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Apple peel extract based formation of organic-inorganic nanoflower with ıntrinsic peroxidase mimic and antimcrobial activities

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Abstract

Purpose: In this study, we developed synthesis of hybrid organic-inorganic nanoflower (NF) composed of flavonoid-rich apple peel extract and Cu^{2+} ions acted as organic and inorganic components, respectively. The apple peel extract based NFs (Ap-NFs) showed peroxidase mimic and antimicrobial activities.
Method: The Ap-NFs were characterized with by Scanning Electron Microscope (SEM) for monitoring morphology (size and shape), Fourier transform Infrared Spectroscopy (FTIR) for bending and stretching vibrations and X-Ray Diffraction (XRD) for elucidation of crystal structure of Ap-NFs.
Findings: In terms of the catalytic activities, Ap-NFs catalyzed oxidation of guaiacol (2-methoxy phenol) to 3,3-dimethoxy-4,4-diphenoquinone in the presence of hydrogen peroxide (H_2O_2) through the Fenton reaction. Benefiting from both the Fenton reaction and porous morphology of Ap-NFs, their antimicrobial activities were tested towards, *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*) and *Candida albicans* (*C. albicans*).
Conclusion: It is worthy to mention that, the Ap-NFs exhibited great antimicrobial activity in the presence of H_2O_2 by killing almost 99% of all microorganisms. The results are quite promising and we claim that Ap-NFs can be ideal candidate for catalytic activity and antimicrobial studies.

Keywords: Apple Peel Extract, Organic-Inorganic Nanoflower, Peroxidase Mimic Activity, Fenton Reaction and Antimcrobial Activity

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Elma kabuğu ekstresi temelli organik-inorganik nanoçiçek ile içsel peroksidaz benzeri ve antimikrobiyal aktiviteler

Özet

Amaç: Bu çalışmada, flavonoid açısından zengin elma kabuğu özütü ve Cu^{2+} iyonlarının sırasıyla organik ve inorganik bileşenler olarak hareket ettiği hibrit organik-inorganik nanoçiçek (NF) sentezini geliştirdik.
Metod: Elma kabuğu özütü temelli NÇ'ler (Ek-NÇ'ler) peroksidaz benzeri ve antimikrobiyal aktiviteler gösterdi. Ek-NÇ'lerin morfolojiyi (boyut ve şekil) izlemek için Taramalı Elektron Mikroskopu (SEM), eğilme ve gerilme titreşimleri için Fourier Dönüşümlü Kızılötesi Spektroskopisi (FTIR) ve Ek-NÇ'lerin kristal yapısını açıklamak için X-ışını kırınımı (XRD) ile karakterize edildi.
Bulgular: Katalitik aktiviteler açısından, Ek-NÇ'ler hidrojen peroksit (H_2O_2) varlığında Fenton reaksiyonu yoluyla guaiacolün (2-metoksi fenol) 3,3-dimetoksi-4,4-difenokinona oksidasyonunu katalize etti. Hem Fenton reaksiyonundan hem de Ek-NÇ'lerin gözenekli morfolojisinden yararlanılarak, antimikrobiyal aktiviteleri *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*) ve *Candida albicans* (*C. albicans*)'a karşı test edildi.
Sonuç: Ek-NÇ'lerin H_2O_2 varlığında tüm mikroorganizmaların neredeyse %99'unu öldürerek büyük antimikrobiyal aktivite gösterdiğini belirtmekte fayda var. Sonuçlar oldukça ümit verici ve Ek-NÇ'lerin katalitik aktivite ve antimikrobiyal çalışmalar için ideal aday olabileceğini iddia ediyoruz.

Anahtar kelimeler: Elma Kabuğu Ekstraktı, Organik-İnorganik Nanoçiçek, Peroksidaz Benzeri Aktivitesi, Fenton Reaksiyonu ve Antimikrobiyal Aktivite

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1. Introduction

NFs can be synthesized using various synthetic strategies. In recent years, the use of plant extracts instead of toxic chemicals in NF synthesis has increased. The easy availability, cheapness and low risk of contamination of plant extracts have increased their use [1-3]. For example, Molina et al. synthesized Ag NFs using *Kalanchoe daigremontiana* extract. They found that the synthesized Ag NFs exhibited high antibacterial activity against *E. coli* and *S. aureus* [4].

The nanoflowers (NFs) composed of enzyme and Cu^{2+} as organic and inorganic components, respectively were reported for the first time by Zare and co-workers [1]. Briefly, Cu^{2+} react with PO_4^{-3} in phosphate buffered saline (PBS) at pH: 7.4 to form $Cu_3(PO_4)_2$ complexes. These NFs especially, metal containing enzyme based NFs exhibit highly enhanced catalytic activity and stability compared to conventionally immobilized and free enzymes. For further studies, different enzymes and metal ions have been used to produce NFs by the researchers and they have been applied in a variety of applications [6-11]. For instance, Wang et al., synthesize amylase@$CaHPO_4$ hybrid NFs, nanoplates, and parallel hexahedrons with great catalytic activities through allosteric effect between α-amylase and Ca^{2+}.[6]. In other study, Goktur et al., successfully reported formation of horseradish peroxidase-based NFs and benefited from their enhanced catalytic activities to polymerize phenol derivatives [12].

In stead of using enzymes, researchers have used different organic and/or biomolecules such as, amino acids, catecholamines and plant molecules to form organic-inorganic NFs [13-18]. These NFs show remarkable peroxidase like catalytic activities in the presence of H_2O_2 (hydrogen peroxide) through the Fenton reaction. The peroxidase like activity relies on copper ions-redox cycling reaction in the presence of H_2O_2 and production of free hydroxyl radicals. For instance, Wu et al., synthesized NFs using 19 natural amino acids and Cu^{2+} and these NFs acted as Fenton agents owing to reduction and oxidation of Cu^{2+} in the NFs by intreaction with H_2O_2. The amino acids based NFs exhibited peroxidase like activity by effectively catalyzing oxidation of ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) via Fenton reaction. In an other example, Koca et al., prepared curcumin-$Cu_3(PO_4)_2$ NFs and examined their catalytic and antimicrobial activities towards guaiacol and fish pathogen bacteria, respectively.

Herein, we developed Ap-NFs with their peroxidase mimic and antimicrobial activities. The Ap-NFs were characterized by various techniques such as, SEM, FTIR and XRD for elucidation of NF formation. The Ap-NFs showed peroxidase like activity by catalyzing oxidation of guaiacol in the presence of H_2O_2 through Fenton reaction. The antimicrobial properties of the NFs were attributed to Fenton reaction and porous morphology of Ap-NFs.

2. Materials and methods

Copper(II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), guaiacol, hydrogen peroxide solution (30% (w/w) in H_2O) and PBS tablet were purchased from Sigma-Aldrich. *Escherichia coli (E. coli)* ATCC 35218, *Staphylococcus aureus (S. aureus)* ATCC 25923 and *Candida albicans (C. albicans)* ATCC 10231 were obtained from the Erciyes University Pharmaceutical Microbiology Laboratory collection). Deionized (DI) water (18.2MΩ; Millipore Co., USA) was used in all experiments.

2.1. Preparation of Apple Peel Extract

Red apples were obtained from local market. The apple peel extract was prepared by following reported study [19]. Briefly, apples were washed and then peeled. 300 mL DI water was poured on 100 g apple peels, then they were cutted into small pieces via a regular kitchen blender. The resulting mixture was homogenized to obtain homogeneous slurry. It was filtered through muslin cloth and filter paper, respectively. The filtered extract solution was heated to 80 °C in a water bath, then the solution was cooled down to room temperature (RT:25 °C) prior to filtration through Whatman No. 1 filter paper. The final collected extract was stored at 4 °C for further use.

2.2. Preparation of Apple Peel Extract Based Nanoflower and Characterization

Ap-NFs was synthesized by modifying reported study [20]. In general, certain amount of apple peel extract solution was added into freshly prepared mixture consisting of 0.8 mM $CuSO_4$ and 10 mM PBS (pH 7.4). The resulting mixture was vortexed for less 1 minute (min), then it was incubated for 3 days under undisturbed condition. After incubation, blue color precipitate known as Ap-NF was washed with DI water by centrifugation to remove excess of apple peel extract. The final Ap-NF powder was dried in an oven at 60 °C. The Ap-NFs were stored at RT for characterization of further use. In terms of the characterization, while the shape and size of Ap-NFs were imaged by SEM (ZEISS, GEMINI 500), elemental composition in the Ap-NF was analyzed by Energy Dispersive X-ray (EDX). The stretching and bending vibrations of Ap-NF was explained by FTIR (Perkin Elmer, 400 FT-IR Spectrometer Spotlight 400 Imaging System) and its crystal structure was analyzed by XRD (Panalytical, Empyrean).

2.3. Peroxidase-Mimic Activity of Apple Peel Extract Based Nanoflower

The peroxidase like activity of the Ap-NF was recorded using UV-Vis spectrophotometer. The certain amount of Ap-NF (3 mg) was mixed with 22.5 mM H_2O_2 and 45 mM guaiacol in 10 mM PBS (pH 6.8) [20]. After 1 hour incubation of the mixture, oxidation of guaiacol catalyzed by the Ap-NF to 3,3-dimethoxy-4,4-diphenoquinone, then the absorbance of product was measured at 470 nm by a UV-Vis spectrophotometer.

2.4. Antimicrobial Activity of Apple Peel Extract Based Nanoflower

Antimicrobial activities of the Ap-NFs towards *E. coli, S. aureus* and *C. albicans* were tested based on Clinical Laboratory Standards Institute (CLSI) guidelines via broth microdilution method (Clinical and Laboratory Standards Institute and National Committee for Clinical Laboratory Standards 2003; Clinical and Laboratory Standards Institute [21, 22].

3. Results

Synthesis of Ap-NFs was completed with three sequential steps including seed, petal and nanoflower formations. In the first step, Cu^{2+} solution was added into PBS, then they combined

PO_4^{-3} to create primary $Cu_3(PO_4)_2$. The apple peel extract solution was mixed with $Cu_3(PO_4)_2$ solution to form Ap-$Cu_3(PO_4)_2$ nanocrystals as seeds. In the second step, Ap-$Cu_3(PO_4)_2$ nanocrystals were grown by addition of Ap extract to produce Ap-$Cu_3(PO_4)_2$ petals. The petals was adhered each other to form flower-shaped hybrid nanostructure called "Ap-NFs". The potential formation mechanism of Ap-NFs was illustrated in Figure 1.

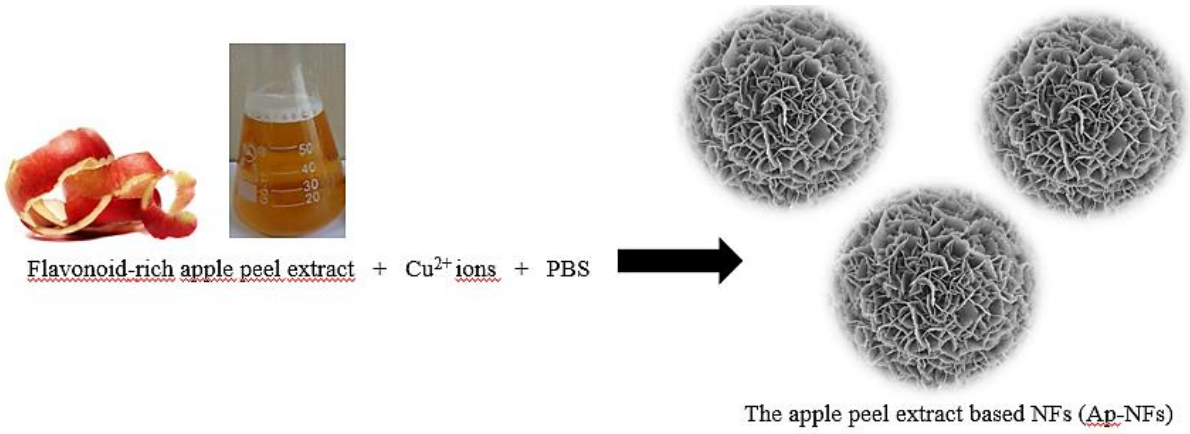


Figure 1. Interaction of apple peel extract, Cu^{2+} and PO_4^{3-} ion with formation mechanism of Ap-NFs

The Ap-NFs were characterized by using SEM, EDX, FTIR and XRD. Figure 2A shows that the Ap-NFs have quite spherical shapes, compact and porous structure with diameters of ~9 µm size. The boundaries of the petals in Ap-NFs was highly rigid and clearly seen in magnified image in Figure 2B. The presence of Cu in the Ap-NF was presented with analysis of elemental composition by EDX (Figure 2C). We claim that appropriate self-assembly was carried out by using flavonoid-rich apple peel extract in Ap-NF formation, which was proved with images of uniform and monodisperse Ap-NF provided by SEM.

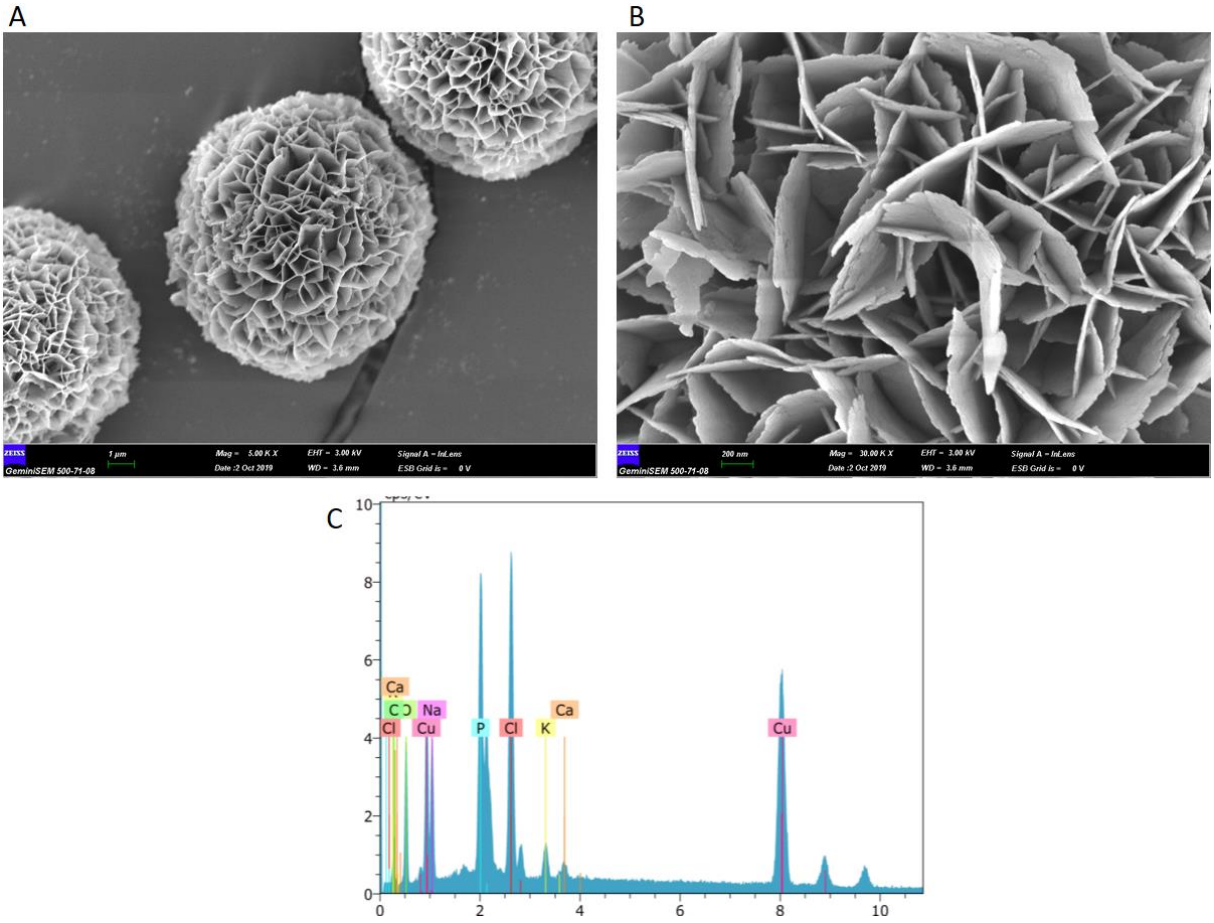


Figure 2. SEM image of Ap-NFs A) with 5.00 KX magnification and B) with 30.00 KX magnification. C) The presence of Cu metal in Ap-NFs on the EDX spectrum

Formation of the Ap-NFs was characterized by FTIR in Figure 3A. The stretching vibrations at 3333.3 cm^{-1} was attributed to hydroxyl bond (-OH). The bending and stretching vibrations of amine groups (-NH_2) and C-N bond in Ap extract were observed at 1617.7 cm^{-1} and at 1141.8 cm^{-1}, respectively. The $Cu_3(PO_4)_2$ primary complex can be considered as backbone of the Ap-NF. The bonds of P-O and P=O in PO_4^{3-} generate stretching vibration peaks at 1034.3 cm^{-1} and 557, 6 cm^{-1}. The crystal structure of Ap-NFs was also revealed by XRD analysis as presented in Figure 3B. The diffraction peaks of $Cu_3(PO_4)_2$ in Ap-NF primary complex mostly matches with the crystal pattern of $Cu_3(PO_4)_2 \cdot 3H_2O$ (JCPDS card (00-022-0548).

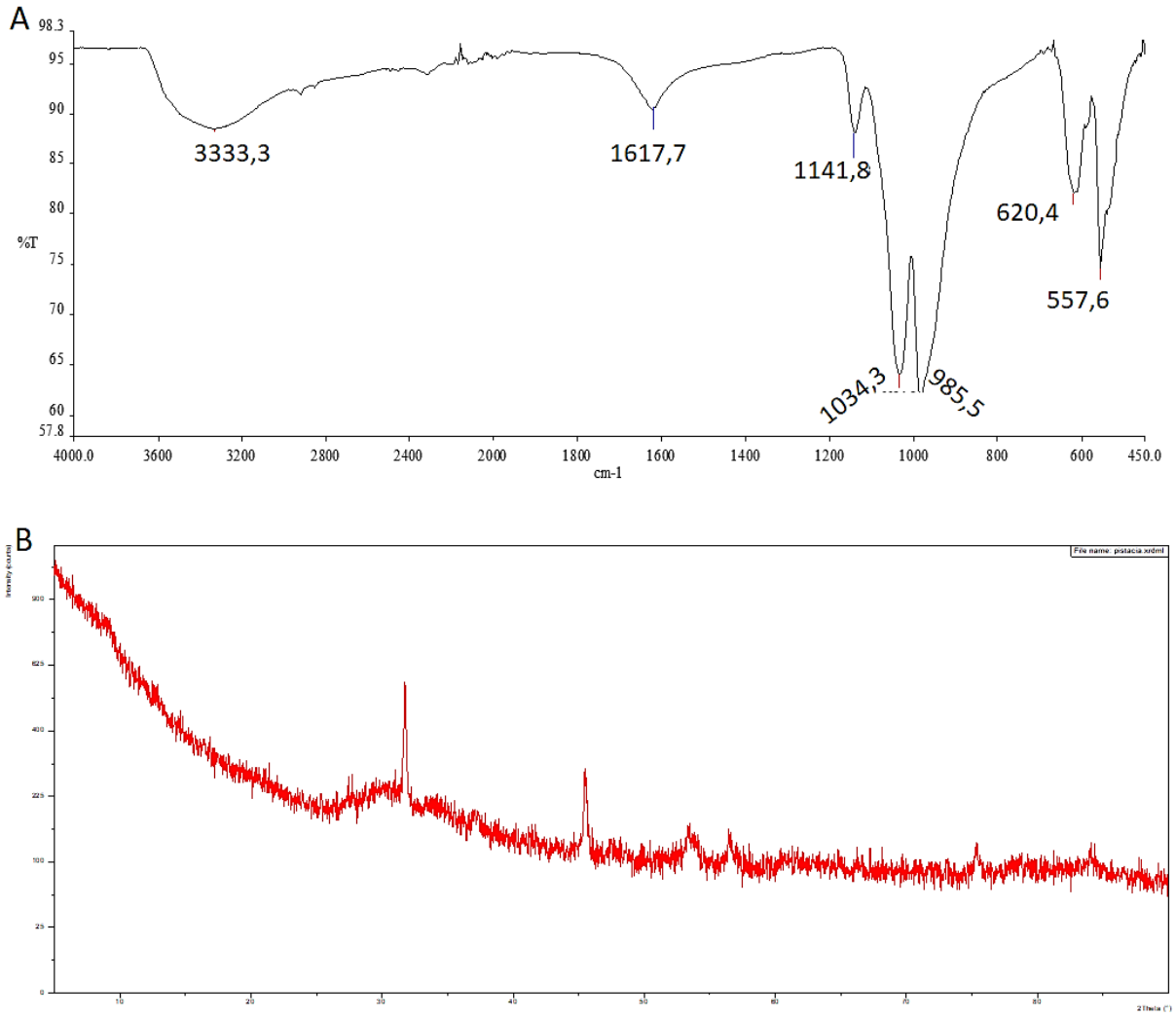
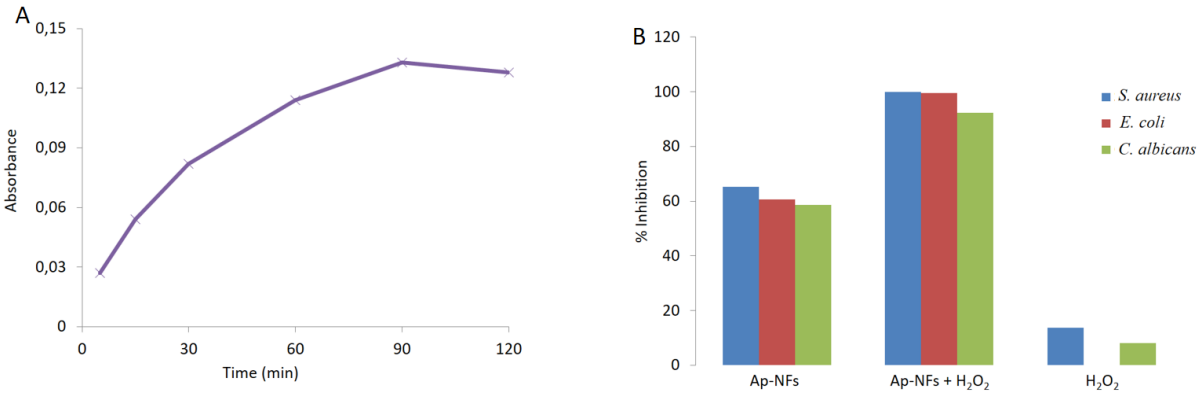


Figure 3. A) FTIR spectrum of Ap-NFs and B) XRD patterns of Ap-NFs

The Ap-NF acted as a Fenton agent and showed peroxidase like activity in the presence of H_2O_2 through Fenton reaction mechanism. The guaiacol was used as a standard substrate and its oxidation was catalyzed by the Ap-NF in order to convert it into 3,3-dimethoxy-4,4-diphenoquinone. The catalysis of guaiacol or its oxidized form was recorded by measuring the characteristic absorbance peak at 470 nm with Uv-Vis spectrophotometer (Figure 4A).

In terms of the antimicrobial study, the antimicrobial activities of the Ap-NF, Ap-NF with H_2O_2, and only H_2O_2 were investigated against model pathogens like *S. aureus*, *E. coli,* and *C. albicans* (Figure 4B). The Ap-NF without H_2O_2 resulted in inactivation between ~58% and ~63 % for all three microorganisms. In addition to that, Ap-NF with H_2O_2 exhibited greatly enhanced antimicrobial activities by killing ~99%, ~99%, and ~92% of *S. aureus*, *E. coli,* and *C. albicans,* respectively. Apart from use of the Ap-NF, only H_2O_2 solution showed weak antimicrobial activity by inhibiting ~15% of *S. aureus* and ~8% of *C. albicans.* Interestingly, no antimicrobial effect was observed with H_2O_2 for *E. coli.* We proposed that antimicrobial activities of the Ap-NFs rely on the morphology of the Ap-NF and reactive hydroxyl radical produced through the Fenton reaction. The peroxidase like and antimicrobial activities of Ap-NFs were well consistant with activities of other non-enzyme based NFs. For instance, Ap-NFs showed similar catalytic activity and antimicrobial properties compared to Viburnum opulus L. Extract based NFs reported by Baldemir and co-workers.

Self assembled snowball like hybrid nanostructures comprising Viburnum opulus L. extract and metal ions for antimicrobial and catalytic applications [20].



C

$$Cu^{2+} + H_2O_2 \text{ ----> } Cu^{1+} + HOO\cdot + H^+$$

$$Cu^{1+} + H_2O_2 \text{ ----> } Cu^{2+} + \cdot OH + OH^-$$

Figure 4. A) Peroxidase-mimic activity of Ap-NF, B) Antimicrobial activity of Ap-NF and C) Potential Fenton reaction

4. Conclusions and discussion

In conclusions, we have demonstrated formation of apple peel extract based nanoflower (Ap-NF) with peroxidase like and antimicorbial activities. The flavonoid-rich apple peel extract induced formation of spherical shape, modosipersed and uniform Ap-NFs. The presence of Cu^{2+} ions acted as inorganic components for formation of the Ap-NFs and showed intrinsic peroxidase mimic activity owing to Fenton reaction. And then, Ap-NFs effectively catalyzed guaiacol to 3,3-dimethoxy-4,4-diphenoquinone in the presence of hydrogen peroxide (H_2O_2). The both production of free reactive radicals through Fenton reaction and flower-shaped morphology made the Ap-NFs superios antimicrobial agents. For instance, the Ap-NFs with H_2O_2 killed ~99%, ~99%, and ~92% of *S. aureus*, *E. coli,* and *C. albicans,* respectively. We belive that these plant extract NFs may have crucial potential to be in fabrication of biosensors, catalytic applications.

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Author contributions: All authors read and approved the final manuscript.

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