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Isolation, Purification and Characterization of Antimicrobial Metabolites from *Aspergillus ibericus*

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ABSTRACT

In the present study, isolation, purification and characterization of antimicrobial compound obtained from rhizospheric soil fungus *Aspergillus ibericus*, was carried out in order to determine the bioactive constituents present in the metabolite which are aactually responsible for the antimicrobial potential of fungus. The fungal metabolite was preliminarily screened for its antimicrobial activity against various test microorganisms. Three solvents of different polarity, ethyl acetate, chloroform and petroleum ether were tested for the extraction of antimicrobial metabolite from culture filtrate. Quantitative analysis of antimicrobial compound was carried out by using Thin Layer Chromatography (TLC). Separation of crude extract was performed by analytical HPLC followed by purification of extract through preparative HPLC. The probable structure of bioactive compound determined by NMR spectroscopy which was found to be 7-hydroxy-3-(methoxy carbonyl)-2-methylene heptanoic acid having molecular formula C10H16O5 with molecular mass 216. The Minimum Inhibitory Concentration of bioactive fraction obtained from *A. ibericus* was found to be significant as 19.5µg/ml against *Pseudomonas aeruginosa*, *Escherichia coli*, *Streptococus pyogenes*, *Candida albicans* and *Candida tropicalis*, moderate as 625µg /ml against *Staphylococcus aureus, Streptococcus mutans* and weak as 1.25mg/ml against *Bacillus subtilis*.

KEY WORDS: *ASPERGILLUS IBERICUS*, ANTIMICROBIAL METABOLITE, THIN LAYER CHROMATOGRAPHY, ANALYTICAL AND PREPARATIVE HPLC, NMR SPECTROSCOPY, MINIMUM INHIBITORY CONCENTRATION.

INTRODUCTION

Opportunistic infection is an infection caused by microbes like bacteria, viruses, fungi, or protozoa that do not cause any disease in healthy host. These opportunists can emerge from normally present in or on human body (innocuous) or from environmentally acquired microbes. These microbes take advantage of an opportunity such as a host with impaired defense system, an altered microbiota or breached integumentary barriers (Cragg and Newman, 2001, Cabrera et al., 2020). Some of the opportunistic organisms include *Candida albicans, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella, Clostridium difficile, Streptococcus pneumoniae, Streptococcus pyogenes* etc (Enoch et al., 2006; Chen et al. 2020).

Antibiotics form the most critical field of microbial biotechnology as they are found to cure various kind of bacterial and fungal infections, but one of the problem in the fight against infectious diseases is the development

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of resistance to the agents used to control them. The phenomenon of resistance in clinical isolates has been known since antimicrobial drugs entered the medicine. Drug resistance, emerging and re-emerging infectious diseases has emphasized the need of search for new strains and compounds with antimicrobial potential (Zinner, 2007; Lakoh et al. 2020).

Natural sources such as bacteria, fungi and plants can be explored for new chemical entities as natural products provide a vast source of chemically diverse biologically active leads for therapeutic agents. Medicinal plants provide enormous secondary metabolites having potential to use as natural drugs in modern medicine. These bioactive secondary metabolites synthesized by medicinal plants can also strongly affect plant-associated microbial communities and their physiological functions as microorganisms live in a world of chemical signals. Surprisingly, not only the plants themselves are able to produce substances with therapeutic properties, but research continues to show that number of natural bioactive compounds are actually produced by their associated microbes (Bull and Stach, 2007; Binyamin et al., 2019). The rhizosphere is defined as the soil zone in vicinity of plant roots, a site of high microbial activity and diversity in comparison to non-rhizosphere bulk soil. Organic compounds released by plant roots may act as basis of chemotaxis to attract some species and repel others, resulting in the existence of different communities. The microbial diversity and selection for competent microbes (for limited nutrients and space) in rhizosphere, makes it potentially an important source of natural products (Berdy, 2005; Shaikh and Mokat, 2017).

In keeping view of the above justifications, for the continuous search of new isolates from rhizosphere soil of medicinal plants, having antimicrobial activity, the present study aimed at the following objectives which included isolation of rhizosphere soil fungi *Aspergillus ibericus* from medicinal plant Ficus religiosa and screening for its antimicrobial activity against various test organisms. The fungus *A. ibericus* belongs to Order *Eurotiales*, Class *Eurotiomycetes* and Family *Trichocomaceae*. The morphological characteristics of fungus *A. ibericus* are black colony color, reverse side yellow, granular texture, biseriate sporulating structure, rough spores with maturity, conidia diameter of 5-5.5µm, conidia head diameter of 55-70 µm, colonies initiate with white hyphae and quickly form jet black conidia (Aneja, 2003). Isolated strain of *A. ibercus* seems to be broad spectrum in its mode of action as it inhibited the growth of all test microbes including gram-positive, gram-negative and yeasts. The work also included purification and characterization of antimicrobial metabolite and determination of minimum inhibitory concentration of antimicrobial metabolite.

MATERIAL AND METHODS

Soil samples were collected from rhizosphere of medicinal plant Peepal (Ficus religiosa) from Botanical garden, Kurukshetra University, Kurukshetra. by removing 1-1.5 inch of top soil with sterilized spatula. The serial dilution agar plate method was used for isolation of *Aspergillus ibericus* from soil sample (Cappucino and Sherman, 1996; Aneja, 2003). Potato dextrose agar (PDA) (CDH) for fungi was used as isolation medium. Fungal colonies appearing on their respective media were transferred to potato dextrose agar plates (one colony on each plate) at 30°C for 4-5 days. The colonies were then transferred on potato dextrose agar slants and incubated at 30°C for 4-5 days and were maintained at 4°C in a refrigerator for further studies.

The antimicrobial activity of *A. ibericus* was evaluated by using agar well diffusion assay (Nandhini and Selvam, 2011). Potato dextrose agar plates were inoculated with 100µl of standardized inoculum (0.5 McFarland Standard) of each test microbe (in triplicates) and was spread with sterile swabs. Wells were made into agar plates containing the test microbe inoculum. 200µl volume of extract was poured into a well of inoculated plates. Uninoculated potato dextrose broth (Hi-Media) was used as negative control. Antibiotics ciprofloxacin (antibacterial) and fluconazole (antifungal) were used as positive control. Then plates were left at room temperature for ten minutes allowing the diffusion of extract into agar. After incubation for 24 hours at 37°C, the plates were observed for inhibition zone surrounding the well containing extract. The zone of growth inhibition was measured and expressed in millimeters (mm). The mean and standard deviation of diameter of inhibition zones were calculated. Three solvents of different polarity viz ethyl acetate, chloroform and petroleum ether were tested for the extraction of antimicrobial metabolite from culture filtrate. The filtrate was solvent extracted with each of the solvent separately, in a separating funnel taking equal volumes of filtrate and solvent (Kekuda et al., 2013). Quantitative analysis of antimicrobial compound was carried out by using Thin Layer Chromatography (TLC). The positions of different spots and solvent (distance it covered) were marked. The relative flow (Rf) value was determined (Rajalakshmi and Mahesh, 2014 with slight modifications).

Separation of crude extract by analytical HPLC (Shimadzu) was performed at CSIR- Indian Institute of Integrative Medicine, Jammu. The analytical column RP-18e chromolith with length of 100mm and internal diameter of 4.6mm with particle size of 5µm was used for separation of crude extract.All components which showed peaks in analytical HPLC were purified by preparative HPLC at CSIR- Indian Institute of Integrative Medicine, Jammu. The preparative HPLC system (Shimadzu UFLC) consisted of pump LC20AD, autosampler SIL20A HT, column oven CTI 10AS and detector PDA SPD M20A. The column Merck Semi-prep RP-18e with length of 250mm and inner diameter of 10mm with particle size of 5µm. The analysis was carried out using a gradient of water with 0.1% formic acid and acetonitrile. Flow rate 2ml/min. and column temperature 45°C was maintained. Characterization of active component showing antimicrobial activity was

performed by Proton (1H), Carbon (13C) and 2D NMR (Heteronuclear Single Quantum Coherence (HSQC), Heteronuclear Multiple Bond Coorelation (HMBC)) at CSIR-Indian Institute of Integrative Medicine, Jammu. A 400MHz Bruker spectrophotometer was used to record the NMR of antimicrobial metabolite. Chemical shift values were given in parts per million (ppm) with tetramethylsilane as internal standard. The solvent used was deuterated methanol.

MIC of antimicrobial compound against all the test microbes was determined by two-fold dilution method. In this method, two-fold serial dilution of antimicrobial metabolite was prepared by first reconstituting the metabolite (10mg/ml) in 10% dimethyl sulphoxide (DMSO). The dilutions were made in 10% DMSO to achieve a decreasing concentration range. A 200µl volume of each dilution was introduced into wells (triplicate) in nutrient agar plates already seeded with 100µl of inoculum of the test microbes. All plates were incubated at 37°C for 24 hours and were observed for the inhibition zones to know the minimum concentration of metabolite which is sufficient to inhibit growth of test microbes (Andrews, 2001; Aneja et al., 2010 with some modifications).

Statistical analysis: The data obtained from various experiments were subjected to analysis of variance (One Way ANOVA) to evaluate the significance of each parameter by estimating p-value and f-value. The level of significance was considered as $p<0.05$ (Pan et al., 2016).

RESULTS AND DISCUSSION

Aspergillus ibericus isolated from rhizosphere soil samples of Ficus religiosa was effective against all test microbes including four gram-positive, two gram-negative and two yeasts. Antimicrobial activity of the fungus against test microbes is shown in Table 1 in terms of zone of growth inhibition. in the current study, *Aspergillus ibericus* showed maximum antimicrobial activity against most of the test microbes with very strong response against *B. subtilis, S. aureus, S. mutans, S. pyogenes, P. aeruginosa, E. coli* and *C. albicans* (24mm, 25mm, 22mm, 25mm, 21mm, 22mm and 26mm, respectively) and strong response against *C. tropicalis* (15mm). The extraction of crude bioactive metabolites from the culture filtrate by solvent extraction method is an important factor to find best solvent that have the potential to extract maximum concentration and most potent antimicrobial compounds (Haque et al., 2017). If the compounds secreted by microorganism are highly soluble in an appropriate water immiscible organic solvent, it can be easily extracted form culture broth (Haque et al., 2017; Kumar and Jadeja, 2018).

Extracellular metabolites are low molecular weight compounds that are secreted by microbial cells into a specific environment, namely the culture media (Pinu and Villas-Boas, 2017). There are some advantages of extracellular metabolite analysis over the analysis of intracellular metabolites. The separation of extracellular metabolites from microbial cells and from their intracellular metabolites can be achieved by simple techniques such as centrifugation for bacterial cells and filtration for fungi, while the extraction of intracellular metabolites from microbial cells is a complicated process (Tredwell et al., 2011; Keller, 2019). The analysis of extracellular metabolites provides invaluable information about the metabolism of different microorganisms that change in response to different environmental conditions.

The culture broth of selected strain *A. ibericus* was extracted with three solvents, ethyl acetate, chloroform and petroleum ether and metabolite was evaporated to dryness. Three solvents of different polarity were used to extract the active compound from filtrate. Antimicrobial activity of metabolites extracted with different solvents was measured in terms of diameter of zone of growth inhibition. Metabolite extracted with chloroform showed activity against only four test microbes mainly *B. subtilis, S. aureus, S. mutans* and *E. coli*. Compound extracted with petroleum ether showed antimicrobial activity against five test microbes

Table 1. Antimicrobial activity of rhizospheric soil fungus *Aspergillus ibericus* strain PIF2

Fungal isolate	Zone of growth inhibition (mm) Test microorganisms							
	Bacteria Gram-positive				Gram-negative			Yeast
	Bs	Sa	Sm	Sp	Pa	Ec	Ca	Ct
PIF2	24.66±0.57	25.00±0.00	22.66±0.57	25.00±0.00	21.00±0.00	22.33±0.57	26.66±0.57	15.00±1.00
Ciprofloxacin	24.00±0.00	NA	25.00±0.00	26.00±0.00	25.00±0.00	23.00±0.00	ND	ND
Fluconazole	ND	ND	ND	ND	ND	ND	NA	NA

Values are mean inhibition zone ± Standard deviation of three replicates
NA: No antimicrobial activity; ND: Not determined; Bs: *Bacillus subtilis*; Sa: *Staphylococcus aureus*; Pa: *Pseudomonas aeruginosa;* Ec: *Escherichia coli*; Sm: *Streptococcus mutans*; Sp: *Streptococcus pyogenes*; Ca: *Candida albicans*; Ct: *Candida tropicalis*

B. subtilis, S. aureus, S. mutans, P. aeruginosa and *C. albicans* with zone of growth inhibition between 14mm and 17mm respectively.

Metabolite extracted with ethyl acetate showed maximum activity against all test microbes with zone of growth inhibition ranging between 20 mm and 28mm. This suggests polar nature of antimicrobial compound extracted from culture filtrate of strain *A. ibericus*. One-way ANOVA analysis at 5% significance level shows calculated F value (5.87) greater than F critical value (3.46) and P value (0.000941) less than 0.05, which indicates that null hypothesis (there is no significant difference between the values) is rejected and there is significant difference between values. Table 2 and Figure 1 shows antimicrobial activity of metabolite extracted with different solvents. Figure 2 shows antimicrobial activity of metabolite extracted with different solvents against test microbes *C. albicans, E. coli* and *S. aureus*. Ethyl acetate as best solvent for extraction of antimicrobial compound from microbes was reported by many researchers (Awla et al., 2016; Ahsan et al., 2017; Haque et al., 2017; Hussaini and Gulve, 2019). The fungal crude extract was subjected to TLC analysis for the separation of the bioactive compounds. Two fractions designated as first and second were observed when developed in dichloromethane: methanol (85:15) on silica gel TLC sheets with Rf values 0.72 and 0.45 respectively (Figure 3).

Table 2. Optimization of solvent and antimicrobial activity

Solvent	Zone of growth inhibition (mm) Test microorganisms							
	Bacteria				Yeast			
	Gram-positive				Gram-negative			
	Bs	Sa	Sm	Sp	Pa	Ec	Ca	Ct
Chloroform	34.66±0.57	22.66±0.57	32.00±1.00	0.00±0.00	0.00±0.00	0.00±0.00	13.33±0.57	0.00±0.00
Ethyl acetate	28.00±0.00	26.33±0.57	26.66±0.57	26.33±0.57	26.33±0.57	22.66±0.57	31.33±0.57	20.00±0.00
Petroleum ether	15.66±0.57	13.66±0.57	15.33±0.57	0.00±0.00	17.00±0.00	0.00±0.00	15.66±0.57	0.00±0.00

Values are mean inhibition zone ± Standard deviation of three replicates
Bs: *Bacillus subtilis*; Sa: *Staphylococcus aureus*; Sm: *Streptococcus mutans*; Sp: *Streptococcus pyogenes*; Pa: *Pseudomonas aeruginosa*; Ec: *Escherichia coli*; Ca: *Candida albicans*; Ct: *Candida tropicalis*

Figure 1: Optimization of solvent and antimicrobial activity Bs: Bacillus subtilis; Sa: Staphylococcus aureus; Pa: Pseudomonas aeruginosa; Ec: *Escherichia coli*; Sm: *Streptococcus mutans*; Sp: *Streptococcus pyogenes*; Ca: *Candida albicans*; Ct: *Candida tropicalis*. When statistically analyzed at significance level 0.05 by One Way ANOVA, proved to be significantly different.

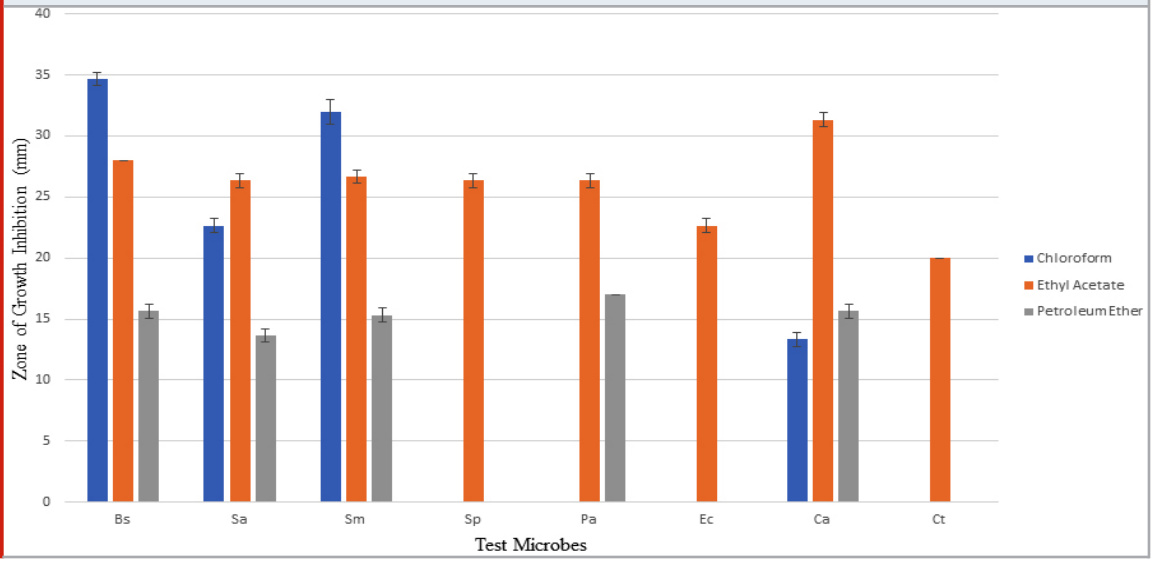
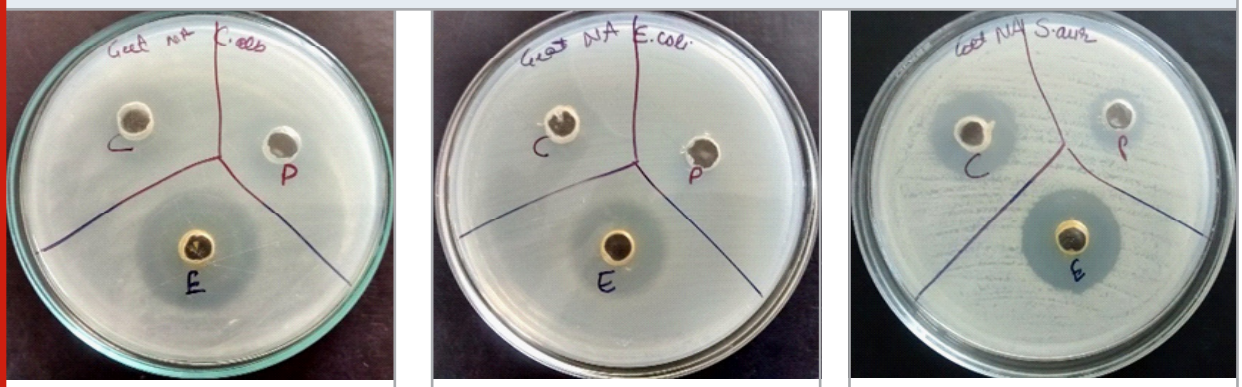
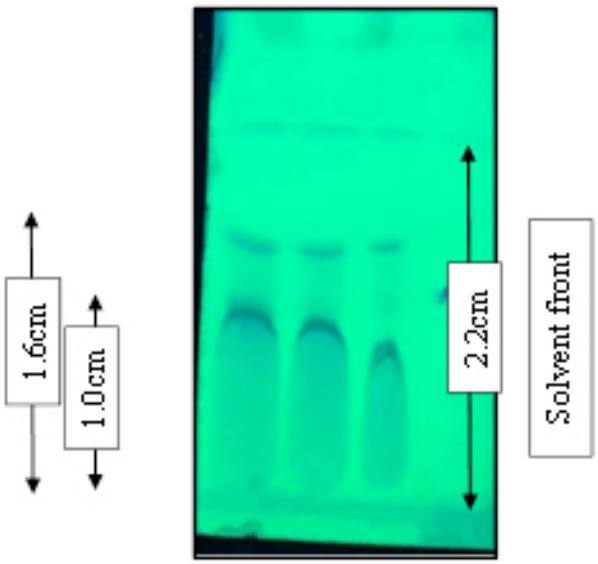


Figure 2: Optimization of solvent and antimicrobial activity against test microbes A) *C. albicans*, B) *E. coli* and C) *S. aureus*; C: Chloroform, P: Petroleum ether and E: Ethyl acetate.



A B C

Figure 3: Thin layer chromatography: Separation of crude extract with dichloromethane and methanol (85:15)



For purification by Preparative HPLC, firstly compound was subjected to analytical HPLC. In analytical HPLC, four main peaks were observed. Peak 1 at retention time 6.129, peak 2 at 6.671, peak 3 at 12.124 and peak 4 at 16.856 was observed with 4.435%, 18.554%, 24.929%, 52.081% area respectively. All four peak fractions were collected in pure form by preparative HPLC. El-Naggar et al. (2001) purified antimicrobial compound produced by Streptomyces violates using analytical and preparative high-performance liquid chromatography (HPLC). Both analytical and preparative HPLC had been performed for purification for antifungal compounds produced by *Lactobacillus plantarum* IMAU10014 by Wang et al. (2012). Analytical HPLC and Preparative HPLC had been performed for purification of ent-pimara-8(14),15-diene from engineered *Aspergillus nidulans* by Bromann et al. (2014). Alshaibani et al. (2016) performed analytical and preparative HPLC techniques for purification of active compounds from Streptomyces sp. SUK 25 with antimethicillin-resistant *Staphylococcus aureus* activity. Preparative HPLC had been performed for purification of antimicrobial substances from endophytic actinomycetes by Sunaryanto and Mahsunah (2013). Reis et al. (2018) characterized secondary metabolites from endophytic fungi *Nodulisporium sp*. isolated from the medicinal plant *Mikania laevigata* through high performance liquid chromatography coupled with mass spectrometry.

Antimicrobial activity of four fractions was analyzed against test microbes and compound showing peak 4 at retention time of 16.856 and with major fraction 52.081% showed antimicrobial activity against all test microbes. NMR spectroscopy had been performed for verification of structure of active compound obtained by purification from engineered *Aspergillus nidulans* by Bromann et al. (2014). Alshaibani et al. (2016) performed 1D and 2D NMR for characterization of active compounds from Streptomyces sp. SUK 25 with antimethicillin-resistant Staphylococcus aureus activity. Recently, Fan et al. (2020) characterized the structures of the compounds produced by seaweed derived fungus *Pyrenochaetospsis* sp. by extensive NMR. The structures of the compounds were elucidated by extensive NMR, H In the present study, purified fraction showing antimicrobial activity was analyzed by NMR spectroscopy (Pretsch et al., 2009). The probable structure of compound was found to be 7-hydroxy-3-(methoxy carbonyl)-2-methylene heptanoic acid having molecular formula C10H16O5 with molecular mass 216.MIC is defined as the lowest concentration of an antimicrobial agent which, under defined in vitro conditions, prevents the appearance of visible growth of a microorganism within a defined period of time. Extract from the isolate should be pure enough to fully characterize the activity of an antimicrobial compound (Lihan et al., 2014, Pelo et al. 2020).

The cut-off value for MIC are significant when MIC ≤ 100µg/ml, moderate in range of 100µg/ml-625µg/ml and weak when MIC ≥ 625µg/ ml. (Kuete, 2010) In the present study, MIC of bioactive fraction obtained from *A. ibericus* was found significant as 19.5µg/ml against *P. aeruginosa, E. coli, S. pyogenes, C. albicans, C. tropicalis, moderate as 625µg /ml against S. aureus, S. mutans*

and weak as 1.25mg/ml against *B. subtilis* and also compared to selected antibiotics fluconazole (showed no activity against any test microbe at any concentration) and streptomycin (120µg for P. aeruginosa, 5µg for *S. mutans, S. pyogenes, E. coli, 0.1µg for S. aureus* and *B. subtilis)*. When compared with selected antibiotics, bioactive fraction showed significant MIC value of 3.9µg against *P. aeruginosa, E. coli, S. pyogenes, C. albicans, C. tropicalis* whereas fluconazole showed no activity and streptomycin showed value of 120µg against *P. aeruginosa,* 5µg against *E. coli*, 5µg against *S. pyogenes* and no activity against two yeasts.

CONCLUSION

It may be concluded that fungus Aspergillus ibericus isolated from rhizosphere soil of medicinal plant Ficus religiosa is a promising source of antimicrobial metabolite. The research work shows rhizospheric soil of medicinal plants is a rich source of clinically important microorganisms. The antimicrobial metabolite produced by the fungal isolate *A. ibericus* was further purified and characterized. Antimicrobial metabolite obtained from A. ibericus is effective against test microbes gram-positive bacteria, *B. subtilis, S. aureus, S. mutans* and *S. pyogenes,* gram-negative bacteria, P. aeruginosa and *E. coli* and yeasts such as *C. albicans* and *C. tropicalis*. The antimicrobial metabolite is thus broad spectrum in nature. It may also be suggested that further research is needed to determine the cytotoxicity and in vivo efficacy against opportunistic pathogens before it is used for commercialization purpose.

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Conflict of Interest: The authors declare that there is no conflict of interests.

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