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Biosynthesis of Copper Nanoparticles Using Aqueous *Thymus daenensis* (Celak) Flora and Investigation of Its Antifungal Activity

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ABSTRACT

Introduction: In recent years, the green synthesis of nanoparticles has received much attention. Green synthesis has several advantages over other methods: cost-effectiveness, simplicity, and non-toxicity. In the present study, we obtained the aqueous extract of *Thymus daenensis* (Celak) flora, biosynthesized the copper nanoparticles (Cu-NPs), and evaluated the antifungal activity. **Methods:** UV-vis spectroscopy analyses, scanning electron microscopy (SEM), and energy-dispersive X-ray (EDX) were used to identify the synthesized nanoparticles. The antifungal activity of the synthesized copper nanoparticles was evaluated using the microdilution method. **Results:** After adding the extract to the copper sulfate solution, the solution color changed from light blue to yellowish-green. A maximum peak at the wavelength of 414 nm confirmed the copper nanoparticles formation. Scanning electron microscopy demonstrated the particle size ranging from 30 nm to 42 nm. The biosynthesized Cu-NPs had an inhibitory effect against *Candida albicans*, *Fusarium solani*, *Aspergillus Niger*, and *Aspergillus flavus*. **Conclusion:** Our findings demonstrated that *T. daenensis* aqueous extract acts as a reducer and stabilizer factor. We successfully synthesized Cu-NPs from copper sulfate using *T. daenensis* (Celak) flora aqueous extract according to the UV-Vis spectrum, FTIR, and SEM results. This research was the first report of Cu-NPs synthesized from an aqueous *T. daenensis* (Celak) flora extract. Our simple, quick, and inexpensive method for biosynthesis of a nanoparticle, which showed antifungal activity, provides a new potential antifungal agent for therapeutic applications.

INTRODUCTION

Green chemistry design, develop and deploy processes and products to reduce or eliminate hazardous substances to humans or the environment [1]. Today, the affordability and cost-effectiveness of a method are no longer enough [2]; the recyclability of raw materials, the biocompatibility of methods, and the safety of products, defined in green chemistry, are other critical dimensions that chemists must consider in addition to the economic justification of the process [3, 4].

Green chemistry is the design and development of chemical products and processes to minimize hazardous materials to the environment [5, 6]. Nanoparticles are synthesized by physical and chemical methods. Green synthesis has recently been considered an alternative environment-friendly method for producing nanoparticles. This approach has several advantages over other methods, including cost-effectiveness, simplicity, non-toxicity, and compatibility with medical and food applications [7, 8]. Nanoparticles are synthesized using all plant parts, such as seeds, stems, flowers, leaves, bark, and fruit [9]. In the green synthesis method, the extracts of some plants are also used as reducing agents and coatings for nanoparticle synthesis.

Due to their catalytic properties, copper nanoparticles have wide applications in medicine and are used as antifungals and antibacterials [10, 11]. Copper nanoparticles act as antibacterial agents in various fields and are toxic to many microorganisms, e.g., *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, and are non-toxic to animal cells [12-14].

Various plants containing phenol and flavonoids can be used to synthesize nanoparticles using the green synthesis method. Tthyme of the mint family is a short stem plant completely wooden at the bottom. The flowering stem height is ~30 cm, and leaves may be overlapping or shorter than internodes [15, 16]. This plant is propagated by seeds and cuttings and comes into flower from June to July [17]. Thyme is widely used in different parts of the world as a beverage, food flavoring, and herbal medicine [18]. Quantitative and qualitative methods confirmed that the functional groups in thyme extract were responsible for reducing copper metal ions and protecting the ions from oxidants during producing copper nanoparticles. Carvacrol and thymol are the main constituents of thyme and the origin of its antifungal properties. Thymol, the primary constituent of thyme, constitutes ~70% of the chemicals in thyme species worldwide [19]. The thyme flowers/leaves extract-based NPs have strong antispasmodic, anti-flatulence, anti-rheumatic, anti-sciatic, and antiseptic effects. It is also used in pharmaceuticals to prepare mouthwashes and cough syrups [20].

In this study, we synthesized copper NPs using *T. daenensis* (Celak) leaves extract and investigated the antifungal activity of synthesized copper NPs.

MATERIAL AND METHODS

Plant collection. *Thymus daenensis* Celak (thyme) specimens were collected in early June from the mountains around Khorramabad, Lorestan. After identifying the samples, thyme leaves were allowed to dry in the shade away from direct sunlight. The dried thyme leaves were ground to a powder using a mill and stored in the refrigerator until used.

Preparation of aqueous extract. 200 ml of deionized water was added to 10 grams of powdered thyme leaves in an Erlenmeyer flask and heated at 70 °C for 50 min. After reaching room temperature, the extract was first passed through filter paper, centrifuged at 10,000 rpm for 150 min, and stored in a refrigerator at 4 °C as described previously [21, 22].

Synthesis of copper nanoparticles. Ten ml of aqueous extract was added to 10 ml of 0.01 M freshly prepared copper sulfate solution (CuSO4.5H2O) mixing on the stirrer, followed by incubation at 60 ºC for 24 h. In the next step, it was centrifuged twice at 12.000 rpm for 20 min to remove all impurities. Nanoparticles started to deposit when the solution color changed from green to amber yellow. The synthesized nanoparticles were heated in an oven at 60 °C for further analyses [23].

Identification of nanoparticles

UV-Vis spectroscopy analysis. The reduction of copper ions to copper nanoparticles was investigated by a UV-visible spectrophotometer (JENWAY6405) exhibiting the surface plasmon resonance (SPR) of copper NPs. Three hundred µl of the sample was diluted with 3 ml of distilled water, and UV-Vis spectroscopy was measured using a spectrophotometer in the range of 300-700 nm.

Scanning electron microscope (SEM). The size and morphology of synthesized NPs were examined using electron microscopy (SEM) (Mira3, Made in Czech) with 15 kV with 1 nm resolution.

X-ray energy diffraction (EDX). X-ray energy diffraction spectroscopy with SEM was used to investigate the presence of copper in SEM images.

Antifungal activity

Fungal strains. Standard strains of *Candida albicans* (PTTC5072), *Fusarium Solani* (UAMH3317), *Aspergillus niger* (ATTC9029), and *Aspergillus flavus* (PPTC 5006) were prepared from the Department of Medical Mycology, Iran University of Medical Sciences, Tehran, Iran. The strains were cultured on sabouraud dextrose agar at 30 °C for 2-3 days.

***In vitro* antifungal activity assessment.** Antifungal effects of Cu-NPs against the four fungi were investigated using the broth microdilution method, according to the modified M27-A3 protocol on yeasts provided by the Clinical and Laboratory Standards Institute (CLSI) [24]. In the first stage, two-fold serial dilutions of each nanoparticle (ranging from 9.7 to 5000 µg/mL) were prepared in 1% dimethyl sulfoxide. Then, 100 µL of nanoparticle solution was pipetted into the first row of the 96-well microplates, and 50 µL RPMI (Sigma–Aldrich) was added to the other wells. Serial dilutions were prepared similarly for each well with 50 µL of the test material in serially descending concentrations. Afterward, 50 µL of broth containing each fungi suspension (5.0×105 CFU/mL) was added to each well. The microplates were sealed with clean film and incubated at 30 °C for 36 h.

Finally, 15 µL of 2, 3, 5-triphenyl tetrazolium chloride (TTC, 0.18%) solution was added to each well and incubated at 37 °C for 1 h. The lowest nanoparticle concentration with no color change was considered positive and defined as minimum inhibitory concentration (MIC). The lowest concentration that yielded negative sub-cultures was considered minimum fungal concentration (MFC) [25-27]. Then, the results were compared with Amphotericin B.

Statistical analysis. Statistical analysis of all experiments was conducted in triplicate, and the values were analyzed in SPSS V. 19 to find any significant differences between the parameters and variables. Results were considered significant if $P < 0.05$.

RESULTS

After adding the extract to the copper sulfate solution, the color of copper sulfate changed from pale blue to yellowish-green, and over time, brown particles began to precipitate. Figure 1 shows this color change, indicating copper nanoparticle synthesis.

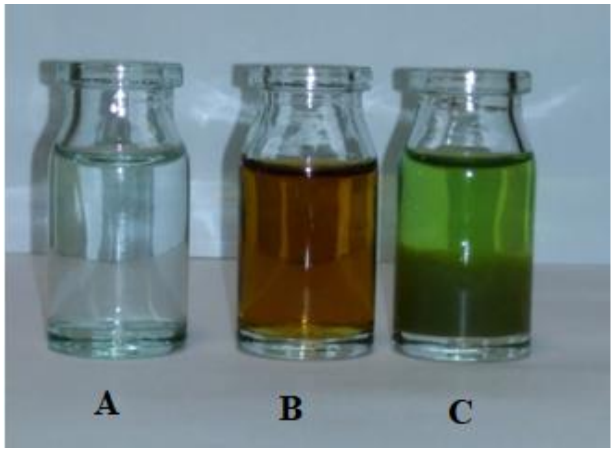

Fig. 1. Discoloration of copper sulfate solution after addition of thyme leaves extract. (A) copper sulfate solution, (B) NPs and plant extract, and (C) plant extract.

UV-Vis spectrum analysis. As shown in Figure 2, the synthesized nanoparticles have an optical absorption of 414 nm within the 300-600 nm range. The characteristic surface plasmon resonance peak (SPR) occurred at 414 nm for copper nanoparticles (Fig 2).

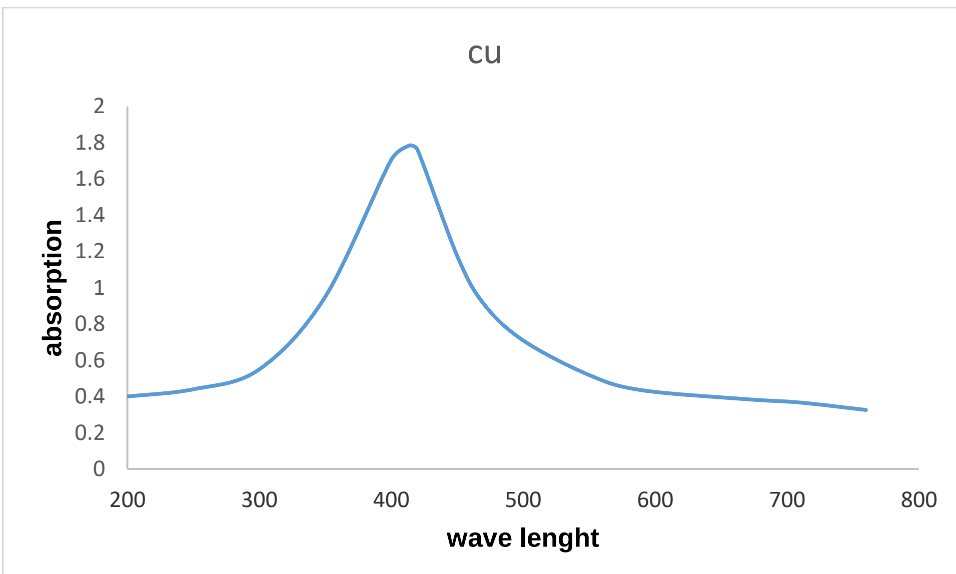

Fig. 2. The absorption spectrum of copper nanoparticles.

Scanning Electron Microscope (SEM). After confirming nanoparticle synthesis using color change, and Vis-UV absorption spectra, investigating the morphology of synthesized nanoparticles by scanning electron microscopy at Lorestan University indicated the particle sizes ranging from 30 nm to 42 nm.

The presence of metallic copper was confirmed by EDX analysis (Fig. 4). Copper nanoparticles have an absorption peak in Kev 1, an indicator for copper metal nanoparticles.

Table 1. Antifungal activity of copper nanoparticles.

Fungi	Cu-NPs MIC (µg/ml)	Cu-NPs MFC (µg/ml)	Amphotericin B MIC (µg/ml)	Amphotericin B MFC (µg/ml)
C. albicans (PTTC5072)	2.5	2.5	0.312	0.625
F. Solani (UAMH3317)	5	5	1.25	2.5
A. niger (ATTC9029)	10	10	2.5	2.5
A. flavu (PPTC 5006)	10	10	0.625	1.25

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Antifungal activity of synthetic nanoparticles. Copper nanoparticles showed antifungal activity against *C. albicans* (PTTC5072), *F. solani* (UAMH3317), *A. niger* (ATTC9029), and *A. flavus* (PPTC 5006) when compared with Amphotericin B (Table1). Even though copper nanoparticles showed lower MICs and MFCs than Amphotericin B, they showed noticeable MICs and MFCs against the four fungi, particularly *C. albicans* and *F. solani* strains.

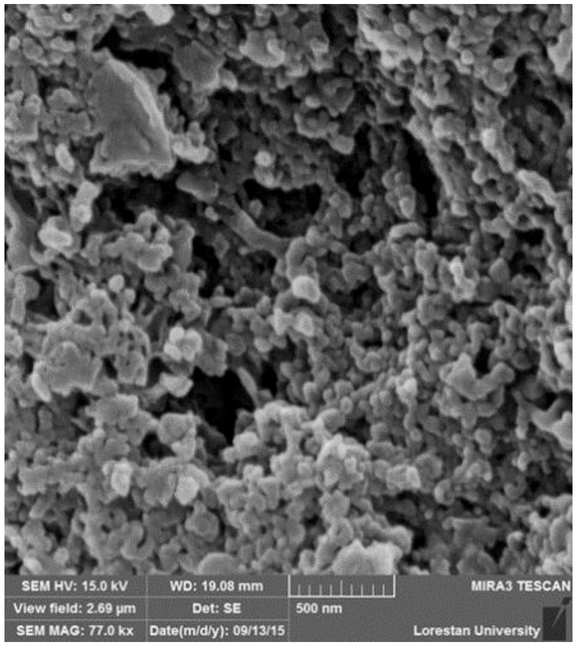

Fig. 3. Electron microscope images of synthesized copper nanoparticles.

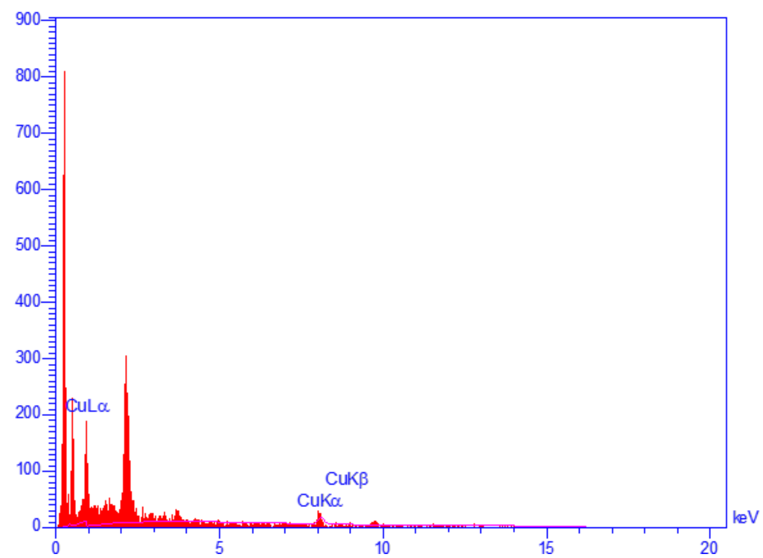

Fig. 4. The spectrum of synthesized EDX copper nanoparticles

DISCUSSION

Green synthesis of copper nanoparticles is a simple, cost-effective, eco-friendly, non-toxic, and non-polluting method. The nanoparticles obtained by this method have shown to be very stable and renewable [2, 6, 28, 29]. Copper nanoparticles have anti-cancer and antimicrobial properties and are used in various medical applications in wound dressing and food packaging [7, 8]. These nanoparticles have industrial applications such as electric capacitors, heat transfer, ultra-strong materials, sensors, and catalysts. One of the items that have received much attention is the therapeutic effects of medicinal plants compared to chemical drugs [7]. Researchers have recently considered synthesizing metal nanoparticles with biological agents, primarily plants [30]. These methods are faster because no chemicals are used [9]. So far,

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copper nanoparticles with various plants, including basil (*Ocimus sanctum*) [31], blind plant (*Capparise zeylanica*) [32], lily flame plant (*Gloriosa superbal*) [33], grape extract (*Vitis vinifera*) [34], *Nerium oleander* [35], *Magnolia Kobus* [36], soybeans [37], ginger and *Syzygium aromaticum* [38], citrus media (*Citrus media* linn) [39] and tea extract (tea) [40] have been synthesized. On the other hand, Savithramma *et al.* found that fungal species were more sensitive to nanoparticles derived from plant extracts than bacteria [41]. So, in this study, we used an aqueous extract of the thyme plant, presenting a simple, fast, cheap, and environmentally friendly method for synthesizing copper nanoparticles that can be developed in any laboratory. This method does not use chemical and toxic reagents, adds no pollution to the environment, and therefore is superior to chemical and physical nanoparticle synthesis methods [2, 28, 29, 42]. Using this method, we synthesized copper nanoparticles with good stability and extensively investigated using techniques such as EDX, SEM, and UV-Visible spectroscopy. Our results showed that the 30-42 nm copper nanoparticles had antifungal activity against *C. albicans*, *F. solani*, *A. niger*, *A. flavus* (particularly, *C. albicans*, *F. solani*) although its effects (MIC and MFC) were lower in comparison with Amphotericin B. So, thyme can be used to synthesize copper nanoparticles in a large scale for many medical applications.

ACKNOWLEDGMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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