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Antifungal and Molecular Assessment of *Azadirachta Indica* and its Silver Nanoparticles Against *Malassezia spp.* Isolated from Iraqi Patients

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ABSTRACT

Objective: *Considering the growing resistance of various fungi to antifungal drugs doses, and side-effects and challenges involved in the use of azole agents, this study aimed to evaluate the antifungal activity of neem seed extract and its green-fabricated silver nanoparticles (NS-AgNPs) against 40 clinical Malassezia species isolates from Wasit Province - Iraq was assessed.* ***Method:*** *The crude extract showed MIC and MFC values of 12.5 and 25 mg/mL, respectively, while AI-AgNPs displayed much greater potency (MIC = 0.125 mg/mL; MFC = 0.250 mg/mL). Correspondingly, inhibition zones reached 12.86 mm for the extract and 15.71 mm for AI-AgNPs.* ***Results:*** *AI-AgNPs also downregulated the lipase gene by ~2.75-fold, indicating reduced virulence, with higher infection rates noted among younger individuals.* ***Novelty:*** *These findings highlight neem-based nanoparticles as a promising, low-cost natural antifungal therapy, warranting further cytotoxicity and clinical validation.*

INTRODUCTION

Cutaneous mycoses due to *Malassezia spp*. are a major problem in the skin conditions of hot and humid places like Iraq [1,2]. These lipophilic yeasts also represent natural inhabitants of the skin, but can turn into pathogens when their host produces excessive sebum, is immunocompromised after organ transplantation or due to hormonal changes, developing diseases like pityriasis versicolor, seborrheic dermatitis and *Malassezia folliculiti*. With over 18 described species, including M. furfur, *M. pachydermatis*, and *M. globosa*, accurate identification and successful treatment are still clinically important features of this organism given increasing reports of relapse with therapy and antifungal resistance [3,4].

Current mode of treatment is dependent temporally on azole antifungals such as ketoconazole, itraconazole, fluconazole and allylamines -terbinafine. However these drugs are commonly plagued with limitations such as hepatotoxicity, high cost, variable efficacy and the problem of resistance particularly in resource-limited settings [5]. The latter has led to an increase in the search for alternative, low-cost and green methods for antifungal treatment.

And in this aspect, green nanotechnology has risen as a hopeful method leader new antimicrobial agents. The synthesis of silver nanostructures through plant extracts is a sustainable process involving no toxic chemical reductants and the phytochemicals work synergistically in this mechanism. In addition, *Azadirachta indica* (neem) is a traditional

medicinal plant known for its antimicrobial, anti-inflammatory, and wound-healing effects. Although most studies focus on the leaves, neem seeds also contain potent bioactive compounds such as azadirachtin, flavonoids, and phenolics [6]. HPLC profiling in this study revealed key constituents particularly rutin and apigenin likely responsible for the extract's antifungal activity.

There are some studies that have showed the possible ways in which nano-formulation can increase the bioactivity of plant extracts by improved solubility, stability and cellular uptake. These phytochemicals not only act as reducing as well as capping agents in the production of nanoparticles but also might be involved in its antimicrobial activity [7]. Despite this potential, there is an obvious scarcity in the literature reviewing the use of *Azadirachta indica* -associated AgNPs against *Malassezia* species especially with respect to their antifungal activity and level of molecular mechanism behind it.

Due to a high numbers of papers published on the synthesis and characterization of nanostructures, In the present study we have reported green synthesis, physicochemical characterisation and antifungal activity of aqueous leaf extract of *Azadirachta indica* (neem) mediated biosynthesized silver nanostructures against clinical isolates of *Malassezia spp*. Herein, the concept of finding a possible alternative means for overcoming widespread fungal infection is pursued using botanical resources inclusive of nanotechnology to construct a locally available, cost-effective and environmental friendly antifungal remedy that may be potential for dermatological topical treatment.

RESEARCH METHOD

A. Study Design and Ethical Considerations

The present experimental study was conducted between January and August 2025 at the Microbiology Laboratory, College of Science, University of Wasit, Iraq. All participants were provided with written informed consent prior to sample collection. Confidentiality was strictly observed by the investigator throughout the study.

B. Clinical Sample Collection and Fungal Isolation

Sixty skin-scraping samples were collected from clinically diagnosed pityriasis versicolor patients at the dermatology clinic of Al-Karama Hospital in Wasit Province. Aseptically taken materials were collected from wound margins on Sabouraud's Dextrose Agar with 1% Olive oil and incubated at 32 °C for 5–7 days. A total of 40 of those samples showed standard *Malassezia* growth, which was isolated and preserved at 4°C for molecular analysis and antifungal tests [8].

B. Phenotypic and Biochemical Characterization of *Malassezia* Isolates

Sixty skin scrapings were collected from patients clinically diagnosed with pityriasis versicolor at dermatology outpatient clinics in Wasit Province. Samples were gently obtained from the active margins of lesions using sterile scalpels and placed in sterile containers. Each sample was cultured on Sabouraud Dextrose Agar (SDA) supplemented with 1% olive oil and chloramphenicol (0.05 g/L) to promote the growth of lipid-dependent *Malassezia* species and to prevent bacterial contamination. The plates were incubated aerobically at 32 °C for 5–7 days. Colonies showing the typical

creamy or pasty appearance of *Malassezia* were sub-cultured for purification and maintained on SDA slants at 4 °C for further analysis [9,10]. Out of the sixty clinical samples, forty yielded *Malassezia* isolates that were included in this study.

C. Molecular Confirmation of *Malassezia* spp. Using ITS-PCR

Molecular confirmation of Malassezia isolates was achieved by amplifying the ITS region with primers ITS1 and ITS4. PCR was performed in a 25 µL reaction containing master mix, primers, and template DNA under the following conditions: 95 °C for 5 min; 35 cycles of 95 °C for 45 s, 52 °C for 1 min, 72 °C for 1 min; and a final extension at 72 °C for 5 min.

Amplicons were separated on 1.5% agarose gels and visualized under UV light, showing a single ~550 bp band confirming genus-level identity as *Malassezia*. The ITS-PCR was used solely for genus confirmation, while species identification relied on phenotypic and biochemical analyses [11].

Table 1. Primers used for ITS-PCR amplification.

Primer	Sequence (5′→3′)
ITS1	TCCGTAGGTGAACCTGCGG
ITS4	TCCTCCGCTTATTGATATGC

D. Preparation of *Azadirachta indica* (Neem) Seed Aqueous Extract

Neem seeds were sourced from a local herbal supplier in Wasit Province. Seeds were washed with distilled water, air-dried for 72 hours, and ground into fine powder using a sterile grinder.

To prepare the aqueous extract, 10 g of seed powder were mixed with 100 mL distilled water and boiled for 30 minutes. After cooling, the mixture was filtered through Whatman No. 1 paper, and the filtrate was stored at 4°C until use [12].

E. HPLC Analysis

HPLC analysis was performed to identify and quantify major phenolic and flavonoid compounds in *Azadirachta* indica seed extract using a SYKAM HPLC system (Germany) with a C18-ODS column (25 × 4.6 mm). The mobile phase consisted of methanol, water, and formic acid (70:25:5, v/v/v) at a 1.0 mL/min flow rate under isocratic conditions, with detection at 280 nm over a 20-minute run. Standard compounds (gallic acid, quercetin, rutin, apigenin, cinnamic acid) were used for calibration ($R^2 > 0.995$). Filtered samples (0.22 µm) were injected (20 µL), and peaks were identified by comparing retention times with authentic standards [13].

F. Green Synthesis of AI-AgNPs

Silver nanoparticles were synthesized through a green-reduction method using neem seed extract as both reducing and stabilizing agent. The extract was mixed with 1 mM $AgNO_3$ (1:9 ratio) under stirring and incubated at 60 °C for 24 h in the dark. The color change from pale yellow to dark brown indicated nanoparticle formation [14].

SEM confirmed nanoscale morphology, while XRD verified crystalline structure. The nanoparticles were collected by centrifugation (10,000 rpm, 15 min), washed thrice with distilled water, and re-suspended to a final concentration of 2 mg/mL [15].

G. Antifungal Susceptibility Testing

Broth Microdilution Assay (MIC and MFC)

Antifungal susceptibility was determined using a modified broth microdilution method for lipid-dependent yeasts. *Malassezia* colonies grown on Sabouraud Dextrose Agar with 1% olive oil were suspended in Modified Dixon Broth (≈1–5 × 10^6 CFU/mL) [16].

Two-fold dilutions of neem seed extract (0.78–100 mg/mL) and AI-AgNPs (0.0156–2 mg/mL) were prepared in 96-well plates; itraconazole and fluconazole (1–512 µg/mL) served as references. Plates were incubated at 32 °C for 72 h [17].

The MIC was the lowest concentration showing ≥50% inhibition, while MFC was defined as the lowest concentration with no colony growth upon subculture on Sabouraud Dextrose Agar with 1% olive oil. All assays were run in quadruplicate, and mean values were used for statistical analysis [18].

Agar Well Diffusion Assay

Antifungal activity was tested using the agar-well diffusion method. *Malassezia* isolates grown on Sabouraud Dextrose Agar with 1% olive oil were suspended in sterile saline (0.5 McFarland ≈ 1–5 × 10^6 CFU/mL) and spread onto fresh oil-supplemented plates [19]. Wells (6 mm) were filled with 100 µL of neem extract (100, 50, 25 mg/mL) or AI-AgNPs (2, 1, 0.5 mg/mL). Itraconazole and fluconazole were tested at 256, 128, and 64 µg/mL, while the solvent served as the negative control [20]. Plates were incubated at 32 °C for 72 h, and inhibition zones (mm) were measured with a digital caliper. All assays were done in duplicate, and mean values were recorded.

Assessment of Lipase Gene Expression Using qRT-PCR

To assess the effect of neem-based silver nanoparticles (AI-AgNPs) on *Malassezia* virulence, the expression of the lipase gene was quantified in six clinical isolates showing strong growth and urease activity. Total RNA was extracted using TRIzol®, and 1 µg was reverse-transcribed to cDNA. qRT-PCR was conducted on a StepOnePlus™ system with SYBR® Green chemistry, targeting lipase and normalizing to ACTIN as the reference gene.

Amplification efficiency was between 94% to 105%, with $R^2 > 0.99$, showing the reliability of the assay [21]. The sequences of the primers are shown in Table 2.

Table 2. Primer sequences used in the study.

Gene	Primer	Sequence (5' → 3')
Lipase	Lip-F	ACCCAACATTTGCTTCGTTC
	Lip-R	TCAATTATCAATGGTCGCGA
ACTIN	Act-F	CTCTCCTTGTACGCCTCTGG
	Act-R	TTGACAAGATGCTCCGTCAG

All primers were synthesized by Macrogen (Korea).

Relative gene expression was calculated using the 2^−ΔΔCT method, where untreated isolates represented baseline expression. The equations applied were:

ΔCT = CT_lipase – CT_ACTIN

ΔΔCT = ΔCT_treated – mean ΔCT_untreated

Fold change = 2^(-ΔΔCT)

Isolates treated with AI-AgNPs were labeled as N2 in the qPCR dataset.

H. Statistical Analysis

All assays were conducted with biological and technical replicates. Results were expressed as mean ± standard deviation (SD). Statistical comparisons were performed using one-way ANOVA followed by Tukey's post-hoc test to evaluate differences in inhibition zone diameters. Pearson correlation and Chi-square (χ^2) tests were used to examine associations among variables. A p-value < 0.05 was considered statistically significant. Analyses were carried out using SPSS version 26.0 (IBM Corp., USA).

RESULTS AND DISCUSSION

Results

A. Identification and Species Distribution of *Malassezia* Isolates

Out of 60 clinical skin samples, 40 *Malassezia*-positive isolates (66.7%) were obtained. ITS-PCR produced a single ~550 bp band confirming genus-level identity [22]. Species differentiation based on phenotypic and biochemical traits identified *M. furfur* (50%), *M. pachydermatis* (30%), and *M. globosa* (20%). *M. furfur* showed growth at 41 °C, while *M. pachydermatis* was able to grow without lipid supplementation [23]. Although *M. pachydermatis* is mainly an animal-associated, lipid-independent species, its detection in human samples may result from transient colonization, indirect contact with animals, or environmental adaptation. These results are consistent with the previously described phenotypic characteristics of *Malassezia* species[24], as presented in Table (3).

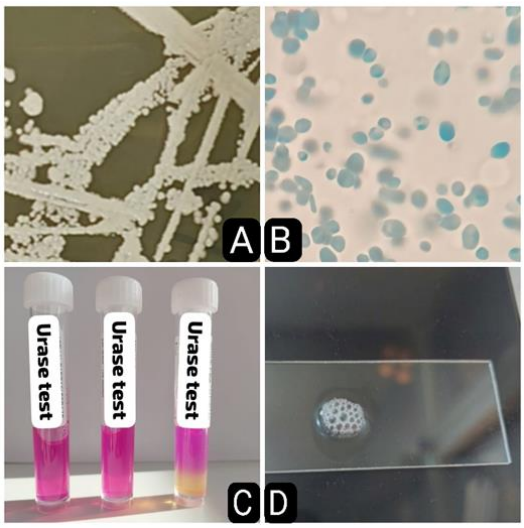

Figure 1. Shows the phenotypic identification of *Malassezia* isolates. (A) Smooth creamy yeast-like colonies grew on Sabouraud agar with olive oil, confirming lipid dependence. (B) Oval budding yeast cells were visible with Lactophenol Cotton Blue stain. (C)

Urease positivity appeared as a pink color on urea medium. (D) Catalase activity was indicated by bubble formation after adding hydrogen peroxide (H_2O_2).

Table 3. Morphological and biochemical characteristics of *Malassezia* species (n = 40).

Characteristic	*M. furfur*	*M. pachydermatis*	*M. globose*
Colonial appearance	Flat,smooth, slightly wrinkled	Flat to convex, occasionally umbonate	Elevated, coarse surface
Microscopic cell shape	Ovoid / cylindrical / spherical	Oval	Spherical
Growth on lipid-supplemented SDA	+	–	+
Growth on non-lipid SDA	–	+	–
Growth at 32°C (mDA)	+	+	+
Growth at 37°C (mDA)	+	+	+
Growth at 41°C (mDA)	+	–	–
Catalase activity	+	+	+
Urease activity	+	+	–
% of isolates	50%	30%	20%

"+" = positive growth/activity; "–" = no growth/negative activity.

B. Molecular Identification of Malassezia spp. Using ITS-PCR

Molecular identification using ITS-PCR produced a single ~550 bp amplicon in all isolates, confirming their identity at the genus level. Since ITS-PCR does not differentiate *Malassezia* species, species assignment in this study was based on phenotypic and biochemical characteristics [25].

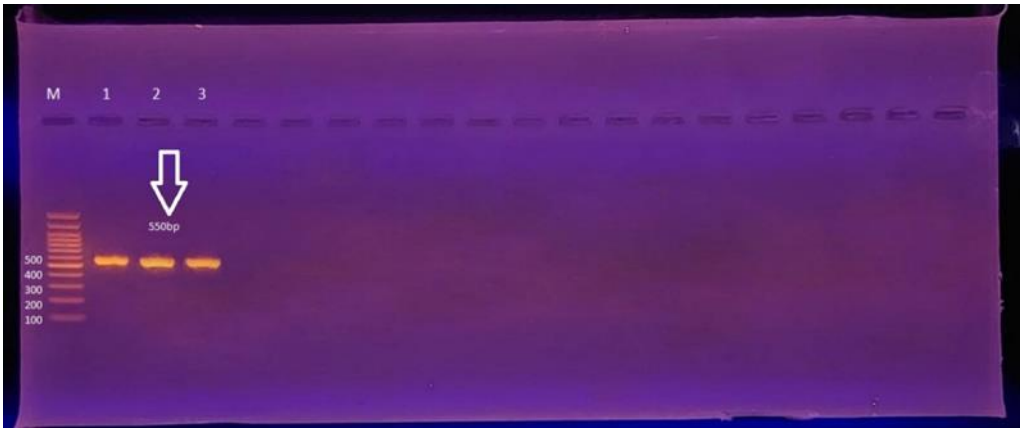

Figure 2. Shows ITS-PCR products of *Malassezia* isolates, where a single ~550 bp band was detected in all samples, confirming their identity. Lane M shows the 100-bp DNA ladder, and lanes 1–3 represent positive isolates.

C. Characterization of Biosynthesized Silver Nanoparticles

X-ray diffraction (XRD) confirmed the crystalline nature of the biosynthesized silver nanoparticles, showing distinct peaks at ~38°, 44°, 64°, and 77°, corresponding to the (111), (200), (220), and (311) planes of face-centered cubic (FCC) silver (JCPDS No. 04-0783) [26] Peak broadening indicated nanoscale crystallite formation, with particle size estimated via the Debye Scherrer equation.

Scanning electron microscopy (SEM) revealed predominantly spherical particles with slight agglomeration, typical of green-synthesized nanoparticles due to phytochemical capping. The average particle size ranged between 45 and 63 nm [27].

Note: Only XRD and SEM analyses were performed to confirm nanoparticle formation; further physicochemical tests (UV–Vis, FTIR, DLS) were not conducted due to technical constraints.

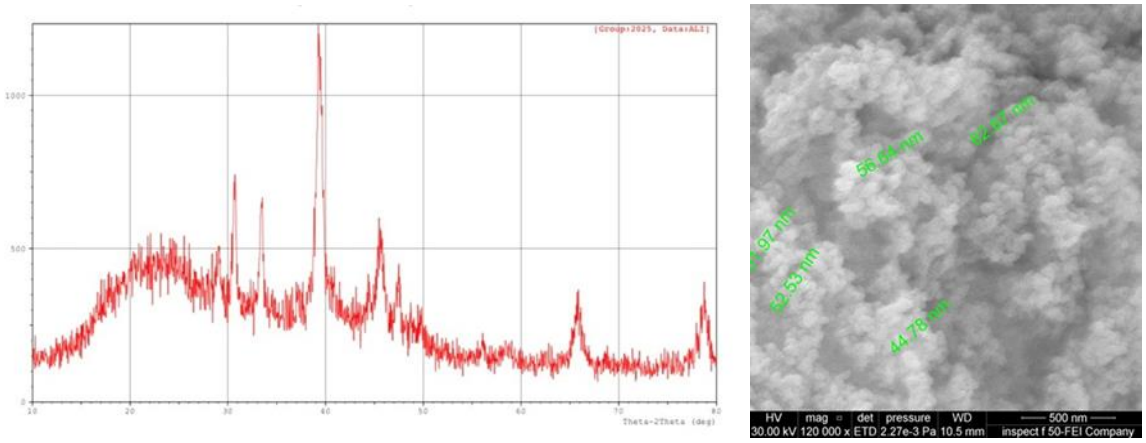

Figure 3. XRD spectrum and SEM picture of silver nanoparticles obtained from neem with crystalline structures peaks and spherical shape particles having sizes in range 45–63 nm.

D. Antifungal Activity of *Azadirachta indica*-Based Formulations

Minimum Inhibitory and Fungicidal Concentrations (MIC/MFC)

All clinical *Malassezia* isolates were inhibited by the aqueous seed extract of *Azadirachta indica* with MIC/MFC values of 12.5/25 mg/mL in all isolates. On the other hand, AI-AgNPs exhibited relatively strong antifungal activity with an average MIC and MFC of 0.125 and 0.250 mg/mL respectively. This enhancement is attributed to nanoscale effects; the smaller particle size and larger surface area produces greater interaction with, and release of, silver ions from cells.

In comparison, the itraconazole yielded MICs (16–32 µg/mL) and MFCs (32–64 µg/mL), whereas fluconazole exhibited both MIC and MFC at 32 and 64 µg/mL. Despite lower MICs for azoles, these are in µg/mL ranges, while plant formulations were measured in mg/mL terms and should be considered as indicating relative instead of absolute efficacy [28]. Collectively, the better efficiency of AI-AgNPs compared to crude extract shows their promise as low-cost antifungals for environmentally friendly applications in drug-poor environments or in cases of increasing resistance.

Table 4. MIC and MFC of test agents against *Malassezia* spp.

Agent	MIC Range	Mean MIC	MFC Range	Mean MFC
A. indica crude extract (mg/ml)	12.5	12.5	25	25
AI-AgNPs (mg/ml)	0.125	0.125	0.250	0.250
Itraconazole µg/ml	16 – 32	20	32 – 64	40
Fluconazole µg/ml	32	32	64	64

Note: 1 mg/ml = 1000 µg/ml. Itraconazole and fluconazole values are reported in µg/ml as per standard practice.

Agar Well Diffusion Assay

The agar well diffusion assay revealed concentration-dependent antifungal activity of *Azadirachta indica* extract, with mean inhibition zones of 12.83, 11.51, and 9.29 mm at 100, 50, and 25 mg/mL, respectively (control: 8.12 mm).

In contrast, AI-AgNPs showed stronger activity at lower concentrations, producing zones of 15.64, 14.15, and 13.55 mm at 2, 1, and 0.5 mg/mL (control: 9.45 mm).

Itraconazole yielded inhibition zones of 26.65, 17.24, and 12.76 mm, while fluconazole showed 23.14, 14.87, and 9.54 mm at µg/mL concentrations; drug controls measured 6 mm. Although azoles remained numerically more potent, AI-AgNPs exhibited notably greater antifungal activity than the crude extract, supporting their potential as a natural, effective topical antifungal alternative [29].

Table 5. Mean inhibition zone diameters (mm) of *Azadirachta indica* extract, AI-AgNPs, and standard antifungal drugs against *Malassezia* spp.

Agent	Concentration	Trial 1	Trial 2	Mean
A. indica crude extract (mg/ml)	100	12.79	12.86	12.83
	50	11.05	11.97	11.51
	25	9.27	9.30	9.29
	Control (C)	8.05	8.18	8.12
AI-AgNPs (mg/ml)	2	15.71	15.56	15.64
	1	14.07	14.22	14.15
	0.5	13.63	13.47	13.55
	Control (C)	9.24	9.65	9.45
Itraconazole(µg/ml)	256	26.66	26.63	26.65
	128	17.25	17.22	17.24
	64	12.77	12.75	12.76
	Control	6.00	6.00	6.00
Fluconazole(µg/ml)	256	23.12	23.15	23.14
	128	14.88	14.86	14.87
	64	9.55	9.52	9.54

Control	6.00	6.00	6.00

Note: Plant extract concentrations are expressed in mg/mL, while itraconazole and fluconazole concentrations are expressed in µg/mL. Direct potency comparison should consider unit differences.

Molecular Response to AI-AgNPs Treatment

To evaluate the molecular impact of AI-AgNPs on *Malassezia*, qRT-PCR was conducted before and after nanoparticle exposure. The virulence-associated lipase gene was the target, with ACTIN as the reference. Expression was analyzed using the 2^−ΔΔCT method, taking untreated samples as controls [30].

Treatment with AI-AgNPs resulted in a mean relative expression of 0.363, corresponding to a ~2.75-fold reduction, with values ranging from 0.083 to 0.667 across isolates. These results indicate consistent downregulation of the lipase gene, suggesting that AI-AgNPs can attenuate *Malassezia* virulence at the molecular level.

Table 6. qRT-PCR analysis of gene expression in *Malassezia* treated with AI-AgNPs.

Sample	CT(target)	CT(reference)	ΔCT	ΔΔCT	Relative Expression	Fold Change
N2-1	32.8	21.1	11.7	2.59	0.167	↓5.99
N2-2	31.8	21.4	10.4	1.29	0.410	↓2.43
N2-3	31.9	22.2	9.7	0.59	0.667	↓1.50
N2-4	33.6	20.9	12.7	3.59	0.083	↓12.05
N2-5	32.7	22.7	10	0.89	0.541	↓1.85
N2-6	32.6	21.8	10.8	1.69	0.310	↓3.22
Mean					0.363	↓2.75

E. HPLC Profiling of *Azadirachta indica* Extract

HPLC analysis of *Azadirachta indica* extract revealed five major phenolic and flavonoid peaks at retention times of 3.75, 4.15, 6.17, 8.84, and 11.76 minutes, confirming the presence of multiple active constituents. The peaks at 4.15 and 8.84 minutes showed the highest areas (22% and 24%), followed by those at 6.17 and 11.76 minutes (20% each), while the smallest appeared at 3.75 minutes (14%). These results indicate a phenolic- and flavonoid-rich profile responsible for the extract's antioxidant and antimicrobial properties, likely contributing to its antifungal effect against *Malassezia spp [31].*

Table 7. HPLC profile of *Azadirachta indica* seed extract showing retention time, peak area, height, and concentrations of major phenolic compounds.

Compound	Retention Time(min)	Area (mAU s)	Height (mAU)	Area %	PeakWidth W05 (min)	Concentration (ppm)
Gallic acid	3.75	2541.09	340.56	14.00	0.05	165.0
Apigenin	4.15	12656.88	805.98	22.00	0.15	70.6
Quercetin	6.17	8520.14	599.80	20.00	0.10	98.7
Rutin	8.84	11456.98	798.08	24.00	0.10	132.5

Cinnamic acid	11.76	7452.09	598.70	20.00	0.10	74.1

Total chromatogram area: 42,636.98 Total peak height: 3,143.25 Total percentage: 100%
Note: Compound identification was performed based on retention time and comparison with authenticated reference standards.

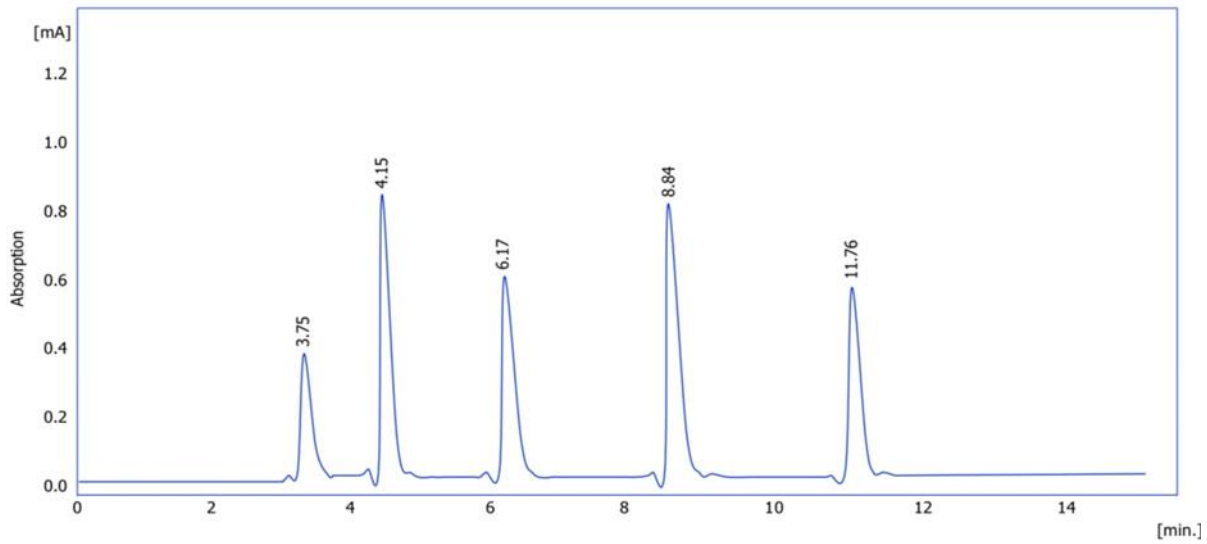

Figure 4. Figure 6: HPLC chromatogram of *Azadirachta indica* seed extract presented with indicating major phenolic and flavonoid compounds at their retention times. The peaks are the major bioactive compounds responsible for the antifungal activity of this extract.

F. Limitations and Future Work

This research verified the desirable anti-fungal implications of extract *Azadirachta indica* and AI-AgNPs; however, some limitations were observed. The sample size consisted of only 40 clinical isolates from one region (Wasit Province) and all assays were performed in vitro, which is not clinically relevant. While SEM and XRD confirmed the synthesis of nanoparticles, no additional analysis (UV-Vis, FTIR, DLS, zeta potential) were carried out nor cytotoxicity tests on human skin cells were performed with a consequent safety unexplored.

Further studies with larger and more diverse samples, complete physicochemical characterization and in vivo safety tests are necessary. In fact, the preparation of topical formulations such as gels, creams and sprays would be desirable and their stability, release rate and skin penetration should be evaluated. Synergistic combinations with natural antifungals along with transcriptomic or proteomic studies may also shed light on mechanisms and improve clinical utility.

Discussion

The present study reports that Antifungal activity of biosynthesized silver nanoparticles from *Azadirachta indica* (AI-AgNPs) is significantly stronger towards clinical *Malassezia* isolates as compared with the neem extract. This increase in efficiency

warrants that the A. indica loaded with SNPs has an enhanced antifungal activity and can serve as a reliable biologic agents to be used against various pathogenic fungi and may become cheaper tool for such risk areas like in Iraq.

A. Species Distribution and Clinical Importance

M.furfur was the most common yeasts with an observational prevalence of 50%, and followed by *M.pachydermatis* (30%) and *M.globosa* (20%) as indicated in Table-3. This pattern is consistent with findings in the Middle East and South Asia, where M. furfur is the most common agent of PV. Its ability to grow at 41 °C and the positive result of urease test also support its pathogenicity.It is worth noting that the animal-associated, lipid-yielding species *M. pachydermatis* was isolated in 30%, which could be explained by local adaptation or transient colonization. No significant association with animal exposure was found for this species ($p = 0.82$), indicating underlyingenvironmental or epidemiological reasons that may play roles on human infections [32].

B. AI-AgNPs Showed Improved Antifungal Activity

The crude *Azadirachta indica* extract exhibited moderate anti-*Malassezia* activity especially against M. furfur, with the MIC and MFC values of 12.5 mg/mL and 25 mg/mL respectively that were observed to be uniform essentially in all fractions, which can be attributed to its phenolic and flavonoid contents. But the use of high concentration is a limiting obstacle for its therapeutic efficacy.

On the other hand, neem-silver nanoparticles (AI-AgNPs) revealed significant efficacy (MIC = 0.125 mg/mL and MFC = 0.250 mg/mL), which was attributed to increased surface reactivity, better controlled release of silver ions, along with oxidative or membrane-damaging activities as well as phytochemical stabilization.

Although itraconazole and fluconazole showed numerically lower MICs (16–32 µg/mL and 32 µg/mL), their values were measured in µg/mL, while plant formulations were tested in mg/mL reflecting relative biological efficiency rather than direct potency [33]. An inverse correlation between MIC values and inhibition zone diameters further confirmed the consistency of results and the superior antifungal potency of AI-AgNPs, highlighting their promise as a natural, nano-enhanced antifungal alternative for areas facing drug resistance or limited access to antifungal therapies [34].

C. Dose-Dependent Inhibition and Zone Dynamics

The agar-well diffusion assay confirmed the superior antifungal activity of neem-derived silver nanoparticles (AI-AgNPs). At 2 mg/mL, AI-AgNPs produced a 15.73 mm inhibition zone, while the crude extract at 100 mg/mL showed only 13.02 mm indicating nearly 50-fold higher efficiency. Both treatments displayed a clear dose-dependent pattern [35].

Itraconazole and fluconazole showed larger zones due to their strong potency, but since they were tested in µg/mL versus mg/mL for plant formulations, this reflects relative biological performance rather than direct potency. AI-AgNPs therefore represent a natural, nano-enhanced antifungal option that could complement existing therapies [36].

D. Molecular Mechanism of Action

qRT-PCR analysis showed that treatment with AI-AgNPs caused significant downregulation of the *lipase* gene in *Malassezia* isolates, with a mean relative expression value of 0.36 (≈2.75-fold decrease) compared to untreated controls. Since lipase enzymes are essential for lipid hydrolysis and skin colonization, this reduction indicates decreased virulence [37,38]. The inhibitory effect of AI-AgNPs may involve oxidative stress, mitochondrial dysfunction, and interference with gene regulation, collectively contributing to the antifungal and anti-virulence action observed in MIC and MFC results.

E. Implications for Local and Global Antifungal Development

This study highlights the potential of neem-derived silver nanoparticles (AI-AgNPs) as a natural, affordable antifungal alternative. *Azadirachta indica* is abundant and traditionally utilized, supporting the feasibility of developing low-cost, eco-friendly, plant-based nanomedicines for pharmaceutical use. The strong inhibitory effect of AI-AgNPs against various *Malassezia* species suggests possible application even in settings lacking species-level diagnostics. Due to their wide-ranging antimicrobial activity, increased potency and natural origin these extracts can be considered a suitable candidates for the development of new topical antifungal preparations. However, further clinical and translation investigations are required to determine the ideal dosage and formulation stability and long-term safety before applying in clinic [38,40,41].

F. Limitations and Future Directions

This study has some limitations. While valuable data was obtained from the analysis of 40 isolates, larger multi-centre studies are required to improve generalisability. Although HPLC validated principal active ingredients of phenolics and flavonoids in neem extract, sophisticated approaches such as LC-MS could be used to unambiguously recognize the precise phytochemicals responsible for NP reduction and stabilization as well as potential synergistic mechanisms.

Moreover, cytotoxicity and biocompatibility testings on human skin cells (such as keratinocytes and fibroblasts) were not included; thus, further studies are required to confirm the safety of AI-AgNPs on topical application. To make these findings clinically applicable, in vivo animal studies and controlled clinical trials are needed for efficacy confirmation, dose establishment, and long-term safety.

CONCLUSION

Fundamental Finding : The present study provides the evidences regarding antifungal potential of *Azadirachta indica* (neem) against clinical *Malassezia* isolates, which was kmandable promoted by green-synthesized silver nanoparticles (AI-AgNPs). The MIC value of the crude extract was 12.5 mg/mL whereas that of AI-AgNPs was significantly lower with a value of 0.125mg/mL and it also formed larger zones even at reduced concentrations. At the molecular level, AI-AgNPs decreased lipase gene expression by approximately 2.75-fold and were suggested to exhibit a dual antifungal/anti-virulence potential through lipid metabolism and colonization pathway

perturbation. Itraconazole and fluconazole were slightly more potent than the neem based nanoparticles but these nanoparticles surpassed the crude extract, and it can be a potential, natural, ecofriendly option for resource-challenged as well as resistance setting with cost effectiveness. **Implication :** Considering the confirmed safety of neem43 and its eco-friendly synthesis route, AI-AgNPs could be considered as a promising candidate for future topical antifungal preparations. **Limitation :** Itraconazole and fluconazole were slightly more potent than the neem based nanoparticles but these nanoparticles surpassed the crude extract, and it can be a potential, natural, ecofriendly option for resource-challenged as well as resistance setting with cost effectiveness. **Future Research :** More cytotoxicity investigation, optimization of the formulation and in vivo validation should be considered for their clinical application.

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