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RESEARCH PAPER

Isolation, Identification and Evaluation of Antifungal Activity of Aspergillus spp. from Cancer Patients During Chemotherapy Treatment in Erbil City.

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A B S T R A C T:
Aspergillus fumigatus and *Aspergillus flavus* are ubiquitous molds, thus infection with them is expected. They are considering as opportunistic molds in immune-compromised patients. Especially in patients undergoing chemotherapy treatment suffer from cancer and patients undergoing hematopoietic stem cell transplantation (HPSCT). 100 specimens were collected from patients suffering from different types of cancers during chemotherapy treatments during the period of 10th of October to 10th of December 2022, the age range was 16 to 80 years, 43 (43%) were males and 57(57%) were females, the specimens were inoculated on Sabouraud's dextrose agar (SDA), the culture result shows that 20 (20%) of the cultures were positive for *Aspergillus* spp growth, among the isolated *Aspergillus* spp. the 9 (45%) were *A. flavus*, 5 (25%) were *A. fumigatus*, 4(20%) were *Penicillium* spp., and 2(10%) were *mycelia sterilia*, the results of the correlation between causative agents and gender showed that all infected cases with *A. fumigatus* were females 5.5% (4), whereas 4.1% (3) females and 6.8% (5) of the males were infected with *A. flavus*., furthermore most of the cases were infected with a different type of hematopoietic malignancies, 5(41.6%) with Acute lymphoblastic leukemia (ALL), 2(16.6%) chronic lymphocytic leukemia (CLL), 2(16.6%) multiple myeloma (MM), and 1 (8.3%) lymphoma. Others were infected with 1 (8.3%) case for each colon cancer and lung cancer. Three cases of *A. flavus* infected patients have ALL, 2 two cases CLL, 1 case has MM, one case has colon cancer and (1) case has lung cancer. On the other hand, two cases with *A. fumigatus* infection have ALL, one has MM, and the last case has Lymphoma. The sensitivity tests performed by using disc diffusion assay and MIC test for isolated *Aspergillus spp*., the results revealed that there are significant differences in the sensitivity of both *A. flavus* and *A. fumigatus* against the four types of antifungal discs (Econazole, Ketoconazole, Miconazole, and Nystatin), the result revealed that Econazole was the most effective antifungal agent against *A. flavus* and *A. fumigatus*, while nystatin has the lowest activity against them, while MIC result shows that Terbinafine has the MIC against both tested fungi at 1.95 mg/ml.

KEY WORDS: *Aspergillus fumigatus, Aspergillus flavus*, Aspergillosis, immunocompromised patients, Antifungal drugs, MIC.
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1.INTRODUCTION :

Aspergillus sp. is popular mold, found everywhere thus infected with them is expected. They inhabit soil and rotten vegetative materials. Factors like (temperatures, humidity, geographical variations in precipitation) are playing important role in the prevalence *of Aspergillus spp*. in the environment, and their relationship to human infections (Cadena *et al*., 2021). 339 species of *Aspergillus* are found of both beneficial and harmful types (Schubert *et al*., 2018).

Aspergillus spp. are remarkable cause of morbidity in both immune-competent and immune-compromised patients (Graham and Nasir, 2019). Patients undergoing chemotherapy for cancer as well as following stem cell and organ transplantation have high risk to infect with *Aspergillus sp*. with high morbidity and mortality rate (Chen *et al*., 2011). Aspergillosis is spectrum of clinical diseases caused by *Aspergillus sp*. ranging from the non-invasive form, asprgilloma (developing of fungal ball), chronic inflammatory and allergic bronco-pulmonary aspergillosis (ABPA)(Latgé and Chamilos, 2019) to invasive aspergillosis (IA) reveals the invasion of lung

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tissue by hyphae and may disseminate to other virtual organ this common in patients with prolonged ganulo-cytopenia following chemotherapy (Kosmidis and Denning, 2015, Cadena *et al*., 2016). The rate of aspergillosis has increased because the number of immunocompromised patients has increased. Aspergillosis occurs following the inhalation of the conidia by immunocompromised patients (Cadena *et al.*, 2021, Latgé and Chamilos, 2019).

The most *Aspergillus spp*. related to human infections are *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus niger* (Nedel and Pasqualotto, 2014). Opportunistic fungi seriously affect public health by causing life threating diseases in immunocompromised patients (Park *et al*., 2017, Seyedmousavi *et al*., 2015). *Aspergillus fumigatus* is a saprotrophic fungus, which commonly reproduce by asexual sporulation furthermore the sexual reproduction is exist *(Latgé and Chamilos, 2019, Pringle et al*., 2005). Among *Aspergillus* genus *Aspergillus fumigatus* is the most important human pathogen because it has the capacity to adapt and reproduce rapidly in antagonistic environment thus resist and survive against the human host defenses(Park *et al*., 2017) , therefore causing lung infection by inhalation of the conidia (Park *et al*., 2017) resulting in different infections such as chronic pulmonary aspergillosis (CPA), allergic broncho-pulmonary aspergillosis (APA), and invasive aspergillosis (IA) in immunocompromised patients (Schubert *et al*., 2018). *Aspergillus flavus* is coming in the second place of the most pathogenic mold (Brauer *et al*., 2020). *Aspergillus flasvus* is ubiquitous and saprophytic fungus (Sugui *et al*., 2014), can infect many crops causing many economic losses. *A. flavus* produce aflatoxin which is carcinogenic mycotoxin that affects liver causing hepatocellular carcinoma and acute aflatoxicosis. *A. flavus* rarely cause infection in healthy individuals, however in immunocompromised individuals (patients undergo cytotoxic, corticosteroid therapy, and the HIV (human-immune deficiency virus) infected persons (Hadrich *et al.*, 2013). The *A.flavus* conidia can enter and access the pulmonary alveoli and germinate leading to many pulmonary infections and invasive aspergillosis (IAs) (Krishnan *et al*., 2009). Up until recently, several drugs used to treat aspirgillosis as amphotericin B (AmB), itraconazole, voriconazole, posaconazole, and caspofungin. Although the anti-fungal resistance has not been reported as great as antibacterial resistance, the number of reported cases has been increased.

Thus the resistance to the antifungal drugs at certain site of infection could increase the mortality rate of these infections*(Hedayati et al*., 2007). The purpose of the study is to determine the incidence of aspergillosis due to *Aspergillus fumigatus* and *Aspergillus flavus* from cancer patients whom their immunity has been suppressed due to chemotherapy treatment in Iraq-Kurdistan region Erbil city. And to determine their susceptibility against antifungal drugs, since there is almost no susceptible tests done for the *Aspergillus spp*. in Erbil city Kurdistan region's hospitals and clinical labs.

2.MATERIALS AND METHODS

2.1. Sample collections:

From Nanakali Hospital in Erbil city, 100 specimens were collected from aspirates, cough, oral swabs from patients suffering from different types of cancers during chemotherapy treatments. The specimens were taken from both sexes randomly, the age ranged between 16 to 80 years, during the period of 10th of October to 10th of December 2022. Demographic and clinical data including, (age, sex, type of cancer, residence, and animals careness), were documented for each subject. The samples obtained by direct cough of patients on Sabouraud's dextrose agar plates. And all the samples subsequently transferred to Mycological laboratory in the College of Science-Department of Biology-Salahaddin University. Incubated at 28-37 oC for 5-7 days.

Purification of samples on SDA medium free of cycloheximide (Guinea *et al.*, 2005).

2.2. Identification:

The positive cultures were identified based on macroscopic (surface morphology and pigment production) on SDA and microscopic (nature of mycelium and conidia formation) characteristics depending on Keys of: (Moubasher, 1993, Watanabe, 2002).

2.3. Identification of Aspergillus species:

Three types of selective media were used, Czapek Yeast extracts Agar (CYA), Malt Extract Agar (MEA), and Glycerol Nitrate Agar (G 25% NA). inoculated with samples from SDA medium and Incubated at three different temperatures (5, 25, and 37 C°) for 10 days then the species were identified as recommended by (Pitt and Hocking, 1997) (Guinea et al., 2005, Ismael, 2009).

2.4. Antifungal susceptibility testing:

2.4.1. Disc diffusion test:

Four microbiology sensitivity antifungal discs were used (Econazole, Itraconazol, ketoconazole, and Nystatin), disc potency (U/d) is 50mg except for Nystatin 100IU (Biorex/UK). Used to evaluate Aspergillus sp. isolates in vitro for antifungal drugs susceptibility By direct application of the discs on mold cultured SDA plates. To establish whether the molds are sensitive or resistance to these antifungals by measuring the inhibition zone of each disc on SDA plate and compared to common interpretive chart (Serrano et al., 2004, FaqeAbdulla, 2022).

2.4.2. Micro titer sensitivity test:

Four antifungal drug (Fluconazole 150 mg, Tindazole 500 mg, Terbinafine 250 mg, and Nystatin 500,000 IU) were tested against A. flavus and A fumigatus, serial dilution method had carried out by melting one pill of each antifungal drug in 10 ml of sterile distilled water to make stock solution, the minimum inhibition concentration (MIC) was obtained by measuring the optical density (OD) value of the wells at 490nm before and after incubation by ELIZA reader (Epson, Biotech, UK). (Berkow et al., 2020).

2.5. Statistical analyses

The distribution of data was analyzed according to the Shapiro-Wilk and Kolmogorov-Smirnov normality test. Then the data were analyzed according to One way ANOVA followed by multiple comparisons Tukey's test for multi-group comparisons of antifungal discs. Using GraphPad Prism (version 8.0.1) P value <0.05 considered statically significant.

3. RESULTS

3.1. Culture results:

During this study 100 specimens were taken from cancer patients during their chemotherapy treatment, as shown in (Figure 1), the results revealed that 43 (43%) of the cases were males and 57(57%) were females.

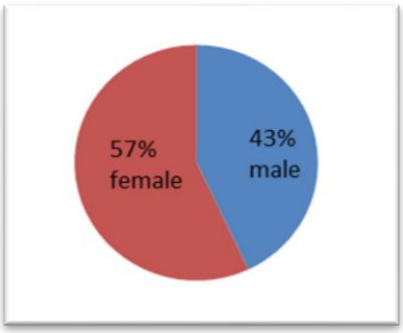

Figure 1: shows the percentage of male and female cancer patients included in the present study.

Cultural examination results showed that 69(69%) of the specimens were culture positive, and 31(31%) were culture negative. among them about 86.95% were fungi: 20(28.98%) were Aspergillus spp., 16(23.18%) cases yield yeast growth on the culture, and 24(34.7%) were Cladosporium, while other isolated Microorganisms were: 5(7.25%) Actinomycetes, and 4(5.8 %) were bacteria as (Table 1) shown.

Table 1: The percentage of isolated and identified microorganisms in positive cultures the present study.

Isolated Microorganisms		Percentage %	
Fungi	*Cladosporium* spp.	34.78	**86.95**
	Aspergillus spp.	28.98	
	Yeast	23.19	
Actinomycetes	**7.25**		
Bacteria	**5.8**		
Total	**100**		

3.2. Direct Microscope examination and selective culture

The mycological identification of the positive mold culture results was based on macroscopic and microscopic examination, as shown in (figure 2), 9 (45%) were *A. flavus*, 5 (25%) were *A. fumigatus*, 4(20%) were *Penicillium*, and 2(10%) were *Mycelia sterilia*.

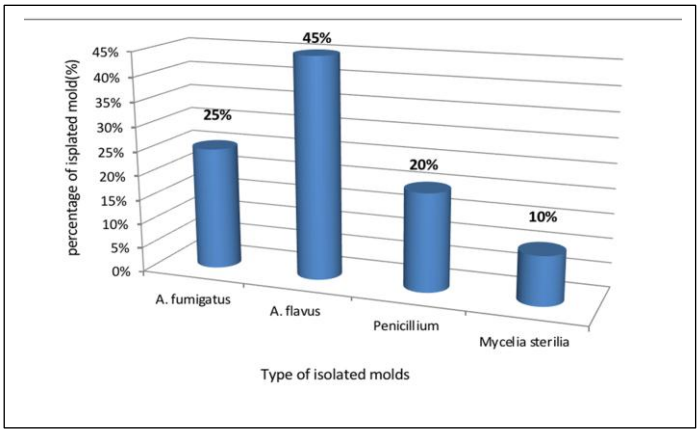

Figure 2: The percentage of isolated molds in the present study

The results of identification of *Aspergillus* spp. by using three selective media were: 40% (n.8) were *A. flavus*, 20% (n.4) were *A. fumigatus*, and 5% (n.1) was *A. oryzae*.

(Figure 3) shows the relation between age groups distribution and *Aspergillus* spp. infections. The age range of infected patients with *Aspergillus* spp. was 16-73 years old were infected with *A. fumigatus*, and *A. flavus*.

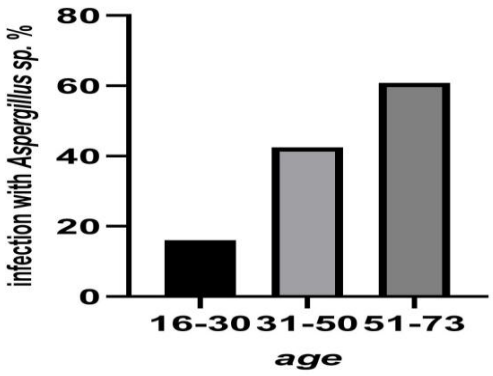

Figure 3: The relationship between the age groups and *Aspergillus spp.* infection.

Table (2) shows the relation between gender and *Aspergillus* spp. infections. The total infected cases with *A. fumigatus* were females 5.5% (4), whereas 4.1% (3) females and 6.8% (5) of the males were infected with *A. flavus*.

Table 2: The percentage of isolated and identified *Aspergillus* spp. related to gender in positive cultures the present study

Aspergillus spp.	Female %	Male %
A. fumigatus	5.5	0
A. flavus	4.1	6.8
Total percentage	9.6	6.8

The percentage of the patients who were infected with *Aspergillus* spp. and living outside Erbil city were 8 (66.6%) while 4(33.3%) reside inside Erbil city. 5(62.5%) *A. flavus* and 3(37.5%) *A fumigatus* was isolated among 8 cases from residences outside of Erbil city ,while 3(75%) *A. flavus* and (25%) *A fumigatus* was isolated within Erbil city as shown in (Table 3).

Table 3: The relation between the percentage of residence and *Aspergillus sp* infections

Aspergillus sp infections	Inside Erbil city (%)	Outside Erbil city (%)
A. fumigatus	25%	37.5%
A. flavus	75%	62.5%

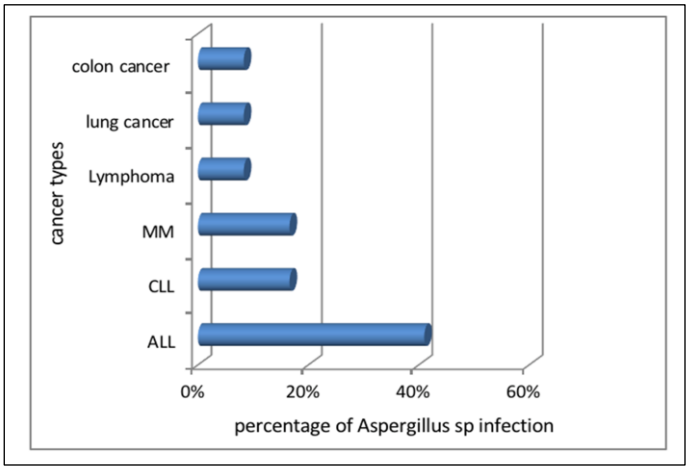

Figure 4: The relation between cancer types and *Aspergillus sp.* infections**.**

The results of infected patients with *Aspergillus* spp. revealed that 4 (33.3%) of infected cases have been in direct contact with pet animals (chickens and cattles) while 8 (66.6%) have not exposed to animals at all. Figure (4) shows the relation between the percentage of cancer type and *Aspergillus* spp. infection cases, the results shows that the highest rate of incidence were infected with different type of hematopoietic malignancies, 5(41.6%) with Acute lymphoblastic leukemia (ALL), 2(16.6%) chronic lymphocytic leukemia (CLL), 2(16.6%) multiple myeloma (MM), and 1 (8.3%) lymphoma. Others were infected with 1 (8.3%) case of colon cancer and 1 (8.3%) case of lung cancer. 3 cases of *A. flavus* infected patients have ALL, 2 cases have CLL, 1 case has MM, one case has colon cancer and 1 case has lung cancer. On the other hands two cases with *A. fumigatus* infection have ALL, one has MM, and the last case has Lymphoma.

3.3. Sensitivity test

3.3.1. Disc diffusion test:

The positive results of *A. flavus and A. fumigatus* were tested against four antifungal discs by measuring the inhibition zone of the growth of the mycelium. The ANOVA test showed that there are significant differences of sensibility of both *A. flavus* and *A. fumigatus* against the four types of tested antifungal discs (Econazole, Ketoconazole, Miconazole, Nystatin). As showed in (figure5), Econazole drug (50mg/ml) has the highest inhibitory activity which inhibits more than 30% of fungal growth of *A. flavus* with P value (<0.0001). The results revealed that there is no significance difference between Ketoconazole and Miconazole inhibitory activity (50mg/ml) as both have almost the same inhibition activity on *A. flavus* growth, around 15% of the mold growth was inhibited by both drugs with P value (<0.0001). While nystatin (100UI) has the lowest inhibitory activity, which inhibited less than 10% of the *A. flavus* growth with P value (<0.0001). *A. fumigatus* (figure 5), Econazole inhibit around 30% of the *A. fumigatus* growth with P value (<0.0001). while Ketoconazole inhibits less than 20% of the mold growth, ketoconazole shows significant difference with Miconazole on *A. fumigatus* opposite to *A. flavus* as 20% of *A. fumigatus* growth inhibited with P value (<0.0001). Nystatin shows the lowest inhibitory activity on *A. fumigatus* about 1% of the mold growth was inhibited with P value (0.0001)

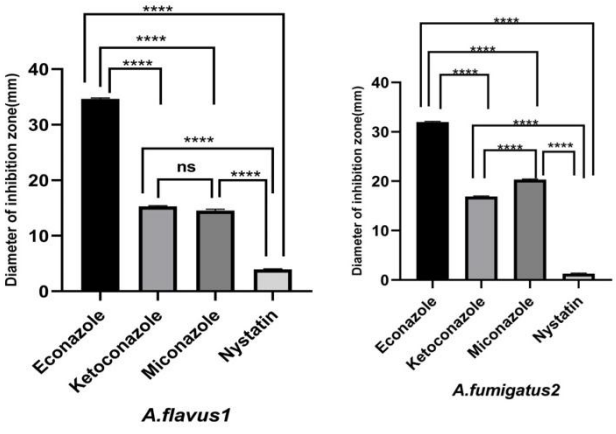

Figure 5: Quantitative measurement of inhibition zone for *A. flavus* and *A. fumigatus* to antifungal discs by disc diffusion method. Data are presented as mean ±SE of three biological replicas. All data were significant at P<0.05 (*), p<0.001 (**) and p<0.0001(***).

3.3.2. Microtiter sensitivity test:

The tested antifungal drugs showed different inhibitory activity against tested fungi. As shown in (table 4), Terbinafine showed the Minimum inhibitory concentration (MIC) against both tested fungi at 6th fold with 1.95mg/ml, followed by Fluconazole with 2,35mg/ml, while nystatin and Tindinazole showed lowest inhibitory activity both were 3.91 mg/ml (table 4).

Table 4: show the minimum inhibitory concentration (MIC) of antifungal drugs on both *Aspergillus fumigatus* and *Aspergillus flavus*

Aspergillus species	Antifungal drugs	MIC
A. fumigatus	Fluconazole	2.35 mg
	Terbinafine	1.95 mg
	Nystatin	3.91 IU
	Tindinazole	3.91 mg
A. flavus	Fluconazole	2.35 mg
	Terbinafine	1.95 mg
	Nystatin	3.91 IU
	Tinidanazol	3.91 mg

4. DISCUSSSION

This study has demonstrated the future risk of developing invasive fungal disease (IFD) especially those caused by *Aspergillus* spp. in patients undergoing chemotherapy treatment of different types of cancer as *Aspergillus* spp. are the most opportunistic molds can cause wide range of diseases particularly in immunocompromised patients (Sugui *et al*., 2014). Multiple risk factors play important role in increasing the chance of infection with *Aspergillus* spp. including; age, certain type of cancer, immunosuppression therapy, organ transplantation, residence and farmers (high spore exposure) (Latgé and Chamilos, 2019). Yet the incidence rate with Aspergillosis differs from one country to the other (Manikandan *et al*., 2019). As recommended in (Chadeganipour and Mohammadi, 2020). Infection with *A. fumigatus* and *A. flavus* reported as the second most prevalent *Aspergillus sp*. in arid regions of the Middle East. Large number of Aspergillus sp, isolated from different clinical sites in Saudi Arabia (Manikandan *et al*., 2019). Allergic broncho-pulmonary aspergillosis (ABPA) by *A. fumigatus* has been reported significantly high in

India (Agarwal, 2014). As our result showed 91.6% of the infected patients there age was between 40-73 years old. Age over 40 years old considered one of the predictive risk factor for aspergillosis. Ages between 41-50 had have the highest frequency of infection with *Aspergillus* species (Chadeganipour and Mohammadi, 2020). (Chadeganipour and Mohammadi, 2020) reports the ratio of infected female was higher than infected men with *Aspergillus sp*. the same our study demonstrate. In opposite side (Yelika *et al*., 2021) shows different result with male to female ratio being 29:27. 83.3% of the infected patients with *Aspergillus sp*. were infected with different type of hematopoietic malignancies (HMs). Currently, about 50% of all invasive aspergillosis (IA) detected with HMs. Primarily, in acute myeloid leukemia (AML), Acute lymphoblastic leukemia (ALL), and allogenic hematopoietic stem cell transplantation (HSCT) recipients. Chemotherapy induced sever neutropenia thus is the most important risk factor for IA (Schmiedel and Zimmerli, 2016). Some of the chemotherapy regimens for ALL occupy high doses of corticosteroid, make those patients predispose to IADs (Stafylidis *et al*., 2022). Despite the usage of antifungal drugs the mortality rate due to fungal infection still at high range 12% to 18% especially in HMs (Ducassou *et al*., 2015). Our study show Econazole is more effective antifungal drug on *A. flavus* and *A. fumigatus* than other antifungal drugs, with high sensitivity effect. 1% solution of econazole was successfully used to treat *A.flavus* and *A.fumigatus* in *Aspergillus sp*. sinusitis (Grigoriu *et al*., 1979). Ketoconazole shows significant effect on *A. flavus* and *A.fumigatus* growth with significant difference compare to econazole. While (Schaffner and Frick, 1985) demonstrate that Ketoconazole alone shows no effect. Also Ketoconazole failed to show any benefit as mentioned in (Wark *et al*., 2001). Our result display that Miconazole has no significant difference with ketoconazole on *A. flavus*, but on *A. fumigatus* significant difference has been shown. Both ketoconazole and miconazole show moderate sensitivity effect on both *A. fumigatus* and *A. flavus*. Similar result for miconazole demonstrated in (Schär *et al*., 1976). Study of (Ahmed *et al*., 2018) demonstrated that 76% of *Aspergillus sp*. were sensitive to miconazole. Nystatin used for treatment candidiasis, but since it is used in Kurdistan region in all type of fungal disease include molds, we established sensibility test against *A. flavus* and *A. fumigatus*. Significant difference in nystatin effect reported on both molds with more effect on *A. flavus*. Study shows liposomal nystatin can be used as salvage therapy for invasive aspergillosis (Offner *et al.*, 2004). Fluconazole at (2.35 mg) show the moderate sensitivity to *A. flavus* and *A.fumigatus*. As (Cornely *et al.*, 2007) demonstrated effective preventing of fluconazole drug against invasive fungal diseases in patients treated with chemotherapy for acute myeloid leukemia and hematopoietic stem cell transplantation, but fluconazole prophylaxis shows significant reduction in invasive aspergillosis and mortality due to any reason in patients go through hematopoietic stem-cell transplantation. TERB has MIC less than 2 mg on *A. fumigatus* and *A. flavus.* High susceptibility of *A. fumigatus* and *A. flavus* has been shown against TERB. On other hand *A. fumigatus* shows less sensitivity against TERB but more potent in vitro effect against *A. flavus* (Moore *et al*., 2001). Our study demonstrates that *Aspergillus spp.* show high susceptibility against azoles especially econazole, which had high significant effect, comparable to other drugs.

5. CONCULSSION

Our pilot study showed that *Aspergillus* infection has significant incidence in cancer patients undergoing through chemotherapy treatment, especially in hematopoietic malignancies. As in ALL, AML, and lymphoma since immune deficiency is the most important risk factor for *Aspergillus* infection. *A. fumigatus* and *A. flavus* are the most etiological source for aspergillus infection as they widely distributed. An Econazoles antifungal drug has the inhibitoriest activity on *A. fumigatus and A. flavus* than other tested drugs.

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Conflict of Interest

Regarding this study, the authors declare that they have no potential conflict of interest.

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