RESEARCH ARTICLE

Antimicrobial potential of newly isolated Aspergillusterreus MK-1: An approach towards new antibiotics

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Abstract

Objective: To attempt discovering new bioactive metabolites from fungal sources.

Methods: The exploratory study was conducted at the Department of Microbiology, Federal Urdu University for Arts, Science and Technology, Karachi from January 2016 to November 2017and comprised of soil samples collected from rhizosphere region of different garden plants from the city. Fungi were screened for production of antibiotics by testing cell-free culture filtrates obtained by Shake-flask fermentation technique. Agar-Well diffusion assay method was used to evaluate antagonistic activity against pathogenic microorganisms.

Results: Bioactive compounds extracted by ethyl acetate and thin layer chromatography revealed mixture of compounds in the crude extract. AspergillusterreusMK-1 showed significant inhibition of medically important test pathogens namely Staphylococcus aureus, Pseudomonas aeruginosa, Escherichiacoli, Salmonella typhi, Micrococcus luteus, Streptococcus epidermidis, Bacillus subtilis, Candida albicans and Aspergillusniger. The best biological activity of crude ethyl acetate extract was observed against Pseudomonas aeruginosa (63mm).

Conclusion: Newly isolated AspergillusterreusMK-1 emerged as a potent candidate for the production of antimicrobial compounds.

Keywords: Soil microbes, Aspergillusterreus MK-1, Antimicrobial activity, Shake-flask fermentation, Thin layer chromatography. (JPMA 69: 18; 2019)

Introduction

Bioactive metabolites, chiefly antibiotics and bacteriocins, are included in bioactive compounds possessing different antimicrobial property. A diverse array of bioactive compounds can be isolated from the extracts of soil organisms. As antimicrobial resistance (AMR) persists, the demand for new antibiotic compounds is rising by the second.

Soil is a colossal reservoir of diverse microbial species and most of the bioactive metabolites including antimicrobial compounds used today in human and veterinary medicine, were isolated from soil *microorganisms*. Despite numerous studies performed on soil micro flora, there are many *microorganisms* which are yet to be identified for the production of bioactive metabolites.[1] Fungi are the most important biotechnologically useful organisms. They are important not only for the production of antibiotics, but also for compounds which are used as food additives and flavouring agents as well as organic acids and enzymes. Penicillium, Cephalosporium, Aspergillus and some members of the fungi imperfecti are of utmost significance. Commercial fungal antibiotics include penicillin G, V, 0, cephalosporin, griseofulvin, fumagillin, variotin, fusidic acid, siccanin and xanthocillin. It is thought-provoking that most of the frequently used antibiotics, like penicillins, cephalosporin C and fusidic acid, are found in the metabolites of various fungal species.[2]

The advancements in the field of biotechnology and industrial microbiology resulted in the production of antibiotics by fermentation technology much more easily and successfully, increasing the production rate as well as greater yield of desired products. Screening and evaluation of different *microorganisms* of various origins may lead to the detection of novel bioactive metabolites with newer spectra of activity. Many of the soil isolates fail to grow in laboratory conditions as their requirements for growth and nourishment are not known. With the provision of optimal environmental conditions and growth requirements we can acquire antibiotics from those isolates that are previously not known for this purpose.

Antibiotic resistance is the natural consequence of selective pressure imposed by antibiotic drugs upon bacterial populations. Antibiotic resistance occurs when pathogenic *microorganisms* inactivate antibiotics or survive under the undesired pressure of antibiotics.[3] Antibiotics epitomise the greatest advancement in contemporary curative medicine. The massive use, misuse and overuse of antibiotics have eroded their efficacy and

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the pipeline of new antibiotics is almost dry. There is a global consensus that AMR poses a profound threat to human health. Estimate of the costs of AMR have been mentioned up to 700,000 deaths annually. If no action is taken, this number could reach 10 million by 2050.[4]

The current study was planned as a miniscule step in the ongoing battle against AMR.

Materials and Methods

The exploratory study was conducted at the Department of Microbiology, Federal Urdu University for Arts, Science and Technology (FUUAST), Karachi from January 5, 2016 to November 25, 2017, and comprised soil samples collected from rhizosphere region of different garden plants from the city. Materials used in study were Sabouraud's dextrose agar (SDA) (Oxoid), nutrient agar (Merck), nutrient broth (Merck), glucose, glycerol, starch, fructose, lactose, maltose, casein, peptone, yeast extract, gelatin, ethyl acetate (LabChem), hydrochloric acid, sodium hydroxide, lipase enzymes (Xyme, Novocor), and protease enzymes (Resopan RN and Resopan AC 3000by SRC (Pvt) Ltd). All the test pathogens i.e., bacteria and fungi, were obtained from the culture collection of Applied Microbiology and Biotechnology Lab, Department of Microbiology, FUUAST.

Five soil samples were collected from rhizosphere regions of various plants by digging into the soil surface for about 10cm. Soil (1gm) was added to each flask containing 100ml sterile distilled water, kept onorbital shaker at 27°C for 15 min and stored as stock cultures.

Isolation of antibiotic-producing fungi was achieved by serial dilution method.[5] From the stock culture, 1ml was transferred aseptically to the 1st tube making the dilution 10-1 and mixed thoroughly using vortex mixer. Further dilutions were made by transferring 1ml from previous tube to the next tube and so on to produce dilutions up to 10-5. From each dilution, 100µl was spread on sterile SDA. The plates were incubated for 7 days at 25-27°C and were observed daily. After 7 days of incubation, fungal colonies were selected, purified and stored at 4oC for further use.

The soil isolate which showed best antimicrobial activity in the primary screening was identified based on colony morphology and microscopic characteristics and was selected for further characterisation.

Sabouraud's dextrose broth medium (1L) was inoculated with a wire-loop full of 72-hours-old culture of isolate coded as MK-1. The inoculated flasks were then incubated on orbital shaker incubator at 25°C for 7 days.[6] The liquid growth medium was subjected to filtration using Whatmann No. 1 filter paper to remove the fungal biomass, followed by centrifugation at 4000 rpm for 20 min to obtain supernatant i.e., cell-free culture filtrate (CCF). The CCF was stored in sterilised glass bottles with airtight screw caps at ± 4°C until further use.

To optimise various physico-chemical parameters for optimum growth and maximum production of the antibiotic, MK-1 CCF was tested for the best carbon source (glycerol, glucose, sucrose, maltose, lactose, fructose and starch), nitrogen source (peptone, yeast extract, gelatin, casein, malt wheat bran and ground nuts), optimal potential of pH (2,4,5,7,9,11) and optimum incubation period (24-192 hours). Employing the method of optimisation[7] each carbon source was added in separate flasks containing 90ml minimal salt medium (MSM) and 10ml total electrolyte solution (TES). Isolate MK-1 was inoculated into the flasks and incubated on shaker incubator at 25°C, for 7 days. Similarly, the optimum nitrogen source, pH and incubation period for maximum production of the antibiotic were recorded.

To examine the sensitivity and stability of the active substance,[8] the CCF was subjected to a range of temperatures (20, 40, 60, 80, 100 and 121°C), acidic and alkaline pH (2-12), and enzyme treatment (protease and lipase).

The antibiotic product(s) produced by fermentation was extracted by solvent extraction method.[9] Ethyl acetate was added into the CCF in equal ratio 1:1 (v/v) and shaken vigorously for 1 hour to facilitate complete extraction. The organic phase having antimicrobial property was dried on Rotavapor (Buchi R-210) at 40°C. The resulting residue was weighed and stored in a cool dry place.

The antimicrobial activity was evaluated by agar well diffusion method.[10] The residue obtained by the evaporation in vacuum of ethyl acetate extract was dissolved in 1ml of ethyl acetate. The test pathogens, P. aeruginosa, S. aureus, B. subtilis, E. coli, S. typhi,

M.luteus, S. epidermidis, C. albicans, and A. niger, were swabbed on nutrient agar and SDA. The wells were bored with sterile cork borer and 100µl of crude ethyl acetate extract was dispensed in the wells. The plates were incubated at 37°C for 24 hours and after incubation, the zones of inhibition around the wells were measured.

Crude ethyl acetate extract was subjected to fractionation using polar and non-polar solvents and their mixtures i.e. hexane, ethyl acetate and hexane 1:1, ethyl acetate, ethyl acetate: methanol 1:1, and methanol. All the fractions were collected in separate glass vials, properly labelled, allowed to air dry, then weighed and tested to determine

the best biologically active fraction.

The best active fraction was subjected to thin layer chromatography (TLC) using silica gel plates using dichloromethane (DCM) and methanol in 8:2 ratio as the solvent system. A tiny spot was spotted with the help of capillary tube on silica gel-coated aluminium TLC card (Merck). The TLC card was placed in the moisture saturated covered TLC tank for development. The TLC plate was then observed in ultraviolet (UV) light for spot location. The retention factor (Rf) values were calculated by measuring the solvent front and the distance covered by the spot.

UV-visible spectrum for the best active fraction was recorded on a UV-1800 (Shimadzu) spectrophotometer equipped with 1.0 cm quartz cells.[11] The width of the excitation slits was set to 1.0 nm. The spectra was collected with subsequent scanning spectrum from 200 to 800 nm to 1.0 nm increments.

Results

A total of 35 fungal colonies were screened for antibiotic production. Based on the results of primary screening, through agar well diffusion method, A. terreus MK-1 was selected because of its promising antimicrobial activity against both test bacteria and fungi, namely B. subtilis, S. aureus, P. aeruginosa, E. coli, C. albicans and A. niger. A. terreus MK-1 showed maximum inhibition against P. aeruginosa.

Rapidly growing colonies with powdery texture that were initially white with buff or cinnamon-brown colour on surface developed at 120 hours and yellow to beige-brown colour on the reverse of the SDA plate due to the production of pigmented secondary metabolites. Isolate MK1 (fungus) was stained by using lacto phenol cotton blue stain and observed under 10x and 40x magnification on light microscope. Based on the morphological and colonial characteristics, soil isolate MK-1 was identified as A. terreus.

Maximum activity of A. terreus MK-1 was recorded using starch as a carbon source. Although all nitrogen sources supported the growth of A. terreus MK-1, gelatin was found to be the optimal nitrogen source. It is an established fact that fungi grow best on acidic pH range and in order to verify this characteristic A. terreus MK-1 was allowed to grow on three different pH values; 5, 7 and 9. The optimal pH observed was 5 at which highest activity was recorded. Maximum fungal growth and maximum production of antibiotic compound was

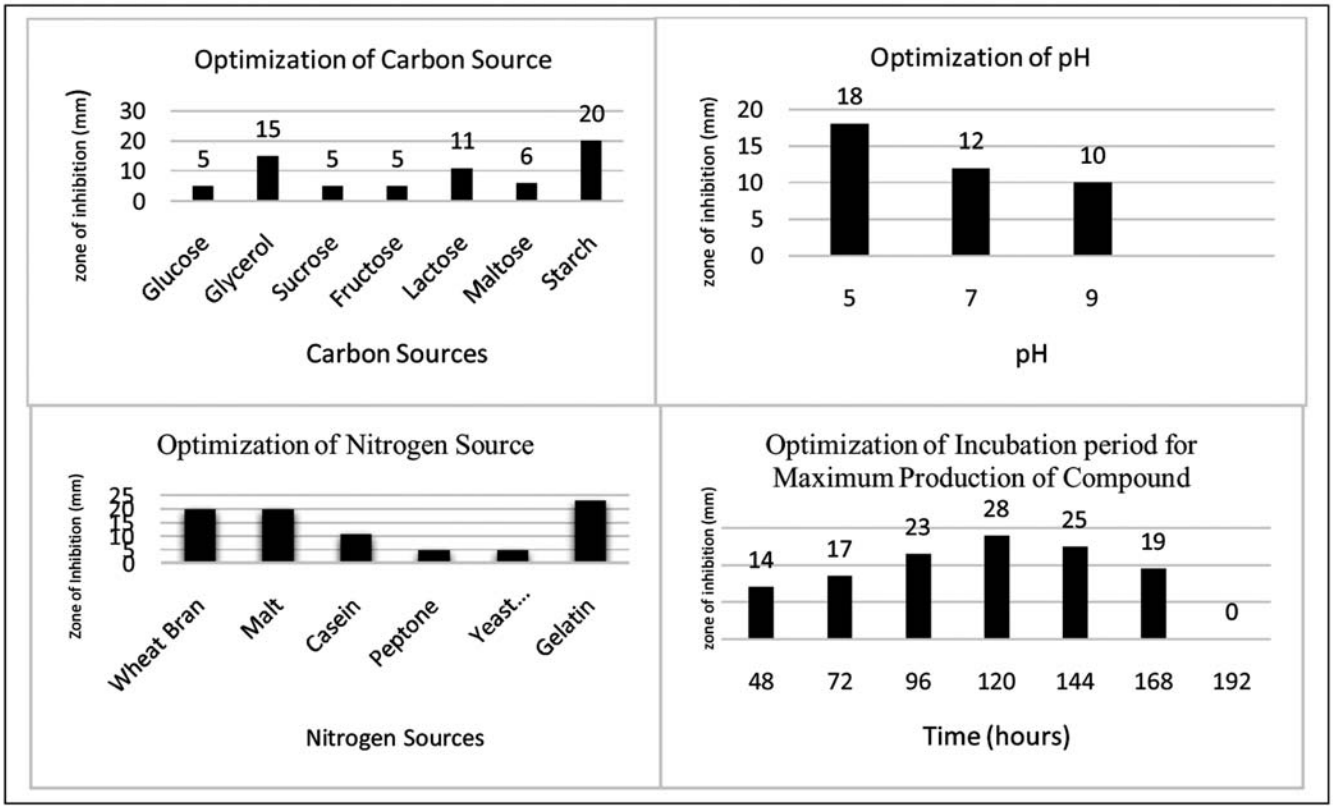

Figure-1: Optimisation of carbon sources, nitrogen sources, optimum pH and optimum incubation period.

investigated over a period of incubation time ranging from 48 hours to 192 hours. The best activity was shown by A. terreus MK-1 after 120 hours (Figure-1).

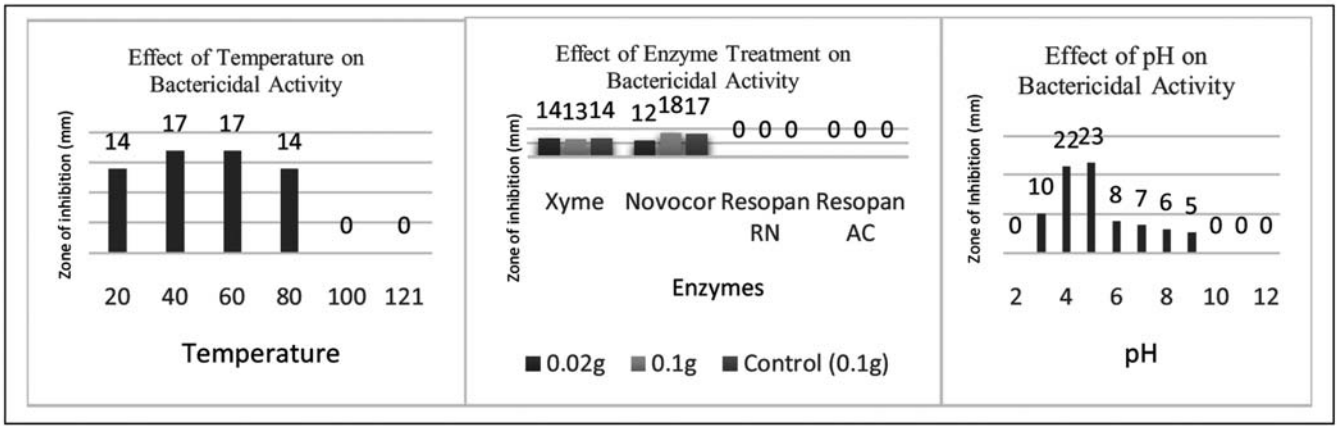

Figure-2: Effect of physico-chemical conditions on the stability of cell-free culture filtrate.

The antimicrobial activity was stable between 40-60°C, but a gradual decrease followed by complete obliteration of antimicrobial activity occurred from 80°C to 120°C. The effect of pH values indicated that the bioactive compound was susceptible to alkaline pH as the activity gradually decreased with the increase in pH. The effect of enzyme treatment on antimicrobial activity revealed that the inhibitory compound was susceptible to commercially available protease enzymes (Resopan RN, and Resopan AC) while lipases (Xyme and Novocor) had no effect on the activity (Figure-2).

After the selection of appropriate solvent, extraction of the fermented product was performed using ethyl acetate. The organic phase was concentrated under pressure. Organic phase was concentrated and dried by the complete removal of solvent. Thus, crude ethyl acetate extract was obtained. The antimicrobial activity of ethyl acetate extract was performed resulting in enhanced antibacterial and antifungal activity due to concentration of bioactive compound (Figures-3,4). From one litre of CCF, after bulk extraction 2gm of ethyl acetate soluble extract was obtained.

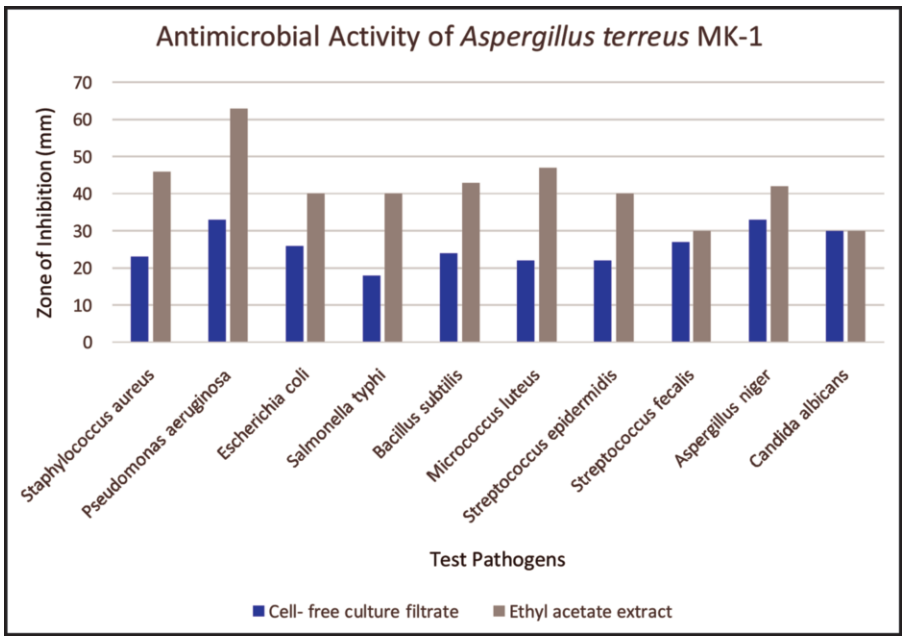

Figure-3: Comparison of antimicrobial activity of cell-free culture filtrate and crude ethyl acetate extract of Aspergillusterreus MK-1.

Crude ethyl acetate extract was checked for its antibacterial and antifungal activity against B. subtilis, S. aureus, P. aeruginosa, E. coli, S. typhi, M. luteus, S. epidermidis, C. albicans and A. niger. Crudely extracted compound(s) showed excellent antagonistic activity particularly against P. aeruginosa (63 mm).

Concentrated crude ethyl acetate extract was further broken down into various fractions to facilitate purification and observation of best active fraction (having maximum antimicrobial activity). Five different fractions were obtained and subjected to agar-well diffusion assay. Ethyl acetate

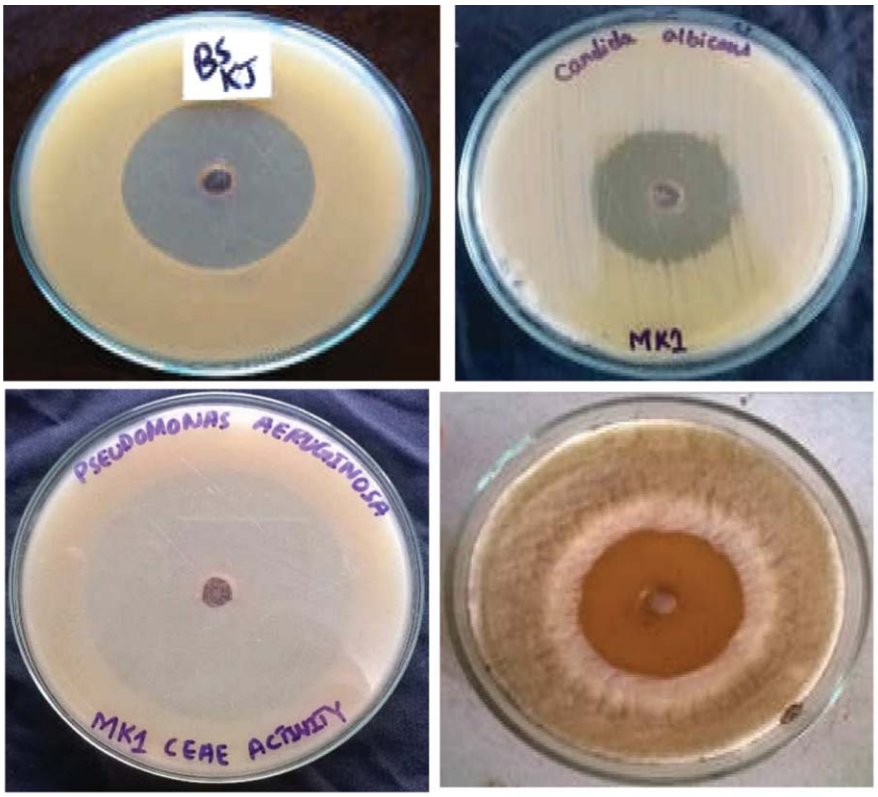

Figure-4: Antimicrobial activity of Aspergillusterreus MK-1 (ethyl acetate extract) against Bacillus subtilis, Pseudomonasaeruginosa, Candida albicans, and Aspergillusniger.

fraction was observed to be the best active fraction, having maximum antimicrobial activity (21 mm). The best active fraction (ethyl acetate) containing bioactive compound showed four spots having Rf values 0.09, 0.20, 0.29 and 0.49.

The maximum absorbance (λ_{max}) of the best active fraction of A. terreus MK-1 was recorded by varying the wavelength 200-800 nm on UV-visible spectrophotometer. The results showed the (λ_{max}) in the region of 208.40 having absorbance spectrum of 2.948 indicating bioactive compound only in UV region.

Discussion

Members of the Aspergillusgenre are widely reported for the production of bioactive metabolites, organic acids, enzymes and various other enzyme inhibitors, antihelminthics, antitumour agents, insecticides, vitamins, immunosuppressant and immunomodulators.[12] Isolate A. *terreus*MK-1 efficiently inhibited the growth of almost all the test bacteria as well as yeast and fungi so it was selected for further studies and characterisation.

Various species of A. genre, especially A. *terreus* strains, are widely reported for the production of amylase enzyme in order to utilise starch as a carbon source.[13] It has been reported[14] that sucrose is the best carbon source for both biomass (78 mg/25mL) and bioactive metabolite production (24mm against E. coli) for the isolate A. *terreus* KC 582297. It has also been reported[15] that gelatine was observed to be optimum nitrogen source at which highest biomass production of the fungus *Trametes versicolour* was observed. Another study[16] described that most of the *microorganisms* have the ability to synthesise antimicrobial compounds at pH ranging from 5.5 to 8.5. A study[17] investigated the influence of pH on the growth and production of bioactive metabolite by an endophyte *Hypocrea spp.* NSF-08 isolated from Dillenia Indica Linn in north east India. C. *gloeosporioides* isolates grew well at pH 5 while pH 6 was found preferred for the sporulation.[18] In the present study, increased antimicrobial metabolites production was observed at pH 5, suggesting the acidophilic nature of A. *terreus* MK-1.

As opposed to the study done on A. *terreus* KC 582297,[19] where the activity of the bioactive compound started to decrease when the temperature was increased from 25°C to 45°C, the bioactive compound in the present study showed activity at 80°C (14 mm) after which it diminished at 100-120°C.

In contrast with a study where the CCF of B. *subtilis*B-FS06 was very heat stable[20] and insensitive to pH alteration, the MK-1 bioactive compound in the present study was sensitive to enzyme treatment.

Treatment of infectious diseases has become more difficult with each passing year. This is especially true for infections caused by the antibiotic-resistant pathogens like *P. aeruginosa* and *S. aureus* with their ability to rapidly develop resistance to multiple classes of antibiotics. These pathogens are particularly problematic for seriously ill patients in intensive care units (ICUs). A. *terreus* MK-1 is capable of producing a bioactive compound(s) that is/are highly antagonistic towards the growth of these resistant pathogens. The highest activity was recorded against P. aeruginosa; 33 mm before extraction and 63 mm after extraction. These observations have shown remarkable increase in antimicrobial activity compared to the study[21] which

reported 25mm activity against P. aeruginosa of crude ethyl acetate extract of another A. *terreusisolate*. The zone of inhibition recorded against S. *aureus* of crude ethyl acetate extract was 46 mm in diameter which is significantly higher than observed in another study[22] which reported 13 mm zone of inhibition against S. Aureususingethyl acetate extract of A. *terreus* MP 15.

Further studies on this bioactive metabolite, like purification and structural elucidation, will provide useful insight about the class of compound and chemical structure of the compound responsible for inhibitory activity. This study was a tiny step in the ongoing battle against AMR. Bioactive compounds produced by *microorganisms* indigenous to the soil of Pakistan may help contribute to the never-ending scope of this field.

Conclusion

Soil isolate A. *terreus* MK-1 was found to be a potent strain for the production of bioactive metabolites against various *microorganisms*. After evaluation of toxicity, this work may provide helpful information about the industrial production and application of antimicrobial compounds from fungi through submerged fermentation.

Disclaimer: Part of this work has been presented twice as a poster at the First International Conference on Life Sciences held at KIBGE on December 28-30, 2015, and Pre-Summit Conference on Innovative Technologies held at FUUAST on December 19, 2017. The abstracts were published in the Abstract Books of the aforementioned conferences.

Conflict of Interest: None.

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References

1. Béahdy J. Recent developments of antibiotic research and classification of antibiotics according to chemical structure. Adv Appl Microbiol. 1974; 18:309-406.
2. Hersbach GJ, Van der Beek CP, Van Dijck PW. In: Hersbach GJ, Van der Beek CP, Van Dijck PW, eds. The Penicillins: Properties, biosynthesis, and fermentation. UK: Marcel Dekker AG, 1984.
3. Valgas C, Souza SM, Smânia EF, SmâniaJr A. Screening methods to determine antibacterial activity of natural products. Brazil J Microbiol. 2007; 38:369-80.
4. De Kraker ME, Stewardson AJ, Harbarth S. Will 10 million people die a year due to antimicrobial resistance by 2050.PLoS Med. 2016; 13:1-6.
5. Turnidge JD, Jorgensen JH, Murray PR, Baron EJ, Pfaller MA, Tenover FC, et al. Antimicrobial susceptibility testing: In: Turnidge JD, Jorgensen JH, Murray PR eds. General considerations. Manu Clini Microbiol, 7th ed. Washington DC: ASM Press, 1999; pp-236 .
6. Pratap Singh A, B Singh R, Mishra S. Studies on isolation and characterization of antibiotic producing *microorganisms* from industrial waste soil sample. Open Nutraceutic J. 2012; 5:169-73.
7. Singh AK, Prakash D, Shahi SK. Decolorization of the textile dye (Brown GR) by isolated Aspergillus strain from Meerut region. Int Res J Environ Sci. 2013; 2:25-9.
8. Zhang T, Shi ZQ, Hu LB, Cheng LG, Wang F. Antifungal compounds from Bacillus subtilis B-FS06 inhibiting the growth of Aspergillusflavus. W JMicrobiol Biotech. 2008; 24:783-8.
9. Bizuye A, Moges F, Andualem B. Isolation and screening of antibiotic producing actinomycetes from soils in Gondar town, North West Ethiopia. Asian Pacific J Trop Dise. 2013; 3:375-81.
10. Podojil M, Blumauerova M, Vanek Z, Culik K. In: Joseph J. Hlavka, James H. Boothe eds. The tetracyclines: Properties, biosynthesis, and fermentation. CRC Press; 1984.
11. Jost JL, Kominek LA, Hyatt GS, Wang HY. In: Jost JL, Kominek LA, Hyatt GS, eds. Cycloheximide: Properties, biosynthesis, and fermentation. UK: CRC Press; 1984.
12. Choudhary MI, Musharraf SG, Mukhmoor T, Shaheen F, Ali S, Rahman AU. Isolation of bioactive compounds from Aspergillusterreus. Zeitsc Naturforsc B. 2004; 59:324-8.
13. Sethi BK, Jana A, Nanda PK, DasMohapatra PK, Sahoo SL, Patra JK. Production of ?-Amylase by Aspergillusterreus NCFT 4269.10 Using Pearl Millet and Its Structural Characterization. Front Plant Sci. 2016; 7:1-13.
14. Mathan S, Subramanian V, Nagamony S. Optimization and antimicrobial metabolite production from endophytic fungi Aspergillusterreus KC 582297. Eur J Exp Biol. 2013; 3:138-44.
15. Bolla K, Gopinath BV, Shaheen SZ, Charya MS. Optimization of carbon and nitrogen sources of submerged culture process for the production of mycelial biomass and exopolysaccharides by Terreusversicolor. Int J Biotech Mol Biol Res. 2010; 1:15-21.
16. Thongwai N, Kunopakarn J. Growth inhibition of Ralstoniasolanacearum PT1J by antagonistic bacteria isolated from soils in the northern part of Thailand. Chiang Mai J Sci. 2007; 34:345-54.
17. Gogoi DK, Boruah HP, Saikia R, Bora TC. Optimization of process parameters for improved production of bioactive metabolite by a novel endophytic fungus Fusarium sp. DF2 isolated from Taxuswallichiana of North East India. W J Microbiol Biotech. 2008; 24:79-87.
18. Kumara KL, Rawal RD. Influence of carbon, nitrogen, temperature and pH on the growth and sporulation of some Indian isolates of Colletotrichumgloeosporioides causing anthracnose disease of papaya (Carrica papaya L). Trop Agri Res Extens. 2010; 19: 7-12.
19. Mathan S, Subramanian V, Nagamony S. Optimization and antimicrobial metabolite production from endophytic fungi Aspergillusterreus KC 582297. Eur J Exp Biol. 2013; 3:138-44.
20. Zhang T, Shi ZQ, Hu LB, Cheng LG, Wang F. Antifungal compounds from Bacillus subtilis B-FS06 inhibiting the growth of Aspergillusflavus. W J Microbiol Biotech. 2008; 24:783-8.
21. Rajalakshmi S, Mahesh N. Production and characterization of bioactive metabolites isolated from Aspergillusterreus in rhizosphere soil of medicinal plants. IntJ Current Microbiol Appl Sci. 2014; 3:784-98.
22. Yin OC, Ibrahim D, Lee CC. Bioactive Compounds from Aspergillusterreus MP15, an Endophytic Fungus Isolated from Swietenia Macrophylla Leaf. Mal J Med Biol Res. 2015; 2:262-72.