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Introduction

Fungi are regarded as the second-largest group of eukaryotic organisms on the planet earth, with an approximate estimation of 1.5 to 5.1 million species (Raja et al., 2017b). They are morphologically, metabolically, ecologically and phylogenetically diverse. They are notorious living organisms that produce various bioactive molecules, which makes them valuable for researchers to pursue the discovery of novel chemical diversity related to industrial, pharmaceutical, agricultural and biotechnological applications (Raja et al., 2017b). Despite their importance in basic (taxonomy, ecology) and applied (bioprospecting, genomics) research, their identification at the species level remains a paramount task for researchers. Identification of fungal diversity is based on morphology and essential for authentication, but sometimes it can be misleading due to several factors including cryptic speciation, hybridization and convergent evolution. As a consequence, DNA-based approaches over morphology-based approaches have emerged for discriminating between the species and among several phyla (Raja et al., 2017a).

A new era of the molecular identification of fungal species initiated in the last decade, which includes DNA barcoding and DNA taxonomy that make the use of the DNA sequences generated from the small subunit (nrSSU-18S), large subunit (nrLSU-26S or 28S) and the 5.8S rRNA gene (Internal Transcribed Spacer region, ITS1 and ITS2). DNA barcoding utilizes a short-standardized region (between 400 and 800 base pairs) to discriminate between species

together with the D1/D2 domain of the Large Subunit (LSU, 26S) nuclear DNA (nrDNA) sequences. The principle of DNA barcoding is that interspecific variation would surpass intraspecific variation and this barcode gap can be exploited for the species-level identification (Kress and Erickson, 2012; Vu et al., 2019). The ITS, nrDNA region has been evaluated as a universal barcode for fungi, which covered seventeen fungal lineages, containing the Basidiomycota and Ascomycota, which are the principal phyla within the kingdom fungi (Raja et al., 2017a). Alternatively, DNA taxonomy identifies an unknown species using an evolutionary relationship with other homologous sequences by employing a phylogenetic approach. The evidence of DNA taxonomy is that genetic variation inherited among sequences of different individuals can be exploited for species-level identification (Raja et al., 2017b).

Fungi produce a variety of secondary metabolites known for their medicinal properties that can be effectively and efficiently used for drug discovery. In this regard, accurate species identification is not only a prerequisite but is also an important parameter, which can unlock the important facts regarding a species and its feasible biochemical properties for the welfare of mankind. This would lead to insights into better screening programs for the development of therapeutic drugs. The secondary metabolites produced by fungi are bioactive and low molecular weight compounds that are formed in response to stress. These secondary metabolites help fungi for their survival by signalling and defence but are generally not requisite by the fungi for their normal growth and reproduction. The most important bioactive metabolite secreted by fungi are polysaccharides belonging to the 1,3-β-glucans family which have antitumor activities achieved by blocking and enhancing cellular immunity pathways. The other bioactive metabolites secreted by fungi having different pharmacological activities include steroids, lectins, lactones, quinones, terpenoids, alkaloids, phenols, coumarins, xanthones, flavonoids, antibiotics and metal-chelating agents (Chaturvedi et al., 2018). These

metabolites possess activities like anticancer, antibacterial, antifungal, anti-parasitic, antioxidants, insecticidal etc (Chaturvedi et al., 2018; Ejaz et al., 2020).

Gujarat state is well known for its varying climatic conditions starting from the moist deciduous forest (south Gujarat), dry deciduous to scrub forest in central and north Gujarat while Rann of Kuchh is known for its typical desert conditions. Despite all such climatic conditions, the fungal diversity of the state is poorly investigated till 2015 (Rajput et al., 2015). However, no special efforts have been made in the state for the documentation of the fungal diversity. Previous studies from our laboratory have collected and identified several fungal species as a part of the Gujarat Biodiversity Board activity for the documentation of fungal biodiversity in Gujarat state (Rajput et al., 2015). During this period several interesting and important fungal taxa have been collected from different forest regions of the state on which no in-depth information is available. Hence, there is an urgent need to identify and explore such important and uncommon fungal species for their therapeutic evaluation.

With this background, the research work was undertaken with the following objectives:

Objectives

1. Collection, isolation, purification and identification of different species of fungi using classical and molecular methods
2. Establishment of DNA barcodes for the identified fungi and utilization of bioinformatics strategies for their taxonomic studies
3. Screening of various fungi to examine their therapeutic applications
4. Characterization of the biomolecule of interest for their therapeutic applications.

Materials and Methodology

Fresh fruiting bodies of various species of fungi growing wild in different forests and on open land were collected in sterile polyethylene bags for their further studies. Field photographs were taken using Canon 1200D. Morpho-taxonomical characters were recorded for both fresh and dried samples. Fruiting bodies were inoculated on a PDA medium for mycelium development to obtain pure cultures. Micro-morphological characters of the mycelium and fruiting bodies were observed using a stereo zoom microscope (Leica, Germany). Distinguishing morphological and microscopical structures were noted using a trinocular research microscope (Leica DM 2000, Germany). Mycelial characters, as well as distinctive features of fruiting bodies were studied and compared with available literature like Martin, 1970; Sen, 1973; Sharma, 1995; Thind and Rattan, 1971; Thind and Waraitch, 1976, 1969; Verma et al., 2008.

Total genomic DNA of the collected fungal fruiting bodies was extracted from the sterilized fruiting bodies as well as pure established pure cultures using Plant/Fungi DNA isolation kit (Sigma Cat# E5038). Subsequently, PCR reactions were carried out using Veriti® thermal cycler (Applied BioSystems) under optimized conditions. The amplification was carried out using primers ITS 1 and ITS 4. The PCR products were visualized on 2% agarose gel and amplified PCR products were purified using the Purelink^{TM} Quick PCR Purification kit (Cat# K310001). Purified PCR products were sent for sequencing to Eurofins Genomics India Pvt. Ltd., Bangalore. The obtained nucleotide sequences were compared with sequences available in the National Centre for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST). All the generated sequences were submitted to GenBank, NCBI. Barcode of Life Data system (BOLD) was used to generate DNA barcodes for nucleotide sequences.

The phylogenetic tree was generated by using separate and combined ITS, LSU, mtSSU, ATP6 and EF 1-α sequence data of the different fungi collected in the present study. Sequence data of other genera and species of closely related fungi were obtained from the NCBI GenBank database. Sequence ambiguities were edited by aligning the forward and reverse sequence in Bioedit 7.2.5. The nucleotide sequences were aligned with Clustal X 2.0 and ClustalW embedded in MEGA X (Kumar et al., 2018)(Kumar et al., 2018). Separate and combined molecular phylogenetic analyses were performed using the Maximum likelihood (ML) method. The concatenated dataset was analysed in Partition Finder to select the best partitioning scheme. The same partition scheme was also selected for ML analyses. ML analyses were employed to infer the phylogenetic relationships in RaxML. An ML analysis was run for 1000 bootstrap replicates under the GTR + I model to assess clade support.

Powders (20g) of each of the selected fungi were soaked in 85% ethanol overnight at 30°C under vigorous shaking conditions (110 rpm). To obtain the dried residue, the ethanol phase was filtered with Whatman No. 1 filter paper and then concentrated using a rotary evaporator. The obtained residues were further dissolved in 10% DMSO and used for the metabolic profiling of bioactive metabolites and the evaluation of their therapeutic effects.

Ethanolic extracts of fruiting bodies were further evaluated for their antioxidant and anticancer potential. Antioxidant activity was measured against 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydrogen peroxide (H_2O_2) free radicals about radical scavenging capability by using ascorbic acid as a positive control. The anticancer potential was performed against human breast adenocarcinoma, MDA-MB-231; human lung cancer cells, A549 and human colorectal cancer cells, HCT116. Cytotoxicity of all the fungal crude extracts against selected cell lines was determined by MTT assay. Profiling of bioactive compounds from the crude extract of selected fungi was carried out via HR-LC/MS analysis (IIT Bombay). Compounds were

identified using their mass spectra and their unique mass fragmentation patterns. Identified potent compounds were further evaluated for antiviral potential using *in silico* analysis.

Results

Fruiting bodies of some unique fungi were collected from different forest regions of Gujarat State. These include Vansda National Park, Vansda; Zand Hanuman, Panchmahal; Wilson hills, Dharampur; Community Science Centre, Vadodara; Bhavnath, Junagadh; Sagai, Narmada; Panjraghat, Narmada; Panas, Valsad; Kheralu, Mehsana; Ratanmahal, Dahod; Jambughoda, Narmada and Gir Forest National Park, Junagadh. The selected fungi were identified as *Clathrus delicatus*, *Trichaleurina javanica*, *Itajahya galericulata*, *Cyathus stercoreus*, *Geastrum triplex*, *Geastrum saccatum*, *Geastrum rufescens*, *Disciseda candida*, *Scleroderma bovista*, *Pisolithus albus*, *Pisolithus tinctorius*, *Dictyophora indusiata* and *Dictyophora multicolor*. The nucleotide sequences of these identified fungi were submitted to the NCBI database using the Bankit nucleotide sequence submission tool with accession numbers, MF506820, MF476196, MH168327, KY706156, MF506821, MF506817, MF506818, MN874209, MK685146, MF510372, MF510373, MF510374 and MF510375, respectively. The nucleotide sequences are also submitted to the BOLD data system to generate DNA barcodes with BOLD ID, KSRF-0015, KSRF-0019, KSRF-0014, KSRF-0002, KSRF-0010, KSRF-0011, KSRF-0013, KSRF-0023, KSRF-0012, KSRF-0007, KSRF-0008, KSRF-0005 and KSRF-0004, respectively. The maximum likelihood cladogram generated for these fungal sequences further confirms the DNA taxonomy of identified fungi.

Itajahya galericulata

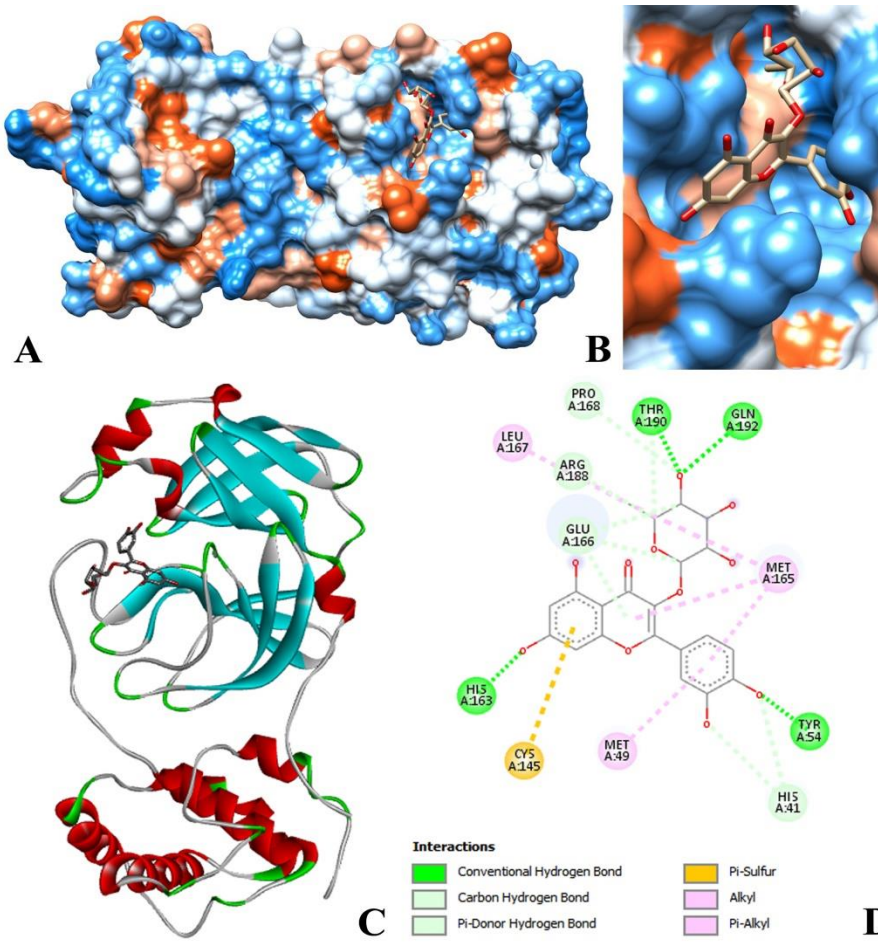
Dictyophora multicolor

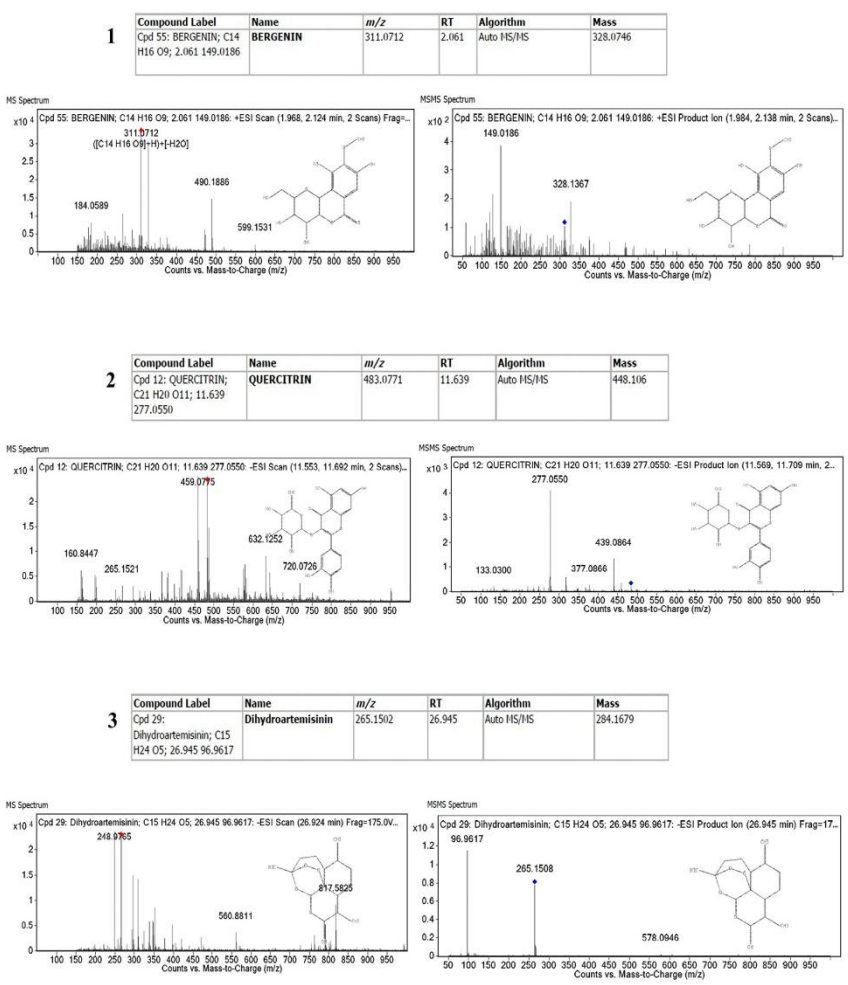
Out of the several fungal strains screened, seven species *viz. P. albus, P. tinctorius, G. triplex, G. saccatum, D. indusiata, I. galariculata* and *C. stercoreus* were used to check their therapeutic potential. The ethanolic extracts of all the fungi in a concentration range of 50-200 µg/ml were tested for antioxidant activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydrogen peroxide (H_2O_2). The results revealed that ethanolic extracts of all the selected fungi possess a concentration-dependent scavenging effect. The scavenging potentials against DPPH were *D. indusiata* > *I. galariculata* > *P. tinctorius* > *P. albus* > *G.triplex* > *G. saccatum* > *C. stercoreus*. The scavenging potentials against H_2O_2 were *D. indusiata* > *I. galariculata* > *P. tinctorius* > *P. albus* > *G.triplex* > *G. saccatum* > *C. stercoreus*. The anticancer potentials of crude extract of seven selected species of fungi that are medicinally important were evaluated against three different cancer cell lines viz MDA-MB-231, HCT-116 and A-549 by MTT assay. Significant inhibition of colorectal cancer cells HCT-116 was found followed by lung cancer cells A-549 and breast cancer cells MDA-MB-231. Dose-dependent anticancer potentials of fungal extracts were observed i.e., an increase in concentration (50, 75, 100, 150 and 200 µg/ml), escalating the potentiality of selected fungus. The crude extract of *D. indusiata* showed the highest inhibition activity in all the three tested cell lines.

Moreover, crude extracts of five selected fungi viz. *P. albus, G. triplex, D. indusiata, I. galariculata* and *C. stercoreus* were used for profiling their bioactive compounds using HR-LC/MS. With the detailed mass spectrum data, absorbance spectra and retention times were compared with the available literature and found that all the fungi possess different bioactive compounds which belong to various classes and have different therapeutic potential. Based on medicinal uses and binding energy, active sites were covered after molecular docking analysis; three potent compounds (bergenin, quercitrin and dihydroartemisinin) were selected for in-depth *in-silico* analysis as potential inhibitors against SARS-CoV-2 M^{pro}. The 6LU7, a protein

crystallographic structure of SARS-CoV-2 M^{pro}, was docked with bergenin, quercitrin and dihydroartemisinin using Autodock 4.2 and the binding energies obtained were -7.86, -10.29 and -7.20 kcal/mol, respectively. The docking analysis, ADMET predictions and medicinal properties indicated that bergenin, quercitrin and dihydroartemisinin are the most significant compounds as potential inhibitors of SARS-CoV-2 M^{pro}, which could be explored further.

In conclusion, the present study was successful in reporting the occurrence, distribution, morpho-taxonomic and molecular identification of some unique fungi from the Gujarat state, and in evaluating their therapeutic properties like antioxidant, anticancer and antiviral potential.





Docking analysis visualization of SARS-CoV-2 M pro binding with Quercitrin

HR-LC/MS analysis

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