Antifungal potency of *Nigella sativa* against toxigenic *Rhizopus stolonifer* and its immunomodulatory effects in aflatoxin-fed mice

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

[1]Biology Department, College of Science, Girls branch, King Khalid University, Abha, KSA
[2]Plant and microbiology Department, College of Science, Girls Branch, Al Azhar University, Cairo, Egypt.

Abstract: The present study describes the antifungal potency of *Nigella sativa* seeds extract and the effect of immunomodulatory of *N. sativa* against aflatoxin- fed mice. Disc diffusion method was used for antifungal efficacy of aqueous extract of *N. sativa*. In animal experiments, lymphoid cell count, total and differential counts of PEC, the phagocytic activity of PEC and detection of the plaque-forming were determined. E-rosette-forming cells (RFC), T-cell mitogenesis assay cells, ALT and AST were detected. The aqueous extract of *N. sativa* (50%) exhibited high inhibition zone with most of isolates of *R. stolonifera*.The results indicated that treatment of mice by using *N. sativa* showed marked rise in the number of cells from thymus and PLN with dose 0.50 g and absolute number and comparative ratio of macrophages ($P < 0.01$) with the doses 0.40 and 0.50 g. There is gradually rise in the scavenger activity of PEC with the dose 0.50 g at 60 min. Serum level of ALT was markedly reduced with dose 0.50 g as compared with a control group. These results indicated that *N. sativa* is promising modifier of biological response.

Keywords: Aflatoxin, *Nigella sativa*, lymphocytes, T-cells, B-cells, macrophages, liver enzymes-spleen cell.

INTRODUCTION

Medicinal plants have many highly effective extracts which has a long history of multiple uses also had been traditionally used to improve health. the therapeutic results of medicinal herbs, focusing on their ability as anti-oxidant, anti-inflammatory, anti-cancer, anti-microbial, and immunomodulatory (Salem, 2005). *N. sativa* (black seed) that belongs to the Ranunculacea family has a large and rich historical background (Goreja, 2003). *N. sativa* is an annual flowering plant, which grows to 20-90 cm tall, leaves finely divided, the leaf segments narrowly linear to threadlike. The flowers are delicate, and they can be white, yellow, pink, pale blue or pale purple, with 5-10 petals. The fruit is a huge and puffy capsule possessed of 3-7 conjoined follicles, each contain huge numbers of seeds (Warrier and Nambiar, 2004). Extract of *N. sativa* seeds are widely used in middle East and South Asian countries to treat or control many diseases, for instance diabetes, hypertension, cancer, leukeamia, liver, lung, kidney, prostate, breast, cervix, kidney disorder, skin inflammation, hepatic disorder, arthritis, cardio vascular, complications and dermatological conditions (Khan *et al*., 2003). Seeds of *N. sativa* contain fixed oils (about 30%) and volatile oils (average 0.5%, maximum 1.5%); this plant is also containing a high level of quinones such as, alkaloids and terpenoids thymoquinone, nigellone, and thymohydroquinone, carvacrol, t-anetholet. *N. sativa* extracts and its oils can be used as natural antibacterial agents to treat infections caused by multidrug resistant bacteria (Saleh *et al*., 2018). In the current study, the effect of *N. sativa* in the treatment of aflatoxin- fed mice is detected by measuring the effect on the number of lymphocytes in different lymphoid organs. Also, phagocytic activity of peritoneal exudate cell (PEC), plaque-forming cells (PFC), E-rosette-forming cells (RFC), T-cell mitogenesis assay cells, ALT and AST were detected.

*Corresponding author: e-mail: Othman_gehan@yahoo.com

MATERIALS AND METHODS

N. sativa seeds were obtained from a local market in Saudi Arabia. *N. sativa* is an annual herbaceous plant that belongs to the Ranuculacea family. Petri dish (100 x 15 mm), a sterile blank paper disk (0.64 cm in diameter), a cell-counting chamber, potato dextrose agar and microplates were obtained from Biology Department, King Khalid University. The international protocol about ethics was followed in this research work.

Preparation of N. sativa seeds extract

Preparation of *N. sativa* seed extract was prepared as the technique carried out Hosseinzadah *et al*., (2013).

Agar diffusion assay

This method was carried out in accordance with that described by Ye *et al.* (2000).

Animals

Male Swiss albino mice (8-10 weeks old, weighing about 20 g each) were used, that kept in the experimental animal unit of faculty of Helwan university, Cairo, Egypt. Male Swiss albino mice were preserved in a quiet room at

28°C, with a light period of 12 hours alternating with a dark period of 12 hours. Mice received laboratory chow and water *ad libitum* and were allowed a period of 10 days, prior to the initiation of experiments, to acclimatize to the laboratory conditions.

Experimental design and treatment regime

Doses of 0.30, 0.40 and 0.50 g of *N. sativa*/100 g body weight were orally administered to aflatoxin- fed mice (0.2 ml/ mouse) every day for following 2 weeks. Mice orally administered with 0.2 ml of distilled water only were used as a control

Determination of lymphoid cell counts

The Determination of lymphoid cell counts was prepared by using method described by El-Shaikh *et al*., (2006).

Harvesting of peritoneal exudate cells (PEC)

To determine inflammatory peritoneal phagocytes, normal, aflatoxin- fed mice and aflatoxin- fed mice treated with dianthus the method described by El-Shaikh *et al.,* (2006) was used.

Carbon clearance assay

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

Detection of the plaque-forming cells (PFC)

The method was done as explained by Brousseau *et al.*, (1999).

Determination of E-rosette-forming cells (RFC)

The method was done as explained by Hsu *et al,* (1975).

T-cell mitogenesis assay

The determination of T-cell mitogenesis was detected as explained by El-Shaikh, *et al.,* (2006).

Determination of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities

AST and ALT activities were determined as described by (Reitman and Frankel, 1957) using BIO ADWIC AST and ALT kits, Egypt.

STATISTICAL ANALYSIS

All *in vivo* results are expressed as the mean ± SD of groups consisting of 8 mice. The *in vitro* data are also expressed as the mean ± SD of groups consisting of four wells. Every experiment was performed independently at least three times. By using Student's *t*-test all data were analyzed for significance. (* Significantly different from aflatoxin- fed group at $P<0.05$ and ** significantly different from aflatoxin- fed group at $P<0.01$).

RESULTS

Antifungal activity of N. sativa extract

The effect of aqueous extract of *N. sativa* (50%) on the growth of the different isolates of *R. stolonifer* shown in table 1. *N. sativa* aqueous extract has exclusive antifungal activity against the most different isolates of *R. stolonifer*. isolates number 1, 2, 4, 11, 12, 14, 15, 22 and 24 recorded the clear inhibition zones ranged from 11-33 mm. the maximum inhibition zone was 33 mm at strain number 22 followed by 25 mm at strain number 23 and 24 mm at strain number 4. The growth of fungal strains number 3, 9, 10, 13, 16, 17, 18, 19 and 24 were not inhibited by *N. sativa* plant extract.

Table 1: Antifungal efficacy of aqueous extract of *N. sativa* (50%) incubation with different isolates of *R. stolonifer* at pH 7.2 and 25°C for 72 hours.

Diameter of inhibition zone (mm)	Fungi Strains
19 ± 1.15	Isolate no 1
22 ± 1.15	Isolate no 2
-	Isolate no 3
24 ± 1.15	Isolate no 4
-	Isolate no 5
-	Isolate no 6
-	Isolate no 7
-	Isolate no 8
-	Isolate no 9
-	Isolate no 10
20 ± 1.15	Isolate no 11
19 ± 1.15	Isolate no 12
-	Isolate no 13
13 ± 1.15	Isolate no 14
11 ± 1.15	Isolate no 15
-	Isolate no 16
-	Isolate no 17
-	Isolate no 18
-	Isolate no 19
-	Isolate no 20
-	Isolate no 21
33 ± 1.15	Isolate no 22
25 ± 1.15	Isolate no 23
-	Isolate no 24

Effect on lymphoid cell count

From table 2 it is clear that the total number of cells from thymus, Spl, PLN, MLN and BM of aflatoxin- fed mice was reduced as compared to those of normal mice. Treatment of aflatoxin- fed mice with *N. sativa* (0.30, 0.40 or 0.50 g/ 100 g BW, every day for 2 weeks) stimulated a sharp increase in the number of cells from spleen and BM compared with aflatoxin- fed mice. Although aflatoxin- fed mice treated with *N. sativa* did not cause any significant effect on the number of cells from MLN, it give rise to a statically significant rise in the number of cells from thymus and PLN with dose 0.50 g ($P < 0.05$).

Table 2: Total number of leukocytes from thymus (Thy), spleen (Spl), peripheral lymph nodes (PLN), mesenteric lymph nodes (MLN) and bone marrow (BM) in normal and in aflatoxin- fed mice. group).

Treatment	Total cell count/ g of 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 + *N. sativa* (0.30 g)	1.05 ± 0.14	1.89 ± 0.09	1.06 ± 0.09	0.97 ± 0.11	1.37 ± 0.22
Aflatoxin- fed + *N. sativa* (0.40 g)	1.34 ± 0.16	2.03 ± 0.16	1.09 ± 0.12	1.00 ± 0.12	1.90± 0.12
Aflatoxin- fed + *N. sativa* (0.50 g)	1.62±0.15*	2.29 ± 0.09	1.22 ± 0.10*	1.06 ± 0.08	2.02 ± 0.24

Table 3: Total peritoneal exudate cells (PEC) count, the absolute number and relative proportion (%) of both macrophages and lymphocytes were assessed in normal and aflatoxin- fed mice.

Treatment	Total PEC count (Mean±SD x10^6)	Macrophages		Lymphocytes	
		Absolute No. (Mean ± SD x10^6)	%	Absolute No. (Mean ± SD x10^6)	%
Normal	6.55 ± 0.96	4.13 ± 0.82	63.05	2.42 ± 0.15	36.95
Aflatoxin- fed + Vehicle	2.9 ± 0.29	1.52 ± 0.08	52.41	1.38 ± 0.36	47.59
Aflatoxin- fed + *N. sativa* (0.30 g)	4.80 ± 0.44	3.02 ± 0.10	62.92	1.78 ± 0.10	37.08
Aflatoxin- fed + *N. sativa* (0.40 g)	5.66 ± 0.24	3.54 ± 0.33**	62.54	2.12 ± 0.33	37.46
Aflatoxin- fed + *N. sativa* (0.50 g)	6.24 ± 0.18	3.93 ± 0.46**	62.98	2.31 ± 0.46	37.02

Table 4: The phagocytic activity of peritoneal exudate cells (PEC), as detected by carbon swallow by PEC and carbon particles remained in the peritoneal fluid in normal and in aflatoxin- fed mice.

Treatment	Carbon swallow by PEC				Carbon particles remained 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 + *N. sativa* (0.30 g)	0.56± 0.03	0.73± 0.04	0.84± 0.06	0.95± 0.05	1.23± 0.09	1.12± 0.14	0.86± 0.02	0.76± 0.04*
Aflatoxin- fed + *N. sativa* (0.40 g)	0.64± 0.04	0.75± 0.04	0.86± 0.03	1.18± 0.11	1.30± 0.08	1.12± 0.09	0.83± 0.04	0.67± 0.03
Aflatoxin- fed + *N. sativa* (0.50 g)	0.66± 0.05	0.90± 0.02	0.97± 0.03	0.93± 0.17*	1.31± 0.07	1.07± 0.11	0.86± 0.05	0.80± 0.07

Table 5: Number of rosette-forming cells (RFCs) and plaque-forming cells (PFCs)/ 10^6 nucleated spleen cells in normal and in aflatoxin- fed mice.

Treatment	No. of RFCs/ million nucleated spleen cells (Mean ± SDx10^3)	No. of PFCs/ million nucleated spleen cells (Mean ± SDx10^3)
Normal	2.3 ± 0.12	1.59 ± 0.21
Aflatoxin- fed + Vehicle	0.86 ± 0.05	0.54 ± 0.05
Aflatoxin- fed + *N. sativa* (0.30 g)	1.58 ± 0.11	1.26 ± 0.08
Aflatoxin- fed + *N. sativa* (0.40 g)	1.88 ± 0.25*	1.30 ± 0.15*
Aflatoxin- fed + *N. sativa* (0.50 g)	2.18 ± 0.15*	1.40 ± 0.10

Table 6: T cell mitogenic response *in vitro*. 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 absence or presence of *N. sativa* (0.03, 0.04 or 0.05 g/ ml) for 72 hours.

Treatment	Optical density (570 nm)		
	Control (Culture medium)	Con A 0.04 µg/ well)	Con A (0.2 µg/ well)
Vehicle	0.23 ± 0.03	0.28 ± 0.04	0.31 ± 0.05
N. sativa (0.03 g / ml)	0.26 ± 0.02	0.43 ± 0.06	0.43 ± 0.03
N. sativa (0.04g / ml)	0.27 ± 0.01	0.61 ± 0.05	0.62 ± 0.08
N. sativa (0.05 g / ml)	0.31 ± 0.02	0.70 ± 0.12**	0.82 ± 0.13**

Table 7: Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assessed in normal and in aflatoxin- fed mice treated orally with vehicle (0.2 ml distilled water) or *N. sativa* (0.30, 0.40 or 0.50 g/ 100 g BW) every other day for 2 weeks.

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 + *N. sativa* (0.30 g)	44.50 ± 4.93	83.75 ± 9.95
Aflatoxin- fed + *N. sativa* (0.40 g)	38 ± 5.60	65.75 ± 7.88
Aflatoxin- fed + *N. sativa* (0.50 g)	19.25 ± 3.40*	33 ± 5.03

Effect on peritoneal exudate cell (PEC) count

Table 3 show that, total number of PEC in addition to the absolute number and the relative proportion of macrophages and lymphocytes of aflatoxin- fed mice were reduced as compared to the normal mice. Treatment of aflatoxin- fed mice with *N. sativa* (0.30, 0.40 or 0.50 g/ 100 g BW, every day for 2 weeks) recovered this effect and caused increase in PEC count, In addition to the absolute number and relative proportion of lymphocytes and a marked rise in the absolute number and the relative proportion of macrophages ($P < 0.01$) with the doses 0.40 g and 50 g as compared with the result of the aflatoxin-fed mice.

Effect on the phagocytic function of peritoneal exudate cells (PECs)

Table 4 indicates that carbon swallow by PEC of aflatoxin- fed mice was reduced as compared with the result of the normal mice. However, treatment of aflatoxin- fed mice with *N. sativa* (0.30, 0.40 or 0.50g/ 100 g BW, every day for 2 weeks) caused a gradual raise in the scavenger activity of PEC ($P<0.05$) with the dose 0.50 g at 60 min as compared with the value of the aflatoxin- fed mice. On the other hand, carbon particles persisted in the peritoneal fluid of aflatoxin- fed mice were raised as compared to normal mice. Treatment of aflatoxin- fed mice with *N. sativa* caused a cumulative reduction carbon contents ($P<0.05$) with dose 0.30 at 60 min as matched to the control group.

Effect on rosette-forming cells (RFCs) count

As illustrated in table 5 it is clear that the number of RFCs in aflatoxin- fed mice was reduced as compared to the control group. Treatment of aflatoxin- fed mice with *N. sativa* seed extract (0.30, 0.40 or 0.50 g/ 100 g BW, every day for 2 weeks) caused a statistically marked raise in the number of RFCs as compared with control group.

Effect on plaque-forming cells (PFCs) count

As outlined in table 5 the number of PFCs in aflatoxin-fed mice was reduced as compared to that of the normal mice. aflatoxin- fed mice treated with *N. sativa* extracts (0.30, 0.40 or 0.50 g/ 100 g BW, every day for 2 weeks) caused a progressive raise in the number of PFCs as matched to that of the corresponding aflatoxin-fed control mice. However, this increase was statistically significant with dose 0.40 g ($P<0.05$).

Effect on T-lymphocyte mitogenesis in vitro

It appears from table 6 that in the absence of Con A mitogen, *N. sativa* by itself elicited a gradual mitogenic effect under the cultured conditions. This effect was statistically significant with the dose 0.05g *($P < 0.01$)* in the presence of Con A (0.04 and 0.2µg/ well).

Effect on Serum Enzyme Activities of the Liver

As illustrated in table 7, serum enzyme levels of ALT and AST of aflatoxin- fed mice were obviously raised as compared to normal mice. Treatment of aflatoxin- fed mice with *N. sativa* (0.30, 0.40 or 0.50 g/ 100 g BW, every day for 2 weeks) recovered this effect and caused a gradual reduce in serum enzyme levels of the liver as compared to the control group. Serum level of ALT was significantly decreased with dose 0.50 g ($P<0.05$) as compared to that of aflatoxin- fed control mice.

DISCUSSION

The results presented in table 1 revealed that *N. Sativa at* concentration 50% was totally effective against the isolated strains. It recorded 33 mm in the strain number 22, 25mm in case of strain number 23, 24mm in case of strain number 4, 22mm in case of strain number 2 and 19 mm in both strain number 1 and 12 while it was less effective with other strains. essential oils and plant extracts against fungi and yeasts varied in their antimicrobial activity. Many of previous studies were focused on the effects of black seed extracts on the various components of the immune system. The majority of the compound have anti-oxidant properties, which aid the body in repairing itself by removing free radicals and this facilitate the immune system in its activities (Oscan and Erkmen, 2001). Thymoquinone which is the main active compound of black cumin seed oil has the ability to inhibit iron-dependent lipid peroxidation in concentration-dependent manner. It is a potent O_2 scavenger activity and Thymoquinone can reduce oxidative stress and raise antioxidant defense in the body. (Mollazadeh and Hosseinzadeh, 2014). *N. sativa* treatment increase antioxidant defense system (Meral *et al.,* 2001).

Also, the data of the current work revealed that *N. sativa* treatment with doses (0.30, 0.40 and 0.50 g/ 100 g BW every day for 2 weeks to aflatoxin- fed mice raised the

cell number of thymus, spleen, PLN and bone marrow lymphocytes. However, either treatment did not affect the cellularity of MLN. The data of this study is agreed with the data of Huang *et al* (2008), Zhou *et al.,* (2013) that indicated many of the drugs and mediators in the treatment of the disease perform their functions by the influence of immune cells, especially lymphocytes. These agents could be body mediators, synthetic factors, or natural elements. This is also consistent with Peter *et al*., (1999), who mentioned that black seed extracts are of vital importance to cancer patients who are immunocompromised. It stimulates the bone marrow leading to increased production of megakaryocytes, macrophages, platelets as well as red blood cells that are necessary to protect the body against opportunistic infections.

Also, the data of this study demonstrated that *N. sativa* treatment to aflatoxin- fed mice raised the number as well as the phagocytic function of peritoneal macrophages as compared to that of control group. These results are in harmony with the results obtained by Akrom, (2017) who found that thymoquinone, is able to increase the activity of macrophages and IL- 12 cytokine is produced by APC, has various functions, and plays an important role in enhancing immune response mediated by CD4Th1 cells. The biological effects of IL- 12 are regulating the proliferation and differentiation of lymphoid, function of macrophages and dendritic cells, and the tolerance memory and lymphocytes homeostasis. *N. sativa* treatment, with doses 0.40 and 0.50 g/ 100 g BW, to aflatoxin- fed mice markedly raised the number of T cells as detected with RFC assay. Moreover, synergistic incubation of splenocytes with dose 0.05 g/ ml *N. sativa* markedly raised the T cell mitogenic response in the presence of Con A mitogen. This result is consistent with the results obtained by Maidalawieh *et al*., (2010) who registered that aqueous extract of *N. sativa* markedly enhanced proliferation of splenocytes in a dose-responsive manner. Furthermore, the aqueous extract of *N. sativa* prefers the secretion of Th2, versus Th1, cytokines by splenocytes. The secretion of IL- 6, TNF- α and NO; key pro-inflammatory mediators by primary macrophages is markedly suppressed by the aqueous extract of *N. sativa*, illustrating that *N. sativa* exerts anti-inflammatory effects in vitro.

In the current work there is a reduce in serum levels of liver enzymes concentration (ALT and AST) and these results are agreed with that of Dollah *et al.,* (2013) who explained that oral administration of *N. sativa* prevented liver damage and has no toxicity in the doses used. The supplementation of *N. sativa* decreases the ALT level and AST level treated rats matched to the control group. This referred to hypo protective effects of *N. sativa* due to some components such as thymoquinone, monoterpenes 2, tocopherols, phytosterols and phenols.

Moreover, Pal *et al.,* (2011) found that Paracetamol in a toxic dose produces hepatic damage to the liver which can be prevented by *N. sativa* oil. Thymoquinone is an efficient cytoprotective agent against chemically induced hepatic damage. Mollazadeh and Hosseinzadeh, (2014) demonstrated that inflammatory responses and activated neutrophils can raise myeloperoxidase activity in the liver tissue. Myeloperoxidase raises lipid peroxidation and formation of free radicals. Thymoquinone reduce inflammation by decreasing malondialdehyde and lipid peroxidation. Inhibition of generates ROS in the liver lead to reduce cytochrome C production from mitochondria that reduced the amount of cytokines. Antioxidant and anti-inflammatory effects of thymoquine are two main mechanisms that parallel to each other protect liver cells from damage. Daba and Abdel Rahman, (1998) found that thymoquinone raised the ratio of helper to suppressor T cells, activated natural killer cell, production of IL- 3 and enhanced macrophages.

CONCLUCION

The results in this study demonstrate that *N. sativa* possess promising modifier of biological response.

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