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ORIGINAL ARTICLE

FTIR Spectroscopy for Identification Aspergillus fumigatus Secondary Metabolites Extract against Multi Drugs Resistance Bacteria Isolated from surgical Site wound Infection

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ABSTRACT

In the current scenario Multi Drug Resistant (MDR) bacterial pathogens are increasing very rapidly due to the misuse of antibiotics to control antibiotic resistance, we need to know the current pattern of antibiotic resistance. Furthermore, new bioactive metabolites are needed for the alternative option to control antibiotic resistance. Fungi have the ability to produce potent metabolites. The production of secondary metabolites such as antimicrobial agents is the most important property of fungi. Antimicrobial activity of extract were noted against different pathogenic (Clinical Isolates) and using agar well diffusion assay method and noted different zone of inhibition of each bacteria, wells were loaded with different concentration's 50 µL and 100 µL. Maximum zone of inhibition were reported at different concentration of different bacteria. Ethyl acetate crude extract at 50 µL concentration's 17 ± 0.15 mm, 18 ± 0.1 mm , 16 ± 0.4 mm, 19 ± 0.26 mm, 17 ± 0.30 mm,16 ± 0.36 mm, zone of inhibition against E.coli sp, Pseudomonas, Klebsiella sp Proteus sp, S,aureus, , and Salmonella sp, respectively. While in 100 µL zone of inhibition was noted, 21 ± 0.15 mm, 22 ± 0.40 mm, 20 ± 0.35 mm, 21 ± 0.26 mm, 22 ± 0.45 mm 19 ± 1.10 mm zone of inhibition against E.coli sp, Pseudomonas, Klebsiella sp Proteus sp, S,aureus, , and Salmonella sp, respectively. In the current study we used FTIR and Plasmon Resonance (SPR) UV-visible spectroscopy observed at 408 nm during UV-visible spectroscopy. FTIR analysis revealed the involvement of phenolic, carboxyl and hydroxyl groups in reduction of Ag^{+} ions to form fungal metabolites while stabilization components of were fungal metabolites amide linkage and amino acid.

Keywords; Aspergillus fumigatus, Secondary metabolites, Ethyl acetate, FTIR, UV-visible spectroscopy, MDR pathogen.

INTRODUCTION

Fungi is one of the largest groups of eukaryotic organisms that play a vital role in ecosystems. Fungi have many applications in industry, agriculture, medicine, and environment (Ramesh et al., 2014). Fungal species are significant producers of antimicrobial agent but due to resistance, they required more attention and research for discovery of new antimicrobial agents and 20 most demandable and very successful drugs are extracted from fungi worldwide. The production of secondary metabolites such as antimicrobial agents is the most important property of fungi. (Pinruan et al., 2007). Some important antibiotics are produce by various fungal species like Penicillium sp., Aspergillus sp, Cladosporium sp, and yeasts (Uzair et al., 2018). Antibiotics produces from fungi include fusidic acid, Cephalosporin and Penicillin are using on large scale for the treatment of many infections. New antibiotics are required to overcome resistance caused by microorganism so that's why pharmaceutical industries are searching fungi to increase the number of antibiotics. Some soil fungi can produce several novel secondary metabolites These metabolites can be exploited for medical therapy and are used as therapeutic agents (Farjana et al., 2014). There are certain classes of secondary metabolites including alkaloids, terpenoids, and polyketides. Which are important sources for drug development. Polyketides is the most abundant fungal secondary metabolite, are a group of bioactive compounds which have applications as anti-cholesterol, anti-cancer and antibiotic agents. Different species of fungi like Aspergillus niger, Aspergillus ochraceus, Aspergillus flavus, Penicillium notatum, Bacillomyces spp, Trichoderma spp, and Cladosporium spp are known for the production of valuable secondary metabolites (Bbosa et al., 2014). Aspergillus fumigatus is fungal specie which belongs to the genus Aspergillus. Aspergillus genera also include Aspergillus oryzae & Aspergillus nidulans. Aspergillus fumigatus produce secondary metabolites in sporulation phase where G protein signaling regulates secondary metabolite production. (Bala et al., 2013) Aspergillus species has potential to produce several varieties of secondary metabolites by optimization of growth factors or environmental condition like temperature, pH and time. These parameters can increase the productivity of secondary metabolites (Khan et al., 2018). Species of Aspergillus are commonly found molds and spread in environment widely (Al-Daamy et al., 2018). In the current scenario Multi Drug Resistant (MDR) bacterial pathogens is

increasing very rapidly due to the misuse of antibiotics to control antibiotic resistance, we need to know the current pattern of antibiotic resistance. Furthermore, new bioactive metabolites are needed for the alternative option to control antibiotic resistance. For this purpose, fungal secondary metabolites are the feasible option to treat SSI infections caused by MDR bacterial pathogens. Available antibiotics in the world are becoming ineffective with the passage of time due to the indiscriminate use of antibiotics (Ang et al., 2004).

MATERIALS AND METHODS

Samples were collected from soil at various locations at Peshawar city in sterilized polyethylene bags by using sterile spatula. After collection of samples, they were screened for fungal isolation by serial dilution method. For serial dilution method ten test tubes and one conical flask along with 9ml distilled water for each sample and Potato Dextrose Agar media (PDA) were sterilized at 121°C for 15 minutes and at 15psi in autoclave (Pena et al., 2010).

Identification of fungal species were performed on the basis of morphology, color, shape & lacto phenol cotton blue staining for microscopic analysis

Batch fermentation: About 500ml of Potato dextrose broth media (PDB) media was prepared in 1000 ml Erlenmeyer flask in Abasyn University labs. Prepared media was sterilized at 121°C for 15 minutes at 15 psi. After sterilization 10 % inoculum was added to the prepared PDB media. Inoculated flask was incubated at 25°C for 2 weeks in a shaking orbital incubator set at 120 rpm.

Extraction of secondary metabolites: The media containing the fungus and metabolites was mixed homogenously in 10% ethyl acetate solution. Solvent extraction technique was used to extract secondary metabolites from the mixture using methanol and ethyl-acetate as extracting organic solvents. Equal volume of each solvent was added to the mixture and was mixed for 10 minutes and then was left steady for 5 minutes. After 5 minutes two clearly immiscible layers was formed in the container. The upper organic layer formed by the solvent was used separated from the lower layer as the lower layer was pellet of fungal crude extract containing our desired product. Rotary evaporator was used to evaporate the organic solvent and the remaining extract collected as desired secondary metabolite

Fourier Transform Infrared Spectroscopy of crude extract: The fungal crude extract was loaded in FTIR and scan at the range of 400 - 4500cm^{-1} with the use of FTIR, different functional groups was interpreted from the graph.

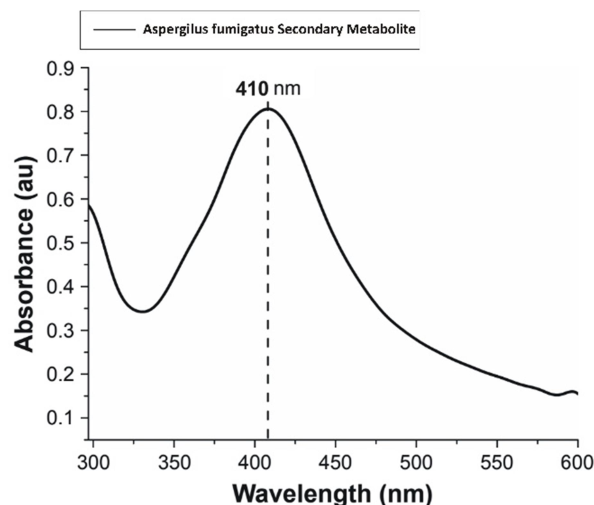
Mycochemical investigation of secondary metabolites: The fungal crude extract was subjected to mycochemical screening, a method used for the detection of different compounds such as flavonoids, phenols, alkaloids, Saponins, tannins and terpenoids.

Surgical wound sample collection: Surgical wound samples were collected from the patient of different hospitals such as Lady Reading Hospital (LRH), Peshawar Pakistan using sterile cotton swabs. The samples were transported to the microbiology laboratory at Abasyn University Peshawar, for culturing and identification of bacteria.

RESULTS

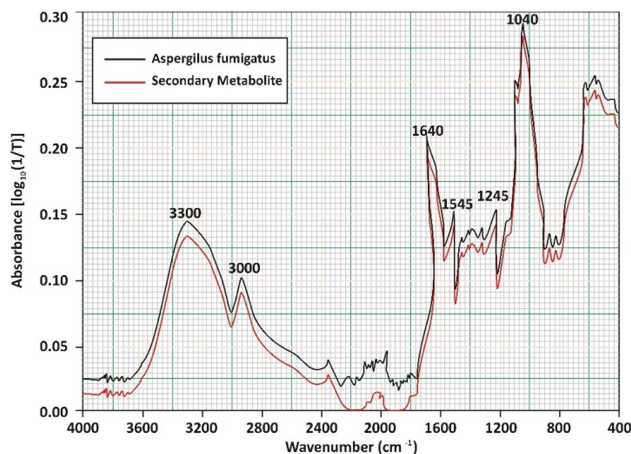
Characterization of Synthesized fungal metabolites from the Extract of A. fumigatus:

UV-visible spectrophotometry: Change in color was observed from pale yellow to grayish after addition of fungal extract to silver nitrate solution. Strong peak specific for the production of AgNPs was observed at 410 nm as shown in Fig. 5.1



Fourier Transform Infrared Spectroscopy (FTIR) of fungal metabolite and synthesize nanoparticles

FTIR spectroscopy analysis was carried out to determine the possible interaction between bioactive molecules which were responsible for the synthesis and stabilization of secondary metabolites. FTIR analysis of crude extract of A fumigatus and fungal secondary metabolites were performed and different peaks were observed. In the FTIR spectrum representing amines, carboxylic acids and alkenes, which played an important role in capping, stabilization and synthesis of fungal secondary metabolites. In the current study. The peaks were in the range in 1040, 1245, 1545,1640, 3000 and 3300 cm^{-1} showed different functional groups such as alcohol, alkanes, carboxylic acid or ester, amide, alkanes, aliphatic amines or phenol and amines respectively.



Mycochemical investigation of secondary metabolites

The fungal crude extract were tested for mycochemical analysis, such as flavonoids, phenols, alkaloids, Saponins, tannins and terpenoids using standard procedure. It was observed that the crude extract of A. fumigatus had different phytochemical's i.e. tannins, flavonoids and terpenoids while phenolic compounds and Saponins were absent in the test extracts as shown in the table 5.2

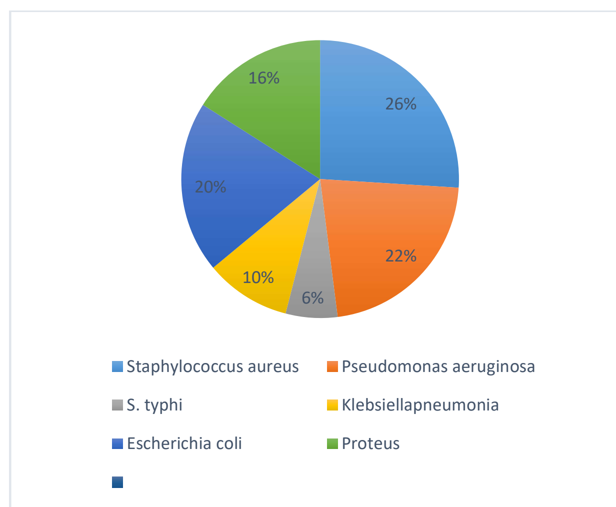
Table 5.2: Detailed Description of Mycochemical investigation of secondary metabolites

Components	Ethyl acetate Crude Extract
Flavonoids	+++
Phenolic Compounds	_
Saponins	_
Tannins	++
Terpenoids	+

Identification of collected Bacterial Isolate: In the current research study among total 50 samples 44 samples were positive for bacterial growth while 6 samples had no growth on culture plates were collected from pathology section and surgical B ward Lady Reading hospital Peshawar Pakistan. Total 44 samples were positive on culture plates for bacterial growth including Gram negative and Gram positive bacteria. Identified bacterial species were Staphylococcus aureus, P. aeruginosa, , K. peneumonia, E. coli, Proteus sp,.and S. typhi . These clinical samples through pure culture techniques including various types of media i.e Nutrient agar (N.A). Blood agar, Mannitol salt agar (MSA), Eosin Methylene blue (EMB), and MacConkey agar. While characterization of these collected isolates were screened out by performing microscopy and different biochemical tests, which showed in percentage in graph. In gram staining Staphylococcus aureus, was Gram positive and remaining isolates were Gram negative. It was observed that E.coli showed acidic with no colour change in TSI test but showed negative response toward oxidase, urease,citrate and coagulase test. E.coli also showed indole positive response while the S. aureus displayed negative respond to indole and oxidase and positive responsed in Urease test as well as towards coagulase test. All positive and negative tests are briefly explained in table

Table 1: Isolated bacterial species from the deep wounds surgical infection.

S.NO	Identified species	No. of isolated species (n)	Percentage
1	Staphylococcus aureus	13	26 %
2	Pseudomonas aeruginosa	11	22 %
3	Klebsiella spp	5	10 %
4	Escherichia coli	10	20 %
5	Proteus	8	16 %
6	S. typhi	3	6%



Identification of collected Bacterial Isolate

Table 5.3: Detailed Description of Microscopy and Biochemical Test Response of Test bacterial Organisms.

Bacterial species identified	Gram Staining	Biochemical tests								
		Lactose	Dextrose	Sucrose	H_2S Test	Indole Test	Citrate Test	Urease Test	Catalase Test	TSI Test
E. coli	-	AG	AG	-	-	+	-	-	+	A/NC
Klebsiella sp.,	-	AG	AG	-	+	+	+	+	+	KK
Pseudomonas sp.,	-	-	-	-	-	-	+	+	+	+
Proteus sp.,	-	-	-	-	+	-	+	+	+	-
S. aureus	+	-	-	-	-	+	+	-	+	N/A
S. typhi	-	-		-	+	-	-	-	+	A

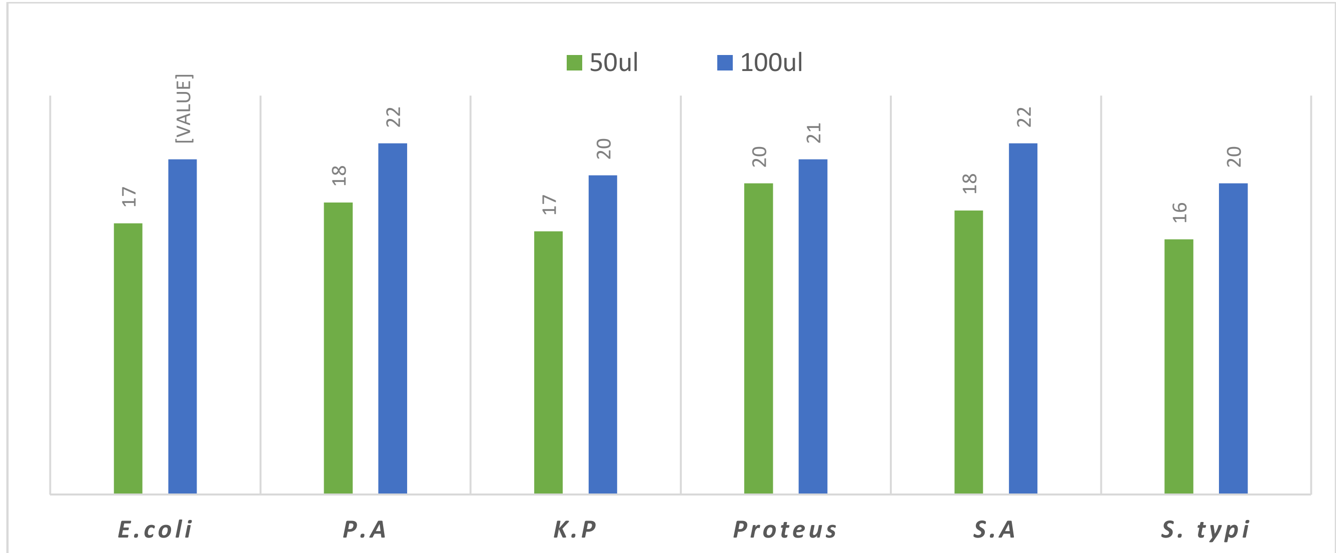
Key: + = Positive reaction, - = Negative reaction, d = Variable reaction, Cat = Catalase, Oxi = Oxidase, Cou = Cougulase, Cit = Citrate utilization, Ind = Indole production, Urea = Urease production, TSI = Triple sugar iron, K = Red, A = Yellow, K = Alkaline, A= Acidic, NA= Not applicable

Antimicrobial Activity of Fungal crude extract: Antimicrobial activity of extract were noted against different pathogenic (Clinical Isolates) and using agar well diffusion assay method and noted different zone of inhibition of each bacteria, wells were loaded with different concentration's 50 µL and 100 µL. Maximum zone of inhibition were reported at different concentration of different bacteria. Ethyl acetate crude extract at 50 µL concentration's 17 ± 0.15 mm, 18 ± 0.1 mm , 16 ± 0.4 mm, 19 ± 0.26 mm, 17 ± 0.30 mm,16 ± 0.36 mm, zone of inhibition against E.coli sp, Pseudomonas, Klebsiella sp proteus sp, S,aureus, , and Salmonella sp, respectively. While in 100 µL zone of inhibition was noted, 21 ± 0.15 mm, 22 ± 0.40 mm, 20 ± 0.35 mm, 21 ± 0.26 mm, 22 ± 0.45 mm 19 ± 1.10 mm zone

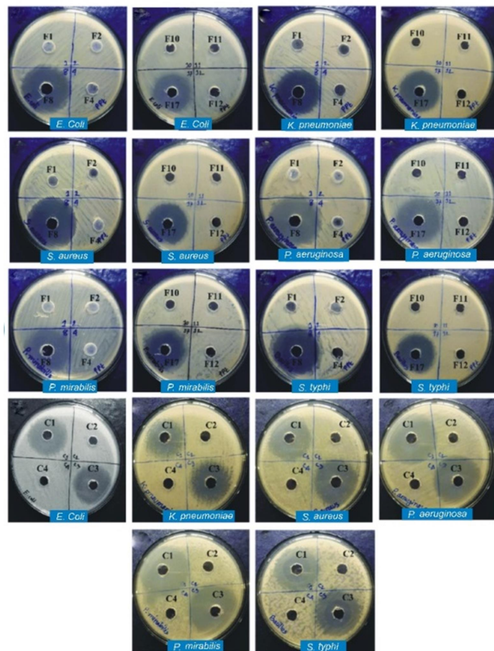
of inhibition against E.coli sp, Pseudomonas, Klebsiella sp proteus sp, S,aureus, , and Salmonella sp, respectively as shown in Fig. 5.6.

Table 5.4: Antibacterial activity of Fungal crude extract of Aspergillus fumigatus

Bacterial Strains	Fungal Metabolites		Standard Deviation 50 µl	Standard Deviation 100 µl	Average 50 µl	Average 100 µl	Ciprofloxacin
	50 µl	100 µl					
E. coli	17	21	0.15mm	0.15 mm	17.3	17.3	23
Pseudomonas	18	22	0.1mm	0.40 mm	18.2	21.63	26
Klebsiella	16	20	0.4mm	0.35 mm	16.8	19.66	23
Proteus	19	21	0.26mm	0.26 mm	19.2	20.6	28
S. aureus	17	22	0.30mm	0.45 mm	17.6	22.4	29
Salmonella	16	19	0.36mm	1.10 mm	15.7	18.33	21



Key: E.coli (Escherichia coli), P.A (Pseudomonas aruginosa), K.P (Klebsiella pneumonia), Proteus, S.A (Staphylococcus aureus), S.typi (Salmonella typhi).



Antimicrobial Activity of Fungal crude extract

DISCUSSION

In the present research work antibacterial activity of fungal crude metabolites in two different concentration i.e. 50 µL and 100 µL were evaluated against all isolated pathogens. Ethyl- acetate extract of crude metabolites produces significant inhibition zones ranging from 16 mm to 22 mm towards all the isolated surgical site wound infection. Significant increase in the inhibition zones were also noted when we increased the concentrations of crude metabolites against these isolated pathogens. Ethyl- acetate extract of fungi exhibited significant antibacterial activity ranging from 19 to 26 mm against various bacterial strains such as E. coli and different bacteria which include K. pneumoniae, S. aureus and P. aeruginosa. In addition to antibacterial activity of ethyl- acetate crude extract, mycosynthesized silver nanoparticles were also utilized against these isolated surgical site wound infection at 50 and 100 µL concentrations. A significant increase in the inhibition zones ranging from 16 to 24 mm were observed against tested isolates.

CONCLUSION

The presence study the Aspergillus fumigatus secondary metabolite have high potency to produce high profile

metabolite to combat Multi Drugs Resistance Bacteria. It's should be commercialized to treat MDR bacteria.

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