International Journal of ChemTech Research
CODEN(USA): IJCRGG ISSN: 0974-4290
Vol.9, No.12 pp 962-972,2016

In Vivo Study on the Hindrance Activity of Cinnamon Extract Against Aspergillusniger in Mice

Randa Mohamed Alarousy[1,4], ShereinIsmail Abd El-Moez[1], Kawkab A. Ahmed[2] and Hany Mohamed Hassan[3]

[1]Department of Microbiology and Immunology, National Research Centre, Giza, Egypt
[2]Department of Pathology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt
[3]Department of Immunology, Animal Reproduction Research Institute
[4]Department of Medical Laboratories, College of Applied Medical Sciences, Majmaah University, KSA

Abstract:
Aim: The antimycotic effect and immune stimulating capacity of natural extract of *Cinnamomum zeylanicum* plant was evaluated against *Aspergillus niger*strain.
Methods and results: the herbal extract was prepared from cinnamon park to be examined against *Aspergillus niger*(ATCC16404)species fungal cell suspension. Mice were injected with both fungal cell suspensions and *Cinnamomum zeylanicum* extract in a certain regime. Histopathological examination was applied on lung, liver and brain tissues extracted from the experimental animals and histopathological findings revealed the strong fungicidal effect of the herbal extract in the mice's tissues. Phagocytic activity, interleukins 2 & 6 (IL 2& IL6) and tumor necrosis factor(TNF) were measured in mice's blood samples as immunity stimulating efficacy parameters and also results revealed the potent immune stimulating efficacy of the extract and its safety*in vivo.*
Conclusion: according to the results of the study, *Cinnamomum zeylanicum* extract has a strong fungicidal activity against *A. niger*(ATCC16404)in addition to potent immune stimulating action confirmed experimentally. Results are supporting the efficacy of *C. zeylanicum* extract as prophylactic agent as well as fungicide.
Significance and impact of the study: our study spotted a light on the advantages of the examined herbal extract as antimycotic substance, the promising results revealed encourages the use of *C. zeylanicum* extract as pharmaceutical preparation for treating and prevention of mycosis in immunocompromised patients instead of using some chemical antimycotic preparations available for commercial use to avoid the disadvantages of these chemically prepared medications.
Keywords: *Cinnamomum zeylanicum*– Interleukin 2 – Interleukin 6 – Antimycotic extract – Immunostimulant. Histopathology.

Introduction

Fungal infection is considered among the main causes of morbidity in immunocompromised patients. The epidemiology of invasive mycotic infections has changed within the last decade (**Lehrnbecher *et al.*,**[1]**).**

About 20 *Aspergillus* species out of 185 have been reported as opportunistic infectious agent in both man and animals (**Stevens *et al.*, [2]**)

Although opportunistic fungal agent leads to life – threatening infection in immunosuppressed patients, fungal infection affects both immunosuppressed patients as well immune-competent persons in developing countries (**Del Poeta and Chaturvedi, [3])** The rising incidence in fungal infections observed in the last decade correlates with increases in invasive medical interventions, long-term hospitalization and with large numbers of immunosuppressed patients due to acquired infection due to human immunodeficiency viral infection or treatment-induced immunodeficiency as prolonged using of cortisone before organ trans-plantation or during anticancer therapy (**Pfaller and Diekema,[4,5])**

It is very hard to distinguish between systemic mycosis from other microbial infection depending on the clinical symptoms. This often leads to delayed appropriate intervention using antifungal therapy**(Pfaller,[6])**

Antimycotic agents are associated with inverse results of systemic aspergillosis; the ascending problems due to azoles – resistance and in addition, currently used antifungals have substantial economic burden (**Karthaus, [7])**.So, presenting safe and economic antimycotic would be an alternative approach.

Certain plant extracts have been examined experimentally to study the immuno-stimulating activities; they revealed great stimulating efficacy of immune system through activating macrophages, T and B lymphocyte, natural killer (NK) cells, as well as interleukins, tumor necrosis factor and interferon**(Puri*et al*.,[8] and Suresh and Vasudevan [9]).**The aim of the present work is to evaluate *invivo* fungicidal and immunity stimulating capacity of *Cinnamomum zeylanicum*extract against *Aspergillus niger* reference strain (ATCC16404)that used to induce an experimental infection of lab. animals.

Materials and methods:

Microorganism:

Aspergillus niger ATCC 16404 referencestrain was used in this study.Strain was spread onto Sabouraud's dextrose agar with Chloramphenicol (0.5 g L-1) to inhibit bacterial growth and incubated at 300C for 1 to 3 days.

Black colonies with fluffy down can be transferred to Czapek's solution agar (Difco Lab, Detroit, MI) for a definitive confirmation. A piece of colony was teased apart with a needle, stained, marked and mounted with a cover slip. Species confirmationwas achieved on the basis of morphological criteria upon microscopic examination (**Richard and Beneke, [10])**

Cell-counting:

Spore concentration of different spore suspensions was adjusted using haemocytometer, according to the classical procedure.

Spores were harvested by flooding the agar surface with PBS (Sigma) and then filtered and suspended in PBS with 0.01 % Tween 80 (Difco), in serial concentrations. Spore suspensions were stored at 4 0C for up to 5 days till use.

Hundred micro liters of fungal stock solutions from *Aspergillus niger* ATCC 16404 spores were inoculated into 10 ml of (YPD) broth and cultured for 48 h. at 30oC with agitation. Fungal pellets were re-suspended in sterile saline solution and were adjusted to 105 - 107 spore/ml using haemocytometer to prepare working solution from microorganism spores.

Experimental infection:

C. zeylanicum extraction from Cinnamon park:

C. zeylanicum, the active principle of cinnamon, was extracted from cinnamon park using steam distillation according to **Nandam and Vangalapati[11]** the extracted material in liquid form was then diluted 10:100 in ddH2O to be injected intravenously in experimental mice.

Lab. Animals:

(experimental infection complied with relevant professional and institutional animal welfare policies)

Eight to ten week-old male mice were purchased from laboratory animal house of Ramad hospital, Giza, Egypt. The mice were provided with clean water and solid feed. All animal studies were performed in accordance with the guidelines and permission of the animal experiment care, feeding, changing bedding and general good health was observed regularly.

Fifty mice were divided randomly into 5 groups in 5 cages each contains 10 mice and treated as follow:

A1: mice were injected I/Vwith 100µl of 105 - 107 spore/ml of *A. niger*ATCC 16404 reference strain as control positive.

A2: mice were injected with 100µl of 105 - 107 spore/ml of *A. niger*ATCC 16404 followed by 100µl of *C. zeylanicum*extract that was prepared previously three days post infection.

A3: mice were injected with 100µl of 105 - 107 spore/ml of *A. niger*ATCC 16404 followed by 100µl of plant extract seven days post infection.

A4: mice were injected with 100µl of 105 - 107 spore/ml of *A. niger*ATCC 16404 followed by 100µl of plant extract ten days post infection.

A5: mice were injected with 100µl of normal saline as negative control group.

Mortalities were determined and recorded daily.

Sampling:

After 14 days, serum and heparinized blood samples were collected via heart puncture from all mice for immunological assays. All mice then were euthanized; lungs, livers and brains of sacrificed mice were removed and fixed in 10% formalin, the organs then were cut and embedded in paraffin then these blocks were cut into 4 µm thick sections and were stained with Hematoxylin and Eosin (H&E) stain for histopathological examination microscopically(**Bancroft *et al*., [12]).**

Blood samples were used to measure certain immunological parameters as phagocytic activity (phage); killing activity of macrophages; lymphocyte transformation (Lyt1); interleukin 2 (IL2); interleukin 6 (IL6) and tumor necrosis factor (TNF).

Lymphocyte transformation:

Lymphocyte transformation assay was carried out as described by **Denizot and Lang [13]** with some modifications**(Maslak and Reynolds, [14]).**

Determination of phagocytic killing and chemotaxis activities of PMN:

Polymorph nuclear cells were isolated from blood by the method described by **Rouse *et al*[15].**The mixture of PMN and bacteria (*S. aureus*) was incubated at 370C for 2 hours with regular stirring and then the mixture was centrifuged at 200 *g* for 5 minutes at 40C. The supernatant was used to estimate the percentage of bacteria phagocytosed **(Woldehiwet and Rowan, [16])**. The mixture of bacteria and PMN was treated with one cycle of freezing and thawing and the percentage of bacteria killed was estimated according to the formula described by **Woldehiwet and Rowan[16].**The chemotactic index of PMN was calculated using the chemotaxis under agarose technique based upon migration patterns to chemotactic factor (*E. coli* filtrate) **Nelson, [17].**

Measurement of IL-2; IL-6 and TNF by enzyme linked immunosorbent assay:

Serum IL-2, IL-6 and TNF-α level was measured by using a polyclonal ELISA kits (Uscn, Life Science Inc., USA) following the manufacturer's instructions. Briefly, the anti-IL-6 capture polyclonal antibody was absorbed on a polystyrene 96-well plate and the IL-6 present in the sample was bound to the antibody coated wells. The biotinylated anti-IL-6 detecting pAb was added to bind the IL-6 captured by the first antibody. Avidin-peroxidase (Sigma, USA) was added to the wells to detect the biotinylated detecting antibody and finally 2,2´- azinobis (ABTS; Sigma, USA) substrate was added and a colored product was formed and measured at optical density 405 nm (OD 405) with an ELISA MICROPLATE READER (MODEL 450, Bio-Rad, Chicago, Illinois, USA). A standard curve was generated and calculated. The measurement of TNF- α and

IL-2 are similar to that of IL-6. All determinations were performed by full-time technical personnel. Statistical analysis was applied using One Way ANOVA SPSS, POSTHOC C ALPHA (0.05).

Results:

The gross findings: liver, lungs and brain were extracted from mice, examined grossly and depicted in Table (1).

Table (1): Showing the gross findings of extracted organs from experimental animals:

Group	Liver	Lung	Brain
A1 (+ve)	**Pale/severe pale**	**Fleshy/semi normal**	**Fragile-granulated-calcified/highly fragile to semi normal.**
A2 (3rd)	**Slight pale**	**Normal**	**Normal**
A3 (7th)	**Slight normal**	**Lobulated lungs**	**Slight normal**
A4 (10th)	**Slight pale**	**Normal**	**Normal**
A5 (-ve)	**Normal**	**Normal**	**Normal**

Remark: Total mortalities were 30%

Histopathological profile:

The histopathological findings of lung tissues from the experimentally infected mice were shown in figures 1, 2, 3, 4, 5 and 6. The profile of liver histopathology is revealed in figures number 7, 8, 9, and 10, while the histopathological findings of mice brain were clear in figures 11, 12, 13 and 14. Comparing the histopathological profiles among the mice groups were applied in order to correlate the fungicidal efficacy of *C. zeylanicum* extract and the intervention time of its use.

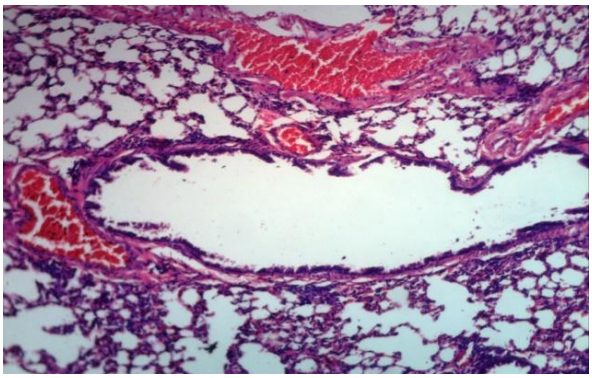

Fig. (1): Lung of mice from group A1 showing marked dilatation and congestion of pulmonary blood vessels (H & E X 100)

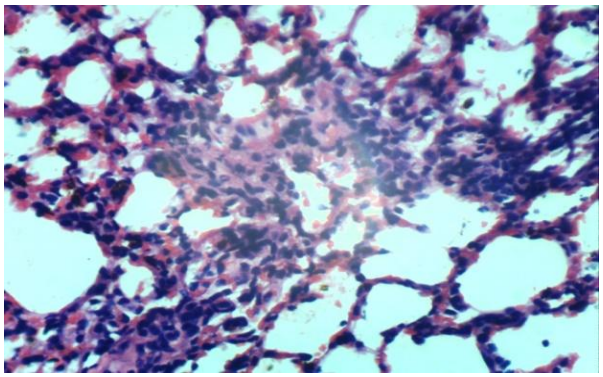

Fig. (2): Lung of mice from group A1 showing focal interstitial inflammatory cells infiltration (H & E X 400).

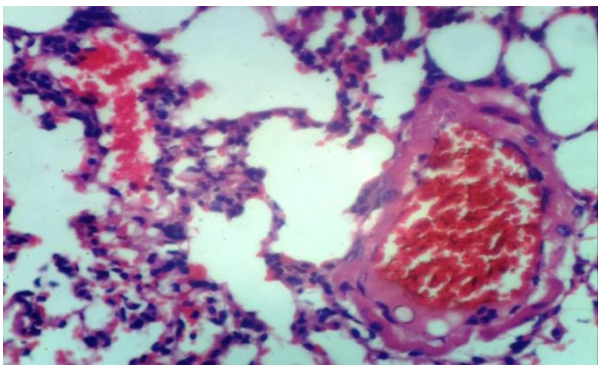

Fig. (3): Lung of mice from group A2 showing dilatation and congestion of pulmonary blood vessels (H & E X 400).

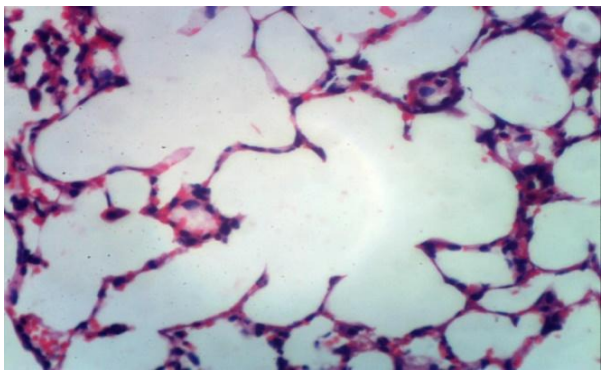

Fig. (4): Lung of mice from group A2 showing focal pulmonary emphysema (H & E X 400).

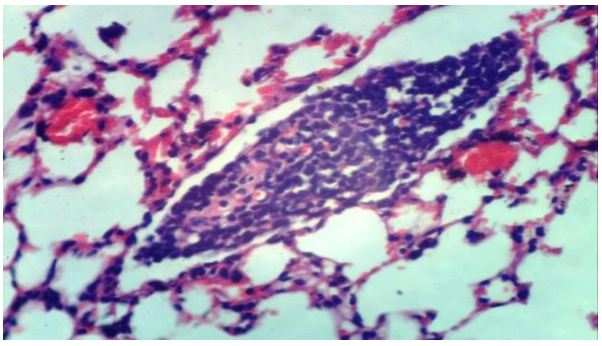

Fig. (5): Lung of mice from group A3 showing congestion of perialveolar blood capillaries and focal mononuclear inflammatory cells aggregation (H & E X 400).

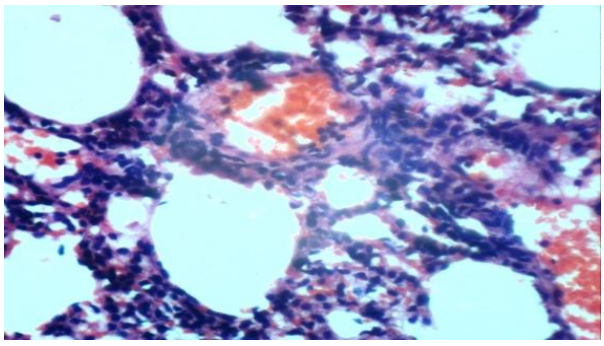

Fig. (6): Lung of mice from group A4 showing congestion of pulmonary blood vessels and interstitial pneumonia (H & E X 400).

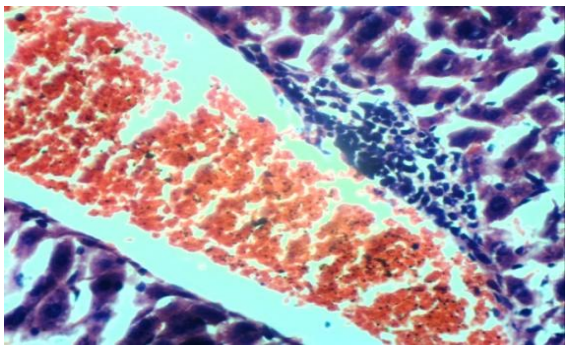

Fig. (7): Liver of mice from group A1 showing congestion of central vein and perivascular inflammatory cells infiltration (H & E X 400).

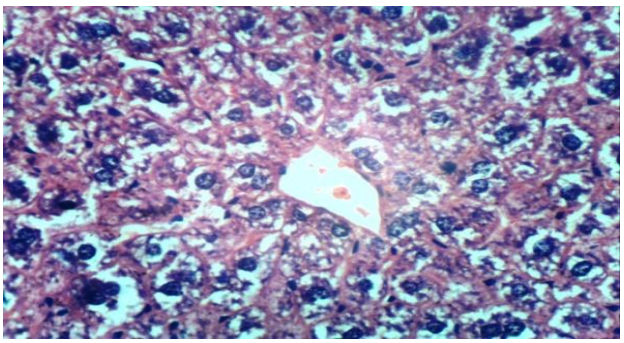

Fig. (8): Liver of mice from group A2 showing hydropic degeneration of hepatocytes(H & E X 400).

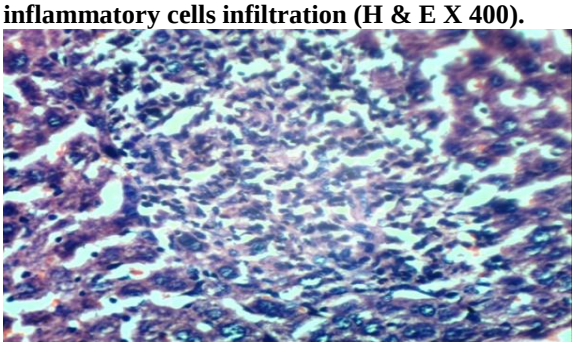

Fig. (9): Liver of mice from group A3 showing focal hepatic necrosis associated with inflammatory cells infiltration (H & E X 400).

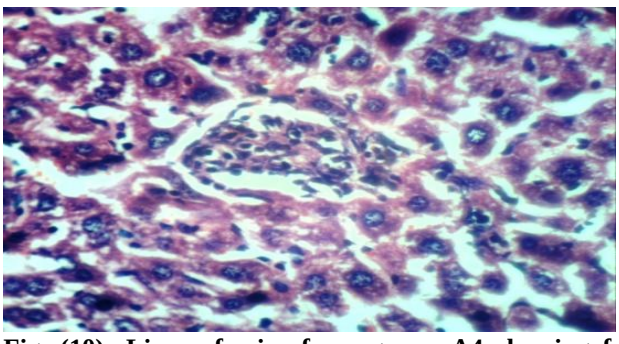

Fig. (10): Liver of mice from group A4 showing focal hepatic necrosis associated with inflammatory cells infiltration (H & E X 400).

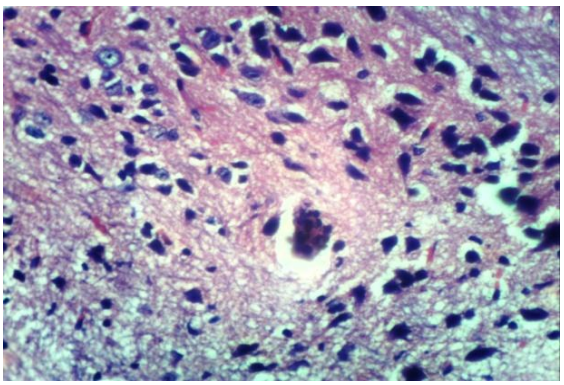

Fig. (11): Brain of mice from group A1 showing necrosis of neurons and neuronophagia (H & E X 400).

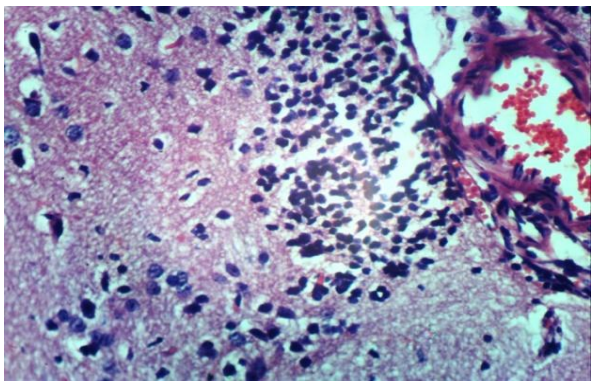

Fig. (12): Brain of mice from group A3 showing focal gliosis (H & E X 400).

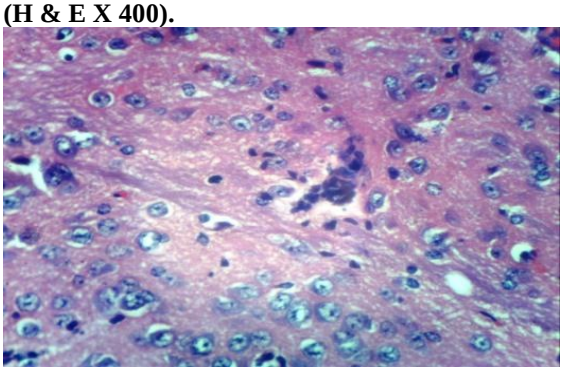

Fig. (13): Brain of mice from group A2 showing focal necrosis infiltrated with glia cells (H & E X 400).

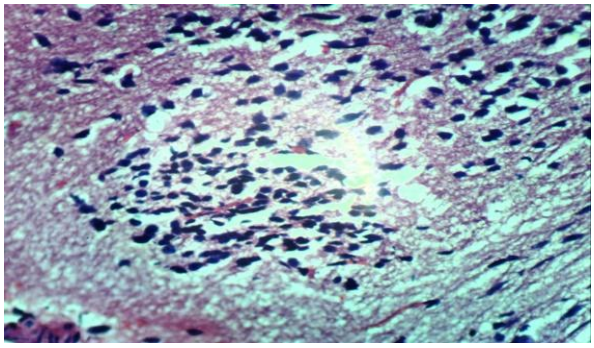

Fig. (14): Brain of mice from group A4 showing focal necrosis of neurons infiltrated with glia cells (H & E X 400).

Immunological profile:

Phagocytic activity percentages (Phage); lymphocytes transformation capacity (Lyt1); values of interleukin 2 (IL2); interleukin 6 (IL6) and tumor necrosis factor (TNF) were measured to assess the immunological stimulation efficacy of *C. zeylanicum* extract and to evaluate the significance of intervention time for treating the mycosis using it, results were depicted in tables 2 and 3.Statistical analysis was applied using one way ANOVA/SPSS.

Table (2): Showing Immunological parameters values among all tested groups:

		A1 (n= 3)	A2 (n= 3)	A3 (n= 3)	A4 (n= 3)	A5 (n= 3)
Phage	Mean	76.4667	76.5067	80.2667	80.8433	89.3333
	SD	3.86307	3.47327	4.42982	4.76593	4.30968
Kill	Mean	70.9333	74.1000	75.5000	76.2667	84.9333
	SD	3.95517	2.75136	5.15655	4.37531	5.21568
Lyt 1	Mean	1.1833	1.2100	1.4033	1.4600	1.6333
	SD	.12662	.08185	.17559	.10149	.61460
IL6	Mean	496.0833	465.4633	374.6167	405.5433	238.8800
	SD	64.98828	.48014	13.83812	14.78130	11.62476
IL2	Mean	193.3133	186.5567	187.4100	179.9467	167.4667
	SD	9.46088	7.75016	11.24827	9.66580	5.09912
TNF	Mean	182.1400	168.7167	164.4667	169.6900	138.7267
	SD	11.26012	10.40734	7.79219	3.09264	10.29692

Table (3): Showing correlation between immunological parameters and intervention time:

Dependent Variable	(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.
Phagocytic Activity	A1 (untreated)	A2	-.00190-	.14282	1.000
		A3	-.18095-	.16159	.908
		A4	-.20841-	.16867	.864
		A5 (Control)	-.61270-	.15912	.104
	A2	A3	-.17905-	.15476	.894
		A4	-.20651-	.16213	.847
		A5 (Control)	-.61079-	.15217	.096
	A3	A4	-.02746-	.17889	1.000
		A5 (Control)	-.43175-	.16992	.316
	A4	A5 (Control)	-.40429-	.17666	.396
Killing Activity	A1 (untreated)	A2	-.13533-	.11888	.899
		A3	-.19516-	.16034	.871
		A4	-.22792-	.14552	.711
		A5 (Control)	-.59829-	.16150	.126
	A2	A3	-.05983-	.14421	1.000
		A4	-.09259-	.12752	.989
		A5 (Control)	-.46296-	.14549	.223
	A3	A4	-.03276-	.16686	1.000
		A5 (Control)	-.40313-	.18096	.417
	A4	A5 (Control)	-.37037-	.16797	.428
Lymphocyte Transformation (Lyt1)	A1 (untreated)	A2	-.02186-	.07135	1.000
		A3	-.18033-	.10245	.620
		A4	-.22678-	.07679	.226
		A5 (Control)	-.36885-	.29696	.848
	A2	A3	-.15847-	.09168	.645
		A4	-.20492-	.06170	.166
		A5 (Control)	-.34699-	.29342	.869
	A3	A4	-.04645-	.09598	.999
		A5 (Control)	-.18852-	.30249	.994
	A4	A5 (Control)	-.14208-	.29479	.999
Interleukin 6 (IL6)	A1 (untreated)	A2	.09892	.12121	.969
		A3	.39240	.12393	.285
		A4	.29249	.12431	.451
		A5 (Control)	.83089	.12314	.070
	A2	A3	.29348*	.02583	**.029**
		A4	.19357	.02758	.073
		A5 (Control)	.73198*	.02170	**.003**
	A3	A4	-.09991-	.03777	.288
		A5 (Control)	.43850*	.03371	**.001**
	A4	A5 (Control)	.53841*	.03507	**.001**
Interleukin 2 (IL2)	A1 (untreated)	A2	.16917	.17679	.955
		A3	.14781	.21247	.993
		A4	.33467	.19552	.640
		A5 (Control)	.64714	.15536	.116
	A2	A3	-.02137-	.19746	1.000
		A4	.16550	.17909	.962
		A5 (Control)	.47797	.13411	.151
	A3	A4	.18686	.21439	.973
		A5 (Control)	.49933	.17853	.308
	A4	A5 (Control)	.31247	.15797	.538
Tumor Necrosis Factor (TNF)	A1 (untreated)	A2	.20977	.13834	.736
		A3	.27619	.12355	.427
		A4	.19456	.10536	.609
		A5 (Control)	.67844*	.13767	**.047**
	A2	A3	.06642	.11730	.998
		A4	-.01521-	.09796	1.000
		A5 (Control)	.46867	.13209	.132
	A3	A4	-.08163-	.07564	.910
		A5 (Control)	.40225	.11651	.153
	A4	A5 (Control)	.48388	.09700	.110

Discussion:

Cinnamon is famous in traditional Chinese medicine as a carminative, it is used for most gastrointestinal complaints **(Nadkarni, [18])**. In addition, it is often present in traditional Chinese formulas to facilitate the action of the other herbs present, cinnamon is frequently used as a cardio-tonic, which helps to stimulate blood flow **Bensky and Gamble[19]**. Antioxidants properties of cinnamon extract were reported in many previous studies confirming its expressed ferric reducing antioxidant power (FRAP)**(Tenore*et al.*, [20]).**

The current study describes the effectiveness of *C. zeylanicum* whole plant extract against *Aspergillus niger* ATCC 16404 reference strain *invivo* in experimental laboratory animals.

Preliminary experiments were carried out *in vitro* using *C. zeylanicum* essential oil on solid medium through solid medium drop diffusion technique in order to evaluate the effect of some essential oils, including it, on the fungal growth **(Alarousy,[21]).** Results showed that *C. zeylanicum* had the strongest fungicidal effect on fungal cells.

Aspergillusniger, that is known as environmental fungal species, was used as a model in this preliminary study and other species will be used in next studies aspathogenic models.

The antimycoticactivity of phytochemicals found in *C. zeylanicum* extractmay involve inhibition ofextracellular enzymes synthesis of the cellwall structure of fungal cells, cellular damage that leads to cellular death **(Brul and Coote, [22]; Burt, [23] and Atanda *et al.*, [24]). Atanda, *et al.*[24] and Rasooli, *et al.*[25]** also reported that *C. zeylanicum*extract is able to interfere with the enzymatic reactions, suchas respiratory electron transport, protein transport and coupledphosphorylation that take place in the mitochondrial membrane.

It was necessary to explore whether the fungicidal effect is restricted on the essential oils only or not, as it would be difficult somehow to assess the fungicidaleffect of *C. zeylanicum* essential oil in the laboratory animals (*in vivo*) due to the difficulties of injecting oils in living bodies. In our study, we tried to test this concept and to approve that the fungicidal effect is due to the entire compounds of the plant as well as its essential oil. So, we used the whole plant extract to inject the pre-infected mice with it in order to track the efficacy of the plant extract inside the animal body for its fungicidal effect as well as its capacity for immune system stimulation. Beside, we designed the experiment to use the extract after the infection of mice with *Aspergillus niger* ATCC 16404 reference strain with different time intervals (three, seven and ten days post infection).

Histopathological profile:

Histopathological examination of lung tissues:

Histopathological lesions are the phenotypic profile representing the direct correlation between defense mechanisms of the host and virulence of infectious agent **(Tochigi*et al.*, [26])**

Results depicted in table (1) revealed that the lungs from group A1 mice who were injected with the fungal spores only, were the most damaged lungs in comparison to the other four groups used in this study, followed by lungs from group A3 and A4 who received the *C. zeylanicum* extract seven days and ten days' post infection respectively. Histopathological results confirmed the harmful effect of the mold on lung tissues, examined lung tissues of mice from group A1 revealed marked dilatation and congestion of pulmonary blood vessels (Fig. 1), focal interstitial inflammatory cells infiltration (Fig. 2) and focal pulmonary emphysema. However, examined sections from group A2, who received the *C. zeylanicum* extract three days post infectionshowed improvement in histopathological picture as the lung showed dilatation and slight congestion of pulmonary blood vessels (Fig. 3) and focal pulmonary emphysema (Fig. 4). Meanwhile, lung of mice from group A3 revealed congestion of perialveolar blood capillaries and focal mononuclear inflammatory cells aggregation (Fig. 5). Examined sections of lung of mice from group A4 showed severecongestion of all pulmonary blood vessels, focal emphysema and interstitial pneumonia (Fig. 6). These findings agreed to those explained by **Tochigi *et al*[26] (2013)**, they identified two distinct patterns of histopathological findings in lung tissues, one of those pattern involves a distinct nodule consisting of demarcated round-shaped coagulative necrosis, while the other pattern involves fused lobular integration, which corresponds to the usual bronchopneumonia which is characterized histologically by the filling of acute inflammatory exudates with a fungal proliferation in alveoli.

Histopathological findings of liver tissues:

findings of group A1 revealed congestion of central vein, perivascular inflammatory cells infiltration (Fig. 7), Kupffer cells activation and focal hepatic necrosis associated with inflammatory cells infiltration. Meanwhile, liver of mice from group A2 revealed no histopathological changes except hydropic degeneration of hepatocytes (Fig. 8). In spite of the slightly normal gross appearance of livers extracted from groups A3& A4, livers showed more or less similar histopathological changes confined as Kupffer cells activation and focal hepatic necrosis associated with inflammatory cells infiltration (Figs. 9 & 10).

No liver abscesses were detected in the current study, in contrary to the expected results. According to **Kumar *et al*., [27]**, the organisms reach the liver either through ascending infection in biliary tract, or vascular seeding, either portal or arterial, or direct invasion of the liver from a nearby source or through penetrating injury**.**

Histopathological profile of brain tissues:

Histological findings of brain tissues were completely different, the brain tissues from the groups: A1, A3 and A4 showed variable degrees of necrosis as shown in figures (11, 12 and 14). However, 20% only of group A2 showed gliosis as shown in figure 13. These results elucidated the limited fungicidal effect of the examined herbal extract in brain. This might be due to rapid growth of lesions caused by Aspergillus species if reached the brain tissue.

The rapiddevelopment of lesions could be explained by three possible mechanisms. First, the origins of the multiple perforator arteries are in proximity along the parent artery and are presumably vulnerable to an extending vasculo-pathy of the parent artery wall. There is an evidence suggesting that the necessary conditions for the vasculo-pathy are present. First, endothelial cells in culture have been shown to engulf the organism **Paris *et al*., [28]** and Aspergillus has been seen to infiltrate and destroy the internal elastic lamina of major cerebral arteries (**Chou*et al*., [29]).** Second, any infarction, thromboembolic or septic, may have associated secondary edema, which increases during the first few days after the arterial occlusion. Third, Aspergillus hyphae were frequently observed within the parenchymal lesions at autopsy; infection of the infarcted tissue may be aggressive, and direct extension into the surrounding brain may progress quickly **(DeLone*et al*., [30])**.

Aspergillosis as an invasive fungal infection has an inflammatory properties as revealed through the histopathological profile of the current studyand*C. zeylanicum*extract showed good fungicidal capacity less or more the expected result, hence cinnamon park extract was reported previously to have strong antimicrobial activity **(Shan *et al*., [31])**.Comparing the histopathological profiles between groups A1 and A5 revealed the fungicidal activity of *C. zeylanicumin vivo* and comparing the histopathological profiles of the three treated groups (A2, A3 and A4) using *C. zeylanicum*extract also showed the significance of early intervention with this herbal extract, as all the results showed the marked improvement of histopathological profile in group A2 which received the treatment three days post infection. Absence of toxicity signs on experimental animals indicated the safety of using *C. zeylanicum*extract *in vivo*.

Immunological profile:

To assess the primary capacity of *C. zeylanicum*extract for stimulating the immune system, values of phagocytic activity (phage); killing activity of macrophage; lymphocyte transformation activity (Lyt1); IL2; IL6 and TNF were measured and compared between the untreated group (A1) and all treated groups (A2; A3; A4 and A5), regardless the intervention time, from one side and withinthe three treated groups (A2; A3 and A4) from the other side.

Results of multiple comparison between all the treated groups (A2; A3 and A4), regardless intervention time, and A1 group were significant except for TNF (p value = 0.047) between A1 and A5 (the accepted mean difference is significant at the 0.05 value). Significance was the least between untreated group (A1) and control

negative group (A5),as for phage (p value = 0.10); for killing capacity (p value = 0.126); for IL6 (p value = 0.07) and for IL2 (p value = 0.116), while for TNF, it didn't show accepted significance (p value = 0.047) as mentioned before. These results were matching with the reported ones by **Carballo*et al*. [32] (1998).**

In the other hand, another factor was correlated to the efficacy of medicinal application of the extract which is the initiation time of using *C. zeylanicum*extract *in vivo*. The relation between the immune stimulating capacity of *C. zeylanicum*and the intervention time of its use was clarified in table (3), as for phage (F= 4.703); killing activity (F= 4.244); Lyt1 (F= 1.178); IL2 (F= 3.689) and TNF (F= 9.296) all values showed significant differences within the three treated groups (A2, A3 and A4). Meanwhile IL6 values were significant only between (A4 & A2) and between (A4 & A3) and there wasn't significance between the values in groups A2 and A3 (P value = 0.029). Hence, these results indicated the capacity of immune stimulation of the extract in correlation to the intervention beginning time and this is a novel character of *C. zeylanicum* extract application medicinally for treating latent and/ or chronic fungal infection as well as its usage as prophylaxis by immunocompromised patients. Further studies will be conducted to investigate deeper the time factor effect on the efficacy of the therapeutic application of the extract.

The conclusion from the current study revealed that *Cinnamomum zeylanicum* extract has strong fungicidal activity and great immune system stimulating efficacy with correlation to intervention time as well. Results proved that the tested natural herbal extract is more advantageous than the synthetic agents due to its biodegradability, potency, absence of toxicity, efficacy as a prophylactic agent for immunocompromised patients and future studies will be done to analyze the herbal components of *Cinnamomumzeylanicum*and compare them with the currently available antimycotic pharmaceutical preparations. *A. niger* ATCC 16404 reference strain was chosen in this study as preliminary tested strain and further investigation will be carried out on other fungal species.

Conflict of interest:
The authors declare that there is no conflict of interest.

References:

1. Lehrnbecher, T., Frank, C., Engels, K., *et al*.(2010)Trends in the postmortem epidemiolgy at a university hospital. J Infect. 61(3), 259-265
2. Stevens, D.A., Kan,v.l, *et al*.(2000)Practice guidelines for diseases caused by Aspergillus. Clin. Infect. Dis. 30, 696-709
3. Del Poeta, M., Chaturvedi, V. (2012) Cryptococcus and cryptococcosis in the twenty-first century.*Mycopathologia*; 173, 283–285
4. Pfaller, M.A andDiekema, D.J.(2007) Epidemiology of invasive candidiasis: a persistent public health problem. *ClinMicrobiol Rev.* 20(1), 133-163.
5. Pfaller, M.A. andDiekema, D.J.(2010)Epidemiology of invasive mycoses in North America. *Crit. Rev. Microbiol.*36, 1–53
6. Pfaller, M.A. (2012)Antifungal drug resistance: mechanisms, epidemiology, and consequences for treatment. *Am J Med.*125(1 Suppl), S3-13
7. Karthaus, M. (2011)Prophylaxis and treatment of invasive aspergillosis with voriconazole, posaconazole and caspofungin-Review of the literature. *Eur. J. Med. Res.*; 16, 145-152
8. Puri, A., *et al*. (1993) Immunostimulant agents from Andrographispaniculata. *J. Nat. Prod.* 56, 995-999
9. Suresh, K., Vasudevan, D.M. (1994)Augmentation of murine natural killer cell and antibody dependent cellular cytotoxicity activities by Phyllanthusemblica, a new immunomoldulator. J. Ethnopharmacol.44, 55-60
10. Richard, J.L. and Beneke, E.S. (1989)Mycosis and Mycotoxicosis. In a Laboratory Manual for the Isolation and identification of Avian Pathogens. Kendall/Hunt Publishing Co., Dubuque, Iowa, 70-76
11. Nandam, S.S. and Vangalapati, M.(2012)Studies on extraction of cinnamaldehyde from cinnamon species. LAP LAMBERT Academic Publishing ISBN-13: 978-3-8383-4979-4
12. Bancroft, D., Stevens, A. and Turner, R.(1996) Theory and Practice of Histological Techniques, Churchill Livingstone, London, UK,4th edition

13. Denizot, F., Lang, R.(1986)Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods.89 (2), 271–277
14. Maslak, D.M. and Reynolds, D.L. (1995)Mitogenic responses of the head-associated lymphoid tissues of the chicken. Avian Diseases. 39(1), 1–8
15. Rouse, D., Mackay, P., Stirnberg, P., Estelle, M., and Leyser, O.(1998)Changes in auxin response from mutations in an Aux/IAA gene. *Science*. 279, 1371–1373
16. Woldehiwet, Z. and Rowan, T.G.(1990)In vitro replication of bovid herpesvirus-1 in macrophages derived from peripheral blood leucocytes of calves. J. Comp Pathol. 103(2), 183-189. doi:10.1016/S0021-9975(08)80174-0
17. Nelson, D.S.(1974)Immunity to infection, allograft immunity and tumor immunity: parallels and contrasts. Transplant. Rev. 19, 226
18. Nadkarni, A.K.(1954) Indian MateriaMedica, with Ayurvedic, Unani and Home Remedies. Revised and enlarged by Nadkarni AK. Reprint. Bombay: Bombay Popular Prakashan PVP. P.1135. 1976
19. Bensky, D. and Gamble, A. (1993) Chinese Herbal Medicine: *Materia Medica*. Eastland Press: Seattle
20. Tenore, G.C., Ciampaglia, R., Arnold, N.A., *et al*.(2011)Antimicrobial and antioxidant properties of the essential oil of Salvia lanigera from Cyprus. Food Chem. Toxicol. 49, 238–243
21. Alarousy, R.M. (2004)Molecular biology studies on *Cryptococcus neoformans*. M. V. Sc. Thesis. Faculty of Veterinary Medicine, Cairo University, Giza, Egypt
22. Brul, S. and Coote, P. (1999) Preservative agents in foods: mode of action and microbial resistance mechanisms. Int. J. Food Microbiol.50, 1-17
23. Burt, S. (2004) Essential oils: their antibacterial properties and potential applications in foods – a review. *Int. J. Food Microbiol.* 94, 223-253
24. Atanda, O.O., Akpan, I., Oluwafemi, F. (2006)The potential of some essential oils in the control of *A. parasiticus*CFR 223 and aflatoxin production. *Food Cont.* 18, 601-607
25. Rasooli, I., Rezaei, M.B., Allameh, A. (2006)Growth inhibition and morphological alterations of *Aspergillus niger* by essential oils from *Thymus eriocalyx* and *Thymus x-porlock*. Food Conti. 17, 359-364
26. Tochigi, N., Yoichiro, O., Tsunehiro, A., *et al*.(2013) Histopathological Implications of Aspergillus Infection in Lung. Mediators of Inflammation. Article ID 809798, 8 pages http://dx.doi.org/10.1155/2013/809798
27. Kumar, V., Cotran, S. and Robbins, S. (2010)Basic pathology . Illustration by James A. Perkins Suunders , 7th .edition , 2003: 610. (Cited in J Fac Med Baghdad. 52 (4), 480 – 483
28. Paris, S., Boisvieux-Ulrich, E., Crestani, B., *et al*. (1997)Internalization of *Aspergillus fumigatus*conidia by epithelial and endothelial cells. Infect Immun. 65, 1510-1514
29. Chou, S.M., Chong, Y.Y., Kinkel, R. A.(1993)proposed pathogenetic process in the formation of *Aspergillus*mycotic aneurysm in the central nervous system. Ann. Acad. Med. Singapore 22 (3), 518-525
30. DeLone, D.R., Goldstein, R.A., Petermann, G.*et al*. (1999) Disseminated Aspergillosis Involving the Brain: Distribution and Imaging Characteristics. AJNR Am J Neuroradiol. 20,1597–1604
31. Shan, L., He, P., and Sheen, J.(2007)Intercepting host MAPK signaling cascades by bacterial type III effectors. Cell Host Microbe. 1, 167–174
32. Carballo, E.1., Lai, W.S. and Blackshear, P.J.(1998) Feedback inhibition of macrophage tumor necrosis factor-alpha production by tristetraprolin. *Science*. 281 (5379), 1001-1005

*****