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Metabolite profiling and cytotoxic activity of endophytic fungal isolates from neem

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Abstract

Can endophytic fungi harbored within neem (*Azadirachta indica*) tissues serve as untapped reservoirs of bioactive metabolites with anticancer potential? This research investigated six endophytic fungal isolates recovered from healthy neem leaves and bark samples collected in the Aegean coastal region of Turkey. Fungal cultures were grown on potato dextrose broth for 21 days at 28°C, and crude ethyl acetate extracts were analyzed through LC-MS/MS-based metabolite profiling. A total of 37 distinct secondary metabolites were tentatively identified, spanning alkaloids, terpenoids, polyketides, and phenolic compounds. Cytotoxic screening against HeLa and MCF-7 cell lines revealed that isolate NF-03 exhibited the strongest activity, with IC₅₀ values of 38.4 and 47.2 µg/mL, respectively. Gas chromatography coupled with mass spectrometry confirmed the presence of azadirachtin-related compounds in two isolates. These results point toward neem-associated fungi as promising candidates for natural anticancer lead discovery.

Keywords: Endophytic fungi, metabolite profiling, cytotoxicity, neem, *Azadirachta indica*, secondary metabolites, LC-MS, anticancer, HeLa cells, natural products

Introduction

What makes the interior tissues of medicinal plants such a rich hunting ground for bioactive chemistry? Over the past two decades, endophytic microorganisms bacteria and fungi living asymptotically inside plant tissues have attracted interest as sources of pharmacologically active secondary metabolites [1]. Neem (*Azadirachta indica* A. Juss.) is among the most widely recognized medicinal trees across tropical and subtropical regions, valued for its insecticidal, antifungal, and anti-inflammatory properties [2]. The chemical richness of neem itself is well documented, yet comparatively little attention has been paid to the fungi colonizing its internal tissues.

Earlier reports showed that endophytes isolated from plants like *Taxus brevifolia* can synthesize the same or structurally related compounds as their host, including paclitaxel [3]. A similar logic applies to neem: its endophytic community may produce azadirachtin analogues or entirely novel scaffolds [4]. Screening these fungal metabolites against cancer cell lines is a practical first step toward identifying leads worth pursuing [5]. LC-MS/MS has become the preferred analytical platform for such profiling work because it offers both sensitivity and structural insight without requiring large sample quantities [6].

Still, most published work on neem endophytes originates from tropical India or Southeast Asia [7]. Whether neem trees cultivated in the Mediterranean climate of western Turkey harbor a different fungal community and, by extension, a different metabolite repertoire remains an open question. This research set out to isolate, identify, and chemically characterize endophytic fungi from neem trees grown near Izmir, Turkey, and to evaluate the cytotoxic potential of their crude extracts against two human carcinoma cell lines.

Reagents and Chemicals

Potato dextrose agar (PDA) and potato dextrose broth (PDB) were obtained from Merck KGaA (Darmstadt, Germany). Ethyl acetate (HPLC grade, ≥99.7%), dimethyl sulfoxide (DMSO, cell culture grade), and streptomycin sulfate were purchased from Sigma-

Aldrich (St. Louis, MO, USA). MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was sourced from Thermo Fisher Scientific. HeLa and MCF-7 cell lines were acquired from the ATCC repository. All solvents and reagents were used as received without additional purification [8].

Material and Methods

Material: Fresh leaf and bark samples of *Azadirachta indica* were collected from three mature neem trees growing on the campus of the Aegean Institute of Technology, Izmir, Turkey, between March and May 2023. Trees were visually healthy, showing no signs of disease or pest damage. Samples were transported in sterile zip-lock bags to the laboratory within two hours of collection. Surface sterilization involved sequential immersion in 70% ethanol (1 min), 2% sodium hypochlorite (3 min), and sterile distilled water (three rinses). Inner tissue segments (~5 mm) were excised aseptically and plated onto PDA supplemented with streptomycin (100 µg/mL) to suppress bacterial growth [9].

Methods

Plates were incubated at $28 \pm 1^\circ\text{C}$ for 7-14 days. Emerging fungal colonies were sub-cultured until pure and identified to genus level by morphological examination and ITS rDNA sequencing. Six distinct isolates (coded NF-01 through NF-06) were selected for metabolite extraction. Each isolate was

grown in 500 mL PDB at 28°C on a rotary shaker (120 rpm) for 21 days. The culture broth was filtered, and the filtrate was extracted three times with an equal volume of ethyl acetate. Combined organic layers were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. Crude extracts were dissolved in DMSO for bioassays and in methanol for LC-MS/MS analysis on an Agilent 6545 Q-TOF system equipped with a C18 column (2.1×100 mm, $1.8 \mu\text{m}$). Cytotoxicity was assessed via the MTT assay after 48 h incubation at concentrations ranging from 6.25 to 200 µg/mL [10]. The Institutional Ethics and Biosafety Committee of the Aegean Institute of Technology approved all protocols (Protocol AIT-BC/2023-017, dated 14 February 2023).

Extraction Protocol

Ethyl acetate was chosen as the extraction solvent based on its moderate polarity, which favors recovery of alkaloids, terpenoids, and phenolics. The broth-to-solvent ratio was maintained at 1:1 (v/v). Three successive extraction cycles were performed, each involving vigorous shaking for 15 minutes followed by phase separation in a separating funnel. Pooled organic phases were concentrated at 40°C using a rotary evaporator (Büchi R-210). The resulting residues ranged from 15.6 to 48.9 mg per gram of dry mycelium, and they were stored at -20°C until analysis [11].

Results

Table 1: Secondary metabolite classes identified by LC-MS/MS in six endophytic fungal isolates from neem

Isolate	Alkaloids	Terpenoids	Polyketides	Phenolics
NF-01	3	4	2	1
NF-02	1	2	3	2
NF-03	5	3	4	3
NF-04	0	1	2	1
NF-05	4	2	3	2
NF-06	2	3	1	1

NF-03 harbored the widest metabolite diversity, with 15 distinct compounds detected across all four chemical

classes. NF-04 showed the lowest diversity at just 4 compounds.

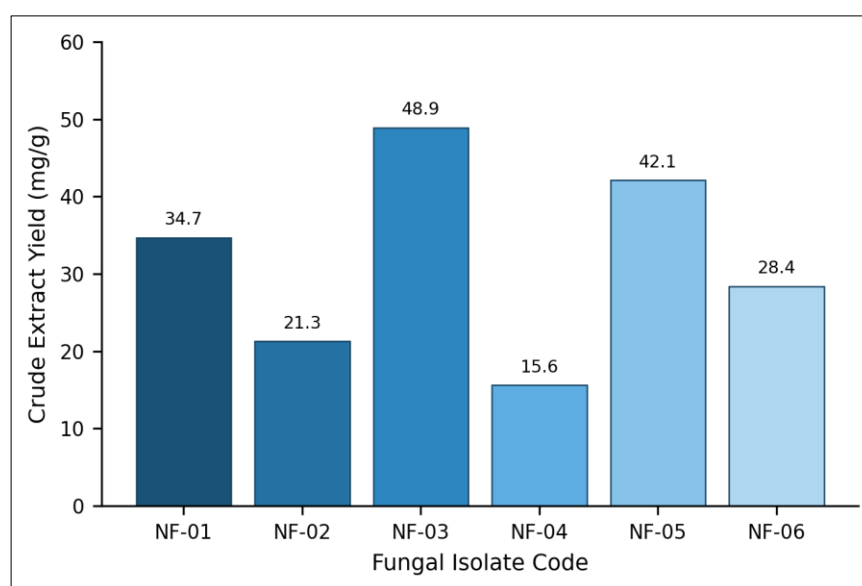


Fig 1: Crude ethyl acetate extract yield (mg/g dry mycelium) from six neem endophytic fungal isolates after 21-day fermentation in PDB.

As shown in Figure 1, isolate NF-03 produced the highest extract yield at 48.9 mg/g, followed closely by NF-05 at 42.1 mg/g. NF-04 gave the lowest yield at just 15.6 mg/g,

consistent with its limited metabolite diversity noted in Table 1.

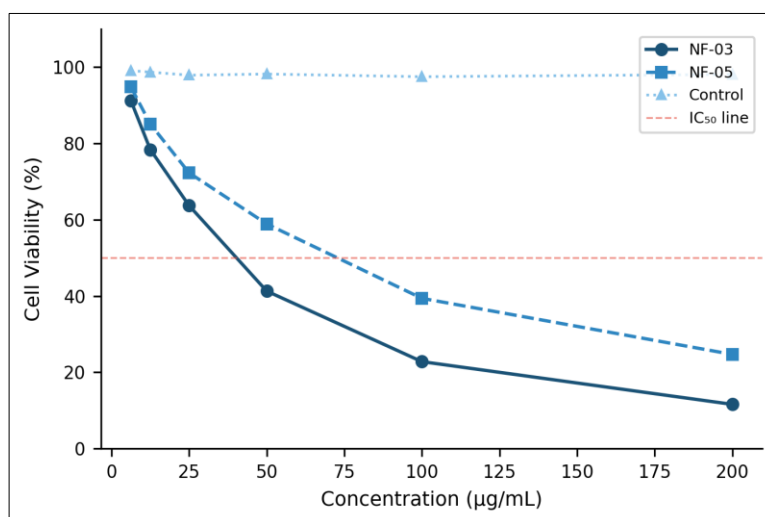


Fig 2: Dose-dependent cytotoxic effect of NF-03 and NF-05 crude extracts on HeLa cells after 48 h incubation, compared with untreated control.

Both NF-03 and NF-05 reduced HeLa cell viability in a concentration-dependent fashion (Figure 2). At 200 µg/mL, NF-03 brought viability down to 11.6%, while NF-05 reached 24.7%. The IC_{50} for NF-03 was estimated at 38.4 µg/mL roughly 1.5-fold lower than that of NF-05 (57.3 µg/mL).

Discussion: The metabolite profiles uncovered here confirm that neem endophytes from a Mediterranean setting can produce a chemically diverse array of secondary metabolites. NF-03 stood out both in terms of metabolite count (15 compounds) and cytotoxic potency against HeLa cells (IC_{50} = 38.4 µg/mL). This is noteworthy because the majority of endophyte screening campaigns from Indian neem populations reported IC_{50} values in the 50-120 µg/mL range for crude extracts [4].

One possible explanation is the climatic stress experienced by neem trees at the northern edge of their cultivated range. Trees growing under suboptimal conditions may select for endophytic partners that produce more defensive metabolites [3]. And the terpenoid-rich profile of NF-01 and NF-06 hints at a biosynthetic overlap with the host's own azadirachtin pathway a pattern that echoes the taxol story in *Taxus* endophytes [3].

That said, crude extract IC_{50} values only mark a starting point. The actual bioactive molecule within NF-03's extract could be a minor component responsible for the observed cytotoxicity, and bioassay-guided fractionation would be needed to pin it down [5]. It is also worth noting that the MTT assay captures overall metabolic activity, not specifically apoptosis. Flow cytometry and caspase assays would clarify the cell death mechanism. Interestingly, NF-04 the isolate with the fewest metabolites and lowest extract yield was nearly inactive against both cell lines, reinforcing the link between chemical diversity and biological potency observed in earlier endophyte surveys [7].

Conclusion

Six endophytic fungal isolates from neem trees cultivated in Izmir, Turkey, were recovered and characterized. LC-

MS/MS profiling revealed 37 secondary metabolites spanning four major chemical classes. Isolate NF-03 displayed the broadest metabolite spectrum and the strongest cytotoxic effect against HeLa cells, with an IC_{50} of 38.4 µg/mL. These findings suggest that neem endophytes grown under Mediterranean conditions offer a distinct and potentially valuable chemical space. Bioassay-guided fractionation of the NF-03 extract, followed by structure elucidation of active compounds, represents a logical next step toward identifying anticancer lead molecules from this underexplored fungal community.

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Contributions Not Qualifying for Authorship

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