

ORIGINAL ARTICLE

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A new approach for green synthesis and characterization of *Artemisia* L. (Asteraceae) genotype extracts -Cu²⁺ nanocomplexes (nanoflower) and their effective antimicrobial activity

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Abstract

In this study, we have demonstrated the fabrication of novel organic-inorganic nanobio-antimicrobial agents called “nanoflowers” (NFs) and elucidate the increase in the antimicrobial activity of NFs. This is the first report that the NFs were formed of plant extracts as the organic components and copper (II) ions (Cu²⁺) as the inorganic component. The *Artemisia* L. (Asteraceae) methanol extracts from three genotypes including *A. absinthium* L. (Aa), *A. vulgaris* L. (Av) and *A. ludoviciana* Nutt. (Al) were selected in the NF synthesis. The effect of the plant extract concentrations on the morphology of NFs was examined. Most regular and uniform flower-shaped morphologies were observed when a concentration of 0.1 mg mL⁻¹ plant extract was used in the synthesis of NFs. The synthesized NFs were characterized with several techniques such as scanning electron microscopy (SEM), Fourier transform infrared spectrometer (FT-IR), energy-dispersive X-ray (EDX) and X-ray diffraction analysis (XRD). The NFs exhibited much antimicrobial activity against the pathogens even at low concentrations compared to the extracts. The MICs and MBCs values for NFs were found to be range between 0.4 to 40 µg mL⁻¹ and 40 to 400 µg mL⁻¹ while those values for Aa, Av and Al extracts were ranged from 500-2000 µg mL⁻¹ and 1000-4000 µg mL⁻¹ for the studied pathogens, respectively.

Keywords: *Artemisia* extracts, hybrid nanoflower, inhibitory property, bacterial and fungal pathogens.

Introduction

The development of nanomaterials has provided many multifunctional versatile tools used in scientific and technical fields [1-3]. Various colloidal nanomaterials (NMs), such as metallic or polymeric, have been synthesized and characterized using several techniques [4-8]. Among the NMs, the biocompatible molecules (DNA, protein, enzyme and plant extract) integrated nano or micro sized materials have been commonly used in last two decades due to much biocompatibility and very less environmental toxicity [9-13].

The plant extracts have been received considerably much attention in NMs synthesis compared to other biomolecules owing to their quite less price, high stability, lack of contamination risk and easy preparation. Up to now, researchers have intensively used various plant extracts as reducing and capping agent in the synthesis of metallic NMs. In typical synthesis, certain amount of plant extract and metal salt are accordingly mixed in aqueous solution and incubated under stirring at various temperatures for different periods of time. The plant extracts are mainly composed of natural chemicals, such as flavonoids and polyphenols. The oxidation of those of which induces the reduction of metal ion and eventual formation of metallic nanomaterials [14-19].

Rather than conventional plant extract based metallic NMs synthesis, we were inspired from an encouraging breakthrough discovered by Zare and co-workers in 2012 for preparation of protein-inorganic nanoflowers (NFs) [20,22]. In several reported

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works, protein, enzyme and amino acid were successfully utilized as organic component and some metal ions were used as inorganic component in the formation of flower-shaped nanostructures. However, to the best of our knowledge, plant extracts have not been previously involved in synthesis of organic-inorganic hybrid nanostructures [22-30]. *Artemisia* L. species are medicinal and aromatic herbs in the Asteraceae family, which has a long history of use in culinary traditions [31]. They have been actively used in various purposes, such as coughs and colds, chills, stomachache, dry dyspepsia, purgative effect; tea, poultice, inhale (vapours from boiling leaves); insect repellent [32]; a vermifuge, in the treatment of chronic fevers and for inflammation of the liver, as an antispasmodic and antiseptic [33,34].

Herein, for the first time, a simple and rational approach is reported for the preparation of novel organic-inorganic nanobio agents called “nanoflowers” (NFs) and elucidate the increase in the antimicrobial activity of NFs. The NFs were formed of plant extracts as the organic component and copper (II) ions (Cu^{2+}) as the inorganic component. The extract of *Artemisia* from three different genotypes including *Artemisia* absinthium L. (Aa), *A. vulgaris* L. (Av) and *A. ludoviciana* Nutt. (Al) were selected in the NF synthesis. The effects of the plant extracts and Cu^{2+} concentrations on the morphology of NFs were systematically examined. The antimicrobial activities of Aa, Av and Al extracts and NFs synthesized from extracts were evaluated against bacterial and fungal pathogens.

Materials and Methods

Chemicals and reagents

Copper sulfate pentahydrate, methanol, phosphoric acid, dimethyl sulphoxide (DMSO) and other chemicals were purchased from Sigma-Aldrich. NaCl, KCl, Na_2HPO_4 , KH_2PO_4 , HCl and NaOH were used to prepare phosphate buffer saline solution (PBS, pH 7.4). Coomassie brilliant blue G-250 were used for buffer solution and prepared using ultrapure water.

Preparation of extracts

Artemisia species were obtained from Zeytinburnu Medicinal Plant Garden, Istanbul, Turkey. Herba of *Artemisia* spp. were washed several times with deionized water and dried at room temperature. Aa, Av and Al herbs were powdered using a blender. 100 g of the each plant powder was added into 500 mL one-necked flask containing 250 mL methanol and incubated at room temperature (RT: 25 °C) for 1 day under stirring. After incubation, the each solution was filtered through a Whatman filter paper No. 1 to collect the extract. This step was repeated twice using the same procedure. The extracts were evaporated under vacuum at 40 °C and stored at -20 °C for further use.

Preparation of Aa, Av and Al- Cu^{2+} hybrid nanoflowers

The extracts incorporated- Cu^{2+} NFs were prepared according to previously reported method with some modifications [23-28]. Briefly, a volume of 0.35 mL CuSO_4 solution (120 mM) was separately mixed with Aa, Av and Al extracts (concentrations increasing from 0.1 and 0.5 mg mL^{-1}) into 50 mL of 10 mM PBS (pH 7.4) The mixtures were vigorously shaken for 30 s to make them homogeneous and then left without disturbing at +4°C for 3 days incubation. The greenish precipitates appeared at the bottom of the solution were washed by centrifugation at 10.000 rpm for

15 min at least 3 times. Finally, the collected NFs were dried under vacuum at 50°C for overnight and stored for further characterization and use. The supernatant of each mixture was kept for Bradford protein assay.

Characterization of plant extract incorporated nanoflowers

The morphologies of the NFs were examined using ZEISS model EVO LS10 scanning electron microscope (SEM). The elemental analysis of the NFs was undertaken by energy-dispersive X-ray (EDX) (ZEISS EVO LS10) to determine weight and atomic percentage of Cu^{2+} in the NFs. The infrared spectra of the NFs were recorded using a Fourier Transform Infrared Spectrometer (FT-IR) (Perkin Elmer Spectrum 400). The 20 mg of NFs was also used for X-ray Diffraction Analysis (XRD) (BRUKER AXS D8). The encapsulation yield of Aa, Av and Al NFs was determined to be ~58%, ~68% and ~48%, respectively via Bradford protein assay using an UV-vis spectrophotometer (HITACHI) [35].

Antimicrobial study

The bacterial strains (*E. coli* ATCC 35218, *S. typhi* ATCC 14028 *P. aeruginosa* ATCC 27853 *C. albicans* ATCC 10231 and *S. aureus* ATCC 25923) were obtained from Medical Microbiology Laboratory, Faculty of Medicine, Inonu University culture collection.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of both the extracts (Aa, Av and Al) and NFs (Aa, Av and Al NFs) were determined via broth microdilution method based on Clinical Laboratory Standards Institute (CLSI) [36,37] guidelines protocol modified by Bazargani and coworkers [38] The MIC assays were carried out in 96-well microtitre plates in triplicate at a two fold serial dilution of the tested materials from 500 $\mu\text{g mL}^{-1}$ to 4000 $\mu\text{g mL}^{-1}$ for the extracts and from 0.4 $\mu\text{g mL}^{-1}$ to 400 $\mu\text{g mL}^{-1}$ for the NFs. The inoculum concentration for the MIC and MBC tests was standardized by using the optical density of the bacterial suspension to a turbidity according to spectrophotometric absorbance. The bacterial suspensions (5×10^5 CFU mL^{-1}) were added in each well. Appropriate antibacterial agent and Mueller Hinton Broth (MHB) + bacterial suspension were used as positive control while MHB served as negative control. The plates were then incubated at 37 °C for 16 to 20 h. After incubation, the plates were visually examined for bacterial growth. No visible growth was observed in each well, then the samples were subcultured on sterile Mueller hinton agar (MHA) plates to determine the MBC value. The plates were then incubated at 37 °C for 24 h. The well containing the lowest concentration with no visible bacterial growth was taken as the MIC value. This is further validated by addition of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) to the wells. 0.2 mg/mL of 4 μL MTT was added to wells and they were incubated at 25 °C for 15 minutes. The pink appeared in the wells was considered as a positive meaning active bacterial growth, while wells with colourless solution were representative of negative for bacterial growth. The well containing the lowest concentration of the colourless solution was interpreted as the MIC. The MBC was interpreted as lowest concentration showing no visible growth on agar subculture [38].

Both the extracts and the NFs were also used to determine their minimum fungicidal concentration (MFC) values via the broth microdilution method according to CLSI, 2008 guidelines [36]. A

mixture of L-glutamine and sodium bicarbonate-free RPMI 1640 broth containing 0.2 % (m/v) glucose was used as media. The pH of the media was adjusted to pH 7.0 by using a solution of 0.165 mol L⁻¹ morpholinepropanesulfonic acid (MOPS). Mc Farland 0.5 inoculum was prepared in 0.85% (m/v) NaCl aqueous solution. Amphotericin B and dimethyl sulfoxide (DMSO) were used as a positive control and a negative control, respectively. Each experiment was performed in duplicate and repeated three times. The plates were examined visually. Sabaraud dextrose agar (SDA) was used to determine the MFC values.

Result

Characterization of Aa, Av and Al-Cu²⁺hybrid nanoflowers

Images of Aa, Av and Al NFs were generated by the SEM. The elemental composition of each NF was analyzed by EDX. The crystal structure and chemical structure of NFs were characterized using XRD and FT-IR spectroscopy, respectively.

The NFs were formed by the combination of each extract with Cu²⁺ ions in PBS buffer. Although all NFs were quite spherical, Aa and Av NFs were much uniform and monodispersed compared to Al NFs. The potential reason can be attributed to contents of each extract. Interestingly, the NFs were formed using 0.1 mg mL⁻¹ of each extract (Figure 1a, 1c and 1e). However, no NFs were formed when 0.5 mg mL⁻¹ extracts were used (Figure 1b, 1d and 1f). The sizes of Aa and Av NFs were around 11±1 µm and 8±1 µm (Figure 1a and 1a), while the size of Al NF was determined in the range of 2 µm and 10 µm (Figure 1e). These differences showed that the content and concentrations of the extracts can be the key point for formation and controlling the size of the NFs.

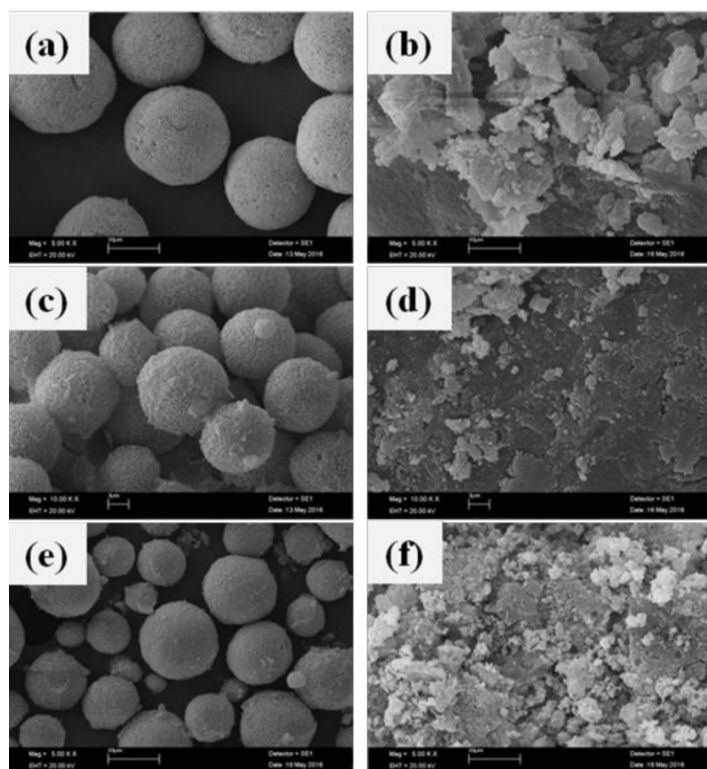


Figure 1. a-b) Aa 0.1 mg and 0.5 mg, c-d) Av 0.1 mg and 0.5 mg, e-f) Al 0.1 mg and 0.5 mg Aa: *A. absinthium*; Av: *A. vulgaris*; Al: *A. ludoviciana*

The presence of Cu metal in NF was analyzed by the EDX as shown in Figure 2a, The diffraction peaks in the NFs corresponding to Cu₃(PO₄)₂ nanocrystals were almost consistent with the JCPDS card (00-022-0548) as presented in Figure 2b, 2c and 2d. The FT-IR spectrums of the only extract and the NFs are illustrated in Fig. 3a and 3b, respectively. The absorption bands (in Figure 3b) at 559, 1032 and 1150 cm⁻¹ in the the NFs were attributed to P-O and P=O vibrations, indicating the existence of phosphate groups (PO₄³⁻) [39]. It is worthy to mention that molecules in the extracts were packed in the NFs with different conformations, which increase localized molecules concentration in the extract and may lead to strong and shifted vibration peaks. The vibration bands of -NH₂ groups were at 1595 cm⁻¹ and 1627 cm⁻¹, for extracts and the NFs, respectively. The stretching bands of -CH₂ and -CH₃ groups in the extract Al were at 2850 and 2918 cm⁻¹. The adsorption bands at 1675 cm⁻¹ and 3232 cm⁻¹ were assigned to carbonyl (>C=O) and hydroxyl (-OH) groups from the compounds in the extract, respectively.

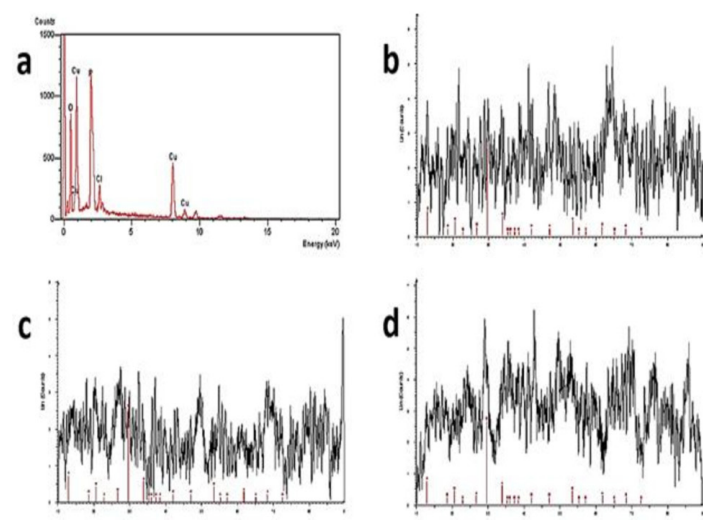


Figure 2. a) EDX analysis of Cu metal in the NFs, b-d) XRD of pattern of Al NFs, Aa NFs and Av NFs. Aa: *A. absinthium*; Av: *A. vulgaris*; Al: *A. ludoviciana*, NFs: Nanoflowers

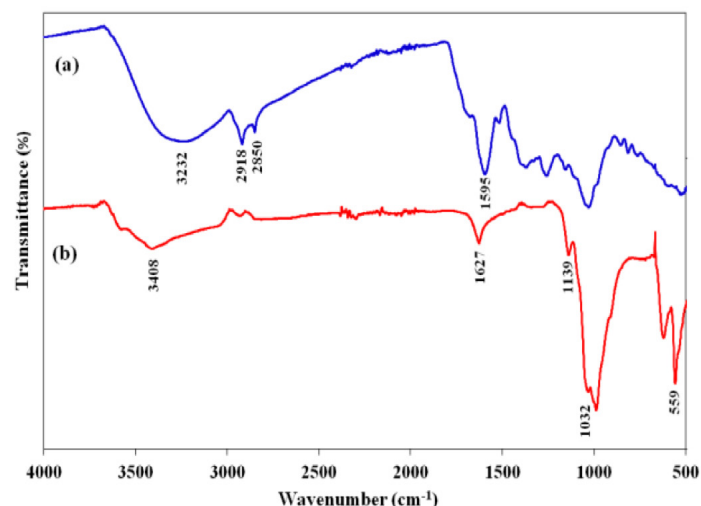


Figure 3. FT-IR spectra of extract Al (a) and the extract Al-Cu²⁺ hybrid nanoflower (b). Al: *A. ludoviciana*

Evaluation of antimicrobial activity of Aa, Av and Al-Cu²⁺hybrid nanoflowers

According to literature, *Artemisia* spp. containing various active molecules (phenolic compounds, essential oil) have high antibacterial and antifungal activity. Previous report revealed that *Artemisia* spp. did not exhibited any antimicrobial effect to Gram negative bacteria but showed moderate and strong activity against Gram positive bacteria and fungi [40-44]. Park et al. [45] used the *A. capillaris* Thunb. extract as reducing agent to synthesis the silver nanoparticles (Ag NPs) and to improve the antimicrobial activity against several Gram negative and Gram positive bacterial strains. Some studies demonstrated that the green synthesis of AgNPs using *Artemisia* extracts and found them as effective antimicrobial agents towards pathogenic bacteria, such as *Staphylococcus aureus*, *Bacillus cereus*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* [46].

In this study, antimicrobial activities of the NFs and extracts of three *Artemisia* genotype (Aa, Av, Al) and were tested against standard pathogens using the broth microdilution method. Subsequently MIC, MBC and MFC values were determined. The NFs showed excellent antimicrobial activity against bacteria and fungi even at very low concentrations (Figure 4,5). The MIC and MBC values for NFs were between 0.4 to 40 µg mL⁻¹ and 40 to 400 µg mL⁻¹ while those values for Aa, Av and Al extracts were 500-2000 and 1000-4000 µg mL⁻¹ for bacterial pathogens, respectively. The extracts did not show any antibacterial activity towards *E. coli*, *S. typhi* and *P. aeruginosa* at 4000 µg mL⁻¹. According to these results, the NFs have very promising antimicrobial properties compared to the extracts.

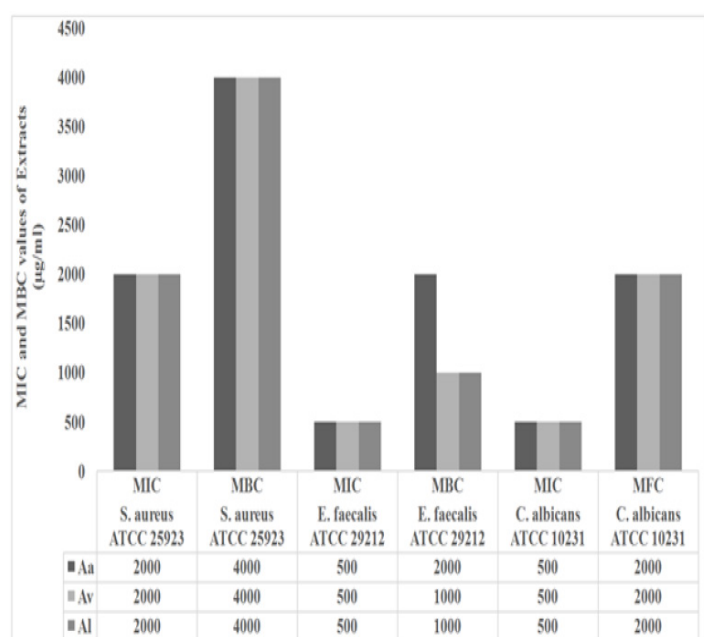


Figure 4. MIC and MBC values (µg/ml) of *Artemisia* sp. extracts (Aa, Al, Av). Aa: *A. absinthium*; Av: *A. vulgaris*; Al: *A. ludoviciana* MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration

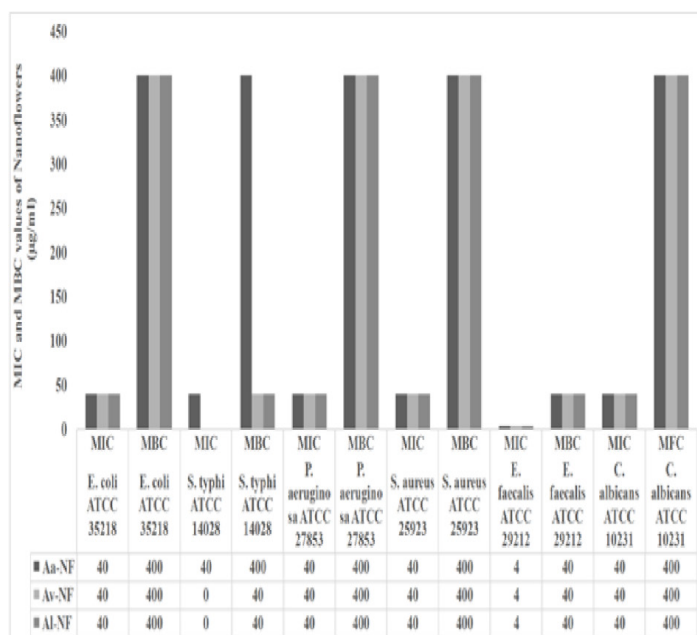


Figure 5. MIC and MBC values (µg/ml) of nanoflowers (NFs). Aa: *A. absinthium*; Av: *A. vulgaris*; Al: *A. ludoviciana* MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration

Discussion

Several plant extracts were used for extracellular synthesis of silver nanoparticles and they can selectively inhibit growth of the Gram negative and Gram positive and they can be considered an interesting versatile biotechnological resource due to their antimicrobial activity [47-50]. The resistance of Gram negative bacteria to plant extract and essential oil have been associated to hydrophilic outer membrane that may block the penetration of hydrophobic compounds into target bacterial cell membrane [51]. The NFs as amphipathic hybrid nanomaterials can attach Gram positive and Gram negative bacteria outer membrane and they may show antibacterial activity. In addition, the NFs exhibited much higher antibacterial activity against Gram positive bacteria than Gram negative.

Conclusion

In this work, we have successfully firstly produced to synthesize the three *Artemisia* genotypes (Aa, Av, Al) extracts-metal ion NFs with very narrow size distribution, high production yield, and also demonstrated their excellent inhibitory property. Differences of the antimicrobial activities of the NFs and extracts may be related to due to the varied amounts of plant extracts of main molecules (proteins, flavonoids, polyphenol) contained in the Aa, Av and Al extracts. Importantly, the NFs exhibited dramatically enhanced antimicrobial activities towards standard bacterial (*E. coli*, *S. typhi*, *P. aeruginosa* and *S. aureus*) and fungal pathogens (*C. albicans*) compared to extracts. The results suggest that green synthesis of the NFs present promising a great potential for development of eco-friendly antimicrobial agents for especially nosocomial human pathogens.

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Competing interests

The authors declare that they have no competing interest.

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Ethical approval

No ethic approval is needed to this research.

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