ISOLATION AND IDENTIFICATION OF ENDOPHYTIC FUNGI FROM MEDICINAL PLANT AND ITS ANTI-OXIDANT,ANTI-MICROBIAL,ANTI-FUNGAL AND ANTI-CANCER ACTIVITY

Mythili T J[1], Swathi S[2,] Priya Dharshini S[3], Dhivya Dharshini U[4]

[1] *Student, Biotechnology, Bannari Amman Institute of Technology, Tamil Nadu, India*
[2]*Student, Biotechnology, Bannari Amman Institute of Technology, Tamil Nadu, India*
[3] *Student, Biotechnology, Bannari Amman Institute of Technology, Tamil Nadu, India*
[4]*Faculty, Biotechnology, Bannari Amman Institute of Technology, Tamil Nadu, India*

ABSTRACT

Endophytic fungi living in plant tissues have received considerable attention due to their many bioactive compounds with potential pharmaceutical applications. This study focuses on the isolation and identification of endophytic fungi from the noni plant (Morinda citrifolia) and evaluates their antioxidant, antimicrobial and anticancer activities. Noni plants were collected from different ecological niches and endophytic fungi were isolated from different plant tissues using surface sterilization techniques. The isolated fungi were grown in a suitable environment and morphological and molecular techniques were used to identify them. Antioxidant activity of endophytic fungal extracts was determined by DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) tests. The results showed a significant antioxidant potential of several extracts, indicating their ability to scavenge free radicals. The antimicrobial activity of the extracts was evaluated against a panel of pathogenic microorganisms, including bacteria and fungi, using agar well-spread and broth microdilution methods. Namely, certain extracts have shown strong antimicrobial activity against both Gram-positive and Gram-negative bacteria and fungal pathogens, suggesting their potential as natural antimicrobial agents. In addition, the anticancer activity of extracts was evaluated using different cancer cell lines by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and cell morphology analysis. Encouragingly, selected extracts have shown significant cytotoxic activity against cancer cells, highlighting their potential as anticancer agents.

Keyword : - *endophytic ,anti-cancer ,anti- microbial,agar well diffusion*

1. INTRODUCTION

Isolation and identification of endophytic fungi from medicinal plants is a promising frontier in natural product development and provides an abundance of bioactive compounds with diverse therapeutic potential. These fungi can be isolated and characterized by a careful process involving surface sterilization of plant tissues and subsequent culture techniques. Their secondary metabolites have shown significant antioxidant, antimicrobial, antifungal and anticancer activities, making them valuable candidates for drug development. Endophytic fungal extracts have shown efficacy in scavenging free radicals, inhibiting the growth of pathogenic microorganisms including bacteria and fungi, and exerting cytotoxic effects on cancer cells by inducing apoptosis and inhibiting proliferation. Exploitation of the bioactive potential of endophytic fungi promises the development of new therapeutic agents for various health problems.

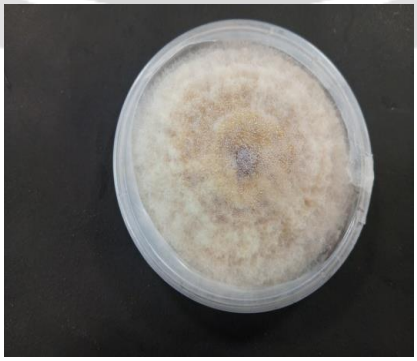
1.2 INTRODUCTION TO PROPOSED METHODOLOGY

The proposed work methodology aims to systematically investigate the isolation and identification of endophytic fungi from medicinal plants and evaluate their bioactive properties, including antioxidant, antimicrobial, antifungal, and anticancer activities. The methodology involves several key steps. Collection and preparation of medicinal plant samples, ensuring proper identification and documentation of plant species. Surface sterilization of plant tissues to remove surface contaminants and isolate endophytic fungi. Culturing of sterilized plant tissues on appropriate nutrient media to promote fungal growth. Isolation and purification of fungal colonies from culture plates for further analysis. Molecular identification of isolated fungal strains using techniques such as DNA sequencing to determine species composition accurately. Extraction of secondary metabolites from fungal cultures for bioactivity assays. Evaluation of antioxidant activity through assays like DPPH and ABTS scavenging assays, as well as FRAP assays. Assessment of antimicrobial activity using agar diffusion assays, broth dilution assays, and determination of MIC values against pathogenic microorganisms. Investigation of antifungal activity through radial diffusion assays, broth microdilution assays, and determination of MFC values against fungal pathogen. Examination of anticancer activity via in vitro assays such as MTT assays, clonogenic assays, to assess apoptosis induction and inhibition of proliferation in cancer cells. This proposed methodology integrates various techniques and assays to comprehensively explore the bioactive potential of endophytic fungi from medicinal plants, laying the foundation for the discovery of novel therapeutic agents with significant biomedical applications.

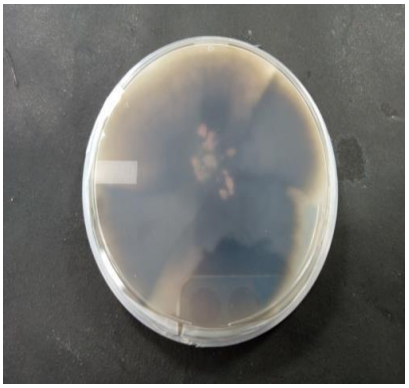
2. ALGORITHMNS AND METHODS:

2.1COLLECTION OF PLANT MATERIAL AND ISOLATION OF FUNGAL ENDOPHYTES:

Fresh plant material was collected from Ooty, Nilgiris district of Tamil Nadu, India in December 2023. The taxonomic identity of the plants was confirmed by the Botanical Survey of India, Southern Circle, Coimbatore, Tamil Nadu, India. Plant materials were washed under running tap water to remove surface contamination and different parts such as leaves were separated mechanically.Collected fresh plant leaves were thoroughly washed with sterile distilled water, cut into m small pieces, washed with 0.5% (v/v) sodium hypochlorite (NaOCl) solution for 2 minutes and then with 70% ethanol (v/v) for 2 mins, washed three times in sterile distilled water for 2 min.It was then placed between folds of sterile filter paper to remove attached water droplets.They were again cut into smaller pieces with sterile knives. Surface-sterilized leaf segments were stacked in petri dishes (3-4 pcs/petri dish) containing potato dextrose agar (PDA) modified with chloramphenicol (150 mg/l).Petri dishes were sealed with parafilm and incubated at 26 ± 2 °C in a light chamber for 12 h, followed by a 12 h dark cycle for 15–20 days.The plates were observed daily to check the appearance of endophytic fungal colonies on the leaf segment. Individual elbows of different fungi emerging from the cut end of a leaf segment were transferred to PDA plates without antibiotics and incubated for 10 days at 27±2ºC. 7. Stock cultures were maintained and subcultured at regular intervals.



(a)

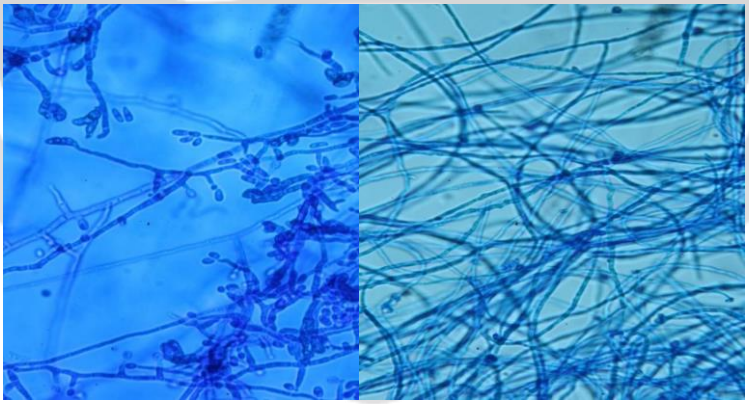


(b)

Fig 2.1 Cultures. (a) subculture (b) pure culture

3. IDENTIFICATION OF ENDOPHYTIC FUNGI

Identification of fungi such as Neofussicum parvum and Pestalotiopsis knightiae from morphological characters requires a detailed study of various properties. First, the structure and color of the mycelium provide the first clues. Secondly, it is important to observe the size, shape, color and all the characteristics of the conidiomata where the spores are produced. In addition, conidial characteristics such as shape, size, color and ornamentation, as well as the size, shape and arrangement of conidiophores, are determining.Cultural characteristics, including growth rate, texture and pigmentation of different materials, provide additional information. Microscopic examination reveals detailed morphology such as the structure of conidia, conidiophores and mycelium. However, accurate identification often requires consultation with a mycologist, reference to the literature, or the use of molecular techniques such as DNA sequencing to determine the exact species, especially for closely related taxa.



(a) (b)

Fig 3.1 (a) *Neofusicoccum parvum* (b) *Pestalotiopsis knightiae*

4. ISOLATION OF GENOMIC DNA:

Genomic DNA of the endophytic fungal strain was extracted according to Miller et al. (1999) with minor modifications. The endophytic fungus was grown in a 250 ml Erlenmeyer flask containing 100 ml of potato dextrose broth by inoculating spores (1 x 10 5 spores/ml) from a 7-day culture. The culture was incubated at 25 ± 1 °C for 7-14 days. 2 g of mycelium from each isolate was separately ground into a fine frozen powder using liquid nitrogen in a sterile mortar. The finely ground tissue was placed in a sterile centrifuge. DNA extraction buffer was added to it, mixed well and incubated in a water bath at 65°C with gentle shaking for 60-90 minutes.

After incubation, an equal amount of chloroform: isoamyl alcohol 24:1 (v/v) was added and gently mixed and centrifuged at 12000 rpm for 20 min at 4 °C. 0.7 volume of isopropanol was added to the upper aqueous phase and the mixture was stored overnight at -20°C. They were then centrifuged at 12,000 rpm for 10 min at room temperature, and the resulting pellets were washed twice with 70% (v/v) ethanol and dissolved (20 µl) in TE buffer and stored at −20°C. Nucleic acid was treated with RNAse to obtain pure DNA suitable for PCR amplification.

5. QUANTIFICATION OF SECONDARY METABOLITES:

5.1 QUANTIFICATION OF PHENOLS:

The total phenolic content was determined according to method described by Siddhuraju and Becker (2003). Fifty microlitre triplicates of the extracts (20 mg/20 mL) were taken in the test tubes and make upto the volume of 1 mL with distilled water. Then 0.5 mL of Folin-ciocalteu phenol reagent (1:1 with water) and 2.5 mL sodium carbonate solution (20%) were added sequentially in each tube. Soon after vortexing the reaction mixture, the test tubes were placed in dark for 40 min. and the absorbance was recorded at 725 nm against the reagent blank. The analysis was performed in triplicate and the results were expressed as gallic acid equivalents.

5.2 QUANTIFICATION OF TANNINS:

Using the same extract, the tannins were estimated after treatment with polyvinyl polypyrrolidine (PVPP) (Siddhuraju and Manian, 2007). 100 mg of PVPP was weighed into a 100 × 12 mm eppendrof tube and to this 1 mL distilled water and then 1 mL of the sample extracts were added. The content was vortexed and kept in the freezer at 4°C for 15 min. Then the sample was centrifuged at 4000 rpm for 10 min. at room temperature and the supernatant was collected. This supernatant has only simple phenolics other than the tannins (the tannins would have been precipitated along with the PVPP). The phenolic content of the supernatant was measured by following the above procedure and expressed as the content of non-tannin phenolics.

Tannins = Total phenolics - Non tannin phenolics

5.3 QUANTIFICATION OF FLAVANOIDS:

The flavonoid contents of the plant extracts were quantified according to the method described by Zhishen ***et al***. (1999). About 100 µL of the plant extracts were taken in different test tubes and 2 mL of distilled H_2O were added to each test tube. A test tube containing 2.5 mL of distilled H_2O served as blank. Then, 150 µL of 5% $NaNO_2$ was added to all the test tubes followed by incubation at room temperature for 6 min. After incubation, 150 µL of 10% $AlCl_3$ was added to all the test tubes including the blank. All the test tubes were incubated for 6 min. at room temperature. Then 2 mL of 4% NaOH was added to all the test tubes which were then made upto 5 mL using distilled water. The contents in all the test tubes were vortexed well and they were allowed to stand for 15 min. at room temperature. The pink colour developed due to the presence of flavonoids was read spectrophotometrically at 510 nm. Total flavonoid content was calculated as quercetin equivalents (mg/g) based on the calibration curve: $y = 0.0255x$, $R^2 = 0.9812$, where x was the absorbance and y was the quercetin equivalent (mg/g).

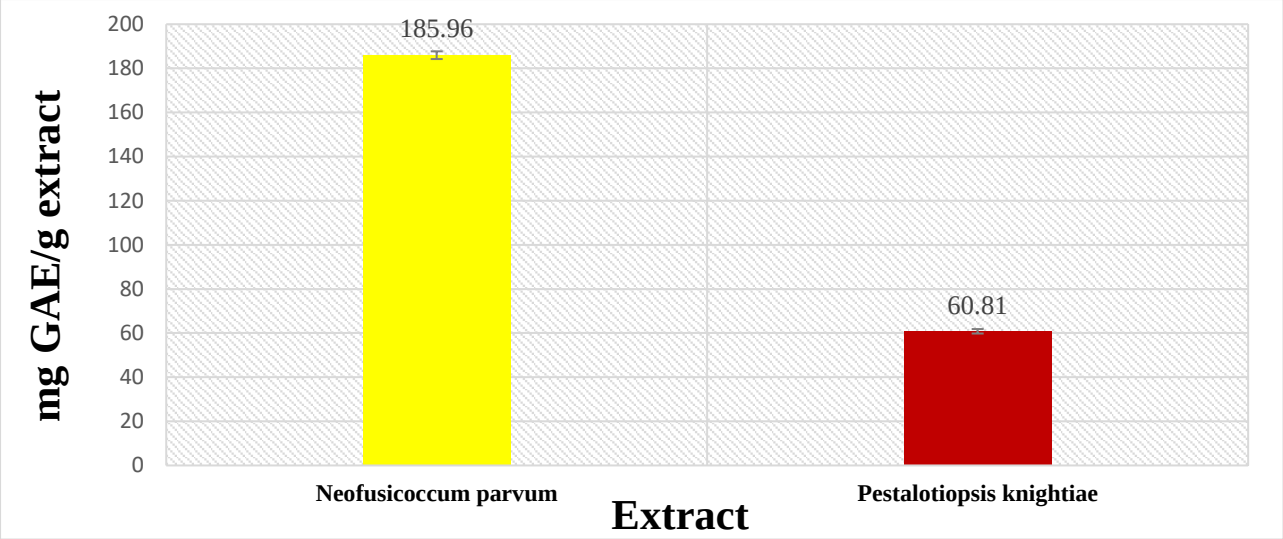


Fig 5.1 (a)Phenol

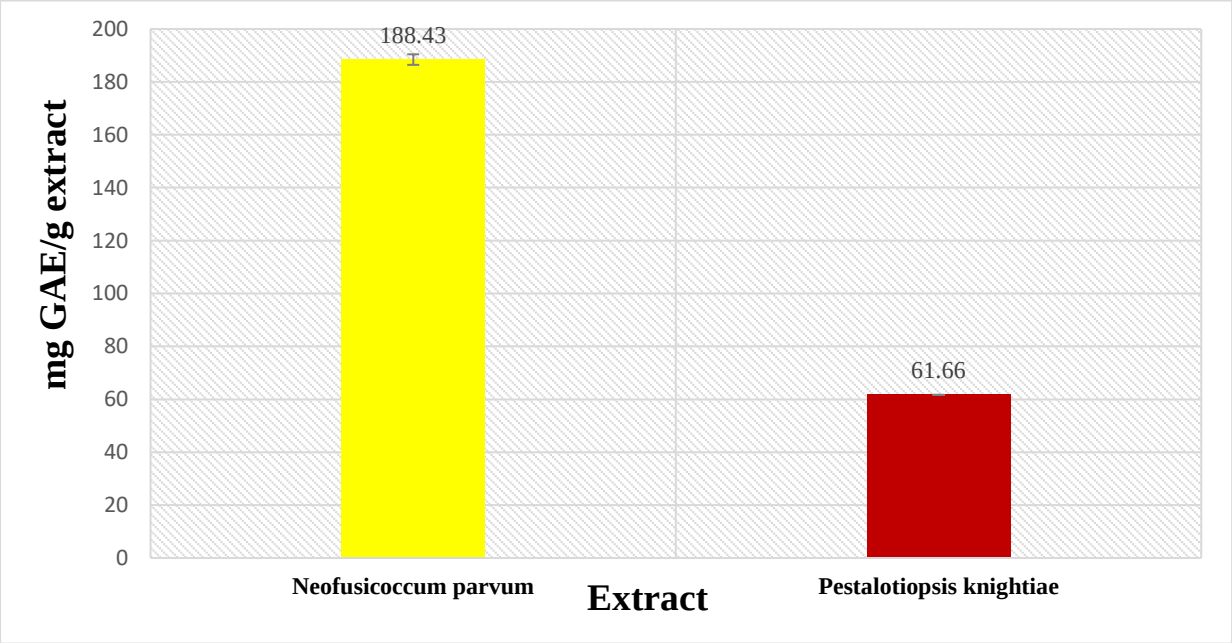


Fig 5.2 (b) Tannins

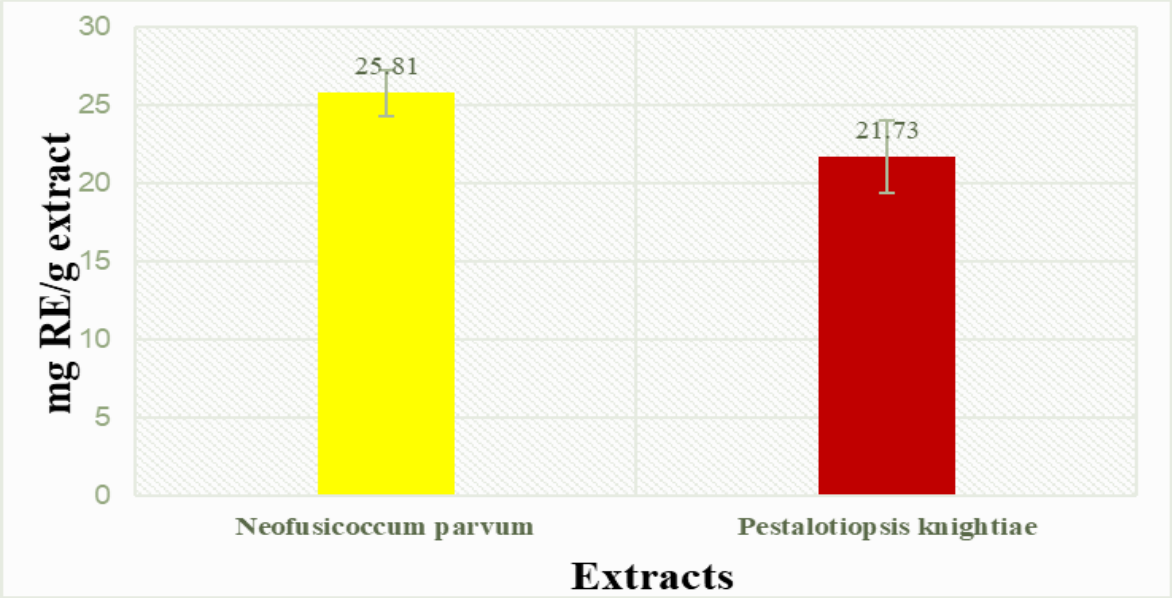
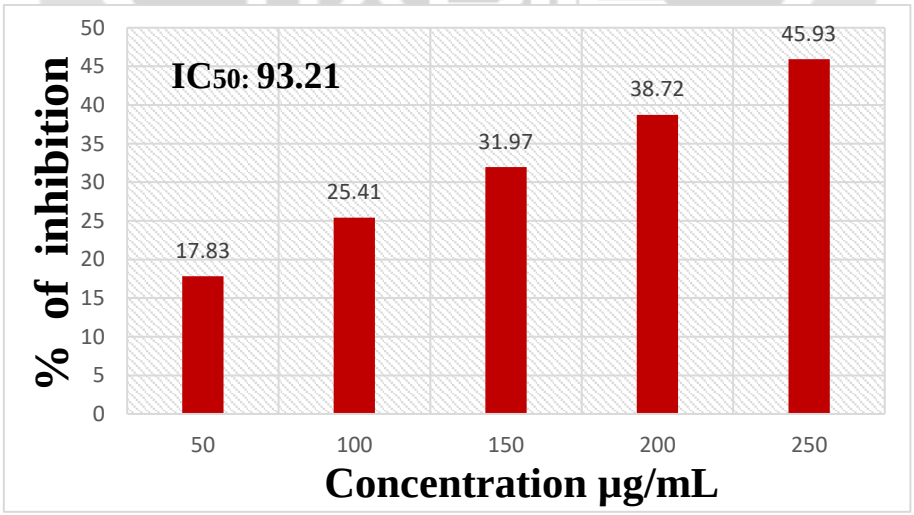


Fig 5.3 © Flavanoids

6. IN-VITRO ANTIOXIDANT ASSAY:

6.1 DPPH ASSAY:

The antioxidant activity of the extracts was determined by hydrogen donation or radical scavenging ability using the stable radical DPPH according to Gursoy et al. according to the method. (2009). Sample extracts were taken in different concentrations (20-100 µl) and the volume was adjusted to 100 µl with methanol. Aliquots of samples and standards (BOD and rutin) were added to approximately 3 mL of a 0.004% methanol solution of DPPH and shaken vigorously. A negative control was prepared by adding 100 µL of methanol to 3 mL of DPPH methanol solution. The test tubes were allowed to stand for 30 minutes. at 27°C. The absorbance of samples and control was measured at 517 nm relative to methanol. The radical scavenging activity of the samples was expressed as the IC50 value, which is the sample concentration required to inhibit 50% DPPH® concentration.



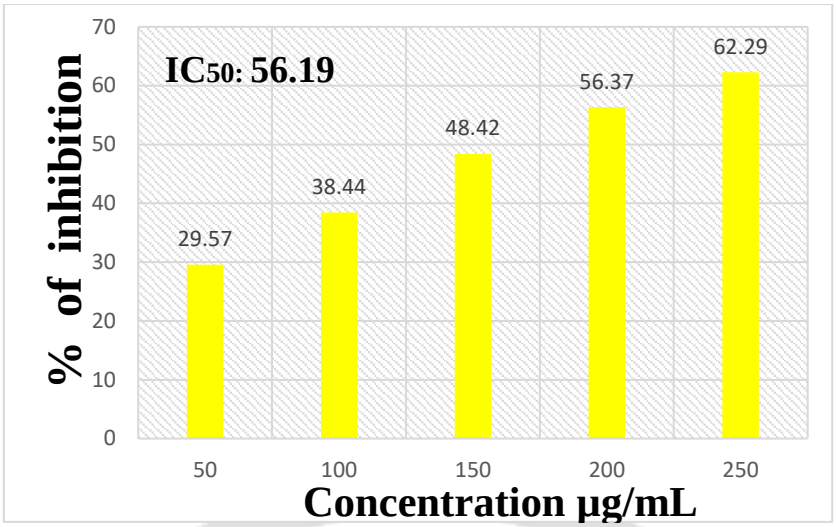


Fig.6.1 DPPH assay

6.2 ABTS assay:

Antioxidant movement was decided by the ABTS radical rummaging measure concurring to Re et al. (1999). The ABTS radical cation was produced by an fluid ABTS (steady radical) arrangement with 2.4 mM potassium persulfate within the dim for 12–16 h. Some time recently investigation, the ABTS arrangement was weakened in ethanol (1:89 v/v) to get an absorbance of 0.700 ± 0.02 at 734 nm. Almost 1 mL of weakened ABTS arrangement was included to almost 30 µL of test arrangement and 10 µL of Trolox (0–15 µM last concentration) in ethanol. A test tube containing 1 mL of weakened ABTS arrangement and 30 µL of ethanol served as a negative control. All test tubes were vortexed well and hatched for precisely 30 minutes at room temperature. After brooding, the absorbance of tests and benchmarks (BOD and rutin) was measured at 734 nm compared to an ethanol clear. The results were taken as the concentration of Trolox with comparing antioxidant movement communicated as µM/g of test extricates

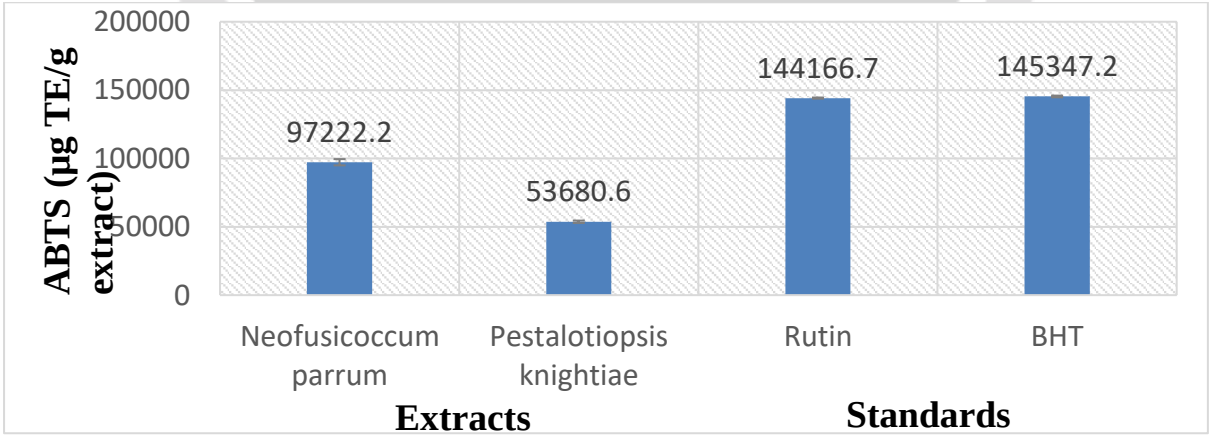
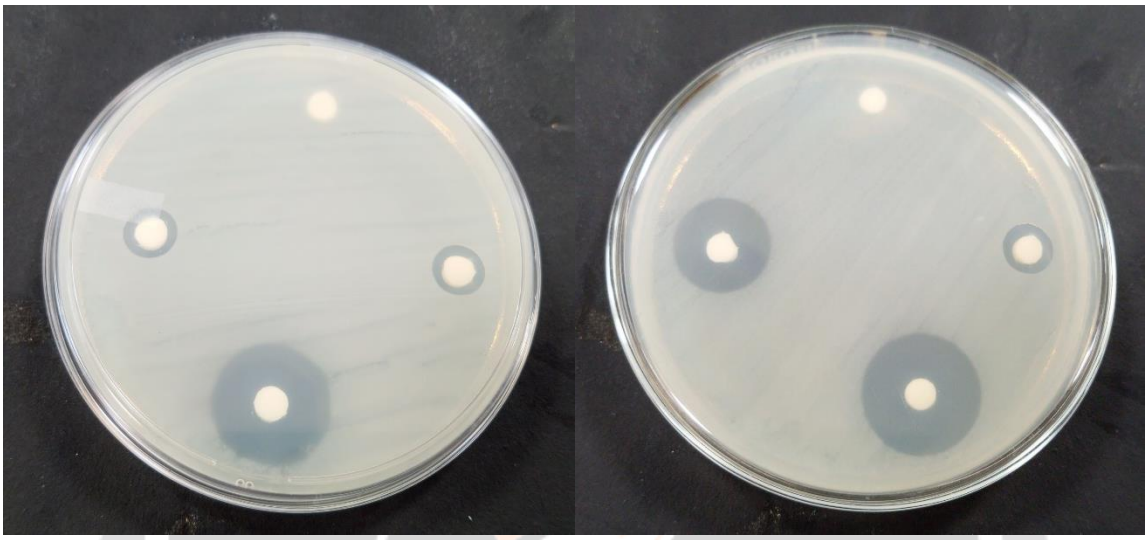


Figure 6.2 ABTS assay

7. ANTI-BACTERIAL ASSAY:

The disk diffusion method is employed to appraise antimicrobial activity of every plant extract. The plant extract residues (20 mg) were dissolved in 1 ml of DMSO. Muller Hinton agar was then prepared under sterile condition. The organisms that were isolated was inoculated in the Nutrient broth and were incubated overnight. Then, swabs were used to lawn the organisms to the Muller Hinton agar plates and then, sterile discs were placed. The plant extract solution of 30 µL were poured into the sterile discs. The plates are incubated at 37ºC overnight (Tendencia, 2004).



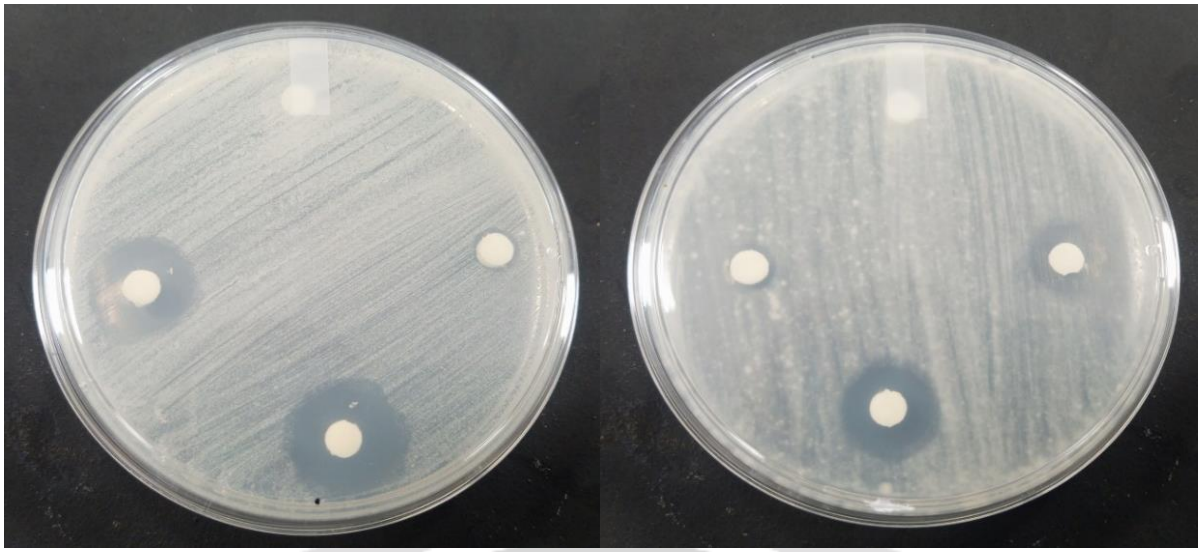
(a) (b)

Figure 7 (a) Gram positive (b) Gram negative

8. ANTI - FUNGAL ASSAY:

Antifungal assay for disk diffusion assay, a 6 mm disc from one week old *C.albicons* and *A. niger* was removed from the growing edges and kept upside down position. A sterile filter paper disc was impregnated with concentration 10 mg/ml of different extraction solution (0.1%) placed in opposite position to the culture disc. For control, sterile water was added. The plates were then incubated at room temperature for two weeks (Heatley N.G et al., 1944). Experiments were done in triplicate and repeated twice.
% of inhibition was calculated using the formula:

$$\% \text{ inhibition} = \frac{\text{mycelial growth in control} - \text{mycelial growth in treatment}}{\text{mycelial growth in control}} \times 100$$



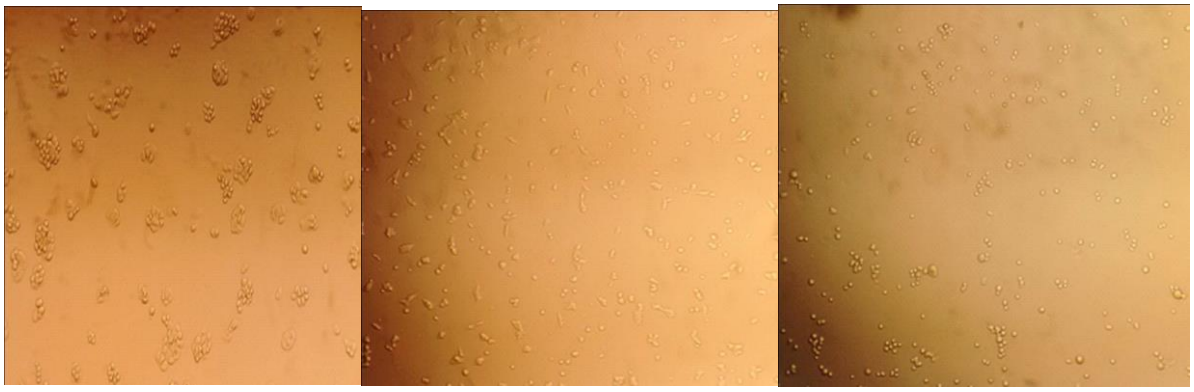
(a) (b)

Figure 8 (a) Candida albicans (b)Aspergillus niger

9. ANTI-CANCER ASSAY:

The anti-cancer assay is a pivotal component in the investigation of endophytic fungi from medicinal plants for their therapeutic potential. This assay encompasses a series of rigorous tests aimed at evaluating the efficacy of fungal extracts or isolated compounds in inhibiting cancer cell growth and inducing apoptosis. Initially, cancer cells are cultured and treated with varying concentrations of fungal extracts or compounds. Subsequently, cell viability is assessed using assays such as the MTT assay, which measures mitochondrial activity, or the clonogenic assay, which evaluates the ability of cells to proliferate and form colonies. Additionally, flow cytometry analysis is employed to assess cell cycle progression and apoptosis induction, providing insights into the mechanism of action of the tested compounds. Through these assays, the anti-cancer activity of endophytic fungi can be elucidated, paving the way for the development of novel therapeutic agents for combating cancer.

(a) (b) (c)



(d) (e) (f)

Figure 9 (a)control (b)18.75 µl (c)37.7 µl (d)37.5 µl (e)150µl (f)300µl

10. RESULT AND CONCLUSION:

Isolation and identification of endophytic fungi from noni plants and evaluation of their antioxidant, antimicrobial and anticancer activities is a versatile study of potential biomedical applications of these microorganisms. Through careful isolation techniques and molecular identification methods, a diverse group of endophytic fungi living in different tissues of the noni plant has been discovered. These fungi have remarkable biodiversity and may be potential sources of pharmaceutically important bioactive compounds. One of the most important results of this study is the clarification of the antioxidant properties of isolated endophytic fungi. Antioxidants play an important role in neutralizing harmful free radicals and ameliorating oxidative stress associated with many chronic diseases such as cancer, cardiovascular disease and neurodegenerative diseases. Evaluation of antioxidant activity by assays such as DPPH and ABTS revealed the ability of these endophytic fungi to scavenge free radicals and provide protection against oxidative damage. Such discoveries highlight the potential of these fungi as valuable sources of natural antioxidants for the development of new medicines and foods.

In addition, the antimicrobial activity of isolated endophytic fungi has been studied, revealing their potential role in the fight against infectious diseases. . Due to the growing threat of antimicrobial resistance worldwide, the search for alternative antimicrobials has become imperative. Antimicrobial screening performed in this study revealed the ability of these mushrooms to inhibit the growth of pathogenic bacteria and fungi, highlighting their potential as reservoirs of new antimicrobial compounds. These findings hold promise for the development of new antimicrobials to address the growing challenge of drug-resistant infections.

In addition, exploring the anticancer potential of isolated endophytic fungi is an important step forward in cancer research and drug development. Cancer remains one of the main causes of morbidity and mortality worldwide, requiring the development of innovative treatment strategiesIn conclusion, the isolation and identification of endophytic fungi from other plants and the evaluation of their antioxidant, antimicrobial and anticancer activities. many bioactive compounds with enormous biomedical potential. These findings highlight the importance of exploiting the biodiversity of endophytic fungi to discover new therapies and develop alternative approaches to combat various diseases. Future research into the mechanisms of action of endophytic fungal bioactive compounds and their safety and efficacy profiles promises to translate these findings into clinical applications that will ultimately benefit human health and well-being

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