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Antifungal resistance modulation of *Aspergillus fumigatus* isolates from brooder pneumonia affected birds by *Eucalyptus globulus* extracts

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Abstract

Brooder pneumonia is a common respiratory problem during brooding period of poultry. It is most frequently caused by *Aspergillus fumigatus*. Use of azole drugs for treatment and prophylaxis results in resistance. The aim of this study was to determine whether the phytochemicals of *Eucalyptus globulus* are modulating resistance in *Aspergillus fumigatus*. Lung samples n = 50 of dead broiler chicks were collected from different farms of Lahore. Out of 50 lung samples 28 % (14) were considered positive for *Aspergillus fumigatus* after observing their macroscopic, microscopic characteristics and by performing PCR by amplifying ITS1-ITS4 gene (597 base pairs) and *RodA* gene (313 base pairs). Kirby-Bauer disc diffusion test was performed 14 isolates (100 %) were resistant towards both Fluconazole and Ketoconazole whereas 11 (78.57 %) were resistant towards Itraconazole. *Eucalyptus globulus* leaves were collected, and these were identified. Three solvent extracts were prepared. Maximum yield was of ethanolic extract. Antifungal activity was evaluated by agar well diffusion method. Highest antifungal activity was shown by *Eucalyptus globulus* Ethanol followed by chloroform and hexane extract showed no activity against any isolate then minimum inhibitory concentration of plant extracts that previously showed antifungal activity against isolates were evaluated by broth microdilution method. Modulation effect was checked by combining antifungal drug one by one with subinhibitory concentration of plant extract evaluated previously by broth microdilution method. 5 isolates were subjected into this experiment which are all sensitive towards *Eucalyptus globulus* ethanol and chloroform extracts tested previously in agar well diffusion test and MIC values were also evaluated. It was concluded that when Itraconazole was combined with *E. globulus* Ethanol extract then resistance was modified. Data of modulation was analyzed by One-way ANOVA and it reported P value of <0.05. It was synergistic inhibitory effect when Itraconazole was combined with *Eucalyptus globulus* Ethanol extract.

Keywords: Aspergillosis; Azole resistance; Phytochemicals; Itraconazole; subinhibitory concentration

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1. INTRODUCTION

Aspergillus fumigatus is considered as major respiratory pathogen of birds. It is filamentous fungus. Fresenius found it in the lungs of bustard (Otis tarda) in 1863. Fungal sporulation and proliferation of *Aspergillus fumigatus* on organic matter creates small sized conidia in air which are inhaled by birds in the respiratory tract and consequently infection occurs. Other species of *Aspergillus* are *Aspergillus flavus, Aspergillus niger*, *Aspergillus nidulans* and *Aspergillus terreus* are less frequently involved in avian Aspergillosis as compared to *Aspergillus fumigatus*[1]. Fungal diseases cause significant economic loses to the poultry industry either because of their infectious nature or by release of mycotoxins which are fungal secondary metabolite produced in poultry feed. Mycotic diseases are responsible for high mortality and morbidity in young birds[2]. In birds *Aspergillus fumigatus* cause brooder pneumonia, which occurs when chicks are hatched from incubators. Chicks suffering from brooder pneumonia develop drowsiness, loss of appetite, and death[3]. Brooder pneumonia is an acute form of aspergillosis. Mortality and Morbidity rates are high in this acute form and can be observed within 24 to 28 hours of infection[4]. Drugs used for treatment of aspergillosis in birds include Itraconazole, Ketoconazole, Fluconazole, Clotrimazole, Miconazole and Amphotericin B[5]. In *Aspergillus fumigatus* azole resistance is increasing dangerously. Azoles are only antifungal drugs which are orally and are major antimycotic agents to treat aspergillosis[6]. In recent years many plant extracts have been recognized to have antibacterial and antifungal properties[7]. Compounds of natural origin are potential sources of new chemical scaffolds for antifungal development but unlike synthetic drugs, they are not related with side effects, are cheaper, come from renewable sources and have greater acceptance due to a long history of use. One manner in which plant-derived compounds exert their potential as antifungals is synergism, a positive interaction created when two agents are combined[8].

2. MATERIALS AND METHODS

2.1 Sample collection

Lungs (n=50) from brooder pneumonia affected morbid chicks were collected from 5 farms of Lahore and were transported to Department of Microbiology, University of Veterinary and animal sciences Lahore for further experiments.

2.2 Isolation and tentative identification of Aspergillus fumigatus:

Lung samples from sterile containers were directly streaked one by one on media plates and plates were incubated at 25 °C for 3 days. From primary culture plates suspected *Aspergillus fumigatus* colonies were sub-cultured on sterile Saboraud dextrose agar plates. After incubation macroscopic features of pure cultures were observed and were used for microscopic examination. Slide culture technique was performed followed by lactophenol cotton blue staining[9].

2.3 Molecular confirmation by Polymerase chain reaction:

Genomic DNA of *Aspergillus fumigatus* was extracted by Exgene™ Plant SV mini extraction kit method. It was done for molecular identification of *Aspergillus fumigatus* isolates by using specific primers targeting ITS1-ITS4 and *RodA* gene. Sequence of primer for ITS1-ITS4 genus specific: forward 5'-TTCGTAGGTGAACCTGCGG-3' Reverse 5'- TCCTCCGCTTATTGATATGC-3' [10]. Primer sequence for *RodA* specie specific: Forward 5'-ACATTGACGAGGGCATCCTT-3' Reverse 5'- ATGAGGGAACCGCTCTGATG -3' [11]. PCR mixture for targeting ITS1-ITS4 and *RodA* (25 µl) each was prepared using nuclease free water (5.5µl), nTaq Master mix (Wizbio solutions) (12.5 µl), forward and reverse primers (1 µl each) and DNA template (5 µl). Prepared reaction mixtures were then placed in T100™ thermal cycler (Bio-Rad) After initial denaturation of DNA at 95°c for 5 minute, each cycle consisted of a denaturation step at 94°c for 30s, an annealing step at 56°c for 30s, an extension step at 72°c for 1 minute and a final extension step at 72°c for 7 minutes following the last cycle amplicons were electrophoresed on 1.5% percent agarose gel at 80 volts for 50 minutes. Gel was visualized in gel documentation system (Cleaver Scientific, UK).

2.4 Kirby-Bauer Disc diffusion method:

Standardized spore suspension of isolates was prepared by counting in neubar counting chamber and 1.0×10^6 is adjusted spore size[12]. 20 m L media was poured in each flask and those flasks were autoclaved 121°C/15mins/15Ibs. When media was cooled down, spore suspension of 100 µl was added in it and poured in pre sterilized Petri plates and when plates were solidified then 4 antifungal discs i.e Itraconazole (10 µg Oxoid), ketoconazole (50 µg Himedia), fluconazole (25 µg Himedia), and voriconazole (1 µg Oxoid) were applied on plate and plates were incubated at 25°C for 3 days and after incubation zone of inhibitions were measured.

2.5 Criterion source for selection of resistant isolates for further experiment:

Interpretative criteria of azole drugs against *Aspergillus species* is ≥18 mm sensitive, 14 to 18 mm intermediate and < 14 mm as resistant[13].

2.6 Plant Extract preparation:

Dried leaves were crushed down and placed in screw cape bottle. The plant extract was obtained by use of 100 grams of powdered plant by soxhlet apparatus at 40 °C using 600 mL of each solvents for 12 hours. The obtained extracts were dried in rotatory evaporator until semisolid extract was obtained[14]. Extract weight was divided with powder weight and multiplied by 100 to get percentage yield. From dried stocks of plant extract, stock solution was prepared whose concentration was 0.1 grams per 1 mL of DMSO.

2.7 Antifungal Activity of Eucalyptus globulus against resistant isolates:

Agar well diffusion test was performed to check antifungal activity of extracts of *Eucalyptus globulus.* For testing three solvent extract of plant, wells were made in inoculum added solidified SDA plates and 100 µl of plant extract stock solution (0.1g/mL) was poured in wells with help of micropipette. After incubation zone of inhibition were measured[15]. MIC of plant extracts those showed antifungal activity against resistant isolates previously in agar well diffusion test was determined by microbroth dilution method. MIC was read as minimum inhibitory concentration of plant extracts inhibiting visible growth of fungus[16].

2.8 Antifungal resistance modulation:

Different sub inhibitory concentrations of plant extract were prepared by using this formula C1V1 = C2V2. Antifungal drug solutions were prepared with respect to concentration of drug mentioned on commercial antifungal disc. All previously mentioned antifungals were dissolved in DMSO as they are soluble in organic solvent[17]. Antifungal drug solutions were stored at -20 °C for further use. Sub inhibitory concentrations of plant extracts for each resistant isolate were mixed one by one with fixed concentration of antifungals in two equal proportions i.e for combined effect each well contain sub inhibitory concentrations combined with antifungal drug. The wells were marked as antifungal alone, plant extract alone and three wells having sub inhibitory concentrations of plant extract along with antifungals. Antifungal (100µl) alone was poured in one well and in the second well 100 µl of plant extract solution (0.1g/mL of DMSO) was poured. In remaining four wells 100 µl having subinhibitory concentration of plant extracts with antifungal drugs (working solution) mixed in two equal proportions was poured. This step was done one by one for each antifungal drugs. These plates were incubated at 25 °C for 3 days. After incubation results were interpreted by measuring zones of inhibition.

2.9 Statistical analysis

Data during this study was analyzed by One way ANOVA at level of significance of 5 % i.e 0.05.

3. RESULTS

3.1. Positive samples:

Out of 50 lung samples, 14 samples were positive for *Aspergillus fumigatus*.

3.2. Tentative identification:

Tentative identification of *Aspergillus fumigatus* was done by confirming its macroscopic and microscopic characteristics. Macroscopic features were powdery texture, blue green to grey with white border from observe side shown in figure 2 A and white to tan from reverse side. Microscopic features were conidia on uniseriate phialides, dome shaped vesicle, conidiophore colorless, septate hyphae 2 B.

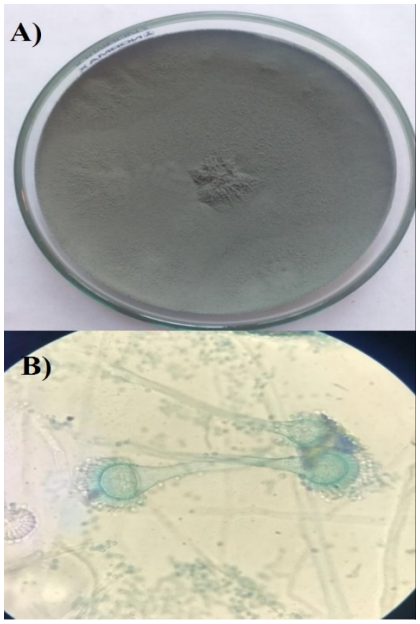

Fig. 2. A) Macroscopic identification, **B)** Microscopic identification

3.3. *Molecular confirmation:*

Isolates of *Aspergillus fumigatus* were confirmed by amplifying genus specific ITS1-ITS4 (597 bp) and specie specific *RodA* (313 bp) shown in figure 2.

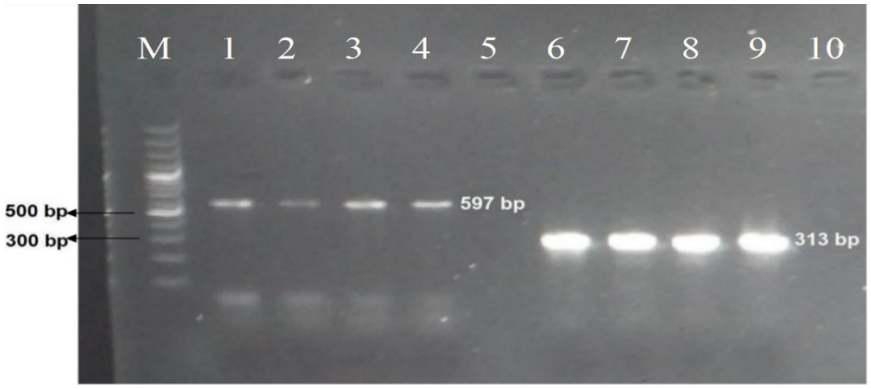

Fig . 2. Representative PCR identification of *Aspergillus fumigatus* ITS1-ITS4 (597 bp) and *RodA* (313 bp) electrophoresed on 2 % agarose gel. Marker denoted by M contains ladder of 1 kb, 1 to 4 contains amplicon of *Aspergillus fumigatus* genus specific ITS1-ITS4 gene (597 bp), 5 is empty, 6 to 9 contains amplicon of *Aspergillus fumigatus* specie specific *RodA* gene(313 bp) and 10 is empty.

3.4. Results of Kirby-Bauer Disc diffusion test:

Out of 14 isolates, all isolates were resistant to fluconazole and ketoconazole. 11 isolates were resistant to

itraconazole. 3 isolates showed zone of inhibition to itraconazole whereas all 14 isolates showed zone of inhibition to voriconazole and it was no voriconazole resistance in isolates. Percentage resistance is shown in figure 4. 14 isolates were considered resistant to fluconazole and ketoconazole and 11 were resistant to itraconazole. Graphical presentation of percentage resistance is shown in figure 3.

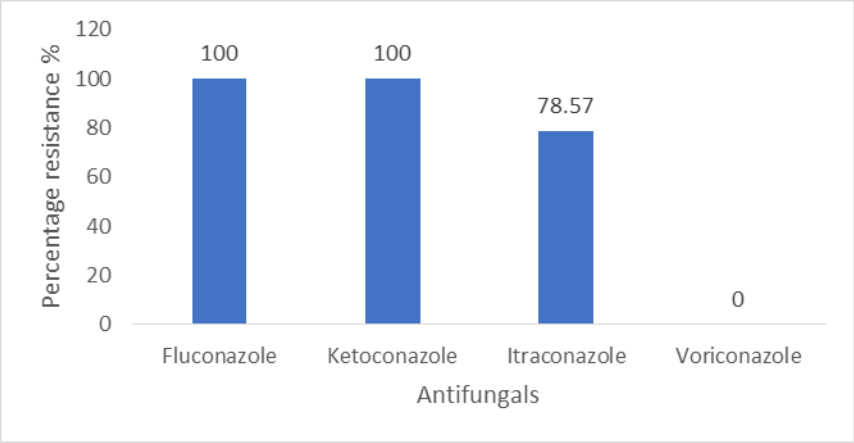

Fig. 3. Percentage resistance graph

3.5. Percentage yield of Extracts:

Highest yield was of Ethanol extract 16.5% followed by chloroform 7 % and yield of hexane was 4.2 %.

3.6. Antifungal activity of Extracts against resistant Aspergillus fumigatus isolates:

Antifungal activity was best shown by Ethanolic extract to all 14 isolates followed by chloroform extract which showed activity against 6 isolates and no inhibitory activity was shown by hexane extract. Results are shown in figure 4 A and B.

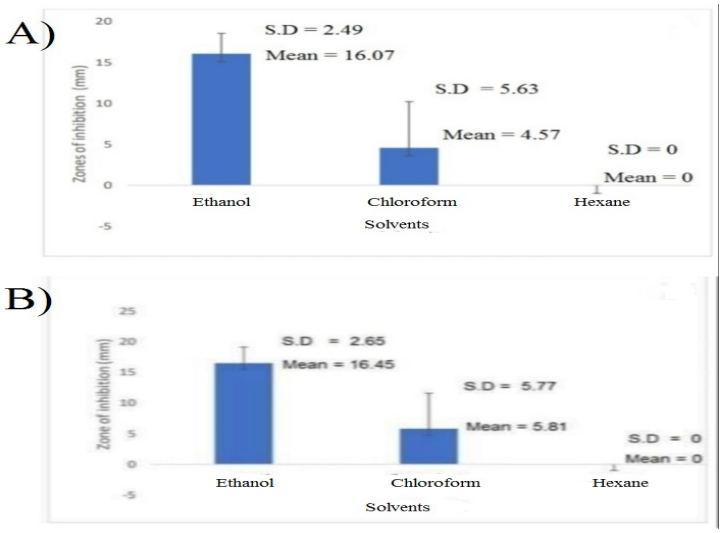

Fig. 4. A) Representative graph of Mean and Standard deviation of zones of inhibition of *Eucalyptus globulus* extracts against 14 resistant *Aspergillus fumigatus* isolates, **B**) Representative graph of Mean and Standard deviation of zones of inhibition of *Eucalyptus globulus* extracts against 11 resistant *Aspergillus fumigatus* isolates.

When means of all 3 groups (Ethanol, chloroform and hexane) was analyzed by One-way ANOVA then P value was lower than level of significance i.e. 0.05 and there was statistically significance in data.

MICs of those plant solvent extracts were determined that previously showed inhibitory activity to resistant isolates in agar well diffusion method. As hexane showed no inhibitory activity so its MICs was not

determined. MICs of Ethanol extract were lower and of chloroform extract were higher. Therefore, Ethanol extract was found more effective as antifungal.

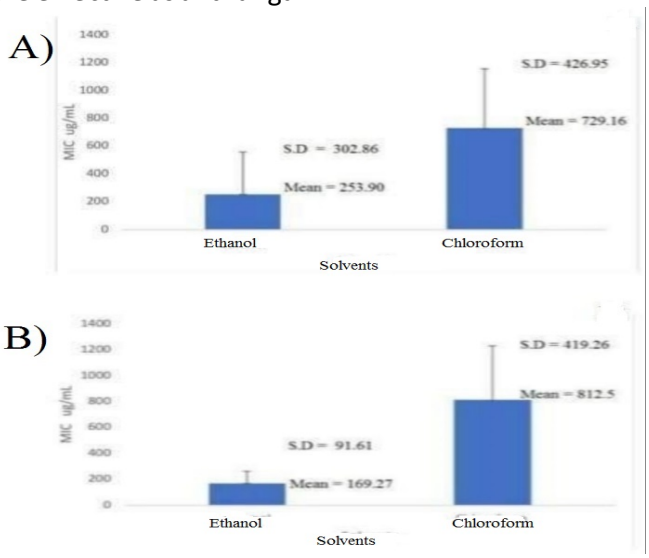

Fig. 5. A) Representative graph of Mean and Standard deviation of MIC µg/mL of *Eucalyptus globulus* extracts against fluconazole and ketoconazole resistant *Aspergillus* fumigatus isolates, **B)** Representative graph of Mean and Standard deviation of MIC µg/mL of *Eucalyptus globulus* extracts against itraconazole resistant *Aspergillus fumigatus* isolates.

3.7. Modulation of resistance:

Subinhibitory concentrations were made for each isolate. 5 isolates (3,6,7,9 & 12) were subjected into this experiment which are all sensitive towards *Eucalyptus globulus* ethanol and chloroform extracts and were tested previously in agar well diffusion test and MIC values were also determined. Overall sub inhibitory concentrations that were prepared are 320 µ g/ml, 160 µ g/ml, 80 µ g/ml, 40 µ g/ml, 20 µ g/ml 10 µ g/ml and 5 µ g/ml. Antifungal activity of plant extracts in combination with Fluconazole, Ketoconazole and Itraconazole was evaluated. None of the isolate showed modulation against combination of fluconazole and plant extracts and ketoconazole with plant extracts. Resistance was also not modulated by chloroform extract of *Eucalyptus globulus* with itraconazole but when itraconazole was combined with *Eucalyptus globulus* ethanol extract resistance was modulated indicating increased diameter. All isolates had shown modulation of resistance when Ethanolic extract of *Eucalyptus globulus* was checked in combination of itraconazole against the isolates and zone of inhibition was observed with increased diameter indicating modulation of those isolates. In **table 1** only results for Itraconazole are shown because it modulated resistance. Antifungal resistance modulatory activity of *E. globulus* ethanol combined with itraconazole against *Aspergillus fumigatus* isolate AFM 6 is shown in **Figure 6.**

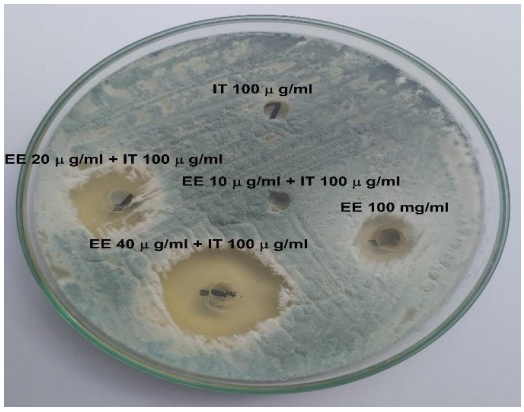

Figure 6: Antifungal resistance modulatory activity of *E. globulus* (Ethanol) with Itraconazole

Table 1. Modulation results of Itraconazole (IT) alone and in combination with *Eucalyptus globulus* Ethanol (EE)

Isolates	Itraconazole (100 µg/ml) alone and in combination with *SICs of *E. globulus* Ethanol	Zone of inhibition (mm)	Mean ± S.D	P value
*AFM 3	Itraconazole 100 µg/mL	0	0 ± 0[a]	
	IT* 100 µg/ml + 40 µg/mL EE	20		
	IT 100 µg/ml + 20 µg/mL EE	14	14 ± 6[c]	
	IT 100 µg/ml + 10 µg/mL EE	8		
AFM 6	Itraconazole 100 µg/mL	0	0 ±0[a]	
	IT 100 µg/ml + 40 µg/mL EE	20		
	IT 100 µg/ml + 20 µg/mL EE	13	11 ± 10.14[b]	
	IT 100 µg/ml + 10 µg/mL EE	0		
AFM 7	Itraconazole 100 µg/mL	0	0 ± 0[a]	
	IT 100 µg/ml + 20 µg/mL EE	17		00.00
	IT 100 µg/ml + 10 µg/mL EE	16	13.33 ± 5.50[c]	
	IT 100 µg/ml + 5 µg/mL EE	7		
AFM 9	Itraconazole 100 µg/mL	0	0 ± 0[a]	
	IT 100 µg/ml + 40 µg/mL EE	19		
	IT 100 µg/ml + 20 µg/mL EE	15	14 ± 5.56[c]	
	IT 100 µg/ml + 10 µg/mL EE	8		
AFM 12	Itraconazole 100 µg/mL	0	0 ± 0[a]	
	IT 100 µg/ml + 80 µg/mL EE	25		
	IT 100 µg/ml + 40 µg/mL EE	18	14.33 ± 12.89[c]	
	IT 100 µg/ml + 20 µg/mL EE	0		

*Subinhibitory concentrations; **Aspergillus fumigatus* *Itraconazole

Means having same Superscripts are non-significant and those having different superscripts are significantly different.

3.8. Data Analysis:

Data of modulation was analyzed by One way ANOVA by comparing mean of Itraconazole alone and mean of Subinhibitory concentrations with itraconazole. P value was less than level of significance of 0.05 indicating statistically significance in data.

4. DISCUSSION

Brooder pneumonia is acute form of aspergillosis caused by *Aspergillus fumigatus* in birds. It causes significant economic loses to the poultry industry with high mortality and morbidity rate in poultry chicks[18]. The postmortem lesion of dead birds were white to yellowish spherical caseous granulomatous nodules of several sizes in whole lungs[19]. In a study 4 dead chicks of cob-500 broiler strain with brooder pneumonia clinical history at 10 days old from Bogra district of Bangladesh and all samples were positive for *Aspergillus fumigatus*[20]. The outbreaks of Aspergillosis in broiler chicks (4 to 15 days old) were observed in 5 different farms in Sulaimania, Iraq. Higher morbidity (76 to 100 %) and mortality rates (62.5 to 100 %) were recorded in five farms[21]. Necropsy of an 11-month-old female ostrich (*Struthio camelus*) revealed white to green mold growth on the walls of caseous thickened air sac membranes in the lungs. Fungal culture of these lesions produced pure growth of *A. fumigatus*[22]. These studies support that *Aspergillus fumigatus* is associated with brooder pneumonia and it is considered as major pathogen of this respiratory disease in poultry.

Amplification of extracted DNA of *Aspergillus fumigatus* from Nigerian food commodities was done using ITS-1/ITS-4 primer pairs for forward and reverse amplification and size of PCR product was 597 base pairs[23]. In Iran an PCR-RFLP method was used to identify opportunistic invasive fungal pathogens including *Aspergillus fumigatus* by using genus primers ITS1-ITS4 and it generated amplicon product of 597 bp in case of *Aspergillus fumigatus*[10]. In Brazil *A. fumigatus* isolates from broilers were confirmed by polymerase chain reaction by using specie specific primer *RodA* gene and after amplification amplicon size was 313 base pairs[23]. In a study multiplex pcr technique was applied using specie specific *A. fumigatus RodA* and βtub and after amplification PCR products of *RodA* and βtub genes were 313 and 153 base pairs respectively[11]. In a study n= 51 *A. fumigatus* strains including environmental, clinical and reference isolates were tested by PCR. PCR amplification of the *RodA* gene resulted in a 313 bp band. The *rodA* gene PCR product exhibited a 100% homology with the associated sequences in the GenBank[25]. PCR results were similar in present study as described in previous studies.

Antifungal susceptibility of fluconazole, ketoconazole and itraconazole was checked by Kirby bauer-disc diffusion test against human strain of *Aspergillus fumigatus* MTCC2550 and result were no zone of inhibition indicating resistance toward fluconazole, ketoconazole and itraconazole[26]. Activity of fluconazole and voriconazole was checked by disc diffusion method against n= 13 isolates of *Aspergillus fumigatus* recovered from a hospital and results were no zone of inhibition towards fluconazole showing 100 % resistance whereas all isolates were sensitive towards voriconazole indicating 0 % or no resistance[27]. In a study activity of voriconazole was checked on n=30 clinical isolates of *A. fumigatus* and all isolates were sensitive towards voriconazole indicating 0 % resistance[28]. In a study a total of 85 strains of *A. fumigatus* were isolated from throat, lungs and drinking water of domestic geese and susceptibility of isolates were checked by disc diffusion technique to voriconazole, ketoconazole and itraconazole and the results were resistance of 84.7 % for ketoconazole, there was 14.1 % of isolates that were resistant toward itraconazole and all isolates were susceptible to voriconazole resistance showing 0 % resistance[29]. These studies support that *A. fumigatus* had developed resistance mechanisms towards fluconazole, ketoconazole and itraconazole but it is susceptible to voriconazole.

In a study ethanol, chloroform and petroleum ether of a *Eucalyptus* leaves were carried out to quantify the biologically active components in plant i.e phytochemicals. The percentage yields obtained through the Soxhlet (Ethanol, chloroform and petroleum ether) extraction for the *Eucalyptus* leaves were 9.34%, 3.82 % and 1.54 % respectively[30]. This study supports that yield of ethanol is more than chloroform and results were similar in present study.

The fraction extracts of the medicinal plant *Veronica biloba* (i.e., water, dichloromethane, *n*-hexane, and ethyl acetate) showed inhibitory activities against *Aspergillus fumigatus* when checked by agar well diffusion test. Maximum inhibition was shown by ethyl acetate extract[31]. The antifungal activity of methanolic extract of *Mimosa pudica* was studied using well diffusion method. The activity was tested against *Aspergillus fumigatus* there was increase in activity with increased concentration of extracts in wells[32]. The antifungal activity of the leaves of *E. globulus* extracted with acetone, ethanol and petroleum ether was studied using well diffusion method against *Aspergillus fumigatus*. Zone of inhibition was of acetone 16 mm followed by 14 mm by ethanol extract and 11 mm zone of inhibition for petroleum ether (Abirami et al., 2017)[15]. Three solvents ethanol, hexane and acetone were used in extraction from rhizome part of plants *Stephania glabra* and *Phoebe Lanceolata.* activity of ethanol extract was highest. Zone of inhibition of ethanolic extract of *Stephania glabra* and *Phoebe Lanceolata* against *Aspergillus fumigatus* was 21 mm and MIC was 50 µg/ml and 12 mm and 100 µg/ml[33]. These studies support that plant extracts can inhibit growth of *Aspergillus fumigatus* and may be used as alternative of antifungal drugs.
In a study zone of inhibition (mm) of ethanol extract of *Sarcococca saligna* against *Aspergillus* species (in absence and in presence of Fluconazole at content of 25 µg/disk). Results were increased zone of inhibition when Fluconazole was combined with plant extract as compared to Fluconazole alone[34]. Phytochemicals in plants can modify resistance[35]. These study supports that resistance can be modified when plant extracts are used with antifungal drugs. Plant extracts can modulate resistance by various mechanisms by inhibition of efflux pump, inhibition of target modification of drugs or by enzymatic inhibition of drugs.

5. CONCLUSIONS

The present study provides data indicating antifungal resistance modulatory activity of Ethanolic extract of *Eucalyptus globulus* leaves combined with Itraconazole tested in vitro by agar well diffusion method against *Aspergillus fumigtaus* isolated from brooder pneumonia affected birds. Test indicated a synergistic effect. So, plant-derived compounds can exert their potential as antifungal is synergism, a positive interaction created when two agents are combined that results in a greater inhibitory effect.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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