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Enhancement of metabolic spectrum and antibacterial activity of endophytic fungi using antibiotics as inducers

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Abstract

Natural resources associated with production of bioactive compounds are getting immense importance in therapeutic fields due to concerns like increasing antibiotic resistance. Endophytic fungi are promising natural source to produce antibacterial agents. In this study, metabolic potential of two endophytic fungi, *Epicoccum nigrum* NFW1 (JX402049.1) and *Chaetomium* sp. NFW8 (KC797170.1), was evaluated using antibiotics (moxifloxacin and clarithromycin) as inducers. Fungal species were under standard cultivation conditions in media supplemented with and without antibiotics. Following incubation, ethyl acetate extract was analysed for antibacterial activity and probable shift in metabolic profile, induced by antibiotics, by high performance liquid chromatography. The results were further verified by thin layer chromatography, bioautography and Fourier Transform Infra-red spectroscopy. In response to antibiotics, endophytic fungi expressed changes in metabolic spectrum. These variations were manifested as phenotypic changes in the growth pattern as indicated by loss of colour by NFW8. Metabolic profiling revealed additional peaks in extracts of media obtained under presence of antibiotics. Considerable changes in antibacterial activity were noted in samples grown in the presence of antibiotics as compared to those grown without antibiotics. This study showed that selective modification of cultivation medium using antibiotics under OSMAC approach could extend the metabolic spectrum of the endophytic fungi in a promising and cost-effective fashion.

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Introduction

Since the discovery of penicillin, chemical scaffolds obtained from varied natural sources have been cherished for the development of potent drugs. Given the alarming increase in antibiotic resistance worldwide, emerging and re-emerging infectious diseases and life-threatening complications (Khalil *et al.*, 2017), there is a general call for natural product discovery leading to effective therapeutics. Among natural sources, endophytic microorganisms are known to harbor novel and potent biochemical entities (Arnold, 2007). These ubiquitous microorganisms reside asymptomatically within the intracellular spaces of higher plants and are often considered as under explored producers of chemical leads like paclitaxel or taxol (Gunatilaka, 2006).

Over the years, several different strategies have been adopted to fast track the discovery of new and novel chemical structures from natural sources including endophytic microorganisms. In addition to exploring microbial sources from novel niches and habitats, several strategies like dereplication, in-silico studies, genome mining, metabolic engineering, mathematical and statistical modeling have been employed for bio-prospecting new compounds (Scherlach and Hertweck, 2009; Teixeira *et al.*, 2019). Additionally, it has been reported that production of biochemical entities by microorganisms is highly influenced by cultivation conditions since many of biosynthetic gene clusters responsible for secondary metabolite production remain unexpressed under standard cultivation conditions (Kusari *et al.*, 2012; Daletos *et al.*, 2017; Ariantari *et al.*, 2019). Therefore, varied approaches are adopted to enhance the biosynthetic potential of microbes. One such methodology is systemic alteration of easily accessible cultivation parameters for extending biosynthetic potential of a particular strain (Bode *et al.*, 2009). This approach is termed as one strain many compounds (OSMAC) approach and involves selective modification of fermentation parameters such as cultivation media, operational parameters (pH, temperature, etc.) and addition of inducer or chemical elicitors (Bode *et al.*, 2009).

The Northern areas of Pakistan are considered a biodiversity hotspot. The endophytic fungi associated with the medicinal plant Taxus fuana of the Himalayan region have been investigated earlier for potential chemo preventive and bioactive compounds with promising findings (Jadoon *et al.*, 2015; Fatima *et al.*, 2016). As part of our ongoing investigations on indigenous endophytes, this study was proposed to extend and reveal the hidden biosynthetic potential of the isolates using OSMAC approach. Therefore, we cultivated the strains by selective modification of the cultivation media and added antibiotics as elicitors of bioactive secondary metabolites. These cost-effective modifications appear instrumental in activating cryptic biosynthetic gene clusters and detecting additional lead compounds which would otherwise be overlooked.

Materials and methods

Endophytic fungal isolates

In the present study, two endophytic fungal isolates *Epicoccum nigrum* NFW1 (JX402049.1) and *Chaetomium* sp. NFW8 (KC797170.1) from medicinal plant *Taxus fuana* were selected for evaluation. The strains were previously isolated and screened as part of ongoing studies on endophytes of *Taxus fuana* from Himalayan region of Pakistan (Jadoon *et al.*, 2015; Fatima *et al.*, 2016). The isolates were refreshed and maintained on potato dextrose agar (PDA, Oxoid, UK.) at 4°C.

Qualitative and quantitative analysis for the growth of fungal isolates on medium with and without antibiotics

Based on OSMAC approach, endophytic fungi were grown on Sabouraud dextrose broth (SDB, Oxoid, UK) with and without the supplementation of antibiotics followed by qualitative and quantitative analysis for the growth of the strains. Two antibiotics, moxifloxacin and clarithromycin were procured from Research and Development Department of Focus & Rules Pharmaceuticals (Pvt. Ltd.) Islamabad. Islamabad and stored at 4°C. Sabouraud dextrose broth (100mL) was prepared and supplemented with antibiotics at concentration of 1 mg/mL. The flasks,

without and with antibiotics, were inoculated with three mycelial agar plugs of (3mm-5mm in size) and incubated at 25°C under shaking (150 rpm) conditions for a period of one week. Following incubation, qualitative analysis was performed by comparing growth pattern for flask with or without antibiotics by making visual observations (with the unaided eye). For quantitative analysis, mycelial mass from all the flasks was collected, dried and weighed to observe the difference in dry mass.

Evaluation of Changes in Metabolic Potential of Fungal Isolates under the effects of antibiotics

Changes in secondary metabolite spectrum under the presence or absence of antibiotics was evaluated by measuring changes in antibacterial activity and further verified by thin layer chromatographic and high-performance liquid chromatography. For this purpose, flasks containing 100 mL SDB were prepared in three sets. Antibiotics; moxifloxacin and clarithromycin (1 mg/mL) were added to one set. All the flasks were inoculated with mycelial agar plugs and incubated at 25°C for 21 days under shaking conditions of 150 rpm. Flaks containing 100 mL SDB and supplemented with antibiotics, without any inoculum, were incubated under same conditions and served as negative control. In third set the 100 mL SDB flask were inoculated with the fungi without supplementation of any antibiotics which also served as control to compare the enhancement of activity due to the supplementation of antibiotics. Following incubation, fermentation broth was filtered, and cell free supernatant was extracted three times with equal volume of ethyl acetate (SIGMA, HPLC grade). While TLC bands scratched after developing a were extracted by methanol. Organic layers were pooled and concentrated to dryness under vacuum. Same procedure was adopted for flask serving as controls and extract was obtained.

Antibacterial activity

Antibacterial activity of all the extracts was evaluated against *Escherichia coli* (ATCC 15224), *Klebsiella pneumonia* (ATCC 13883), *Bacillus* sp. (ATCC *6633)* and *Staphylococcus aureus* (ATCC25923) using agar well diffusion assay (Ref) for extracts in concentration of 1 mg/mL. While disk diffusion assay (Haq *et al.*, 2013) was performed for the compounds extracted from TLC bands. The activity was expressed as zone of inhibition and diameter of zones measured in millimeters (mm).

HPLC based metabolic profiling of fungal extracts

The metabolic shift in fungal isolates growth with and without antibiotics was analyzed by high performance liquid chromatography. All the extracts were dissolved in methanol and filtered (0.2micron) to remove suspended particles. The separation of the samples was achieved on an HPLC instrument (Agilent 1100 series; Agilent Technologies, Santa Clara, CA, USA) using a 5-μm Capcell Pak C_{18} YMC column (150 mm×3.0 mm ID; Japan).

The instrument was equipped with UV detector. The injection volume was 20 μL, detected at 254 nm. The mobile phase constituted 100% water (solvent A) and 100% acetonitrile (solvent B). Linear gradient elution was performed from 10% solvent B and (90% solvent A (*t*=0 min) to 100% solvent B at *t*=35 min. The flow rate was 1 mL/min and the column oven temperature was 30°C. The chromatograms in each case were comparatively analyzed to identify peaks in each extract.

TLC based analysis of fungal extracts

For TLC analysis, aluminum plates coated with silica (silica gel 60, MERCK Germany) were employed. Various combinations of solvents (acetonitrile, aqueous ammonia, ethyl acetate, isopropyl alcohol dichloromethane and methanol) (SIGMA, HPLC grade) were used as mobile phase.

The plates were run in mobile phase and developed. The spots were visualized under Ultraviolet (UV) lamp (long as well as short wavelength) and evaluated for antibacterial activity against *Escherichia coli* (ATCC 15224), *Klebsiella pneumonia* (ATCC 13883), *Bacillus spizizenii* (ATCC *6633)* and *Staphylococcus aureus* (ATCC25923) using Disc Diffusion Assay (Balouiri *et al.*, 2016).

FTIR of active compounds

Samples obtained from TLC analysis and showing promising antibacterial activity were evaluated by Fourier Transform Infrared Spectroscopy for its chemical moieties. The FTIR instrument (Tensor 27, Bruker, equipped with ZnSe ATR) was used in the range of 4000-400cm-1. Extracted bioactive samples were positioned on the sample tray and infrared spectrum was obtained. The FTIR spectrum and peak values labelling were gained by using software OPUS65.

Results and discussion

Growth of endophytic fungal isolates on antibiotics supplemented medium

The growth of endophytic fungal isolates was screened by supplementing moxifloxacin and clarithromycin in SDB medium and then compared with their respective controls.

The isolates showed visible changes in growth pattern under the effect of antibiotics supplemented media.

Table 1. HPLC Peaks ascribing metabolites that were inhibited in the presence of clarithromycin.

S. No.	Peak No.	Retention time in minutes
		Peaks vanished in clarithromycin presence
1	1	4.7
2	3	10.1
3	4	10.7
4	7	12.7
5	8	13
6	22	36.7
7	23	37
8	24	40
9	25	42

Table 2. HPLC Peaks attributing metabolites that were induced only in the presence of clarithromycin.

S. No.	Peak No.	Retention time in minutes
		Peaks induced by clarithromycin presence
1	8	17.2
2	9	18.3
3	10	18.7
4	12	19.7
5	14	20.5
6	19	25
7	21	26.5
8	22	27.5
9	23	28
10	24	28.3
11	25	28.7
12	26	29
13	29	31.5
14	31	35.5
15	32	39

The fungi grow as mycelial balls under standard conditions while under the effect of antibiotics, mycelial balls dissociated into thread like structures. Similar observations were made for both *Epicoccum* sp. NFW1 as well as *Chaetomium* sp. NFW8 in the presence of both the antibiotics. Additionally, under the effect of antibiotics, NFW8 lost it peach like colour. However, no significant change in biomass (300 mg per 100 ml ± 10mg dry weight) was noted in flask with or without antibiotics. Similar observations were made in case of NFW1 which showed slight change in colour (Fig. 1). No changes in biomass, as noted by dry weight of mycelial mass (325mg ± 10mg per 100ml medium), were observed.

Table 3. HPLC Peaks attributing metabolites that were inhibited in the presence of moxifloxacin.

S. No.	Peak number	Retention time in minutes
		Peaks inhibited by moxifloxacin presence
1	13	19.5
2	14	20.5
3	16	21.5
4	17	24.5
5	18	29.7
6	19	30
7	25	42

Table 4. HPLC Peaks attributing metabolites that were induced only in the presence of moxifloxacin.

S. No.	Peak No.	Retention time in minutes
		Peaks induced by moxifloxacin presence
1	1	3.5
2	3	5.5
3	4	7.5
4	5	9
5	6	9.7
6	15	17.5
7	18	23.5
8	19	27
9	20	28.5
10	21	31.5
11	22	32
12	24	33
13	25	33.5
14	27	34
15	28	34.5
16	31	39.5
17	33	41.5

Antibacterial activity of isolates

Ethyl acetate extract of *Epicoccum nigrum* NFW1 and *Chaetomium* sp. NFW8 showed mild antibacterial activity when grown on SDB at 25°C without the supplementation of antibiotics against all the test bacterial strains. Most notable of these effects were expressed by NFW1 against *S. aureus* with a diameter of zone of inhibition 11mm (Fig. 2a).

Table 5. Antibacterial activity of compounds separated on TLC produced by *Epicoccum nigrum* NFW1 only in the presence of clarithromycin and moxifloxacin in SDB (bioautography).

Endophytes grown on antibiotic supplemented SDB	Rf value	Zone of inhibition in mm			
		E. coli	*Klebsiella pneumonia*	*Bacillus sp.*	*S. aureus.*
NFW1 + clarithromycin	0.14	10	9	11	10
	0.41	11	10	11	10
NFW1 + moxifloxacin	0.45	11	10	12	11
	0.87	15	14	16	14

Key: NFW1+clarithromycin = *Epicoccum nigrum* NFW1grown only in the presence of clarithromycin

NFW1+moxifloxacin = *Epicoccum nigrum* NFW1grown only in the presence of moxifloxacin.

The isolates expressed enhanced antibacterial activity when growth in media supplemented with antibiotics (Fig. 2b, 2c). A percentage enhancement of up to 54% was noted in case of NFW1, against when grown in the presence of moxifloxacin. Therefore, *Epicoccum nigrum* NFW1 was prioritized for further analysis. Similar findings were reported by Bode and colleagues where changes in cultivation conditions led to the production of additional bioactive compounds (Bode *et al.*, 2002).

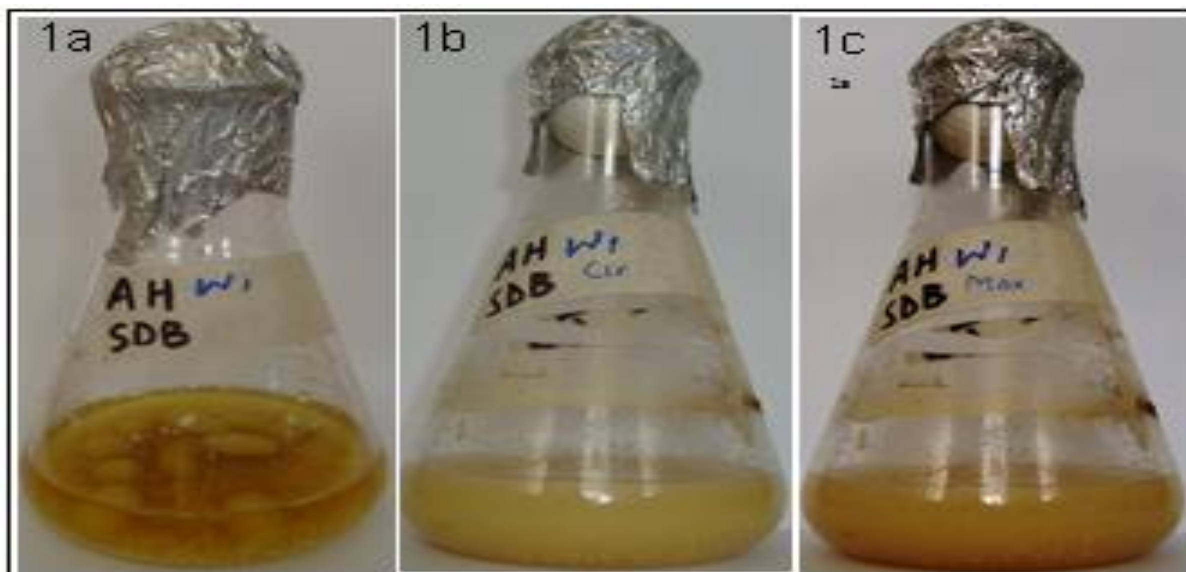

Fig. 1. Growth of NFW1 on SDB supplemented with and without antibiotics (1a) on SDB without antibiotic supplementation (1b) SDB supplemented with Clarithromycin (1c) SDB supplemented with Moxifloxacin.

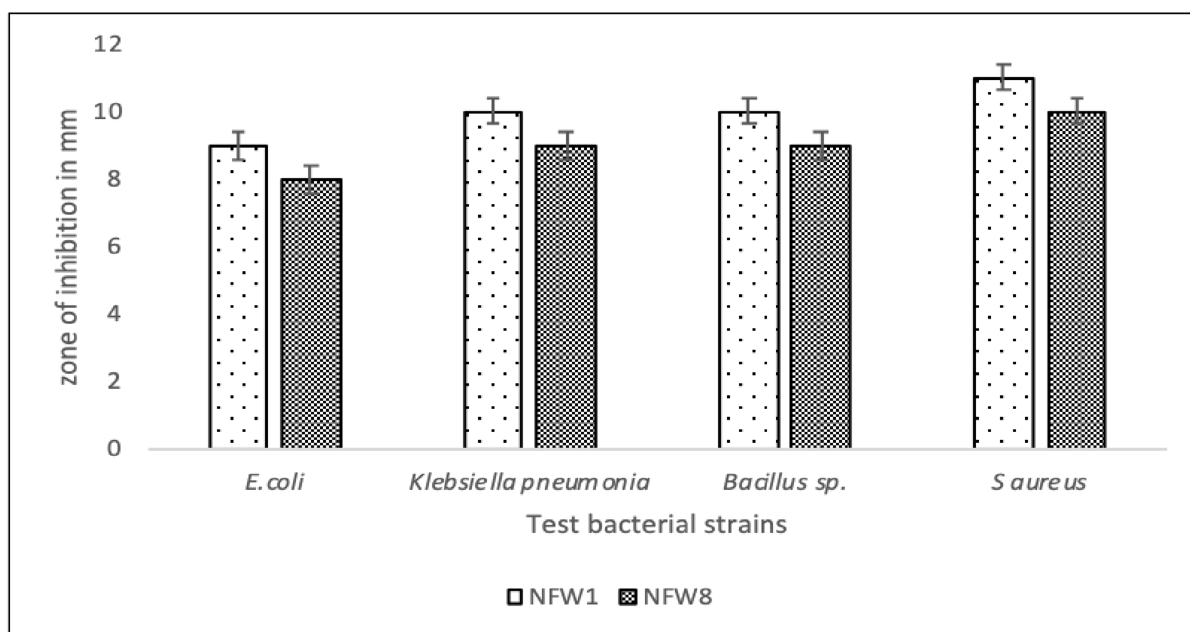

Fig. 2a. Antibacterial activity of endophytic fungal isolates against test bacterial strain.

Metabolic profiling of Epicoccum nigrum NFW1 with and without antibiotics supplementation

HPLC analysis

Fungal endophyte NFW1 showed significant shift in its metabolic profile under the presence or absence of antibiotics. Additionally, both clarithromycin as well as moxifloxacin showed varied effects on metabolism as indicated by HPLC chromatogram (Fig. 3a, 3b, 3c). Clarithromycin inhibited the production of certain metabolites in NFW1 as indicated by a total number

of nine peaks which are detected in the absence of clarithromycin but remain undetected otherwise (Table 1). Likewise, additional 15 peaks were noted in the chromatogram when the strain is cultivated in the presence of Clarithromycin (Table 2).

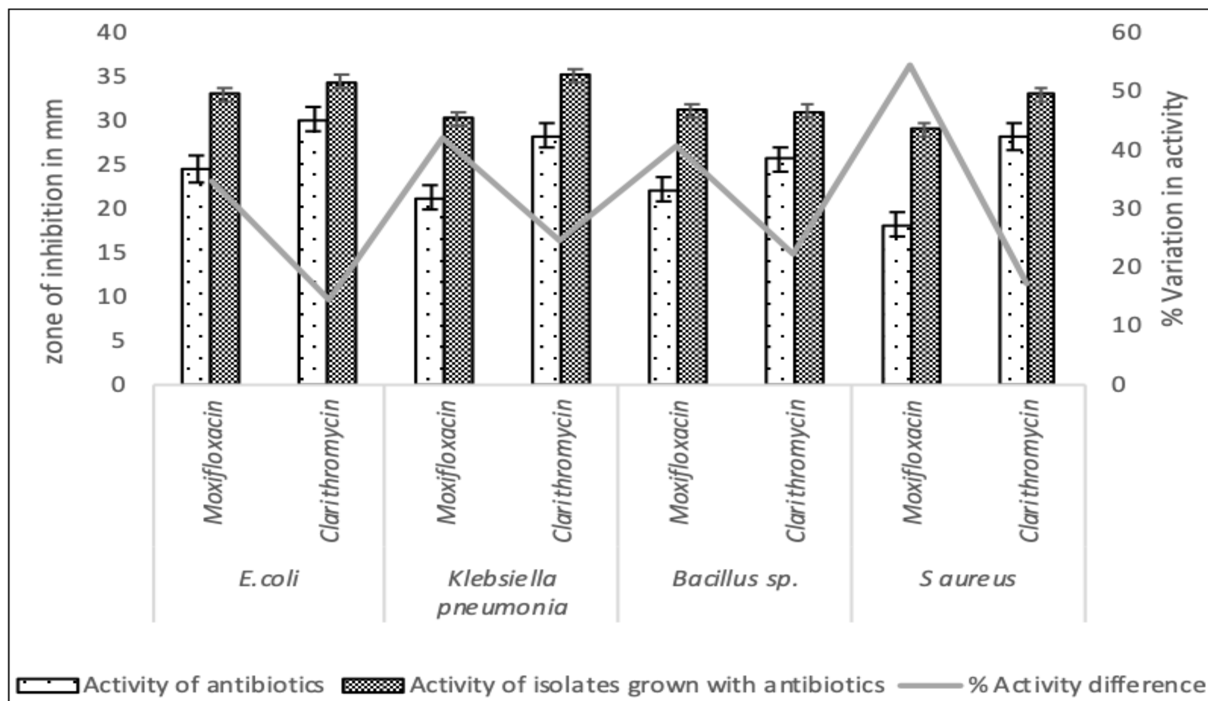

Fig. 2b. Antibacterial activity of ethyl acetate extract of control antibiotic and *Epicoccum nigrum* NFW1 grown with antibiotics.

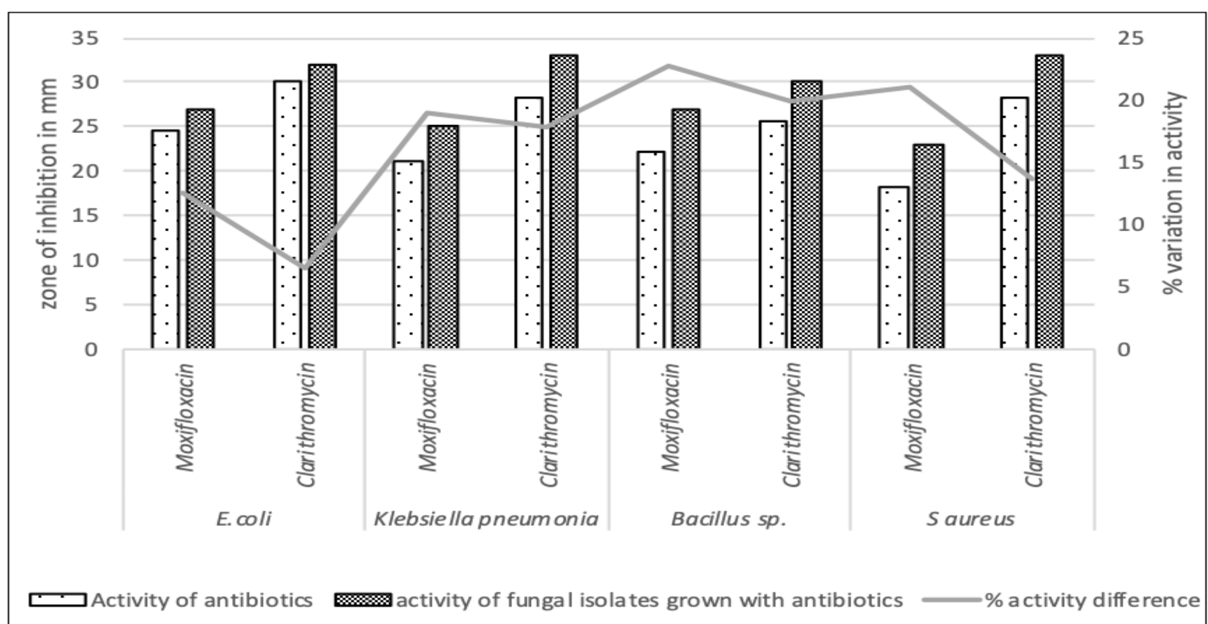

Fig. 2c. Antibacterial activity of ethyl acetate extract of control antibiotic and *Chaetomium sp.* NFW8 grown with antibiotics.

These peaks ascribing the secondary metabolites of fungus induced by antibiotic and some among them may be the transformed compounds originating from antibiotics. Further, there is variation in concentration of metabolites under the supplementation of antibiotic as indicated by

variations in peak intensities at similar retention times (Fig. 3b). Similar observations were noted in case of endophytic fungus *Bulgaria inquinans* where changes in cultivation media under OSMAC approach led to the expansion in chemical diversity of the isolate (Ariatari *et al*., 2019).

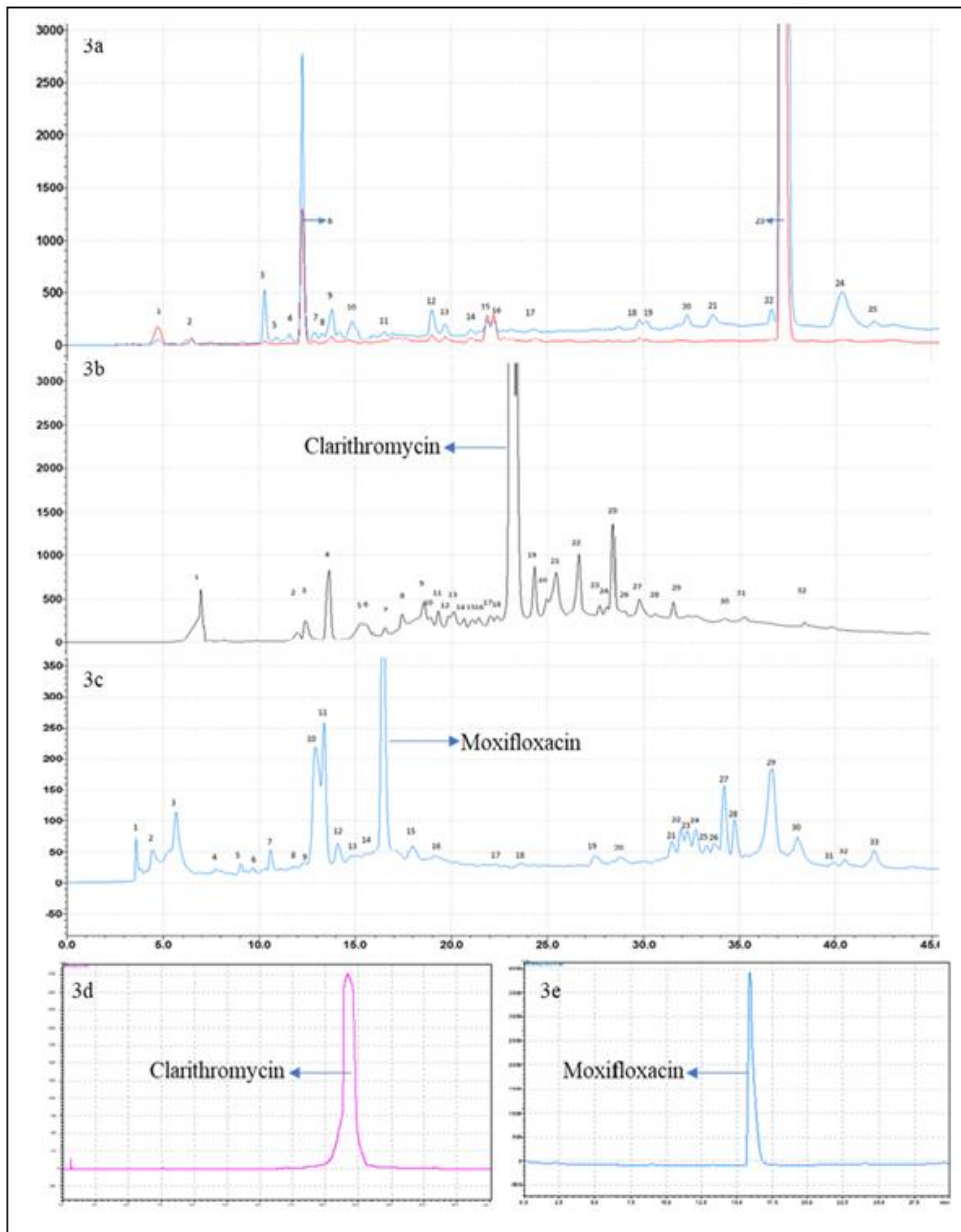

Fig. 3. HPLC Chromatograms of fungal isolates *Epicoccum nigrum* NFW1 grown with and without antibiotics and control antibiotics a) grown on SDB medium without antibiotics (UV 254 nm and 216 nm) b) grown on SDB medium with clarithromycin UV = 254 nm c) grown on SDB medium with moxifloxacin (UV 254) d) Clarithromycin UV 254nm e) Moxifloxacin UV 254 nm.

Moxifloxacin, on the other hand, also showed remarkable effects on metabolic profile of NFW1, the metabolites affected were different as compared to those in case of Clarithromycin supplementation (Fig. 3c). A total of seven peaks disappeared (Table 3) when NFW1 was grown in the presence of moxifloxacin, leading to appearance of another seventeen peaks not detected under standard cultivation conditions (Table 4) which are the secondary metabolites of fungus induced by antibiotic and some among them may be the transformed compounds originating from antibiotics These findings confer that the antibiotics act not only as inducers and activate certain biosynthetic gene clusters but could also be inhibitors to certain metabolic pathways as indicated by Scherlach and coworkers (Scherlach and Hertweck, 2009).

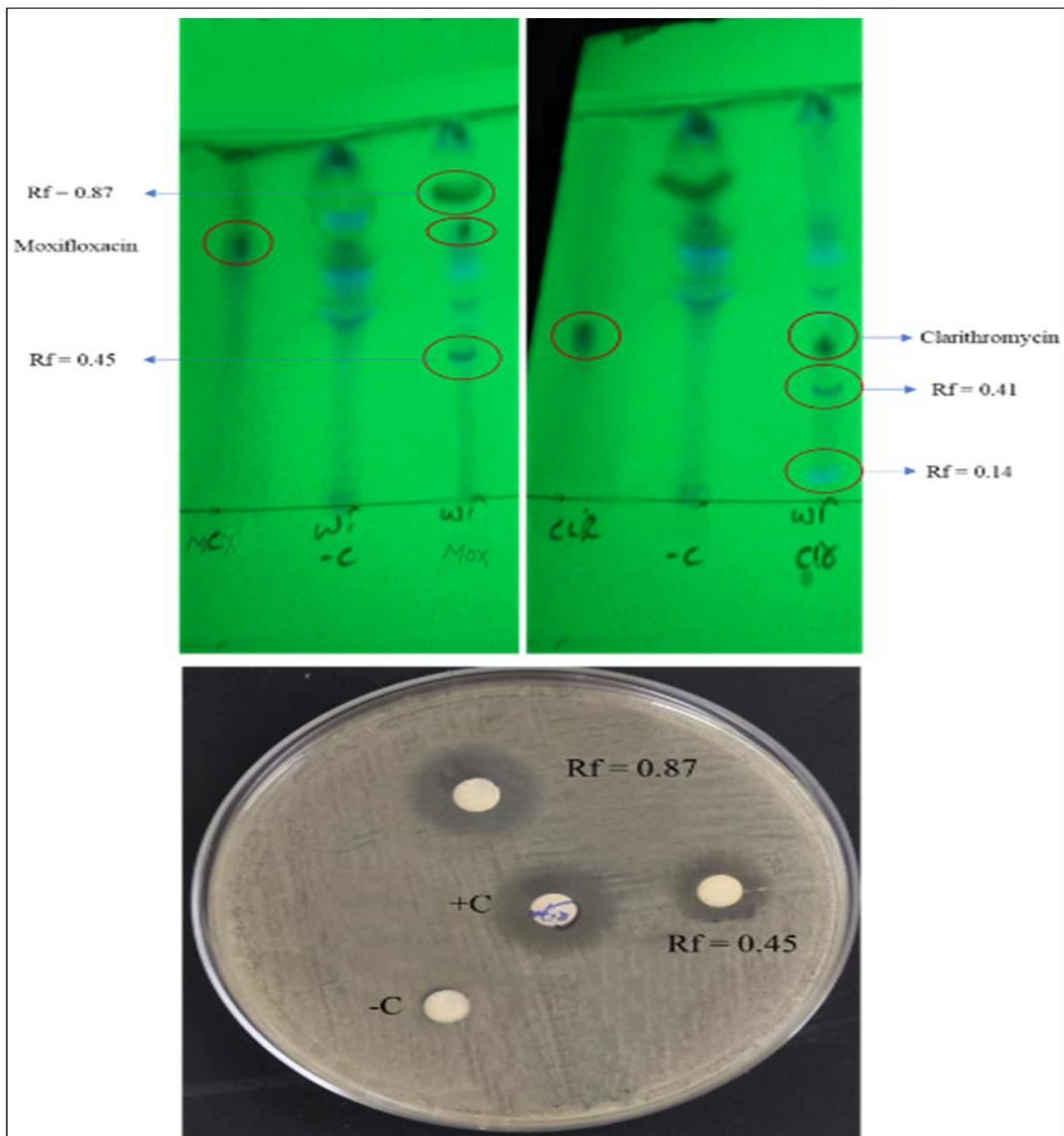

Fig. 4. TLC of extract of *Epicoccum nigrum* NFW1 grown with and without supplementation of antibiotic a, moxifloxacin b, clarithromycin while c, represent antibacterial activity of compounds isolated from TLC which were only present with moxifloxacin, +C = ciprofloxacin 10µg, -C = methanol on disk (disks placed after evaporation of solvents).

TLC analysis

TLC analysis for the extracts of *Epicoccum nigrum* NFW1 could be correlated to HPLC profiling. The plates showed variation in the samples grown with or without antibiotics as indicated under UV light (Fig. 4a and 4b). As noted earlier in HPLC, additional spots were detected in samples grown under antibiotic supplementation. Also, variations in intensity and visible spots was noted for those corresponding to antibiotics.

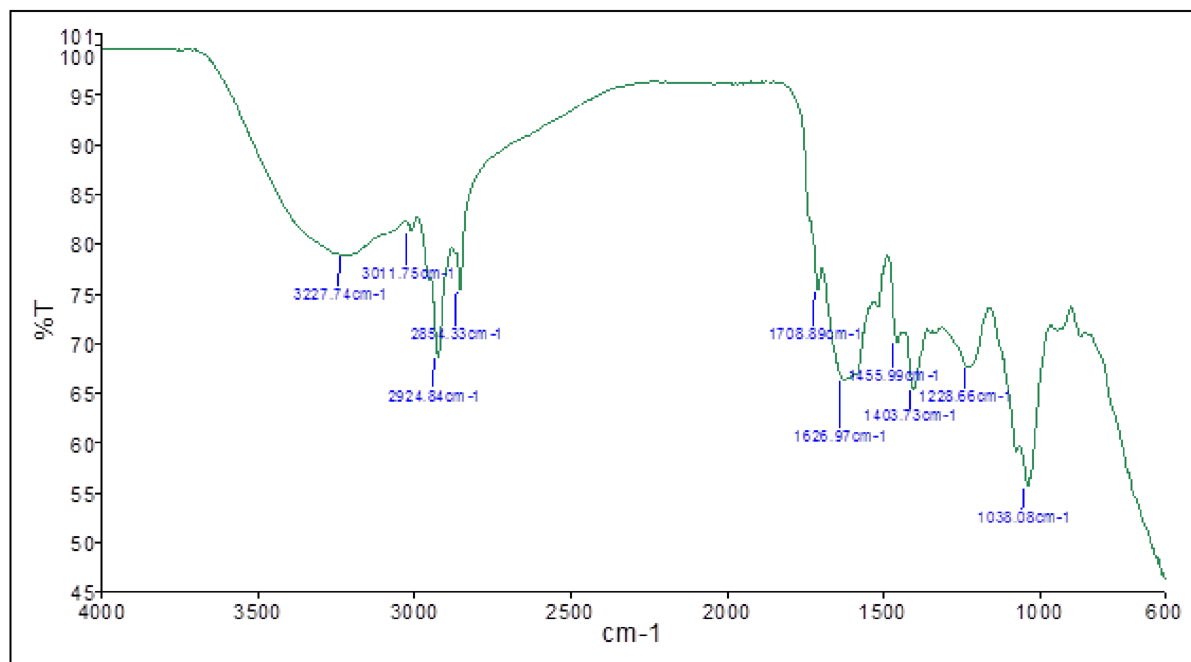

Fig. 5. FTIR spectrum of compound produced by *Epicoccum nigrum* NFW1 in the presence of moxifloxacin in growth medium appeared on TLC with Rf value 0.45 with antibacterial activity.

These findings show that antibiotics are not only affecting the biosynthetic potential of the endophytic fungus NFW1 but are also being utilized or degraded by the fungus. Thus, the additional bands with Rf value 0.45 and 0.87 in case of moxifloxacin while in case of clarithromycin bands with Rf value 0.14 and 0.41 could correlate with the fungal secondary metabolites induced by antibiotics or the transformed compounds originating from antibiotics as a result of biotransformation.

These observations are consistent with reports describing biotransformation of antibiotics by fungi as a result metabolic changes and enzymatic activity (Cvancorova *et al.*, 2015; Shang *et al.*, 2016). The TLC bands which were absent without antibiotic presence were subjected to antibacterial activity using bioautography and showed promising inhibition of gram positive as well as gram negative bacteria. Table 5 summarizes the antibacterial activity of these compounds. These results confirm that there is significant enhancement of bioactive potential of the isolates when grown under antibiotic supplementation.

FTIR analysis

FTIR analysis of the bands isolated from TLC was performed to identify the chemical moieties present in the samples. The spectrum (Fig. 5) showed the presence of broad peaks at 3227cm^{-1} representing the presence of O-H group from alcohols and carboxylic acids. Sharp peaks at 3011 cm^{-1}, demonstrate the C-H stretch of alkenyls, another one at 2924 cm^{-1} indicate CH_2 stretch from alkyls. Presence of aldehyde group is indicated by peak at 1708 cm^{-1} (C=O stretch), while peak at 1626 cm^{-1} corresponds to C=C of amides. Finally, peak at 1455 cm^{-1} denotes the O-H group of aromatics. The IR pattern indicates that the immense variety of functional groups are attributed to secondary metabolite of fungus rather than any

transformed product of antibiotic. Similar results were observed by Prabha and co-workers (Prabha *et al*., 2018).

Conclusion

In conclusion, antibiotics supplementation to the cultivation media under OSMAC approach could significantly change the biosynthetic potential of endophytic fungi. Since, this is preliminary investigation on the effects of antibiotics as elicitors, further studies are strongly recommended for the comprehensive elucidation of the extended metabolic spectrum of the isolate.

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References

Khalil AT, Muhammad A, Fauzia T, Muhammad O, Muhammad I, Zabta KS, James EH. 2017. Emerging viral infections in Pakistan: issues, concerns and future prospects. Health Security **15(3),** 163-164. http://dx.doi.org/10.1089/hs.2016.0072

Arnold AE. 2007. Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. Fungal Biology Reviews **21,** 51–66.

Gunatilaka AAL. 2006. Natural products from plant-associated microorganisms: Distribution, structural diversity, bioactivity and important implication of their occurrence. Journal of Natural Products **69,** 509-526. http://dx.doi.org/10.1021/np058128n

Scherlach K, Hertweck. 2009. Triggering cryptic natural product biosynthesis in microorganisms. Organic & Biomolecular Chemistry **7(9),** 1753-60. https://doi.org/10.1039/B821578B.

Teixeira TR, Santos GS, Armstrong L, Colepicolo P, Debonsi HM. 2019 Antitumor Potential of Seaweed Derived-Endophytic Fungi. Antibiotics **8,** 205. https://doi.org/10.3390/antibiotics8040205

Ebrahim W, El-Neketi M, Lewald LI, Orfali RS, Lin W, Rehberg N, Proksch P. 2016. Metabolites from the fungal endophyte *Aspergillus austroafricanus* in axenic culture and in fungal–bacterial mixed cultures. Journal of natural products **79(4),** 914-922. http://dx.doi.org/10.1021/acs.jnatprod.5b00975.

Bode HB, Bethe B, Höfs R, Zeeck A. 2002. Big effects from small changes: possible ways to explore nature's chemical diversity. ChemBioChem **3(7),** 619-62. http://dx.doi.org/10.1002/14397633(20020703)3:7<619:AID-CBIC619>3.0.CO;2-9

Kusari S, Hertweck C, Spiteller M. 2012. Chemical ecology of endophytic fungi: origins of secondary metabolites. Chemistry & biology **19(7),** 792-798. http://dx.doi.org/10.1016/j.chembiol.2012.06.004.

Ariantari NP, Daletos G, Mándi A, Kurtán T, Müller WE, Lin W, Proksch P. 2019. Expanding the chemical diversity of an endophytic fungus *Bulgaria inquinans*, an ascomycete associated with mistletoe, through an OSMAC approach. RSC advances **9(43),** 25119-25132. http://dx.doi.org/10.1039/C9RA03678D

Jadoon M, Fatima N, Murtaza S, Chang LC, Ali N, Ahmed S. 2016. Production of antimicrobial peptides by *Epicoccum* sp. NFW1: an endophyte of *Taxus fuana*. Acta Poloniae Pharmaceutica – Drug Research **73(6),** 1555-1563.

Fatima N, Kondratyuk TP, Park EJ, Marler LE, Jadoon M, Qazi MA, Mirza HM, Khan I, Atiq N, Chang LC, Ahmed S, Pezzuto JM. 2016. Endophytic fungi associated with *Taxus fuana* (West Himalayan Yew) of Pakistan: Potential bio-resources for cancer chemopreventive agents. Pharmaceutical

Biology **54(11),** 2547-2554.
http://dx.doi.org/10.3109/13880209.2016.1170154

Haq IU, Mirza B, Kondratyuk TP, Park EJ, Burns BE, Marler LE, Pezzuto JM. 2013. Preliminary evaluation for cancer chemopreventive and cytotoxic potential of naturally growing ethnobotanically selected plants of Pakistan. Pharmaceutical biology **51(3),** 316-328.
http://dx.doi.org/10.3109/13880209.2012.728612

Balouiri M, Sadiki M, Ibnsouda SK. 2016. Methods for *in vitro* evaluation of antimicrobial activity: a review. Journal of Pharmaceutical Analysis **6(2),** 71-79.
http://dx.doi.org/10.1016/j.jpha.2015.11.005

Cvanacrova M, Moeder M, Filipova A, Cajthaml T. 2015. Biotransformation of fluoroquinolone antibiotics by ligninolytic fungi-metabolites, enzymes and residual antibacterial activity. Chemosphere **136,** 311-320.
http://dx.doi.org/10.1016/j.chemosphere

Shang Z, Salim A A, Khalil Z, Bernhard PV, Capon RJ. 2016. Fungal Biotransformation of Tetracycline Antibiotics. Journal of organic chemistry **81(15),** 6186-6194.
http://dx.doi.org/10.1021/acs.joc.6b01272

Palanichamy P, Krishnamoorthy G, Kannan S, Marudhamuthu M. 2018. Bioactive potential of secondary metabolites derived from medicinal plant endophytes. Egyptian Journal of Basic and Applied Sciences **5(4),** 303-312.
http://dx.doi.org/10.1016/j.ejbas.2018.07.002