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Original Article

Bioprospecting of Endophytic Fungi from *Phyllanthus acidus* Linn. Leaf, Identification of Secondary Metabolite: Quercetin

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ABSTRACT

Phyllanthus acidus (L.), a medicinal plant, shows numerous pharmacological properties like antioxidant, anti-inflammatory, hypoglycemic effects, neuroprotective, anti-diarrheal, anti-pyretic, and analgesic and anaesthetic effects which may be attributed to the bioactive compounds produced by *Phyllanthus acidus* or due to associated endophytic fungi. In the current work, *Phyllanthus acidus* L. leaf samples were analysed for the presence of endophytic fungi and to produce quercetin, a beneficial chemical, from them using an optimal surface sterilising process and analytical techniques., five fungal endophytes were isolated and they were preliminarily identified by morphology. According to morphological features of isolated endophytes identified as *Aspergillus* sp. Aff and *Chaetomium* sp. Aff. Interestingly, only leaves were used to isolate all five fungus, and in response to selected plant samples, the overall colonisation frequency from surface sterilised leaves was found to be 7.5%. Shake flask culture approach used for fermentation followed by extraction and purification process. Analytical and thin-layer chromatography, HPLC of ethyl acetate extract of isolated fungal endophytes PAEF3 showed a distinct phytochemical fingerprinting profile.The fact that quercetin, a key component of *Phyllanthus acidus* L., is used for a variety of properties like Antiviral, Anticancer, and Antidiabetes, makes this study of practical significance as well.

Keywords: Explant, Surface sterilization, Endophytic fungi, Morphological features, Statistical analysis.

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INTRODUCTION

Endophytes are microbes that grow intracellularly in plant tissues and are a significant source of biological natural product synthesis.[1] Bacteria or fungus colonise the interior plant tissues underneath the epidermal cell layers known as endophytes, which do not appear to harm host which show symbiotic association. The endophytic fungi of plants were a significant source of secondary metabolites, bioactive substances that are beneficial for the pharmaceutical industry and other sectors like Food and textile industries. These molecules include insecticides, antimicrobials, antioxidants, and antipyretics.[2] Numerous distinctive bioactive metabolites, such as alkaloids, benzoquinones, flavanoids, phenols, stereoid, terpenoids, tetralones, and xanthones, have been discovered to be produced by endophytic fungi.[3,4] Botanical characteristics of *Phyllanthus acidus* Linn. explained in Table 1.

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MATERIALS AND METHODS

In the present study, endophytic fungi were isolated from healthy/ disease free leaf tissue of *Phyllanthus acidus* Linn. or *Acidus distichus* [phyllanthaceae family] endophytic from 18.5204° N, 73.8567° E and A survey to the fields were done and healthy-looking plants explat: leaf part were collected and in sterile bags, they were transported to the lab and processed there for a second round of isolation within two hours. A voucher specimen of the plant species has been deposited at Agharkar Research Institute Pune, Maharashtra, India.

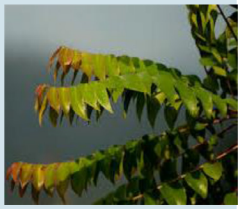
Optimization of surface sterilization methodology for endophytic fungi isolation

By using a modified technique and an enhanced surface sterilisation process, the endophytic fungi were separated whole process shown in Figure 1.[6]

Optimization of Surface sterilization of *Phyllanthus acidus* Linn. Leaf and isolation of endophytic fungi

As per shown in above Figure 1 different surface sterilizing agents like 70% ethanol, 4% sodium hypochlorite and 0.1% mercuric

Table 1: Botanical characteristics of *Phyllanthus acidus* Linn.[5]

Phyllanthus acidus Linn is a small, glabrous tree up to 10 m		
Branch	Phyllanthoid bark rough, grey, lenticels prominent.	
Leaf [Selected for isolation of endophytic fungi]	Pinnate, 20-40 cm long. Leaflets alternate, simple, entire, shortly petiolate, broadly ovate to ovate-lanceolate, apex acute, petiole 2.5-4 mm long, stipules triangular-acuminate.	
Flowers	Small, pink.	
Fruit	Drupaceous, oblate, 6 to 8 lobed, greenish yellow.	
Seeds	Grooved stone.	

chloride solution were used, from above surface sterilizing agents and various time of treatment; 1% mercuric chloride ($HgCl_2$) solution for 30 sec after 70% ethanol was chosen. due to no evidence found for contamination when experiment caried out as shown in Table 4.[7]

Statistical analysis for isolated endophytic fungi colony

As shown in Figure 1 the isolation process carried for endophytic fungi and the colonization, relative frequency were calculated as per follows[8] by observation of PDA plates.

Colonization frequency

It was done as follows to calculate the colonisation frequency percentage (CF%).

$$CF(\%) = \text{Number of species isolated} \div \text{Total number of segments screened} \times 100$$

This formula was used to determine the EIR percentage:

$$EIR(\%) = \text{Number of infected segments of leaf} \div \text{Total number of segments screened} \times 100$$

Morphological identification of isolated endophytic fungi by microscopy methodology[9,10] isolates of fungi The morphological characteristics of PAEF1, PAEF2, PAEF3, PAEF4, and PAEF5 have been identified, comprising colony growth, the presence or absence of aerial mycelium, colony colour, the presence of wrinkles and furrows, and pigment production etc. in reference to Barnett, 1992.

With the use of lactophenol with a 5 min heating treatment, isolated endophytic fungi were examined under a microscope with 40X and 100X resolution using Labomed microscope.

Preservation of isolated endophytic fungi

Isolated fungal Colonies were preserved for a long time at -80°C in tubes containing 15% sterile glycerol.[11,12]

Fermentation and metabolites extraction[13-16]

The isolated endophytic fungi PAEF1, PAEF2, PAEF3, PAEF4, PAEF5 were cultivated in Potato Dextrose Broth (PDB) fermentation further used for extraction process details as shown in Figure 2.

After the pre-determined incubation period, the culture broth was filtered using sterile Whatman filter paper to remove the mycelia. In a solvent extraction process, the metabolites of the endophytic fungi PAEF1, PAEF2, PAEF3, PAEF4, and PAEF5 were extracted using ethyl acetate as an organic solvent. Equal parts of ethyl acetate were added to the filtrate, shaken vigorously for 15–20 min, and then set aside until two distinct clear immiscible layers arose. The upper layer i.e organic layer containing the extracted biochemicals was separated using a separating funnel. The solvent was evaporated to yield the crude metabolite, and the product that was extracted was then dried in a rotator vacuum evaporator.

Preliminary phytochemical screening for isolated endophytic fungi extract for PAEF1, PAEF2, PAEF3, PAEF4, PAEF5[17,18]

The formed Endophytic fungi extract for PAEF1, PAEF2, PAEF3, PAEF4, PAEF5 utilised for phytochemical analysis of alkaloids, flavonoids, glycosides, volatile oils, saponins.

A survey of the literature revealed that *Phyllanthus acidus* has a wide range of phytoconstituents, including alkaloids, tannins,

Figure 1: Stepwise procedure for surface sterilization of leaf part of Phyllanthus Acidus Linn for isolation of endophytic fungi.[6]

flavonoids, and terpenoids,all of which have been demonstrated to have advantageous biological effects, such as antioxidant, anti-inflammatory, hypoglycemic effects, neuroprotective, anti-diarrheal, anti-pyretic, and analgesic and anaesthetic effects.[19-24]

Numerous potential bioactive molecules like quercetin, kaempferol, epicatechin, coumaric, hypogallic acid, gallic acid, adenosine and cinnamic acids were identified in *Phyllanthus acidus* leaves.[25,26]

Characterization of extracts[27-29]

Therefore the extract obtained after fermentation process further used for UV analysis, Infrared Spectroscopy (IR), Thin Layer Chromatography (TLC), High Performance Thin Layer Chromatography.

PAEF3 extract was used for further processing since it contains Quercetin, according to preliminary testing and UV analysis. Steps for separation as follow:

- Concentration and hydrolyzation by 7% H_2SO_4 (5 hr).
- Filteration and extraction with ethyl acetate (1:1/ 3 times).
- Concentration to get the crude form Quercetin.
- 10% ethanol utilized for Crystallization.

Table 2: Optimization of solvent system used for isolation of quercetin from PAEF03[25-28]

Solvent system used for TLC development	Ration for solvent
N butanol-Acetic Acid-Water	4:1:5
Ethyl Acetate Saturated with Acetic Acid: Water	6:4
Acetic Acid, HCl, Water	10:3:30
Butanol, Acetic Acid, Water	4:1:5
Ethyl Acetate, Toluene, Formic Acid	4:3.5:0.5

Table 3: HPLC Conditions.

Sl. No.	Parameter	Set Value
1	Injection Volume	10 ul
2	Run Time	30 min
3	Column name	C185 u
4	Flow Rate	1 mL/min
5	Detection	PDA 220nm

UV Spectroscopy

Quercetin solution: made by dissolving 10 mg of quercetin in 10.0 mL of ethanol in a volumetric flask. Which used for comparison with extract prepared from isolated endophytic fungi.

Table 4: Surface sterilization method optimization for *Phyllanthus acidus* Linn for isolation of endophytic fungi.

Sl. No.	Surface sterilization agents used for isolation of endophytic fungi	Time of treatment [sec]	Observations (In term of successful treatment for isolation of endophytic fungi with no contamination in petriplate which compare with control)
1	0.1% mercuric chloride ($HgCl_2$) solution	30 sec	+
2	0.1% mercuric chloride ($HgCl_2$) solution	60 sec	+
3	4% sodium hypochlorite (NaOCl)	180 sec	+
4	70% ethanol +4% sodium hypochlorite (NaOCl)	60 sec + 180 sec	+
5	70% ethanol + 0.1% mercuric chloride ($HgCl_2$) solution	30 sec each	-
6	70% ethanol	30 sec	+

*+ indicate presence of contamination after surface sterilization.
*- indicate absence of contamination after surface sterilization.

Table 5: Statistical analysis for isolated endophytic fungi colony.

Sl. No.	Name of the Plant	Number of infected segments	EIR (%)	CF (%)	Total number of segments	Number of sporulative species	Endophytic fungi isolated
1	*Phyllanthus acidus* (L.)	25	25/35*100= 71.42	5/35*100=17.5	35	5	5

Infrared Spectroscopy (IR)

The sample was made by combining the separated portion of quercetin with KBr (1:100), and then it was analysed using infrared spectroscopy.

Thin Layer Chromatography (TLC)[27-29]

The 3.0 × 8.0 cm precoated TLC plate was activated in a hot air oven for 30 min at 105°C before being cooled to room temperature. Along with extracted Quercetin, standard Quercetin was dissolved in ethanol and applied 1 cm above the edge of the pretreated TLC plate. In an airtight chromatography chamber with optimised solvent mixture of ethyl acetate, toluene, and formic acid (4:3.5:0.5), this plate was run for identification process. The developed plates 1% ethanolic aluminum chloride solution were air dried and visualized under UV. The TLC plates were further sprinkled by reagents for flavonoids such as ferric chloride and aluminium chloride. Report the R_f value and compare with standard quercetin. Optimization of solvent system explained in Table 2.

High Performance Thin Layer Chromatography (HPTLC)

The prepared extract was subjected to HPLC studies. HPLC conditions are given in Table 3.

RESULTS AND DISCUSSION

Optimization of Surface sterilization of *Phyllanthus acidus* Linn. Leaf and isolation of endophytic fungi

Different surface sterilizing agents like 70% ethanol, 4% Sodium hypochlorite and 0.1% Mercuric chloride ($HgCl_2$) solution were used, and their efficacy of action shown in Table 4.

70% ethanol for 30 sec followed by 0.1% mercuric chloride ($HgCl_2$) solution 30 sec was selected due to no evidence found for contamination when experiment caried out as shown in Table 4.

Statistical analysis for isolated endophytic fungi colony

Relative frequency and colonisation were computed as mentioned in Table 5.

Morphological identification of isolated endophytic fungi by microscopy methodology

Total 05 endophytic fungi isolated from *Phyllanthus acidus* fresh leaves as per above mentioned optimised procedure and code given PAEF 01, PAEF 02, PAEF 03, PAEF 04, PAEF 05.

Morphological characterisation of isolated endophytic fungal colonies mentioned in Table 6.

Table 6: Morphological characterisation of isolated endophytic fungal colonies.

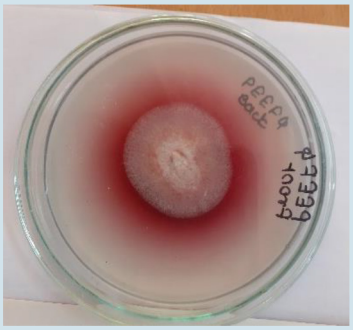
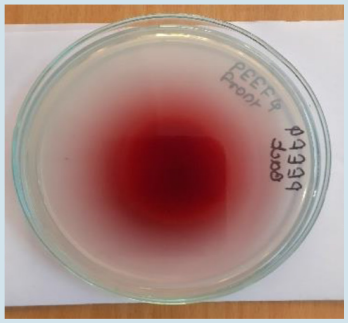
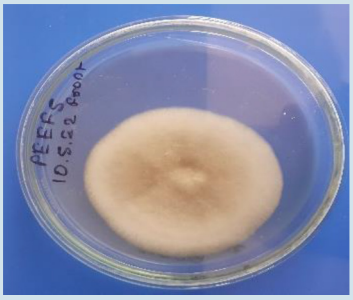
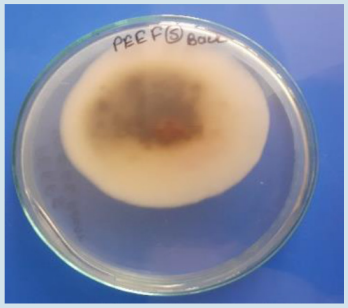
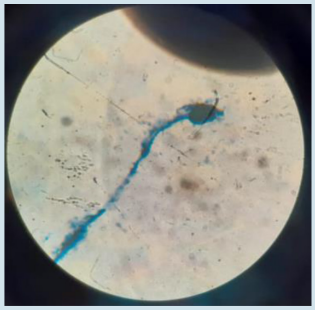
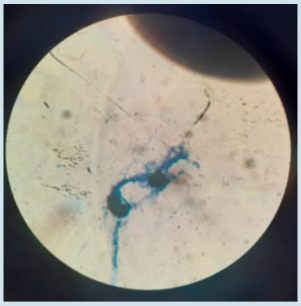
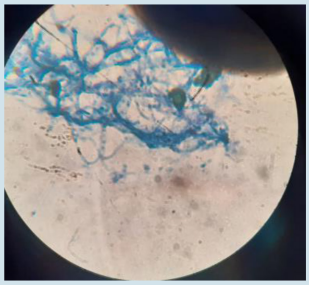
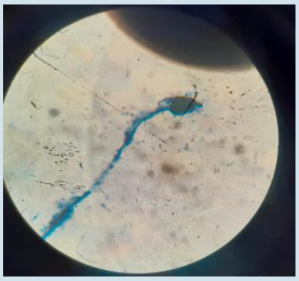
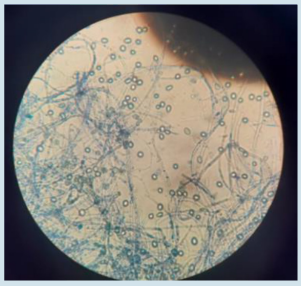
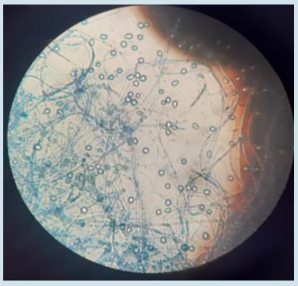
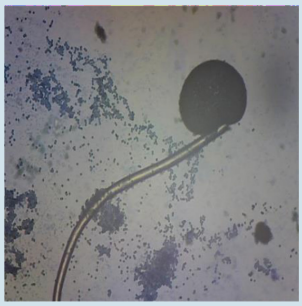
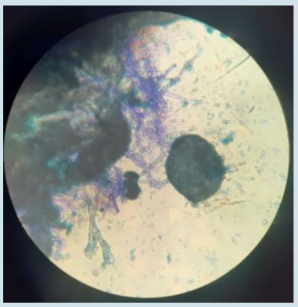
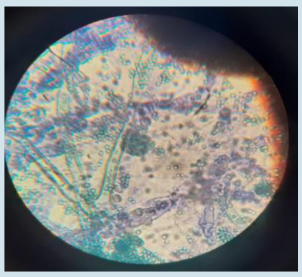
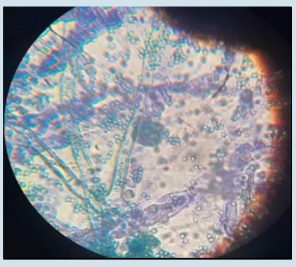
Sl. No.	Endophytic fungi code	Front view Isolated endophytic fungal colonies	Back View Isolated endophytic fungal colonies	Isolated endophytic fungi colony Description
1	PAEF1			Filamentous fungus, smooth, hair-like soft tufts, filiform margin, translucent, cottony texture.
2	PAEF2			Whitish black color colony, irregular and lobate margin.
3	PAEF3			Brown/yellow color round granular/ powdery colony, entire margin.
4	PAEF4			White color velvetycolony and red color backside, entire margin,red pigment observed at bottom of colony.
5	PAEF5			White color cottony colony, smooth and entire margin. Black coloration develop at centre of colony [backside]. Rised elevation, unbonated.

Table 7: Microscopic observation details for PAEF1, PAEF2, PAEF3, PAEF4, PAEF5.

Sl. No.	Endophytic fungi code	Microscopic view		Microscopic Observation
1	PAEF1			Hyaline and smooth-walled conidiophore stipes. Conidia have smooth walls, are globose to ellipsoidal in shape, and range in size from 1.5 to 2.5 m.
				
2	PAEF2			Ascospores are limoniform and 9–12 x 8–10 × 6–8 m in size. One lateral face is noticeably flattened, and the ends are weakly apiculate.
3	PAEF3			Conidial heads are small, columnar, biseriate, and have a diameter of up to 500 x 30–50 m. Hyaline and smooth-walled conidiophore stipes. Conidia have smooth walls, are globose to ellipsoidal in shape, and range in size from 1.5 to 2.5 m.
				

continued...

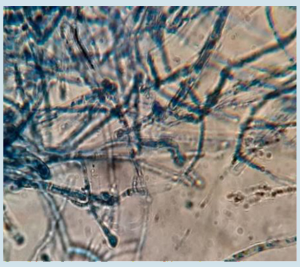
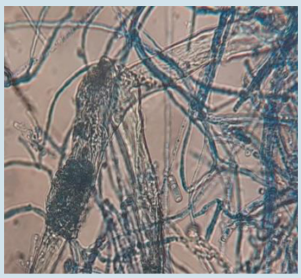
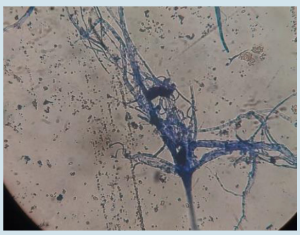
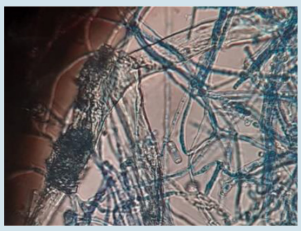
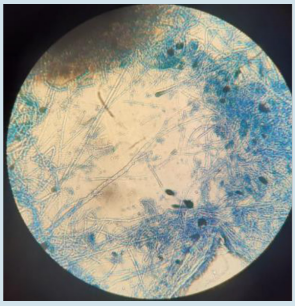
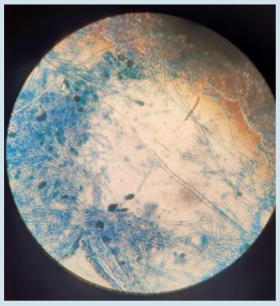
4	PAEF4			Ascomata 160–300 mm in diameter, spherical to ovoid.
				
5	PAEF5			Asci 30-40 x 11–16 mm, clavate, long-stalked, 8-spored.

Table 8: Morphological identification details for PAEF1, PAEF2, PAEF3, PAEF4, PAEF5.

Sl. No.	Endophytic fungi code	Identification Remarks	Family
1	PAEF1	*Aspergillus* sp. Aff A. Terreus	Aspergillaceae
2	PAEF2	*Chaetomium* sp. Aff C. Globosum	Chaetomiaceae
3	PAEF3	*Aspergillus* sp. Aff A. Terreus	Aspergillaceae
4	PAEF4	*Chaetomium* sp. Aff C. Globosum	Chaetomiaceae
5	PAEF5	*Chaetomium* sp. Aff C. Globosum	Chaetomiaceae

Isolated endophytic fungi used for microscopic observation by use of lactophenol with description mentioned in Table 7.

Based on the preliminary morphological features of all isolated endophytic fungi, were selected for further confirmation for Microscopical characteristics utilized for identification of endophytic fungi by Agharkar Research Institute, department of science and technology, Pune 04, Maharashtra, India. And identification data mentioned in Table 8.

Fermentation and metabolites extraction

The isolated endophytic fungi PAEF1, PAEF2, PAEF3, PAEF4, and PAEF5 were grown in 200 mL of Potato Dextrose Broth (PDB) and incubated at 28°C in a BOD shaking incubator for two weeks at 120 rpm. The filtrate was then used for extraction and phytochemical research, which are detailed in Table 6 below.

Preliminary phytochemical screening for Isolated Endophytic fungi extracts for PAEF1, PAEF2, PAEF3, PAEF4, PAEF5

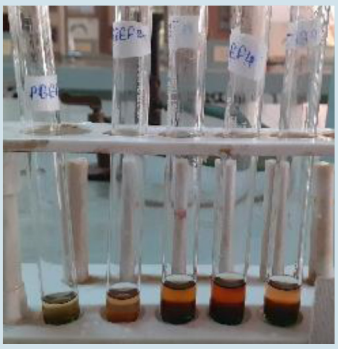
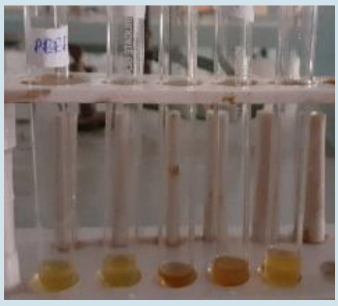
Endophytic fungi extract for PAEF1, PAEF2, PAEF3, PAEF4, and PAEF5 is used for phytochemical analysis, which reveals the presence of glycosides, phenolic compounds, flavonoids, proteins, amino acids, carbohydrates, and saponins. Results of analysis mentioned in Table 9.

Characterization of extracts

UV Spectroscopy

The formed Endophytic fungi extract for PAEF1, PAEF2, PAEF3, PAEF4, PAEF5 utilised for UV analysis from data obtained regarding λ_{max}, PAEF3 showed presence of quercetin. Quercetin

Table 9: Phytochemical analysis of *Phyllanthus acidus* endophytic fungal extracts.

Sl. No.	Constituent	Test/Reagent	PAEF1	PAEF2	PAEF3	PAEF4	PAEF5
	Alkaloids	Dragendroffs reagent Mayers reagent	-	-	+ +	+	+
1	Carbohydrate	Molish's test Barfoeds test 	+	-	+	+	+
2	Flavonoids	Shinoda test 	-	+	+	+	-
3	Tannins	Ferric Chloride test 	-	-	+	+	-
4	Proteins	Biuret test Xanthoproteic test 	+	+	+	+	-
	Saponins	Foam test	-	-	+	-	-
	Steroids	Liebermann Burchard test Salkowski reaction	-	+	+ -	+	+

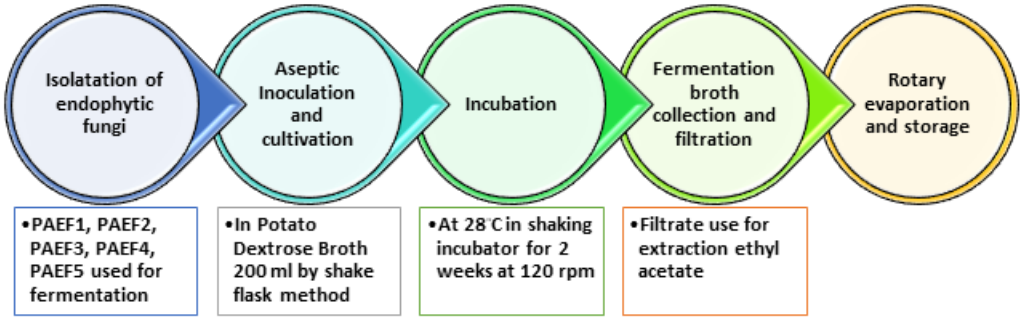


Figure 2: Stepwise procedure for fermentation and extraction of isolated endophytic fungi.

Figure 3: Fermentation process for Isolated Endophytic fungi for PAEF1, PAEF2, PAEF3, PAEF4, PAEF5.

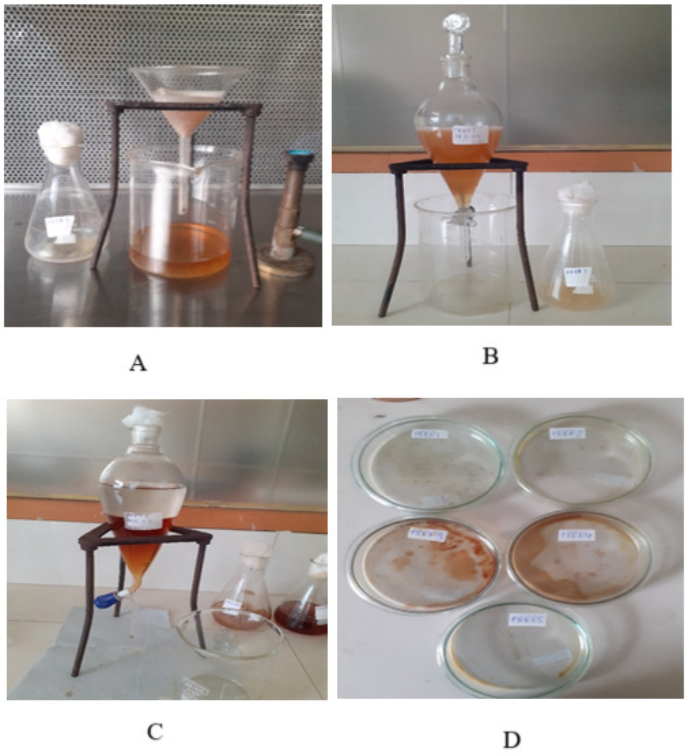


Figure 4: Extraction process for Isolated Endophytic fungi for PAEF1, PAEF2, PAEF3, PAEF4, PAEF5 [A: Filtration of Fermented broth in aseptic condition ;B: Solvent Extraction mechanism ; C: Distinct two clear immiscible layers formed after Solvent Extraction mechanism];D: Extract formation after evaporator treatment

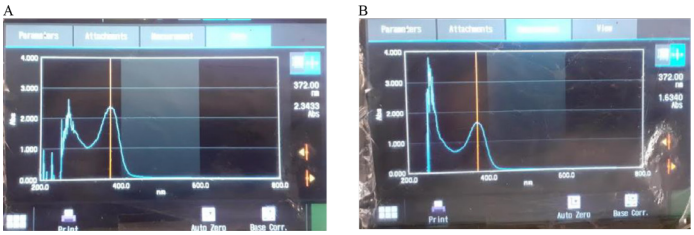


Figure 5: UV-spectroscopy of standard quercetin[A] and PAEF3 extract[B].

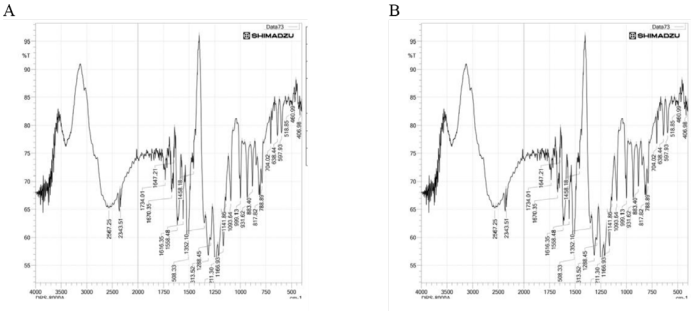


Figure 6: IR spectra of standard quercetin [A] and PAEF3 extract [B].

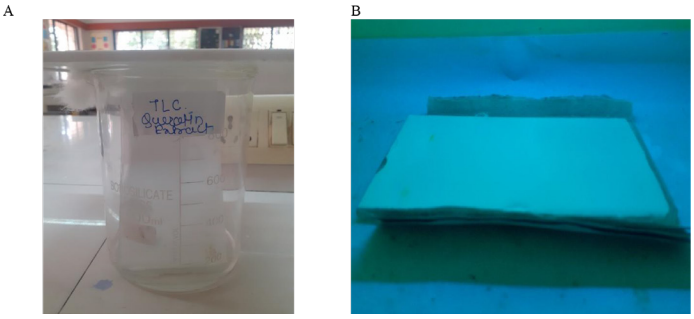


Figure 7: Thin Layer Chromatography for PAEF3.

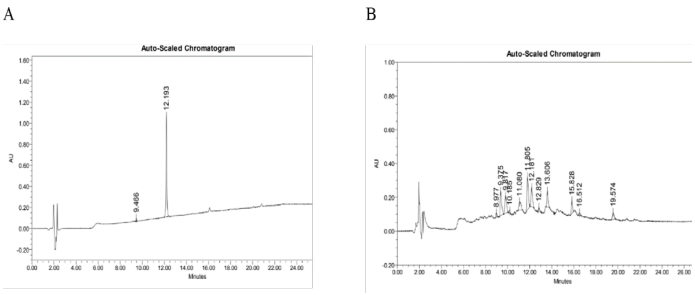


Figure 8: HPLC of standard quercetin [A] and PAEF3 extract [B].

and the isolated extract PAEF3 λ_{max} were found to be identical as shown in Figure 5.

Infrared Spectroscopy (IR)

It was discovered that the characteristic IR peaks matched those of their respective standard reference component of quercetin, IR graphs as mentioned in Figure 6.

Thin Layer Chromatography (TLC)

For PAEF3 extract quercetin, a solvent mixture of ethyl acetate, toluene, and formic acid (4:3.5:0.5) produced the best results. The standard quercetin appears in spots on the TLC plate that was generated under UV light. The R_f value of the isolated quercetin from the sample was identical to the R_f value of the standard quercetin i. 0.43. Set put for TLC shown in Figure 7.

High Performance Thin Layer Chromatography (HPTLC)

Comparable HPTLC spectra were obtained for isolated and standard quercetin. HPLC analysis of separated quercetin extract revealed retention times of 12.18 and 12.19 min, which were identical to those of standard quercetin as shown in below Figure 8.

CONCLUSION

Quercetin is a key component that has numerous benefits, including those for preventing cancer, controlling diabetes, protecting the liver, and fighting free radicals. was identified using a quick, easy, and simple isolation technique from the endophytic fungus PAEF03. The structure of standard and isolated quercetin was confirmed after the separated fraction was characterised using sophisticated methods of investigation from physicochemical, spectroscopic, and chromatographic studies.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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