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In vitro inhibitory activity of spice-derived essential oils for multi-drug resistant *Aspergillus fumigatus* recovered from poultry feed

Atividade inibitória *in vitro* de óleos essenciais derivados de especiarias para *Aspergillus fumigatus* multirresistente obtido de ração de aves

Alishba Naeem[1], Muhammad Adnan Ashraf[1*], Aftab Ahmad Anjum[1], Ali Ahmad Sheikh[1], Tehreem Ali[1], Rabia Manzoor[1]

[1]Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore, Punjab, Pakistan
[*]Corresponding author: adnan.ashraf@uvas.edu.pk
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ABSTRACT

Aspergillus fumigatus is a respiratory pathogen, which has the potential to cause brooder pneumonia in poultry chicks and aspergillosis in birds and farmers. Frequent and irrational use of antifungals has led to the development of resistance in the fungal species. The present study aimed to evaluate the antifungal activity of essential oils (EOs) from four different spices against multi-drug resistant (MDR) *A. fumigatus* isolated from 40 poultry feed samples. Twenty fungal isolates were identified to be *A. fumigatus* based on the macroscopic and microscopic characteristics, which were confirmed by targeting and amplifying the species-specific *rodA* gene (313 bp). Five (5/20) isolates were found to be MDR by the disk diffusion method. EOs were tested as an alternative strategy for 5 MDR isolates of *A. fumigatus*. Clove EO provided better activity by 6 $\log_{10}$ reduction for 30-90 min exposure as compared to other EOs, therefore, shortlisted for well-diffusion and minimum inhibitory concentration (MIC) method. Clove EO provided promising results as compared to fluconazole, amphotericin B, ketoconazole, nystatin and voriconazole as its mean ZOI (39.6 ± 6.34) were better than all other commercial antifungals ($p < 0.001$). Mean MIC was reported to be 0.195 ± 0.11 µl/ml. Safety testing of the EO produced 74-82% cell viability for the range of 0.390 – 0.097 µl/ml as compared to DMSO. Findings of the present study highlight that clove EO can be source of a good therapeutic agent for infections of MDR isolates of *A. fumigatus*.

Index terms: Antifungal activity; clove; minimum inhibitory concentration; cytotoxicity assay.

RESUMO

Aspergillus fumigatus é um patógeno respiratório, que tem o potencial de causar pneumonia incubadora em pintos e aspergilose em aves e criadores. O uso frequente e irracional de antifúngicos tem levado ao desenvolvimento de resistência nas espécies fúngicas. O presente estudo teve como objetivo avaliar a atividade antifúngica de óleos essenciais (OEs) de quatro diferentes especiarias contra A. fumigatus multirresistente (MDR) isolado de 40 amostras de ração de aves. Vinte isolados fúngicos foram identificados como *A. fumigatus* com base nas características macroscópicas e microscópicas, que foram confirmadas pelo direcionamento e amplificação do gene rodA espécie-específico (313 pb). Cinco (5/20) isolados foram considerados MDR pelo método de difusão em disco. OEs foram testados como uma estratégia alternativa para 5 isolados MDR de *A. fumigatus*. Clove EO forneceu melhor atividade por redução de 6 log10 para exposição de 30-90 min em comparação com outros OEs, portanto, pré-selecionado para difusão e método de concentração inibitória mínima (MIC). Clove EO forneceu resultados promissores em comparação com fluconazol, anfotericina B, cetoconazol, nistatina e voriconazol, com ZOI médio (39,6 ± 6,34) melhor do que todos os outros antifúngicos comerciais ($p < 0,001$). A MIC média foi de 0,195 ± 0,11 µl/ml. O teste de segurança do EO produziu 74-82% de viabilidade celular para a faixa de 0,390 – 0,097 µl/ml em comparação com DMSO. Os resultados do presente estudo destacam que o EO de cravo pode ser fonte de um bom agente terapêutico para infecções de isolados multirresistentes de *A. fumigatus*.

Termos para indexação: Atividade antifúngica; cravinho; concentração inibitória minima; ensaio de citotoxicidade.

INTRODUCTION

Many fungal species act as contaminants in the food and feed sources leading to the spoilage and deterioration of the nutritive values. The release of mycotoxins followed by the ingestion can further aggravate the disease conditions. Increased humidity favours the fungal growth in the environment. Fungal species infect plants, which produce the grains for the animal feed. Improper storage conditions increase the mycobiota of the feed acquiring fungus from the environment and plant disease (Liu et

al., 2020; Sugiharto, 2019). The poultry industry is the second largest industry in Pakistan; therefore, preparation, handling and storage of poultry feed is required on a large scale. Poultry birds, farmers, poultry veterinarians and farm workers remain the health hazard of contracting fungal infection due to their exposure to feed. Amongst the fungal infections, aspergillosis is an important zoonotic disease for the chickens as well as human beings in the poultry farm setting (Whitehead; Roberts, 2014).

Fungal species, such as *Aspergillus*, *Penicillium*, *Fusarium*, and others, can contaminate poultry feed and produce mycotoxins. Fungal growth and mycotoxins can affect the palatability of feed, leading to reduced feed intake by poultry. This can result in decreased nutrient intake and subsequently affect the growth and performance of birds (Greco et al., 2014). Certain mycotoxins can impair the digestion and absorption of nutrients, leading to poor nutrient utilization by poultry. This can result in reduced growth rates, decreased egg production, and suboptimal feed conversion efficiency. Specific mycotoxins, such as aflatoxins and ochratoxins, can cause damage to various organs in poultry, including the liver, kidneys, and immune system organs. This can impair the overall health and performance of birds (Murugesan et al., 2015).

Treatment of brooder pneumonia, aspergillosis and other fungal diseases involves the use of antifungal drugs, which comprise the azole and polyene groups. Apart from clinical use, these antifungals are being used in agriculture and environmental hygiene. Irrational use has led to frequent reports of the development of resistance to the antifungal drugs including azole and polyene groups (Rhodes et al., 2022; Serrano-Lobo et al., 2022; Zvezdanova et al., 2022). Furthermore, antifungal drugs lead to side effects including hepatotoxicity and gastrointestinal problems (Houšť; Spížek; Havlíček, 2020). Alternatively, the given problem can be tackled by plant essential oils (EOs) as these have good fungicidal potential and carry multiple allied benefits such as antioxidant, anti-inflammatory, antibacterial, antiviral, anti-coccidial, and psychogenic reliefs (Awaad et al., 2016; Imran; Alsayeqh, 2022; Ramsey et al., 2020). The fungicidal mechanism of EOs includes the contraction of the fungal cell wall, distortion of the hyphae, cytoplasm coagulation and disruption of ATP synthesis (Das et al., 2021). Amongst plant EOs, spice-derived EO can have alternative antifungal potential as these have been used in traditional medicines since historic times. Keeping in view the background, the EOs of *Syzygium aromaticum* (clove), *Curcuma longa* (turmeric), *Elettaria cardamomum* (cardamom) and *Ferula asafetida* (asafetida) were evaluated for antifungal activity for the multi-drug resistant (MDR) isolates of *Aspergillus fumigatus*.

MATERIAL AND METHODS

Isolation of fungi

Isolates of *A. fumigatus* were isolated from 40 poultry feed samples by using the spread plate method. A 25-gram poultry feed was added to the peptone water to make the total volume 250 ml. The prepared suspension was serially diluted from 10^{-1} to 10^{-4}. A 100 µl from each dilution was spread on the Sabouraud dextrose agar (SDA) plates. Plates were incubated at 25 °C for 3 days. Then purification of colonies was done from primary culture by spot plate method. Identification was performed by macroscopic study and microscopy of pure culture. Characters of growth were observed from both obverse and reverse sides. Hyphae, spores shape, spores arrangement and other special structures were examined by slide culture method.

Confirmation of fungal isolates

DNA was isolated by using a DNA extraction kit. Concentration and purity of the extractions were checked by Nanodrop. Fungal isolates were confirmed by polymerase chain reaction (PCR) using specie-specific primers (Forward Primer 5′-ACATTGACGAGGGCATCCTT-3′ and Reverse Primer 5′-ATGAGGGAACCGCTCTGATG-3′) for *rodA* gene (Zarrin et al., 2017). PCR conditions were programmed for 25 µl reaction volume. Following conditions were set, which include pre-incubation at 95 °C for 15 min, denaturation at 94 °C for 30 sec, annealing at 69 °C for 90 sec, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. PCR product was visualized using agarose gel electrophoresis and DNA bands were visualized under UV.

Antifungal resistance testing

The resistance profile for commonly used antifungals was determined by the disk diffusion method as described by CLSI guidelines (M 51-A document) (Clinical and Laboratory Standards Institute - CLSI, 2010; Gupta et al., 2015). The inoculum of each fungal isolate was swabbed on SDA media. Different antifungal drugs *i.e.*: fluconazole (25 µg; Oxoid Ltd. England), voriconazole (1 µg; Oxoid Ltd. England), ketoconazole (17.25 µg/ml), amphotericin B (8.5 mg/ml) and nystatin (100 units; Oxoid Ltd. England) were used to check their efficacy against fungal isolates. Plates were incubated at 25 °C for 48 hr and results were recorded as one of inhibition in mm. Slight

portions of hyphae extending within the zone of inhibition (ZOI) were ignored while measuring.

Log_{10} reduction assay

Antifungal activity of turmeric, clove, cardamom and asafetida EOs was determined by log_{10} reduction method for resistant isolates. The EOs and spore suspension were mixed to achieve 10^6 spores/ml and incubated at 25 °C for 30-, 60- and 90-mins. A 500 µl was taken after incubation and was added to the normal saline tube, and serially diluted till 10^{-5}. A 100µl from each dilution was poured on pre-solidified SDA plates and was spread, followed by incubation at 25 °C for 3-5 days. Colony-forming unit (CFU) per ml was calculated from the plate having 15-150 colonies. Each CFU/ml was performed in five replicates and results were expressed as in Log_{10} CFU/ml values (STAATT, 2005).

In vitro activity of essential oils

In-vitro activity of EOs for resistant isolates was checked by the well diffusion and minimum inhibitory concentration (MIC) assays. The spore suspension was swabbed in sterile molten media. Wells were made in it and sealed by molten SDA. The essential oil was added to these wells and incubated at 25 °C/3-5days, and ZOI were measured. For MIC, a two-fold serial dilution of EO was made in a microtiter plate ranging from 100 – 0.19 µl/ml. Standard spore suspension 10^6 spores/ml was used to determine the inhibitory activity. The spore suspension was prepared in Sabouraud dextrose broth, and 100 µl of inoculum was added to each well of the microtiter plate keeping one well for the positive and negative controls. The plates were incubated at 25 °C for 5 days. The minimum concentration of the oil, which inhibited the growth of the fungus was noted as MIC (Perveen et al., 2018).

Cell viability assay

Safety of EO was estimated by colourimetric method using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) following de Oliveira et al. with some modifications (De Oliveira et al., 2015). Analysis was performed spectrophotometrically based on the ability of living cells to reduce MTT yellow-coloured dye to a purple-coloured formazone product as compared to controls. Vero cell lines were used, which were cultured in Glasgow minimum essential medium supplemented with 10% fetal calf serum for the assay. A 3×10^4 cells/well were cultured in the microtiter plate, followed by the incubation at 37 °C in a 5% CO_2 incubator for 72 hr. A 100 µl of decreasing concentrations of EO (100 – 0.19 µl/ml) were added by two-fold serial dilution while keeping replicate wells for DMSO negative and positive controls. After 72 hr, the essential oil was removed and 10 µl of MTT reagent was added to each well, followed by incubation. After incubation, the medium was discarded and 200 µl methanol was added to solubilize crystals of formazone. MTT reduction was measured by determining the optical density (OD) values by ELISA reader at 570 nm wavelength. Cell survival percentage was calculated by applying the Equation 1.

$$\text{Cell survival \%} = \left[\frac{\text{OD Experimental} - \text{OD Negative Control}}{\text{OD Positive Control}}\right] \times 100 \quad (1)$$

Gas chromatography-mass spectrometry

Components of clove EO were determined by Gas chromatography-mass spectrometry (GC-MS) analysis. The analysis was performed on Agilent 6890N gas chromatography coupled to Agilent 5973N mass selective detector model having fused silica capillary column HP-5MS column (Kamaliroosta et al., 2012). The injector and detector were kept at 240 °C and 300 °C, respectively. For gas chromatography, the temperature was set at 60 °C for 1 min, followed by the programming at 8 °C /min to 200 °C, 200 °C for 2 min, followed by an increase in temperature by 10 °C/min to 230 °C and held there for 5 min. The final temperature was programmed to be 260 °C for 5 min. Phytochemicals were determined by comparing the retention indices of constituents.

Statistical analysis

Data obtained from the antifungal activity were analysed by one-way analysis of variance (ANOVA) followed by Dunnet's test using Graphpad Prism 8.0.2. Significance of results were reported at $p < 0.05$.

RESULTS AND DISCUSSION

Hot and humid conditions in the long summer season promote the growth of fungi in poultry feed, particularly in storage conditions. Different *Aspergillus* species are commonly present as a common contaminant in the ambient environment of poultry farm settings (Arné et al., 2011). Antifungal therapy and acquired genetic resistance have led to an increase in the MDR isolates of *A. fumigatus*. The irrational use of antifungals has led to GIT side effects as well. In contrast to commercial antifungals, plant EO can be used as a biological weapon to overcome resistance problems and side effects (Mutlu-Ingok et al., 2020). Therefore, the present study is designed

to study the antifungal activity of clove EO for MDR isolates of *A. fumigatus* as compared to commercially used antifungals. Clove EO proved its potential as compared to all the antifungals including fluconazole, amphotericin B, ketoconazole, nystatin and voriconazole. Inhibitory activity was promising in both *in-vitro* assays including well diffusion assay as well as MIC method. Clove EO had a better activity, which was statistically highly significant as compared to the other antifungals.

Preliminary identification

Typical isolates (n=20) of *A. fumigatus* were identified from poultry feed by macroscopic, microscopic and molecular identification methods. Immature colony characters were observed after 2 days while mature characters were observed after 5 days. Initial colour of the fungal growth was white, then it turned to bluish-green having white edges and white on reverse side on the 3rd day. Texture of the immature colony was cottony while the mature growth was dusty in texture with slate grey colour having folds on the obverse side as were observed by (Afzal et al., 2013). Reverse side was pale in colour with ridges on it. Microscopic characters such as conidial heads were typical columnar and uniseriate hyphae, Conidiophores were short, smooth-walled have a conical shape terminal vesicle. Hyphae were septate and hyaline (Figure 1). Twenty isolates were presumptively identified to be *A. fumigatus*.

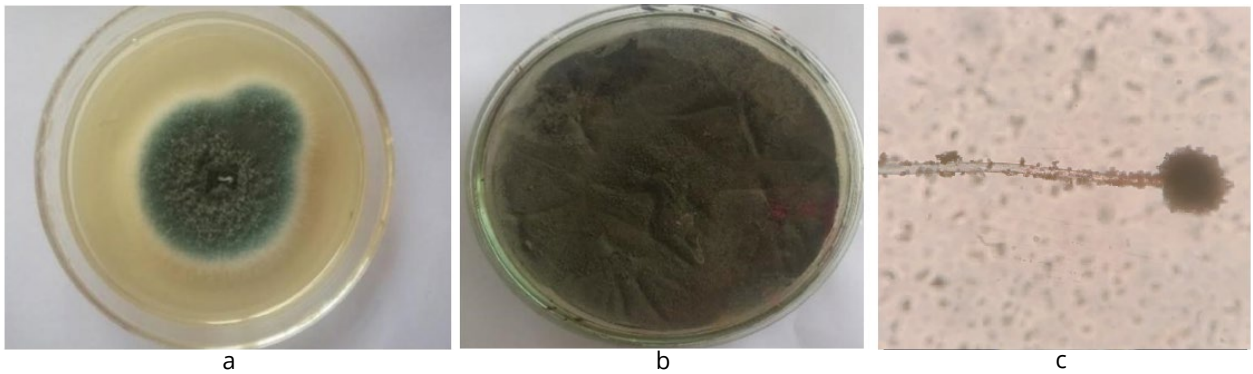
The isolation suggested the common occurrence of infectious *A. fumigatus,* which can cause respiratory disease in chickens, and humans handling poultry feed. The fungal specie is prevalent in the ambient environment of poultry feed storage godowns and is commonly reported in poultry feed in moist environment (Saleemi et al., 2010; Sana et al., 2019). Detection of the specie from the lung tissues of the chicken evidenced the involvement in respiratory infection (Nawrot et al., 2019). Another study from Pakistan reported Aspergillus sp. to be the causative organism for lung infection in broiler. Retained moisture in the litter of saw dust was one of the main risk factor associated with Aspergillus presence in the environment (Ali, M. et al., 2022).

Molecular confirmation of *A. fumigatus*

The purity of extracted DNA was evaluated by determining the OD *via* nanodrop. The 260/280 ratio for absorbance was indicative of good extraction. Gel electrophoresis was performed to visualize DNA in form of bands. Amplification of the amplicons by specific primers resulting in the bands of 313 bp, confirmed the isolates to be of *A. fumigatus*. All the isolates (n=20) were confirmed by molecular identification while the amplification of three representative isolates is shown in Figure 2. Amplifying the species-specific *rodA* gene confirmed the isolates to be *A. fumigatus*. The *rodA* gene codes for hydrophobic polypeptide rodlet A, present on the surface of conidia. Its amplification is a reliable technique for the identification of *A. fumigatus* isolates (Giray et al., 2016; Spanamberg et al., 2016; Zarrin et al., 2017).

Antifungal resistance profile:

Antifungal activity of various antifungals was checked on isolates of *A. fumigatus* (n=20) through the disk diffusion method. For fluconazole, 19 out of 20 isolates (95%) were resistant and one isolate was categorized as intermediate based on CLSI guidelines. Amphotericin B was the second antifungal in the list to which more isolates (75%) were resistant. Seven out of 20 isolates were sensitive, while 13 isolates (65%) were resistant to ketoconazole. For voriconazole, 16 isolates (80%) were sensitive and 4 (20%) were resistant. Five isolates (25%) were sensitive, 6 were intermediate and 9 (45%) were resistant to nystatin (Table 1).



a b c

Figure 1: Preliminary identification and confirmation of *A. fumigatus* (a: Immature obverse side, b: Mature obverse side, c: Microscopic structure.).

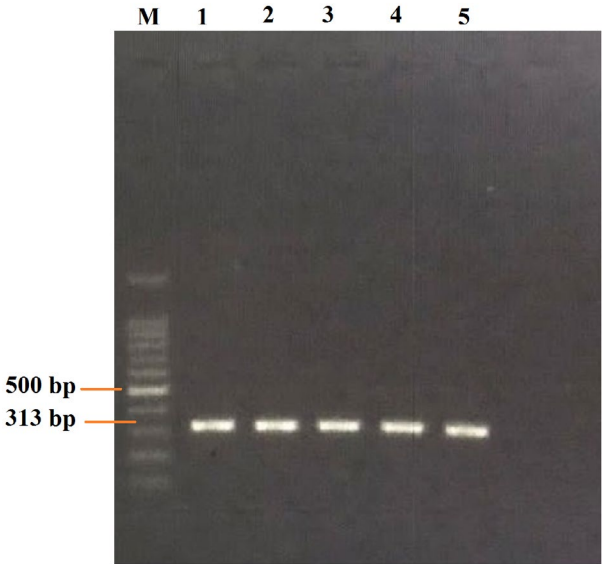


Figure 2: PCR-based detection of *A. fumigatus*. Lane M represents marker, Lanes 1, 2, 3, 4 and 5 represents the *rodA* gene amplicons of the representative isolates of *A. fumigatus*.

Table 1: Resistance and sensitivity patterns of *A. fumigatus* isolates (n=20) recovered from poultry feed for various antifungal agents.

Antifungal	Sensitive		Resistant	
	N	%	N	%
Fluconazole	0	0	19	95
Amphotericin B	3	15	15	75
Ketoconazole	7	35	13	65
Nystatin	5	25	9	45
Voriconazole	16	80	4	20

The present study found five isolates (S3, S4, S12, S14 and S18) to be categorized as MDR, as those were resistant to more than three antifungals. The increase in resistance to the isolates of poultry feed origin is a matter of grave concern for poultry veterinarians and farmers as the pathogen has high zoonotic importance. The data on the resistance pattern of *A. fumigatus* in poultry farm settings are lacking in Pakistan. Azole-based drugs are frequently used in clinical, agricultural and environmental settings. Therefore, the azole resistance range is reported high. Before 2000, the azole resistance was found to be rare but in the last decade, 5-10 % isolates have been found to be resistant across Europe, Africa, South America and Asia (Fraaije et al., 2005; Resendiz-Sharpe et al., 2021; Yerbanga et al., 2021). Similarly, the present study reported (5/20) isolates to be resistant to multiple drugs for *Aspergillus* from poultry feed. The fungus is developing resistance due to mutation in the azole target (cyp51A). Surprisingly, a study in Poland, identified 60 isolates from chicken lungs but only one isolate was found to be resistant to the azole group (Nawrot et al., 2019).

Comparative activity of EOs by Log_{10} reduction of *A. fumigatus*

Five MDR isolates of *A. fumigatus* were shortlisted to determine the antifungal activity of cardamom, clove, asafetida and turmeric EOs by log_{10} reduction assay. Log_{10} CFU reduction of all the EOs was reduced or remained at the maximum level with the increase in exposure time. The use of different EOs in log_{10} reduction assay revealed clove EO to be having the highest log_{10} reduction of 6 ± 0 at the exposure time of 30 min, which remained the same at 60 and 90 min as well. The lowest activity was reported for cardamom EO; it achieved the log_{10} reduction of 3.76 $\pm$ 1.11 at 30 min, while improving the log_{10} reduction to be 4.98 $\pm$ 0.73 at 90 min. Turmeric and asafetida EOs revealed close activity with each other, which was comparatively better than that of cardamom EO. Log_{10} reduction of turmeric and asafetida was 5.6±0.76, 5.76 $\pm$ 0.6 at 30 and 60 min of exposure while asafetida EO had 5.88±0.4 and turmeric had 6±0 log_{10} reduction at 90 min (Figure 3).

The EOs have been more commonly studied for the bacterial pathogens in the literature, however, the antifungal activity is also promising. The antimicrobial activity for *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella*, *Enterococcus faecalis* has been encouraging (Ali et al., 2022a; Peret-Almeida et al., 2008; Raja et al., 2016; Yasmin et al., 2020). Furthermore, antifungal activity for multiple fungal species has been also reported based on the active components of EOs which include carvacrol, thymol, eugenol, menthol, α-terpineol and geraniol (Medeiros et al., 2011; Miri et al., 2023; Qin; Zhang, 2023; Raut; Mahatma; Vaniya, 2023). Studies having the log_{10} reduction of filamentous fungi using relevant EOs were not available in the literature review. Although, the results of the present study were supported by the literature where cardamom was reported to be less active as compared to clove (Naveed et al., 2013). Similar as well as contradictory results were reported by another study where turmeric and cardamom EO had less activity as compared to clove EO but asafetida EO was found to be equally effective (Kamble; Patil, 2008).

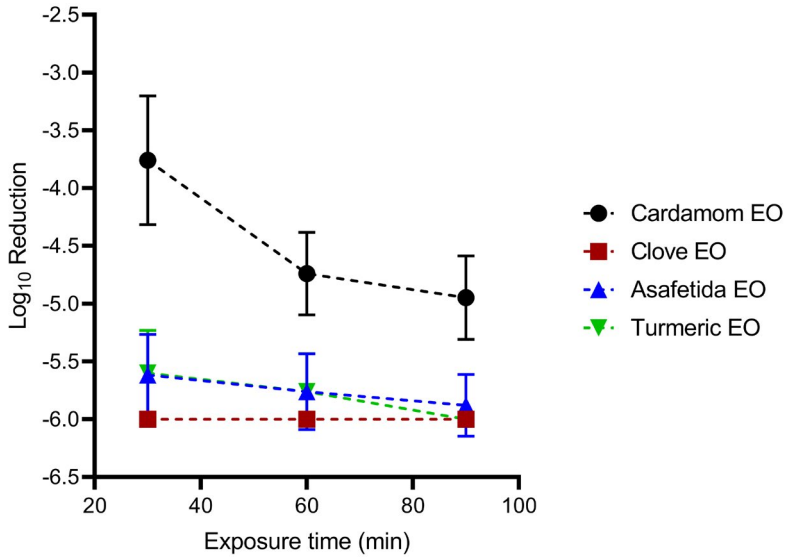


Figure 3: Graphical representation of Comparative activity of EOs by Log_{10} reduction of *A. fumigatus.* Clove EO provided better log reduction than other EOs.

In-vitro activity of essential oil

Five isolates of *A. fumigatus* were short-listed based on the high resistance profile for the previously described commercial antifungals. As clove EO provided better results, the antifungal activity of clove EO was determined by well diffusion assay and MIC. For well diffusion assay wells were made for EO of the size of commercial antifungal disks. After measuring ZOI, the sensitivity was compared to the commercial antifungals as controls. ZOI of clove EO for MDR isolates ranged from 34-50mm, averaging 39.6 ± 6.34 mm. The inhibitory activity of clove EO was better significantly as compared to all the antifungals including fluconazole, amphotericin B, ketoconazole, nystatin and voriconazole ($p = 0.001$) (Figure 4). Antifungals from the azole and polyene group were not able to inhibit the growth of MDR isolates. One of the main strengths of the EOs is that these can be used for drug resistant pathogens as resistance far less likely to develop for EOs as compared to commonly used antifungals (Lang; Buchbauer, 2012). Alternatively, Amphotericin B and itraconazole are two of the earliest antifungals recommended for *Aspergillus* infection, their frequent use had led to the development of high resistance (Ashu et al., 2018; Verweij et al., 2020). The resistance for amphotericin B was evidenced in the present study, however, the unavailability of the itraconazole disks was the limitation of the study.

In MIC, isolate S3 had maintained inhibitory activity till 8th well with MIC 0.390 µl/ml, isolates S4 and S12 showed growth inhibition up to 9th well with MIC 0.195 µl/ml and isolates S14 and S18 had inhibitory activity up to 10th well with MIC of 0.097 µl/ml. The mean MIC for the MDR isolates was calculated to be 0.195 ± 0.11 µl/ml (Table 2). A similar study was reported in which the activity of clove EO was explored for the five isolates of *C. albicans*. MIC for all the isolates was found to be 1.25 µl/ml (Alshaikh; Perveen, 2017). A decade ago relatable studies were conducted in India and Portugal, where the activity of clove EO was comparatively less as compared to commercial antifungals fluconazole. Contrary to that study, the present study reported minimal activity for fluconazole while the activity of the EO was better than other antifungals used as well. The difference in the inhibitory concentration can be due to different constituents of EO and the different nature of isolates or species. Components of one essential oil may enhance activity in combination with other essential oil based on the synergism. Furthermore, the combination of EOs and IR treatment enhances the antifungal potential (Ji; Li; Fan, 2022). Another study from Portugal supported the results of our study where clove EO was able to inhibit the *Candida* spp and dermatophytes better than Fluconazole and amphotericin B (Pinto et al., 2009). The MIC value for *A. fumigatus* isolates was found to be 0.32 µl/ml, which is comparable to the inhibitory concentration of 0.390 for the S3 MDR isolate.

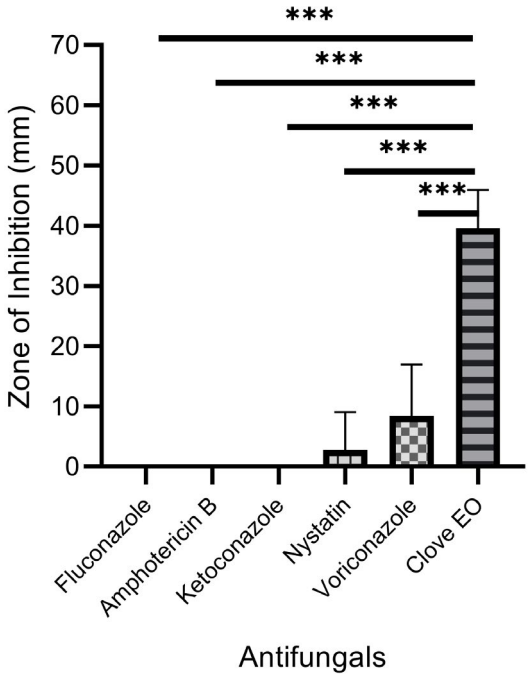


Figure 4: Graphical representation of a comparison of the antifungal activity of clove essential oil (EO) with fluconazole, amphotericin B, ketoconazole, nystatin and voriconazole. Clove EO has significantly better activity as compared to all commercial antifungals used.

Table 2: Minimum inhibitory concentration (MIC) of clove EO for five multi-drug resistant (MDR) isolates of *A. fumigatus*.

Sample IDs of MDR isolates of *A. fumigatus*	MIC of clove EO (µl/ml)	Mean MIC (µl/ml)
S3	0.390	
S4	0.195	
S12	0.195	0.195 ± 0.11
S14	0.097	
S18	0.097	

Determination of safe concentration of clove essential oil

Clove EO is widely used in pharmaceutical, cosmetic and flavouring agents. It has known antimicrobial, antioxidant and cytotoxic properties. The cytotoxic potential is high at a high concentration of EO with the particular component of eugenol. The percentage of components of EO may vary based on the environment and nutrients under which plants grow (Lang; Buchbauer, 2012). In the present study, the cytotoxic potential of the EO was evaluated by MTT assay on Vero cell lines. The EO concentrations were diluted from 100 – 0.097 µl/ml, while 0.097 µl/ml was found to provide cell viability to 82%. The concentration range from 0.390 - 0.097 µl/ml proved to be promising as it resulted in the inhibition of five MDR isolates of *A. fumigatus* (Figure 5). MTT assay on Vero cell lines is an equally reliable technique to test the safety of EOs for their antifungal or antibacterial potential when compared with Hela and Hepii cell lines (Ali et al., 2022b; Rahimifard et al., 2009).

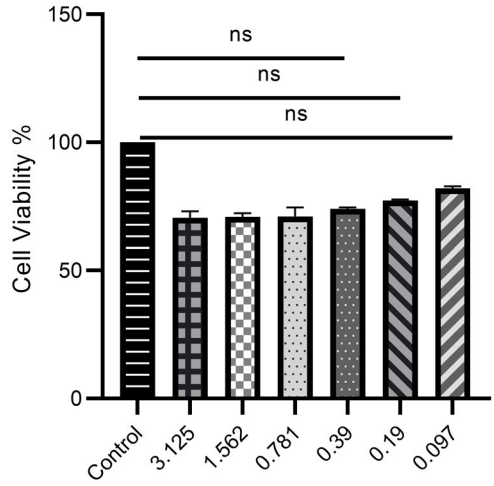


Figure 5: Dose-dependent cytotoxicity profiling of clove essential oil (EO) on Vero cell culture. Cell viability increased with the decrease in the concentration of clove EO. DMSO was used as a control.

Table 3: Fatty acid profile of clove EO analyzed by GC-MS, steam-distilled from clove buds.

Sr. No	Compound	Retention time (min)	Amount (%)
1	Stearic acid	28.21	3.33
2	Eugenic acid	21.36	66.32
3	Linoleic acid	25.6	4.03
4	Octadec- 11-enoic acid	24.5	19.15
5	Butyric acid	7.3	3.7
6	3 -bis 1,1 dimethylethyl	19.5	1.5

Composition of clove EO

Steam distillation of clove buds produced straw-coloured oil with low viscosity. Chemical composition

was analysed by GC-MS analysis, which revealed 6 components to be present in the EO. Amongst all the compounds, the percentage of eugenic acid was the highest (66.32%) followed by octadec- 11-enoic acid (19.15%). The lowest percentage was found to be for 3 -bis 1,1 dimethylethyl (1.5%) (Table 3).

Results of the present study related to many studies performed on clove EO where eugenol was the major component. Studies in literature have demonstrated that eugenol has better antifungal potential as compared to 21 active components of EO and their derivatives. Eugenic acid plays its role by inhibiting the cell wall synthesis or assembly of components (Carrasco et al., 2012). Furthermore presence of stearic acid enhances the fungal activity as well (Bhaskaran et al., 2022). Although the percentage of eugenic acid (66.32%) varied from those of Alshaikh and Perveen (74.6%), Matta (2010) (62.1%) and Marya et al. (2012) (58.29%). The number of compounds identified was also different as 5 and 11 different compounds were identified by Alshaikh and Perveen, and Marya et al. (2010) respectively (Alshaikh; Perveen, 2017; Marya et al., 2012; Matta, 2010). The varied number of compounds and percentages was presumptively due to variations present in the plant species and the soil nutrients and environment these grow in.

Limitations and Future Perspectives

The EOs of spices have shown good potential for antifungal activity. However, there are few limitations of this study and for the overall use of EOs. One of the limitations of the study is that we did not study the activity of EO in the poultry feed system. Results of antifungal activity of the given concentration of EO may differ when applied to the poultry feed system as the feed contains its own lipid, carbohydrates, vitamins and proteins which may interfere the activity of EO on fungal species present on it. Another limitation is the absence of optimization of EO components in dissolving agents other than DMSO. Furthermore, combination of EO and activity of purified components were not tested in this study which may provide some further understanding of the EO activity.

Prospective researchers may explore the activity of individual components and combination of the EO. Furthermore nano-encapsulation nano-emulsion of EO can be tested on animal feed system to control the source of fungal pathogen to poultry industry particularly and animal feed industry generally. Working on these options may provide a good alterative solution to prevent fungal contamination and diseases.

CONCLUSIONS

Multiple drug resistance for azole groups was found in *A. fumigatus* isolated from poultry feed in Pakistan. Clove EO has promising antifungal activity for MDR *A. fumigatus* isolates when compared to turmeric, cardamom and asafetida EOs and commercial antifungals. Therefore, clove EO is recommended to be used and explored further as a bio-fungicide.

AUTHOR CONTRIBUTION

Conceptual idea: Anjum, A.A.; Methodology design: Naeem A., Ali, T., Manzoor, R.; Data collection: Sheikh, A.A. Naeem A., Ali, T., Manzoor, R.; Data analysis and interpretation: Ashraf, M.A., Sheikh, A.A.; Writing and editing: Ashraf, M.A., Anjum, A.A.

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