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The Green synthesis of gold nanoparticles from synephrine extracted of citrus auriantum and its Potential effect against dermatophytes in vitro

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ABSTRACT
The prevalence of skin infections caused by fungi, especially dermatophytes species, has been on the rise recently. Patients with impaired immune systems are at increased risk for these infections, and the prospect of medication resistance is a real worry due to the current pharmacological landscape. Hence, new antifungal medications are required. Synephrine, a chemical with antibacterial and antifungal characteristics, was isolated from citrus sinensis peels. In order to verify the presence of synephrine, the extract was examined using thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC). A green synthesis approach was also employed to produce gold nanoparticles (AuNPs) using the extract. Energy dispersive spectroscopy (EDS), field-emission scanning electron microscopy (FESEM), and ultraviolet-visible spectroscopy were used to analyze the produced AuNPs. The findings demonstrated that the AuNPs were spherical in shape with a diameter between 9.7 and 31 nm. Dermatophyte isolates were obtained from patients and identified by means of polymerase chain reaction (PCR), macroscopic and microscopic examination, and other methods. Some of the isolates were Microsporum canis, Trichophyton rubrum, and Trichophyton mentagrophytes.

Keywords: Synephrine, AuNPs, Dermatophyte

INTRODUCTION

There has been an upsurge in the occurrence of fungal skin manifestations, particularly dermatophytes species, as reported by (Chanyachailert et al. 2023). It is more common for patients with compromised immune systems to get fungal infections (Rayens and Norris, 2022). This includes people receiving treatment for cancer, organ transplants, or who are HIV positive. This concerning trend is especially worrisome in light of the current lack of antifungal drugs (Vitiello et al., 2023). The reason behind this is because using antifungal medication before a fungal infection could lead to the emergence of resistant strains. So, the medical community is in dire need of new antifungals.

Micropsorum, Trichophyton, and Epidermophyton fungus are the causative agents of the zoonotic disease known as dermatologtophytosis. The dermatophyte species, agent virulence factors, host immune condition, age, and sex are all elements that influence dermatophytosis (Paryuni et al., 2020). A significant role in dermatophyte infection is reportedly played by elevated levels of cortisol and pro-inflammatory cytokines (Hamdy, 2024).

A number of plant secondary metabolites have potential as therapeutic agents (Rahimi et al., 2024). Synephrine is a proto-alkaloid that stands out among the secondary metabolites identified in citrus fruits (Hosseini et al., 2024). P-Synephrine is a phenyl-ethylamine derivative that has a hydroxyl group in the para position on the benzene ring and a chemical formula of C9H13NO2 (Jadou and Albahrani, 2024). Chemically and structurally, p-synephrine, m-synephrine (phenylephrine), and p-octopamine (nor-synephrine) are similar to ephedrine, as stated by Costa et al. (2022). One of the proto-alkaloids discovered in citrus fruit extracts is p-synephrine, the content of which tends to decrease as the fruit ripens. This compound possesses a wide range of pharmacological actions, such as those against microbes, fungi, antioxidants, cancer, and inflammation. Research by Karthika et al. (2024), Riar and Panesar (2024), and Mahato al. (2019) indicated that synephrine concentrations in orange juice vary between 53.6 and 158.1 µg/L, whereas in dried orange peel, the range is 1.2 to 19.8 mg/g. It has long been known that gold and its compounds have antibacterial properties. Nano-Au has just come to light as a possible antibacterial agent due to advancements in the research of metal nanoparticles. The production of uniform nano-sized gold particles with exact physical and chemical properties, as well as

exact dimensions and geometries, is thus of great relevance in the creation of new therapeutic treatments. The in vitro anti-fungal activity of synephrine extract and AuNPs biosynthesized from orange peel synephrine was examined against the most prevalent and common fungal species associated with skin diseases, namely Trichophyton rubrum, Trichophytonmentagrophytes, and Microsporumcanis.

MATERIALS AND METHODS

1. Extract preparation

1.1. Collection of plant samples

Citrus sinensis peels sourced from Iraqi marketplaces were utilized in this investigation. The plants were identified in the University of Baghdad's herbarium, which is part of the Department of Biology in the College of Science. Before further research, the plant peels are dried and ground into a powder using a mechanical grinder. The powder is then stored at4 C°.

1.2. Extraction of Synephrine

One hundred grams of powdered citrus sinensis fruit peels were defatted with n-hexane for 24 hours before being left to dry at room temperature. In a thimble, the defatted plant material was placed, and the soxhlet extractor was filled with it. A soxhlet extractor was used to dissolve 500 ml of 80% methanol in a 1 liter round bottom flask. It was decided to keep extracting for another twelve hours. First, the extract was mixed with 2N hydrochloric acid in a water bath, then filtered. Then, it was concentrated under decreased pressure to dryness using a rotary evaporator while the temperature remained below 40°C. Shaking the filtrate with chloroform further removed any remaining impurities. The alkaloidal bases were extracted from the acidic water layer by raising the pH with ammonia. Then, the water layer was concentrated under vacuum and went through the processes of separation, purification, and identification(Jasim et al .,2016).

1.3. Using Thin Layer chromatography (TLC)

To investigate plant content, on prepared silica gel GF254 (20 x 20 cm) plates with a 0.25 mm thickness from MERCK, the extract was analyzed using TLC. A n-butanol spraying reagent containing 2% ninhydrin was employed for detection. Chengdu Biopurify Phytochemicals was the manufacturer of the synephrine standard.

1.4. Using High-performance liquid chromatography (HPLC)

The HPLC method was used to determine the synephrine's identity. The Develosil ODS-5 column was used, which had a particle size of 5 m, an internal diameter of 4.6 mm, and dimensions of 250 mm × 4.6 mm. Along with a 2:98 acetonitrile/water mixture containing 10 mM phosphoric acid, 0.8 mL of water was added every minute to make up the mobile phase. A constant 223 nm eluent monitoring allowed the detector to take data between 200 and 360 nm. There were 5 liters in the injected sample. The temperature at which the column was operated was 35 degrees Celsius. Synephrine was located by comparing its retention time and ultraviolet (UV) spectra to a database reference standard. The chemical concentration was calculated by adding the sample's and standard's peak regions. Each treatment was repeated three times, and the results are shown as the mean.

1.5. Biosynthesisofgoldnanoparticles

Using a biosynthetic method that involves plant peels, gold nanoparticles are created. The citrus peels are rinsed in distilled water, then chopped into small pieces and cooked in the same universal solvent to obtain the extract. Additional purification of the extract is possible by employing several techniques, including filtration and centrifugation. Creating nanoparticles from the extract and auric salts solution is an easy, quick, and environmentally friendly process that only requires mixing the two at ambient temperature. Because phytochemicals accomplish the dual purpose of decreasing and stabilizing, no further external capping or stabilizing agents are required. A further incubation of the reaction mixture is performed to eliminate the chloroauric acid HAucl4 metal salt, and the color change is used for visual monitoring.

1.6. AuNPs Characterization

An essential stage in the biosynthesis of gold nanoparticles is the characterization of the particles. Gold nanoparticle synthesis is frequently distinguished by the stability, dispersion, size, shape, and surface area of the particles. Three methods used for characterizing nanoparticles are UV-visible spectroscopy, Field – Emission Scanning Electron microscopy (FESEM) and Fourier Transform Infrared (FTIR) spectroscopy.

1.6.1. Ultraviolet-visible spectroscopy

Absorption spectroscopy is referred to as a UV-Vis spectrophotometer. From 400 to 800 Wave length, UV-VIS double beam spectrophotometers were used to measure the samples.

1.6.2. Field – Emission Scanning Electron microscopy (FESEM)

Examine the surface of objects using electron probes instead of light microscopes. A large electrical field gradient is used to accelerate electrons that are created by the field emission source. A high-vacuum column concentrates the main electrons, and an electronic lens focuses the blasted electrons onto individual objects. This is how all of the objects release their secondary electrons. Surface properties have a significant impact on secondary electron velocity and angle. These electrons are captured by a detector, which then generates an electrical signal. This signal is used to create a video scan. An image that can be seen on a screen and preserved for future use is created by converting this signal to video scan (Ismail, Salleh and Yusof, 2020).

1.6.3. Energy DisperSpectro (EDS)

Laboratory services for spectroscopy is a set of tests that looks at how different materials respond to energy, usually light. Spectroscopic examination: comprehensive Spectroscopy tests can determine the presence or absence of certain compounds and components in a sample, as well as the degree to which deterioration has occurred.

Dermatophyte isolates preparation

1.7. Collection of specimens

The study examined 60 samples collected from patients between January and May of 2021 at Al-Kindy and Al-Karama Teaching Hospitals. Participants were selected and enrolled in the study once informed consent was obtained. Each patient had a comprehensive medical history recorded in accordance with the established process. Documentation also included the patient's name, gender, the affected body part, whether or not an inflammatory margin was present, symptoms, and the duration of the disease. After taking a full history, a comprehensive clinical examination was performed to identify the site of infection, the presence or absence of an inflammatory margin, and any other relevant factors. After taking aseptic samples of hair, nails, and skin, the area was cleansed with 70% ethyl alcohol to remove any remaining debris or environmental pollutants, such as bacteria. Then, the area was allowed to dry completely. A sterile scalpel was used to scrape the area surrounding the infection in order to get the skin sample. While cutting the nails using a sterile clipper, we used sterile scissors to gather hair samples. Placing the specimens on folded paper ensured that they remained dry during collection and transmission, preventing the growth of any potential bacterial contamination. The subsequent phase was to fold the paper and fasten it with a clip (Al-Hmadani, AlDhalimi and AbdulhusienAlrufae, 2014). In order to study the effects of nanoparticles and synephrine extract on the fungus, they were cultivated in Sabouraud Dextrose Broth for a long time.

1.8. Culturing of isolates, Preservation and storage

Isolates were stored at room temperature in a suspension in distilled water until testing. Subculturing each isolate on SDA twice ensured its viability and purity. The results that were obtained after incubating the plates in an incubator at 25°C for 7 days following the culture of the swabs in the lab were considered negative and rejected. Subcultures were subsequently introduced to any colonies that had grown throughout this period until only pure colonies remained (Amit and Wulff, 2022). Using 5 ml of sabouraud dextrose agar as slants, the isolates were cultured in screw-capped tubes and maintained in the fridge at 4°C for three months (Arthy, 2019).

1.9. Identificationofdermatophytes

1.9.1. MacroscopicExamination

The size, color, texture (fluffy, cottony, wiry, or suede-like), surface topography (folded, flat, rugose, or plicate), back side (medium pigmentation), and rate of development of the colony are all explored. Some of the fungus identified in this study are known to cause disease in humans. (Husseinetal.,2024)

1.9.2. MicroscopicExamination

A tiny sample of the colony was delicately removed off the slide and stained with a drop stain (lactophenol cotton blue) using either a straight wire or an inoculating needle that had been bent with flames. A cover slip was gently applied and examined under both low and high power magnification. (HashooshandAL-Araji,2023).

1.9.3. IdentificationofFungalIsolatesbyPolymeraseChainReaction(PCR)

Using universal primers (ITS), the rRNA gene and ITS sections were amplified. These regions are conserved in all eukaryotic organisms.The thermal cycling was conducted under these conditions: The thermal cycling protocol followed the steps outlined in Table 1. The first step was a 5-minute initial denaturation at 95 °C. Then, there were 35 cycles of 95 °C for 45 seconds, 52 °C for 1 minute, and 72 °C for 1 minute. The second extension was carried out at 72 °C for 5 minutes (Ranalli et al., 2022). Both the Master Mix and the GoTaq® Green Master Mix were utilized for the PCR. .Thefollowingprimerswereused:

Forward primer 5′- TCCGTAGGTGAACCTGCGG -3′

Reverse primer 3′- CCGCAGGTTCACCTACGGA -5′

Table 1. The Optimum Condition of Detection.

No.	Phase	Tm(°C)	Time	No.ofcycle
1-	InitialDenaturation	95°C	5min.	1cycle
2-	Denaturation	95°C	45sec.	35cycle
3-	Annealing	52°C	1min.	
4-	Extension	72°C	1min.	
5-	Extension	72°C	5min.	1cycle

The PCR results were separated using 1.5% agarose gel electrophoresis, and after being stained with safe red stain, they were seen under ultraviolet light.

1.1 Estimation anti-Fungal activity for Synephrine and gold nanoparticles against fungi

1. Mixed the three constration of synephrine and nano solution (10,15,20) mg/ml with the media.
2. Taken touch from pure colony by needle, and cultured in middle petri dish.
3. Incubated at (25) C° for (7) days.
4. Study the effect of gold nanoparticles on mold by measured the diameter of growth zone (Muhannad& Ahmed, 2020).

RESULTS

1. Thin layer chromatography (TLC)

Applied drops of solvent to the plate containing the sample and sorbent layer, the procedure was termed drop chromatograghy (Bagatela et al., 2015). TLC of the Citrus sinensis extract obtained from dried peels, confirms the presence of Synephrine in all extraction portions in comparison with standards. As shown in Fig. (4-2).

Figure 1. TLC Detection (a) Citrus auriantum

2. High performance liquid chromatography (HPLC)

Synephrine was estimated both qualitatively using HPLC. Using (Knauer/Germany), HPLC analysis was performed. By comparing the retention durations obtained from examined materials with genuine standards under identical chromatographic conditions, identifications were made. The column utilized was C18 (150mm 4.6mm/5um), the mobile phase was acetonitrile: water: orthophosphoric acid 0.1% acid (30:70), and the flow rate was 0.5 ml/min with UV detection220 nm detector . the stander retention time (6.5) min, the Citrus sinensis (6.2) as shown in Figs. (4-3), (4-4) (Jasim, Hussein and Nasser, 2016).

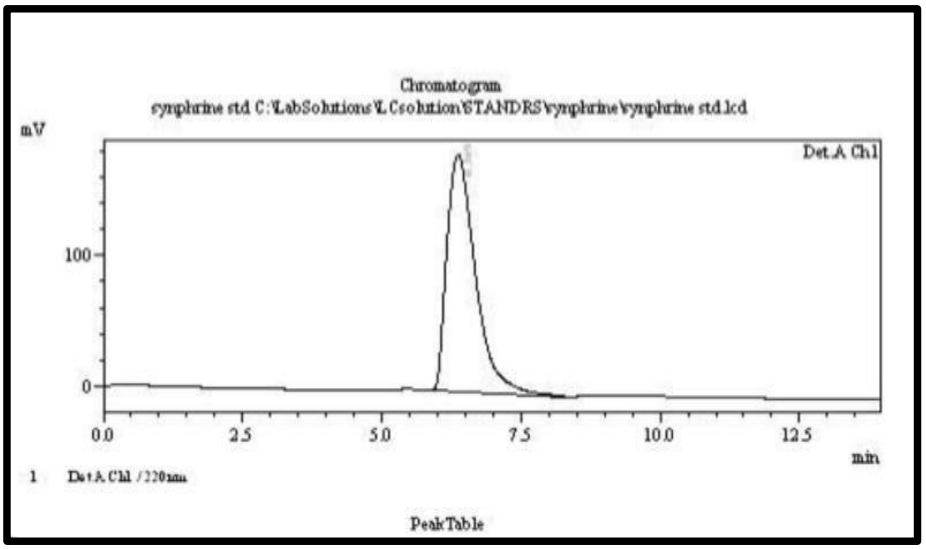


Figure 2. HPLC of Synephrine Stander.

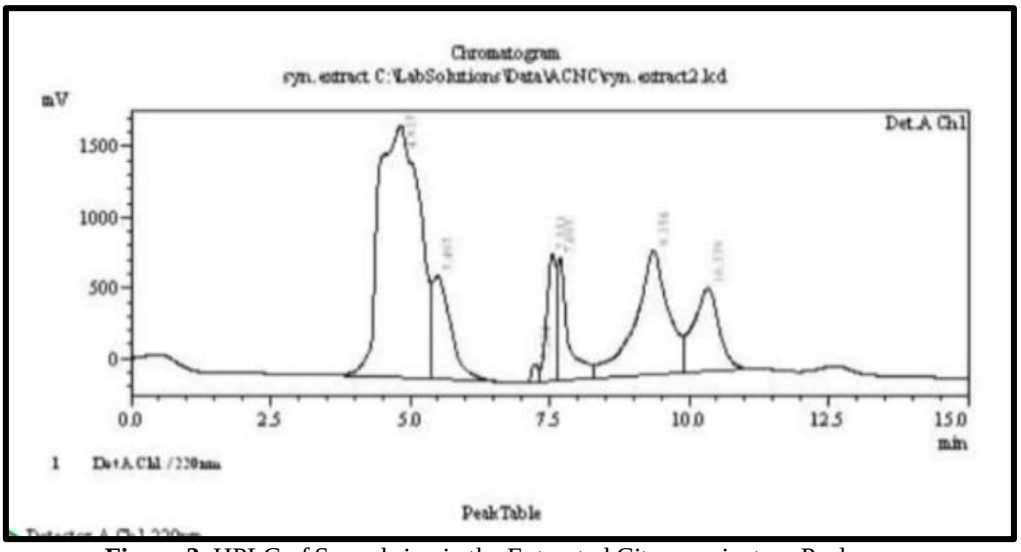


Figure 3. HPLC of Synephrine in the Extracted Citrus auriantum Peels.

3. Green Synthesis of Gold Nanoparticles

Citrus peels are employed as sources in the biosynthesis of AuNPs from the plant. HAuCl4 and plant extract are typically combined for the metal salt solution at room temperature. Alkaloids, flavonoids, proteins, polysaccharides, cellulose, and phenolic compounds are only a few of the organic chemicals found in plant extracts that are used to create nanoparticles These can function as stabilizing agents and include the bio reduction of metallic ions to NPsProteins with functionalized amino groups (NH2) found in plant extracts are capable of actively taking part in the reduction process of AuNPs Functioning groups

4. AuNPs Characterization

Synthesized AuNPs were initially identified in the change in reaction colour formation of brown to red color

4.1. Characterization Using UV-Visible Spectroscopy

The initial step was to characterize the gold nanoparticles. The reduction of gold ions to nanoparticle form was monitored by measuring the UV-Visible spectra of the solutions after dilution with deionized water. Using a Shimadzu UV-1601 spectrophotometer, which operates at a resolution of 1 nm from 400 to 700 nm, the UV-visible spectra were recorded using materials in quartz cuvettes. (Sujitha and Kannan, 2013), as Figs 4

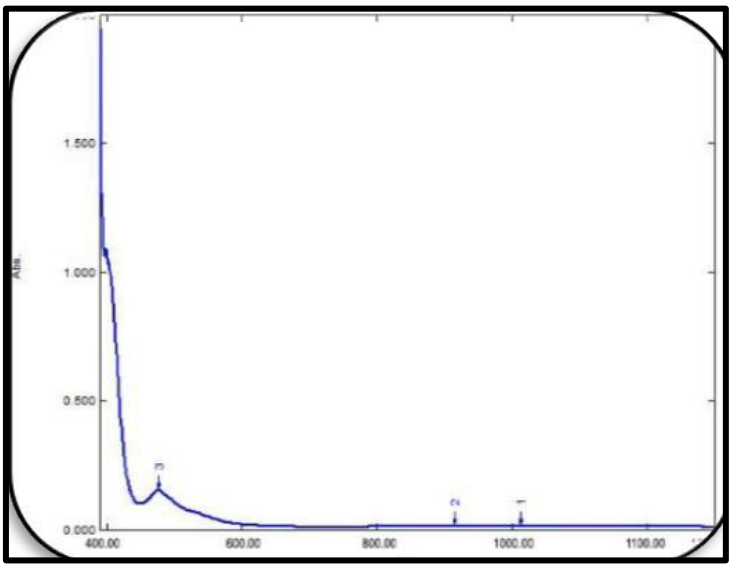


Figure 4. UV–visible spectrum of synthesized Au NPs FROM Citrus auriantumshowed peak at 465nm

4.2 Characterization of AuNPs using field – emission scanning electron microscopy (FESEM)

Using magnifications between 10,000 and 300,000x, field-emission scanning electron microscopy (FESEM) provides topographical and elemental data useful for characterizing AuNPs. Images produced by FESEM are sharper, less impacted by electrostatic distortions, and have better spatial resolution than those from traditional scanning electron microscopy (SEM). It requires no coating of insulating materials with conducting materials and may acquire high-quality, low-voltage images with only a small amount of electrical charging.

Table 2

No	Solution of AuNp	Diameter (nm)
1	AuNps biosynthesis by Citrus auriantum	20.8-34.3(nm) rounded to oval shape

Plant extract contains various phytochemicals, but no specific mechanism for manufacturing nanoparticles has been identified. Variations in reducing agent content and concentration in Shapes, sizes, and morphologies might vary when plant extracts are used. The most efficient biosynthesis was mediated by Citrus sinensis, and FESM analysis revealed spherical, solitary, or aggregated AuNPs. Possible biological uses for these chemical-free methods.

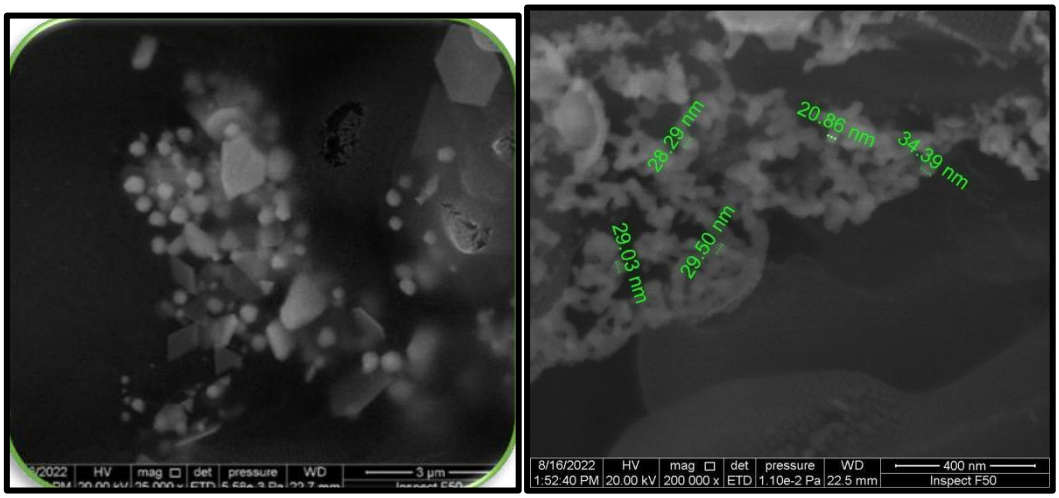
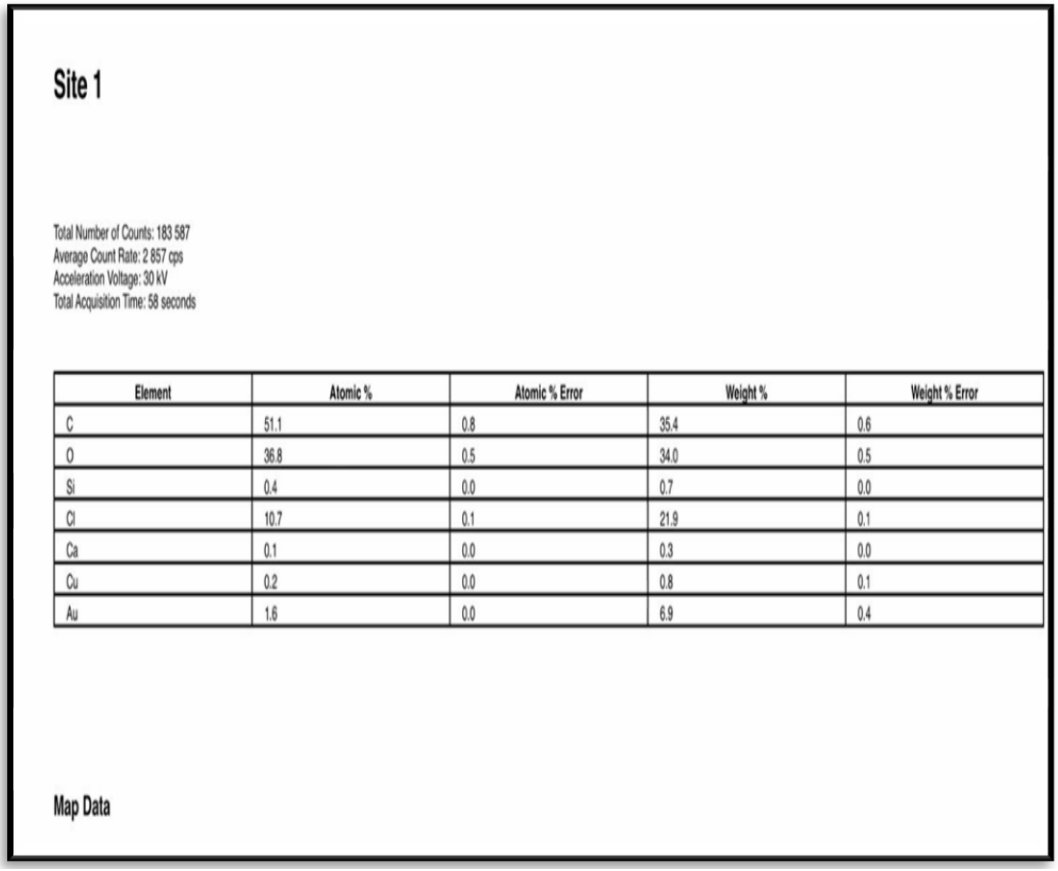


Figure 5. Show the Spherical Morphology Appearance and Diameter of (a) AuNps (b) Citrusauriantum.

4.3 Energy Disper Spector (EDS)

Quantitative and qualitative examination of components in samples can be achieved with the use of the Energy Dispersion Spectrometer (EDS). All elements and calibration standards had their line intensities measured.

Because it helps with identification and compares with recorded spectra,the ED spectrometer is great for qualitative analysis.



Element	Atomic %	Atomic % Error	Weight %	Weight % Error
C	51.1	0.8	35.4	0.6
O	36.8	0.5	34.0	0.5
Si	0.4	0.0	0.7	0.0
Cl	10.7	0.1	21.9	0.1
Ca	0.1	0.0	0.3	0.0
Cu	0.2	0.0	0.8	0.1
Au	1.6	0.0	6.9	0.4

Figure 6. Show the Result of Energy DisperSpectro for AuNp Biosynthesis fromauriantum**.**

5. Morphological diagnosis of isolated dermatophytes

In this experiment, colonies of Trichoderma glabrata, Trichodermarubrum, and Microsporumcanis were cultured on Sabouraud dextrose agar. Colonies of T. mentagrophytes grew rapidly, had surfaces that were powdery to granular, and generated conidiophores that were branching. In contrast to the white to buff, granular, or fluffy colonies of T. rubrum, the flat, slightly grooved colonies of Microsporumcanis ranged from white to yellowish. Their external cell walls were robust, and they possessed septate hyphae and lengthy macroconidia.

5.1 identification of dermatophytes by Polymerase Chain Reaction (PCR)

The identification of dermatophytes species was confirmed by PCR, which yielded three isolates. The findings showed a purity level of 1.4–1.8 and a concentration ranging from 37.1–72.5 g/ml. To ensure the DNA was pure, agarose gel electrophoresis was employed. For the molecular characterisation, each dermatophytes isolate had its nuclear rDNA region covering the ITS and 5.8S rRNA genes amplified. Each and every one of the examined isolates had its own distinct product size of about 550-650 bp, and this was achieved by amplifying the intergenic spacer region. With each isolate, we were able to produce an amplicon of this area that ranged in size from 565 bp.

6. EvaluatetheAntifungalAgentInVitro

Table 3: The MIC of Citrus auriantumAgainst Dermatophytes.

Genus	**Alkaloids from Citrus auriantum Inhibition rate %**			**Antifungal Candstatin**	**Control diameter of growth**	Mean±
	10 mg/ml	**15 mg/ml**	**20 mg/ml**			
Trichophytonmentographytes	30.6	40.8	67.3	59.1	4.917	49.5
Trichophyton rubrum	49	66	69.8	62.2	5.333	61.7
Microsporumcanis	48.4	62.5	84.3	73.3	6.433	67.1

lsd5%	0.889*					NS
Mean±	42.3	56.1	73.8	64.8	5.561	
p-value	0.165	0.001	0.020			

Table 4: MIC OF Gold Nanoparticles Biosynthesis from Citrusauriantum Against Dermatophytes.

Genus	**Au Np with Citrus auriantum Inhibition rate %**			**AntifungalCandstatin**	**Control**	Mean±
	10 mg/ml	**15 mg/ml**	**20 mg/ml**			
Trichophytonmentographytes	41	41.4	52.8	71	7.000	51.2
Trichophyton rubrum	59	52.3	57.1	52.3	4.233	55
Microsporumcanis	40	50	55	50	4.033	41.2
lsd5%	0.889*					0.421**
Mean±	46.6	47.6	54.7	2.028	5.561	
P-valeu	0.001	001	0.04			

DISCUSSION

The highest quantities of synephrine are found in Citrus auriantum(bitter orange) (Leeuwen et al., 2017), so the effect of alkaloids extracted from peels ofCitrusauriantumagainst the three types of fungal showed in Table (the statistics show that in 20mg/ml give the highest value of inhibition against Microsporumcanis, Trichophytonrubrum and Trichophytonmentographytes, respectively . Trichophyton rubrum in 15,20 mg/ml more effect than the antifungal, so there is a significant differences in compare with control. These results are reliable with Tripathy where was proved in 2017 that the extract of the Citrus auriantumhave inhibition effect on microorganisms. That's agree with The effect of Alkaloids on isolated fungi. It was gave the highest inhibitory rate comparable to control(Obead AL-Biati, 2023), synephrine has higher water solubility due to its amphoteric properties. The small relative molecular weight of synephrine enables it to readily penetrate the cell membranes. Synephrine exhibits a wide range of pharmacological activity without obvious adverse effects (Huang et al., 2022).The effect of gold nanoparticles and the control on the inhibition of molds growth used in this study was observed. When the gold nanoparticles were applied to the samples, the diameter of the mold colonies was less than the diameter of the colonies over which control was applied and the inhibition zone was larger than that of control.

When bacteria and fungi come into contact, it can have a negative impact on cellular metabolism, leading to the inhibition of cell growth.

Additionally, it inhibits respiration, the basal metabolism of the electron transfer system, and the transportation of the substrate into the microbial cell membrane. It has been found that nanoparticles act by increasing the permeability of the cell wall reactivate the oxygen atom interactively, thus disrupting cellular components and thus cell death mechanism (R. Wang et al., 2022).

CONCLUSION

Our investigation concludes that synephrine, an active ingredient with anti-dermatophyte qualities, is present in Citrus auriantum peels. Moreover, synephrine extracted from peels of Citrus Sinensis shows the capacity to form nanoparticles. Trichophyton rubrum, a dermatophyte fungus, reacts more favorably to orange peel extracts. Furthermore, the production of gold nanoparticles (AuNPs) from Citrus Sinensis extracts has stronger antifungal capabilities than the conventional antifungal medications utilized in the study. Furthermore, AuNPs generated from Citrus Sinensis extract have therapeutic properties, suggesting promise as a medication.

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