

Cell suspensions were prepared by squeezing the respective organs between two slides in Hank's balanced salt solution (HBSS), followed by filtration through a nylon sieve. The respective cell suspensions were washed many times, suspended in HBSS, and the cells were counted in a hemocytometer [14].

Carbon clearance assay

The phagocytic activity of PEC was determined using Pelikan special biological ink (Pelikan-Werke, Hannover, Germany).

Determination of plaque-forming cells (PFC)

Immune responses against SRBC were determined after one intraperitoneal injection of $1x10^{8}$ SRBC in 0.2 ml saline. After 5 days, normal and treated groups were sacrificed, and the spleens were excised and used for preparation of cell suspensions. The suspensions were plated onto a slide chamber and incubated for 30 - 45 min at 37 ^{o}C. Plaques were scored under microscope and calculated per million mononuclear cells [15].

Determination of E-rosette-forming cells (RFC)

Spleens from normal and treated animals were excised. Cell suspensions were prepared, washed twice by centrifugation at 200 g and suspended in HBSS at a concentration of 2×10^{6} cells, with shaking to suspend the cells in the

pellet. The rosettes were counted in hemocytometer [16].

Assay of T-cell mitogenesis

Normal and treated mice were sacrificed and the spleens were removed and dispersed gently. The splenocytes were then seeded into 96-well culture plates at a density of 1.5 x 10^5 splenocytes/well in RPMI-1640 medium supplemented with 5 % fetal calf serum, 50 µM β-mercaptoethanol and antibiotics. The dark blue crystals were dissolved by the addition of 150 µl of 0.04 M HCl/isopropanol, and the plates were read in a spectrophotometer [14].

Determination AST and ALT activities

Blood samples were collected and centrifuged at 3000 rpm for 30 min, and the serum samples were kept at -20 °C prior to assay of AST and ALT activities using assay kits (BIO ADWIC, Egypt) [17].

Statistical analysis

Results from the *in vivo* and *in vitro* studies are expressed as mean ± SD. All data were analyzed for statistical significance with Student's *t*-test, using SPSS ver. 14.0. Statistical significance was assumed at $p < 0.05$ and $p < 0.01$).

RESULTS

Antifungal activity of *S. aromaticum* (*Dianthus*) extract

The effect of 50 % aqueous extract of *Dianthus* on the growth of the different isolates of *R. stolonifer* is shown in Table 1. The aqueous extract exhibited strong antifungal activity against most of the isolates of *R. stolonifera*, with isolate numbers 1, 2, 4, 8 and 10 having the largest inhibition zones, especially isolate number 4. However, there were no statistically significant ($p < 0.05$) inhibition of isolates 3, 5, 6 and 7.

Table 1: Antifungal effect of 50 % aqueous extract of *Dianthus* against different isolates of *R. stolonifer* at pH 7.2 and 25 °C for 72 h.

Fungi strain	Diameter of inhibition zone (mm)
Isolate 1	19 ±1.1
Isolate 2	22 ±1.1
Isolate 3	-
Isolate 4	24 ±1.1
Isolate 5	-
Isolate 6	-
Isolate 7	20 ±1.1
Isolate 8	19 ±1.1
Isolate 9	-
Isolate 10	13 ±1.1

Effect of aqueous extract of *Dianthus* on lymphoid cell count

Table 2 shows that the aflatoxin exposure produced significant reductions in the number of leukocytes from thymus (Thy), spleen (Spl), peripheral lymph nodes (PLN) and mesenteric lymph nodes (MLN), when compared to the control group. However, daily treatment of the aflatoxin-fed mice with *Dianthus* (0.30, 0.40 or 0.50 g/100 g for 2 weeks) resulted in sharp increases in the number of cells from thymus and BM, relative to those of untreated aflatoxin-fed mice. Although treatment of the aflatoxin-fed mice with *Dianthus* did not have any significant effect on the number of cells from PLN or MLN, it led to significant increases in splenocytes at a dose 0.30 g ($p < 0.05$).

Table 2: Effect of *Dianthus* extract on number of leukocytes from thymus (Thy), spleen (Spl), peripheral lymph nodes (PLN), mesenteric lymph nodes (MLN) and bone marrow (BM)

Treatment	Total cell count/g tissue (mean±SD)				
	Thy x10^9	***Spl x10^9***	***PLN x 10^9***	***MLN x10^9***	***BM x10^6***
Normal	2.15 ± 0. 24	2.38 ± 0.26	1.37 ± 0.11	1.15 ± 0.10	2.05 ± 0.11
Aflatoxin-fed + vehicle	0.73 ± 0. 09	1.77 ± 0.12	1.05 ± 0.10	0.95 ± 0.07	0.93 ± 0.04
Aflatoxin-fed + *Dianthus* (0.30 g/100 g bw)	0.83 ± 0.12	1.83 ± 0.13*	1.07 ± 0.18	0.93 ± 0.05	1.15 ± 0.15
Aflatoxin-fed + *Dianthus* (0.40 g/100g bw)	0.96 ± 0.11	1.53 ± 0.17	0.95 ± 0.19	0.95 ± 0.07	1.92 ± 0.12
Aflatoxin-fed + *Dianthus* (0.50 g/100g bw)	1.14 ± 0.18	2.18 ± 0.36	1.17 ± 0.05	1.04 ± 0.11	2.04 ± 0.15

Effect of dianthus extract on peritoneal exudate cell (PEC) count

The total number of PEC, and the absolute number and relative proportion of macrophages and lymphocytes in aflatoxin-fed mice were significantly decreased, when compared to those of normal mice (Table 3). However, this was reversed by treatment of aflatoxin-fed mice with dianthus (0.30, 0.40 or 0.50 g/100 g bw every other day for 2 weeks), which also increased PEC, lymphocyte and macrophage counts, as well as the absolute number and relative proportion of lymphocytes and macrophages ($p < 0.05$), relative to the untreated aflatoxin-fed mice.

Effect of *Dianthus* extract on phagocytic function of peritoneal exudate cells (PECs)

Table 4 shows that carbon uptake by PEC in aflatoxin-fed mice was significantly decreased, when compared with the control group. However, treatment of the aflatoxin-fed mice with *Dianthus* (0.30, 0.40 or 0.50 g/100 g) for 2 weeks led to a progressive rise in the scavenging activity of PEC ($p < 0.05$), when compared with the corresponding values from untreated aflatoxin-fed mice. Furthermore, carbon particles in the peritoneal fluid of aflatoxin-fed mice were increased, relative to normal mice. However, treatment of aflatoxin-fed mice with *Dianthus* caused gradual and significant decreases in carbon contents at doses of 0.30 and 0.40 g/bw at 15 and 30 min ($p < 0.05$), relative to the control group.

Effect of *Dianthus* extract on rosette-forming cell (RFC) count

The number of RFCs in aflatoxin-fed mice was significantly decreased, when compared to the control group (Table 5). However, treatment of the aflatoxin-fed mice with *Dianthus* (0.30 and 0.40 g/100 g) every day for 2 weeks caused statistically significant increases in the number of RFCs, when compared with corresponding values for the aflatoxin-fed control mice ($p < 0.05$; $p < 0.01$).

Effect of *Dianthus* extract on plaque-forming cells (PFCs) count

As shown in Table 5, the number of PFCs in aflatoxin-fed mice was reduced, compared with control group of mice. However, when the aflatoxin-fed mice were treated with *Dianthus* extract (0.30, 0.40 and 0.50 g/100 g every day) for 2 weeks, there were progressive increases in the number of PFCs, relative to the aflatoxin-fed control mice. The increase was statistically significant at a dose 0.50 g/100 g ($p < 0.05$).

Table 3: Effect of *Dianthus* extract on peritoneal exudate cells (PEC) count, absolute number and relative ratio (%) of macrophages and lymphocytes

Treatment	Total PEC count (10^6) (mean ± SD)	Macrophage		Lymphocyte	
		N (mean ± SD)	%	N (x10^6) (mean ± SD	%
Normal	6.55 ± 0.96	4.13 ± 0.82	63.05	2.42 ± 0.15	36.95
Aflatoxin-fed + vehicle	2.90 ± 0.29	1.52 ± 0.08	52.41	1.38 ± 0.36	47.59
Aflatoxin-fed + *Dianthus* (0.30 g/100g body bw)	5.00 ± 0.29	2.85 ± 0.45	57.00	2.15 ± 0.17	43.00
Aflatoxin-fed + *Dianthus* (0.40 g/100 g bw)	5.41 ± 0.83	3.07 ± 0.41	56.75	2.34 ± 0.58	43.25
Aflatoxin-fed + *Dianthus* (0.50 g/100g bw)	6.25 ± 0.90	3.26 ± 0.25*	52.16	2.99 ± 1.00	47.84

Table 4: Effect of *Dianthus* extract on phagocytic activity of peritoneal exudate cells (PEC) (determined by carbon uptake by PEC and carbon particles remaining in the peritoneal fluids)

Treatment	Carbon uptake by PEC				Carbon particles remaining in fluid			
	15 min	*30 min*	*45 min*	*60 min*	*15 min*	*30 min*	*45 min*	*60 min*
Normal	0.83±0.07	1.08±0.07	1.14±0.08	1.54±0.10	0.95±0.13	0.82±0.05	0.50±0.09	0.41±0.06
Aflatoxin-fed + Vehicle	0.36±0.08	0.46±0.06	0.54±0.06	0.75±0.08	1.70±0.12	1.55±0.09	1.39±0.12	1.19±0.11
Aflatoxin-fed + *Dianthus* (0.30 g/100 g)	0.44±0.08	0.57±0.04	0.74±0.06	0.83±0.06	1.50±0.17*	1.36±0.11*	1.17±0.15	0.88±0.07
Aflatoxin-fed + *Dianthus* (0.40 g/100g)	0.57±0.03	0.78±0.04	0.88±0.11	0.96±0.04	1.28±0.05	1.21±0.05*	0.85±0.16	0.81±0.06
Aflatoxin-fed + *Dianthus* (0.50g/100g)	0.67±0.05	0.70±0.08*	0.83±0.05	0.96±0.04	1.28±0.12	1.15±0.08	0.92±0.04	0.61±0.03

Table 5: Effect of *Dianthus* extract on number of rosette-forming cells (RFCs) and plaque-forming cells (PFCs) per 10^6 nucleated spleen cells in normal and in aflatoxin-fed mice

Treatment	RFCs/million nucleated spleen cells (mean ± SD) x10^3)	No. of PFCs/million nucleated spleen cells (mean ± SD) x10^3
Normal	2.3 ± 0.12	1.59 ± 0.21
Aflatoxin-fed + vehicle	0.86 ± 0.05	0.54 ± 0.05
Aflatoxin-fed + *Dianthus* (0.30 g/100 g bw)	1.38 ± 0.08*	1.24 ± 0.12
Aflatoxin-fed + *Dianthus* (0.40 g/100g)	2.06 ± 0.16**	1.38 ± 0.09
Aflatoxin-fed + *Dianthus* (0.50 g/100g)	2.31 ± 0.11	1.48 ± 0.21**

Table 6: Effect of *Dianthus* extract on T-cell mitogenic response *in vitro*

Treatment	Optical density at 570 nm		
	Control (Culture medium)	Con A 0.04 µg/well)	Con A (0.2 µg/well)
Vehicle	0.23 ± 0.04	0.41 ± 0.10	0.37 ± 0.08
Dianthus (0.03 g/mL)	0.24 ± 0.05	0.63 ± 0.09	0.46 ± 0.05
Dianthus (0.04 g/mL)	0.26 ± 0.04	0.84 ± 0.03	0.63 ± 0.05
Dianthus (0.05 g/mL)	0.29 ± 0.06	0.85 ± 0.17**	0.69 ± 0.13*

Cultured splenocytes (1.5 x10^5 cells/ well) were exposed to culture medium (control), Con A (0.04 µg/well) or Con A (0.2 µg/ well) in the presence or absence of dianthus (0.03, 0.04 or 0.05 g/ml) for 72 h

Table 7: Effect of *Dianthus* extract on serum levels of ALT and ALT in normal and in aflatoxin-fed mice

Treatment	ALT (Ref range: up to 32 U/L)	AST (Ref range: up to 31 U/L)
Normal	12 ± 2.36	17 ± 4.57
Aflatoxin- fed + vehicle	51 ± 9.91	111 ± 2.15
Aflatoxin-fed + *Dianthus* (0.30 g/100g bw)	46 ± 8.14*	74 ± 1.65
Aflatoxin-fed + *Dianthus* (0.40 g/100g bw)	39 ± 3.11	82 ± 1.24
Aflatoxin-fed + *Dianthus* (0.50 g/100g bw)	36 ± 6.56	58 ± 1.33

Effect of *Dianthus* extract on T-lymphocyte mitogenesis *in vitro*

The results in Table 6 show that in the absence of Con A mitogen, the *Dianthus* extract elicited a gradual mitogenic effect under the culture conditions. This effect was statistically significant at the dose of 0.05 g/ml *($p < 0.05$; $p < 0.01$)* in the presence of Con A (0.04 and 0.2 µg/well).

Effect of *Dianthus* extract on serum activities of liver enzymes

Serum levels of ALT and AST in the aflatoxin-fed mice were markedly elevated, relative to the control group (Table 7). This effect was however reversed by treatment of the aflatoxin-fed mice with *Dianthus* (0.30, 0.40 or 0.50 g/100 g bw) every day for 2 weeks. In addition, the extract caused progressive decreases in serum ALT and AST levels, with the decreases significant at a dose of 0.30 g/100 g bw ($p < 0.05$), relative to the untreated aflatoxin-fed control mice.

DISCUSSION

The results obtained in the present study revealed that daily treatment of aflatoxin-fed mice with *Dianthus* at doses of 0.30, 0.40 and 0.50 g/100 g for 2 weeks led to increased cellularities in thymus, spleen and bone marrow lymphocytes. However, the treatment did not affect the cellularity of peripheral and mesenteric lymph nodes. These results are consistent with those reported earlier [18, 19]. Most of the drugs and mediators used in the treatments perform their functions through their influence on immune cells, especially lymphocytes [20].

The present results have demonstrated that *Dianthus* administration to aflatoxin-fed mice progressively raised the number of peritoneal macrophages and their phagocytic function, relative to the untreated, aflatoxin-fed control mice. These results are in agreement with the results of an *in vivo* study which showed that ethanol extract of eugenol and aqueous extract of clove exerted effects on macrophages [21]. In

the present study, *Dianthus* extract did not produce any cytotoxic effects, but it exhibited potential antioxidant and pro-oxidant effects.

The effect of the extract on macrophage cytokine release also displayed a dose-related pattern. The extract at doses 0.30 and 0.40 g/100 g bw increased the number of T cells in aflatoxin-fed mice. Incubation of splenocytes with the extract at a dose of 0.05 g/ml significantly improved the number of T cells in aflatoxin-fed mice, and raised the T cell mutagenic response in the presence of Con A mitogen. These findings are in agreement with the results obtained by previous workers who reported that in cyclophosphamide-immunosuppressed mice, essential oil from clove increased the white blood cell count and enhanced delayed-type hypersensitivity responses [22].

Enhancement of T cell responses implies restoration of immune function, which indicates that clove modulates T cell cellularity and function. Furthermore, clove restored cellular immunity, which hints at its beneficial applications. Estimation of the proliferation of lymphocyte subtypes showed that higher concentrations (100 mg/mL and 1000 mg/ mL) of clove oil condensed PHA-stimulated splenocytes (T cell) proliferation and enhanced LPS-stimulated (B cell) or unstimulated splenocytes [20]. In another study, aqueous extract of clove reduced PHA-stimulated splenocyte (T cell) proliferation only, whereas it had no effect on the other two lymphocytes [19].

The results obtained in the present study showed that the number of PFCs in aflatoxin-fed mice treated with *Dianthus* was significantly increased in a dose- dependent manner, relative to that of aflatoxin-fed control mice, when mice were immunized with SRBCs 4 days before sacrifice. In another study, humoral immunity was assessed by foot-pad thickness, and the results showed that *Dianthus* extract modulated immune response by augmenting humoral immunity and decreasing cell-mediated immunity [23].

In the current study, the observed decreases in serum levels of liver enzymes (ALT and AST) are consistent with the results reported in another study which reported decreased activities of serum ALT, AST and ALP in diabetic rats treated with olive oil at doses of 300 and 600 mg/kg. [24]. The decrease in ALP activity in the diabetic rats given clove oil is an indication of the hepatoprotective effect of clove oil, probably due its content of flavonoids and polyphenolic compounds.

CONCLUSION

The findings in this study demonstrate that *Dianthus* possesses a potential as a modifier of biological responses. It exerts this effect via enhancement of the efficiency of the immune system, activation of phagocytosis, immune response, and prevention of liver damage.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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