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Original Article

ISOLATION AND CHARACTERIZATION OF ENDOPHYTIC FUNGI FROM MEDICINAL PLANT *CRESCENTIA CUJETE* L. AND THEIR ANTIBACTERIAL, ANTIOXIDANT AND ANTICANCER PROPERTIES

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ABSTRACT

Objective: The present study was aimed to isolate endophytic fungi from ethnomedically important plant *Crescentia cujete* L. in view to screen their bioactive principles towards different pharmacological applications.

Methods: A total of four morphologically distinct endophytic fungi were isolated and identified via analyzing their ITS region of 5.8s rRNA and sequences were submitted in Genbank. The recovered four isolates were further cultivated in Czapek-Dox broth, from this extra cellular bioactive metabolites has been extracted using ethyl acetate for their different biological activities. DPPH assay was performed to measure the free-radical scavenging activity of extracts and antibacterial property was assessed through disc diffusion method. On the other hand, the cytotoxic potential of fungal extracts against hepatocellular carcinoma cell lines (HepG2) was studied by MTT assay, AO-EB and Hoechest staining methods under *in vitro* condition. Most importantly, the active compounds present in the solvent extracts were identified through GC-MS analysis.

Results: The fungal extracts showed a strong growth inhibitory effect against bacterial human pathogens and excellent free radical scavenging activity. It also exhibits excellent antiproliferative effect against hepatocellular carcinoma cells, further it was observed that the cell death was primarily mediated by apoptosis. The active compounds present in the extracts were identified through GC-MS analysis, which depicts the presence of aspirin and diethyl phthalate as the major constituents.

Conclusion: Overall, this study strongly suggests that extracts of isolated endophytic fungi from *C. cujete* L. can be developed as a lead/drug molecule in view of pharmaceutical context.

Keywords: *Crescentia cujete* L., Endophytic fungi, GC-MS analysis, Antibacterial activity, Scavenging activity, Apoptosis.

INTRODUCTION

The wide span of natural bioactive compounds derived especially from the plant-associated microbes has been largely unexplored [1]. Fungal endophytes protect their hosts from contagious agents and withstand at adverse conditions by discharging active metabolites [2]. These creatures were drawing a great attention after the discovery of fungi *Taxus brevifolia*, producing the anti-cancer drug taxol [3]. It was assumed that medicinal plants and their fungal endophytic communities produce similar therapeutic products. Medicinal plants are known to harbor endophytic fungi that are believed to be associated with the production of pharmaceutical products [4]. Endophytic organisms that exist in the tissues of living plants are potential resources of novel natural products for exploitation in pharmaceutical and agricultural industries [5].

Moreover, consumption of fungal endophytes for the production of beneficial active metabolites has been on the rise [6]. While microbial production of drugs, especially from endophytic fungi will be an immediate interest of pharmaceutical importance than from plants. Therefore, it is necessary to investigate the fungal endophytic community exist in medicinal plants. *C. cujete* L. was commonly known as calabash tree which is an evergreen tree native to America, India and the rest of the sub-continents [7].

This tree possesses stupendous biological activities such as expectorant, antitussive, laxative, ulcers, wound healing, anti-viral, cancer and stomach disorders. Traditionally, the leaves, fruits of *C. cujete* L. practiced as a medicine in ancient times in Indian and Vietnamese folk medicine [8]. But to our knowledge, there is no report on endophytic fungus isolation from this particular plant. Hence, the present study was aimed to isolate fungal endophytes from the leaves of *C. cujete* L. and to explore their bioactive potentials like antioxidant, antibacterial and anticancer activities towards biomedical applications.

MATERIALS AND METHODS

Isolation and solvent extraction of Endophytic fungi

Healthy leaf samples of *C. cujete* L. were collected from Bharathidasan University campus. Isolation of endophytic fungi from leaf segments was carried out as per the method of Petrini [9]. Briefly, the leaf samples were initially surface sterilized using sodium hypochlorite to remove microbial contaminates. After sterilization and inoculation process, mycelium growth was observed on the fifteenth day from the cut end of sterilized leaf segments assumed as an indefinite. Then, each fungal culture (CC1, CC2, CC3 and CC4) was checked for purity and transferred to fresh PDA plates. The purified fungal isolates were transferred separately and maintained in a PDA slant at 4 °C. Fungal genomic DNA was extracted by the CTAB method and DNA fragments were separated by electrophoresis on a 0.8% agarose gel [10]. For PCR amplification, 1 µl of diluted genomic DNA, Primers (ITS-1 and ITS-4) and Ampliqon (Taq polymerase) were used to make the final reaction mixture. After the reaction, PCR products were purified using QIAGEN–QIA quick PCR purification Kit and sequenced (Macrogen, Korea). The completed ITS sequences were aligned and their closest relative sequences were ascertained using BLAST search and multiple sequence alignment was done using CLUSTAL X program. Phylogenetic dendogram was constructed by the neighbor joining method and tree topologies were evaluated through bootstrap analysis of 1,000 data sets using MEGA 5.2 and submitted to Gen Bank database.

Fermentation and extraction

Isolated fungal endophytes were inoculated into 1000 ml Erlenmeyer flask containing Czapek-Dox broth. After 15 d growth under stationary condition, the fungal culture was filtered to remove mycelium. Subsequently, the filtered broth was extracted twice with an equal volume of ethyl acetate solvent [11]. From this the organic

phase was evaporated to dryness under reduced pressure at 35 °C using the rotary vacuum evaporator and weighed to constitute a fungal crude extracts. GC-MS was employed to analyze the active ingredients present in the final extract.

Preparation of inoculums

Bacterial inoculums were prepared from 24 h. old pure culture grown on nutrient agar for bacteria. Bacterial colonies were pre-cultured in nutrient broth medium and kept overnight, then centrifuged at 10, 000 rpm for 5 min. The pellet was resuspended in sterile distilled water and cell turbidity was assessed spectroscopically along with 0.5 McFarland standards (approximately 1.5×10^8 CFU/ml). Then the inoculums were used for the antibacterial assay [12].

Antibacterial activity

Antibacterial activity of fungal solvent extracts was determined using a modified Kirby Bauer disc diffusion method [13]. For that, the fungal solvent extracts were dissolved in a ratio of 1 mg/ml using DMSO and tested against Gram negative organisms such as *Salmonella typhi* (ATCC-51812), *Shigella flexneri* (MTCC 1457), *Enterococcus faecalis* (ATCC-29212), *Klebsiella pneumoniae* (ATCC-432), *Pseudomonas aeurginosa* (ATCC-27853), *Escherichia coli* (ATCC-25922) and Gram positive bacterial strains of *Bacillus subtilis* (ATCC-441), *Staphylococcus aureus* (ATCC-25923). The bacterial cultures (inoculum size 10^5 CFU/ml) were plated in Muller-Hinton agar media. The solvent extracts were loaded to the sterilized sterile 6 mm discs (Whatman No 3 discs), allowed to dry and then the impregnated discs with 25 µl/disc placed on the surface of inoculated medium. The plates were incubated at 37 °C for 24 h. The diameters of the inhibition zones were measured in mm. All the assays were done in triplicate and the results were given in mean±SD. DMSO and chloramphenicol (30 µg/ml) was used as a negative and positive control respectively.

DPPH scavenging assay

Free radical scavenging effect of isolated fungal extracts was determined by the DPPH method as described by Brand-Williams *et al.*, [14] with minor modifications. Appropriate dilutions of the crude extracts (20 µg/ml to 100 µg/ml) were mixed with, 1 mL of 0.135 mM methanolic solution of DPPH radical. Absorbance was measured at 517 nm after 30 min of incubation at dark conditions. BHT was used as the reference standard and the inhibition percentage was calculated using the following formula:

% of inhibition= (Abs control-Abs sample/Abs control) x 100

Where Abs of control=Mean OD of untreated cells, Abs of Sample =Mean OD of treated cells

***In vitro* anticancer activities**

Cell lines

Human liver cancer cell line (HepG2) was obtained from the National Center for Cell Science (NCCS), Pune, India. The cells were maintained in DMEM medium supplemented with 10% FBS (Sigma-Aldrich, St. Louis, Mo, USA), and with 100 U/ml penicillin and 100 µg/ml streptomycin as antibiotics (Himedia, Mumbai, India) in a humidified atmosphere of 5% CO_2 and 95% air in a CO_2 incubator (Heraeus, Hanau, Germany).

MTT assay

The cytotoxicity of fungal extracts was measured using MTT assay (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) [15]. The (HepG2) human hepato carcinoma cells were seeded in 96-well tissue culture plates at a 1.5 x 104 cells/well allowed to attach for 24 h at 37 °C h in 5% CO_2 humid atmosphere and treated with different concentrations of fungal extracts (20–500 µM/ml). After 24 and 48 h of incubation, 20 µl of MTT solution (5 mg/ml in phosphate-buffered saline (PBS) was added to each well and the plates were covered with aluminum foil and incubated for 4 h at 37 ℃. The purple formazan product was dissolved by the addition of 100 µl of 100% DMSO to each well. The absorbance was monitored at 570 nm (measurement) and 630 nm using a 96-well plate reader (Bio-Rad, Hercules, Calif, USA). Data was collected for four replicates each and used to calculate the means and the standard deviations. The percentage inhibition was calculated using the following formula:

% of cytotoxicity= (Abs of control– Abs of Sample/Abs of control) × 100

Where, Abs of control=Mean OD of untreated cells; Abs of Sample =Mean OD of treated cells

The IC_{50} value was determined as the concentration of the extract that is required to reduce the absorbance to half that of the control. All the assays were done in triplicate and the results were given in mean±SD

Acridine orange (AO) and ethidium bromide (EB) staining

The cell suspension of each sample containing 5×10^5 cells were treated with 25 µl of AO and EB solution (3.8 µM of AO and 2.5 µM of EB in PBS) and examined with a fluorescent microscope (Carl Zeiss, Germany) using a UV filter (450–490 nm). Three hundred cells per sample were counted in triplicate for each dose point.

The cells were scored as viable, apoptotic or necrotic as judged by the staining, nuclear morphology and membrane integrity and their morphological changes were also observed and photographed. The percentage of apoptotic and necrotic cells was then calculated.

Hoechst 33528 Staining

Cells (HepG2) were treated with IC_{50} concentration of fungal extracts. After 24 h incubation, the treated and untreated cells were harvested and stained with Hoechst 33258 (1 mg/ml, aqueous) for 5 min at room temperature. A drop of cell suspension was placed on a glass slide, and a cover slip was laid over to reduce light diffraction. At random 300 cells, in duplicate, were observed at 400× in a fluorescent microscope (Carl Zeiss, Jena, Germany) fitted with a 377-355 nm filter, and the percentage of cells reflecting pathological changes was calculated.

GC–MS analysis

GC-MS analysis was employed to study the active constituents present in fungal extracts using Shimadzu QP2010Plus with Thermal Desorption System TD 20. The samples were introduced into glass injector working in split mode; carrier gas was He 99.9995% pure with a Linear Velocity Pressure: 81.7 kPa. Rtx-5 MS fused silica capillary column (30 meters X 0.25 mm. i.d. X 0.25 um film thickness). The following temperature: Column Oven Temp: 80.0 °C, Injection Temp: 270.00 °C. The constituents were identified using commercial libraries.

Statistical analysis

The values of DPPH and MTT assays were expressed as mean±SD for three replicate. One-way ANOVA and the separation of means were carried out with least significance difference test (SPSS). Post-hoc testing was performed for inter grouping comparisons using the least significance difference test. Values of $P<0.001$.

RESULTS

Isolation of endophytic fungi

In this study four fungal endophytes (CC1, CC2, CC3 and CC4) were isolated from *C. cujete* L. (fig. 1). We used conidia and mycelia morphology to identify the endophytes and further confirmed by rDNA sequence analysis. The sequence was then aligned with the CLUSTAL W program and aligned sequence was subjected to phylogenetic tree construction based on the maximum parsimony method encompassing with 1000 bootstrap replications (fig. 2).

In the phylogenetic tree the species were clustered into their respective genus clade. This revealed that the isolated fungal sequences showed 99% homology with *Nigrospora sphaerica, Fusarium oxysporum, Gibberella moniliformis,* and *Beauveria bassiana* species from NCBI nucleotide database. The sequences were then submitted in GenBank, NCBI database with the corresponding accession numbers KJ190247, KJ190249, KJ190248 and KJ190250.

Fig. 1: *C. cujete* L. A-leaves, B-Branches, C-Whole tree

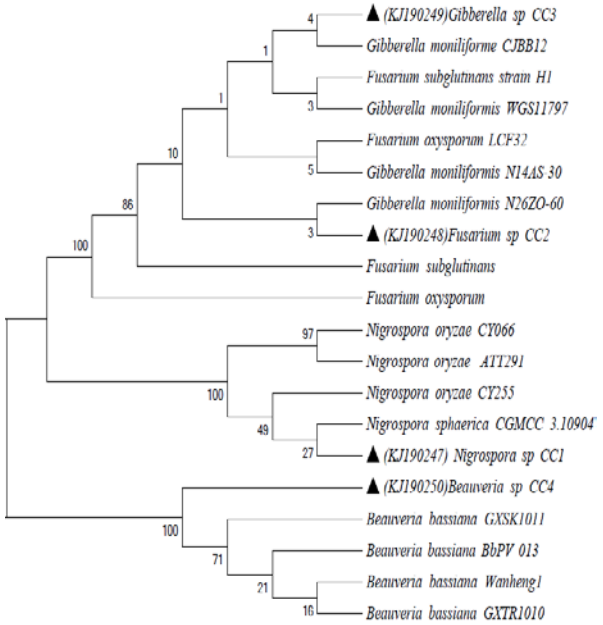

Fig. 2: Phylogenetic analysis of endophytic fungal communities isolated from the leaves of *Crescentia cujete* from India. The phylogenetic tree was constructed based on ITS-1 and ITS-4 sequences using the maximum parsimony method in MEGA ver. 5.2. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches

Antibacterial activity

Antibacterial activity of fungal extracts was evaluated against gram positive and negative bacteria (fig. 3). All the isolated strains showed an excellent inhibitory effect against human pathogenic bacteria. Among the tested strains, crude extract of CC4 was having effective growth inhibition against all the human pathogens except *S. aureus.* The extract of CC2 showed strong growth inhibition against *S. typhi*, *Shigella sp.* Strain CC1 and CC3 crude extract displayed moderate inhibition activity against all the human pathogens except *P. aeurginosa* and *S. aureus*. The results revealed that strain CC2 and CC4 showed a prominent inhibitory activity against the human pathogens.

Antioxidant activity (DPPH assay)

DPPH scavenging activity of tested fungal extracts was shown in fig. 4. The scavenging activity rate increase proportionately with the different concentration of the fungal extracts (20-100µg/ml). From these results, it was identified that the extract of strain CC4 exhibits effective scavenging activity at 13 to 46%. The observed fungal extracts activities were lower than of standard BHT at 17 to 70%.

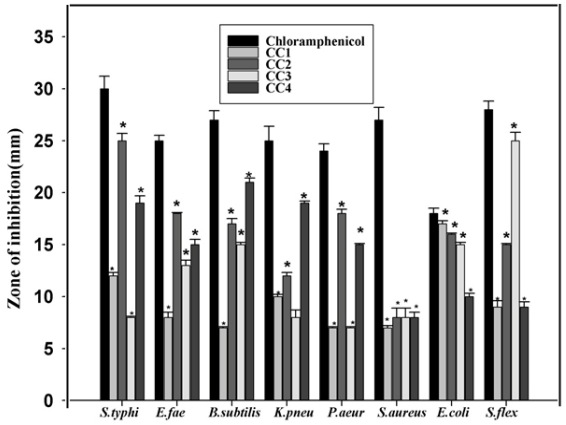

Fig. 3: Antimicrobial activity of fungal extracts of CC1, CC2, CC3 and CC4 was evaluated against gram positive and negative bacteria. *indicates that significantly equal to control, *indicates that significantly not equal. The test was performed in triplicate and the results were given in mean±SD

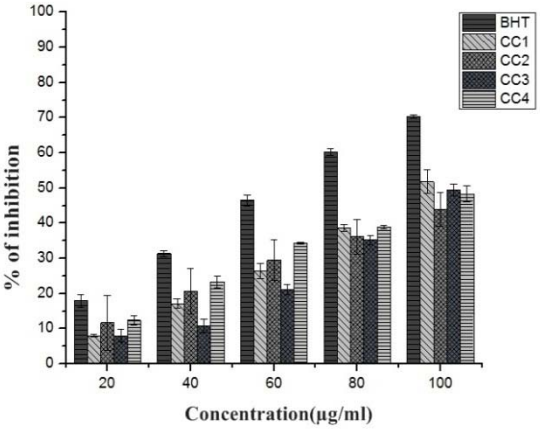

Fig. 4: DPPH scavenging activity of BHT, CC1, CC2, CC3 and CC4. The assay was done in triplicate and the results were given in mean±SD

In vitro anticancer activities

Cell viability (MTT assay)

Cytotoxicity of fungal extracts was examined against HepG2 cell line by MTT assay. The mode of action and interaction of these extracts with the cancer cell varied with their potency (CC4>CC1>CC2>CC3). The cytotoxic effect of strain CC4 is higher, among other isolates with IC_{50} values 158.5µg/ml. However, extracts of CC1 and CC2 strain were found to be exhibiting cytotoxicity at concentrations lower than strain CC3 extracts (Fig.5). The IC_{50} values were obtained by plotting the cell viability against the concentrations of the fungal extracts. Hence, the inhibitory concentrations IC_{50} for the endophytic strains were fixed at CC1 175.76 µg/ml, CC2 188.21 µg/ml and CC3 199.96 µg/ml. Further experiments have been carried with IC_{50} values of four endophytes.

Analysis of apoptosis

AO/EB and Hoechst 33528 staining methods was performed to verify whether the inhibitory action of crude extracts on HepG2 cells is due to apoptosis. The cytological changes and morphological features of chromatin condensation were observed in the treated cells based on the fluorescence emission and AO/EB stained nuclei. Under microscopic examination clear apoptotic cells were visualized with the scoring rate of 47, 51, 48 and 58 for extracts CC1, CC2, CC3

and CC4 respectively (fig. 6). Therefore, it was confirmed that extracts of the four isolates trigger HepG2 cellular death via apoptosis. Hoechst 33258 staining was used to visualize the changes in the nucleus and formation of apoptotic bodies which are characteristic features of apoptosis. Fig. 6 indicates the apoptotic nuclear morphology induced at IC_{50} concentration by each of the extracts after 24h treatment. Strain CC4 extract displays 56% of abnormal nuclei which is higher than others, whereas the strain CC2 extract was shown 55% of abnormal nuclei and strain CC1 and CC3 extracts were the least potent with 49% and 48%.

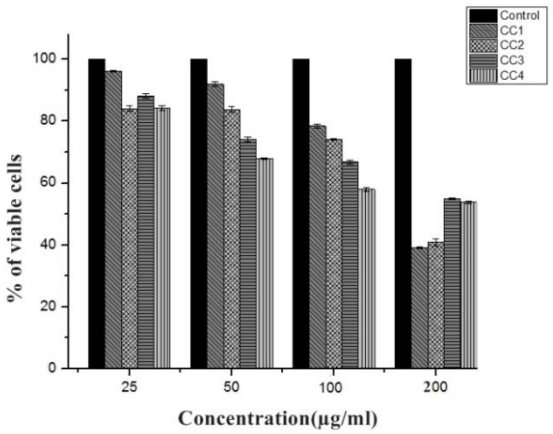

Fig.5: MTT assay for EtoAc extract of fungal endophytes of CC1, CC2, CC3 and CC4 against HepG2 cell line for 24h. All the assays were done in triplicate and the results were given in mean±SD.

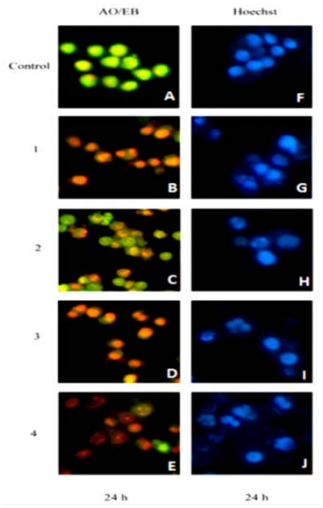

Fig. 6: Results of AO/EB staining. A: Control (Untreated cells), B, C, D and E: treated with ETOAC of isolates CC1, CC2, CC3 and CC4. Results of Hoechst staining: Control (Untreated cells), G, H, I and J: treated with EtoAc extracts of CC1, CC2, CC3 and CC4 strains.

GC-MS analysis

GC-MS analysis shows the active ingredients present in the ethyl acetate extracts of all four isolates. The following compounds were identified homogeneously viz: 1,2-Benzenedicarboxylic Acid; Diethyl Ester; Benzyl Benzoate; Benzoic acid, 2-hydroxy-, phenyl methyl ester; Dodecanoic acid, 1, 2, 3-propanetriyl ester; Tetratetracontane; Heneicosane; Hexacosane; 1,2-Benzenedicarboxylic Acid; (-)-Isolongifolol; n-Hexadecanoic acid; Hexatriacontane; 1,6-methanonaphthalen-1(2h)-ol, octahydro-; 1-Phenanthrene carboxylic acid, 1,2,3,4,4a,4b,5,6,7,8,10,10a-d; Benzoic acid, 2-hydroxy-, phenyl methyl ester; 9-Octadecenoic Acid (Z)-; Octadecanoic acid; Tetracosane; Tetratetracontane; Squalene; 1-Tetradecanol, acrylate; 1-Stearoyl-1h-Imidazole; 2-tert-Butyl-4,6-bis(3,5-di-tert-butyl-4-hydroxybenzyl)phenol (fig. 7, 8, 9 and 10).

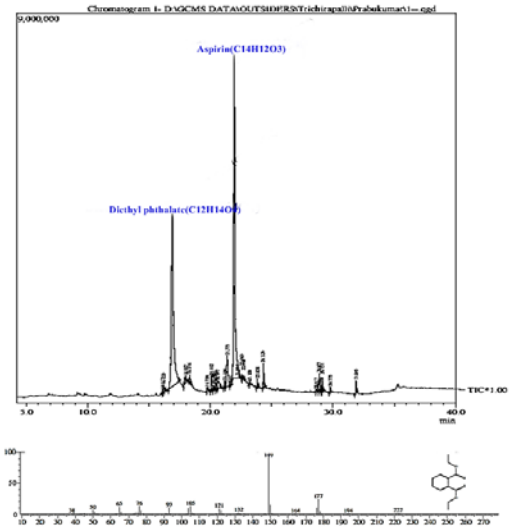

Fig. 7: GC-MS chromatogram results for EtOAc of CC1and MS spectrum for the Benzyl salicylate (Aspirin)

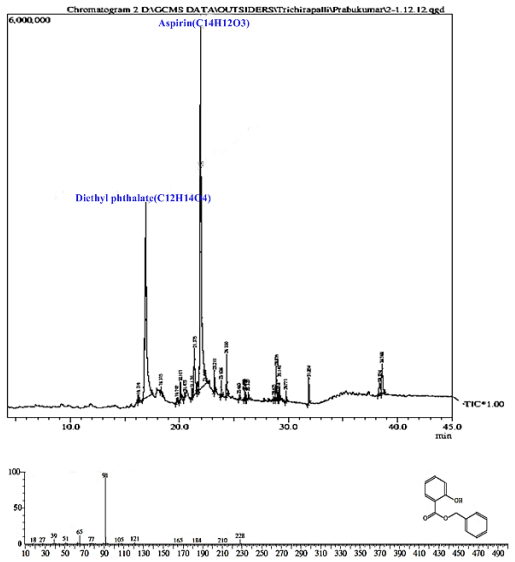

Fig. 8: GC-MS chromatogram results for EtoAc of CC2 and MS spectrum for the Diethyl phthalate

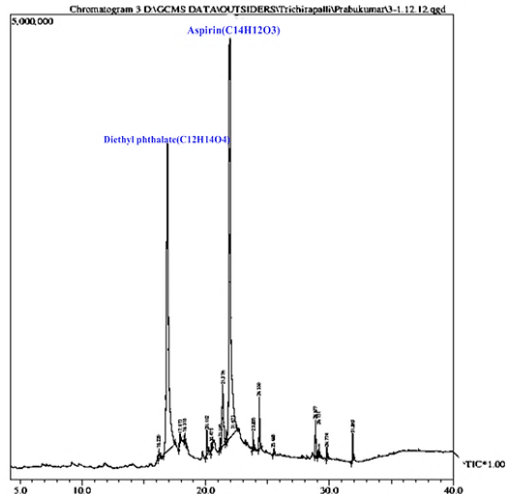

Fig. 9: GC-MS chromatogram results for EtoAc extracts of CC3

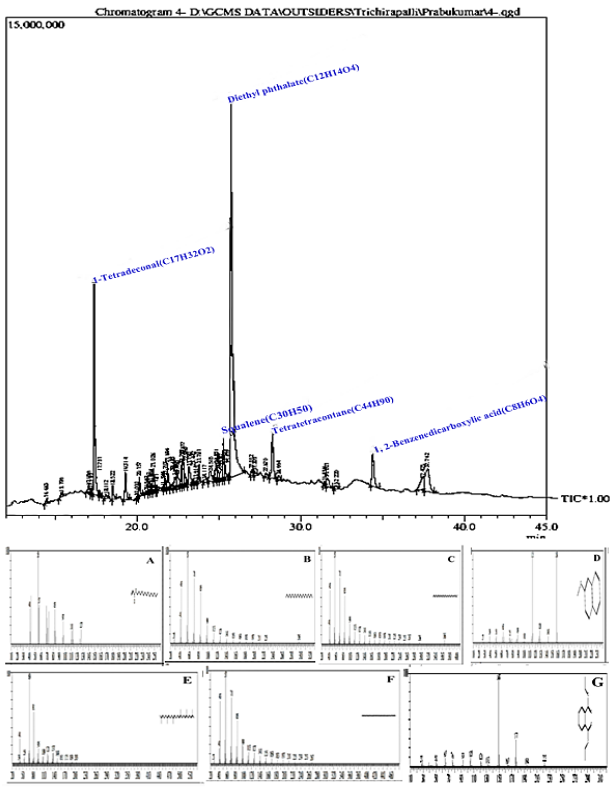

Fig. 10: GC-MS chromatogram results for EtoAc of CC4 and MS spectrum for A: 1-Tetradecanol, B: Heneicosane, C: Hexacosane, D: 2-methoxynaphthalene, E: Squalene, F: Tetratetracontane, G: 1, 2-Benzenedicarboxylic Acid

DISCUSSION

Generally, endophytic fungi were widely distributed in all plant domains. It produces valuable plant secondary metabolites and exploited for medical, agricultural and industrial uses [16]. Plants having an ethnobotonical history or used by local indigenous people for any therapeutic purpose will provide the best opportunities to isolate novel endophytic fungi and to make use of them to produce novel bioactive products [17]. With this rationale, the medicinal plant *C. cujete* L. was chosen for the isolation of endophytic fungi from its leaves. *Nigrospora* and *Fusarium* sp. were the common endophytes in medicinal plants reported previously from *Taxus globosa* [18] and *Tripterygium wilfordii* [19] respectively. *Beauveria bassiana* was an entomopathogenic fungus possessing a broad spectrum of insecticidal activity against crop pests by discharging active beauvericin [20]. *Gibberella moliniformis*, the teleomorph of *Fusarium verticillioides* reported as endophytic fungi from medicinal herb adlay [21]. The active ingredients identified in the above mentioned isolates were shown excellent therapeutic values.

The crude fermentation broth of endophytic fungi has effective antagonistic activity as a whole or should possess them in high concentrations of vigorous principles that result in the exposition of positive biological activities [22]. The fungal extracts were more potent against Gram positive bacteria when compared to Gram negative bacteria. This is due to the more rigid and complex cell wall structures in Gram negative bacteria [23]. Several studies have reported that endophytes are factories for producing a wide spectrum of bactericidal, fungicidal and cytotoxic compounds [24]. Similarly the ethyl acetate extract of fungal endophytes such as *Acremonium sp*, *Aspergillus fumigatus*, *Botryodiplodea theobromae*, *Fusarium sp*. *Fusarium verticillioides*, *Trichoderma sp* were reported to possess excellent antibacterial activity against both gram positive and gram negative human pathogens [25]. Our results revealed that the extracts of fungal endophytes can be developed as potential antimicrobial agent against different infectious diseases.

The DPPH scavenging activity method is an archetypal, simple and hasty of assessing antioxidant activity [26]. Hydroxyl free radical is one of the reactive oxygen species causing damage to the cell membrane [27]. Antioxidants are compounds that inhibit or delay the oxidation process by preventing the initialization or propagation of oxidizing chain reactions. In the present study, extracts of endophytes exhibited potent DPPH radical scavenging activity. Antioxidant compounds also posse's anti-inflammatory, anti-atherosclerotic, antitumor, antimutagenic, anticarcinogenic and antibacterial activities [28].

The demand for anticancer drugs was mainly due to the existence multiple drug resistance (MDR), increasing costs of chemotherapy and adverse side effects. The collective effect of all these factors leads to the destruction of endangered medicinal plants for the identification of alternative drugs [11]. Therefore, there is the growing need to develop alternative chemotherapeutic agents. For that screening and evaluating novel anticancer compounds from endophytic fungi will be an effective and time consuming alternative to other sources [29]. Several studies have been carried out to find out novel drugs from endophytes of various medicinal plants for cancer therapy. In this study, the cytotoxic activity of fungal extracts was investigated on human hepato carcinoma cell line (HepG2) by using MTT assay. A similar phenomenon was also carried out in HepG2 cell line by the endophytic fungi *Penicillium sp.* displays good cytotoxicity, isolated from plant *Hopea hainanensis* [30]. The main role of anticancer drug was to Hang-up the proliferation and/or induction of apoptosis [31]. Apoptosis is a biological code of cell death classified by morphological structures, genetic destruction and condensation. In this study, the morphological features of apoptosis was confirmed by using acridine orange/ethidium bromide (AO/EB) staining and Hoechst 33258 staining. Recently, it was reported that nonsteroidal anti-inflammatory drug asprin induce cytotoxicity against HepG2 human hepatocellular carcinoma cell line. It was also noticed that aspirin triggers cellular death via extrinsic and intrinsic pathways, i.e. DNA damage, alteration in the Bax/Bcl-2 ratio, caspase cascade mediated mitochondrial dysfunction [32]. Similarly, diethyl phthalate present in the grape seeds has shown potential anticancer effect against human skin cancer cells (A431) [33].

The Gas Chromatography-Mass Spectrometry turns as a basic and important key for metabolic profiling of microbes [34]. In our study, we have identified different bioactive compounds with various pharmaceutical values, such as antibacterial, antioxidant and anticancer activities. It was already demonstrated that the compound diethyl phthalate has antimicrobial, acetylcholinesterase and neurotoxic activity [35]. Phthalates are stated to have antagonism against pathogens and other pharmacological activities. Until now phthalic acid has been known to produce by *Gibberella fujikuroi* [36]. Raza *et al.* [37] demonstrated that Benzoic acid, 2-hydroxy-, phenyl-methyl ester (Aspirin) treatment (5-10µmol/ml) induces oxidative stress, cell cycle arrest in the G0/G1 phase, apoptosis and mitochondrial dysfunction against HepG2 cells. Benzyl benzoate is having a good antimicrobial activity [38]. Kotan *et al.* [39] was proved that the hexane extract of Turkish *Achillea, Satureja* and *Thymus* sp. which contains mainly hexacosane and heneicosane and extract has shown potent antibacterial activity over a board spectrum against 25 phytopathogenic bacterial strains. Christinah *et al.* [40] reported that methanolic extract fraction of *Garcinia kola* (Heckel) seeds were having potent antibacterial activity mainly due to the presence of 1, 2-Benzenedicarboxylic acid. The derivatives of 1-Phenanthrenecarboxylic acid, 1,2,3,4,4a,4b,5,6,7,8,10,10a-d show antibacterial, cardiovascular and antioxidant activities [41]. Li *et al.* [42] demonstrated that 2-tert-Butyl-4, 6-bis (3, 5-di-tert-butyl-4-hydroxybenzyl) phenol having the excellent antioxidant activity.

CONCLUSION

To summarize, in this study fungal endophytes have been successfully isolated from the medicinal plant *C. cujete* L. Four isolates were identified and molecularly characterized as *Nigrospora sphaerica, Fusarium oxysporum, Gibberella moniliformis,* and *Beauveria bassiana*. The extracts of all four fungal isolate have shown prominent antibacterial, antioxidant and anticancer activities. Therefore, further deep investigation should be needed to exploit their potentiality to develop as an antimicrobial/ chemotherapeutic agent.

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CONFLICT OF INTERESTS

Declared None

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