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Impact of *Irpex lenis* and *Schizophyllum commune* endophytic fungi on *Perilla frutescens*: enhancing nutritional uptake, phytochemicals, and antioxidant potential

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Abstract

Background Endophytic fungi (EF) reside within plants without causing harm and provide benefits such as enhancing nutrients and producing bioactive compounds, which improve the medicinal properties of host plants. Selecting plants with established medicinal properties for studying EF is important, as it allows a deeper understanding of their influence. Therefore, the study aimed to investigate the impact of EF after inoculating the medicinal plant *Perilla frutescens*, specifically focusing on their role in enhancing medicinal properties.

Results In the current study, the impact of two EF i.e., *Irpex lenis* and *Schizophyllum commune* isolated from *A. bracteosa* was observed on plant *Perilla frutescens* leaves after inoculation. Plants were divided into four groups i.e., group A: the control group, group B: inoculated with *I. lenis*; group C: inoculated with *S. commune* and group D: inoculated with both the EF. Inoculation impact of *I. lenis* showed an increase in the concentration of chlorophyll a (5.32 mg/g), chlorophyll b (4.46 mg/g), total chlorophyll content (9.78 mg/g), protein (68.517 ± 0.77 mg/g), carbohydrates (137.886 ± 13.71 mg/g), and crude fiber (3.333 ± 0.37%). Furthermore, the plants inoculated with *I. lenis* showed the highest concentrations of P (14605 mg/kg), Mg (4964.320 mg/kg), Ca (27389.400 mg/kg), and Mn (86.883 mg/kg). The results of the phytochemical analysis also indicated an increased content of total flavonoids (2.347 mg/g), phenols (3.086 mg/g), tannins (3.902 mg/g), and alkaloids (1.037 mg/g) in the leaf extract of *P. frutescens* inoculated with *I. lenis*. Thus, overall the best results of inoculation were observed in Group B i.e. inoculated with *I. lenis*. GC-MS analysis of methanol leaf extract showed ten bioactive constituents, including 9-Octadecenoic acid (Z)-, methyl ester, and hexadecanoic acid, methyl ester as major constituents found in all the groups of *P. frutescens* leaves. The phenol (gallic acid) and flavonoids (rutin, kaempferol, and quercetin) were also observed to increase after inoculation by HPTLC analysis. The enhancement in the phytochemical content was co-related with improved anti-oxidant potential which was analyzed by DPPH (% Inhibition: 83.45 µg/ml) and FRAP (2.980 µM Fe (II) equivalent) assay as compared with the control group.

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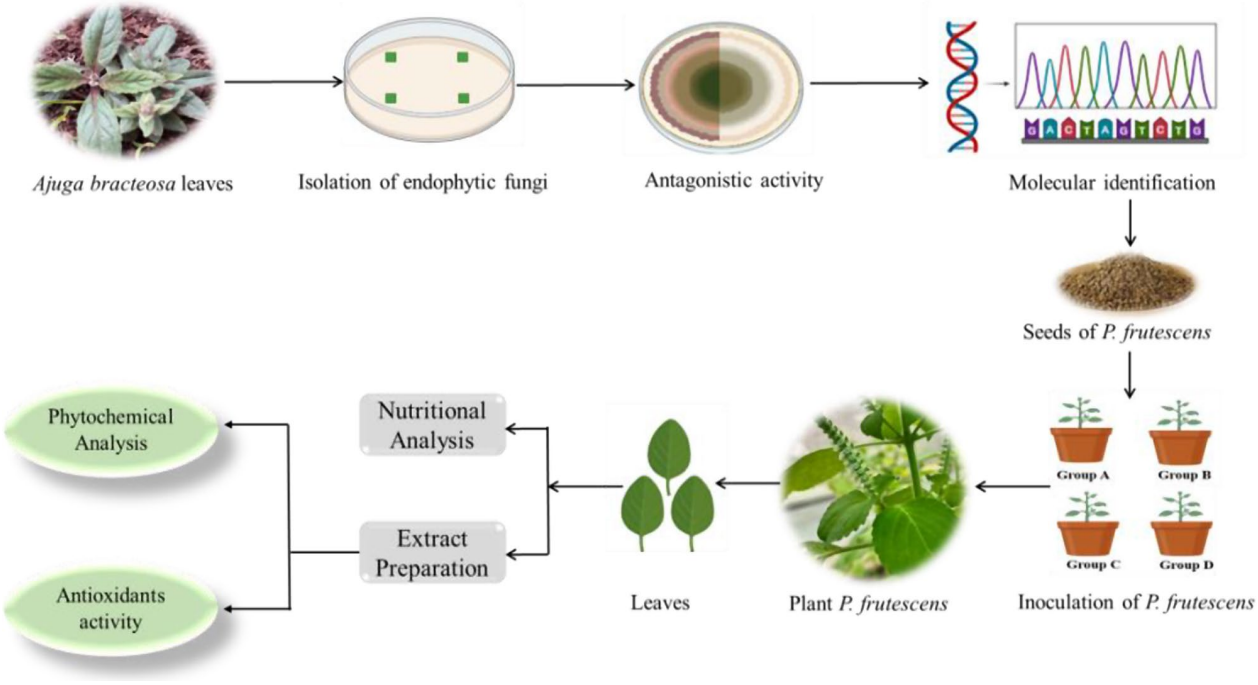
Conclusion Inoculation with *I. lenis* significantly enhances the uptake of nutritional constituents, phytochemicals, and antioxidant properties in *P. frutescens*, suggesting its potential to boost the therapeutic properties of host plants.

Graphical Abstract



Keywords Endophytic fungi, Isolation, Inoculation, Gene sequencing, Nutrient up-take, Phytoconstituent profiling

Background

The term "endophyte" was coined by Anton de Bary in 1866 [1] and refers to the microorganisms that inhabit plants throughout or a portion of their life without inducing any outward symptoms of disease [2]. Their ability to multiply within plant tissue makes them more likely to interact with their hosts. Consequently, they become better at meeting their nutritional requirements and are protected from unfavorable changes in the rhizosphere and phyllosphere [3]. Plants provide ample opportunities for microbial colonization. Various parts of plants, particularly those with high moisture and nutrient content, provide an optimal environment for the proliferation and thriving of microorganisms [4], while the host plant benefits from the metabolic substances produced by endophytes in terms of competitiveness and increased resistance to pathogens, herbivores, and diverse environmental stresses [5, 6]. They are also found to produce a variety of bioactive constituents that are highly valuable in pathology, medicine, and ecology [7, 8]. Novel immunosuppressive substances, volatile antibiotic mixtures, novel anticancer agents, antioxidants, and, most recently, hydrocarbons connected to fuel are included in this list. In many significant scientific domains, it seems that the field is ready for scientific invention and discovery [9].

Endophytic fungi (EF) live in close association with plants throughout the world [8]. They receive protection from the harsh natural environment as well as nourishment and shelter from the host plant [10]. In exchange, EF benefits the host plant, particularly in the case of phytochemical synthesis [11], and increases the host plant's resistance to biotic or abiotic challenges [10]. Plants contain a variety of EF, and there is a favorable correlation between the biological activity of these linked plants and endophytes. Unique secondary metabolites with potential therapeutic benefits have been found to potentially originate from EF [12]. The anti-diabetic, antiviral, antioxidant, antibacterial, anticancer, immunomodulatory, and immunosuppressive qualities that these secondary metabolites possess are assessed as benefits of the host-endophyte interaction [13]. The molecular processes through which endophytes leave an impact on plants remain unclear, despite considerable progress. Fortunately, more research on the biological characteristics, structure-activity correlations, and mechanisms of action of endophytes and plants can support more exploration of their interactions. This will make it easier to investigate

the molecular processes and associated signaling pathways that endophytes use to influence the production of secondary metabolites in plants [14]. At present, there are four hypothesis about the mechanisms: (a) Endophytes encourage plants to store more photosynthetic material. (b) Genes linked to secondary metabolites in plants are expressed differently in response to endophytes. (c) The introduction of endophytes causes compounds to modify the genetic makeup of plants. (d) Plant biosynthetic pathways are impacted by the production of distinct secondary metabolites by endophytes [15]. Studies indicate that natural products could significantly contribute to the drug innovation and development procedure by serving as a rich and innovative reservoir for future pharmaceuticals [16]. The field of endophyte research in natural products has the potential for advance drug innovation and development due to the currently developing realization that a significant amount of natural products either originate from microbes or result from microbial host interactions [17, 18].

Over the past decade, EF inhabiting medicinal plants has shown promise in producing pharmacologically active bioactive compounds that closely resemble those of their host plants [17, 19]. The medicinal properties of the plants may be due to their endophyte's capacity to produce physiologically active bioactive compounds [17]. The Lamiaceae (Labiatae) family, commonly referred to as the mint family, holds significant importance as a medicinal and aromatic plant family [20]. The family is notable for being among the most diverse and extensively spread plant families in terms of ethnomedicine [21]. *Ajuga bracteosa* Wall. ex Benth is commonly found in hilly regions [22]. It is prescribed in Ayurveda as a remedy for various health conditions as astringent, anthelmintic, anti-inflammatory, and antimicrobial and is incorporated into traditional medicine [23, 24]. *Perilla frutescens* (L.) Britton is part of a vast botanical family that encompasses 236 genera and over 7000 species [25]. In folk medicine, *P. frutescens* leaves, seeds, and stems are employed to cure a range of health conditions. Historically it is prescribed for treating depression, vomiting, anxiety, phlegm, colds, abdominal discomfort, flu, fever, nasal congestion, headache, coughs, asthma, tumors, allergies, chest congestion, constipation, indigestion, and intoxication. Additionally, it also functions as an antiabortive, sedative, and analgesic [26].

Recent years have witnessed a significant increase in interest in fungi, as seen by the various studies that have been carried out using a variety of approaches [27]. The most frequently brought-up topics have to do with elucidating certain facets of the relationship between endophytic fungi and their host plants [28]. In natural ecosystems, a symbiotic relationship consistently exists between plants and EF [29]. EF which inhabits diverse medicinal plants has been revealed to host a variety of bioactive compounds [30]. The EF in medicinal plants, especially those implicated in the creation of specific compounds with human health benefits, is believed to be linked to the generation of diverse phytoconstituents [31]. Presently, there is extensive ongoing research on EF derived from medicinal plants, and the reported medicinal capabilities of these EF have garnered widespread attention [32]. In the present study, we hypothesized that EF inoculation may enhance the nutritional constituents, phytochemicals, and antioxidant potential of *P. frutescens* leaves. However, there have been no studies conducted to assess the capabilities of EF isolated from *A. bracteosa.* Therefore, the objective of the current research was to isolate EF from the leaves of *A. bracteosa* and evaluate the effect of EF inoculation on chlorophyll content, nutritional constituents, minerals, phytochemicals, and antioxidant potential of *P. frutescens*. Moreover, comparative phytochemical analysis with GC-MS and HPTLC was done in both inoculated and non-inoculated plants of *P. frutescens*.

Materials and methods

Sampling

Leaves of *A. bracteosa* were collected from the vicinity of Shoolini University of Biotechnology and Management Sciences district Solan, Himachal Pradesh, and *P. frutescens* seeds were collected from the village of Sirmaur district of Himachal Pradesh.

Isolation of EF from medicinal plant *A. bracteosa*

Samples of healthy, asymptomatic *A. bracteosa* leaves were meticulously cleaned with water to eliminate any dust particles on the leaf surface. After that, the leaves were rinsed three times in sterile distilled water. They were then submerged in 4% sodium hypochlorite solution for 2–3 min, and subsequently in 70% ethanol for 1 min. Finally, the explants underwent a triple rinsing in sterile distilled water and were subsequently dried on filter paper to eliminate any remaining moisture. Following surface sterilization, the leaf segments were dissected into roughly 0.5 cm small segments which were then sterilized with an open flame. Approximately 3–4 of these segments were deposited onto individual Petri dishes filled with potato dextrose agar (PDA) medium enriched with streptomycin (250 µg/L) to inhibit bacterial proliferation. The Petri dishes were wrapped using parafilm and placed in an incubator at 25 °C for 7 days. Once the EF developed from the explants, the uncontaminated cultures were preserved and kept on PDA slants at a temperature of 4 °C for subsequent research purposes [33].

Antagonistic activity of EF

The assessment of fungal endophyte's antagonistic action against pathogenic fungi was conducted by dual culture technique using the method by Vinayarani and Prakash [34]. In this approach, a fungal endophyte culture measuring 5 mm^2 and an equivalent-sized culture of the test pathogen of the same size were placed 1 cm apart along the edge of a petri dish containing PDA media, with each placed in opposite directions. The petri dish inoculated solely with the pathogen without any antagonistic endophytes served as the control, and the experiment was conducted in triplicate with incubation at 25±2 °C until the pathogen mycelia fully colonized the control plates. To calculate the mycelial growth inhibition (I), the radial mycelial growth of the pathogen towards antagonistic fungus (T) and that on control plate (C) were measured, and the following formula was applied:

$$I = C - T \times 100/C$$

The pathogenic fungi i.e., *Fusarium oxysporum* (accession no. SR266-9) and *Rosellinia necatrix* (accession no. ON652311) were procured from the School of Applied Sciences and Biotechnology in Shoolini University, Solan.

Molecular identification

The EF from *A. bracteosa* was subjected to molecular characterization using the Cetyltrimethylammonium bromide technique to extract genomic DNA (gDNA). After homogenizing the sample with extraction buffer, isoamyl alcohol, phenol, and chloroform (24:25:1) were added. Following a 15-minute centrifugation at 14,000 revolutions per minute (rpm), the top aqueous phase was transferred to a fresh tube. After precipitating DNA with sodium acetate and isopropanol, the mixture was centrifuged once more. After wiping with ethanol, the DNA pellet was allowed to air dry. Afterward, the DNA was resuspended in TE buffer (Tris-Cl 10 mM, pH 8.0, EDTA 1 mM) and treated with RNAse A to eliminate any leftover RNA [35]. Using a Thermo Scientific Nano Drop 1000 spectrophotometer set at 260 nm, the extracted gDNA was measured. By employing 0.8% agarose gel electrophoresis, its quality and appropriateness for Random Amplified Polymorphic DNA (RAPD) study were assessed. A PCR was conducted using 100 ng of gDNA, a 10 mM dNTPs mix, a random decamer oligo template OPA-1 (5′-CAGGCCCTTC-3′), and 1 µL of dNTPs (5 U/µL) in a 25 µL reaction volume (Sigma-Aldrich) to assess RAPD. Using an Eppendorf master cycler, amplification was performed with a three-minute initial denaturation at 94 °C, thirty cycles of one minute at 94 °C, one minute at 50 °C, and two minutes at 72 °C, and a 10-minute final extension at 72 °C [36]. DNA sequences were aligned using the ClustalW algorithm in MEGA 11 software. The National Center for Biotechnology Information (NCBI) GenBank database's BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify homologous sequences. Using the neighbor-joining (NJ) technique, position estimation, and bootstrapping with 1000 iterations, a phylogenetic tree was built with MEGA 11. The rRNA sequences' partial ITS sections were uploaded to GenBank.

Inoculation of EF in *P. frutescens*

Seeds of *P. frutescens* were surface sterilized for five minutes using 1.5% NaOCl, followed by three sterile water washes and drying under laminar airflow [37]. In potato dextrose broth, both EF were grown using fungal discs as inoculants. These EF were cultured for 10 days at 30±2 °C with continuous shaking at 100 rpm. After being extracted, the fungal mycelium was cleaned using sterile water. A mechanically homogenized fungal culture in sterile water was mixed with about 10 g of surface-sterilized seeds at a 1% inoculation concentration [38]. The coated seeds were planted 1.5 cm deep in pots, with an average of four seeds germinating per pot. The experiment took place in a greenhouse, where the pots were organized into four groups i.e., group B: inoculated with *I. lenis*; group C: inoculated with *S. commune* and group D: inoculated with both EF.

Physicochemical analysis

Determination of chlorophyll

The chlorophyll content was estimated following the methodology of Huang et al. [39]. Crushed dry leaf samples were combined with 80% acetone (1:10 w/v) and centrifuged for 10 min at 7000 rpm. The process was continued until the residue lost all color. Using acetone as a blank, the absorbance of the supernatant was measured at 645 and 663 nm. The supplied equation was then used to determine the amounts of chlorophyll:

Chlorophyll a: 12.7 (A663) – 2.69 (A645).
Chlorophyll b: 22.9 (A645) – 4.68 (A663).
Total Chlorophyll: 20.2 (A645) + 8.02 (A663).

Nutraceutical analysis

Protein estimation

A 1 g leaf sample was powdered in 5–10 ml of phosphate buffer and centrifuged for 5 min at 7000 rpm. The collected supernatant was used. Working standard solutions of 200, 400, 600, 800, and 1000 µl and the sample extracts were placed in test tubes, each adjusted to 1 ml with water as a reference. 5 ml of alkaline copper solution was added, stirred, and allowed to sit for ten minutes. The Folin-Ciocalteau reagent (500 µl) was then added and combined. A spectrophotometer was used to measure the blue color that developed at 660 nm during

30 min of incubation at room temperature. The information obtained from the standard graph was then used to determine the sample's protein content [40].

Determination of carbohydrate content

In boiling water with 5 ml of 2.5 N hydrochloric acid, a 1 g leaf sample was hydrolyzed for three hours. After cooling, Na_2CO_3 was added to neutralize the solution. After adjusting the capacity to 100 ml, the centrifuge was run for five minutes at 10,000 rpm. For analysis, aliquots of the supernatant were collected. With '0' serving as the blank, standard glucose solutions (0, 0.2, 0.4, 0.6, 0.8, and 1 ml) were made. Samples were mixed with 4 ml of newly made anthrone reagent (200 mg anthrone in 100 ml of ice-cold 95% sulfuric acid) after being diluted to 1 ml with sterile water. After eight minutes of heating in boiling water, the combination was cooled, and the dark green color that had developed was detected at 630 nm [41].

Determination of crude fat

The crude fat content was assessed using the method by Unuofin et al. [42] with slight adjustments. After shaking five grams of powdered leaves at 120 rpm for a day, 100 mL of diethyl ether was used for the extraction. Filtered extract was gathered in a beaker that had been previously weighed (W1). Another 24-hour extraction using 100 mL of diethyl ether was performed on the residue. After being evaporated in a steam bath, the diethyl ether was dried in an oven at 40°–60°C. Next, the beaker was weighed once again (W2). The crude fat content was calculated as follows:

$$\%crude\,fat = \frac{\mathrm{W2} - \mathrm{W1}}{\text{Weight of original sample}} \times 100$$

Measurement of ash content

The method by Unuofin et al. [42], with minor modifications, was used to determine crude ash content. The crucible was weighed (W1) and allowed to cool in a desiccator after an hour of drying at 105°C. The crucible was weighed once again after two grams of finely powdered leaf powder were added (W2). In a muffle furnace, the sample was ashed for one hour at 250°C and then for five hours at 550°C. After cooling, the samples were weighed (W3). The ash% was calculated as follows:

$$\%ashcontent = \frac{\mathrm{W2} - \mathrm{W3}}{\mathrm{W2} - \mathrm{W1}} \times 10$$

Determination of crude fibre

A minor modification was made to Unuofin et al. [42] method, where 2 g of the sample was digested in 1.25% sulfuric acid for 30 min and then filtered. After four hot water rinses, the residue was digested using 100 ml of 1.25% NaOH solution. After cooling in a desiccator and drying at 100 °C, the residue was weighed (C1). After five hours of ignition at 550 °C in a muffle furnace, it was cooled in a desiccator and weighed once more (C2). The % of crude fibre was calculated as follows:

$$\%crude\,fibre = \frac{\mathrm{C1} - \mathrm{C2}}{\text{Weight of original sample}} \times 100$$

Mineral analysis

Plant samples were washed, dried at 40 °C, and ground into powder. After mixing H_2O_2 and HNO_3 with 0.5 g of powder, the containers were sealed and allowed to stand at room temperature for a whole day. Following microwave digestion, cooling, filtering, and dilution to 50 mL, the samples were examined by ICP-OES with the use of an approved reference material. 1 g of powder was cooked in ultrapure water, filtered, and then reduced to ten milliliters. It was then treated with HNO_3, microwave-digested, filtered, diluted, and subjected to ICP-OES analysis [43].

Phytochemical analysis

Preparation of plant leaf extract

The leaves of *P. frutescens* were carefully gathered, dried in the shade for seven to ten days, powdered finely, and then sealed in airtight receptacles. Plant extracts were prepared using methanol solvent, and quantitative analyses of phytoconstituents were conducted [44, 45]. To prepare extracts, approximately 10 g of shade-dried powder of leave were separately combined with 100 ml of methanol solution. After being shaken for two days at 150 rpm, the mixtures were filtered through Whatman Filter Paper No. 1 and allowed to dry for 24 to 48 h in a hot air oven set at 37 °C. After that, the crude extracts were kept for further chemical analysis at 4 °C [46].

Total phenolic estimation

The total phenolic content was determined using the Folin-Ciocalteu reagent technique. 500 µl of diluted Folin-phenol reagent, 2.5 ml of 20% sodium carbonate, and around 20 mg of extracts were combined with 1 ml of distilled water. The liquid was stirred and then allowed to develop color for 40 min in the dark. At 725 nm, absorbance was measured. The calibration curve or standard graph was used to determine the total phenolic content in milligrams equivalent to gallic acid (mg/g of gallic acid) [47].

Total flavonoid estimation

1 ml of plant extract was diluted with 200 µl of distilled water to measure the flavonoid concentration. 150 µl of sodium nitrite (5%), then. 150 µl of 10% aluminum chloride was added after five minutes, and the mixture was left for 6 min. By adding 2 ml of 4% sodium hydroxide and distilled water, the volume was raised to 5 ml. At 510 nm, the pink coloring indicative of flavonoids was observed after 15 min at room temperature. Using a standard curve, the flavonoid concentration was calculated as milligrams of rutin equivalent per gram (mg/g of rutin) of dry extract [46].

Total tannin estimation

In a 10 ml volumetric flask, 1 ml of the sample extract was combined with 7.5 ml of distilled water, 0.5 ml of Folin-Ciocalteu reagent, and 1 ml of 35% sodium carbonate. After adding distilled water to make 10 ml, the mixture was allowed to sit at room temperature for half an hour. Tannic acid reference standards were made similarly, with 200–1000 µg/ml. Tannin concentration was determined using a standard curve and expressed as milligrams of tannic acid equivalent per gram of extract (mg/g of TAE) based on absorbance measured at 725 nm [48].

Total saponin estimation

To determine total saponin content, 50 µl of plant extract was combined with 200 µl of distilled water, followed by the addition of 250 µl of vanillin reagent (800 mg vanillin in 10 ml of 99.5% ethanol). Then, 2.5 ml of sulfuric acid (72%) was well combined. After 10 min of incubation at 60 °C and 10 min of cooling in ice-cold water, the mixture's absorbance at 544 nm was determined. The final results were calculated utilizing a standard curve and presented as diosgenin equivalent (mg/g of Dg) [49].

Total alkaloid estimation

A spectrophotometric method utilizing bromocresol green (BCG) was used to determine the alkaloid content. After dissolving the plant extract (1 mg/ml) in 2 N hydrochloric acid, 0.1 N NaOH was used to filter the mixture, and phosphate buffer was used to neutralize it. In a separating funnel, 1 ml of the mixture was mixed with 5 ml of BCG solution and phosphate buffer. Chloroform was used to extract the complex, gather it in a flask containing 10–20 ml, and dilute it to the required level. At 470 nm, absorbance was measured. The experiment was conducted three times, and the extract's caffeine concentration (mg/g of caffeine) was calculated using a caffeine standard solution [46].

Gas chromatography mass spectrometry (GC-MS) analysis

GC-MS analysis of the leaf extract of *P. frutescens* was conducted using the method described by Dhalaria et al. [50] with minor modifications. A Thermofisher Trace 1300/TSQ Duo GC-MS/MS Spectrometer equipped with a TriPlus RSH autosampler was utilized in the experiment. Chromatography was carried out using helium gas at a rate of 0.7 mL/min on a Thermofisher TG-5MS capillary column (40 m length, 0.15 mm ID, and 0.15 µm film thickness). The temperatures of the injector and detector were 250 °C and 270 °C, respectively. With a 2-minute equilibration interval, the oven temperature program began at 70 °C (6 min hold), climbed to 250 °C (18 min hold), and then to 270 °C at 7 °C/min (20 min hold). A split-less approach was used to inject 1 µL of 1% extract in methanol throughout a 50-minute run period. X Calibur software and the NIST/EPA/NIH Mass Spectral Library, version 2.2, were used to evaluate the data.

High-performance thin-layer chromatography (HPTLC) analysis

Preparation of extract and standard stock solutions

P. frutescens leaf extracts (5 mg/5 ml) were made in methanol for HPTLC analysis. Subsequently, each solution was filtered through a 0.45 µm membrane before chromatography.

Estimation of rutin, gallic acid, kaemferol and quercetin

The HPTLC analysis of leaf extract of *P. frutescens* extract was done following the methodology of Prashar and Patel [51]. Using a Linomat V sample applicator and TLC scanner, the CAMAG HPTLC system was employed in this procedure. After being cleaned with methanol and heated to 60 °C for 15 min, aluminum TLC plates (20×20 cm, 0.20 mm thickness) covered with silica gel 60 and a fluorescent indicator from Loba Chemie were ready. On the plates, 4 µl of 1 mg/mL methanol extracts were applied in 5 mm broad bands separated by 6 mm intervals. At room temperature, rutin, gallic acid, kaemferol, and quercetin were separated using solvent systems of toluene, ethyl acetate, and formic acid (6:4:0.8) and (7:3:1), respectively. Following separation, plates were allowed to dry before the spots were examined at 254 nm in a UV chamber. Chromatograms were densitometrically analyzed using WinCATS software (Appendix 4).

Antioxidant activity

DPPH radical scavenging activity

The scavenging activity of the samples was determined by following a method by Jamkhande et al. [52] with minor modifications. A 0.1 mM DPPH solution was prepared by dissolving 3.94 mg of DPPH in methanol and adjusting the volume to 100 ml. After incubating in darkness for 30 min, 1 ml of this DPPH-methanol solution was combined with 1 ml of methanolic extract (at concentrations ranging from 20 to 100 µg/ml). The mixture was shaken

thoroughly and allowed to stand at room temperature in the dark for half an hour. At 517 nm, spectrophotometric analysis was carried out with methanol serving as a blank and ascorbic acid as a reference. The calculation for free radical scavenging activity was performed as follows:

Scavenging effect (%) = [(Absorbance of blank - Absorbance of sample)/Absorbance of blank] × 100.

Ferric reducing antioxidant potential (FRAP) assay

The assay assessed the ability of the extracts to reduce a colorless ferric complex to a blue ferrous complex under acidic conditions, aided by antioxidants that contribute electrons. The reduction process was shown by the change at 593 nm. The reagent was made up of 10 mM TPTZ (2,4,6-Tripyridyl-S-triazine) in 40 mM HCl, 300 mM acetate buffer (pH 3.6), and 20 mM ferric chloride. Before usage, fresh solutions were made. After being combined with 3 mL of FRAP reagent, samples (100 µL at mg/mL) were incubated for 30 min at 37 °C. After measuring the absorbance at 593 nm, FRAP values were computed by deducting the blank absorbance. The ferric chloride standard curve was utilized to translate the FRAP result into milligrams of Fe^{2+} per gram of the sample [53].

Statistical analysis

The data were analyzed using one-way and two-way analysis of variance (ANOVA) and presented as mean ± standard deviation, with values determined in triplicate. To detect significant variances, the Bonferroni multiple comparison test was employed, and statistical analysis was conducted using Graph Pad Prism software.

Results and discussion

Isolation and morphological identification of EF

There were ten EF which were isolated from the leaves of plant *A. bracteosa* as shown in Fig. 1. The EF were morphologically identified by observing various macro-morphological features such as colony color, colony margins, and hyphal diameter. Based on morphological identification, EF (S1-S10) isolated from *A. bracteosa* were recognized as *Irpex* sp., *Absidia* sp., *Agaricus* sp., *Fusarium* sp., *Sclerotina* sp., *Rhizopus* sp., *Phythium* sp., *Rhizoctnia* sp., *Schizophyllum* sp., and *Trichoderma* sp., respectively. Simultaneously the plant was also identified at Botanical Survey of India (BSI), High altitude western Himalayan regional centre, Nauni, Solan, Himachal Pradesh with accession number- 00670.

Antagonistic assay

Antagonism in the context of plant pathology refers to the active interference or suppression exerted by any organism on the normal growth and functions of plant pathogenic fungi. From the antagonistic assay, two fungi were selected i.e., S1 and S9 which showed the highest antagonistic effect against *F. oxysporum* and *R. necatrix* respectively (Fig. 2). S1 showed 37.04% inhibition against *F. oxysporum* whereas S9 showed 51.92% inhibition against *R. necatrix* as shown in Table 1.

In a similar study, EF were isolated from *Zataria multiflora* and assessed for their antagonistic potential against *Monosporascus cannonballus* using dual culture technique. The isolated EF namely *Nigrospora sphaerica*, *N. sphaerica*, *Subramaniula cristata*, *Polycephalomyces sinensis*, *P. sinensis* showed 81.7 ± 5.5%, 66.1 ± 1.9%, 38.7 ± 3.7%, 80.6 ± 11.2% and 75.8 ± 9.3% inhibition against *M. cannonballus* individually [54]. In another study, the antagonistic potential of *Chaetomium globosum* was observed against various phytopathogenic fungi using a dual-culture method. The tested fungi included *F. oxysporum*, *Sclerotinia sclerotiorum*, *R. necatrix*, and *Cladosporium xanthochromaticum* [55].

Molecular identification of isolated EF

Two endophytic isolates with potential antagonistic activity against phytopathogenic fungi were subjected to molecular characterization to validate the reliability of morphological identification i.e., S1 and S9. Molecular characterization was based on the amplification of

Fig. 1 Ajuga bracteosa plant

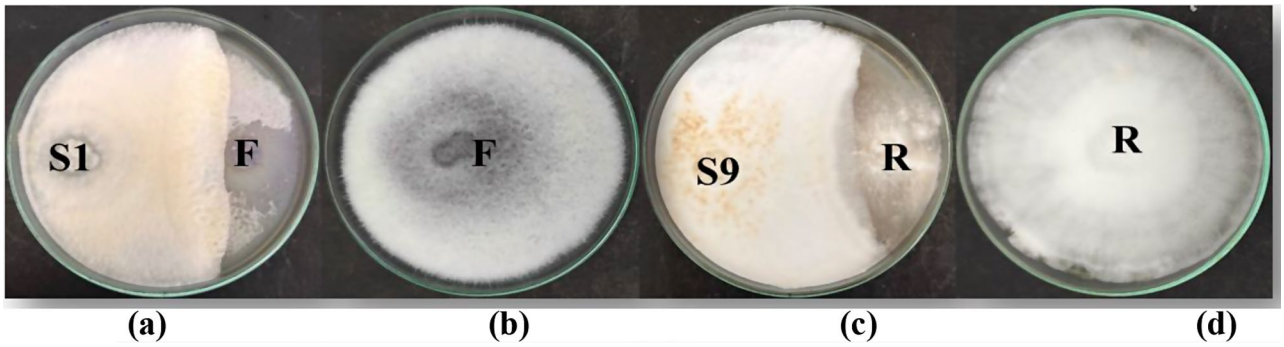


Fig. 2 Pictures of antagonistic assay of (**a**) EF against *Fusarium oxysporum*(**b**) *Fusarium oxysporum* control (**c**) Antagonistic assay of EF against *Rosellinia necatrix* (**d**)*Rosellinia necatrix* control

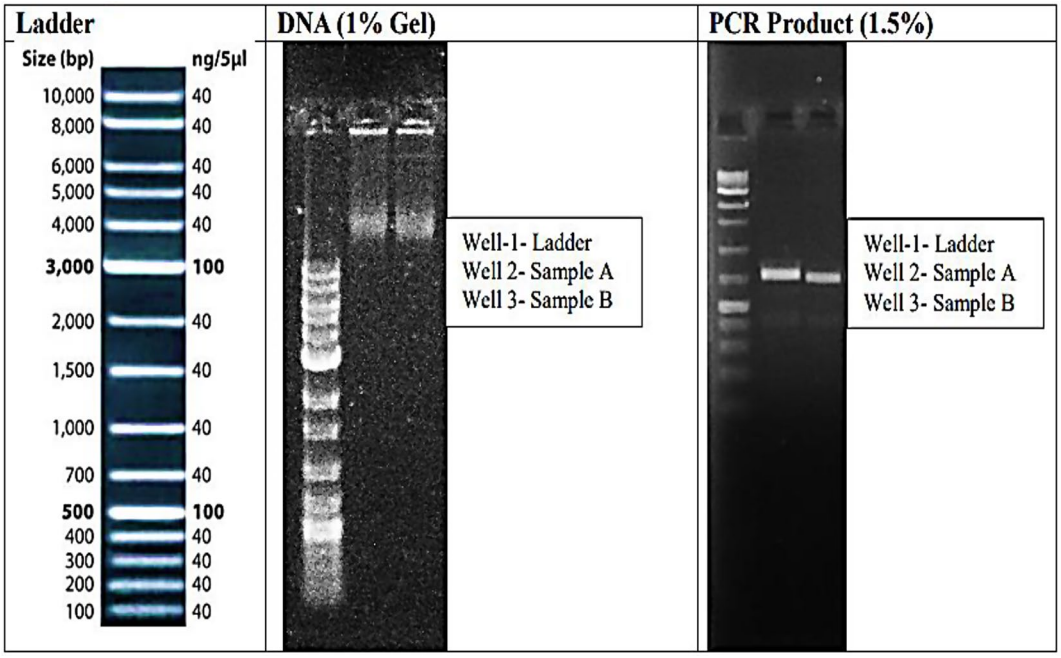


Fig. 3 Polymerase chain reaction results of ribosomal DNA (~ 500 bp amplicons) of EF

ITS nuclear rDNA intervening 5.8 S rRNA gene and 16 S rRNA gene sequence analysis for fungi as shown in Fig. 3.

Phylogenetic tree of isolated EF

Phylogenetic trees represent the evolutionary history of two selected EF which showed the highest antagonistic activity against plant pathogenic fungi i.e., *F. oxysporum* and *R. necatrix* as shown in Figs. 4 and 5.

Molecular study of S1 strain

The genomic sequence of S1 fungus is given as follows:

NANTGAAAAAAAGGTTTTAGAACGGGTTG-TAGCTGGCCTCTCACGAGGCATGTGCACGCCTG-GCTCATCCACTCTTAACCTCTGTGCACTTTAT-GTAAGAGAAAAAAATGGTGGAAGCTTCCAG-GATCTCGCGAGAGGTCTTTGGTTGAACAAGC-CGTTTTTCTTTCTTATGTTTTACTACAAAC-GCTTCAGTTATAGAATGTCAACTGTGTATAACA-CATTTATATACAACTTTCAGCAACGGATCTCTTG-GCTCTCGCATCGATGAAGAACGCAGCGAAATGC-GATAAGTAATGTGAATTGCAGAATTCAGTGAAT-CATCGAATCTTTGAACGCACCTTGCACTCCTTG-GTATTCCGAGGAGTATGCCTGTTTGAGTCT-CATGGTATTCTCAACCCCTAAATTTTTGTAAT-GAAGGTTTAGCGGGCTTGGACTTGGAGGTTGT-GTCGGCCCTTGTCGGTCGACTCCTCTGAAATG-CATTAGCGTGAATCTTACGGATCGCCTTCAGT-GTGATAATTATCTGCGCTGTGGTGTTGAAGTATT-TATGGTGTTCATGCTTCGAACTGTCTCCTTGC-CGAGACAATCATTTGACAATCTGAGCTCAAAT-CAGGTAGGACTACCCGCTGAACTTAAGCATAT-CAATAAGCGGAA.

After BLAST analysis of the genomic sequence of the S1 fungus strain in the NCBI database, it was revealed

Table 1 Results of antagonistic assay of EF against *F. oxysporum* and *R. Necatrix*

Sample	Inhibition against *F. oxysporum* (%)	Inhibition against *R. necatrix* (%)
S1	37.04	30.28
S2	30.4	22.90
S3	30.62	36.65
S4	16.06	17.07
S5	17.28	19.85
S6	28.15	40.97
S7	12.84	14.24
S8	20.02	13.99
S9	35.33	51.92
S10	34.95	20.86

Where S1: *Irpex* sp.; S2: *Absidia* sp.; S3: *Agaricus* sp.; S4: *Fusarium* sp.; S5: *Sclerotina* sp.; S6: *Rhizopus* sp.; S7: *Phythium* sp.; S8: *Rhizoctnia* sp.; S9: *Schizophyllum* sp. and S10: *Trichoderma* sp. %: Percent

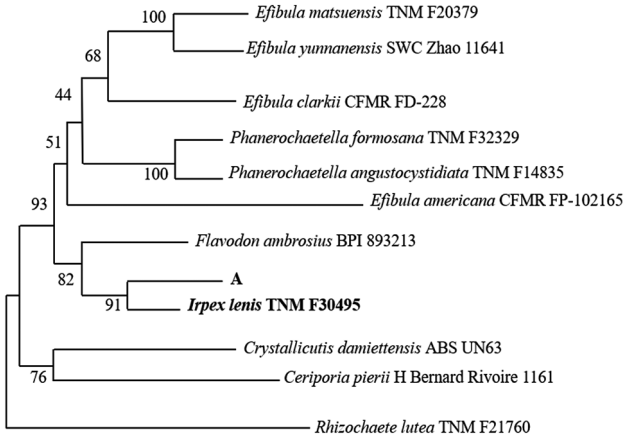


Fig. 4 Phylogenetic tree derived from NJ analysis showing the evolutionary relationship of S1 (*Irpex lenis*)

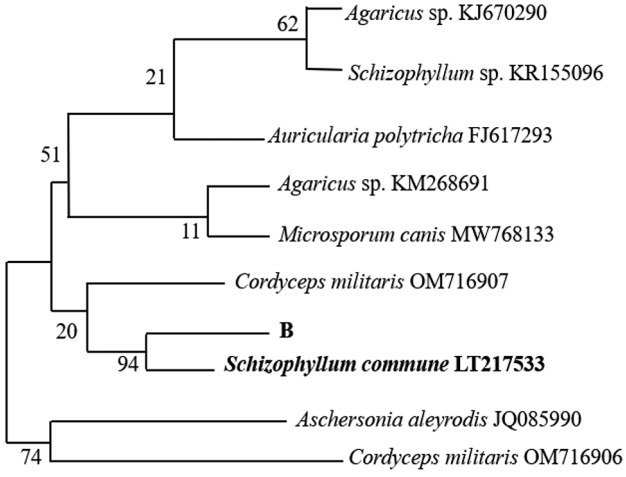


Fig. 5 Phylogenetic tree derived from NJ analysis showing the evolutionary relationship of S9 (*S. commune*)

that *Irpex lenis* showed a high similarity of 94.25% to our sample, indicating a close relationship. On the other hand, its genomic sequence was found to be distinctly related to *Rhizochaete lutea* with a similarity of 82.56%. The Basic Local Alignment Search Tool (BLAST) analysis allowed us to identify these related fungal species and gain insights into the genetic similarities and differences between them as shown in Fig. 4. After that the genomic sequence was submitted in the NCBI gene nucleotide sequence databases (http://www.ncbi.nlm.nih.gov) and the accession number of EF was also obtained (OQ520290).

Molecular study of S9 strain

The second endophytic fungus with potential antagonistic activity was S9 and the genomic sequence of the same is given as follows:

ACTAANGTGNATCCTANGTATCTGGATCCGGTGCACCTTATGTATGTGCCCAAAGCCTTCACGGACGGCCGGTTGACTACGTCTACCTCACACCTTAAAGTATGTTAACGAATGTAATCATGGTCTTGACAGACCCTAAAAAGTTAATACAACTTTCGACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCCCTTTGGTATTCCGAGGGGCATGCCTGTTTGAGTGTCATTAAATACCATCAACCCTCTTTTGACTTCGGTCTCGAGAGTGGCTTGGAAGTGGAGGTCTGCTGGAGCCTAACGGAGCCAGCTCCTCTTAAATGTATTAGCGGATTTCCCTTGCGGGATCGCGTCTCCGATGTGATAATTTCTACGTCGTTGACCATCTCGGGGCTGACCTAGTCAGTTTCAATAGGAGTCTGCTTCTAACCGTCTCTTGACCGAGACTAGCGACTTGTGCGCTAACTTTTGACTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGA.

The genomic sequence of the S9 fungi in the NCBI database was subjected to a BLAST analysis, which allowed us to obtain details on closely related fungi. Our analysis demonstrated a high similarity of 98.97% between our sample and *Schizophyllum commune*, indicating a close relationship.

In contrast, the genomic sequence of our sample was distinctly related to *Archersonia aleyrodis*. The BLAST analysis enabled us to identify these related fungal species and provided valuable insights into the genetic similarities and differences between them, as depicted in Fig. 5. After that we submitted the genomic sequence in the NCBI gene nucleotide sequence databases (http://www.ncbi.nlm.nih.gov) and OQ982428 was received as accession number for *S. commune.*

Inoculation of EF in host plant

The EF which exhibited the highest antagonistic activity against the plant pathogenic fungi i.e., *F. oxysporum* and *R. necatrix* were identified and inoculated on the seeds of plant *P. frutescens.* The host plant selected in the present study was also identified from BSI Nauni with accession number- 00671 and a herbarium was submitted in the herbarium of SBES at Shoolini University.

Physicochemical analysis

Chlorophyll content

In this study, it was noted that EF-inoculated plants exhibited an increase in chlorophyll concentration as compared with an uninoculated group of plants. In the leaves of Group A plants, the concentration of Chl. a, Chl. b, and total chlorophyll content was recorded as 3.01 mg/g, 2.67 mg/g, and 5.68 mg/g, respectively. In the leaves of Group B plants, the values for Chl. a, Chl. b, and total chlorophyll were 5.32 mg/g, 4.46 mg/g, and 9.78 mg/g respectively. The leaves of Group C exhibited concentrations of 3.18 mg/g for Chl. a, 2.95 mg/g for Chl. b, and 5.97 mg/g for total chlorophyll. Similarly, leaves of Group D displayed concentrations of 4.02 mg/g for Chl. a, 3.82 mg/g for Chl. b, and 7.84 mg/g for total chlorophyll. Group B inoculated with *I. lenis*, had the highest chlorophyll content of all the inoculated groups followed by group D plants. There was significant variation in the values of chl. between group A and group B as shown in Fig. 6.

In conclusion, EF inoculation positively enhanced the chlorophyll content. Similar studies were also conducted and it was observed that application of EF enhanced the chlorophyll content in paddy leaves [56]. Another study also got the same results. The EF *Paecilomyces formosus* (*P. maximus*) was inoculated in cucumber plants and then after a certain time the inoculated plants were observed with improved chlorophyll content (32.9±0.13 SPAD) as compared with the control (29.9±0.16 SPAD) [57]. Waqas et al. [29] discovered that the inoculation of *Phoma glomerata* (31.4±0.36 SPAD) and *Penicillium* sp. (29.43±0.89 SPAD) led to an increase in chlorophyll content as compared with the control (22.86±1.3 SPAD).

Nutritional analysis

In this study, it was observed that EF-inoculated plants showed a higher concentration of nutritional constituents as compared with the uninoculated group of plants. Protein (Group A: 65.67±0.74 mg/g, Group B: 68.52±0.77 mg/g, Group C: 66.35±1.34 mg/g and Group D: 67.06±1.35 mg/g), carbohydrates (Group A: 117.12±4.76 mg/g, Group B: 137.88±13.71 mg/g, Group C: 126.08±6.82 mg/g, Group D: 130.83±7.92 mg/g), crude fat (Group A: 3.13±0.80%, Group B: 3.333±0.37%, Group C: 3.966±0.40%, Group D: 4.766±0.40%), ash content (Group A: 11.80±0.20%, Group B: 12.17±0.23%, Group C: 12.46±0.17%, Group D: 12.73±0.25%), crude fiber (Group A: 16.73±1.00%, Group B: 21.53±2.19%, Group C: 17.80±0.91%, G Group D: 18.73±1.33%) were observed in leaves of *P. frutescens* plants inoculated with EF. Plants inoculated with *I. lenis* i.e., group B exhibited the highest protein carbohydrate and crude fiber content. However, in case of crude fat and ash content, group D exhibited the highest content as compared with control group plants. In conclusion, inoculated plant showed an increase in all nutritional values as compared with control group of *P. frutescens* leaves. Thus, inoculation with EF has significant impact on nutritional content of Perilla plant as shown in Fig. 7 (a-e).

Among all the inoculated plants, nutritional content was significantly enhanced in plants inoculated with *I. lenis*. Researchers conducted a similar study and found that proteins, carbohydrates, and fibers exhibited significantly higher content in EF-inoculated plants [58].

Mineral analysis

Leaves of the plant *P. frutescens* were evaluated for their mineral content. In the current study the content of phosphorus (Group A: 801.92±99.55 mg/kg, Group B: 1605.16±106.10 mg/kg, Group C: 1023.96±53.66 mg/kg, Group D: 1254.29±100.98 mg/kg),

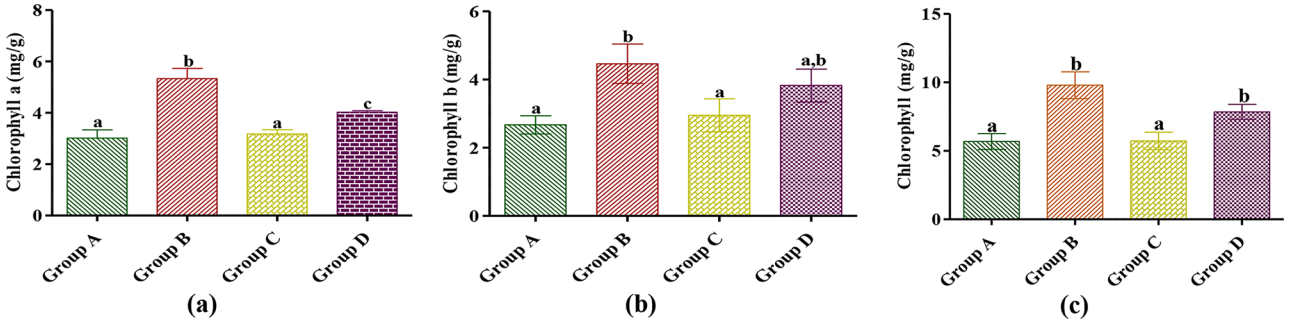


Fig. 6 (**a**) Chlorophyll a (**b**) Chlorophyll b (**c**) Total chlorophyll content (mg/g) as observed in leaves of *P. frutescens* inoculated with EF. Different letters were used to express significant variations ($P < 0.05$), while the same letters were used for non-significant variations. Where Group A: control group; Group B: Inoculated with *I. lenis*; Group C: Inoculated with *S. commune* and Group D: inoculated with both EF

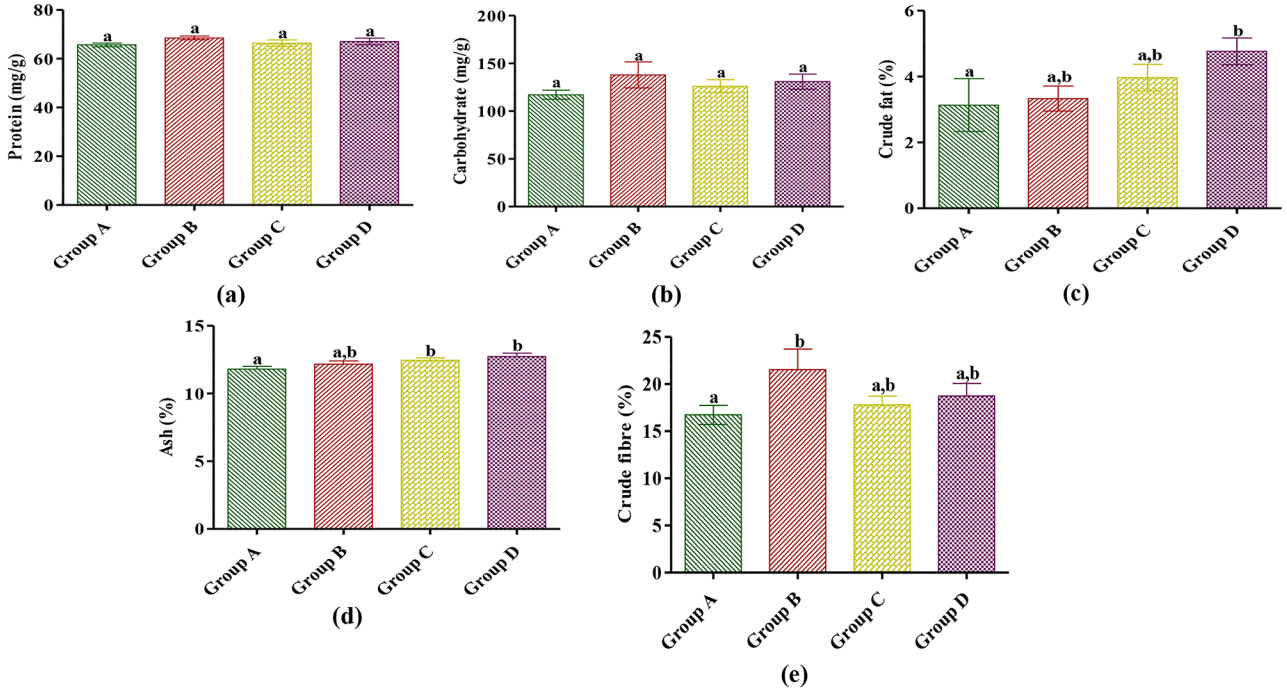


Fig. 7 (**a**) Protein content (mg/g) (**b**) Carbohydrate content (mg/g) (**c**) Crude fat content (%) (**d**) Ash content (%) (**e**) Crude fiber (%) in leaves of *P. frutescens* inoculated with EF. Different letters were used to express significant variations ($P < 0.05$), while the same letters were used for non-significant variations. Where Group A: control group; Group B: Inoculated with *I. lenis*; Group C: Inoculated with *S. commune* and Group D: inoculated with both EF

magnesium (Group A: 3856.06±374.74 mg/kg, Group B: 4964.32±284.37 mg/kg, Group C: 3970.01±310.05 mg/kg, Group D: 4660.59±305.49 mg/kg), calcium (Group A: 22214.60±913.34 mg/kg, Group B: 27389.40±732.08 mg/kg, Group C: 22506.70±501.23 mg/kg, Group D: 22872.90±869.50 mg/kg), iron (Group A: 448.76±56.06 mg/kg, Group B: 579.03±71.33 mg/kg, Group C: 605.39±17.59 mg/kg, Group D: 590.54±30.49 mg/kg), manganese (Group A: 57.87±2.94 mg/kg, Group B: 86.88±8.87 mg/kg, Group C: 59.68±6.13 mg/kg, Group D: 66.90±5.00 mg/kg), zinc (Group A: 26.77±1.14 mg/kg, Group B: 31.78±2.09 mg/kg, Group C: 32.47±2.54 mg/kg, Group D: 34.34±1.50 mg/kg), copper (Group A: 14.40±1.50 mg/kg, Group B: 15.59±0.84 mg/kg, Group C: 16.88±1.71 mg/kg, Group D: 18.20±1.06 mg/kg) were analysed. Group B which was inoculated with *I. lenis* demonstrated the highest content of P, Mg, Ca, Mn. Conversely, for Zn and Cu, group D displayed the highest content. In the case of Fe, group C exhibited the highest content. In conclusion, when compared with group A i.e., control group, all parameters were increased under fungal inoculation as shown in Fig. 8.

It was observed that mineral content increased under all inoculated conditions with EF as compared with uninoculated plants. However, among the inoculated plants, those inoculated with *I. lenis* showed a significant enhancement in mineral content. A study found that the nutritional quality of all inoculated crops was enhanced, with significantly higher levels of Ca, Fe, Mg, and P as observed in the inoculated plants as compared with those that were not inoculated [58]. It is also found that the nutritional quality of all inoculated crops with EF was enhanced as compared with uninoculated plants [59]. Similarly, a study by Santamaria et al. [60] included inoculation of four EF i.e., *Sordaria fimicola*, *Sporormiella intermedia*, *Stemphylium* sp., *Fusarium* sp., in *Ornithopus compressus.* After inoculation, they found that minerals like Cu, Mg, Mn, Zn enhanced in inoculated plants as compared with uninoculated plants. In comparison, inoculated plants with different EF, it was observed that those inoculated with *Ornithopus compressus* exhibited the highest concentration of Zn (96.4±6.3 mg/kg) and Cu (6.9±0.3 mg/kg), whereas those inoculated with *Sordaria fimicola* (E071) displayed the highest concentration of Mg (5.3±0.1 mg/kg) and Mn (307.4±17.5 mg/kg) as compared with the control plants Cu (5.0±0.5 mg/kg), Mg (4.5±0.3 mg/kg), Mn (223.8±25.8 mg/kg), Zn (66.0±8.0 mg/kg). Thus, different EF had different impacts on nutrient acquisition in plants.

Phytochemical analysis

The quantitative analysis of phytochemicals in *P. frutescens* leaf extract was done with methanol solvent. All the compounds increased in all the inoculated groups as shown in Fig. 9. The flavonoids (Group A:

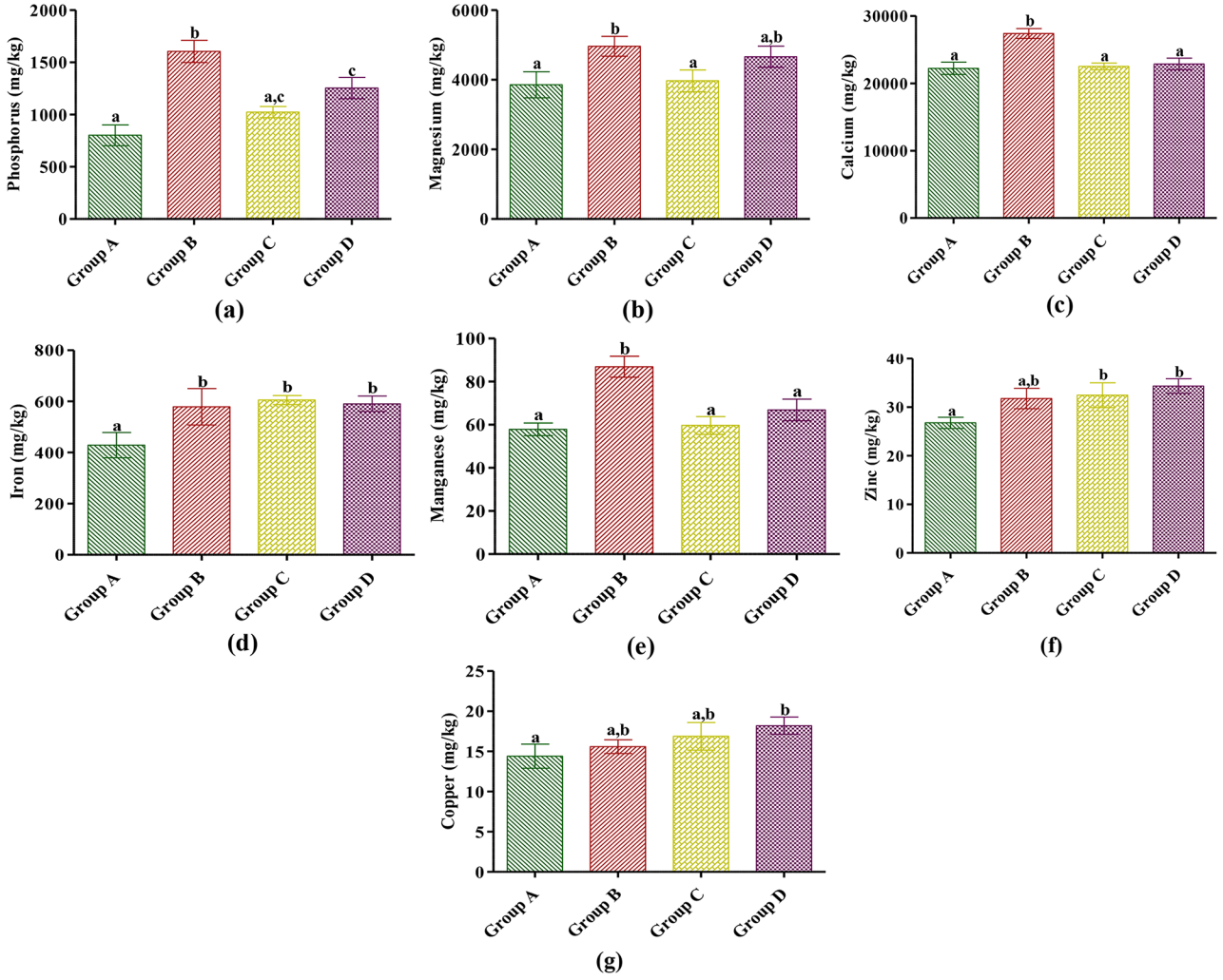


Fig. 8 (**a**) Phosphorus (mg/kg) (**b**) Magnesium (mg/kg) (**c**) Calcium (mg/kg) (**d**) Iron (mg/kg) (**e**) Manganese (mg/kg) (**f**) Zinc (mg/kg) (**g**) Copper (mg/kg) content in *P. frutescens* leaves extract of different groups which were inoculated with EF. Different letters were used to express significant variations ($P < 0.05$), while the same letters were used for non-significant variations. Where Group A: control group; Group B: Inoculated with *I. lenis*; Group C: Inoculated with *S. commune* and Group D: inoculated with both EF

1.120±0.08 mg/g, Group B: 2.347±0.15 mg/g, Group C: 2.026±0.12 mg/g, Group D: 2.133±0.13 mg/g), phenols (Group A: 1.991±0.07 mg/g, Group B: 3.086±0.06 mg/g, Group C: 2.239±0.05 mg/g, Group D: 2.162±0.06 mg/g), tannins (Group A: 2.449±0.15 mg/g, Group B: 3.902±0.12 mg/g, Group C: 3.110±0.09 mg/g, Group D: 2.716±0.07 mg/g), saponins (Group A: 2.883±0.10 mg/g, Group B: 4.981±0.12 mg/g, Group C: 4.362±0.13 mg/g, Group D: 2.965±0.10 mg/g), and alkaloids (Group A: 0.579±0.11 mg/g, Group B: 1.037±0.09 mg/g, Group C: 0.841±0.11 mg/g, Group D: 0.835±0.10 mg/g) content were observed in *P. frutescens* leaves. In conclusion, flavonoids, phenols, tannins, and alkaloids, showed an increase in group B which was inoculated with *I. lenis* as compared with the control group. But saponin was observed in the highest concentration in group C which was inoculated *S. commune.*

All the EF-inoculated plants exhibited an increase in all phytochemicals as compared with the uninoculated plants. Similarly, earlier research work also noted an increase in the overall phenols and flavonoids contents in plants when they were inoculated with EF [61]. *Rhizopus oryzae*, was introduced to *Glycine max* and *Helianthus annuus* plants and was observed with enhanced concentrations of phenolics and flavonoids [62]. An experiment was conducted where *Oryza sativa* was inoculated with four different EF i.e., *A. flavus*, *T. pinophilus*, *T. zelobreve*, and *Trichoderma* sp. After inoculation, an increase in phenolic content in the inoculated plants was observed. Notably, among these EF, *O. sativa* inoculated with *T. zelobreve* exhibited the highest concentration of phenolic content, measuring 122.32 mg/g, as compared with control plants which measured 92.84 mg/g [63].

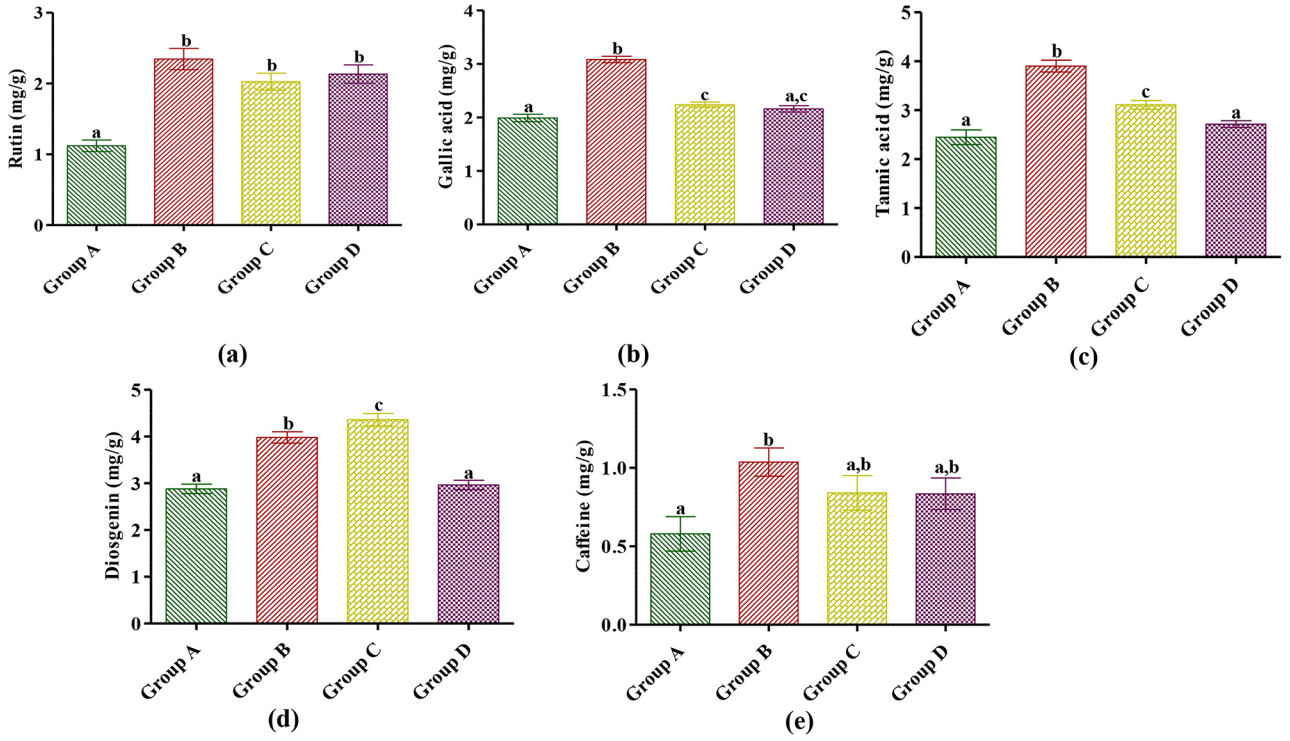


Fig. 9 (**a**) Flavonoids (mg/g), (**b**) Phenols (mg/g), (**c**) Tannins (mg/g), (**d**) Saponins (mg/g) and (**e**) Alkaloids content (mg/g) in leaves of *P. frutescens* in different groups inoculated with EF. Different letters were used to express significant variations ($P < 0.05$), while the same letters were used for non-significant variations. Where Group A: control group; Group B: Inoculated with *I. lenis*; Group C: Inoculated with *S. commune* and Group D: inoculated with both EF

GC-MS analysis

The methanol extract of *P. frutescens* leaves was screened for GC-MS analysis and various phytochemicals were observed in the leaves extract as shown in chromatograms (Fig. 10a-d; Table 2). The criteria for identification of the compounds were based on the peak area of the compounds representing the percentage area of that compound, chemical formula, molecular formula and molecular weight. The main bioactive compounds identified in methanol extract of *P. frutescens* leaves were octadecenoic acid (Z)-, methyl ester and hexadecanoic acid, methyl ester, both exhibiting the highest peak area % in all the groups at the retention time of 25.50 and 23.09 respectively.

GC-MS analysis revealed that compound concentrations increased after the EF inoculation. Among the plants inoculated with *I. lenis* highest peak area % of 9-octadecenoic acid (Z)-methyl ester (24.45%) and hexadecanoic acid, methyl ester (10.31%) was observed. 9-Octadecenoic acid (Z)-, methyl ester has anti-oxidant and anti-cancer properties while hexadecanoic acid, methyl ester has anti-oxidant properties and decreases blood cholesterol and anti-inflammatory properties [64]. A study found a total of 118 compounds in plant extract which included a diverse range of substances such as sugars, fatty acids, phenolic acids, terpenoids, amino acids, organic acids, flavonoids, and various other compounds [65]. Another study discovered that the primary volatile constituent in plants was perilla ketone, constituting 43.34% of the total phytoconstituents. This was followed by myristicin with 16.3% and beta-caryophyllene with 11.88%. Additionally, other notable compounds, including apiol, alpha-humulene, trans-alpha-bergamotene, elemicin, and phytol, exhibited relative concentrations exceeding 1%. Conversely, the concentration of the remaining identified components was observed to be below 1% [66]. In a study, researchers found 1-cyclohexane-1-carboxaldehyde (3.38%), β-caryophyllene (10.15%), and asarone (23.91%), as the main volatile compounds in the perilla plant [67].

HPTLC analysis

In HPTLC analysis the leaf extracts of *P. frutescens* which was inoculated with EF were studied. Four standard compounds were selected which included rutin, kaempferol, gallic acid, and quercetin as shown in Fig. 11. The analysis includes TLC fingerprint profiles as well as the estimation of chemical markers and biomarkers. It was revealed that all the compounds were present in all the groups in the methanol extract of *P. frutescens* leaves. The observed phytoconstituents increased after inoculation with EF, in leaves of *P. frutescens.* However, the findings revealed that the concentrations of rutin, gallic acid, and quercetin were highest in group B, which was inoculated with *I.*

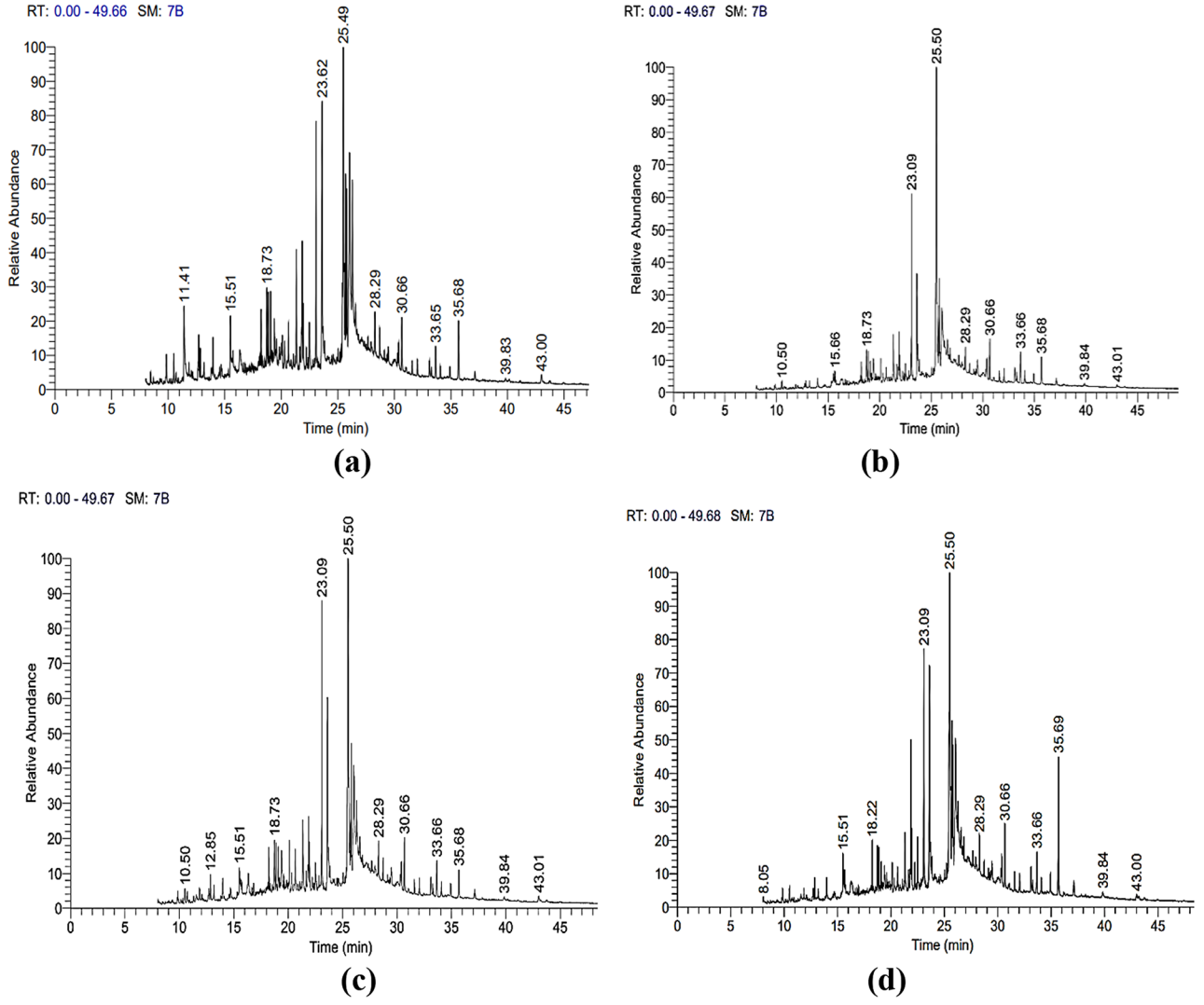


Fig. 10 GC-MS analysis of phytoconstituents present in (**a**) control (Group A) (**b**) In plants inoculated with *I. lenis* (**c**) In plants inoculated with *S. commune* (**d**) In plants inoculated with both the EF in 50:50 of methanol extract of *P. frutescens* leaves

Table 2 GC-MS based identification and characterization of phytoconstituents in different groups of *P. frutescens* leaves

Compound name/ chemical formula	Molecular weight	RT	Area %			
			Group A	Group B	Group C	Group D
9-Octadecenoic acid (Z)-, methyl ester ($C_{19}H_{36}O_2$)	296	25.49	13.18	24.45	18.75	17.87
Hexadecanoic acid, methyl ester ($C_{17}H_{34}O_2$)	270	23.09	6.34	10.23	10.31	7.63
Methyl stearate ($C_{19}H_{38}O_2$)	312	25.80	3.91	4.98	4.75	4.13
Docosanoic acid, methyl ester ($C_{23}H_{46}O_2$)	354	30.66	1.59	2.63	2.29	2.43
Caryophyllene oxide ($C_{15}H_{24}O$)	220	18.22	1.40	1.05	1.49	1.58
Eicosanoic acid, methyl ester ($C_{21}H_{42}O_2$)	326	28.29	1.39	-	1.64	1.50
Tetracosanoic acid, methyl ester ($C_{25}H_{50}O_2$)	382	33.65	1.18	2.29	1.86	1.90
9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z, Z,Z) ($C_{21}H_{36}O_4$)	352	26.58	0.94	1.71	1.44	1.36
Methyl 11-docosenoate ($C_{23}H_{44}O_2$)	352	30.38	0.91	1.54	1.41	1.36
2,3,3a,4,5,6,7,7a-Octahydro-1 H-cyclopenta[a]pentalen-7-ol ($C_{11}H_{16}O$)	164	12.85	0.70	-	0.92	0.72

(Where RT: Retention time, -: Absent; Group A: control group; Group B: inoculated with *I. lenis*; Group C: inoculated with *S. commune*; Group D: inoculated with both EF)

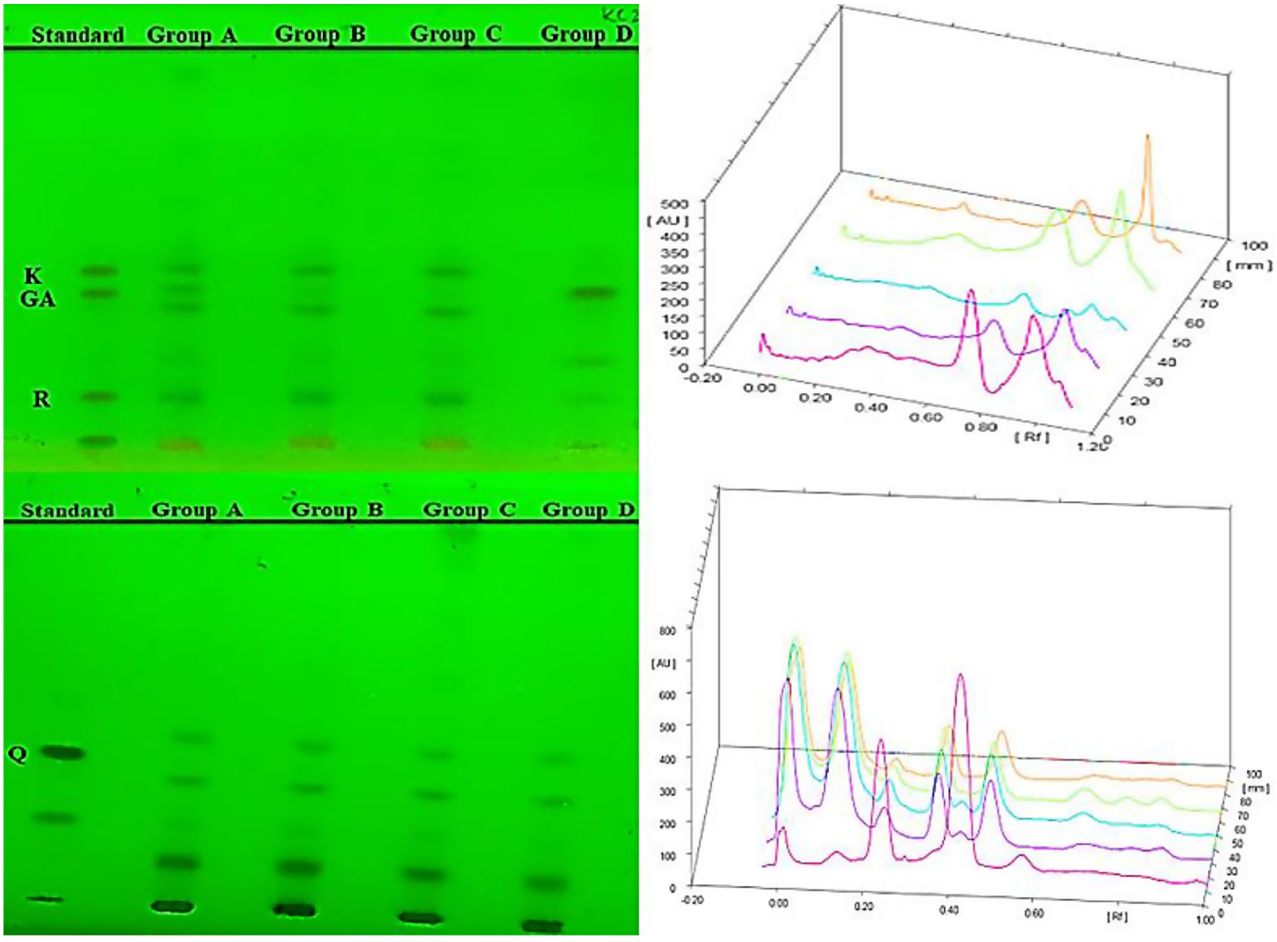


Fig. 11 TLC and HPTLC estimation of rutin, kaempferol, gallic acid, and quercetin in methanol extracts of *P. frutescens* leaves where K: kaempferol, GA: gallic acid, R: rutin, Q: quercetin. Where Group A: control group; Group B: Inoculated with *I. lenis*; Group C: Inoculated with *S. commune* and Group D: inoculated with both EF

Table 3 HPTLC estimation of rutin, kaempferol, gallic acid, and quercetin in methanol extracts of *P. frutescens* leaves under different inoculation conditions

Groups	Rutin (g/100 g)	Kaempferol (g/100 g)	Gallic acid (g/100 g)	Quercetin (g/100 g)
Group A	3.350	0.841	2.500	0.628
Group B	5.716	1.069	4.302	0.778
Group C	4.018	1.105	2.675	0.637
Group D	3.360	0.875	2.643	0.698

(Group A: control group; Group B: inoculated with *I. lenis*; Group C: inoculated with *S. commune*; Group D: inoculated both EF in 50:50)

lenis, while kaempferol was detected in group C, inoculated with *S. commune* as shown in Table 3.

In HPTLC analysis it was observed that all the compounds exhibited an increase after inoculation. A similar investigation was done on *Salvia abrotanoides* plants inoculated with *Penicillium canescens*, where the concentration of caffeic acid was found to be increased by 64.00%, while that of rosmarinic acid increased by 69.00% as compared with the control group [68]. Another study revealed that *Stevia rebaudiana* plants inoculated with *Fusarium fujikuroi* exhibited concentrations of rutin and syringic acid as 20.879 mg/L and 5.438 mg/L, respectively, which was observed to be more than control plants [10].

Anti-oxidant activity

DPPH assay

The antioxidant potential of the leaves of *P. frutescens* was observed in different groups through DPPH assay and presented in Fig. 12. The antioxidant activity in leaf extract increased correspondingly with increased concentration of plant extract. Additionally, no activity was observed in the negative control, indicating that the leaf extract was responsible for the observed antioxidant effects. Maximum antioxidant potential was observed in positive control viz., ascorbic acid. Additionally, the leaf extract tested at the highest concentration exhibited the highest scavenging activity. Specifically, the extract from group B which was inoculated with endophytic fungus *I. lenis* exhibited the maximum inhibition value of 83.45%.

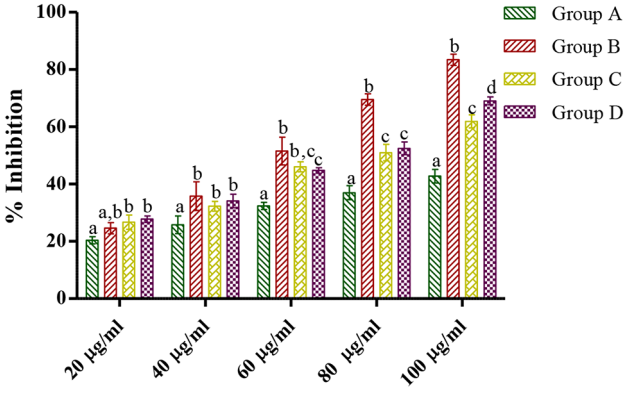


Fig. 12 Percentage inhibition of free radicals with *P. frutescens* leaf extracts which was inoculated with endophytic fungi; where group A: control group; group B: inoculated with *I. lenis*; group C: inoculated with *S. commune*; group D: inoculated with both EF. Bars with different letters in the same concentration group were used to express significant variations ($P < 0.05$), while the same letters were used for non-significant variations

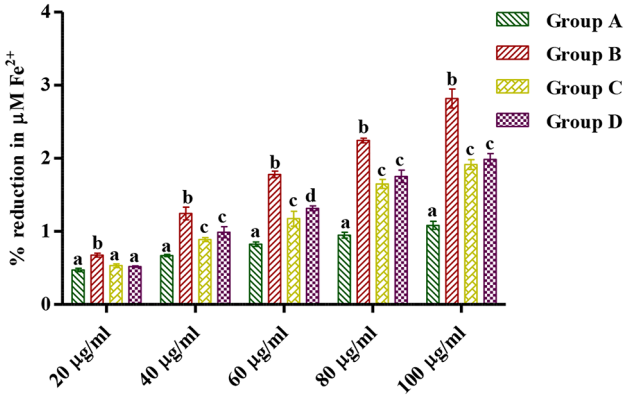


Fig. 13 Percentage reduction of free radicals with *P. frutescens* leaf extracts which was inoculated with endophytic fungi; where group A: control group; group B: inoculated with *I. lenis*; group C: inoculated with *S. commune*; group D: inoculated with both EF in 50:50 ratio. Bars with different letters in the same concentration group were used to express significant variations ($P < 0.05$), while the same letters were used for non-significant variations

In contrast, the extract derived from group A i.e., control produced the minimum value of 42% (% RSA value) at 100 µg/ml.

FRAP assay

The FRAP assay results are presented in Fig. 13. The antioxidant potential of *P. frutescens* leaf extract in different groups ranged from 0.471 to 2.980%. Upon assessment of the antioxidant activity of the leaf extract, it was observed that with increasing concentrations of leaf extracts from 20 to 100 µg/ml, there was a corresponding increase in antioxidant potential. Additionally, no activity was observed in the negative control, indicating that the leaf extract was responsible for the observed antioxidant effects. Maximum antioxidant potential was observed in positive control viz., ferrous sulfate. Additionally, the leaf extract at the higher concentration revealed significant scavenging activity. Specifically, the extract from the group B sample exhibited a maximum value of 2.980% at 100 µg/ml concentration. In contrast, the extract derived from the group A sample produced the minimum value of 0.471% at 20 µg/ml concentration.

In the present study, from both DPPH and FRAP assays, we found that inoculated plants have higher antioxidant potential than non-inoculated plants. When the correlation coefficients (r) were analyzed between DPPH and FRAP assays, a positive correlation was observed with r value 0.991. In leaves higher antioxidant activity was found in plants inoculated with *I. lenis*. Numerous investigations have demonstrated that EF inoculation enhances antioxidant potential in plants [57]. Rice treated with *Trichoderma zelobreve* also exhibited a notable increase in antioxidant potential which was higher than non-inoculated control, and also higher than the chemical fertilizer [63]. In another study it was found that antioxidant potential of *Bacopa monnieri* enhanced after inoculation with *Piriformospora indica* [69].

Conclusion

Fungal endophytes have been gaining increased attention because of the numerous benefits they can offer directly to the host plant with the intimate interaction established during the colonization of the plant tissues [70]. However, it is believed to possess supporting ecological and physiological benefits for the plant [71]. EF plays an essential role in maintaining plant health. Inoculation of EF significantly enhances the medicinal and nutritional value of *P. frutescens*. The main aim of this research was to enhance the phytochemical content which thereby improves the therapeutic potential of the plant. The current research demonstrated that EF-inoculation improved the chlorophyll content, nutrient acquisition, and mineral content which further attributed to improved biosynthesis of metabolites in the leaves of *P. frutescens*. Among all the groups, plants inoculated with *I. lenis* exhibited the highest concentration of all the phytochemicals. Furthermore, increased antioxidant potential was observed in *I. lenis* inoculated plants can be correlated with a higher content of bioactive constituents such as polyphenols, thereby acting as effective free radical scavengers. Further, with changes in the environment, urbanization, and industrial growth, agricultural land is shrinking day by day. Inoculation of *P. frutescens* with EF has improved the nutritional and therapeutic benefits of *P. frutescens* which is valued for both food and medicinal significance. This method increases the development and synthesis of bioactive compounds in the plant, providing a sustainable way to get the most out of the limited amount of agricultural land. This article contributes to

future studies by highlighting the potential of using EF to enhance the nutritional and medicinal value of *P. frutescens*. It promotes more studies regarding the use of EF for other medicinal plants and crops to maximize agricultural output in terms of increasing beneficial components in plants. It also emphasizes how crucial it is to combine biotechnological developments with sustainable practices to lessen the negative effects of urbanization and environmental shifts on agriculture and to get more benefits from medicinal plants. Additionally, further research is needed to identify the potential mechanisms of action by which EF enhances the phytochemicals.

Abbreviations

ANOVA	Analysis Of Variance
BLAST	Basic Local Alignment Search Tool
BSI	Botanical Survey of India
CRM	Certified Reference Material
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EF	Endophytic fungi
FRAP	Ferric reducing antioxidant potential
gDNA	Genomic DNA
HPTLC	High-Performance Thin-Layer Chromatography
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometry
NCBI	National Center for Biotechnology Information
NJ	Neighbor-joining
PDA	Potato Dextrose Agar
PTFE	Polytetrafluoroethylene
r	Correlation coefficients
rpm	Revolutions Per Minute
RT	Retention Time
TPTZ	2,4,6-Tripyridyl-S-triazine

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Author contributions
KS performed the experimental work. RV designed the research plan. DK statistical analysed the results. VK have done the editing and final drafting of the manuscript.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors have given their consent for the publication of manuscript.

Competing interests
The authors declare no competing interests.

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Impact of Irpex lenis and Schizophyllum commune endophytic fungi on Perilla frutescens: enhancing nutritional uptake, phytochemicals, and antioxidant potential

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