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SVP-2334

"Microbial biochemical characterization and bioactivity"

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Title: "Microbial biochemical characterization and bioactivity"

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Signature of Mentor

Certificate of Originality

This is to certify that the aforementioned student from Sri Venkateswara College has participated in the summer project SVP-2334 titled "**Microbial biochemical characterization and bioactivity**". The participants have carried out the research project work under my guidance and supervision from 15 June 2023 to 15th September 2023. The work carried out is original and carried out in an offline mode.

Signature of Mentor

Acknowledgments

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TABLE OF CONTENTS

INTRODUCTION

Plants are inhabited internally by a wide range of fungi, which can overcome the endodermis barrier without being targeted by plant immune signals, moving from the root cortex to the vascular system, and then living as endophytes in plant organs (Lu H et al.,2021). Endophytes are present in all kinds of plants, and microbes from various environments have adopted an endophytic existence. Endophytes have been discovered to serve as beneficial variables in plant symbiotic interactions. It is widely established that mutualistic fungi may provide various host-specific benefits, including host tolerance to biotic and abiotic stress conditions such as drought, disease, herbivory, temperature, and nutritional stress, plant growth improvement, and enhanced reproductive capacity (Rai et al.,2016).

Endosymbionts also produce secondary metabolites for their persistence in the host, which are necessary for their adaptation. These compounds yield properties like improved health and decreased incidence of pathogenic attack on the host, with the microbe receiving food and shelter in return. Endosymbiotic microbes of plants and animals have attracted much attention due to their bioactive compounds having therapeutic properties (Eleftherianos et al., 2013).

The ability of microbes to produce different secondary metabolites, which are also biologically active substances are a major source of anticancer, antioxidant, antidiabetic, immunosuppressive, antifungal, anti-oomycete, antibacterial, insecticidal, and antiviral agents, is what distinguishes endophytic microbes from other types of microbes. In addition, endophytes have a role in the genetic regulation of the development of symbiosis as well as signalling, defence, and other mechanisms. Endophytes can affect their plant host's secondary metabolism in addition to producing secondary metabolite molecules (Hardoim et al., 2015).

Endophytes are appealing sources of physiologically active chemicals for drug development and other industrial uses because some of the secondary metabolites they produce are equivalent to those of the host plant and others are not. In other words, endophytic microorganisms are reservoirs of novel bioactive compounds with prospects in different industrial sectors such as food, agriculture and pharmaceutical (Falade et al., 2021). The discovery of the paclitaxel-producing endophytic fungus *Taxomyces andreanae*, isolated from the pacific *yew Taxus brevifolia*, is the most prominent known example of the presence of "phytochemicals" in endophytic fungi (Stierle et al. 1993).

Phytohormone production by endophytes is probably the best-studied mechanism of plant growth promotion, leading to morphological and structural changes in plant hosts. Salicylic Acid, Jasmonic Acid, and Ethylene are phytohormones which are recognized to play vital roles in modulating plant defensive responses against diverse pathogens. In general, Salicylic Acid signalling activates resistance to biotrophic and hemibiotrophic pathogens, whereas Jasmonic Acid and Ethylene signalling activates resistance to necrotrophic pathogens. All of these hormones are components of a wider signalling network that integrates environmental inputs and provides resistance to microbial manipulation. Auxins, abscisic acid, gibberellic acids, and brassinosteroids have also been demonstrated to have a role in plant immunity and to optimize plants' growth and development. The ability to produce auxins and gibberellins is a

typical trait for root-associated endophytes. It was proposed that indole-3-acetic acid, a member of the auxin class, increases colonization efficiency, possibly via interference with the host defense system, and the production of this compound or related compounds may be an important property for plant colonization by endophytes. (Xiaotang et al.,2016, Long HH et al., 2008, Bastian et al., 1998)

Furthermore, some fungi are capable of functioning as beneficial endophytes in one setting and pathogenic in another. Our knowledge of the interactions between plants, endophytes, and pathogens continues to evolve, but it will continue to expand in the future to provide more effective and long-lasting therapeutic resolutions. In recent years, research on microbiomes, especially that of the endosphere, has elevated significantly, but the findings are mostly descriptive rather than prescriptive. By utilizing standard molecular genetics and biochemical approaches and functional examinations, we can gain an improved understanding of how the microbiome affects plant growth, development, and stress resistance (Collinge et al., 2022).

Our research will continue to employ these studies to gain further insight into this enigmatic way of life and implement them for drug design in order to combat agricultural development, overpopulation, urbanization and climate change.

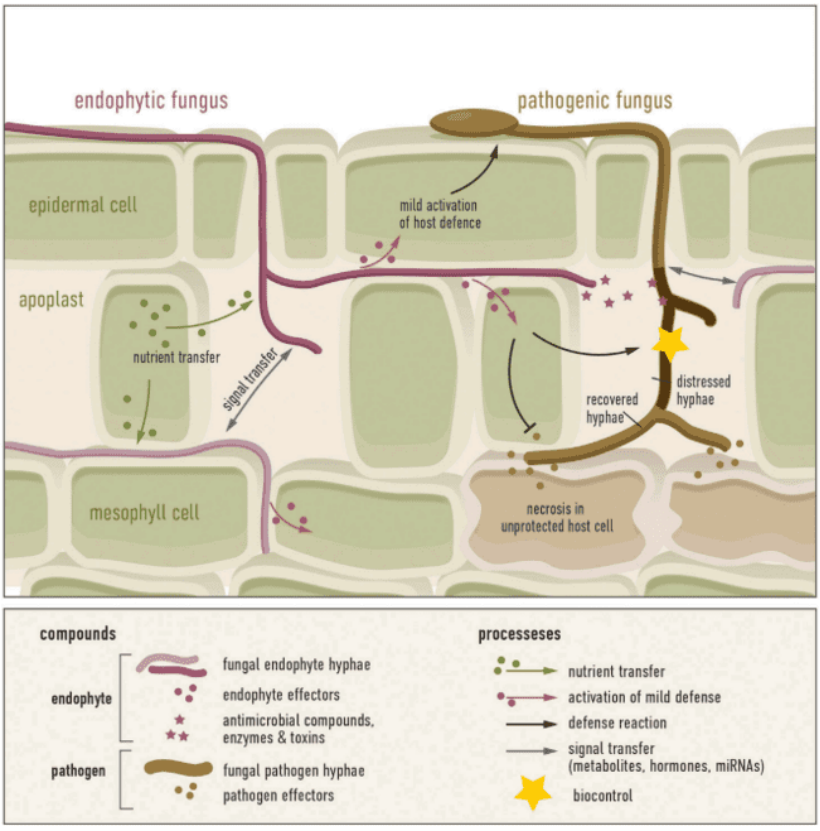


Figure 1: A conceptual model for endophytic interactions (Collinge et al., 2022)

OBJECTIVES

The following were the objectives of our study:

1) To subculture and identification fungal endophytes, present in medicinal plants.
2) To evaluate phytochemicals, present in endophyte extracts.
3) To determine extracellular enzymes in the isolated endophytes.
4) To determine the antioxidant, anti-inflammatory and anti-microbial activity in cell-free extract and aqueous extract of endophytes.
5) To determine the production of glucosinolates for role in plant defense in aqueous extract of endophytes.
6) To determine the production of phytohormones for role in plant growth promotion in aqueous extract of endophytes.

METHODOLOGY

1. Subculturing and Identification of fungal endophytes, present in medicinal plants

● Subculturing of Fungal Endophytes

The fungi extracted from plant source were sub-cultured in a Potato Dextrose Agar (PDA). The medium was sterilized by autoclaving at 121°C and 15 psi for 20 min for complete dissolution and homogeneity. Thereafter, it was allowed to cool to room temperature. Streptomycin was added to PDA so as to inhibit bacteria growth. The petri dishes with PDA were streaked and incubated for 72hrs at 28 °C for fungal growth (Ezeonu et al., 2022).

● Identification Biochemical characterisation of Fungal Endophytes

The colonies growing on the plates were identified macroscopically and microscopically. Colony colour, type (compact, loose, aerial hyphae), texture (velvety, cottony, coarse) shape and growth pattern were observed. Direct observation of culture under the light microscope (low power) by careful preparation of slides, and staining with cotton blue-in lactophenol was done.

Biochemical reactions can reveal the vital information necessary for accurately identifying the genera of various fungi within a sample. By their nature, fungi produce large volumes of enzymes, and it is these enzymes that allow for their identification via biochemical methods. Though molecular methods are required for an optimal identification, for that, sequencing the ITS regions of the rDNA is well recommended for fungi (Aslanzadeh, J. et al., 2006).

Various biochemical assays performed with slight modifications according to as per Varghese (et al., 2014) -

● Triple Sugar - Iron Agar Test

The triple sugar- iron agar test is designed to differentiate among the differences in carbohydrate (Glucose, Lactose. Sucrose) fermentation patterns and hydrogen sulfide production by the various groups of intestinal organisms. Using sterile technique, the test organism was inoculated into the TSI Agar Slants by means of stab and streak inoculation. An uninoculated tube was kept as control. All tubes were incubated at 37°C for 24 hours, the reaction was observed and inferences were made according to the colour change.

● Catalase Test

Catalase is an enzyme, which is produced by microorganisms that live in oxygenated environments to neutralize toxic forms of oxygen metabolites and H2O2. The catalase enzyme neutralizes the bactericidal effects of hydrogen peroxide and protects them. Anaerobes generally lack the catalase enzyme. Transfer a small amount of colony to a surface of clean, dry glass slide

using a loop or sterile wooden stick and lace a drop of 3% H_2O_2 on to the slide and mix. A positive result is the rapid evolution of oxygen (within 5-10 seconds) as evidenced by bubbling.

- **Oxidase Test**

It is useful for speciation and identification of microbes those have to use oxygen as the final electron acceptor in aerobic respiration. The enzyme cytochrome oxidase is involved with the reduction of oxygen at the end of the electron transport chain. The test organisms impregnated on filter paper were rubbed with 1% Kovac's Oxidase reagent using sterile glass rod. Uninoculated filter paper was kept as control. The change in colour to purple within 10 seconds indicates a positive result and no colour change indicates negative result.

- **Citrate Test**

It is used to differentiate among isolates on the basis of their ability to utilize / ferment citrate as the sole carbon source. In the absence of glucose or lactose some microorganisms utilize citrate as a carbon source. Using sterile technique, Simmons Citrate Agar slant was inoculated with the test organism by means of a stab and streak inoculation. An uninoculated tube was kept as control. All tubes were incubated at 37°C for 72 hours. The colour change from green to blue indicate positive and no colour change indicate negative result.

- **Urease Test**

Urease is a hydrolytic enzyme that attacks nitrogen and carbon bond in amide compounds such as urea and forms the alkaline end products ammonia. Using sterile technique, the test organism was inoculated in Christener's Urea Agar slant by means of loop of inoculation. An uninoculated tube was kept as control. The tubes were incubated at 37°C for 72 hours, development of pink colour indicator a positive test and no colour change shows a negative test.

- **Salt Tolerance**

The basal test medium was a Potato Dextrose Agar (PDA) was supplemented with a graded series of NaCl concentrations (1M and 2M), these plates were streaked with test samples in duplicates. Cultures were incubated for 10 days at 28 C, at which time determinations were made of the maximum level that any growth occurred as well as any unusual physiological or morphological effects (Tresner et al., 1971).

- **pH Tolerance**

pH treatments were prepared by adjusting the pH of PDA with 1N H_2S04 and 1 N NaOH to provide pH 4 and 10 respectively, as determined with a pH paper. These plates were streaked with test samples in duplicates. Cultures were incubated for 10 days at 28 C, at which time determinations were made of the maximum level that any growth occurred as well as any unusual physiological or morphological effects (Hung et al., 1983).

2. Phytochemical Assays

- **Saponins**

The presence of saponins was determined by Frothing test. The crude fungal extract was vigorously shaken with few drops of olive oil and was allowed to stand for 10 min. No froth indicates absence of saponins and stable froth more than 1.5 cm indicated the presence of saponins.

- **Tannins**

The presence of tannins was determined by adding 2-3 drops of $FeCl_3$ to 1 mL endophyte extract. The occurrence of Blackish-blue or Blackish-green colour indicated the presence of tannins (Photolo et., 2020).

- **Flavonoids**

The presence of flavonoids was determined by adding 2-3 drops of NaOH to 1 mL endophyte extract. Formation of yellow colour indicated the presence of flavonoids (Sasidharan.,2017).

- **Steroids**

The presence of steroids was determined by adding 1 mL of $CHCl_3$ and 2-3 drops of conc. H_2SO_4 to 1 mL endophyte extract. Formation of Reddish-brown ring indicated the presence of steroids. If there is immediate formation of ring, it indicates presence of **Terpenoids**.

- **Alkaloids**

The presence of alkaloids was determined by adding 2-3 drops of Wagner's reagent to 1 mL endophyte extract. Formation of Reddish-brown precipitate indicated the presence of alkaloids.

- **Gums (Polysaccharides)**

The presence of polysaccharides was determined by adding few drops of Molisch's reagent to 5 mL endophyte extract, thereafter a small volume of concentrated sulphuric acid was allowed to run down the side of the test tube to form a layer without shaking. The interface was observed for a purple colour as indicative of positive test (Auwal et al., 2014).

- **Reducing Sugars**

The presence of reducing sugars (monosaccharides and some disaccharides), which have free ketone or aldehyde functional groups was determined by adding 5mL Benedict's reagent to 1 mL endophyte extract and it was consequently boiled for 5 minutes. The change in colour of the solution to blue, green orange, brick red indicates absence, trace, moderate, large amount of reducing sugars respectively.

- **Cardiac glycosides (Deoxysugars)**

  Keller-Kiliani test was performed to assess the presence of cardiac glycosides. The crude fungal extract was treated with 1 mL of FeCl3 reagent (mixture of 1 volume of 5% FeCl3 solution and 99 volumes of glacial acetic acid). To this solution a few drops of concentrated H2SO4 was added. Appearance of greenish blue colour within a few minutes indicated the presence of cardiac glycosides (Devi et al.,2012).

- **Anthraquinone Glycosides**

  Borntrager's reagent test was performed wherein 1 mL of extract was heated with equal volumes of 10% ferric chloride solution and conc. HCl and boiled in water bath for 10 minutes. The content was cooled and organic layer was filtered with chloroform. The trichloromethane extract was further extracted with strong ammonia. Pink or deep red coloration of aqueous layer indicate the presence of anthraquinones (Onwukaeme et al.,2007).

3. Production of Extracellular Enzymes

Extracellular enzyme assays were conducted to investigate the production of enzymes by the endophytic fungi. It was assessed by digestion of suspended or dissolved substrate in agar plates after inoculation with 3 mm mycelia plugs and incubation for 3-5 days at 37°C.

- **Laccase**

  Laccases (benzenediol: oxygen oxidoreductases; EC 1.10.3.2) exist widely in nature. They are predominantly found in higher plants and fungi (Mayer and Staples 2002) It is also used in the design of biosensors, biofuel cells, as a medical diagnostics tool and bioremediation agent to clean up herbicides, pesticides and certain explosives in soil. Laccases have the ability to oxidize both phenolic and nonphenolic lignin-related compounds as well as highly recalcitrant environmental pollutants (Shekher et al., 2011).

  GYP agar medium amended with 1-napthol, 0.005% at pH 6 were prepared and fungus was inoculated and kept for incubation. On oxidation of 1-napthol by laccase, the medium change from clear to blue (Devi et al., 2012).

- **Lipase**

  Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) catalyse the hydrolysis of long chain triacylglycerol substrates (with carbon number greater than 8). These lipolytic enzymes can be isolated from plants, animals, and microorganisms, however, microbial lipolytic enzymes are reported to be supposedly more robust in nature than plant or animal enzymes (Ramnath et al, 2017).

The fungus was grown on peptone agar medium supplemented with Tween 20. A clear zone around the colony indicates lipase positive fungus (Devi et al., 2012).

- **Protease**

Proteases are one of the most useful and powerful enzymes since they are capable of breaking down complex protein compounds into smaller amino acids and peptides chains via cleaving the peptide bonds (Flint et al., 2014).

The fungus was grown on GYP agar medium amended with 0.4% phenol and adjusted with pH 6. After incubation, plates were flooded with saturated aqueous ammonium sulphate. The undigested phenol was precipitated with ammonium sulphate and digested area around the colony was clear (Devi et al., 2012).

- **Amylase**

Starch-degrading, amylolytic enzymes are required for the hydrolysis of starch polysaccrides into its respective monomeric glucose units. These enzymes include alpha-amylase, glucoamylase, beta-amylase, alpha-glucosidase, pullulan-degrading enzymes, exo-acting enzymes yielding alpha-type end products, and cyclodextrin glycosyltransferase (Vihinen et. al, 1989).

The activity of amylase was measured by growing the studied fungi on glucose yeast extract peptone agar (GYP) with 0.2% soluble starch. After the incubation period, the dishes were flooded with reagent material consisting of iodine (1%) and potassium iodide (2%). The transparent circle surrounding the colony indicates the production of amylase (Sunitha et al., 2013).

- **Polyphenol Oxidase**

Polyphenol oxidases (E.C. 1.10.3.1) are widely distributed in plants, containing enzymes having two binding sites for phenolic substrates. PPO is mainly located in the chloroplast bound to thylakoid membranes and is activated after its release into the cytosol when plant tissues undergo physical damage such as bruising, cutting or blending. PPO has been reported from several plants, animals, fungi and bacteria (Gupta et al., 1981).

It was determined according to the Bavendamm's reaction (Conceição et al. 2005). Fungus was grown on AAYE medium. The dishes were incubated with a photoperiod of 12 h of light, for five days. The formation of an amber halo around the colony in the culture medium indicated a qualitative production of polyphenol oxidases (Cattelan 1999).

4.1 Antioxidant Activity

Many living things depend on oxidation and reduction reactions to produce the energy needed for biological processes. However, the ongoing production of oxygen free radicals and other reactive oxygen species (ROS) in vivo leads to tissue damage and cell death. These species have the ability to interact with biological materials including DNA and proteins, causing a variety of illnesses like cancer, diabetes, cardiovascular conditions, aging, arthritis, and atherogenesis (Halliwell 2007).

Antioxidants are essential compounds that shield living things from harm brought on by the unchecked generation of ROS and the ensuing lipid peroxidation, protein damage, and DNA strand rupturing. Recently, it has been demonstrated that a number of anti-inflammatories, antinecrotic, neuroprotective, chemo-preventive, and hepatoprotective medications also include antioxidant and radical scavenging mechanisms that contribute to their efficacy. Restoring the body's antioxidant reserves may help avoid oxidative stress and cancer (Lin et al.,2000).

- **DPPH Free Radical Scavenging Assay**

  DPPH or 2,2-diphenyl-1-picrylhydrazyl is a synthetic dark-coloured compound, consisting of stable free radical molecules and is widely used in monitoring reactions involving anti-oxidant activity.

  To test the same for endosymbionts in our study, DPPH assay was performed using their cell-free and aqueous extracts. To begin with, 0.1 mM DPPH was prepared in 95% ethanol. The standard solution prepared for reference value validation was composed of 1.5 ml of L-ascorbic acid (1mg/mL) and 1.5 ml DPPH solution, accounting for a 3ml total solution volume. The sample solutions for cell free extracts contained the 20µl cell-free extract in 1.48ml ethanol whereas, for aqueous extracts, the solution was prepared with 200µl aqueous extract in 1.3ml ethanol, to which 1.5ml DPPH was added later. In order to prepare the control, 1.5ml alcohol while 200µl NAM broth in 1.3 ml of ethanol was taken for CFE and aqueous respectively. All of these samples were well vortexed before incubating for 30 minutes in a dark environment. Anti-oxidant activity of all the cell-free extracts was then determined by their ability to decolourize DPPH, measured spectrophotometrically at 517nm (λ max). The experiment was performed in triplicate to ensure the reproducibility of the result (Senthil et al.,2017).

  The percentage of DPPH scavenging activity was calculated using the standard and the formula:

$$\% \text{ DPPH activity} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

  where, $A_{control}$ = Absorbance value observed for the controls of respective solutions
  A_{sample} = Absorbance value observed for samples and standard

4.2 Anti-inflammatory Activity Assay

A number of chronic diseases, including cancer, diabetes, neurological diseases, and arteriosclerosis, are directly linked to inflammation. Fungi have long been used extensively to alleviate inflammation because of their outstanding medicinal and nutritional properties. They are abundant in polysaccharides, phenol and indole compounds, steroids, fatty acids, carotenoids, vitamins, and metals, all of which have anti-inflammatory properties. They are employed because of their antagonistic effects on free radicals, cholesterol, viruses, tumors, and blood sugar and blood (Anusiya et al., 2021).

Numerous biologically active compounds, particularly polysaccharides, are discovered to have immune regulatory properties, which constitute a unique treatment strategy for immunotherapy against inflammation. Because autoantigens are produced during in vivo investigations, proteins are denaturized, and substances that prevent denaturation could be valuable in the search for new anti-inflammatory medications.

- **Protein denaturation Assay**

  The inhibitory effect on the denaturation of proteins can be accessed by using a heat-induced protein denaturation process using diclofenac sodium as the standard drug (Kamat et al., 2020).

  The anti-inflammatory activity was tested in aqueous and cell free extracts of the endosymbionts. The standard solution (0.5ml) contained 0.45ml bovine serum albumin (BSA, 5% w/v solution) and 0.05ml of diclofenac sodium. The stock concentration of diclofenac was 50mg/ml from which different concentrations (50, 100, 150, 200, 250, 300 µg/ml) were prepared. However, the samples were taken in concentrated form only to make the test solution. A test control solution (0.5ml) was prepared and consisted of an aqueous solution of 5% w/v bovine serum albumin (0.45ml) and distilled water (0.05ml). The product control solution (0.5ml) consists of (0.05ml) the test solution and (0.45ml) of distilled water. Using 1N HCl, all the solutions were adjusted to a pH of 6.3 and further incubated for 30 minutes at 37 C. After this, the samples were heated for 3 minutes at 57 C. 2.5 ml of phosphate buffer was then added after cooling the samples. Lastly, the absorbance was measured at 416 nm. The experiment was performed in triplicate to ensure reproducibility of the result. The results of the test sample were compared with diclofenac sodium (Ogunbiyi et al.,2021).

  The percentage inhibition of protein denaturation was calculated using the formula:

$$\% \text{ Inhibition} = (100 - A_{control} - A_{sample}) / A_{control}] \times 100$$

where, $A_{control}$ = Absorbance value observed for the control solution
A_{sample} = Absorbance value observed for samples/standard solution

4.3Anti-microbial Assay

An increase in the number of individuals in the world having health problems caused by various malignancies, drug-resistant bacteria, parasitic protozoans, and fungus is a reason for alarm. Finding new and innovative antimicrobials to treat human diseases has become more important as a result of the emergence of several drug-resistant bacteria. Endophytes are a fresh source of potentially beneficial pharmaceutical chemicals, and there is currently a significant effort on to find newer and more potent drugs to cope with these disease problems (Newman et al. 2003).

The anti-microbial assay is utilised to establish the inhibition of microbes on one another. This determines confirms the susceptibility to chosen antimicrobial agents and detects resistance in individual bacterial isolates. The method in use provides qualitative assessment of the property into consideration. The resistance mechanisms enable the microbiota to conquer and localise in a region alongside which it promotes co-evolution based on microbe-host interaction requirements. To establish the same, we can replicate the settings by preparation of a standard microbe lawn (Reller et al., 2009).

These studies aim to determine the antimicrobial activity of the extract fractions endophytic fungus against microbes. Long-term goal acquires potent new antimicrobial finding efficacious and safe so that it can be used as raw material for medicine.

- **Agar well diffusion method**

Antibacterial activity of secondary metabolites extracted from fungal endophytes was screened against Gram-negative bacterial pathogen *E. coli* using agar well diffusion method. A bacterial pathogen was spread on Muller Hinton agar (MHA) plates. Then wells were bore on the agar plates and 20 µL of aqueous extract were poured in separate wells with varying concentrations of 50%, 75%, 100%. Antibacterial activities were detected after an incubation of 96 hrs at 37 °C. The presence of zone of clearance on plates was used as an indicator of bioactive nature of the strain. As positive control, 1mg/mL streptomycin was used. The inhibition zone was measured in mm after incubation was complete (Sharma et al.,2016).

Activity index with respect to standard drug was calculated by using the following formula:

$$\text{Activity index} = I_{\text{sample}} / I_{\text{standard}} \times 100$$

where, I_{sample} = Inhibition zone by extract in mm

I_{standard} = Inhibition zone by standard drug in mm

5. Role in Plant Defence

Glucosinolates are organic substances found in plants in the Brassicaceae family, including mustard, cabbage, and horseradish. These come from the cysteine or the products of the cysteine biosynthesis pathway. These plants' pungency is caused by the presence of glucosinolates. These are in charge of protecting plants from herbivorous animals. Glucosinolates are degraded by the enzyme myrosinase to form isothiocyanates, which are harmful to insect herbivores because they bind to their digestive enzymes.

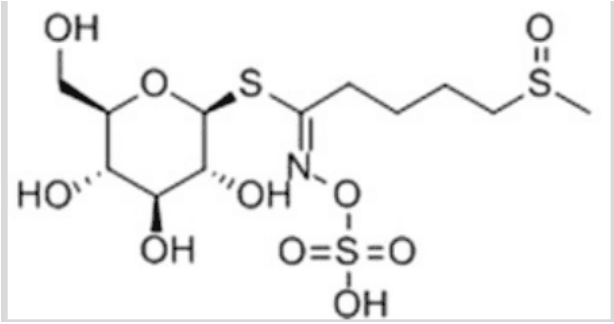


Figure 2: Structure of Glucosinolates

Some isothiocyanate derivatives are volatile, so insects steer clear of these plants by smelling them. The distinctive odour of *Brassica sp.* is caused by these volatile substances. Myrosinase and glucosinolates are kept in distinct compartments, but when tissue is injured, they mingle. In the human liver, glutathione detoxifies isothiocyanates (Bhatla et al., 2018).

- **Reduction of Ferricyanide Method**

  This method is based on the reduction of ferricyanide by the breakdown product, 1-thioglucose produced by the alkaline treatment of glucosinolates, and measurement of the absorbance at 420 nm.

  Take 500 mg ground sample in a glass test tube and pipette 7.5 mL of near boiling acetate buffer (0.2 M, pH 4.2). Keep the mixture in a boiling water bath for 15 min. After cooling (5 min), mix it with 1.5 mL of the barium and lead acetate solution and vortex thoroughly. Add to it about 0.4 g of insoluble polyvinylpolypyrrolidone, stir, and incubate the mixture at room temperature for 15 min. Then add 1.5 mL of sodium sulphate solution while stirring. Vortex it and centrifuge the content for 10 min at 4000 g and collect supernatant. For a blank run the above procedure, without the sample.

  Determination of Glucosinolates was done by mixing 0.9 mL of the clear supernatants (test and blank separately) with 0.9 mL of 2 M of NaOH, incubate it for 30 min at room temperature, and then add 0.138 mL of concentrated HCl (37%) to neutralize the solution. Centrifuge the resulting mixture (4000 g for 10 min). Take 0.5 mL of the supernatant and mix with an equal volume (0.5 mL) of potassium ferricyanide (2 mM) prepared in phosphate buffer (pH 7, 0.2 M). Vortex the mixture, centrifuge at 4000 g for 3 min, and measure the absorbance of the supernatant at 420 nm within 15 s against the blank. Convert the absorbance to the concentration using the following calibration curve.

  Preparation of the Calibration Curve was done by dissolving 10 mg sinigrin in 2 mL distilled water. From this, take 0 to 500 µL and add the phosphate buffer to reach the final volume of 500 µL. Add 500 µL of potassium ferricyanide in each tube, mix thoroughly, and centrifuge at 4000 g for 3 min. Collect the supernatant and measure the absorbance at 420 nm against the phosphate buffer pH 7.0 (Makkar et al.,2007).

6. Role in Plant Growth Promotion

Phytohormones are chemical messengers having small molecular size and are secreted in low concentrations. They are known to act as versatile regulators of growth, developmental, and defense processes in plants. Based on the status and developmental level of cellular context, phytohormones interact both synergistically and antagonistically. Functionally phytohormones can be distinguished into two categories: (1) developmental hormones, i.e. cytokinins, brassinosteroids, auxins, strigolactones and gibberellins, and (2) stress-related hormones, i.e. salicylic acid, jasmonic acid, abscisic acid and ethylene.

Endophytic fungi can produce auxins, gibberellins and cytokinins. The potential of phytohormone production by endophytic fungi is underexplored, especially for gibberellins, even though these molecules are as important as chemical signalling and messengers for plant growth in different conditions (Khan et al.2015).

● Indole-3-Acetic Acid

The main auxin produced by fungi is indole-3-acetic acid (IAA). Auxins are the main regulators of plant growth and present several positive effects on shoot and root development, such as the responses of tropism, division and cell elongation, differentiation of vascular tissue and initiation of the root formation process. IAA produced by fungi can induce lateral root and root hair development. In IAA produced by fungi can defeat pathogenic strains and disease progression by enhancing the plant's immune response. Auxin is suggested to play a role in the cross-talk between plant and fungal signalling during ectomycorrhizal establishment (Cosoveanu et al.,2021).

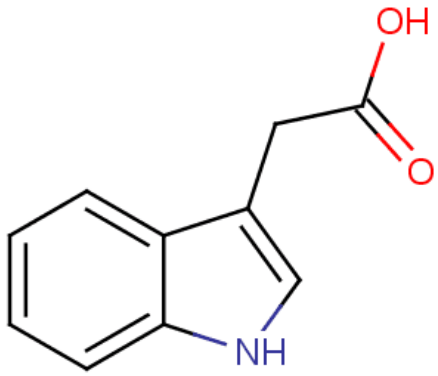


Figure 3: Structure of IAA

Modified Salkowski's Method

To determine the amount of IAA produced by each endophyte, a spectrophotometric technique was performed using the modified Salkowski's method (Bhagobaty et al.,2009). The endophytes were cultured in 100 mL of broth with 1000 µg/ml of L-tryptophan and incubated at 30 ± 2 °C at 120 rpm in shaking incubator until optimum growth. The broth cultures were centrifuged at 5000 rpm, 4 °C for 25 minutes, 4 mL of Salkowski's reagent (3 ml of 0.5 M $FeCl_3 \cdot 6H_2O$, 100 ml of distilled H_2O, 60 ml of concentrated H_2SO_4) and 1 mL of supernatant were suitably mixed. (Junaidi et al., 2017). As a negative control, 1 mL of uninoculated broth with 4 mL of Salkowski reagent was taken. After 30 minutes incubation in the dark at room temperature, the colour change from colourless to pink demonstrated a positive reaction in the extract, the absorbance was measured at 530 nm using a UV-vis spectrophotometer.

IAA present in potential endophytes was calculated using the standard curve of known IAA concentrations (20 –900 µg/mL) prepared in methanol and analysed identically against OD. The complete experiment was conducted in triplicates (Maheshwari et al.,2020).

- **Gibberellic Acid**

Gibberellic acids (GAs) are ubiquitous plant substances, eliciting various metabolic functions needed in plants' growth and development. Chemically, gibberellins are diterpenoid carboxylic acids; GA1 and GA4 are predominant functional forms. GA influences the physiological processes of seed germination, leaf expansion, stem elongation, flower and trichome initiation, and fruit development.

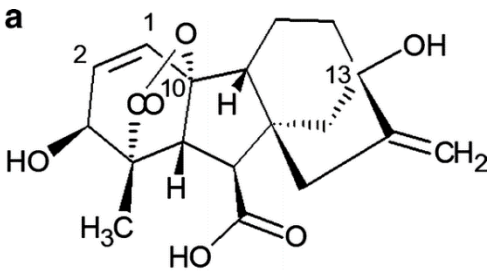


Figure 4: Structure of GA3

It is an important phytohormone that can impart stress tolerance in salt, heat, cold and drought stress in many crop plants (Cosoveanu et al.,2021).

Gibberellins produced by plant growth promoting endophytes promotes the plant growth and increases yields of many crop plants. Among all, 136 GAs have been identified from higher plants (128 species), 28GAs from fungi (7 species), and only 4GAs (GA1, GA3, GA4, and GA20) from bacteria (7 species). Gibberellic acid (GA3), the main product of gibberellins in fungi and bacteria, is a terpenoids hormone that is an important phytohormone regulating plant growth and development (MacMillan, 2002).

DNPH (2,4-Dinitrophenyl hydrazine) Method

For estimation of Gibberellic Acid, for GA3 estimation equal volume of aqueous extract was dissolved in absolute alcohol (Zeigler et al., 1980). 2 ml of this suspension was mixed with 1 ml of DNPH and incubated at 100 °C for 5 min and cooled in water bath. To this, 5 ml of 10% potassium hydroxide was added and allowed to stand till red wine colour developed. 15 ml of sterile distilled water was added and finally, the content was diluted to 1:2 using sterile distilled water. Colour intensity was measured at 430 nm in UV-vis Spectrophotometer. For standard curve, different aliquots of standard gibberellic acid (0.8 mg/ml) were prepared using absolute alcohol and estimated similarly (Desai et al.,2017).

RESULTS

1.1Identification of Fungal Endophytes

Colony Characteristics / Fungi	Growth on PDA in 72 hrs	Shape/Form	Elevation	Margin	Colour		Texture
					Body	Margin	
PWEL 1031	+	Circular	Convex	Filiform	Green	Creamish-Yellow	Coarse
PWEL 1032	+	Circular	Raised	Filiform	Green	Creamish-Yellow	Coarse
PWEL 1033	+	Circular	Convex	Filiform	Green	Creamish-Yellow	Coarse
PWEL 1034	+	Circular	Raised	Entire	Green	Green	Coarse
PWEL 104	+	Filamentous	Flat	Undulate	Green	Green	Velvety

Table 1 – Macroscopic Morphology of Fungal Isolates

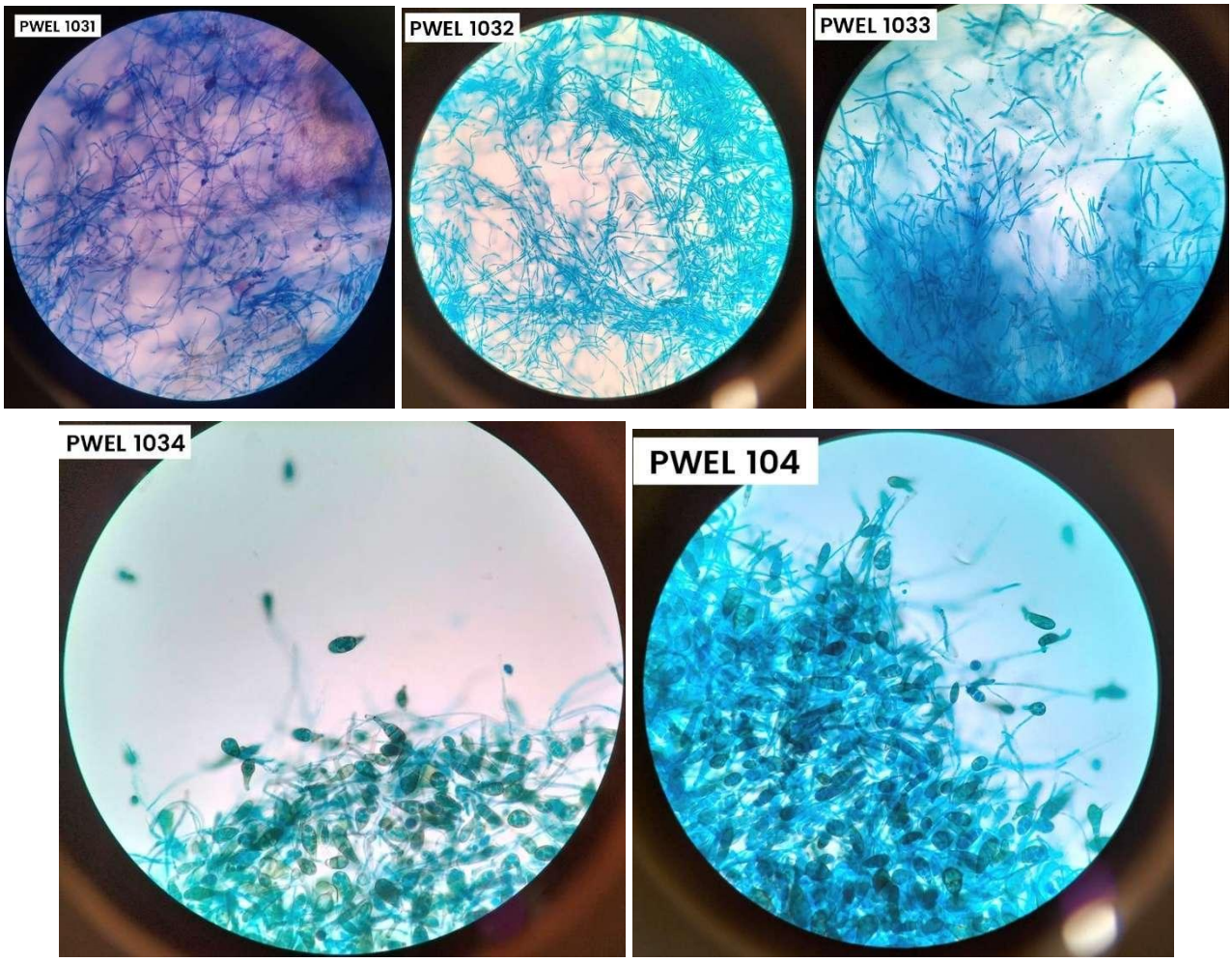


Figure 5 - Fungal Isolates stained by lactophenol cotton blue under Microscope

1.2 Biochemical characterisation

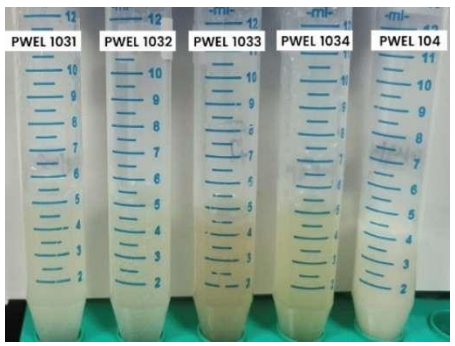
Biochemical Tests/ Fungi	PWEL 1031	PWEL 1032	PWEL 1033	PWEL 1034	PWEL 104
Triple Sugar - Iron Agar Test	-	-	-	-	-
Catalase Test	+	+	+	+	+
Oxidase Test	-	-	-	-	-
Citrate Test	+	-	+	-	+
Urease Test	+	-	-	+	+
1M Salt Tolerance	-	-	-	-	-
2M Salt Tolerance	-	-	-	-	-
pH 4 Tolerance	-	-	-	-	-
pH 10 Tolerance	-	-	-	-	+

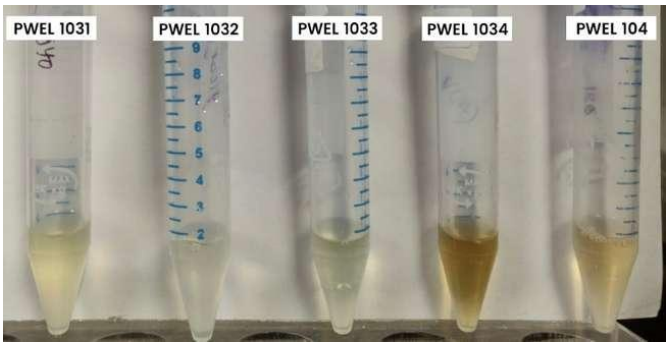
Table 2 – Observations and Results of Biochemical Tests of Fungal Endophytes

2. Phytochemical Assays

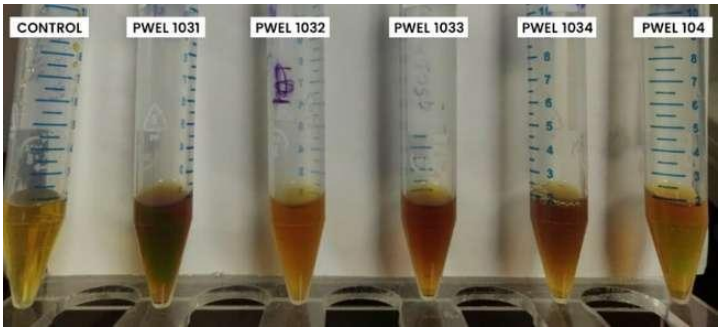
Phytochemical Tests/ Fungi	PWEL 1031	PWEL 1032	PWEL 1033	PWEL 1034	PWEL 104
Saponins	-	-	-	-	-
Tannins	+	-	-	-	+
Flavonoids	+	-	-	+	+
Steroids	+	+	+	-	-
Alkaloids	+	+	+	+	+
Gums (Polysaccharides)	+	+	+	-	+
Reducing Sugars	+	+	+	-	+
Cardiac glycosides (Deoxy Sugars)	+	+	+	-	-
Anthraquinone Glycosides	+	-	+	+	-

Table 3 – Observations and Results of Phytochemical Tests of Fungal Endophytes

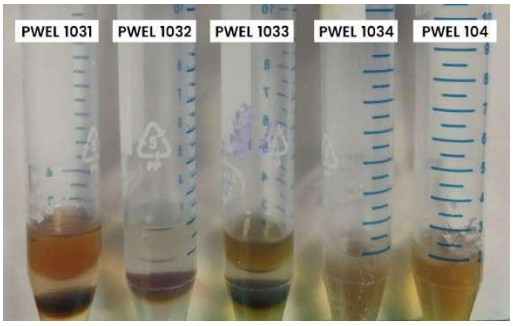




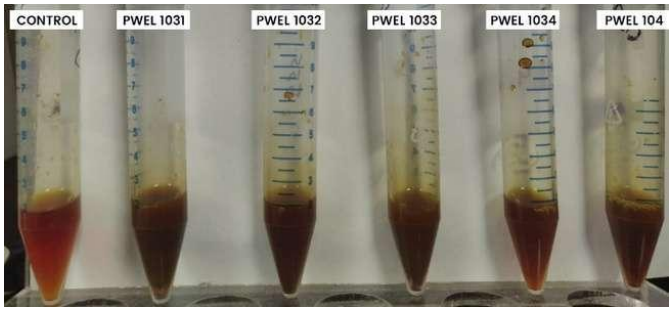
Saponins



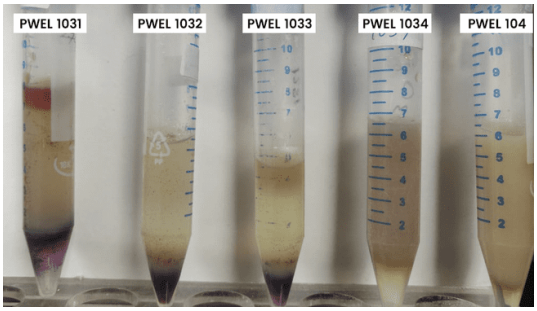
Tannins



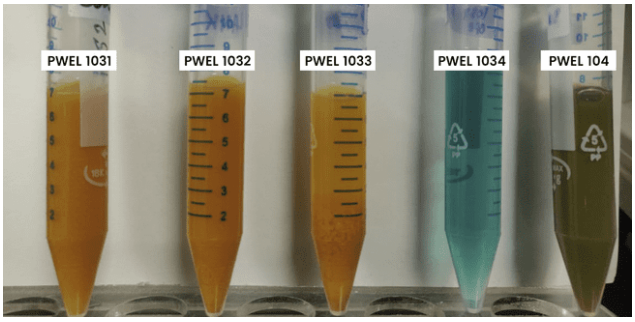
Flavonoids



Steroids

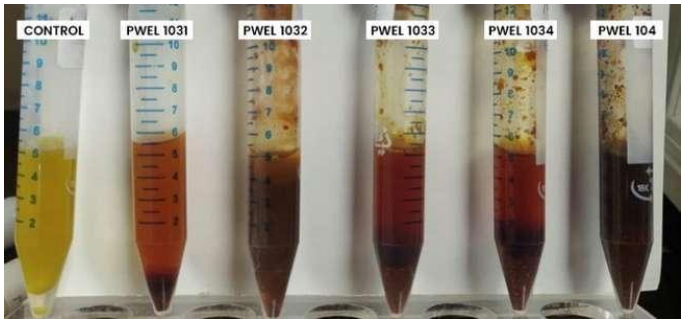


Alkaloids



Polysaccharides

Reducing Sugars



Anthraquinone Glycosides

Figure 6 – Results of Phytochemical Assays on Fungal Endophytes

3. Production of Extracellular Enzymes

Biochemical Tests/ Fungi	PWEL 1031	PWEL 1032	PWEL 1033	PWEL 1034	PWEL 104
Laccase	+	+	+	+	+
Lipase	-	-	-	-	+
Protease	-	-	-	-	-
Amylase	+	+	+	-	+
Polyphenol Oxidase	-	-	-	-	+

Table 4 – Observations and Results of Production of Extracellular Enzymes in Fungal Endophytes

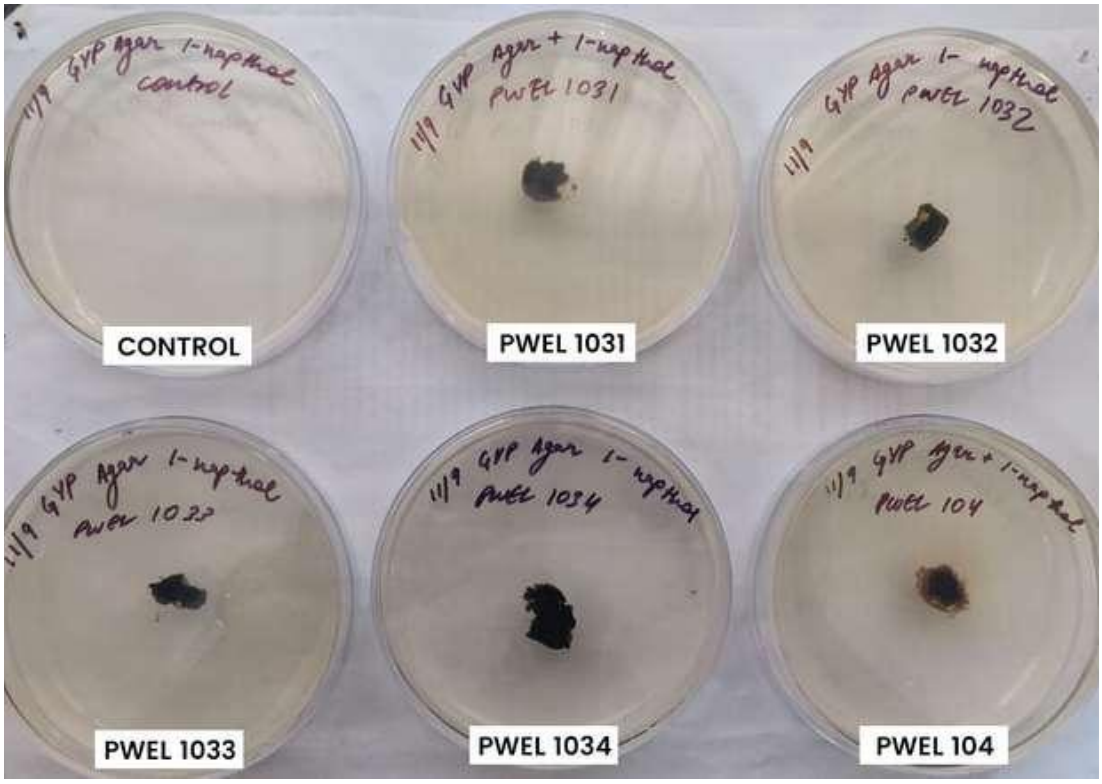


Figure 7 – Results of Laccase Activity in Fungal Endophytes

Figure 8 – Results of Lipolytic Activity in Fungal Endophytes

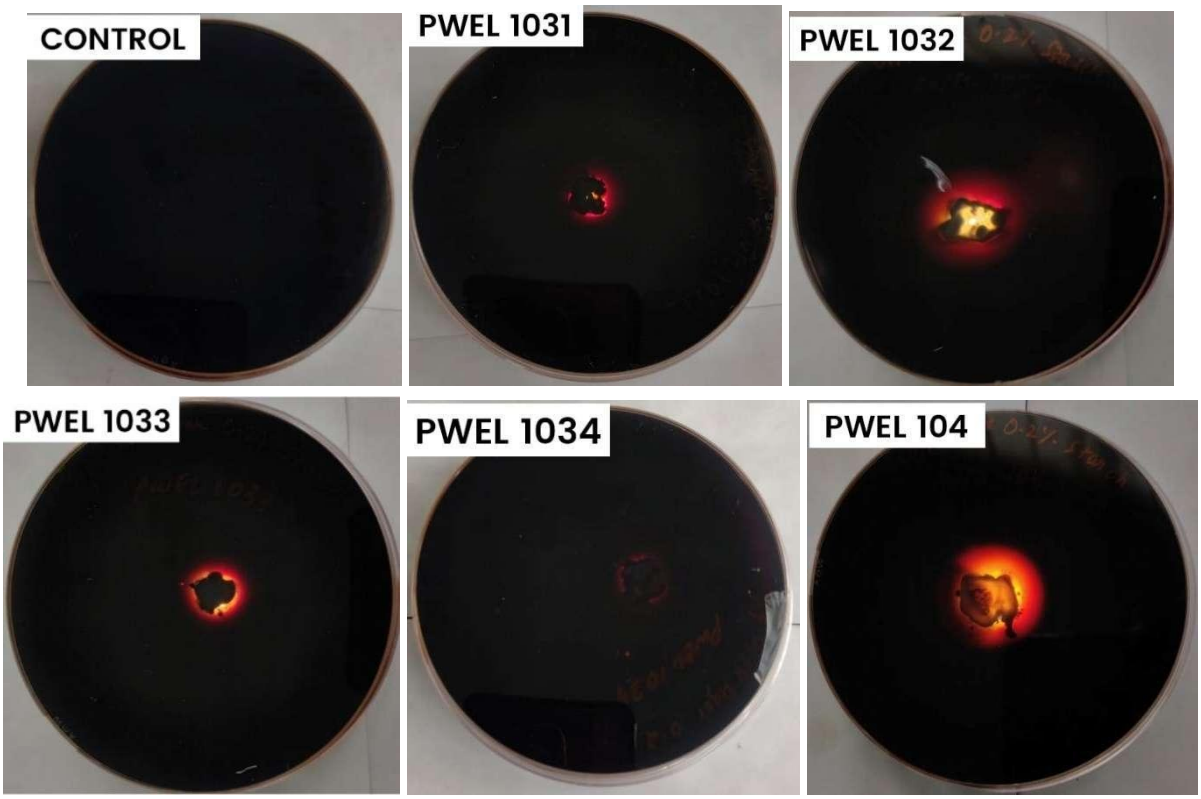


Figure 9– Results of Amylolytic Activity in Fungal Endophytes

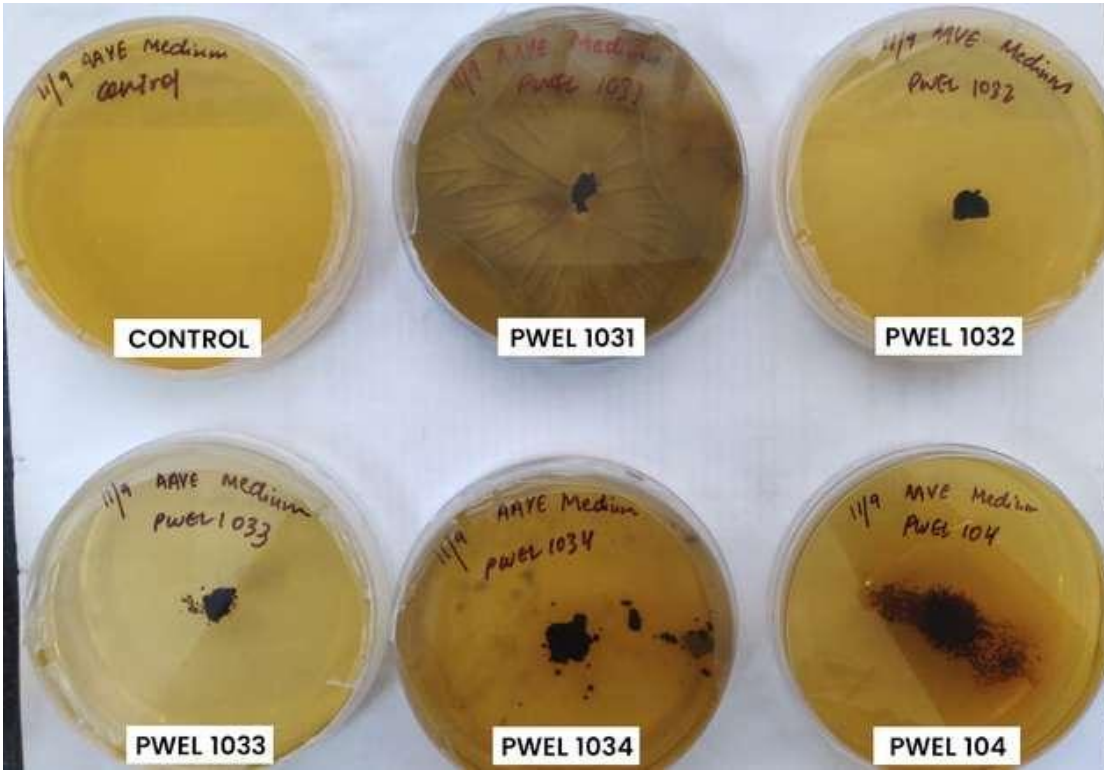


Figure 10– Results of Polyphenol Oxidase Activity in Fungal Endophytes

4.1Antioxidant Activity

Test Sample	Absorbance @517nm	% Radical Scavenging	Absobance @517nm	% Radical Scavenging	Absorbance @517nm	% Radical Scavenging	Average % Radical Scavenging
	R1		R2		R3		
AqE							
PWEL1031	1.308	-131.915	1.456	-158.156	1.366	-142.199	-144.090
PWEL1032	0.698	-23.759	0.773	-37.057	0.684	-21.277	-27.364
PWEL1033	0.678	-20.213	0.711	-26.064	0.658	-16.667	-20.981
PWEL1034	0.143	74.645	0.133	76.418	0.222	60.638	70.567
PWEL104	0.789	-39.894	1.447	-156.560	0.677	-20.035	-72.163
CFE							
PWEL1031	0.77	-36.525	0.746	-32.270	0.71	-25.887	-31.560
PWEL1032	0.676	-19.858	0.747	-32.447	0.825	-46.277	-32.861
PWEL1033	0.134	76.241	0.127	77.482	0.131	76.773	76.832
PWEL1034	0.613	-8.688	0.689	-22.163	0.734	-30.142	-20.331
PWEL104	0.699	-23.936	0.669	-18.617	0.827	-46.631	-29.728

Control	0.564						
Standard	0.061						89.184

Table 5 – Observations and Results of the DPPH assay for Aqueous extracts (AqE) and cell-free extract (CFE)

From the above observations, one can certainly observe that the cell-free extract of PWEL 1033 and the aqueous extract of PWEL 1034 has the highest inhibition activity. While other cell free extracts and aqueous extracts have minimal to no inhibition activity.

4.2Anti-inflammatory Activity Assay

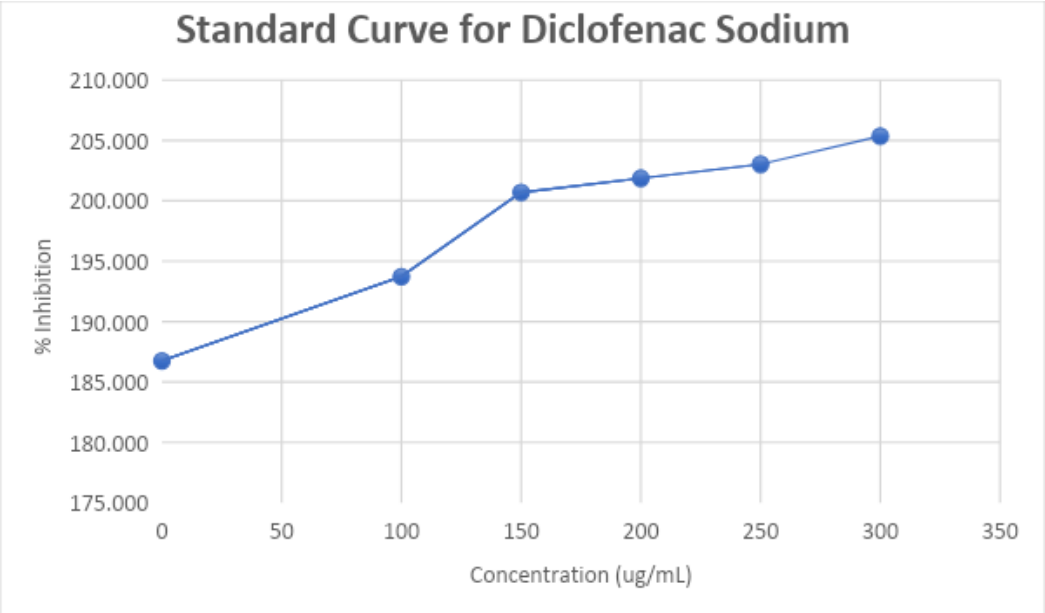
Test Samples	Absobance @416nm	% Inhibition		% Inhibition		% Inhibition	Average % Inhibition
	R1	R1	R2	R2	R3	R3	
AqE							
PWEL1031	0.099	206.516	0.096	203.028	0.094	200.702	203.416
PWEL1032	0.086	191.400	0.085	190.237	0.094	200.702	194.113
PWEL1033	0.093	199.540	0.103	211.167	0.098	205.353	205.353
PWEL1034	0.103	211.167	0.097	204.191	0.101	208.842	208.067
PWEL104	0.09	196.051	0.087	192.563	0.09	196.051	194.888
CFE							
PWEL1031	0.082	186.749	0.084	189.074	0.09	196.051	190.625
PWEL1032	0.083	187.912	0.079	183.260	0.085	190.237	187.136
PWEL1033	0.095	201.865	0.093	199.540	0.095	201.865	201.090
PWEL1034	0.086	191.400	0.088	193.726	0.086	191.400	192.175
PWEL104	0.081	185.586	0.082	186.749	0.081	185.586	185.974
Control	0.086						191.400

Table 6 – Observations and Results of the Protein Denaturation assay for Aqueous extracts (AqE) and cell-free extract (CFE)

Concentration (ug/mL)	Absorbance @416nm	% Inhibition
0	0.082	186.749
100	0.088	193.726
150	0.094	200.702
200	0.095	201.865
250	0.096	203.028

300	0.098	205.353

Table 7 – Observations for Standard Curve for Diclofenac Sodium



Graph 1 - Standard Curve for Diclofenac Sodium

From the above observations, one can certainly observe that most of the cell free extract and aqueous show % Inhibition equivalent to the standard drug. The cell free extract of PWEL 1033 and the aqueous extract of PWEL 1034 has the highest inhibition activity.

4.3Anti-microbial Assay

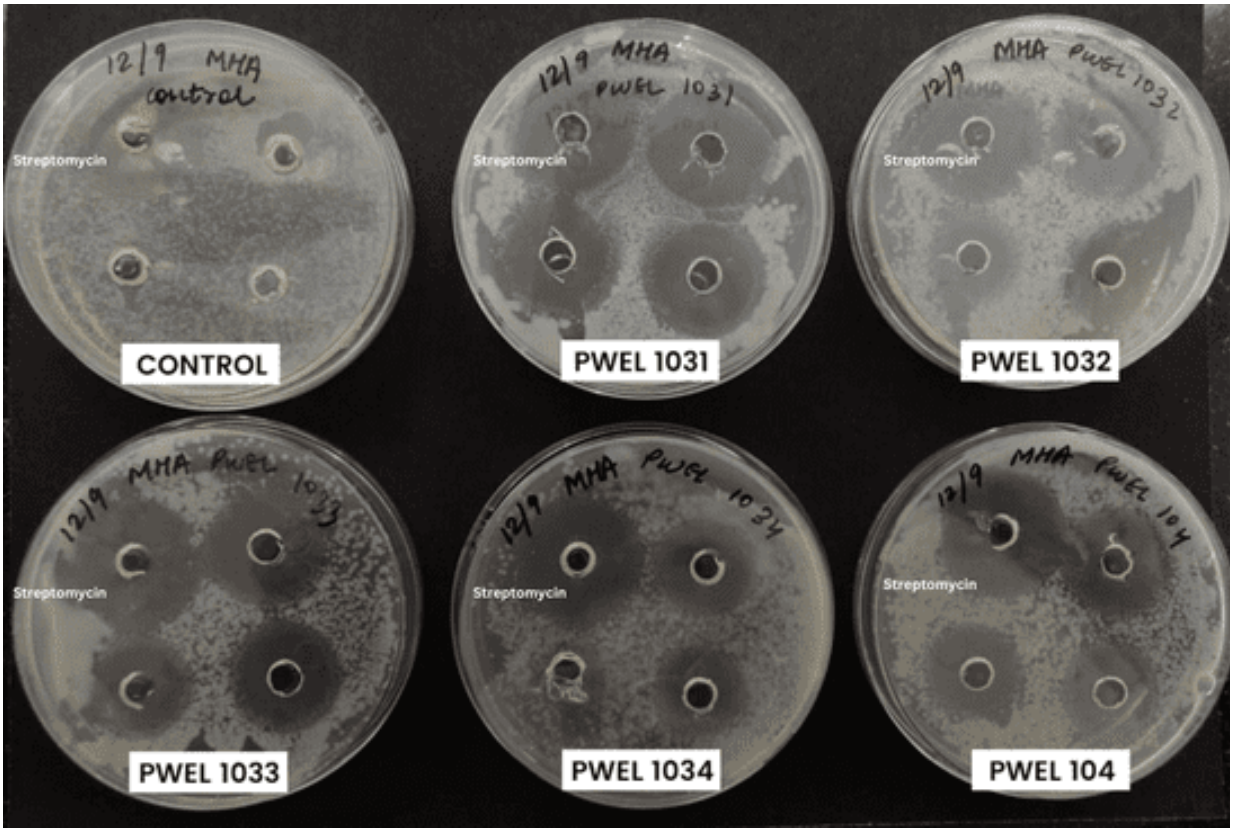


Figure 11 – Observations of the anti-microbial assay (against *E.coli*)

Fungal Endophytes	Inhibition Zone(mm) Streptomycin 1mg/mL	Inhibition Zone(mm) 100% Extract	Activity Index	Inhibition Zone(mm) 75% Extract	Activity Index	Inhibition Zone(mm) 50% Extract	Activity Index
PWEL1031	30	31	103.333	29	96.667	24	80.000
PWEL1032	30	28	93.333	22	73.333	20	66.667
PWEL1033	31	30	96.774	26	83.871	21	67.742
PWEL1034	30	21.5	71.667	20	66.667	14	46.667
PWEL104	30	28	93.333	22	73.333	21	70.000

Table 8 – Results of the anti-microbial assay (against *E.coli*), all concentrations showing considerable activity relative to standard drug

5. Glucosinolates

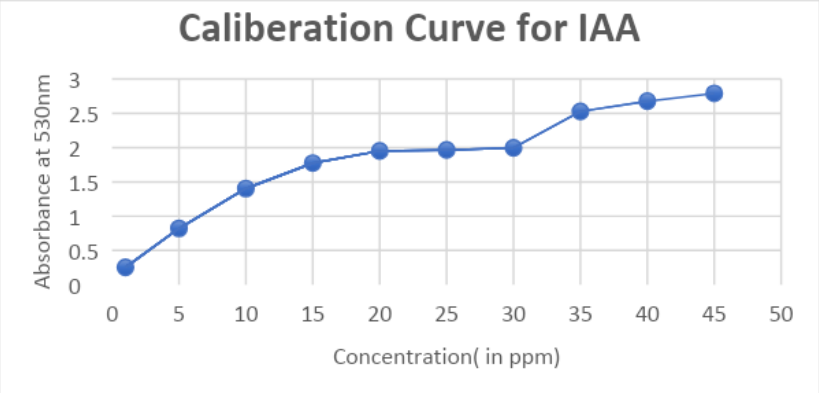
Test Samples	Absorbance at 420nm	Presence of Glucosinolates
PWEL1031	-0.006	-
PWEL1032	0.13	-
PWEL1033	0.026	+
PWEL1034	-0.019	+
PWEL104	0.142	+
Positive Control (Plant Source)	0.038	+

Table 9– Observations and Results of Reduction of Ferricyanide Method for Aqueous extracts (AqE) according to the calibration curve of the standard drug (Sinigrin)

6.1Indole-3-Acetic Acid

Test Samples	Absorbance at 530nm	Presence of IAA	Calculated concentration (ppm)
PWEL1031	0.368	+	19.909
PWEL1032	0.503	+	22.535
PWEL1033	0.7	+	26.368
PWEL1034	0.401	+	20.551
PWEL104	0.141	-	15.492
Control	0.126		15.200

Table 10– Observations and Results of Modified Salkowski Method for Aqueous Extracts (AqE)



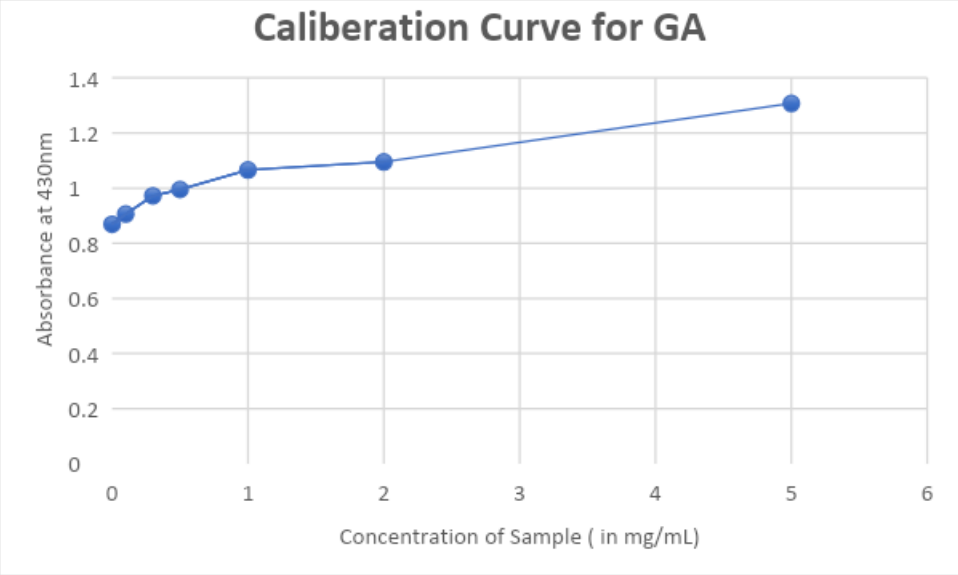
Graph 2 – Standard Curve for IAA
y = 0.0514x + 0.6553
R^2 = 0.9081

From the above observations, one can certainly observe that 4 out of 5 endophytes' aqueous extracts show production of indole acetic acid equivalent to the standard drug. The extract of PWEL 1033 has the highest activity.

6.2 Gibberellic Acid

Fungal Endophytes	Absorbance at 430nm	Presence of GA	Calculated concentration (mg/mL)
PWEL1031	1.295	+	13.062
PWEL1032	1.272	+	13.039
PWEL1033	1.239	+	13.006
PWEL1034	3.027	+	14.794
PWEL104	1.74	+	13.507
Positive Control	1.243		13.010
Blank	0.838		12.605

Table 11– Observations and Results of **DNPH** Method for Aqueous Extracts (AqE)



Graph 3 – Standard Curve for GA
$y = 0.079x + 0.9296$
$R^2 = 0.9271$

From the above observations, one can positively observe that all extracts of endophytes show the production of gibberellic acid equivalent to the standard drug and a better activity than positive control. The extract of PWEL 1034 has the highest activity.

DISCUSSION

The interaction between the endosymbionts and their host organism tends to co-facilitate with each other and provides both nutritional support and environmental protection against stress. They demonstrate characteristics including enzyme degradation, phytochemical, secondary metabolites, phytohormones production, anti-oxidant, anti-inflammatory, and anti-microbial action to maintain competitiveness within numerous microbial colonies and contribute to their functionalization.

The anti-microbial activity allows a microbe to colonize and preferentially access the source of nutrition. In our analysis, all fungal strains observed in the leaves of *Brassica* have been identified with the proposed property. These strains also show proteolytic, lipolytic as well and amylolytic activities which supposedly contribute to the overall anti-microbial tendencies. Leaf microbes protect the organism from pathogenic attacks and add to the overall medicinal property of the plant.

These endophytic fungal compounds revealed antioxidant activity. Therefore, the compounds produced by these endophytic fungi may be considered safe and can become alternatives to commercial antimicrobial agents.

The strains also exhibit a significant degree of protein denaturation that regulates anti-inflammatory activity. Hence anti-arthritic activities of strains were concentration dependent which confirms the anti-arthritic activity of the extract against the denaturation of protein. It may be well recognized that free radicals are critically involved in various pathological conditions like cancer, arthritis, inflammation, and liver diseases (Senthil et al.,2017).

The results of the preliminary phytochemical study are given in Table 3 and indicated some secondary metabolites found in fungal extract. Glycosides play an important role in predation by microorganisms, insects, and herbivores. Eventually, saponins can affect bacterial growth due to their ability to cause leakage of proteins and certain enzymes from the cell. Tannins have antidiarrheal properties and may precipitate proteins on enterocytes, inhibiting peristaltic action and intestinal output. Flavonoids have antioxidant action, which protects cells from oxidative damage and lowers the chance of developing certain malignancies. Steroids are capable of decreasing inflammation and reducing the activity of immune response and most strains are positive for it (Auwal et al., 2014).

In conclusion, the microbes under consideration showcase a wide range of attributes that not only enhance their chances of survival but can be a beneficial tool for aid in plant growth and defense and also be utilized for therapeutic purposes as well.

Future advancements in medicine could greatly benefit from the microbiological world of plants. The ability of fungal endophytes to produce bioactive compounds with a variety of properties, including those that are antimicrobial, anticancer, antioxidant, anti-inflammatory, antidiabetic, immunomodulatory, and cardio-protective, has drawn significant attention from the scientific community (Gupta et al.,2023). This demonstrates how important fungal endophytes are as a bioresource for the creation of new medicines and other biotechnology-based products.

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