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Translating Endophytic Fungal Research Towards Pharmaceutical Applications

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ABSTRACT

Fungi living without any symptoms within the tissue of upper subgroups of *Plantae* are known as endophytic fungi. Since the discovery of Taxol, these fungi have received immense attention both from mycologists and microbiologists that resulted in the discovery of cryptocandin, camptothecin, vincristine and several other clinically useful small molecules. The Indian subcontinent, owing to its rich biodiversity, offers a great opportunity to discover unexplored fungi for pharmaceutical applications. For several years at Basic Research Centre of Hoechst Marion Roussel Limited and Research Centre of Piramal Enterprises, Mumbai, the prime interest was to use these fungi for drug discovery purposes using various enzymes, cell and target based screening to discover anti-cancer, anti-inflammatory and anti-microbial compounds. Various approaches including epigenetic tools, co-culture strategy, biotransformation and gene-editing tools, which are not routinely used in drug discovery programs, are also briefly discussed.

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President, Mycological Society of India (2016-2017)

INTRODUCTION

The most promising source of 'first-in-class' drugs has been derived from the natural source like plants and microorganisms which are the most productive and consistent sources (Newman and Cragg, 2007). The endophytic fungi based discovery of pharmacological agents has drawn the attention of scientific community remarkably (Strobel and Daisy, 2003). The untapped potential of these endophytic fungi has not been used adequately due to less exploration. Schulz *et al.* (1993) observed that these organisms have made their presence in almost all plant tissues studied so far without showing any symptoms. They play a role in plant growth, physiological activities and enable them to cope with biotic stresses (Carroll, 1988; Hallmann and Sikora, 1996; Azevedo *et al.*, 2000; Azevedo and Araujo, 2007). They also promote the growth of plants by enhancing their nitrogen fixing capabilities (Verma *et al.*, 2001; Rahman and Saiga, 2005). These fungi are a reservoir of pharmaceutically important bioactive secondary metabolites (Deshmukh and Verekar, 2009; Kharwar *et al.*, 2011; Deshmukh *et al.*, 2015; Deshmukh *et al.*, 2017) and would be helpful in developing new drugs of clinical importance and management of plant disease (Murray *et al.*, 1992; Kusari *et al.*, 2013; Kumar *et al.*, 2013; Chowdhary and Kaushik, 2016).

Smith *et al.* (2008) reported that endophytes are present in one or more number in nearly 300,000 species of terrestrial plants. Strobel and Daisy (2003) rightly remarked that endophytes are a goldmine for a diverse class of bioactive metabolites. *Pestalotiopsis* sp. can be considered as the "*E. coli* of the rain forests". Considering the range of metabolites obtained from *P. microspora*, it was called as a "microbial factory". A range of compounds with diverse structures has been described from these fungi that include ambuic acid, cryptocandin, taxol, torreyanic acid, subglutinol A and B and several others.

Newer strains and novel metabolites derived from endophytes have great potential to inhibit or eradicate variety of infections caused by pathogens like fungi, bacteria, viruses and protozoa that infect animals and humans (Tan and Zau, 2001). Endophytes in suspension culture produce secondary metabolites during growth. Parameters like temperature, media composition and aeration rate are known to affect the quantity as well as the variety of compounds produced (Strobel *et al.*, 2004), like steroids, xanthones, phenols, isocoumarins, perylene derivatives, quinines, furandiones, terpenoids, depsipeptides and cytochalasins (Zhang *et al.*, 2006; Gunatilaka, 2006; Kharwar *et al.*, 2011; Deshmukh *et al.*, 2015; 2017; Bedi *et al.*, 2018). Dreyfuss and Chapela (1994) estimated that approximately one million endophytes exist in nature. The discovery of such a large number of novel bioactive metabolites is possible because fungal endophytes display genetic diversity that can be exploited for the production of bioactive compounds (Gunatilaka, 2006). Some of the metabolites isolated from endophytic fungi at Basic Research Centre of Hoechst Marion Roussel Limited and Research Centre of Piramal Enterprises, Mumbai are listed in **Table 1** and **Figure 1** and **2**.

Metabolites with Anticancer activity:

PM181110 (1) (Fig. 1) a novel depsipeptide, was isolated from *Phomopsis glabrae* (**PM0509732**), an endophytic fungus harboring within the leaves of *Pongamia pinnata* (L.)

Pierre, collected from Karnala Bird Sanctuary of Maharashtra. The compound displayed the mean IC_{50} value of 0.089 µM towards a set of 40 human cancer cell lines and *ex vivo* efficacy (mean IC_{50}= 0.245 µM) towards 24 human tumor xenografts (Verekar *et al.*, 2014).

Ophiobolin A (2) (Fig.1) was derived from the endophytic fungus *Bipolaris setariae* of the *Parthenium hysterophorus* collected from Mumbai, India. It showed IC_{50} of 0.4-4.3 µM and restricted cell growth of haematological cancer. In contrast, IC_{50} of 20.9 µM was recorded for normal cells. Ophiobolin A was also observed to check the phosphorylation of S6, ERK and RB, the effector proteins of PI3K/mTOR, Ras/Raf/ERK and CDK/RB pathways, respectively. It checked the progress of cell cycle and induced cell death in MDAMB-231 cancer cell line with simultaneous inhibition of pS6, pAKT, pERK, pRB and cyclin D1 proteins. The anti-cancerous property was as a result of simultaneous blockage of different cancer regulatory pathways like PI3K/mTOR, Ras/Raf/ERK and CDK/RB (Bhatia *et al.*, 2016).

Altersolanol A (3) (Fig. 1), a derivative of anthraquinone was obtained from *Phomposis* sp. isolated from *Nyctanthes arbor-tristis* which was collected from Mumbai. Altersolanol A showed activity against 34 human cancer line *in vitro* with mean IC_{50} (IC_{70}) values of 0.005 µg /mL (0.024 µg /ml), respectively (Mishra et al., 2015). The cellular activity of Altersolanol A has been studied in detail, which inhibits kinase that follows caspase-dependent pathways for inducing apoptosis. Altersolanol A inhibited a variety of kinases which suggested that the kinase inhibition might be the mode behind cytotoxic activity (Debbab *et al.*, 2009). Altersolanol A blocks NF-B transcriptional activity (Teiten *et al*., 2013).

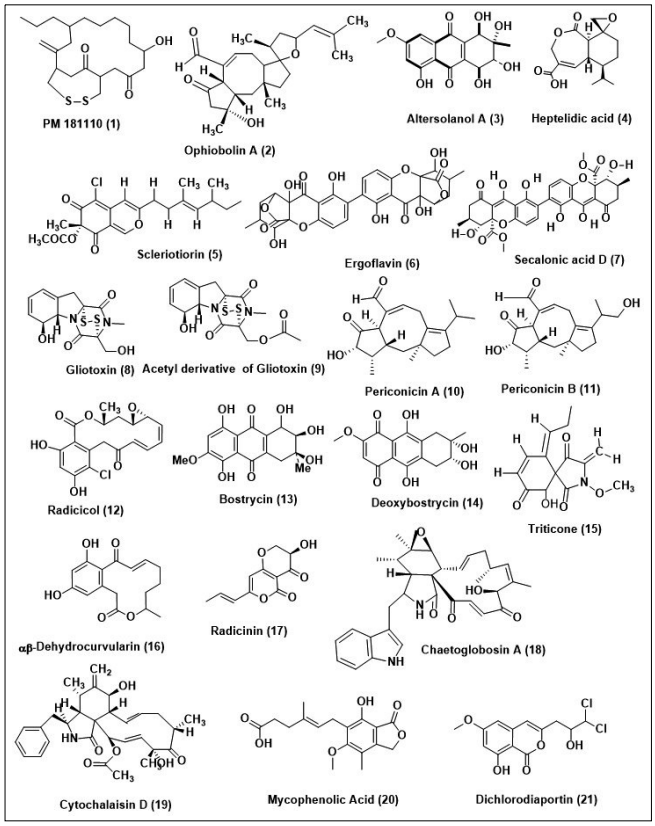

Fig. 1. Structures of anticancer metabolites obtained from Endophytic fungi.

Heptelidic acid (4) (Fig. 1) is a sesquiterpene lactone, also known as Koningic acid (KA) was derived from *Trichoderma* sp. of *Azadirachta indica*. The compound exhibited moderate activities towards T47D, SKOV-3, KM-12, NAMALWA, MDAMB-231, NCI-H460, HOP-62, Colo-205, TK10, Ovcar-3, BXPC3, HL-60, WM-266-4, DU145, HCT-116, A549 cancer cell lines with IC_{50}< 1 µg/mL (Rahier *et al.*, 2015). Early on, KA was found as a glycolytic pathway inhibitor that inhibited ATP synthesis, thus inhibiting glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which is involved in the conversion of glyceraldehyde 3-phosphate to 3-phosphoglycerate (Endo *et al.*, 1985). KA permanently blocks GAPDH via blocking the active site of the enzyme with a cysteine residue (Sakai *et al.*, 1988, 1990, 1991; Kato *et al*., 1992; Cane and Sohng, 1994).

Sclerotiorin (5) (Fig. 1) was isolated from *Cephalotheca faveolata*, occurring within the petiole of *Eugenia jambolana.* It showed cytotoxicity against HCT-116, H460, ACHN, Panc-1 and Calu-1 cell lines with the IC_{50} value of 0.63, 1.6 1.2, 1.6 and 2.1µM, respectively, while in MCF10A, it showed an IC_{50}> 10µM. Sclerotiorin induced apoptosis in HCT116 cells via the triggering of BAX and downregulation of Bcl-2 that results in stimulation of cleaved caspase-3 thereby causing the death of cancerous cells (Giridharan *et al.*, 2012).

An unidentified fungus (PM0651480) isolated from the leaves of *Mimosops elengi* yielded **Ergoflavin (6) (Fig. 1).** Ergoflavin showed IC_{50} values of 1.9 and 1.2 µM for TNF-α and IL-6, respectively. The compound showed IC_{50} value of 1.2, 4.0, 2.4, 8.0, 1.5 µM against ACHN, H460, Panc1, HCT16, Calu1 cancer cell lines, respectively (Deshmukh *et al.*, 2009).

Secalonic acid D (SAD) **(7) (Fig. 1)** was produced from *Aspergillus aculeatus*, harboring within a marine sponge *Cinachyra cavernosa* collected from Mandapam, Tamilnadu. It was cytotoxic against Panc-1, H-460, ACHN, Calu-1, HCT-116 and WI -38 cell lines with IC_{50} value of 0.2,0.2, 0.19, 0.2, 0.2 and 4.9 µg/mL (Deshmukh, unpublished data). Previously it was isolated from *Paecilomyces* sp. (tree 17), a mangrove endophytic fungus that showed cytotoxicity against KB cells with an IC_{50} <1 µg/mL and checked activity of topo isomerase I with an IC_{50} of 0.16 µM (Guo *et al*., 2007). SAD was earlier isolated from mangrove-derived endophytic fungus No. ZSU44 and exhibited cytotoxicity against HL60 and K562 cells, with IC_{50} values of 0.38 and 0.43 µM, respectively. PI assay/Annexin V-FITC and western blot also confirmed its apoptosis inducing activity. It also checked G1 phase progress resulting in cell cycle arrest due to downregulation of c-Myc. The reduction in the activity of c-Myc; cell-cycle arrest occurred by the activation of GSK-3β followed by β-catenin degradation (Zhang *et al*., 2009). *Penicillium oxalicum*, isolated from the roots of *Catharanthus roseus* was observed to produce SAD that possesses potent antitumor activity. SAD was observed to inhibit tumor formation by targeting

Table 1: Bioactive metabolites isolated from endophytic fungi.

Sr. No.	Endophytic fungal strain	Host plant(s)	Plant part or tissue/ Locality of host plants	Isolated metabolite	Biological activity	Tested systems	Activity response	References
1.	*Phomopsis glabrae*	*Pongamia pinnata*	Leaf, Karnala Bird Sanctuary, Raigad (MS), India	PM 181110 **(1)**	Anti-cancer	40 human cancer cell lines 24 human tumor xenografts	Mean IC_{50} value 0.089 µM Mean IC_{50} value 0.245 µM	Verekar *et al.*,2014
2.	*Bipolaris setariae*	*Parthenium hysterophorus*	Leaf, Mumbai, India	Ophiobolin A **(2)**	Anti-cancer	A2780, Pc3, MDAMB-231, MCF-7, MM1R, RPMI8226, U266B1 68 and Jurkat cell lines	Mean IC_{50} value 0.4–4.3 µM	Bhatia *et al.*, 2016
3.	*Phomopsis* sp.	*Nyctanthes arbor-tristis*	Leaves, Mumbai India	Altersolanol A **(3)**	Anti-cancer	34 human cell lines	Mean IC_{50} value 0.005 µM	Mishra *et al.*, 2015
4.	*Trichoderma* sp	*Azadirachta indica*	Leaf, Mumbai, India	Heptelidic acid **(4)**	Anti-cancer	T47D, SKOV-3, KM-12, NAMALWA, MDAMB-231, NCI-H460, HOP-62, Colo-205, TK10, Ovcar-3, BXPC3, HL-60, WM-266-4, DU145, HCT-116, A549 cell lines	IC_{50} value 0.21 0.34 0.41 0.45 0.45 0.49 0.51 0.63 0.66 0.69 0.76 0.76 0.84 0.89 0.93 0.99 µM,	Rahier *et al.*, 2015
5.	*Cephalotheca faveolata*	*Eugenia jambolana*	Leaf, Mumbai, India	Scleriotiorin **(5)**	Anticancer	ACHN, Panc-1, Calu-1, HCT-116, and H460 cell lines MCF10A cell lines	IC_{50} value 1.2, 1.6, 2.1, 0.63, and 1.6 µM, 0.63 to 2.1 µM IC_{50} > 10 µM	Giridhran *et al.*,2012
6.	Unidentified fungus	*Mimosops elengi*	Leaf, Mumbai, India	Ergoflavin **(6)**	Anti-inflammatory Anti-cancer	Inhibited TNF- α and IL-6 ACHN, H460, Panc1, HCT16, Calu1 cell lines	IC_{50} value 1.9 1.2µM IC_{50} value 1.2, 4.0, 2.4, 8.0, 1.5 µM	Deshmukh *et al.*, 2009
7.	*Aspergillus Aculeatus*	Marine spong *Cinachyra cavernosa*	Mandapam, Tamilnadu, India	Secalonic acid D **(7)**	Anti-cancer	Panc-1, H-460, ACHN, calu -1, HCT-116 and WI -38 cell lines	IC_{50} value 0.2,0.2, 0.19, 0.2, 0.2 and 4.9 µg/ml	(Deshmukh *et al.* unpublished Data).
8.	*Dichotomyces ceipii*	*Annona squamosa*	Bark, Thane, India	Gliotoxin **(8)**	Anticancer	ACHN, H460, Panc1, HCT16, Calu1 cell lines	IC_{50} in the range of 0.1-0.4 µM	(Deshmukh *et al.* unpublished Data).
9	*Dichotomyces ceipii*	*Annona squamosa*	Bark, Thane, India	Acetyl derivative of Gliotoxin **(9)**	Anticancer	ACHN, H460, Panc1, HCT16, Calu1 cell lines	IC_{50} in the range of 0.7-1.6 µg/mL	(Deshmukh *et al.* unpublished Data).
10.	*Periconia* sp.	Unidentified plant	Tejpur, Assam.	Periconiasin A **(10)**	Anticancer	ACHN, H460, Panc1,HCT16, Calu1 cancer cell lines	IC_{50} in the range of 0.8 to 1.5 µg/mL	(Deshmukh *et al.* unpublished Data).
11.	*Periconia* sp.	Unidentified plant	Tejpur, Assam.	Periconiasin B **(11)**	Anticancer	ACHN, H460, Panc1,HCT16, Calu1 cancer cell	IC_{50} in the range of IC_{50}, 1 -3 µg/mL in different cancer cell lines	(Deshmukh *et al.* unpublished Data).
12.	*Humicola fuscoatra*	*Mangifera indica*	Mulund Mumbai. India	Radicicol **(12)**	Anticancer	ACHN, Panc1,Calu 1, H460, HCT 116, MCF 10A cell line	IC_{50} values of 0.29, 0.45, 0.41, 0.27, 0.29 and 2.7 µM respectively.	(Deshmukh *et al.* unpublished Data).
13.	Unidentified fungus	*Avicennia marina*	Thane creek.	Bostrycin **(13)**	Anticancer	ACHN, Panc1,Calu 1, H460, HCT 116, MCF 10A cell line	IC_{50} in the range of 1.2-3.5µg/mL	(Deshmukh *et al.* unpublished Data).
14.	Unidentified fungus	*Avicennia marina*	Thane creek.	Deoxybostrycin **(14)**	Anticancer	ACHN, Panc1,Calu 1, H460, HCT 116, MCF 10A cell line	IC_{50} in the range of 2.2-5.7µg/mL	(Deshmukh *et al.* unpublished Data).
15.	Unidentified fungus	*Physalia angula*	Mumbai, India	Triticone **(15)**	Anicancer	pERK pS6 ACHN, Panc1,Calu 1, H460, HCT 116, MCF 10A cell line	IC_{50}, 1.9 µM, 1.9 µM, IC_{50}, ~0.1µg/mL	(Deshmukh *et al.* unpublished Data).
16.	*Curvularia* sp.	*Citrus sinensis*	Mumbai, India	αβ-Dehydrocurvularin **(16)**	Anicancer	pERK, pS6 Proteasome pRb, ACHN, Panc1,Calu 1, H460, HCT 116, MCF 10A cell line	IC_{50} , 30;, 7.4; 29.3; > 30; µM, In the range of 1-3 µg/mL	(Deshmukh *et al.* unpublished Data).

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Sr. No.	Endophytic fungal strain	Host plant(s)	Plant part or tissue/ Locality of host plants	Isolated metabolite	Biological activity	Tested systems	Activity response	References
17.	Unidentified fungus	*Butea monosperma*	India	Radicinin **(17)**	Anticancer	pERK, pS6, proteasome, pRb ACHN, H460, Pancl, HCT16, Calul cell lines	IC_{50}, 5.5; 8.9; 25.1; > 100µM; IC_{50}, In the range of 1-3 µg/mL	(Deshmukh *et al*. unpublished Data).
18.	*Chaetomium* sp.	*Phyllanthus* sp.	Stem, Thane, India	Chaetoglobosin A **(18)**	Anticancer	pERK, PS6, proteasome	IC_{50}, <3 µg/mL >3µg/mL, >3µg/mL	(Deshmukh *et al*. unpublished Data).
19.	*Xylaria juruensis*	*Nyctanthes arbortristis*	Leaf, Mumbai, India	Cytochalaisin D **(19)**	Anticancer	ACHN, H460, Panc1, HCT16, Calu1 cell lines	IC_{50} in the range of 0.3-1 µg/mL	(Deshmukh *et al*. unpublished Data).
20.	*Penicillium* sp.	Unidentified plant	Mumbai, India	Mycophenolic Acid **(20)**	Anticancer	ACHN, H460, Panc1, HCT16, Calu1 cell lines	IC_{50} between 0.8 to 1.5 µM in different cancer cell lines	(Deshmukh *et al*. unpublished Data).
21.	Unidentified fungus	*Embelia ribes*	Mumbai, India	Dichlorodiaportin **(21)**	Anticancer	ACHN, H460, Panc1, HCT16, Calu1 cell lines	< 1 µg/mL	(Deshmukh *et al*. unpublished Data).
22.	Unidentified fungus	*Thevetia* sp.	Mumbai, India	PR-Toxin **(22)**	Anticancer	ACHN, H460, Panc1, HCT16, Calu1 cell lines	IC_{50}, 0.3-3 µg/mL in different cancer cell lines	(Deshmukh *et al*. unpublished Data).
23.	*Trichoderma* sp.	*Azadirachta indica*	Mumbai, India	Viridiol **(23)**	Anticancer	ACHN, H460, Panc1, HCT16, Calu1 cell lines	IC_{50}, <1 µg/mL	(Deshmukh *et al*. unpublished Data).
24.	*Mycoleptodiscus terrestris*	*Vallisneria* sp.	Mumbai, India	A52688 A **(24)**	Anticancer	ACHN, H460, Panc1, HCT16, Calu1 cell lines	IC_{50}, 1-3 µg/mL	(Deshmukh *et al*. unpublished Data).
25.	*Lepidosphaeria nicotiae*	Unidentified plant	Rajkot, India	Mutolide **(25)**	Anti-inflammation	TNF-α and IL-6	IC_{50}1.27 and 1.07 µM	Shah *et al*.,2015
26.	*Humicola fuscoatra*	*Ficus glomerata*	Thane, India	Brefeldin A **(26)**	Antiinflammation	TNF-α and IL-6	IC_{50}, 0.3 and 0.02 µg/mL	(Deshmukh *et al*. unpublished Data).
27.	*Trichurus* sp.		India	Trichurusin A **(27)** Trichurusin B **(28)**	Antiinflammation	TNF-α and IL-6	Ic_{50}, and 0.3µg/mL IC_{50} and 0.8µg/mL	(Deshmukh *et al*. unpublished Data).
28.	*Dendryphion nanum*	*Ficus religiosa*	Leaf, India	Herbarin A**(29)**	Anti-inflammatory and Anti-diabetic	TNF-α and IL-6 GUA	IC_{50}0.60 and 0.60 µM IC_{50}, 0.3 µg/mL Toxicity > 10 µg/mL	Mishra *et al*.,2013
29.	*Alternaria longissima*	*Sphaeranthus* sp.		Ustilaginoidin **(30)**	Antidiabetic	GUA	IC_{50}, 0.1 µM; Toxicity > 1 µM	(Deshmukh *et al*. unpublished Data).
30.	*Arthrinium phaeospermum*	Unidentified grass	Not reported, India	Arthrichitin **(31)**	Anti-fungal	Fungicidal efficiency against *Pyricularia oryzae* infection of riceand *B. cineria* infectionof cucumber at 5000 ppm	75 and 85%	Vijayakumar *et al.*, 1996

angiogenesis in human MCF-7 breast tumor xenografts and human umbilical vascular endothelial cells (HUVEC) by impeding HIF-1α/VEGF interaction. Studies suggested that SAD inhibited cell viability through the Akt/mTOR/p70S6K pathway. The addition of neutralizing VEGF antibodies or by Akt inhibitors perifosine and GSK69069, reversed the anti-angiogenic effects of SAD. It exhibited both extrinsic and intrinsic apoptotic properties. It was observed to alter the

amount of G1/S transition phase proteins, thereby leading to cell cycle inhibition. For ascertaining SAD as a potent cancer-specific therapeutic agent, deciphering its mode of action and preclinical evaluation has been recommended (Guru *et al*., 2015).

Gliotoxin (8) and its **acetyl derivative** have been **(9) (Fig. 1)** isolated from endophytic fungus *Dichotomyces ceipii* residing in *Annona squamosa*. Gliotoxin displayed IC_{50} values between 0.1-0.4 µM against different cancer cell lines, whereas acetyl derivative of gliotoxin exhibited IC_{50} values in the range of 0.7-1.6 µg/mL in different cancer cell lines (Deshmukh, unpublished data). Gliotoxin was originally isolated from *Gliocladium fimbriatum* (Johnson, *et al*., 1943), later on isolated from *Aspergillus fumigatus* (Glister and Williams, 1944) and *Penicillium terlikowskii* (Johnson, *et al*., 1953). Gliotoxin is a proven cytotoxic fungal-derived metabolite which belongs to epipolythiodioxopiperazine (ETP) class of compounds (Vigushin *et al*., 2004). The histone methyltransferase activity was inhibited by dimeric ETPs (Iwasa *et al*., 2010). It was also reported from *Penicillium* sp. It showed inhibitory activity against HMT G9a with IC_{50} value 2.6 µM and cytotoxicity on P388 Cell lines with the IC_{50} value of 0.056 µM (Sun *et al*.,2012).

Cytotoxic compounds **Periconiasin A (10)** and **B (11) (Fig. 1)** were identified from *Periconia* sp. isolated from an unidentified plant collected from Tejpur, Assam. Periconiasins A exhibited activity against ACHN, H460, Panc1, HCT16, Calu1 cancer cell lines with IC_{50} in the range of 0.8 to 1.5 µg/mL. Similarly, periconiasin B exhibited cytotoxicity against ACHN, H460, Panc1, HCT16, Calu1 cancer cell lines with IC_{50} in the range of IC_{50}, 1-3 µg/mL in different cancer cell lines (Deshmukh, unpublished data). Both the compounds were previously isolated from *Periconia* sp. F-31 obtained from *Annona muricata*. Periconiasin A selectively inhibited HCT-8 and BGC-823 cell lines with IC_{50} values of 0.9 and 2.1 µM, respectively. Periconiasin B displayed selective inhibition of growth in BGC-823, Bel-7402 and HCT-8 with IC_{50} values of 9.4, 5.1 and 0.8µM, respectively (Zhang *et al*., 2013).

Radicicol (12) (Fig. 1) was isolated from *Humicola fuscoatra* of *Mangifera indica* collected from Mulund, Mumbai. Radicicol exhibited cytotoxicity against ACHN, Panc1,Calu 1, H460, HCT 116, MCF 10A cell line with IC_{50} values of 0.29, 0.45, 0.41, 0.27, 0.29 and 2.7 µM, respectively. Based on the efficacy of pancreatic cancer cells, radicicol was further profiled for molecular signature in Panc-1 cells using high content screening tools. The results revealed that, radicicol up-regulates p21 and p53 significantly and also showed notable downregulation of NFkB and STAT3 protein levels at 6 hrs in Panc-1 cells. In addition, the levels of $pAKT^{S473}$, pRB^{S780} were significantly downregulated in Panc-1 cells. Radicicol also showed upregulation of caspase3 when compared to untreated control cells (Deshmukh, unpublished data). Radicicol is an antifungal macrolactone antibiotic that inhibits protein tyrosine kinase. Radicicol induces the differentiation of HL-60 cells into macrophages, blocking cell cycle at G1 and G2. It suppresses NIH 3T3 cell transformation by diverse oncogenes such as *src*, *ras*, and *mos* and also suppresses the expression of mitogen-inducible cyclooxygenase-2. As a cell differentiation modulator, radicicol has anti-angiogenic activity *in vivo*, inhibiting the proliferation of and plasminogen activator production by vascular endothelial cells (Kwon *et al*,. 1992; Oikawa *et al*.,1993; Zhao *et al*., 1995; Shimada *et al*., 1995; Chanmugam *et al*., 1995; Pillay *et al*., 1996; Schulte *et al*., 1999; Wu *et al*., 2013).

An unidentified mangrove endophytic fungus of *Avicennia marina* collected from Thane creek yielded **Bostrycin (13) (Fig. 1)** and **Deoxybostrycin (14) (Fig. 1).** Bostrycin exhibited cytotoxicity against ACHN, Panc1, Calu 1, H460, HCT 116, MCF 10A cell lines with IC_{50} in the range of 1.2-3.5µg/mL in different cancer cell lines. Similarly deoxybostrycin exhibited cytotoxicity against ACHN, Panc1,Calu 1, H460, HCT 116, MCF 10A cell lines with IC_{50} in the range of 2.2-5.7µg/mL in different cancer cell lines (Deshmukh, unpublished data). Mangrove fungus No. 1403 also yielded bostrycin. *Saccharomyces cerevisiae* was used as a model wherein at G1 phase cell cycle was ceased, finally leading to time- and dose-dependent cell death by inhibiting cell proliferation using bostrycin. Bostrycin also leads to mitochondrial destruction by decreasing mitochondrial membrane electric potential, during apoptosis. The cell death was induced in YCA1 null yeast strain but was partially rescued in AIF1 null mutant both in respiratory media and fermentative. This strongly suggests that cell death is induced by mitochondria facilitated but caspase-independent pathway (Xu *et al*., 2010). Both bostrycin and deoxybostrycin were also obtained from marine fungus *Nigrospora* sp. (No. 1403) occurring in *Kandelia candel* wood. Bostrycin showed cytotoxicity with IC_{50} values of 2.64, 5.39, 5.90, 4.19, 6.13, and 6.68 µM against A549, Hep-2, Hep G2, KB, MCF-7, and Adr, cell lines respectively (Xia *et al*., 2011).

Some other compound viz. **Triticone (15) (Fig. 1)** was extracted from unidentified fungus of *Physalia angula*. Triticone displayed IC_{50} for pERK=1.9 µM, pS6=1.9 µM and cytotoxicity in different cancer cell lines having IC_{50} of ~0.1µg/mL (Deshmukh *et al*., unpublished data). Previously triticone with phytotoxic properties was isolated from plant pathogenic fungus *Drechslera tritici* which also inhibits enzymes with the functional group carrying SH as a component of the active site, e.g. the protease-ficin (Kenfield *et al*.,1988; Sugawara *et al*., 1988).

αβ-Dehydrocurvularin (16) (Fig. 1) was isolated from *Curvularia* sp. residing in *Citrus sinensis*. The compound exhibited IC_{50} for pERK = 30; pS6 = 7.4; Proteasome = 29.3; pRb > 30 µg/mL; and cytotoxicity in different cancer cell lines with IC_{50} value between 1-3 µg/mL (Deshmukh, unpublished data). Previously it was reported from *Alternaria macrospora* as a phytotoxic compound (Robeson *et al*., 1985).

Radicinin (17) (Fig. 1) was isolated from an unidentified fungus obtained from *Butea monosperma*. This compound displayed *in vitro* potency IC_{50}, pERK = 5.5; pS6 = 8.9; proteasome = 25.1; pRb > 100 M and cytotoxicity in range of 1-3 µg/mL against different cancer cell lines (Deshmukh,

unpublished data). It was isolated earlier from *Alternaria radicina* and *Bipolaris coicis* as a phytotoxic compound (Nakajima *et al*., 1997; Solfrizzo *et al.,* 2004).

Chaetoglobosin A (18) (Fig.1) was obtained from endophytic fungus *Chaetomium* sp. harboring stem of plant *Phyllanthus* sp. The compound exhibited IC_{50} of <3µg/mL for pERK and >3µg/mL for pS6 and proteasome (Deshmukh, unpublished data). It was previously reported as cytotoxic compound and was isolated from the same fungus (Sekita *et al.*, 1973; Umeda, 1975).

Other cytotoxic compound **Cytochalasin D (19) (Fig. 1)** was isolated from *Xylaria juruensis*, an endophyte residing in *Nyctanthes arbor-tristis*. The compound displayed IC_{50} values between 0.3-1 µg/mL in different cancer cell lines (Deshmukh, unpublished data). It was previously reported from *Xylaria arbuscular* an endophytic fungus from healthy tissues of *Cupressus lusitanica* (Amarala *et al*., 2014). Cell-permeable cytochalasin D, causes cell arrest at G1-S transition by activation of p53 dependent pathways and checks actin polymerization. It prevents polymerization of actin monomers by binding to the F-actin polymer (Heptinstall *et al*., 1998).

Investigation of the endophytic fungus *Penicillium* sp. obtained from an unidentified plant lead to the isolation of an anticancer compound, **Mycophenolic Acid (20) (Fig. 1).** The compound exhibited cytotoxic activity with IC_{50} values between 0.8 to 1.5 µM against different cancer cell lines (Deshmukh, unpublished data). Mycophenolic acid was discovered for the first time from *Penicillium glaucum* (now called *P. brevicompactum*) from spoiled corn and possess broad-spectrum antiviral, antifungal, antibacterial, anticancer, and anti-psoriasis properties (Silverman *et al*., 1997). Mycophenolatemofetil is a prodrug for mycophenolic acid, an immunosuppressive agent that is in use for transplant recipients and used to treat several inflammatory conditions (Moder, 2003). It is also used for curing autoimmunological diseases like lupus nephritis (Appel, 2012).

Dichlorodiaportin (21) (Fig. 1) was obtained from an unidentified fungus from *Embelia ribes.* It exhibited cytotoxicity against different cell lines and showed IC_{50} values of < 1 µg/mL. It was previously reported from *Penicillium nalgiovense* (Larsen and Breinholt, 1999). It was also reported from *Trichoderma* sp. 09 and endophytic fungus of *Myoporum bontioides* with anti fungal activity against *Colletotrichum musae* and *Rhizoctonia solani*. (Li *et al*., 2016).

PR-Toxin (22) (Fig. 2) was isolated from an unidentified fungus from *Thevetia* sp. The compound showed IC_{50} values between 0.3-3 µg/mL in different cancer cell lines (Deshmukh *et al*., unpublished data).

Viridiol, a steroidal antibiotic **(23) (Fig. 2)** was isolated from *Trichoderma* sp. harboring in *Azadirachta indica.* Viridiol exhibited cytotoxicity with IC_{50}, <1 µg/mL. It was earlier reported from *Trichoderma viride*. Viridiol exhibit phytotoxic (Shows necrotic activity on plants) and antifungal properties. It is also a Phosphatidylinositol 3-kinase (PI3K) inhibitor (Moffatt *et al.,* 1969;, Hanson *et al.,* 1988; Jones and Hancock, 1987; Jones *et al.*; 1988; Andersson *et al.*, 2010; Cao*et al*., 2010).

A52688 (24) (Fig. 2) was obtained from *Mycoleptodiscus terrestris*, from the leaf of *Vallisneria* sp. A52688 exhibited cytotoxic activity towards different cell line with IC_{50} values between 1-3 µg/mL. It was earlier reported from the same fungus with antibacterial and anti-neoplasticactivity (Anderson *et al*., 1985).

Metabolites with Anti-inflammatory activity:

An anti-inflammatory compound **Mutolide (25) (Fig. 2)** was obtained from *Lepidosphaeria nicotiae*, of an unidentified plant and also from a coprophilous fungus *Lepidosphaeria* sp. In LPS-induced inflammation, the compound checked cytokines TNF-α and IL-6 secretion from THP-1 and mononuclear cells of the human peripheral blood (IC_{50}1.27 and 1.07 µM , respectively). In anti-hCD3/anti-hCD28 stimulated hPBMCs, the compound was found active to inhibit release of pro-inflammatory cytokine IL-17. NF-κB has prominent role involved in the release of pro-inflammatory cytokines including IL-17. Mutolide was found effective in inhibiting NF-κB activation and translocation. However, the compound was not much effective in checking the activity of p38 MAPK enzyme, a serine/threonine kinase responsible for cytokine secretion. In the LPS-induced acute model of inflammation in Balb/c mice, a dose of 100 mg/kg of mutolide was found to inhibit secretion of TNF-α (Shah *et al*., 2015).

Brefeldin A (26) (Fig. 2), a lactone was isolated from *Humicola fuscoatra*, an endophyte residing in *Ficus glomerata.* The compound inhibited TNF-α and IL-6 with IC_{50} of 0.3 and 0.02 µg/mL, respectively (Deshmukh unpublished data). It was previously reported from *Eupenicillium brefeldianum* as an antiviral agent (Tamura *et*

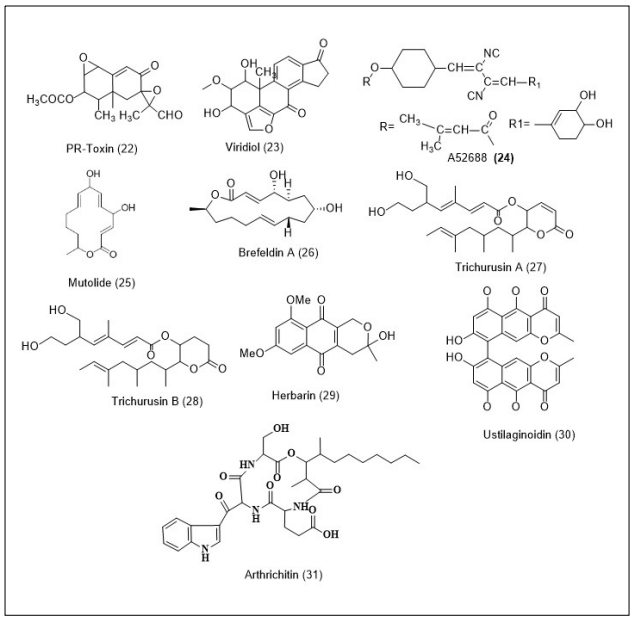

Fig. 2. Structures of anticancer, anti-inflammatory, antidiabetic and antifungal metabolites

al., 1968; Weber *et al*., 1971). It stops the joining of COP-I coat to the Golgi membrane thus checking protein transport from the endoplasmic reticulum to the Golgi apparatus (Helms and Rothman, 1992). In recent times, it is dominantly being used in research to study protein transport.

Trichurusin A (27) (Fig. 2) and Trichurusin B (28) (Fig. 2) were isolated from *Trichurus* sp. of *Calotropis procera*. These compounds inhibited IL-6 (IC_{50}, 0.3 µg/mL and 0.8 µg/mL, respectively) and TNF-α (IC_{50}, 1.0 µg/mL and 6.0 µg/mL, respectively) (Deshmukh unpublished data). These compounds were also reported from *Trichurus terrophilus* and exhibited considerably high immunosuppressive activities (Akiyama *et al*., 2003, 2005; Fujimoto *et al*., 2005).

Metabolites with anti-diabetic activity:

Herbarin (29) (Fig. 2) a naphthoquinone with anti-inflammatory and anti-diabetic activity was detected from *Dendryphion nanum* harboring leaf of *Ficus religiosa*. Herbarin displayed anti-inflammatory activity by inhibiting IL-6 (IC50 0.60 µM) and TNF-α (IC50 0.60 µM) and antidiabetic activity in GUA assay with IC_{50}value of 0.3 µg/mL and Toxicity at > 10 µg/mL (Mishra *et al*., 2013).

Another antidiabetic metabolite, **Ustilaginoidin A (30) (Fig. 2)** was detected from *Alternaria longissima* from *Sphaeranthus* sp. Ustilaginoidin A exhibited IC_{50} , GUA 0.1 µM; Toxicity > 1 µM (Deshmukh Unpublished data). It was reported from *Claviceps virens* with weak antitumor cytotoxicity to human epidermoid carcinoma (Koyama and Natori, 1988; Koyama *et al*., 1998).

Metabolites with Antifungal activity:

A cyclic depsipeptide **Arthrichitin (31) (Fig.2)** was obtained from *Arthrinium phaeospermum* derived from unidentified grass, and also from (as LL156256g) the marine fungus *Hypoxylon oceanicum* (Vijayakumar *et al*., 1996; Schlingmann *et al*., 1998). This compound showed antifungal activity against *Candida* sp., *Trichophyton* sp. and several phytopathogens. It has low *in vitro* potency to be used in the clinic, but analogs with improved activity could be developed (Vijayakumar *et al*., 1996). Arthrichitin **(31)** also displayed fungicidal efficiency of 75 and 85% against *Pyricularia oryzae* infecting rice and *Botrytis cineria* infecting cucumber at 5000 ppm (Vijayakumar *et al*., 1996).

Some of the strategies of cultivation of these fungi:

The cultivation of fungi for production of secondary metabolites needs optimizing the parameters that involve many strategies. Different approaches like changes in pH, aeration, temperature, the design of culture flask, modulation of medium to flask volume ratio, media composition, or by biotic elicitation through co-culture, abiotic stimulation using physical and chemical stress or by epigenetic modulation (Bode *et al*. 2002; Cichewicz, 2010; Pettit, 2011; Marmann *et al*. 2014; Bertrand *et al*. 2014). Some of the approaches used for getting chemical diversity are described briefly here.

One factor at a time (OFAT):

One factor at a time approach is a classical way for finding optimum condition for the processes. Response Surface Methodology (RSM) and Plakett-Burman design are a most accepted way for the optimizing processes. The Plackett-Burman design (Plackett and Burman, 1946) constitutes two level factorial design that reduces the number of trials and finds out the most prominent factor that affects productivity. Response surface methodology (RSM) (Box and Behnken, 1960) is another approach based on polynomial regression fitting, significance analysis and stationary point location that determines the best concentration of chosen factor in order to get desired response. In different organism and systems, it has been implemented to optimize most critical factors that affect the level of secondary metabolite produced (Garyali *et al*.,2014; Luo and He, 2004; Srivastava and Srivastava, 2012; Wang *et al*., 2013; Xu *et al*., 2006).

Garyali *et al*. (2014) reported the increased production of taxol using OFAT approach in fermentation by using *Fusarium redolens*. Due to the use of RSM, fungal taxol production rate increased by 3 folds compared to un-optimized medium.

Chemical epigenetic modifiers:

In fungi, certain genes remain silent and are not expressed during the entire life in normal condition, but under stress condition their expression takes place. These silent genes or cryptic genes can be induced to express using epigenetic modulators. Epigenetic modifiers act without acting changes in DNA sequence but alter expression levels. Chemical inhibitors like HDAC or DNMT are effective in regulating gene cluster such that there is a significant increase in the production of metabolites. DNA methyltransferase (DNMT) inhibitors like procaine, hydralazine, 5-azacytidine, 5-aza-2′-deoxycytidine, and procainamide and/or histone deacetylase (HDAC) inhibitors like suberoyl anilidehydroxamic acid (SAHA), valproic acid and sodium butyrateare commonly used epigenetic modifiers (Williams *et al*., 2008; Takahashi *et al*., 2016).

Simultaneous feeding of the HDAC inhibitor, SAHA, and the DNMT inhibitor, 5-azacytidine, to the growth media of *Pestalotiopsis acaciae* resulted in significant changes in the production of secondary metabolites, and led to production of three novel aromatic compounds, 2'-hydroxy-6'-hydroxymethyl-4'-methylphenyl-2, 6-dihydroxy-3-(2-isopentenyl) benzoate,4,6-dihydroxy-7-hydroxymethyl-3-methyl coumarin and 4,6- dihydroxy-3,7-dimethyl coumarin, along with five known polyketides endocrocin, pestalotiollide B , pestalotiopyrone G , scirpyrone A and 7-hydroxy-2-(2-hydroxypropyl)-5-methylchromone (Yang *et al*., 2013).

Brominated resorcylic acid lactones, along with other four already known, aigialomycin B,11 zeaenol,12 LL-Z1640-1, 12,13 and LL-Z1640-2 were isolated from the marine-derived fungal strain *Cochliobolus lunatus* (TA26-46) culture was treated with sodium butyrate a HDAC inhibitor (Zhang *et al*. 2014). There was 10 fold increase in the production of fumiquinazoline C when *Aspergillus fumigatus* (GA-L7) culture was treated with valproic acid, an epigenetic modifier,

Magotra *et al.* (2017).

Co-culture of different strains

One of the promising strategies to obtain chemically different compounds is the co-culture strategy. This is because the microorganisms live in an extremely biodiverse community in their natural habitats. Sharing the same niche, interferes with their morphology, growth, adaptation, and development patterns, that may also include changes in secondary metabolite production as a result of chemical interaction among the organisms (Kusari *et al.*, 2014; Pamphile *et al.*, 2017). A promising and complex environment is created by co-culturing for new secondary metabolites production due to intercommunication between different organisms. The compound austradixanthone and sesquiterpene (+)-austrosene along with other five deciphered compounds were demonstrated by Ebrahim *et al.* (2016) obtained from the EtOAc extract of axenically cultured *Aspergillus austroafricanus*, a fungus that harbors the leaves of aquatic plant *Eichhornia crassipes*. There was an increase up to 29 times for several diphenyl ethers, including the new austramide, in mixed cultures where the same strain was grown with *Streptomyces lividans* or with *Bacillus subtilis*.

FUTURE PERSPECTIVES

Endophytic fungi from the Indian subcontinent have immense potential to produce diverse metabolites. The true potential of these fungi has not been fully explored. In the Indian subcontinent, one third of the plants are endemic, and there are nine phytogeographic zones, two hotspots of biodiversity, six wetlands and 8000 km of coastal areas with endemic mangroves. India is a country which has different geographical regions and climate zones ranging from tropical to alpine (Himalayas) and has cold and hot deserts. Exploration of the diversity of endophytic fungi will give a boost to the natural product drug discovery.

We need to study the whole genome sequence of microbes and generate bioinformatics data based information. It will help to predict the presence of genes/gene clusters responsible for the synthesis of novel classes of chemical scaffolds.

The genome editing system (CRISPR/Cas9) is a powerful tool to manipulate genomes of different organisms. Only a few studies exist that employ genome editing approach in filamentous fungi (Shi *et al*., 2017). In *Trichoderma reesei* CRISPR/Cas9 generated site specific alteration in target genes (Liu *et al*., 2015). The system also offered an opportunity for simultaneous manipulation of multiple genes. The observations recorded from CRISPR/Cas9 mediated genome editing in *T. reesei* are promising and extend the opportunity for other filamentous fungi.

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