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Study on Antioxidant, Antidiabetic and Antibacterial Activity of Rhizospheric Fungi from Achanakmar Biosphere Reserve, Bilaspur

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FUNGAL derived bioactive compounds can be beneficial for the human immune system. They act as synergistic or agonistic molecules in the therapy of various human diseases. A total 18 rhizospheric fungi (ABRF1-ABRF18) were identified from rhizospheric soil of the medicinal plants of Achanakmar Biosphere Reserve, Bilaspur, India. The capacity of the fungi to produce metabolites with therapeutic potential was examined. Crude extract from these fungi demonstrated potent *in vitro* antioxidant activity with various antioxidant assays including Ferric reducing power, Phosphomolybdenum.2,2-diphenyl 1-picrylhydrazyl, and 2,2'-azino-bis3-ethylbenzthiazoline-6-sulfonic. Among different isolated fungi, four, (ABRF1-*Fusarium oxysporum,* ABRF2-*Talaromyces purpureogenus,* ABRF3-*Penicillium citrinum* and ABRF4- *Aspergillus carneus)* exhibited significant antioxidant potential. Active metabolites of the rhizospheric fungi obtained by extraction with solvents increasing in order of polarity, i.e. Toluene, Chloroform, Ethyl acetate, Methanol, Ethanol and Acetonitrile were examined for antibacterial activity. Variable zones of inhibition against the bacteria *Bacillus circulans, Bacillus subtilis, Escherichia coli, Staphylococcus aureus* and *Ralstonia eutrophae* were observed. Further, relatively purified extracts found in the ethyl acetate fraction of column chromatography demonstrated significant antidiabetic activity (up to 93.28±0.12) as measured by α amylase inhibition assay. The secondary metabolites extracted from these species may thus provide potential therapeutic ingredients for pharmaceutical applications worthy of future study.

Keywords: Antimicrobial activity, Antioxidant, Rhizospheric, Secondary metabolite.

Introduction

Medicinal plants have been used for treatments of various ailments in alternative medicine and as a source of bioactive pharmaceutical agents (Akerele et al., 1991). The growth, development and productivity of a medicinal plant is influenced by the soil quality and the organic and inorganic nutrients available to the plant through its roots. Rhizospheric regions of plants have a diverse array of microorganisms that may affect the growth period, cells and nutrient criteria (Simova-Stoilova et al., 2008). The rhizospheric regions are relatively active regions because microbes interact with plant cells as well as with other microbes to compete for food and growth. Utilization of microbes like fungi and bacteria for commercial production of secondary metabolites is an important research area, as microorganisms are easy to handle for any large-scale processes (Cragg & Newman, 2013). Fungi are eukaryotic microorganisms, easy to grow in the laboratory and known for secondary metabolite production. The fungi associated with plant roots are called rhizospheric fungi. Secondary products with therapeutic potential are formed by fungi to inhibit other microorganisms present in the rhizospheric range of a plant (Hibbing et al., 2010). Asperlicin, obtained from *Aspergillus alliaceus*, is effective in treating neurological conditions (Butler, 2008); strobilurin has been found to be effective as a fungicide (Reuveni, 2000); kojic acid is an antioxidant molecule (Pandit et al., 2018), and Lovastatin, isolated from *Aspergillus terreus* is hypocholesterolemic

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(Jahromi et al., 2012). Antimicrobial molecules, Patulin (Demain & Fang 2000) and Terrecyclic acid A, from *Aspergillus terreus* (Bok et al., 2006) are recognized as potent anticancer and antibacterial agents. These secondary metabolites may exhibit a beneficial role in protection from diseases. It has been found that the rhizospheric fungi make a significant number of antioxidants which activate the antioxidant mechanism that scavenges the reactive oxygen species originated from cell disruption or oxidative stress. The current study focused on a total of 18 rhizospheric fungi isolated from the rhizospheric region of medicinal plants (found in the Achanakmar biosphere reserve that is native to Chhattisgarh, India. Successive solvent extractions were performed to obtain secondary metabolites. Selected and screened fungi were assessed for antimicrobial and antioxidant potential. Based on the screening results, metabolites of rhizospheric fungi ABRF1-ABRF4 were found to produce potent antimetabolites toward pathogenic bacteria. ABRF1-ABRF4 were subjected to partial sequencing analysis to identify their species. The culture parameters and incubation condition were optimized. The extracted metabolites were partially purified by column chromatography to obtain relatively pure compounds for further studies.

Materials and Methods

Chemicals and reagents

Malt extract, Yeast extract, Potato dextrose agar, Potato dextrose broth, and Czapekdox agar were procured from Himedia, India. 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2' azinobis (3-ethyl benzthiazoline-6-sulphonic acid) (ABTS+), potassium persulfate, were purchased from Sigma-Aldrich, USA. All reagents and chemicals used were of analytical reagent grade.

Methods

Isolation of the fungal strains

Fungal strains were isolated from the rhizospheric soil in densely forested regions of the Achanakmar biosphere reserve, Chhattisgarh, India. The soil samples were collected by excavating twelve inches below the surface of the ground near the rhizospheric area with known medicinal plants. The soil was collected in poly bags and was brought to the laboratory. Subsequently, serial dilution was performed for isolation of the fungi (Dick, 1997; Bridge & Spooner, 2001). The soil sample, 1g, was serially diluted with distilled water from 10^{-1} to 10^{-9} and then 100µl each of 10^{-1}, 10^{-2},10^{-3}, 10^{-4} dilutions was spread on Potato Dextrose Agar (PDA) plates and the plates were incubated for 72 hours at 27±2°C. After incubation, various colonies were observed and 18 different colonies were segregated based on their growth characteristics, morphological appearances (*Isolate ABRF*1-18), and Spore was identified by using lactophenol cotton blue staining (Barnett & Hunter, 1972). Selected fungi were periodically subcultured and preserved on PDA slants and stored at 4°C.

Characterisation of fungal isolates

The effective strains based on preliminary screening (fungal isolates ABRF1-4) were characterized and identified, based on morphological characterization using a compound microscope (AXIO SCOPE.A1 HBO 50, Zeiss, Germany). Genomic DNA was separated and isolated with the help of gel electrophoresis and amplification of the ITS region of the fungal isolates was carried out through polymerase chain reaction (PCR). The sequencing of internal transcribed spacer (ITS) regions was performed (Chromous Biotech Pvt. Ltd., Bangalore, India). Briefly, DNA of each fungus was isolated using a DNA isolation kit (Invitrogen, USA). PCR amplification of ITS region was performed with primers specific for fungal ITS region 1 and ITS 2 and with minimum degeneracy (forward primer 5'-TCMGTAGGTGADCCWBCGS – 3' and reverse primer 5'- TCCTNCGYTKATKGVTADGH -3') (Ahirwar & Parihar, 2019; Beeck et al., 2014). These are degenerate primers used for identification purposes. The obtained amplified ITS sequence was aligned with similar sequences of other fungi using the BLASTN program of NCBI. Mega 6 software was used for the construction of the phylogenetic tree with many aligned sequences of fungi using maximum likelihood analysis and Tamura 3-parameter nucleotide substitution methods.

Source of microorganisms for antibacterial studies

Five bacterial strains were used for determining the antibacterial activity of fungal metabolites. These bacterial strains, namely, *Bacillus cerculans (*MTCC-7906 Gram-positive, rods), *Bacillus subtilis* (MTCC 441, Gram-positive, rods), *E. coli (*MTCC-739 Gram-negative cocci), *Ralstonia eutrophae*

(MTCC-2487 *Gram-positive* Rhodococcus), and *Staphylococcus aureus* (MTCC-96 Gram-positive cocci) were procured from the Microbial-Type Culture Collection (MTCC, CSIR-IMTECH, Chandigarh, India) and used in the investigation of antimicrobial properties of secondary metabolites. All five bacterial cultures were grown overnight on Luria–Bertani agar (LB) slants and maintained at 4°C for further experiments.

Screening and Preparation of fungal extracts

Five screening liquid nutrient media (100ml) with different constituents (CzapekDox Broth (CDB), CzapekDox Yeast Broth (CDYB), Malt Extract Broth (MEB) (Pinkerton & Strobel, 1976), Potato Dextrose Broth (PDB) and Yeast Extract Sucrose Broth (YESB) (Peterson & Bridge, 1994)) were chosen for the development of fungal biomass and secondary metabolite production. Static fermentation was preferred for secondary metabolite production as it was established to be more effective than constant shaking (Lin et al., 1976; Wei et al., 1991). All the strains were incubated in the incubator at 25±2°C for 15 days with the formation of fungal mats indicating complete growth of the strain. The fermented culture was harvested for the extraction of secondary metabolites. For the preparation of crude extracts, all the cultured flasks containing nutrient media with secondary metabolites were treated with 1.5% Triton X-100 post-incubation period with shaking for 30min at ambient temperature for homogenous cell wall disruption of the fungal biomass and consequent release of intracellular secondary metabolites into media. The fungal biomass was removed by filtration to obtain intracellular and extracellular secondary metabolites (Alkhulaif et al., 2019). The filtrates were collected and concentrated up to 20% of the original volume (v/v) in a vacuum rotary evaporator at 50±5°C. The residues were further concentrated and stored appropriately and diluted to working solution in sterilized distilled water for subsequent analysis.

Solvent extraction process and purification of ethanolic extract

The selected air-dried fungal biomass was initially subjected to Soxhlet extraction with ethanol to obtain most of the secondary metabolites. The crude ethanolic extracts were mixed with toluene (1:1 w/v) and subjected to adsorption chromatography on a glass column packed with silica gel (60-120 mesh size) with different eluents according to the increasing polarity of toluene, chloroform, ethyl acetate, methanol and acetonitrile. Since preliminary experimental results suggested that the maximum activity was found in the ethanolic extracts, they were further purified to obtain pure compounds. In brief, fungal biomass was processed through a Soxhlet extractor unit with pure ethanol. Extracted ethanol fraction was concentrated and dried under sterilized conditions.

Therapeutic potential of the isolated fungi

The antioxidant and antimicrobial functional characteristics of the isolated crude extracts from fungi were evaluated in triplicates with appropriate blanks and controls (ascorbic acid for antioxidant and streptomycin for antibacterial activity) in all the experiments. *In vitro* α amylase inhibition assays were also used to determine carbohydrate consumption to detect the presence of metabolites with antidiabetic activity.

Antioxidant assay

Four antioxidant assays based on the electron transfer (ET) system (FRAP, DPPH free radical, ABTS+ and Phosphomolybdenum assay) were used to evaluate the antioxidant capacities of all the isolates.

Measurement of the Ferric reducing antioxidant power (FRAP) assay: To determine the reducing power of fungal isolates, FRAP assay was performed by the method as described by Benzie & Strain (1996) and Barapatre et al. (2016) with slight modifications. Briefly, when a ferric tripyridyltriazine (FeIII-TPTZ) complex is reduced to the ferrous (FeII) form at low pH, an intense blue colour appears. Low pH is responsible for maintaining iron solubility and a decrease in the ionization potential that drives electron transfer and increases the redox potential. The working reaction mixture for FRAP assays was prepared by mixing of three solutions [10mM TPTZ dissolved in 40mM HCL, 20mM $Fecl_3 \cdot 6H_2O$ dissolved in distilled water, 0.3M acetate buffer 3.1gm sodium acetate dissolved in 16 ml acetic acid /L of buffer solution (pH 3.6)]. Then 2900µL of reaction mixture solution and 100µL of the sample were added in each test tube prepared in triplets. Reaction mixture without sample was used as negative control and 1mg/ml L-ascorbic acid was used as a positive control (standard). The reaction mixture was incubated for 30min in the dark and absorbance was read at 593nm wavelength. The results are expressed in terms of µmol (Barapatre et al., 2016).

Also, a calibration curve was created using an aqueous solution of ferrous sulphate $FeSO_4$ over a concentration series from 25–1100µM. The FRAP values were expressed on a fresh weight basis as µmol of ferrous equivalent Fe (II)/ L of sample. The scavenging activity was calculated as follows:

Scavenging Activity (%) = $[A_{30}\text{-}A_0] / [A_0] \times 100$

where $[A_0]$ was the absorbance of the control and $[A_{30}]$ was the absorbance of a sample with a FRAP solution (Barapatre et al., 2016). FRAP scavenging activity and IC50 values were calculated as described in statistical analysis.

2-diphenyl 1-picrylhydrazyl (DPPH) activity radical scavenging activity: The DPPH (0.2mM) radical scavenging activity was measured according to a previously reported method (Blois, 1958) with modification (Dhale & Vijay-Raj, 2009). The activity was evaluated using various concentrations (31.25–125µg/ml) of purified compound at 517nm and calculated according to the formula :

DPPH Scavenging Activity (%)= $[A_0\text{-}A_{30}]/[A_0]\times100$

where $[A_0]$ was the absorbance of the control (DPPH without sample) and $[A_{30}]$ was the absorbance of the sample with DPPH after 30 min (Barapatre et al., 2016).

The scavenging effect of crude extract on DPPH free radicals was determined by the method reported by Barapatre et al. (2016). The DPPH working assay was performed through the working solution of 2mg DPPH dissolved in 54 ml of methanol and fungal extract solution (100mg/ml) in autoclaved distilled water. 100µL of the sample and 2900µL of DPPH solution were taken in each test tube to obtain absorbance of 1.1±0.05 at 517nm. Each sample was prepared in triplicate. Controls, prepared as 100µL of autoclaved distilled water and 2900µL of DPPH solution in test tubes, were incubated for 30 minutes in the dark. Subsequently, absorbance was taken at 517nm wavelength using L-ascorbic acid (1mg/ml) as the positive control. The scavenging activity was calculated as follows :

Scavenging Activity (%)= $[A_0\text{-}A_{30}]/[A_0]\times100$

where $[A_0]$ was the absorbance of the control (DPPH without sample) and $[A_{30}]$ was the absorbance of a sample with DPPH scavenging activity and scavenging activity values were calculated as described in the statistical analysis.

Measurement of the total antioxidant capacity 2'-azino-bis3-ethylbenzthiazoline-6-sulfonic (ABTS assay): ABTS (2, 2'-azino-bis3-ethylbenzthiazoline-6-sulfonic) radical cation decolorization assay is based on the inhibition by antioxidants of absorbance imparted by radical cation 2,2- azinobis-(3-ethylbenzothiazoline-6-sulphonate) ($ABTS^{*+}$). Test samples were mixed with the $ABTS^{*+}$ solution, incubated for 2hrs in dark, followed by reading of absorbance at 734nm (Aadil et al., 2014).

A total antioxidant capacity assay was performed with the improved ABTS method proposed by Shan et al. (2007) with slight modifications. 100µL of the sample was mixed with 2900µL of ABTS solution. The absorbance was determined by measuring the UV absorbance at 734nm against deionized water blanks. After incubation at 37°C for 30min in the dark, the absorbance was read when the sample was replaced with methanol as the blank sample. The antioxidant capacity was expressed as the percent decrease in absorbance at 734nm, which was calculated using the formula:

ABTS*+ scavenging activity (%)= $(A0 - Ai)/A0\times100$

where A0 was the absorbance of the control, and Ai was the absorbance of the sample.

Measurement of the phosphomolybdenum assay: Phosphomolybdenum assays were utilized for the spectrophotometric quantitation of total antioxidant capacity by combining the sample with 1mL of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate) followed by incubation at 95˚C for 90min. The samples were cooled to room temperature, and the absorbance of the test solution was measured at 695nm against a blank (Sowndhararajan & Kang, 2013). The total antioxidant assay is based on the reduction of Phosphate-Molybdenum (VI) to Phosphate Molybdenum (V). The incubation of extracts with Molybdenum (VI) will indicate the presence of antioxidant components in the extract, which can be assessed by recording the absorbance at 695nm (to detect the reduced green molybdenum complex). So, this essay is particularly useful for predicting the antioxidant activity of crude extracts on a total basis.

The reducing capacity of extracts was calculated by using the formula :

% inhibition= (1- Absorbance of sample/ absorbance of control) ×100

This method is utilized for the spectrophotometric quantification of total antioxidant capacity and employs cost-effective reagents.

Antimicrobial tests

Agar well diffusion assay: The antibacterial activity assays were carried out using the agar well diffusion method (Balachandran et al., 2016) against test pathogenic organisms. About 1mg of the column fractionated sample was placed into an agar well and was used to determine antibacterial activity. Plates were incubated at 37°C for 24hrs and the zone of inhibition was measured. Ethanol was used as a vehicle control and Streptomycin (1mg/ml) as a positive control.

α amylase inhibition assay for antidiabetic activity

3, 5-dinitrosalicylic acid (DNS) is an aromatic complex that reacts with reducing sugars to form 3-amino-5-nitrosalicylic acid, which powerfully absorbs light at 540 nm. The mixture of 250µL of column Fungal fraction (1mg/mL) and 250µL of 20mM phosphate buffer containing α amylase was incubated at 25ºC for 10min. After pre-incubation, 250 µL of the starch solution was added and incubated at 25ºC for 10min. Acarbose was taken as a reference drug in the concentration of 100µg/mL. The reactions were stopped by the addition of 0.5mL of DNS reagent. The tubes were kept in boiling water for 10min and cooled at room temperature. The reaction mixture was diluted with 3.75mL water and the absorbance was measured at 540nm.

% inhibition = {(OD Control - OD Sample) /OD Control}×100

where OD control- Reaction mixture excluding column fraction; OD sample- Reaction mixture with column fraction.

Statistical analysis

All experimental data obtained are the means of three independent experiments under the same environmental conditions and the results are expressed as mean with standard deviation (mean ± SD). One way analysis of variance (ANOVA) was used to test significance between mean values of control and samples and comparison among means was carried out using Tukey-Kramer multiple comparisons test with the help of Graph Pad In Stat software (version 3.0). The level of significance $P< 0.05$, $P< 0.01$ and $P< 0.001$were denoted by *, ** and *** respectively, considered as statistically significant.

Results and Discussion

Isolation and characterization of rhizospheric fungi

A total of 18 pure fungal species were obtained from the soil of the rhizospheric region of the Achanakmar Biosphere Reserve. All isolates showed different morphology and characteristic features (variation size and shape of hyphae and spores), that were used for preliminary identification and characterization (Gautier et al., 2016; Wyatt et al., 2013). Isolates ABRF2, ABRF3, ABRF6, ABRF7, ABRF9, ABRF11, ABRF14, and ABRF15 were identified as *Penicillium sp*, ABRF4, ABRF5, ABRF8, ABRF10, ABRF12, ABRF13, ABRF16, ABRF17 and ABRF18 were of *Aspergillus sp* and ABRF1 is a *Fusarium sp* (Table 1). The morphological characteristics of isolate ABRF1 were, creamy white mycelia, mycelium which was initially white but subsequently turned purple, with discrete orange sporodochia, flask-shaped monophialides, and densely branched clusters. ABRF2 had soft, cottony fruit bodies, red-green mycelia, dense sporulation and tightly interwoven hyphae. ABRF3 had green mycelia dense sporulation, ellipsoidal conidia septate, hyaline hyphae, smooth-walled conidiophores, biverticillate and ampuliform phialides. ABRF4 was characterized by its yellow, thick-walled hyphae biseriate, white-black mycelia and dense sporulation shown in Fig.1.

Phylogenetic analysis of fungal strain

Molecular characterization of the most potent fungi (Isolates ABRF1-ABRF4) was performed using the PCR amplified ITS sequencing. The size of the PCR products observed for ABRFs1-4 was 554, 422, 528 and 540 base pairs, respectively (Fig. 2 A, B; 3 A, B; 4 A, B and 5 A, B). The amplicons were sequenced and the similarity sequence was analysed using the BLASTN program of NCBI. The maximum similarity of 100% was observed for ABRF1 amplified sequence with *Fusarium oxysporum,* ABRF2 with *Talaromyces purpureogenus,* ABRF3 with *Penicillium citrinum* and ABRF4 were observed with *Aspergillus carneus* (Fig. 2 C, 3 C, 4 C and 5 C). The fungal species were further confirmed by correlation with growth pattern in nutrient medium and morphological appearances. The sequence (NCBI accession numbers for ABRF1-4 are MN250029, MG905442, MK0271338

and MN250168 respectively) was submitted to the GenBank database. Construction of the phylogenetic tree was done using Mega 6 software by maximum likelihood and neighbour-joining method of selected taxa.

Evaluation for therapeutic potential

Rhizospheric fungi are present in the soil. They are a relatively unexploited source of novel value-added compounds. These compounds may be alkaloids, flavonoids, polyphenols, quinines *etc*. categorically known as secondary metabolites. Soil environment provides a special microenvironment that facilitates the organisms to produce novel secondary metabolites with promising applications in the expansion of the drug delivery system against a variety of diseases (Peterson & Bridge, 1994). Rhizospheric fungi have been studied in this article to determine their antioxidant, antimicrobial and antidiabetic potentials.

TABLE 1. Comparative details of 18 rhizospheric fungi used in the present study.

S. No.	Pure fungal strain (name of isolate)	Appearance in color	Pigment produce in media	Microscopic observation	Genus identification
1	ABRF-1	Creamy white mycelia	White viscous	*Fusarium* sp.	*Fusarium oxysporum*
2	ABRF-2	Red-green mycelia dense sporulation	Dark red	*Penicillium* sp.	*Talaromyces purpureogenus*
3	ABRF-3	Green mycelia dense sporulation ellipsoidal conidia	Yellow Pigment	*Penicillium* sp.	*Penicillium citrinum*
4	ABRF-4	White black mycelia	Black pigment	*Aspergillus* sp.	*Aspergillus carneus*
5	ABRF-5	Green mycelia, ovoid conidia	Light yellow	*Aspergillus* sp.	NA
6	ABRF-6	Green white mycelia	Milky white	*Penicillium* sp.	NA
7	ABRF-7	White light green mycelia, whitish edge	white	*Penicillium* sp.	NA
8	ABRF-8	Green mycelia	white	*Aspergillus* sp.	NA
9	ABRF-9	Green-yellow mycelia ellipsoidal conidia	Yellow pigment	*Penicillium* sp.	NA
10	ABRF-10	Cottony white mycelia	Purple white	*Emercellia* sp.	NA
11	ABRF-11	White green mycelia, whitish edge	white	*Penicillium* sp.	NA
12	ABRF-12	Cottony white mycelia	Yellow pigment	*Aspergillus* sp.	NA
13	ABRF-13	Black mycelia, ovoid conidia	Black	*Aspergillus* sp.	NA
14	ABRF-14	Brown white mycelia, plane surface colony	white	*Penicillium* sp.	NA
15	ABRF-15	Pink mycelia, ellipsoidal conidia	white	*Penicillium* sp.	NA
16	ABRF-16	Black mycelia, dense sporulation, plane surface colony	white	*Aspergillus* sp.	NA
17	ABRF-17	Black mycelia	white	*Aspergillus* sp.	NA
18	ABRF-18	Black mycelia dense sporulation ovoid conidia	white	*Aspergillus* sp.	NA

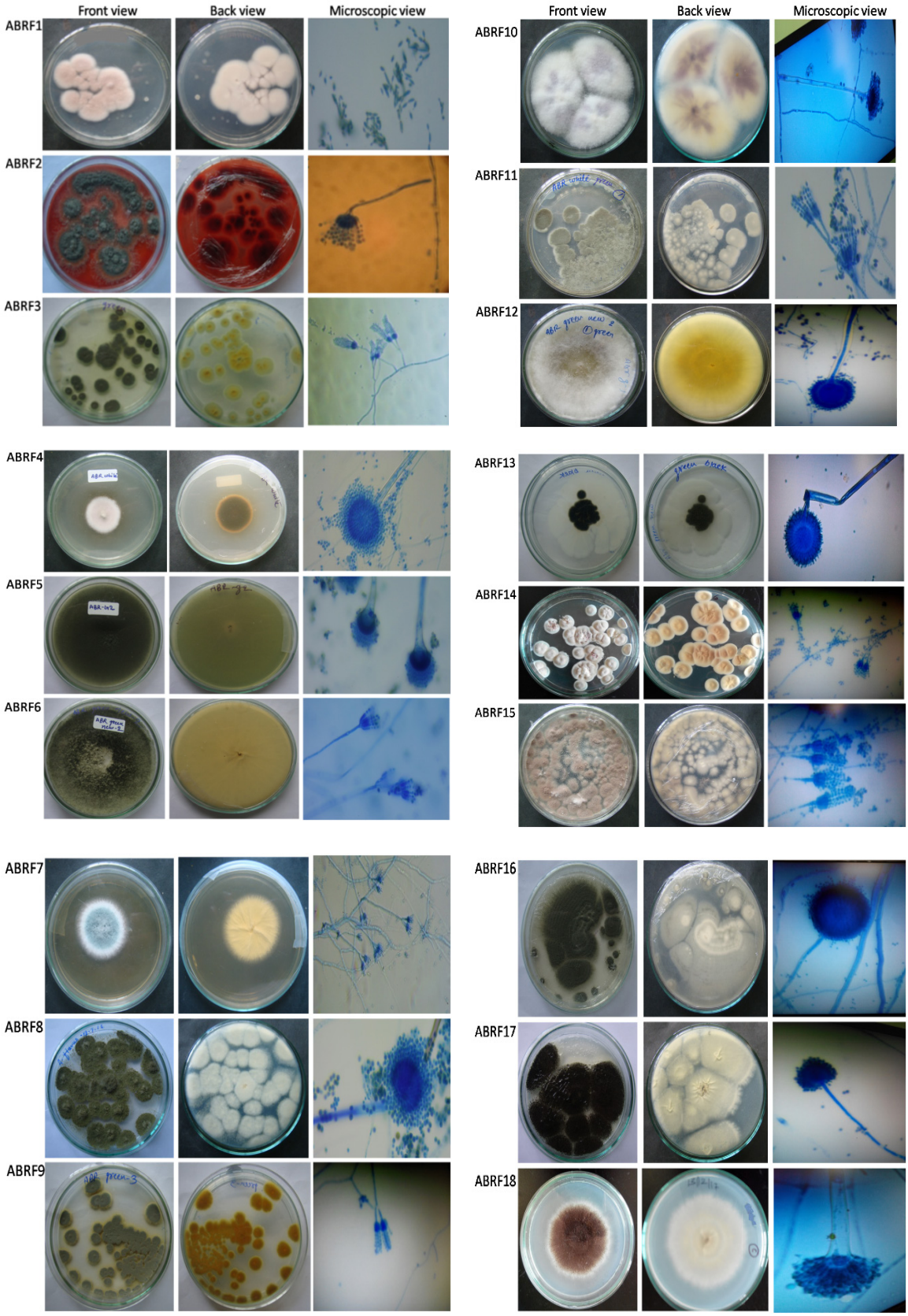

Fig. 1. Morphological and microscopic analysis of fungal strains from Achanakmar Biosphere Reserve (ABRF 1-18) .

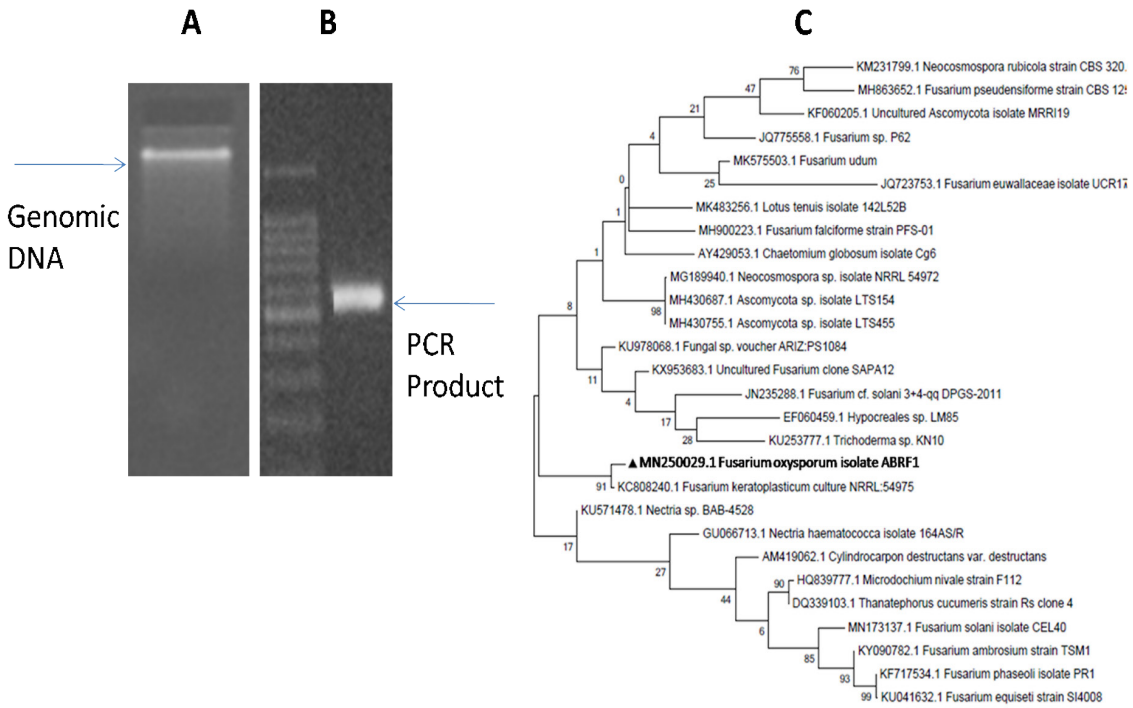

Fig. 2. The PCR products of isolated fungal strain and Phylogenetic tree of the fungal ITS region from ABRF1 (*Fusarium oxysporum*) [A: Isolation of Genomic DNA, B: PCR amplification of the ITS region from a fungal sample. The size of the PCR amplified product is ~ 554bp, C: Phylogenetic tree created using Mega 6 software].

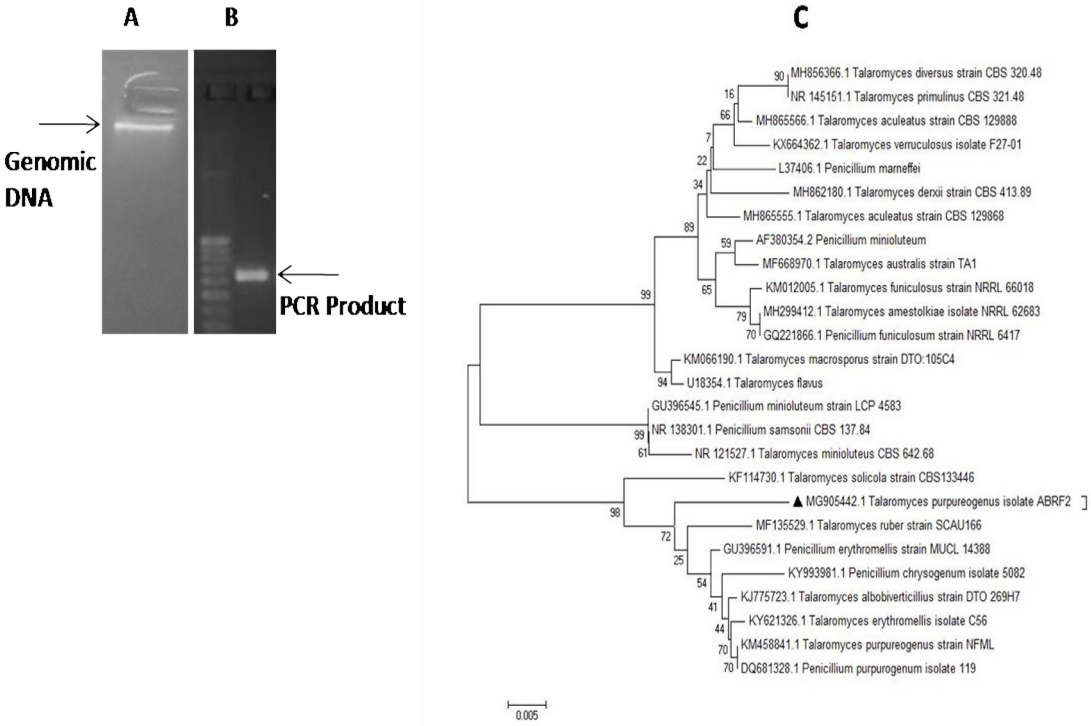

Fig. 3. The PCR products of isolated fungal strain and Phylogenetic tree of the fungal ITS region from ABRF2 (*Talaromyces purpureogenus*) [A: Isolation of Genomic DNA, B: PCR amplification of the ITS region from a fungal sample. The size of the PCR amplified product is ~ 422bp, C: Phylogenetic tree created using Mega 6 software].

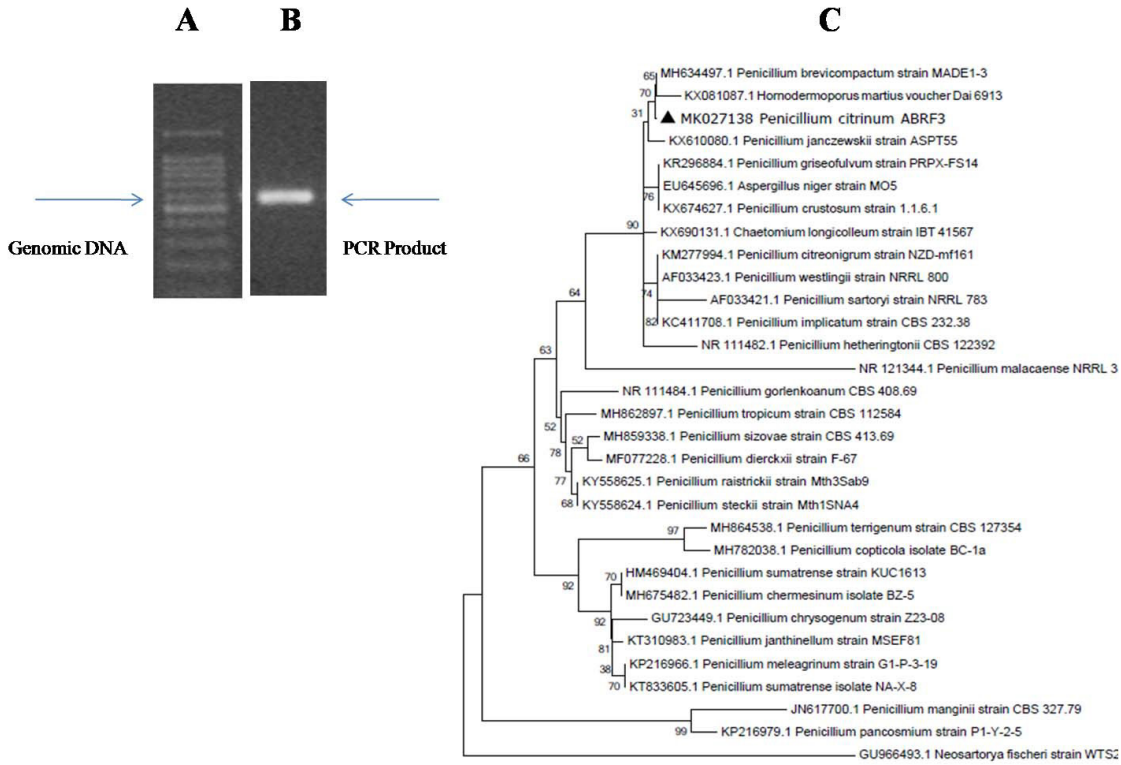

Fig. 4. The PCR products of isolated fungal strain and Phylogenetic tree of the fungal ITS region from ABRF3 (*Penicillium citrinum*) [A: Isolation of Genomic DNA, B: PCR amplification of the ITS region from a fungal sample. The size of the PCR amplified product is ~ 528bp, C: Phylogenetic tree created using Mega 6 software].

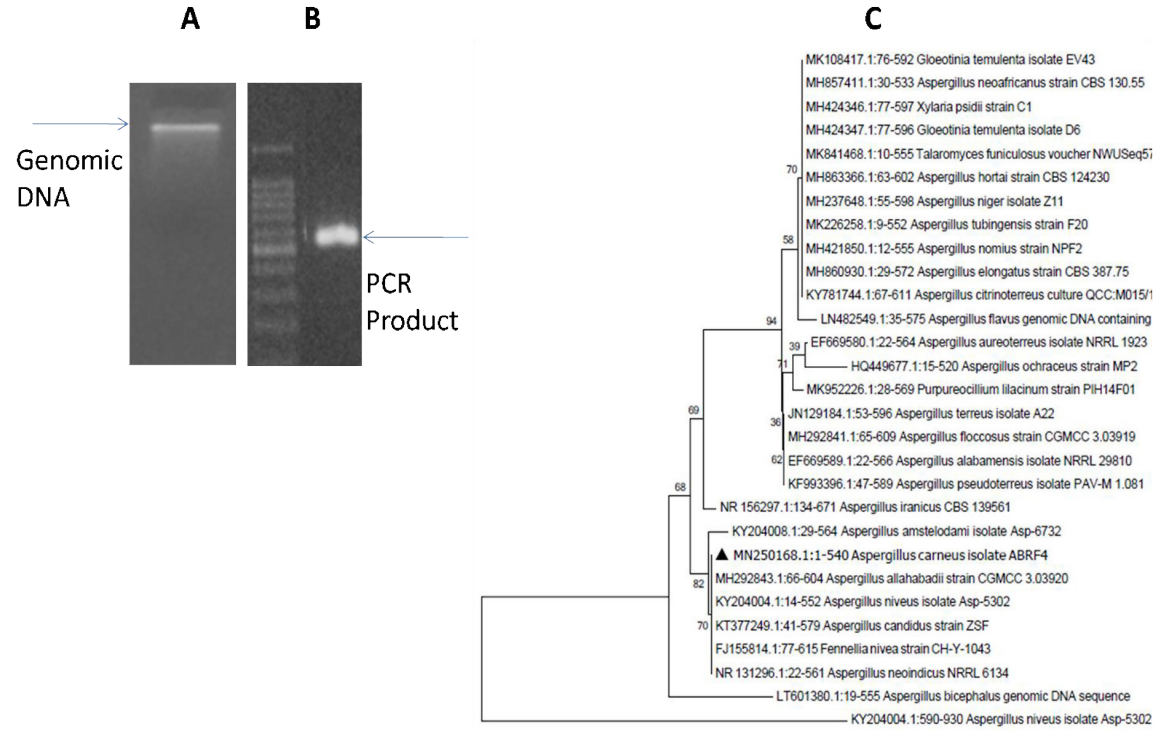

Fig. 5. The PCR products of isolated fungal strain and Phylogenetic tree of the fungal ITS region fromABRF4 (*Aspergillus carneus*) [A: Isolation of Genomic DNA, B: PCR amplification of the ITS region from a fungal sample. The size of the PCR amplified product is ~ 540bp, C: Phylogenetic tree created using Mega 6 software].

Fungi can produce natural compounds with varying antioxidant activity, which is overly complicated to evaluate with a single antioxidant assay. The antioxidant potential of the fungal fraction was determined by several methods including the 2,2-diphenyl 1-picrylhydrazyl (DPPH) radical scavenging assay, 2, 2'-azino-bis3-ethylbenzthiazoline-6-sulfonic (ABTS), Ferric reducing power (FRAP) and Phosphomolybdenum assay (PM). The fungal fractional with antioxidant property were further evaluated for antibacterial activity and antidiabetic potentiality (Zhai et al., 2016). Results indicated that fungal strains from the Achanakmar biosphere reserve are a potential therapeutic resource worthy of further *in vivo* study.

Screening of the optimum nutrient media

It was observed that Yeast extract sucrose broth nutrient medium was (YESB) optimum for all the isolates based on the maximum growth and development of fungus biomass and production of secondary metabolites at 26°C over 15 days compared with other nutrient media. In the preliminary screening, the YESB extract containing metabolites exhibited higher antioxidant and antimicrobial activity, compared with the extract of other media.

Potential reductones present in FRAP assay

In the oxidative metabolism, free radicals generated by toxic heavy metals cause cellular disruption. Antioxidants and natural secondary metabolites quench these free radicals and protect biomolecules from oxidative damage. Iron overload may lead to tissue damage through formation of toxic free radicals. (Siah et al., 2005). The release of hydroxyl radicals and hydroperoxyl radical from hydrogen peroxide in the Fenton reaction is catalysed by ferrous ion and ferric ions (Liochev & Fridovich, 2002). In this study, 18 fungal crude extracts were subjected to determine FRAP values and compared with standard L-ascorbic acid. FRAP assay measures the reduction of ferric ion during reaction with an antioxidant: strong antioxidant molecule has higher FRAP value (Rabeta & Faraniza, 2013). Our results demonstrate the presence of high amounts of potential reductones in YESB crude extract and significantly differ from other extracts. Thus, FRAP reaction is correlated to varying concentrations of the antioxidants and is reproducible (Fig.6).

The FRAP percentage scavenging values of strains ABEF1-ABEF 4 was higher among the 18 isolates (83%± 2.16, 76.91%± 2.46, 75% ± 0.33and 74%± 0.91, respectively at 1mg mL^{-1}) and comparable to positive control 88%± 0.31. In brief, the all fractions from the ABRF17 isolate had the least activity (22% ± 0.44) among other fractions (Fig.6).

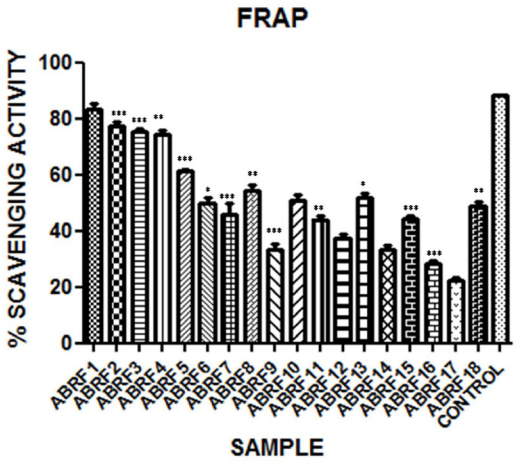

Fig. 6. Ferric ion-reducing antioxidant power (FRAP) of crude extract from isolated fungal strains (ABRF1-ABRF18) [Activity is represented as percentage activity, data exhibit the mean ± standard deviation (SD) of three independent experiments (n=3), statistically significant *P< 0.05, **P< 0.01, ***P< 0.001].

Synergistic effect found by DPPH scavenging assay

DPPH scavenging assays of crude extract of all 18 isolates gave results in the range of 17-78%. ABRF1 grown on YESB media crude extract exhibited maximum DPPH scavenging at 78%± 1.74 as compared with the L-ascorbic acid control. The results of our findings were like the DPPH scavenging activity shown by *T. purpureogenus* CFRM02 extract (Loggini et al., 1999; Pandit et al., 2018). The variation in the antioxidant activity in the different experimental protocols (Fig.7) may reflect diversity in functional groups across the molecular structures of active metabolites.

DPPH percentage scavenging values of the extract from strain ABEF2, ABRF3 and ABRF4 in YESB fraction was determined (70.50± 1.54, 71.88± 0.25 and 74.32± 0.93, respectively) to be lower than the positive control (81.96± 0.56) while ABRF7 strain had the lowest activity 18%.

Scavenging capacity observed by ABTS activity

Metabolic processes and irradiation of oxygen may lead to the generation of primary reactive oxygen species (ROS). These then interact with biomolecules to generate secondary ROS

(Loganayaki et al., 2013). Existing free radicals may harm some molecules and other cellular machinery. However, antioxidants protect cellular molecules (Re et al., 1999). We also observed that ABRF3 YESB extract with 79% scavenging ability was quite potent in quenching of cation-free radical for other extracts, while ABRF6, ABRF14 and ABRF16 have -18%, -24.5% and -11% activity, respectively, suggesting the presence of relatively hydrophobic reductones in other extracts (Fig.8).

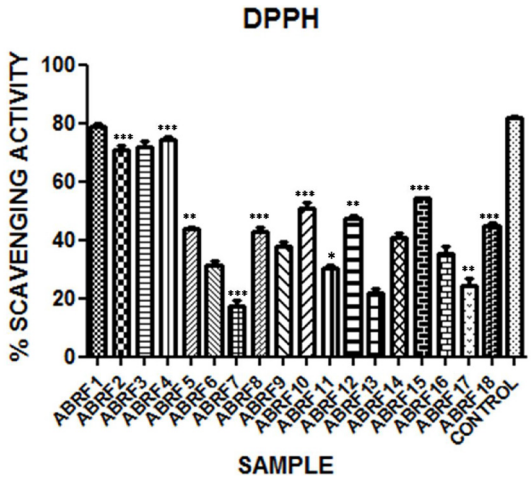

Fig. 7. DPPH scavenging assay of crude extract from isolated fungal strains (ABRF1-ABRF18) [The activity was represented as percent scavenging activity, data exhibit the mean ± standard deviation (SD) of three independent experiments (n= 3), statistically significant *P< 0.05, **P< 0.01, ***P< 0.001].

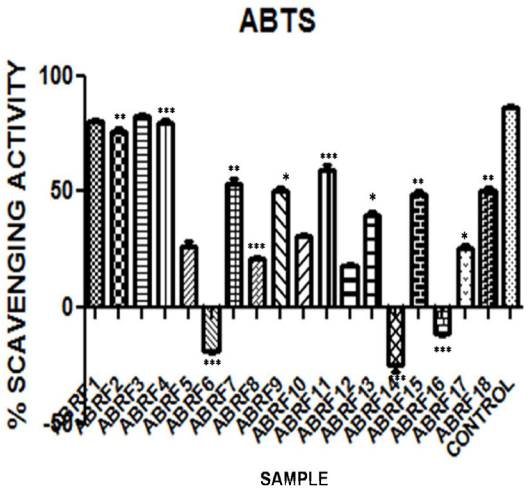

Fig. 8. ABTS scavenging activity of crude extract from isolated fungal strains (ABRF1-ABRF18) [Data exhibit the mean ± standard deviation (SD) of three independent experiments (n=3), statistically significant *P<0.05, **P<0.01, ***P<0.001].

$ABTS^{+}$ scavenging activity versus the positive reference L-ascorbic acid for ABRF1 ABRF2 and ABRF4 was 86.5, 80, 75.5 and 79, respectively.

Potent free radical scavenging activity by Phosphomolybdate assay

Phosphomolyb date assay is based on the development of a greenish-blue d complex at acidic pH due to conversion of Phosphomolybdate (VI) into Phosphomolybdate (V). The phosphomolybdate method is a common method for determining the reducing ability of fungal originated antioxidant molecules (Prieto et al., 1999). The highest percentage of scavenging activity was found in YESB extract of ABRF2, at 85% while the lowest observed was 16.5% against a control level of 89%. The percentage scavenging activity 82%, 85%and 79%, respectively for ABRF1 ABRF3 and ABRF4 YESB fungal crude extracts was significant and compared well with L-ascorbic acid. Therefore, data suggests YESB extracts have effective free radical scavenging activity and therapeutic potential (Fig.9).

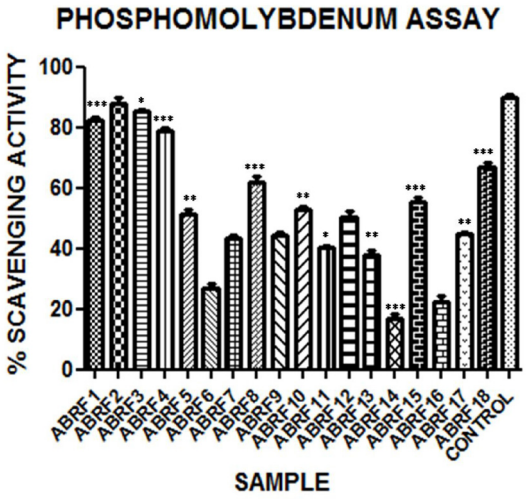

Fig. 9. Phosphomolybdenum assay scavenging capacity of crude extract from isolated fungal strains (ABRF1-ABRF18) [Data exhibit the mean ± standard deviation (SD) of three independent experiments (n= 3), statistically significant *P< 0.05, **P< 0.01, ***P< 0.001].

By contrast, phosphomolybdenum assay is a method for determining free radical scavenging capacity in hydrophilic medium (Saghaie et al., 2013). We observed that control L-ascorbic acid had maximum scavenging activity with 89%. Thus, from this data, it could be concluded that YESB extract of ABRF1-4 contains good free radical scavenging components.

In vitro antimicrobial activity of potent fungus

The column purified fractions containing secondary metabolites from YESB extract were also tested for antimicrobial activity based on inhibition of growth of *B. Cerculans (*MTCC-7906), *B. Subtilis (*MTCC-441), *E. coli (*MTCC-739), *S. Aureus (*MTCC-96) and *Ralstonia eutropha (*MTCC-2487), (Sahu et al., 2020) at a maximum concentration of 100µg/mL for qualitative assay (Fig. 10 and Table 2). The results were compared to the standard antibiotic streptomycin (Sarker et al., 2007). The Acetonitrile fraction of ABRF 1, Toluene and Ethyl acetate fraction of ABRF 2, Ethyl acetate fraction of ABRF3 and Toluene, Methanol and Acetonitrile fraction of ABRF4 showed antibacterial activity with suppression of the expansion of various pathogenic bacteria as seen in Fig. 10 and Table 2.

Antidiabetic activities of the fungal secondary metabolites

Breakdown of a complex carbohydrate like starch to simple sugars like glucose is accomplished by α-amylase. Thus, the suppression of this enzyme can slow down carbohydrate degradation and decrease the rate of glucose assimilation (Asgar, 2013). Column fractionation of extracts for four rhizospheric fungal species was investigated for inhibition of α-amylase activity at 1mg/mL concentration. Percent inhibition of 96.31± 0.14 was exhibited by positive control. The entire fraction showed significant inhibition activity of α-amylase (Table 3). The ethyl acetate fraction of all three fungal species showed comparatively high inhibition. The ethyl acetate fraction of ABRF2 showed the highest inhibition percent of 93.28± 0.12 at 1mg/mL concentration and at a corresponding concentration ABRF1, ABRF3 and ABRF4 inhibition was 90.66± 0.08, 92.61± 0.18 and 91.34± 0.2, respectively. Inhibition percent range of all fractions ranged from 63.89± 0.21 to 93.28± 0.12. Hydroxyl group methylation enhances the affinity of secondary metabolites for α-amylase and an increase in the number of hydrogen atom donor/acceptor in metabolites might have decreased the affinity for α-amylase.

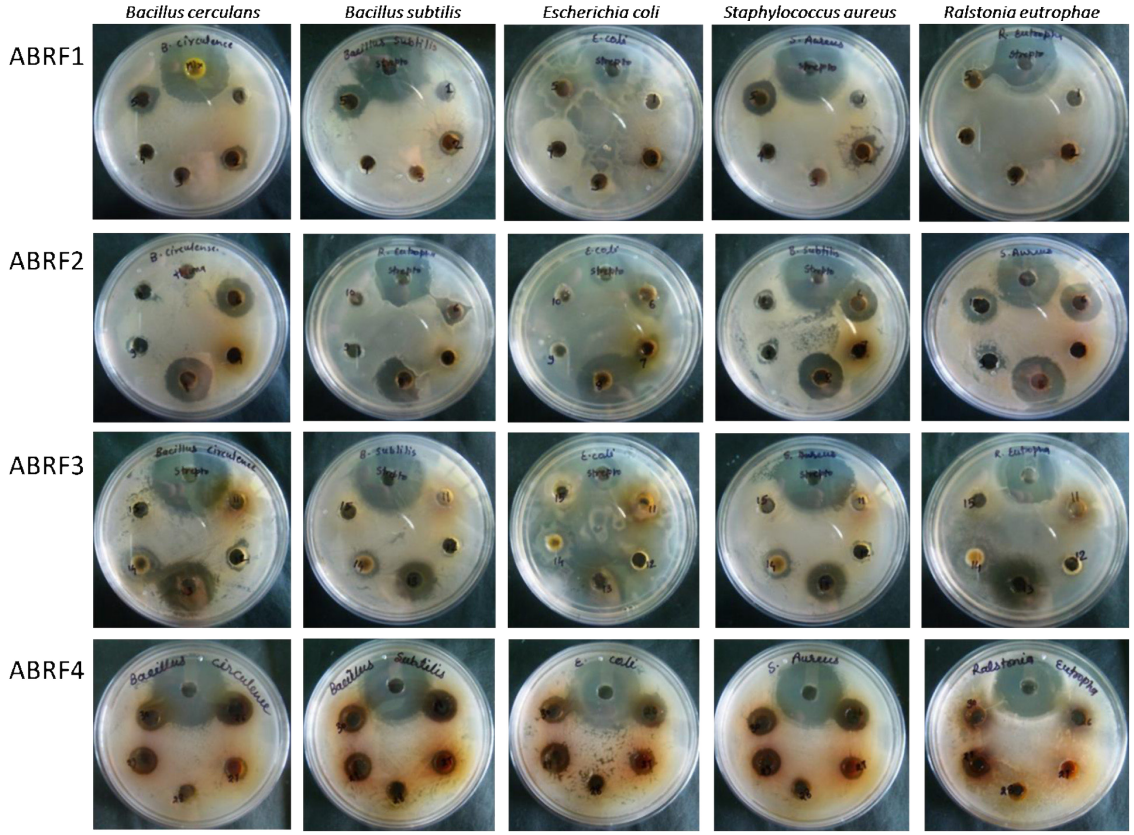

Fig. 10. Antibacterial activity with a variable inhibition zone of relatively purified extract of isolates ABRF1-4 against pathogenic bacteria strains [A crude extract of ABRF1-4 purified through column chromatography, column chromatography solvent fractions. Clockwise wells in each plate containing nonpolar to polar solvent extracts viz. Toluene, Chloroform, Ethyl Acetate, Methanol, and Acetonitrile against *Bacillus circulans*, *Bacillus subtilis, E. coli, S. aureus*, and *R. eutropha*].

TABLE 2. Antibacterial activity of fungal column fraction.

Fungal strain	Bacterial pathogen	Zone of inhibition [a] Column fraction of Ethanolic extract of the strains					Positive control Streptomycin
		Toluene	Choloform	E. Acetate	Methanol	Acetonitryle	
ABRF1 *(Fusarium oxysporum)*	*Bacillus circulans* (Gram +ve)	Nd	07.34 ± 1.3	Nd	Nd	11.16 ± 1.1	24.28 ±1.25
	Bacillus subtilis (Gram +ve)	Nd	06.22± 0.9	Nd	Nd	13.81± 1.8	24.21 ± 0.93
	Escherichia. coli (Gram –ve)	Nd	Nd	Nd	Nd	Nd	23.85 ± 1.25
	Staphylococcus aureus (gram +ve)	Nd	07.18 ± 1.3	Nd	Nd	14.16 ± 0.21	24.62 ± 1.05
	Ralstonia eutropha (Gram –ve)	Nd	Nd	Nd	Nd	Nd	25.33 ± 1.7
ABRF2 *(Talaromyces purpureogenus)*	*Bacillus circulans* (Gram +ve)	14.12 ±1	Nd	18.11 ± 1.4	Nd	Nd	24.28 ±1.25
	Bacillus subtilis (Gram +ve)	12.66 ± 0.57	Nd	17.66 ± 1.2	Nd	Nd	24.21 ± 0.93
	Escherichia. coli (Gram –ve)	12.15 ± 0.92	Nd	18.31 ± 0.82	Nd	Nd	23.85 ± 1.25
	Staphylococcus aureus (Gram +ve)	14.76 ± 1.4	Nd	18.36 ± 1.6	Nd	10 ±	24.62 ± 1.05
	Ralstonia eutropha (Gram –ve)	14.42 ±0.9	Nd	18.44 ± 1.9	Nd	12 ±	25.33 ± 1.7
ABRF3 *(Penicilliumcitrinum)*	*Bacillus circulans* (Gram +ve)	Nd	10	17.4 ± 1.25	13.1 ± 1.5	15.95 ± 1.03	24.28 ±1.25
	Bacillus subtilis (Gram +ve)	Nd	Nd	15.7 ± 1.15	12.44 ± 1.8	Nd	24.21 ± 0.93
	Escherichia. coli (Gram –ve)	Nd	Nd	Nd	Nd	Nd	23.85 ± 1.25
	Staphylococcus aureus (Gram +ve)	Nd	Nd	15 ± 1.3	12.14 ± 0.89	Nd	24.62 ± 1.05
	Ralstonia eutropha (Gram –ve)	Nd	Nd	17.1 ± 0.9	Nd	Nd	25.33 ± 1.7
ABRF4 *(Aspergillus carneus*)	*Bacillus circulans* (Gram +ve)	13.12 ±1.7	11.66 ± 1.2	Nd	15.55 ±2.1	15.41 ± 1.2	24.28 ±1.25
	Bacillus subtilis (Gram +ve)	13.43 ±.1.1	11.31 ± 0.9	Nd	15.16 ± 0.9	15.32 ± 0.78	24.21 ± 0.93
	Escherichia. coli (Gram –ve)	13.6 ± 0.9	12.1 ±0.48	Nd	15.11 ± 0.23	15.1 ± 1.1	23.85 ± 1.25
	Staphylococcus aureus (Gram +ve)	13.11 ± 1.3	11.38 ±0.98	Nd	15.16 ± 0.32	13.43 ± 1.65	24.62 ± 1.05
	Ralstonia eutropha (Gram –ve)	Nd	Nd	Nd	15.44± 1.5	13.90 ±1.09	25.33 ± 1.7

- Nd-not detected
- The superscript letters are significantly different (P< 0.05).[a]Inhibition zone excluding good size.

TABLE 3. Percent inhibition of the test sample in an alpha-amylase inhibition assay.

Solvents	% inhibition			
	ABRF1	ABRF2	ABRF3	ABRF4
Toluene	68.33± 0.11***	72.12± 0.91***	63.89± 0.21***	69.25± 0.32***
Choloroform	72.78± 0.4 **	69.98± 0.34 ***	68.23± 0.55**	70.91± 0.6 ***
Ethyl acetate	90.66 ± 0.08*	93.28 ± 0.12*	92.61 ± 0.18*	91.34 ± 0.2*
Methanol	83.61± 0.31***	84.87± 0.21***	86.57± 0.32***	81.10± 0.41 **
Acetonitrile	78.22± 0.06***	75.32± 0.22**	72.44± 0.25***	73.55± 0.22*
Water	70.88± 0.19**	68.52± 0.65***	71.58± 0.31*	65.11± 0.87***
Metformin	95.78± 0.09			
Acarbose	96.31± 0.14			

Data exhibit the mean ± standard deviation (SD) of three independent experiments (n=3), statistically significant *P<0.05, **P<0.01, ***P< 0.001.

Conclusion

Fungal crude extract from ABRF1- ABRF4 identified as *Fusarium oxysporum, Talaromyces purpureogenus, Penicillium citrinum* and *Aspergillus carneus* respectively exhibited modest antioxidant activity with significant antibacterial activity. These column fractions of the particular solvent fraction may have specific metabolites responsible for antioxidant and antimicrobial activity. Potential purified extract further needs to be tested for other therapeutic activities like anticancer and antiaging potential via *in vitro, in vivo* and *in silico* approaches.

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Ethical Statement

The above-presented work does not include any studies performed with human participants or animals. The authors affirmed no conflict of interest.

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