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Anti-inflammatory and anti-obesity activity of endophytic fungal isolates of *Nardostachys jatamansi*

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The present study underpins the anti-obesity and anti-inflammatory activity of fungal endophytes inhabiting *Nardostachys jatamansi* of the high-altitude region of Uttarakhand. The study entails the recovery of 15 endophytic fungi from the leaves, inflorescence, and stem of *N. Jatamansi,* showing maximum colonisation in plant leaves. The anti-obesity and anti-inflammatory potential of the isolates was investigated via phenol red plate assay, L-asparaginase production, and albumin denaturation assay. The maximum albumin denaturation inhibitory activity of 74.8% was observed in -22JTM(I), followed by 70.3 and 67.6% in -6(b)JTM(L) and-7JTM(S), respectively. Three isolates were found to demonstrate potent L-asparaginase production, viz. -22JTM(I),-7JTM(S) and -25JTM(I). Interestingly, partial purification of the L-asparaginase demonstrated maximum enzyme production in -22JTM(I). In the phenol red plate assay, six isolates demonstrated anti-obesity activity, of which four isolates viz, -6(b)JTM (L), -7JTM (S), -23JTM (I) and -25JTM (I) exhibited 100% lipase inhibitory action. The free radical scavenging potential of the isolates as a contributor to the observed bioactivities was investigated. The isolates exhibited potent antioxidant activity with 70.9% by -23JTM (I) followed by -14JTM (L) and -22JTM (I) with 67.4 and 60.1%, respectively. The most potent isolate, -22JTM (I), was identified as *Fusarium sp.* by morpho-taxonomic tools. The present study showcases the promising therapeutic potential of fungal endophytes from *N. jatamansi*, demonstrating the repository of potent anti-obesity and anti-inflammatory chemistries.

Keywords: Anti-inflammatory, Anti-obesity, Endophytic fungi, *Fusarium* sp., Lipase inhibition

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Introduction

Endophytes are ubiquitous and diverse groups of microorganisms with imperceptible existence within the internal tissues of host plants for at least a part or whole of their life cycle[1,2]. Endophytes are reported to exhibit biological diversity coupled with their potential to emanate a plethora of novel bioactive chemistries to be utilised in various therapeutic settings[3]. The discovery of bioactive compounds such as paclitaxel, podophyllotoxin, deoxypodophyllotoxin, camptothecin, and azadirachtin has ushered the era of tapping the novel chemistries from endophytes. Many industrially relevant enzymes viz lipase, asparaginase, papain, and pectinase have been purified from fungal endophytes, mainly from *Aspergillus niger*, *Alternaria sp., Fusarium sp.*[4-7].

Fungal endophytes represent an abundant resource of novel antioxidant, anticancer, anti-obesity, antidiabetic, and antimicrobial compounds by either producing certain enzymes or by blocking specific enzymes[8-10]. Among the various enzymes to be used as therapeutic tools, L-asparaginase is considered a potential enzyme involved in exhibiting the anti-inflammatory and antitumor potential against acute lymphoblastic leukaemia (ALL), acute myeloid leukaemia (AML), and non-Hodgkin's lymphoma. L-asparaginase derived from prokaryotic sources suffers from greater allergic and adverse immunological reactions[11,12]. Considering the above effects, fungal endophytes emerged to be a reliable source of producing L-asparaginase for the treatment of leukaemia. Few studies have documented the L-asparaginase production from a variety of fungal endophytes viz. *Fusarium proliferatum*, *Colletotrichum*, *Phoma* and *Penicillium* sp[13,14].

Among the inflammation-associated disorders, obesity has become the prevalent condition that further manifests the progression of adverse human health conditions such as diabetes, cardiovascular disorders,

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arthritis, insulin resistance, and certain cancer types. As per the records of WHO[15], 13% of the total global population is obese and at risk of developing many serious health conditions[16]. Obesity is a multifactorial set of disorders characterised by an anomalous or extreme accumulation of lipids. Many targets have been utilised to prevent and treat the condition of obesity. However, pancreatic lipase (PL) is an effective and valid target due to the least adverse effects[17]. Human PL (EC 3.1.1.3) catalyses the hydrolysis of triglycerides to fatty acids. The major strategy to prevent obesity is to decrease the absorption of fatty acids by inhibiting the pancreatic lipase in the digestive tract. Orlistat is the only synthetic FDA-approved drug being administered as a PL inhibitor to treat obesity[18]. Due to the hepatotoxicity associated with the intake of orlistat, there is an utmost need for novel and safer drugs with lesser side effects. Fungal endophytes, the treasure trove of novel and diverse bioactive molecules and their significant contribution to drug discovery and development need to be utilised to explore many therapeutic moieties[19-21]. The rationale for isolating the novel medicinal chemistry containing endophytic fungi lies in the rationale of selecting the host plant for the isolation. *Nardostachys jatamansi* is a pivotal medicinal plant exhibiting the potential to treat respiratory system disorders, urinary system disorders, neurological disorders, insomnia, epilepsy, hysteria, syncope, convulsions, and mental weakness in Ayurveda. It has been reported to demonstrate antioxidant, antiarrhythmic, anticonvulsant, lipid peroxidation, and antidepressant activities. Considering the above facts, the present study was undertaken to systematically prospect fungal endophytes inhabiting *N. jatamansi* and to tap the repository with lipase inhibitory activity, anti-inflammatory, and antioxidant activity.

Materials and Methods

Plant sample collection and isolation of fungal endophytes

Healthy plant parts (leaf, stem, and inflorescence) of *N. Jatamansi* (D.Don) DC. (Caprifoliaceae Juss.: Dipsacales Batsch) were identified and deposited (Hb/101-NJ) in the Department of Botany, Shri Guru Ram Rai University, Dehradun, Uttarakhand, were collected from the Yamanotri region located at 31.0140° N, 78.4600° E in Uttarakhand in the month of December 2019. The collected plant parts were surface sterilised by dipping them in 0.1% sodium hypochlorite for 2-3 min, followed by 70% Ethanol for 1 min, and then subsequent washing by dipping in 30% ethanol for 30-45 sec. Further, the surface-sterilised sample was cross-sectioned into 1-2 mm pieces, followed by inoculation onto pre-sterilized Potato Dextrose Agar (PDA) plates aseptically. The inoculated plates were incubated at 26±2°C for 8-10 days and were regularly observed for any fungal growth[22]. The fungal hyphae emerging from the section were transferred to a fresh PDA plate aseptically with the help of an inoculation loop to obtain pure culture[23].

Production of culture filtrate

Each fungal endophyte was subjected to submerged fermentation by inoculating 5 mm mycelial plug of 7 days actively growing culture into 100 mL pre-sterilised PDB broth in Erlenmeyer flasks aseptically followed by incubation on the rotatory shaker at 120 rpm, 26°C for 7-10 days. After the culmination of the incubation period, the fungal mycelium was separated from broth by filtration through Whatman filter paper No. 4, followed by centrifugation at 10,000 rpm for 10 min to get cell-free culture filtrate[24].

Screening for pancreatic lipase inhibitor production

For assessing the lipase inhibitory potential of fungal endophytes, phenol red olive oil plate assay as described by Singh *et al.*[25] was followed. Briefly describing, the phenol red-olive oil agar (pH-7.0) plates comprising 2.5% olive oil, 0.01% filter sterilised phenol red and 0.01% Tween 80 and molten agar (2%) were prepared. Subsequently, 50 µL of the master mix containing pre-incubated 15 µL of Porcine Pancreatic Lipase (Stock- 100 U/mL) and 35 µL of culture filtrate was dispensed into 5 mm wells prepared by sterile cork borer followed by incubation at 37°C for 24 h. The control comprised 15 µL of PPL and 35 µL of sterile PD broth. The appearance of a yellow halo against a red background indicated the PPL activity in the control, while the reduction in the diameter of the halo as compared to the control indicated PPL inhibition.

Antioxidant assay

The antioxidant potential of fungal endophytes was assessed by following the method of Ho *et al.*[26] employing a DPPH (2, 2-diphenyl-1-picrylhydrazyl hydrate) assay. Briefly, the reaction mixture comprised 800 µL of 0.1M TrisHCl (pH 7.8) and 200 µL of the crude fungal extracts. To this test solution, 1 mL of freshly prepared DPPH (Stock-1

mg/mL) solution was added to initiate the reaction. The tubes were mixed well and stored at room temperature in dark conditions for 30 minutes. Blank was prepared by adding 800 µL of TrisHCl to 1.2 mL of methanol. DPPH solution was used as a control. After the culmination of the reaction period, the absorbance was recorded at 517 nm. Ascorbic acid (1 mg/mL) was used as positive control.

Anti-inflammatory assay

The anti-inflammatory activity of all the fungal endophytes was assessed by two methods: L-asparaginase production and albumin denaturation assay.

Screening for L-asparaginase production

Primary screening

Briefly describing, asparagine-phenol red agar plates comprising L-asparagine- agar media (1%) supplemented with filter sterilised 0.09% phenol red were prepared. Further, a 5 mm mycelial plug of actively growing culture was inoculated onto the asparagine-phenol red agar plate, followed by incubation at 28±2°C until the profuse fungal growth was observed. Un-inoculated plate served as control. The plates were regularly monitored for the pink zone formation, indicating the hydrolysis of L-asparagine around the colony. The presence of the asparaginase enzyme hydrolyses the asparagine into aspartic acid, and due to aspartic acid formation, which is basic in nature, thereby indicating the pH change of phenol red in the form of the pink zone. The asparaginase activity was measured by recording the pink zone diameter[27].

Secondary screening

In the secondary screening, the positive isolates from the primary screening were subjected to an agar well diffusion assay. The L-asparagine-phenol red agar plates were prepared as described above. Briefly, 40 µL of each culture filtrate was dispended into the 5 mm well-prepared wells, followed by incubation at 37±2°C for 24 hours. After the culmination of incubation, the plates were observed for the appearance of a pink zone around the well, indicating L-asparaginase production. Un-inoculated media was used as control. The asparaginase activity was measured by recording the zone diameter.

Albumin denaturation assay

The anti-inflammatory activity was assessed by employing an albumin denaturation assay as described by Kumari *et al.*[28] with minor modifications. Briefly, the reaction mixture comprised 200 µL of Bovine serum albumin (stock- 1 mg/mL), 1400 µL of Phosphate Buffered Saline (PBS), and 1000 µL of test extract. The tubes were incubated at 37°C for 15 min and then heated at 70°C for 5 min. After cooling the tubes, the absorbance was recorded at 660 nm using a UV-VIS spectrophotometer (Systronics, India). Negative control comprised of 1000 µL distilled water in place of test extract. Diclofenac (1 mg/mL) was used as positive control. The percentage inhibition of protein denaturation was calculated by the following formula

$$\% \text{ Denaturation Inhibition} = \frac{Abs_{control} - Abs_{test}}{Abs_{control}} \times 100$$

$Abs_{Control}$ is the absorbance of negative control, and Abs_{Test} is the reading of test extracts under study.

Partial purification of L-Asparaginase enzyme from potent fungal endophyte

The fungal endophyte exhibiting maximum L-asparaginase production was subjected to mass fermentation of 5 L by inoculating 10 mm active mycelia plugin Erlenmeyer flasks containing pre-sterilised PDB medium and processed to obtain cell-free filtrate as described in the previous section.

For the partial purification of the enzyme of interest from cell-free filtrate, the salting out method by ammonium sulphate was employed[29]. Briefly, solid ammonium sulphate was slowly added to the culture broth to achieve saturation with slow and continuous stirring at 4°C. After achieving the threshold of saturation, the mixture was incubated overnight at 4°C followed by centrifugation at 12,000 rpm for 15 min at 4°C to recover the crude protein precipitate. The obtained precipitate was dissolved in a minimum volume of 1M phosphate buffer (pH- 7.2) and reanalysed for L-asparaginase production as described previously. A phosphate buffer was used as control while evaluating the L-asparaginase production of partially purified enzymatic extract.

Identification of potential endophytic fungi

The potential isolate exhibiting L-asparaginase activity was identified by classical taxonomic tools. The culture was grown over different media and observed for morphological characteristics, viz. colony size, texture, colour, growth rate, and microscopic characteristics for tentative identification.

Size and septation of microconidia and macroconidia were observed as the morphological parameters, and further identification was with the FusaHelp database (A site program for the morphological identification of Fusarium species, www.fusahelp.com)[30].

Statistical analysis

The statistical analysis was carried out by employing Analysis of Variance (ANOVA) in Graph Pad Prism 5 software followed by post hoc analysis by Tukey's test (P<0.05). All the data is represented in triplicates, and their mean and SD are calculated. Three independent experiments were performed for each test.

Results

Isolation of fungal endophytes

A total of 15 fungal endophytes with significant variation in tissue colonisation were recovered from *N. jatamansi* after three independent isolation experiments from leaf, stem and inflorescence. Out of 15 endophytic fungi, maximum colonisation of fungal endophytes was observed in the leaf (60%), followed by inflorescence (26.6%) and stem (13.3%) (Fig. 1).

Anti-obesity assay: Screening for pancreatic lipase inhibitor production

Among all the endophytic isolates, six were found to inhibit the activity of porcine pancreatic lipase. As per One Way ANOVA analysis [F (7,14) = 233.7, P<0.001] and post hoc analysis by Tukey's test, four isolates viz.-6(b) JTM (L), -7 JTM (S), -23 JTM (I) and -25 JTM (I) exhibited maximum relative lipase inhibitory activity of 100%. Further, -21 JTM (I) and -9 JTM(S) displayed a weak relative lipase inhibitory activity of 37.7 and 21.9%, respectively, with a zone size of 6.67 and 8.33 mm, respectively, in comparison to control with a zone size of 10.67 mm (Table 1; Fig. 2).

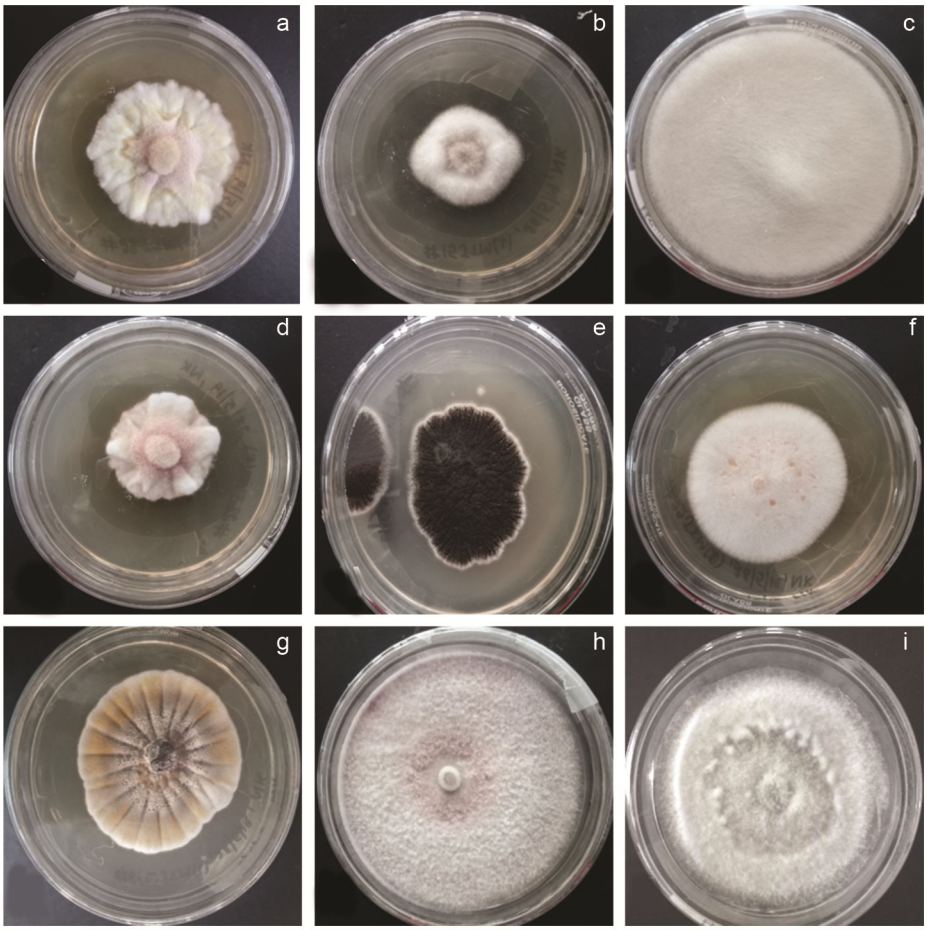

Fig. 1 — Fungal endophytes obtained from the medicinal plant *N. jatamansi*, a) -23 JTM(I); b) -12 JTM(L); c) -9 JTM(S); d) - 23 JTM; e) -7 JTM(S); f) -22JTM(I); g) -6(b) JTM(L); h) -14 JTM (L); and i) - 15JTM(L). The culture coding nomenclature includes JTM-*N. jatamansi*, I- inflorescence, L-Leaf, and S-stem.

Anti-inflammatory activity

L-asparaginase production and partial purification

In the preliminary screening for L-asparaginase production, five isolates viz. -22 JTM (I), -15 JTM (L), -9 JTM (L), -25 JTM (I) and -7 JTM(S) were found to be potent producers due to the formation of the pink-coloured zone around the colony, resulted due to the pH shift of neutral to alkaline by the hydrolysis of L-Asparagine into aspartic acid and ammonia (Fig. 3).

Table 1 — Lipase inhibitory potential of fungal endophytes by Phenol red agar plate assay

S. No.	Culture code	Relative pancreatic lipase inhibition (in % age)
1	-6BJTML(L)	100[a]
2	-7JTM(S)	100[a]
3	-25JTM(I)	100[a]
4	-23JTM(I)	100[a]
5	-21JTM(I)	37.5[b]
6	-9JTM(S)	21.9[c]
7	Control	0[d]

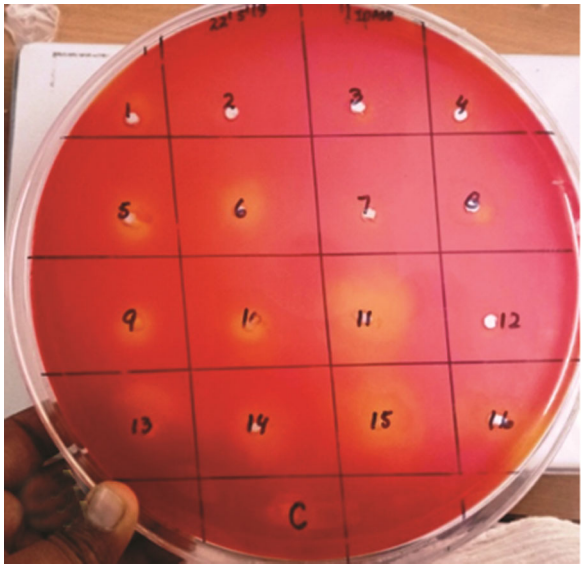

Fig. 2 — Porcinepancreatic lipase inhibitory activity exhibited by culture filtrates of different fungal endophytes over olive oil-phenol red agar plate assay.

The positive isolates from primary screening were screened using the agar well diffusion method. Out of the five selected isolates, only three isolates displayed the L-asparaginase production. As per One Way ANOVA analysis [$F(3,6)=134.0$, $P<0.001$] and post hoc analysis by Tukey's test, maximum L-asparaginase production was observed in -22 JTM (I) with zone size of 13.6 mm followed by -7JTM(S) and -25 JTM (L) exhibiting zone size of 13 mm and 10.6 mm respectively (Fig. 4).

Further, the partial purification of L-asparaginase enzyme from -22 JTM (I), -25JTM (I) and -7JTM (S) displayed a relative shift in the increase of enzyme activity. Maximum enzyme production was exhibited by the crude protein of -22JTM (I) with a zone size of 20.6 mm (Fig. 5).

Albumin denaturation assay

In the albumin denaturation assay, relative inhibition of protein denaturation was chosen to select the isolates with anti-inflammatory potential. Of all the isolates under investigation, as per one-way ANOVA analysis [$F(10,20)= 20.63$, $P<0.001$] and Tukey's post hoc analysis, maximum denaturation inhibition of 74.8% was exhibited by -22 JTM (I) followed by 70.2±0.1 and 67.2±0.3% by -6(b) JTM(L) and -7 JTM (S), respectively. Diclofenac disodium exhibited 68.4±1.4% protein denaturation inhibitory potential (Table 2).

Antioxidant assay

In the DPPH free radical scavenging assay, six isolates exhibited free radical scavenging activity greater than 50%, followed by four isolates in the range of 40-50% (Table 3). As per one-way ANOVA [$F(14,28)= 372.2$, $P<0.001$] and Tukey'spost hoc analysis, the

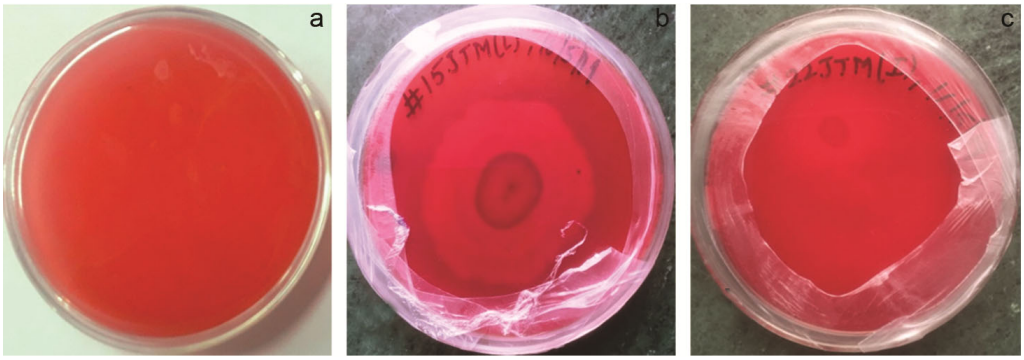

Fig. 3 — L-asparaginase enzyme production by different fungal endophytes, a) Control plate; b) - 15JTM(L); and c) -22JTM (I).

maximum free radical scavenging activity of 71.1±0.2% was observed in -23 JTM (I) followed by -14 JTM (L) and -22 JTM (I) with 67.4±0.3% and 58.2±0.8% scavenging activity respectively. No activity was observed in -23 JTM (L) and -24 JTM (L).

Identification of potential endophytic fungi

The colony of -22 JTM (I) over PDA medium was white from the front and light orange from the back, moderately growing with aerial hyphae (Fig. 6a). Its appearance was velvety to woolly. The fungus produces light orange soluble pigment without odour (Fig. 6b-c). Among microscopic characteristics, hyphae were thick, septate (3 to 7), multinucleate and branched. Macro and microconidia were observed with size ranges of 33-42 and 8-11 µM, respectively. These were arranged in the chains of 6–18 (Fig. 6d-f). Hence, the potential endophytic fungus, - 22 JTM (I), was tentatively identified as *Fusarium sp.*

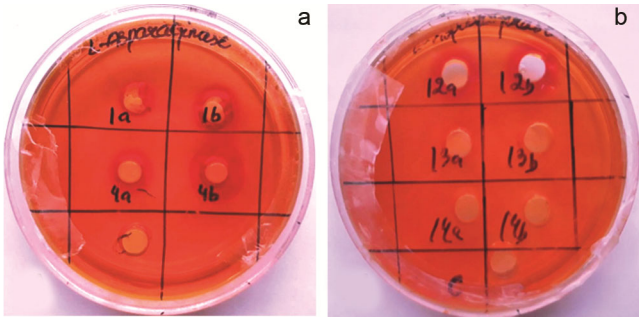

Fig. 4 — L-asparaginase enzyme production by culture filtrates of different fungal endophytes, a) well 1a-1b: 25JTM(L); well 4a-4b: 22JTM (I); and b) Well 12a-12b: -7JTM(S).

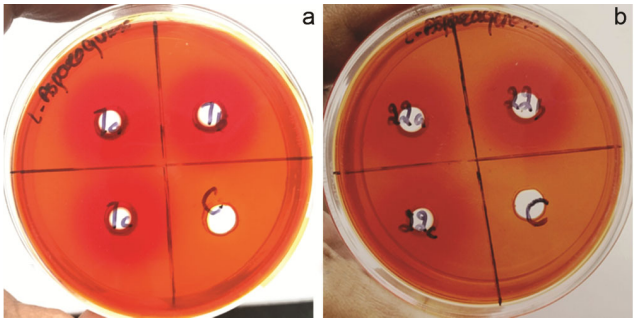

Fig. 5 — L-asparaginase production potential of partially purified protein extract, a) -7JTM (S); and b) -22 JTM (I).

Discussion

Endophytic fungi, forming symbiotic associations with host plants, have emerged as a valuable source of novel compounds with therapeutic potential. They are known to produce bioactive metabolites similar to those found in the host plant, making them promising candidates for various medicinal applications. The pharmaceutical and therapeutic fields have witnessed a revolution with the discovery of anti-inflammatory, anticancer, antifungal, and anti-diabetic agents from

Table 3 — Antioxidant potential of fungal endophytes by DPPH radical scavenging assay

S.No.	Culture code	Average	% age Free Radical Scavenging
1	-4JTM (L)	0.494±0.001	54.2^{d}±1.3
2	-6BJTM (L)	0.524±0.002	51.4^{e}±1.2
3	-7JTM (S)	0.562±0.003	47.9^{g}±1.1
4	-9JTM (S)	0.489±0.002	54.6^{d}±1.1
5	-10JTM (L)	0.677±0.019	37.2^{j}±1.4
6	-14JTM (L)	0.351±0.006	67.4^{b}±0.3
7	-15JTM (L)	0.545±0.005	49.4^{f}±0.7
8	-21JTM (L)	0.623±0.004	42.2^{i}±1.1
9	-21JTM (I)	0.677±0.018	37.2^{j}±1.2
10	-22JTM (L)	0.573±0.015	46.8^{h}±1.0
11	-22JTM (I)	0.451±0.007	58.2^{c}±0.8
12	-23JTM (I)	0.312±0.004	71.1^{a}±0.2
13	-25JTM (I)	0.714±0.050	33.8^{k}±7.0
14	Control	1.089±0.018	-

Table 2 — Anti-inflammatory potential of fungal endophytes by albumin denaturation assay

S.No.	Culture code	Absorbance at 660 nm				% age Protein denaturation
		A	B	C	Average	
1	-6BJTML(L)	0.053	0.049	0.051	0.051±0.002	70.2^{b}±0.1
2	-7JTM(S)	0.053	0.056	0.058	0.056±0.002	67.2^{d}±0.3
3	-9JTM(S)	0.069	0.073	0.084	0.075±0.007	56.1^{e}±4.6
4	-21JTM(L)	0.104	0.1	0.109	0.104±0.004	39.2^{h}±0.3
5	-22JTM(L)	0.088	0.085	0.081	0.084±0.003	50.8^{f}±0.5
6	-22JTM(I)	0.047	0.038	0.045	0.043±0.004	74.8^{a} ±2.6
7	-23JTM(L)	0.168	0.16	0.176	0.168±0.008	1.75^{i}±1.3
8	-25JTM(I)	0.09	0.088	0.08	0.086±0.005	49.7^{g}±1.7
9	Positive control (Diclofenac disodium)	0.054	0.053	0.055	0.054±0.001	68.4^{c}±1.4
10	Negative Control	0.176	0.163	0.176	0.171±0.007	-

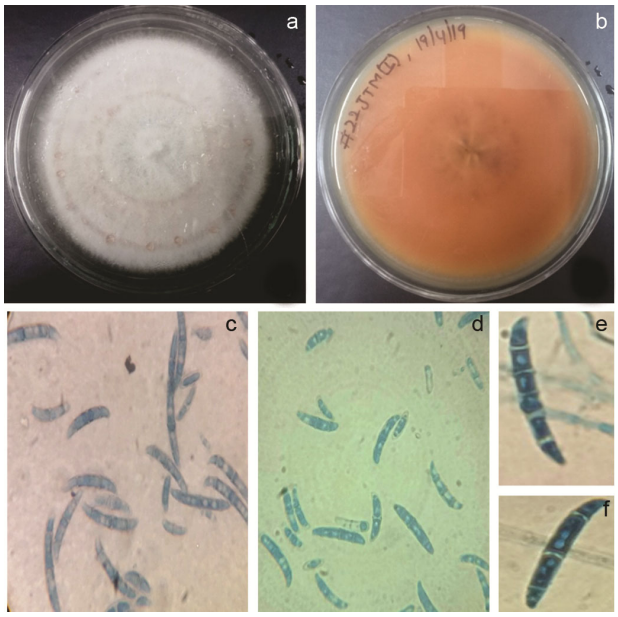

Fig. 6 — Morphological and microscopic identification of potential fungal endophyte -22 JTM(I) over PDA medium, the moderately growing colony with aerial hyphae, a) white in colour from front; b) light orange in colour from the back; and c-f) branched, septate and multinucleate hyphae with the presence of macro and microconidia.

endophytic sources. Although many aspects of endophytic fungi have been explored, there are still reports highlighting their anti-inflammatory and proteolytic capabilities. This study focused on investigating the anti-inflammatory and anti-obesity potential of fungal endophytes residing within *N. jatamansi.* Among the recovered isolates in the present study, four isolates displayed promising 100% lipase inhibitory activity, comparable to previous findings in endophytes from other plant species, viz. *Aegle marmelos*[31]. Two isolates also exhibited significant anti-inflammatory potential, particularly in terms of L-asparaginase enzyme production and protein denaturation capacity similar to that of L-asparaginase production study on fungal endophytes of *Rhododendron arboreum* by Kapoor *et al.*[5]. Certain isolate displayed promising anti-inflammatory activity in terms of protein denaturation comparatively better regarding a study conducted by Shoba and Sathiavelu[32] in which methanol extracts of *Cochliobolus* sp. from *Aervalanata* exhibited 62.5% of protein denaturation. Additionally, all the fungal endophytes demonstrated antioxidant activity, which aligns with previous studies conducted by Uzma and Chowdappa[33]. Further, crude protein residue recovered post partial purification of selected isolates displayed potent enzymatic activity in the -22JTM (I), contemplating the presence of the extracellular enzyme in the isolated endophytic fungi. Further, the potent isolate was identified as *Fusarium* sp. with microscopic examination. Microscopic observation revealed the presence of septation fusiform microconidia and macroconidia. Observations were similar to the identification done by Hafizi *et al.*[34] and Hlaiem *et al.*.[35] using the same parameters and identification keys[36]. As mentioned earlier, the present study stands as the first-ever report on the anti-inflammatory and anti-obesity potential of fungal endophytes derived from *N. jatamansi.* This novel discovery underscores the significance of exploring the untapped therapeutic capabilities of these fungal inhabitants within the medicinal plant. However, to harness the full potential of fungal endophytes for therapeutic purposes, further research is essential. Optimisation of the purification process is crucial to isolate and identify desirable bioactive molecules from the diverse repository of fungal endophytes. This step is pivotal in unlocking the medicinal properties of these compounds and may pave the way for the development of novel drugs and therapies.

Conclusion

The fungal endophytic flora of *N. jatamansi* inhabiting the high-altitude regions of Uttarakhand was screened for bioactivities viz anti-inflammatory and anti-obesity. Among the 15 isolates, the most potent bioactivity was exhibited by -22 JTM(I), identified as *Fusarium sp.,* using classical taxonomical tools. The isolate significantly inhibited the porcine pancreatic lipase, a key enzyme for curbing obesity. In addition, -22 JTM(I) exhibited potent anti-inflammatory potential via L-asparaginase production and albumin denaturation assays. Partial purification of L-asparaginase enzyme from -25JTM(I) and -22 JTM(I) provided new insights for the further purification and optimisation of desirable bioactive for therapeutic utilisation. In conclusion, this pioneering study sheds light on the untapped potential of endophytic fungi from *N. jatamansi* as a valuable source of bioactive compounds with significant anti-obesity and anti-inflammatory properties. These findings open new avenues for drug discovery and underscore the importance of harnessing nature's hidden treasures for human health.

Conflict of interest

There is no actual or potential conflict of interest.

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