Synthesis of *Axinyssa digitata* Extract Directed Hybrid Nanoflower and Investigation of Its Antimicrobial Activity

Baris Karsli, Ibrahim Seyda Uras, Belma Konuklugil, and Ayse Demirbas

***Abstract*— First time in this study, the antibacterial effects of *Axinyssa digitata* sponge extracts and *Axinyssa digitata*-based cupper hybrid nanoflowers (Cu hNFs) were evaluated. Herein, hybrid nanoflowers (Cu hNFs) were produced by combining *Axinyssa digitata* sponge extract with Cu^{2+} ions in Phosphate-buffered saline (PBS) (at pH 7.4) at room temperature for three days using green synthesis method. The shape and size of hNFs were evaluated using scanning electron microscope (SEM) images. Energy dispersive X-ray spectroscopy (EDX) mapping was used to determine the presence of Cu metals and other components. X-ray diffraction (XRD) is a non-destructive analysis method that was used to determine of the crystallographic properties of materials and the phases they contain. Fourier-transform infrared spectroscopy (FT-IR) peaks were used to discuss the presence of functional groups that played a key role in the synthesis. The Cu-hNFs had antimicrobial activity against selected microorganisms. This research is expected to provide knowledge on hNFs synthesis and antimicrobial activity application investigations using *Axinyssa digitata* rather than biomolecules obtained through costly and time-consuming methods.**

***Index Terms*— Antimicrobial activity, *axinyssa digitata*, green synthesis, hybrid nanoflower.**

I. Introduction

NEW viruses are always evolving, posing a global threat. In addition to developing new antiviral medications to prevent viral spread, creative strategies must be developed to enhance the effectiveness of current antiviral medicines and additional techniques [1]. Antivirulence medicines are most successful because they can decrease, eliminate eliminate with, or even reverse the selective pressures common to conventional antibiotics that have made broad-spectrum antibiotics so ineffective. However, virulence targets would have been missed by phenotypic assays developed to test for growth abnormalities normally used to discover antibiotics. As a result, virulence factors are brand-new targets, and different antivirulent substances can be found in chemical libraries that already exist. Additionally, virulence factors that are secreted or exposed on the surface are good targets for biological substances that may not reach cells, such naturally occurring and artificial antibodies [1]. Thus, nanotechnology-based techniques have recently been studied for their antibacterial and antiviral capabilities [2], [3], [4], [5], [6], [7], [8], [9], [10], [11], [12]. Chemical, physical, and biological (natural precursors) investigations have been carried out to design and synthesize nanoparticles with the necessary size, shape, and functions [13], [14], [15]. During the last decades, "green synthesis" method was more widespread for new multifunctional nanotechnological design on application in many fields such as biocatalysts, cancer therapy, drug delivery, food safety, and environmental decontamination [16], [17], [18], [20]. Toxic solvents, chemical precursors, and extra reducing agents are not used in these synthesis routes [21]. In comparison to existing materials and processes, biologically inspired green synthesis offers substantial benefits. This includes (1) a one-pot, one-step process that saves time and energy; (2) gentle and simple processing using non-hazardous, environmentally friendly chemicals; and (3) improved control of product attributes for performance optimization [22], [23], [24]. Bioavailability, low cost, and a natural simplistic chemical environment are all advantages of using microorganisms (microscopic organisms), and also organic ingredients (fruit and vegetable juice, plant seed, leaves, and peel extracts, and so on) that contain abundant hydrogen atoms for nanoparticle biosynthesis [22], [23], [25], [26]. Green nanoparticles have been produced with amino acids, phytochemicals, polysaccharides, polyphenols, and vitamins [27], [28], [29], [30].

Marine organisms are also employed in the creation of nanoparticles using a variety of approaches [31], [32], [33], [34], [35], [36], [37], [38], [39], [40]. Researchers have completed significant research on metal nanoparticles generated from marine species extracts, which proved to be both biocompatible and nontoxic. These nanoparticles have antiviral,

Manuscript received 16 June 2022; revised 5 October 2022; accepted 18 October 2022. Date of publication 21 October 2022; date of current version 30 June 2023. *(Corresponding author: Ayse Demirbas.)*

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Digital Object Identifier 10.1109/TNB.2022.3216355

antibacterial, and anticancer activities and can be used to deliver target drugs in recent studies [41], [42], [43], [44], [45], [46], [47], [48], [49], [50].

Studies on drug discovery from marine organisms are ongoing to find cure for most problematic diseases. *Axinyssa digitata (syn. Pseudaxinyssa digitata)* sponge belongs to Halichondriidae family. 26-methylhalistanol sulfate and 25-demethylhalistanol sulfate derived from *P. digitata* showed basically full protection against the cytopathic consequences of HIV-1 infection in The National Cancer Institute (NCI) main screen, according to McKee et al. [51] and Kim and Van Ta [52]. In another study, antimicrobial activity of *Axinyssa digitata* sponge collected from Turkey coast was examined. *A. digitata* extract showed strong activity against both of *Cryptococcus neoformans* and *Cryptococcus gattii* [53]. In recent years, increasing interest in the green synthesis of nanostructures such as nanorods, nanowires, nanoparticles, nanoflowers, nanosponges etc. has lead to the use of some marine species in this field. Therefore, the motivation of this study is being utilized the advantages of marine organisms such as high biomass, tolerance to metal ions and high accumulation ability. According to some research, marine organisms such as lichens, sponges, algeas can be utilized in place of expensive biological components (DNA, protein, enzymes, etc.) in the manufacture of organic-inorganic hybrid nanostructures that are used in biological applications.

In this work, Cu hNFs were produced utilizing *Axinyssa digitata* extract instead of amino acids, phytochemicals, polysaccharides, polyphenols, vitamins, enzymes, bacteria, and DNA. SEM, EDX, XRD, and FT-IR analyses were used to characterize Cu hNFs. The obtained Cu hNFs were found to have antimicrobial properties. The work demonstrates that *Axinyssa digitata* extracts can be used to create organic/inorganic hybrid Cu hNFs in a cost-effective and efficient manner. It is envisioned as a roadmap for nanotechnology and multidisciplinary research fields in this subject in connection with producing environmentally approachable and economic Cu hNFs creation and claims.

II. Material and Methods

A. Materials and Reagents

Axinyssa digitata sponge was collected by scuba-diving from Aegean Sea (Kömür Limanıdepth: 20 m) in August 2018 and was identified by Dr. Bülent Gözcelioğlu. A voucher specimen was deposited at the Pharmacognosy Department of Faculty of Pharmacy, Ankara University. All reagents and chemicals were bought from Sigma-Aldrich Products, USA.

B. Extraction of Axinyssa Digitata

The sponge material was sliced by a knife into small pieces, (approximately 50 g) and then extracted with 100% methanol (500 mL) for four times. Obtained methanol phase were filtered and evaporated in vacuum until dryness. The obtained dry methanol extract was kept at 4 °C until its use.

C. Synthesis of Cu hNFs

1 g of dried sponge sample was filled in 10 ml purified water for 1 hour at room temperature, then filtered using Whatman No#1 filter paper and centrifuged (10.000 rpm, 10 min). Sponge extracts at 1 mg/L (Cu hNFs) with 0.8 mM copper sulfate ($CuSO_4$) in 10 mM PBS buffer were used to build organic-inorganic Cu hNFs (pH 7.4). Vortexing was used to achieve a smooth reaction, which was subsequently incubated at room temperature for three days. The precipitates were centrifuged for 15 minutes at 12,000 rpm and then rinsed with distilled water [54], [55]. The resulting nanoflowers were characterized by SEM, XRD, EDX mapping, and FT-IR analysis.

D. Instrumentation

Buchi R-300 (Switzerland) rotary evaporator systems were used for evaporating solvents and obtain dry crude fungi extract. SEM (Jeol 6510), XRD (Rigaku smartlab), EDX (Panalytical/ Epsilon 5), and FT-IR/4700 (PerkinElmer) were used for the characterization of Cu hNFs.

E. Antimicrobial Activity

1) Bacterial Strains and Growth Conditions: The microorganisms used in this study were *Salmonella enterica* ATCC 13076, *Escherichia* coli ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Aeromonas sobria* ATCC 43979, and *Aeromonas hydrophila* ATCC 7966. The bacteria were first sub-cultured on Mueller Hinton Agar (MHA) plates at 37 °C for 24 h, and then the microorganisms were standardized to the 0.5 McFarland standards (approximately 10^8 CFU/ml) for use in antimicrobial susceptibility assays [56].

2) Disk Diffusion Assay: The antimicrobial activity of the *A. digitata* extracts and nanoflower was determined using the disk diffusion assay according to the method described by Bauer et al. [57]. Briefly, sterilized MHA medium was poured into sterile plates, and allowed them to solidify. The respective plates were inoculated with bacterial suspensions adjusted to 0.5 McFarland standard turbidity using sterile cotton swab. Then, sterile paper disks (6 mm diameter) were loaded with 20 μL of each extract and placed on the agar plates. The inoculated plates were then incubated overnight at 37 °C in biological incubator. After incubation, the diameter of the inhibition zones was measured.

3) Determination of Minimum Inhibitory Concentration (MIC): MICs were determined by microdilution method which was performed in 96-well microtiter plate at different dilutions (from 1024 to 0.25 μg/mL) of the tested extracts. 100 μL of Mueller Hinton Broth (MHB) was poured into each well, and then 10 μl of bacterial suspensions adjusted to 0.5 McFarland standard turbidity were inoculated in microwells containing serial two-fold broth dilutions of *A. digitata* extracts. Only MHB for negative control and MHB + bacterial suspension for positive control were used. After 24 hours of incubation at 37 °C, the lowest concentration of antimicrobial agent inhibiting visible growth was considered as the MIC value [23].

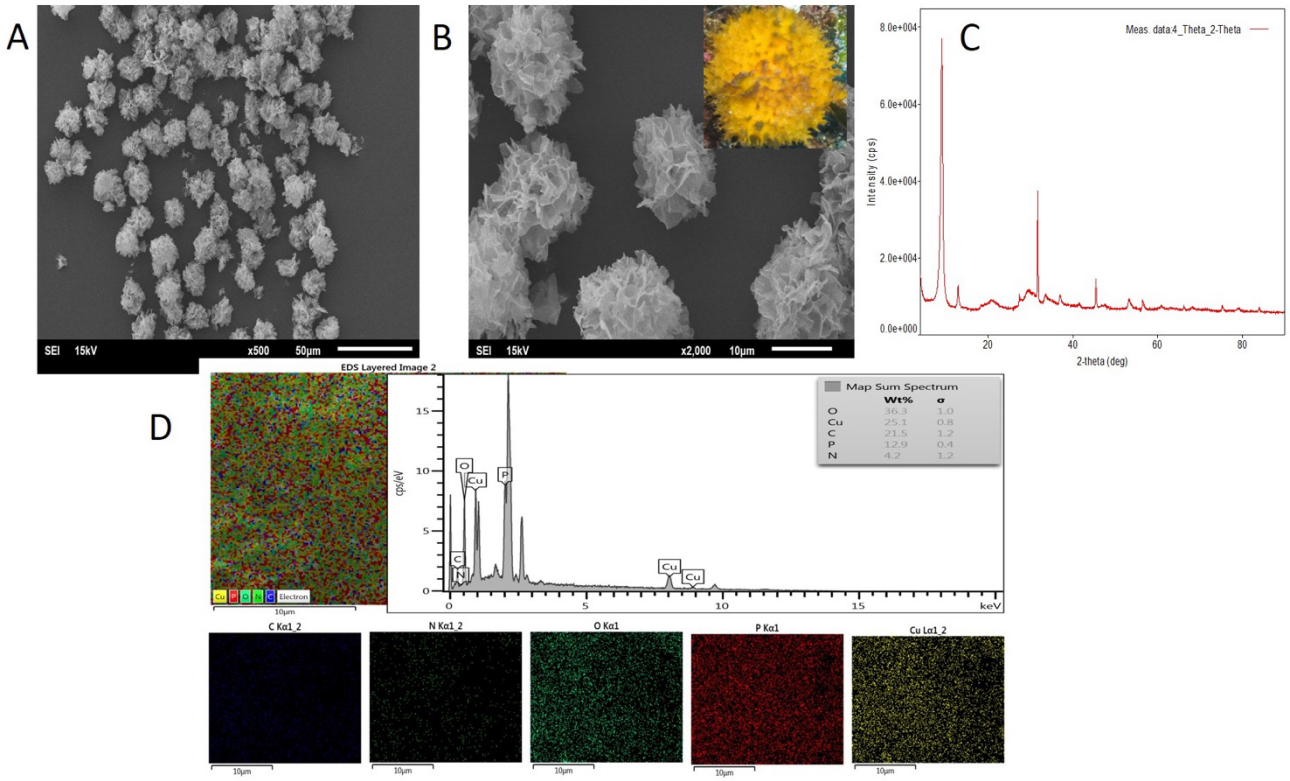

Fig. 1. SEM images (A-B) of *Axinyssa digitata* extract (∼13-16 μm). Inset of B: a photo of *Axinyssa digitata*, C) EDX mapping of nanoflower and D) XRD of nanoflower

III. Results and Discussion

A. Synthesis and Characterization of Organic-Inorganic Hybrid Nanoflowers

The effects of experimental conditions such as pH, storage temperatures, concentrations of metal ions, types of compounds as stabilizing agents, and reaction time on the activity and stability of hNFs were evaluated in several studies [22], [24], [26]. Baldemir et al. [22] examined the concentration effect of extracts and standard molecules of green tea obtained using different solvents such as water and ethanol on the synthesis of organic-inorganic nanoflowers. Their obtained SEM images show that the GTe-Nf, GTw-Nf, ct-Nf and cf-Nf have spherical morphologies with narrow size distribution (GTe-Nf, green tea ethanol extract nanoflower; GTw-Nf, green tea water extract nanoflower; ct-Nf, catechin nanoflower; cf-Nf, caffeine nanoflower) [22]. The diameters of the Nf are ∼5.5 μm. It is worth to mention that the morphology of GTe-Nf and ct-Nf are very similar each other, while morphology of the GTw-Nf and cf-Nf are not exactly similar. They speculate that the major content of the extract obtained in ethanol can be ct, which is less soluble in water compared to cf. Furthermore, they found that GTw-Nf are quite compact due to the contribution of both the hydrophobic and hydrophilic component obtained in water extraction. They emphasized that the cf-Nfs gives blooming structures, which can be due to water solubility of cf. Their results provided SEM image of $(Cu_3SO_4)_3$ crystal. They observed that only debris of no $(Cu_3SO_4)_3$ crystal was observed but no Nfs was formed. They presents that the Nfs are formed when caffeine and catechin standard molecules or plant extracts as organic parts and copper ions as inorganic parts are present in the PBS [22].

Koca et al. [26] assessed the impact of media pH on the morphology of Thymol extract-hNFs using SEM images. Image shows that nanoflower synthesis was achieved in a pH 5 condition and that nanoflower formation was seen in a pH 7 condition, however the final stage of nanoflower synthesis was not completed and that nanoflower formation was not seen in SEM images.

The size of hNFs and petals generated as a result of the reaction of 1 ml *A. digitata* extract and 0.8 mM $CuSO_4$ in 100 ml of PBS were ∼ 13-16 μm, according to the SEM images (Fig. 1A-B). Under pH 7.4 conditions of PBS, blue precipitate was formed as unique flower shape after three days incubation at room temperature. The creation mechanism of NFs has been extensively studied in the past.

The nucleation phase of the process, which begins through the creation of major phosphate crystals due to Cu^{2+} ions reacting with the bioextracts (nucleation phase), ends with the arranging of the petals in the mechanism, which consists primarily of nucleation, growth, and final phases [23], [24], [25], [26], [58], [59], [60], [61]. The diameters of produced hNFs with bioextracts were measured in the range of 2-10 μm by Kilic et al. [60]. Furthermore, it has been reported that while hNF is formed when 0.1 mg/ml bioextract is used in the procedure, hNF production was not detected when 0.5 mg/ml bioextract was used. Compared to our results with the past studies, we were observed similar size and shape on the

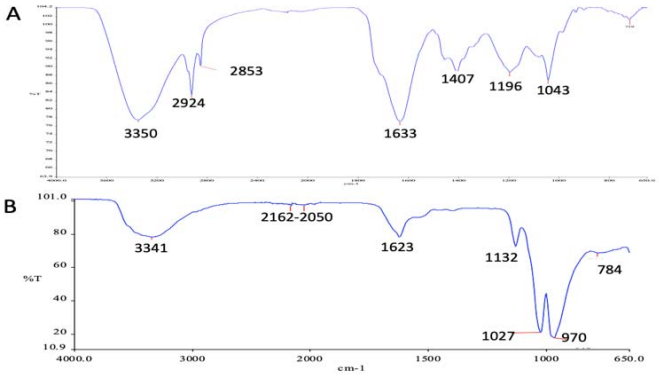

Fig. 2. FT-IR of *Axinyssa digitata* (A) *and Nanoflower* (B).

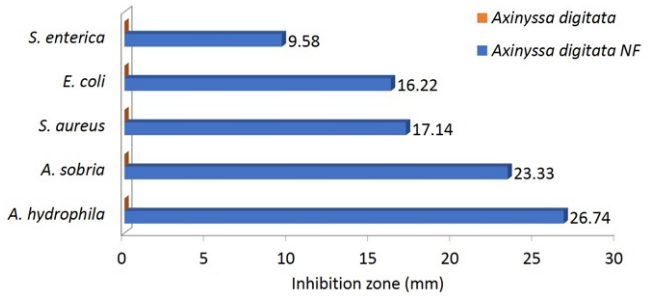

Fig. 3. Antibacterial activity of *A. digitata* extracts against pathogen bacteria. NF: Nanoflower.

TABLE I
MINIMUM INHIBITORY CONCENTRATION (μg/mL) OF *A. digitata* EXTRACT AND NANOFLOWER AGAINST PATHOGEN BACTERIA

Extracts	*S. enterica*	*E. coli*	*S. aureus*	*A. sobria*	*A. hydrophila*
A. digitata	+	+	+	+	1024
***A. digitata* NF**	64	32	32	32	16

+: Growth in all concentrations. NF: Nanoflower.

production of NFs [24], [58], [59], [60]. EDX (Fig. 1C) and FT-IR (Fig. 2A-B) studies were used to evaluate the synthesized hNFs, respectively. EDX spectrum (Fig. 1C) and EDX mapping show the occurrence of Cu^{2+} and other constituents in the structure of NFs. EDX mapping was used to prove the distribution of four important elements and Cu in NF: C (blue), O (green), P (red), N (light green) and Cu (yellow) elements were examined via mapping by representing distinct colors in NFs. Fig. 1D is the XRD spectrum of the Cu hNFs which is compatible well with the results of previous studies [24], [58], [59], [60]. The diffraction peaks of Cu hNFs confirmed the good crystallization of the nanoflower. The results also indicate that the Cu^{2+} ions were an inorganic component of Cu hNFs (Fig. 1D). FT-IR analysis was used to determine the functional groups. The presence of C-H (alkane groups) was discovered at wavenumbers of 3350 cm^{-1}, 3341 cm^{-1}, 2924 cm^{-1}, 2853 cm^{-1}, 2050 cm^{-1}, and 2162 cm^{-1}. The amine (-NH) and aliphatic ether (C-O) peaks at 1633 cm^{-1}, 1623 cm^{-1}, and 1407 cm^{-1}, respectively, correspond to amine (-NH) and aliphatic ether (C-O). Peaks at 1196 cm^{-1}, 1043 cm^{-1}, 1132 cm^{-1}, 1027 cm^{-1}, 970 cm^{-1}, and 784 cm^{-1} were found in core phosphate crystals produced in PBS (Fig. 2A-B). Description peaks revealed the development of NFs in PBS, along with their shape.

B. Analysis of Antimicrobial Activity

As shown in Table I, extract of *A. digitata* did not show any antibacterial activity against all bacteria tested. However, nanoflower from *A. digitata* had greater antibacterial properties than non-nanoflower extract. According to disk diffusion assay, *A. digitata* NF was more effective in inhibiting the growth of *A. hydrophila,* but less effective against *S. enterica.* These results were also supported by the MIC results. The MIC values of extracts against five pathogen bacteria were given in Fig. 3.

No growth was observed in negative control wells. Also, there was growth in all positive control wells. While *A. digitata* extract did not show antibacterial activity on *S. enterica*, *E. coli*, *S. aureus*, and *A. sobria*, it had a low antibacterial activity on *A. hydrophila* at an MIC of 1024 μg/mL. On the other hand, the nanoflower produced by *A. digitata* exhibited strong antibacterial activity against all pathogenic bacteria tested, and *A. digitata* NF had MIC value in the range of 16-64 μg/mL. AgNPs are known to be excellent antimicrobial and anti-inflammatory agents [62]. Also, the results of both in vitro antimicrobial susceptibility tests in the present study supported that silver nanoparticles have antimicrobial. Since the biosynthesized AgNPs from *A. digitata* showed considerable antibacterial activity, they can be widely used as new antimicrobial drugs in clinical applications. Dogan et al. [53] reported that *A. digitata* extract against all tested microorganisms showed antimicrobial activity (range: 8 - 64 μg/mL) and they also were found a similar MIC value (32 μg/mL) against *S. aureus* compared to our results. The antimicrobial activity of marine sponges such as *A. digitata* is thought to be due to their rich bioactive ingredients [53].

IV. CONCLUSION

The specific flower shape Cu NFs morphology were produced at pH 7.4 with the organization of Cu and *Axinyssa digitata* sponge extract. SEM, XRD, EDX mapping, and FT-IR analyses were used to characterize Cu hNFs, which were synthesized utilizing sponge extract, which is natural, inexpensive, effective, and simple rather than expensive and toxic chemical compounds. The produced hNFs were found to have high antimicrobial activity against *Salmonella enterica* ATCC 13076, *Escherichia* coli ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Aeromonas sobria* ATCC 43979, and *Aeromonas hydrophila* ATCC 7966. The results of this study are likely to be used in nanotechnology as a guide for biotechnology, biomedical, and environmental applications.

ACKNOWLEDGMENT

The authors gratefully acknowledge use of the services and facilities of Technology Research and Implementation Center

of Recep Tayyip Erdoğan University. They would like to thank Dr. Bulent Gozcelioglu for contributing to the work by collecting marine organisms.

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