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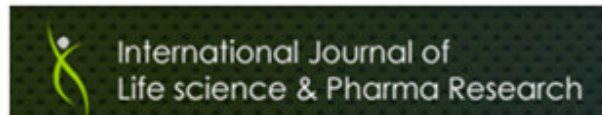
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INVITRO STUDY OF ASPERGILLOSIS AND THE EFFECT OF SELECTED TRADITIONAL PLANTS AGAINST THE PATHOGENIC FUNGAL ORGANISM OF *ASPERGILLUS FUMIGATES*

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ABSTRACT

The present study was designed to obtain the information about the allergenic proteins present in *Aspergillus fumigatus* from mycelial extracts. *A. fumigatus* was isolated from the decayed vegetables and mass cultivated done for 14 days. The experimental crude extracts were prepared from *A. fumigatus*, estimated the proteins by ammonium precipitation method. The fungal spores were subjected to experimental rabbit and the serum sample was collected from the infected rabbit for further analysis such as the immunological techniques are Immuno-electrophoresis, single radial immuno diffusion and western blotting technique were completed for determined the specificity and molecular weight of the specific antigen present in the fungal sample affected in experimental rabbit. The current result showed the presence of the specific antigen on mycelial extract against antiserum. Through, the immune blot technique the molecular weight of antigen from mycelial extract contained 42k Da compared with molecular weight of different antigenic protein obtained by SDS-PAGE. The effect of various plant extract were observed through Kirby Baeyer technique and by direct intake of the infected rabbit. The *Coriandrum sativum* showed greater effect against *A.fumigatus*. Although, among the six plants *C. sativum* possessed significant effect against the experimental pathogens. So the important phytoactive compounds are elucidated by the GCMS analysis as well as the following important bioactive constituents were identified such as 11-tetra decenoic acid, carpic acid (12.7%), undecyl alcohol (6.4%), tri decenoic acid (5.5%), undecenoic acid (7%). The presence of tri decenoic acid was found to be too low asphenols, fixed fats and oils also been noted. Hence, the present study was concluded that the medicinal plant of *C. sativum* comprised superior antifungal consequences against the pathogenic fungi of *A. fumigates*. Therefore, *C. sativum* is potential used for pharmaceutical purpose for arrest or destroy the aspergillosis.

Key words: Aspergillosis, SDS-PAGE, Immunoblot, GCMS, *Aspergillus fumigatus*

INTRODUCTION

Aspergillosis is one of the forms of hypersensitivity and is called as type I or immediate hypersensitivity .It is characterized by excessive activation of certain white blood cells called mast cells and basophiles by a type of antibody known as Ig-E, resulting in an extreme inflammatory response (Tonnel *et al.*,

2005). The most common types of fungi that causes allergic like asthma belong to Ascomycetes such as *Aspergillus*, *Pencillium*, *Cladosporium* and *Alternaria*, Among this *Aspergillus* is a ubiquitous mould in the environment. Ig-E reactivity to glucoamylase, cellulose and hemicellulase from *Aspergillus* was established by skin test and immune blotting (Bhanuet *al.*, 2003) Most patients are

symptomatic and present with poorly controlled asthma, low grade fever, weight loss, malaise, and wheezing, bronchial hyper reactivity, expectoration of brownish- black mucus plugs, haemoptysis or productive cough (Ashok.*etal.*,2008). Plants generally produce many secondary metabolites which constitute an important source of microbicides, pesticides and many pharmaceutical drugs. Antimicrobial activity of plant essential oils also used recently (Thenmozhi *et al.*, 2011).The study was to analyze *A. fumigates* mycelial extracts from short term shake cultures by SDSPAGE and immunological studies for components able to bind to antibodies from experimental rabbit antiserum.In this study different plants were examined against *A. fumigates*. Coriander (*Coriandrum sativum* L.) also called as “cilantro” is an annual herbaceous plant originally from the Mediterranean and Middle Eastern regions, cultivated for its culinary, aromatic and medicinal use (Mildner-Szkudlarz *et al.*, 2009). This plant is of economic importance since it has been used as a flavoring agent in food products, perfumes, cosmetics and drugs. This culinary and medicinal plant widely distributed and mainly cultivated for the seeds which contain an essential oil (ranges between 0.3% and 1.1%) (Neffati *et al.*, 2011).

MATERIALS AND METHODS

Sample collection and Isolation of *A. fumigatus*: Decayed vegetable samples were collected and 1g of vegetable sample was serially diluted upto 1:100000. 0.1ml aliquots of sample from 3rd, 4th and 5th dilution were plated on sabaoraud dextrose agar medium by spread plate method.

Identification of *A. fumigatus*

Slide culture technique:Take a sterile Petri plate and place a piece of moisten cotton. Place the fungal agar block slide set up on the Petri plate and incubates at 24°C for 48 hours. After the visual growth of fungal, remove the agar block from the slide and add a drop of lacto phenol cotton blue stain on the slide. Lacto phenol Cotton Blue Staining: Place a drop of LPCB reagent on a clean glass slide. Remove the small portion of colony and placed it in a drop. Place the cover glass and apply gently pressure. Examine the preparation microscopically.

The Cultivation of *A. fumigatus* was carried out in 150ml sterilized Erlenmeyer flasks containing 50ml of Potato Dextrose Broth medium. Flask was inoculated with 1ml of suspension of *A. fumigates* spores to a final concentration of 1×10^3 spores/ml. The pressed wet weight of the mycelium also measured each day. Biomass estimation and filtration: Fungal biomass was measured gravimetrically. Culture medium was filtered through Whatmann No.1 filter paper. The mycelium retained was washed with a saline solution and each day samples were dried to constancy at 60°C for 48 hours. Fungal colonies were separated from medium for all specified days by using Whatmanns No.1 filter paper in sterile condition were washed 3 times with sterile PBS. The *fumigatus* cultue were spread on to PDA plate. After the growth of fungi three wells were made and the extracts fo *C. sativun* extract were added as amount of 100, 150, 200 μ l were added. Subsequently after three days result was observed. Antifungal activity of the plant extract was tested on *A. fumigatus*. One ml of fungal suspension was inoculated in 20 ml of Potato dextrose agar and was poured into the germ culture plates. The holes were then created by the punch in the medium and were filled by the plant extract. The plates were incubated for 7 days at 30°C to 35°C and the results were recorded during this period. Cell fractionation: Cell disruption was performed using glass beads (1mm dm) on a vortex mixture disrupter for one minute until 80 to 90% cells were disrupt (Kim *et al.*, 1978). Preparation of Mycelial extracts antigens and estimation of protein: The organism was grown in Potato Dextrose Broth in aerated culture. After the incubation period mycelium was obtained by filtration, washed 3 times with PBS and homogenized in a vortex mixture. Microscopic examination of the preparation revealed <80% breakage of the hyphae. The extract obtained was centrifuged at 10000rpm for 30 minute and the supernatant were collected. The mycelia proteins were estimated by ammonium precipitation method. The Mycelia extract was purified by dialysis method.

Experimental exposure of *A. fumigatus* spores for inhalation to rabbit

Rabbit were exposed to the inhalation of *A. fumigatus* spores by aerosol. The tests were carried out with the circulating blood samples taken at post exposure and pre exposure with one week intervals. In this experiment female white rabbits weighing 2.5Kg divided into 2 groups. A) Test animal. B) Control. 3ml of blood collected from Rabbit before the exposure of spores. A total 10gm of fine dust consisting of the spores of *A. fumigates* was collected from PDA plates. In Erlenmeyer flasks, the spores were dried for 10 days at 37°C. 5gm of spore powder was placed in a sack made from sterile gauze hung underneath the lid and the spores were dispersed into the air of the cage for 15 minutes. The experiment was repeated after 6 days. Blood samples were collected for precipitation tests from the ear vein of each rabbit are tested before and after the first and second exposure. The physiological changes were observed through 2 weeks daily.

Technical studies

Determination of mycelial protein by SDS PAGE: SDS-PAGE was conducted using a minigel system to determine the molecular weight of mycelial extract followed by the methodology (Veronica *et al.*, 1996). Electrophoresis was conducted using a discontinuous buffer system (Laemli1989). Determination of allergenic proteins done by western blotting and enhanced chemiluminescence (Kricka, 2003; Kurien, 2006). Single radial immune diffusion (Shirley *et al.*, 1976) were done to analyze the specific antibodies and Immuno electrophoresis (Chaparasetal., 1978) was performed to detect specific antigen. Through immune blot technique and enhanced chemiluminescence the molecular weight of antigen were detected.

Effect of Selected traditionalplants against *A. fumigatus*

The extract of *Eucalyptus acaciiformis* (H. Deane and Maiden), *Azadirachta indica* (A. Juss), *Biophytum sensitivum* (L.), *Centella asiatica* (L.), *Pergularia daemi* (Forssk.), *Coriandrum sativum* (L.) was treated against *A. fumigates*

GC MS analysis of Screened plant extract

Among the six plants, *C. sativum* showed greater effect against *A. fumigatus*. The components of *C. sativum* were done using GC MS analysis.

RESULTS

From the vegetable samples the fungi were isolated and identified. The macroscopic and microscopic observation of *A. fumigatus* shown in (Fig-1). The wet weight of mycelium revealed active growth at first two days and slightly decreased and then increased. In mycelial extract *A. fumigatus* contained 0.71-1.95µg of protein/ml of mycelium. The result was represented in (Fig-2). The morphological study showed various changes in infected rabbit (Fig-3). In addition it showed physiological changes including weight loss, increasing body temperature, loss of appetite, nasal secretion and later showed aspergillosis infection (Table-1). Owing to infection the body weight of experimental rabbit was decreased day by day (Fig-4). In single radial immune-diffusion technique, specific antigen antibody complex showed precipitation reaction with zone formation (Fig-5). The result for SDS-PAGE profile clearly the molecular weight of antigen in 14th day of mycelial extract is 42kDa. The results exposed to the X-ray sheet indicate the black lines given the confirmatory report of antigenic molecular weight (Fig-6). The antifungal effect of ethenolic extract of selected plants were treated against *A. fumigates* (Table-2). Among this *C. sativum* showed greater inhibition effect on *A. fumigatus* by well diffusion method (Fig-8). Through GCMS analysis (Fig-9) the phytochemical screening of the

components of *C. sativum* are detected as the presence of 2-decenoic acid were found to be greater, i.e, 30.8%, when compared with other components like 11-tetra decenoic acid, carpic acid (12.7%), undecyl

alcohol (6.4%), tri decenoic acid(5.5%), undecenoic acid (7%). The presence of tri-decenoic acid was found to be too low (Table3).

Figure 1
The appearance of the experimental organism of A. fumigates (A) colony structure (B) Microscopic observation

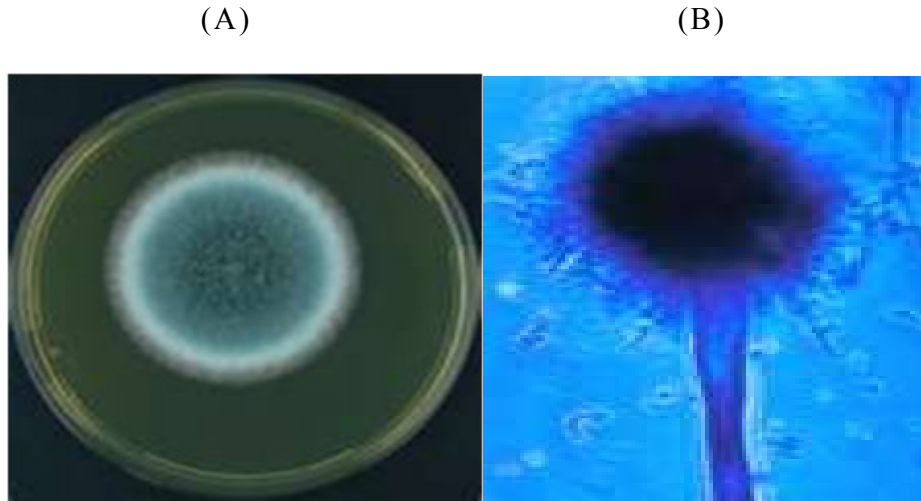


Figure 2
Wet weight of mycelium extract (A) and Protein estimation in mycelial extract (B)

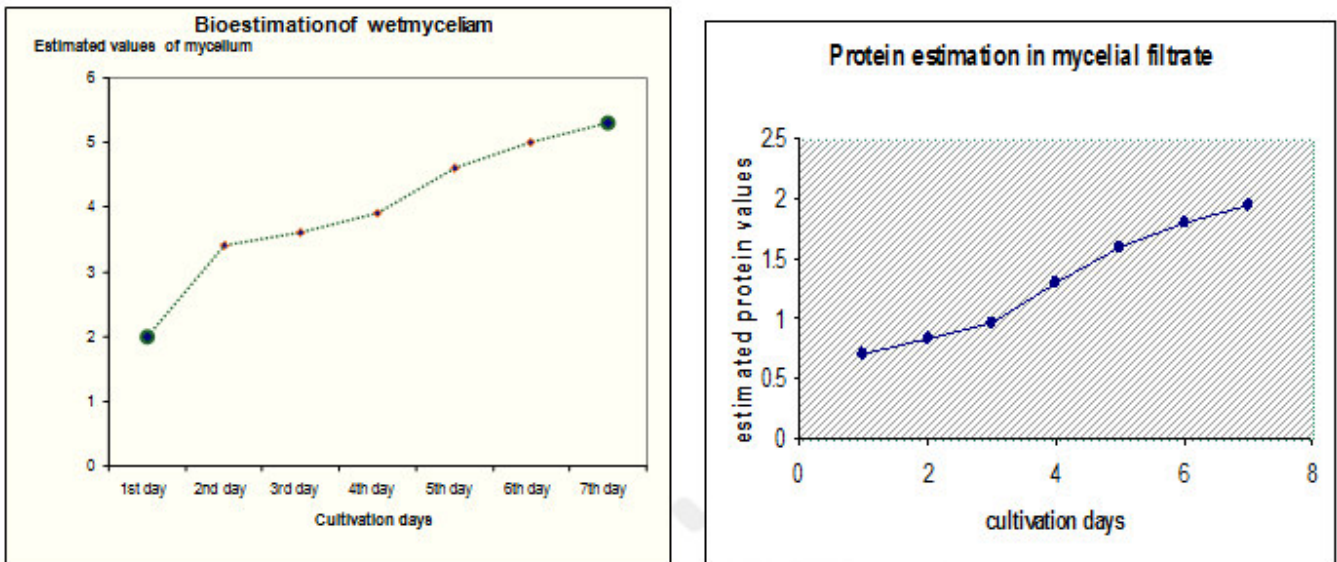


Figure 3
The morphological changes of Experimental animal of rabbit show
(A) Before treated (B) Treated (C) After treated (After recovered)

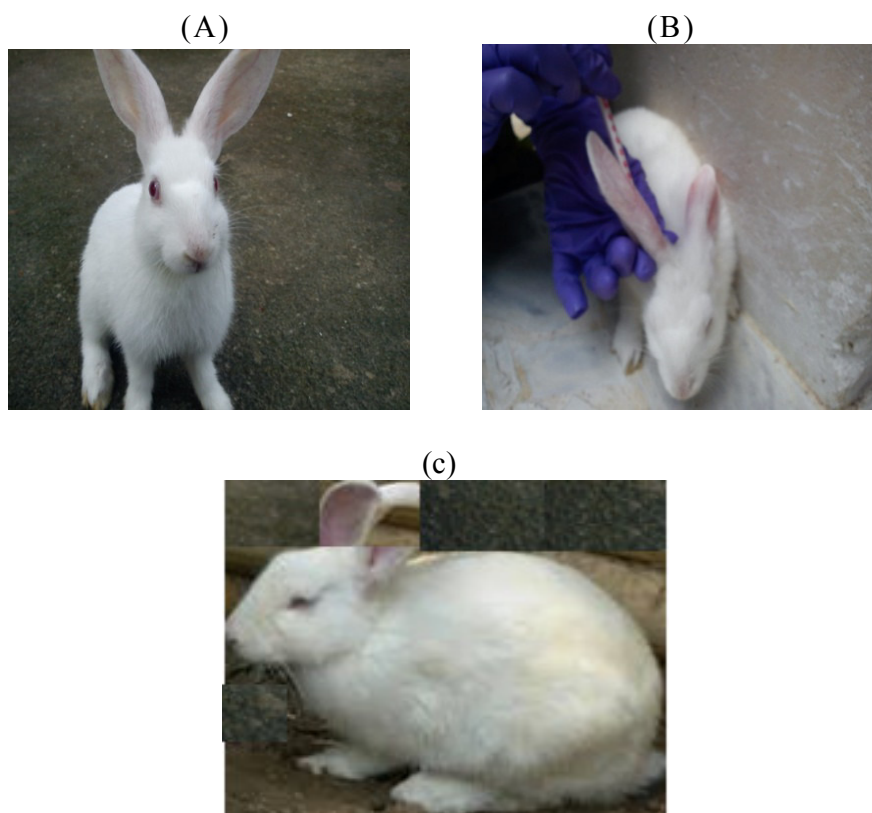


Table 1
Physiological Changes in Rabbit

Total days of Experiment	Exposure of spores to rabbit	Morphological changes occur in rabbit	Loss of weight occurred in infected rabbit
1 Day	1 st exposure	Sneezing	2.5kg
2 Day	NO	Nasal mucus secretion	-
3 Day		-	-
4 Day		Rapid weight loss	2.1kg
5 Day	-	Increased body temperature	-
6 Day	-	-	-
7 Day	2 nd exposure	Rapid breathing and weight loss	-
8 Day	-	Increased body temperature	1.6kg
9 Day	-	Breathing very slow	-
10 Day	-	Ruffled fur	-
11 Day	-	Loss of appetite	1.4kg
12 Day	-	Bloody nasal discharge	-
13 Day	-	Increased body temperature	1.2kg
14 Day	-	<i>Allergic aspergillosis</i>	1.2kg

Figure 4
Shows infected rabbit has decreasing body weight

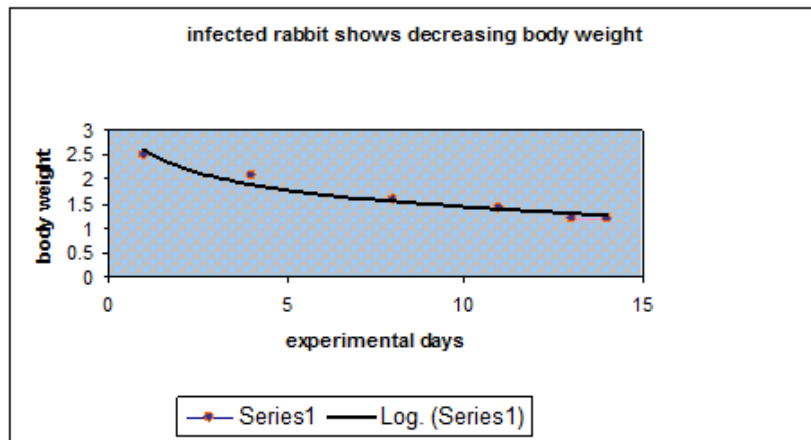


Figure 5
Single radial immune-diffusion shows specific antigen and A. fumigates

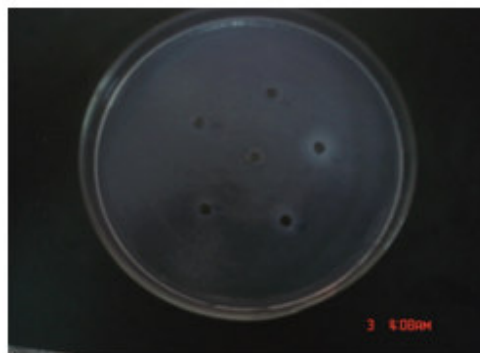


Figure 6
SDS-PAGE mycelial extract antigen from *A. fumigatus* (A) BSA Standard and Mycelial Extract (B) chemiluminescence Antigen

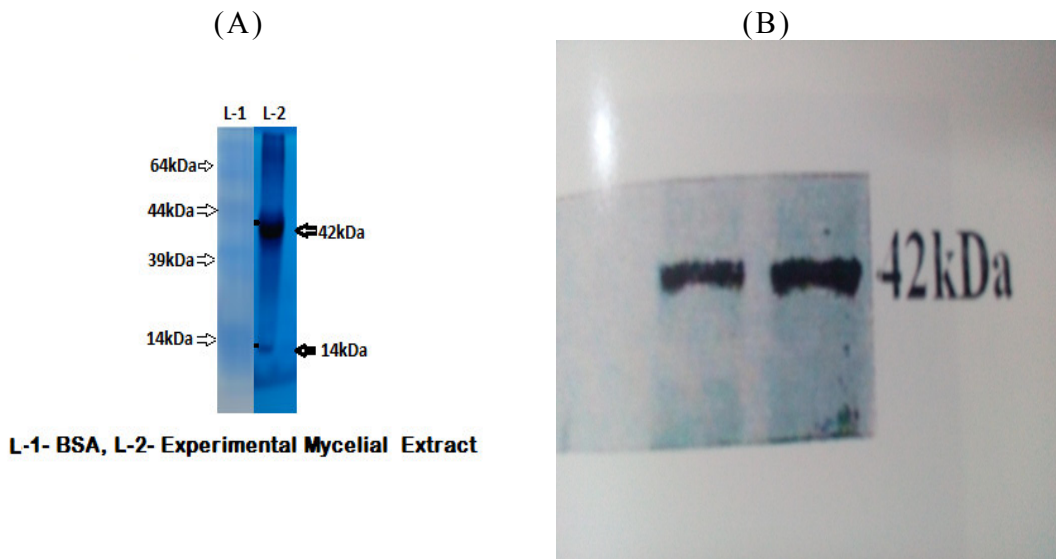


Table 2
Antifungal effect of ethanolic extract of selected plants against *A. fumigatus* by well diffusion method

Sl.No	medicinal plants	Zone of inhibition shows at different concentration in mm diameter.		
		100µl	150µl	200µl
1	<i>E. acaciiformis</i>	13	15	18
2	<i>A. indica</i>	10	16	22
3	<i>B. sensitivum</i>	12	14	16
4	<i>C. asiatica</i>	22	27	32
5	<i>P. daemi</i>	6	18	24
6	<i>C. sativum</i>	24	28	35

Figure 8
The antifungal activity of *C. sativum* show inhibition on *A. fumigatus* by well diffusion method



Figure 9
Compound elucidation of leaf from the C. sativum plant through GC-MS Chromatogram Analysis

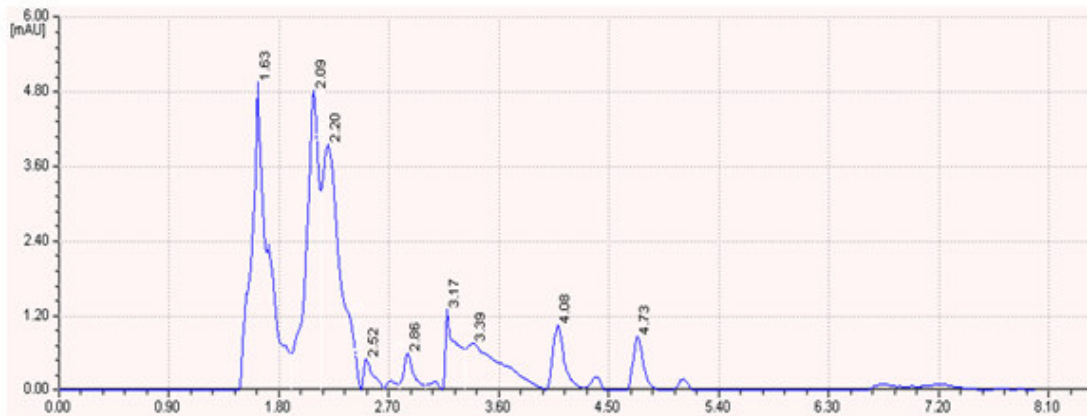


Table 3
Analysed Phytoactive compounds through GCMS –Analysis

Parameter	Result	Group Result	Sample Inf						
ID	Name	RT(min)	Height	Area	Conc	Half Width(s)	Res	Theo Plate	Tail Fact
1		1.625	510	4972.2	27.0950	9.75	0.00	553.70	1.09
2		2.090	496	3772.9	20.5598	7.61	1.89	1504.76	0.66
1	Item 1	2.200	410	4016.0	21.8847	9.80	0.45	1005.40	3.22
5	Item 5	2.517	63	416.5	2.2696	6.61	1.36	2888.37	2.11
6	4	2.858	73	490.7	2.6742	6.72	1.81	3603.30	1.53
6		3.173	144	972.3	5.2984	6.75	1.65	4402.46	2.56
7		3.392	88	1995.3	10.8729	22.67	0.52	445.97	4.89
8		4.078	117	921.6	5.0219	7.88	1.59	5343.34	1.58
9		4.732	102	793.4	4.3234	7.78	2.95	7375.79	1.69
		∑i%AE:	iAE=2003	iAE=18350.9	iAE=100.0000				

DISCUSSION

The present study was designed to obtain the information about the allergenic proteins present in *A. fumigatus* from mycelial extracts. In this experiment observed the following results: In this study, the immunoelectrophoresis shows numerous precipitin lines by treated with mycelial antigens and infected rabbit serum. This result was correlated with previous studies. Self crossedradio immune eletrophoresis of the 1, 3, 7 and 14 day extracts showed that IgE binding components were released into the medium within 24hr (Harvey and Longbottom, 1987) A range of molecules which differed

in apparent molecular mass from 14, 30 and 42 kDa was seen with the extract. Few of these major allergens have been investigated in this experiment and understand their specific role in the pathogenesis of allergic aspergillosis. Three major allergens of *A. fumigatus* were investigated in this experiment. The SDS-PAGE gel shows, two band was obtained from mycelial extract of *A. fumigatus* from 14th day culture, molecular weight is 14 and 42kDa antigens. Strong immune reactive was observed in the low pH range of the immunoblots at molecular weight 60kDa, in particular for animal after the infection (Abdul *et al.*, 2010), the 18kDa antigen is the first *Aspergillus* antigen

which has been purified to homogeneity. This antigen produced by all strains of *A. fumigatus* tested (Jean *et al.*, 1991). Analysis of the crude extracts by Western blotting using sera from infected rabbit with asthma reveals band which correspond in size to known antigenic components. Immunoblot technique showed that rabbit with aspergillosis produce antibody against a total of two components of *A. fumigates* extracts. This result is correlated with previous studies. The filtrate antigens formed stronger bands in immunoblots of urine sample of infected rats with the strongest band being in the 27kDa. (Bessie *et al.*, 1990). The immunoblot technique showed that patients with pulmonary aspergillosis produced antibody against a total of nine components of *A. fumigatus*, ranging in molecular weight from 88000 to 33000 Daltons (Ruthet *al.*,1985). Using serum of the patients with asthma showed that *A. fumigatus* with 12 allergic bands and the maximum band from 18 to 120kDa (Saeednejad *et al.*, 2010) The data revealed that significant reduction in growth of *A. fumigatus* was observed with extracts of eight medicinal plants and the extract showed significant differences in their efficacy. Among all the ethanolic plant extract in well diffusion method, all plants showed the inhibition of mycelial growth of *A. fumigates* and four plants that are *Coriandrum sativum*, *Centella asiatica*, showed exceptionally prominent activity. The extract of plant *C. ativum* showed maximum activity even lower concentration at 100 μ l in 24mm in diameter. The other plant shows moderate activity against *A. fumigates* such as *Eucalyptus acaciiformis*, *Pergularia daemi*, *Biophytum sensitivum*, *Azadirachta indica* even at high concentration (200 μ l). The following result agrees with the finding of them. In forty nine methanolic extract of plants, 86% plants showed inhibition of mycelial growth of *Aspergillus niger* and four plants *Grewia arborea*, *Melia azedarach*, *Peltophorum pterophorus*, *Terminalia*

chebula, showed exceptionally prominent activity. The extract of plant *Grewia arborea* showed maximum activity even at low concentrations. (Varaprasad *et al.*, 2009). The medicinal plant oils tested exhibited different degrees of antifungal activity against *A. fumigatus* and *Aspergillus niger*. The maximum antimycotic activity was shown by *C. martini* followed by *C. citratus*, *Eucalyptus globulus* and *Cinnamomum zyleneicum*. Essential oil extracted from *C. zyleneicum* demonstrated strong antifungal activity on both species of *Aspergillus* (Sunitha and Mahendra, 2008). The present finding suggests that *A. fumigatus* is susceptible to the ethanolic extract of medicinal plants. Therefore, this study suggests that ethanolic extracts of screened plants would be helpful in treating allergic aspergillosis in animals and human beings caused by *A. fumigatus*. Medicinal plant extract are important source of fungi toxic compounds and they may provide a renewable source of useful fungicides that can be utilized in antimycotics against *A. fumigates* infection in patients suffering from aspergillosis.

CONCLUSION

From this study it was concluded that the *A. fumigatus* has the ability to cause aspergillosis diseases. Some selected plants such as *E. acaciiformis*, *A. indica*, *B. sensitivum*, *C. asiatica*, *P. daemi*, and *C. sativum* were treated against *A. fumigatus*. Totally the six experimental medicinal plants *C. sativum* showed the peak effect than other plants. Since the current research clearly depicted that *C. sativum* has confirmationally proved that due to the presence of the phytoactive compounds such as 2-decenoic acid, 11-tetra decanoic acid, carpic acid, undecyl alcohol, tri-decanoic acid it has the ability to control aspergillosis so it can be used for the probable pharmaceutical medicinal value

against the pathogenic respiratory disease causing fungal organism of *A. fumigatus*.

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