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Original Article

Phytocosmetics and beyond: the enzyme inhibitory activities of two rosaceae plant extracts and their cupper-nanoflowers

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Abstract

Background: In this study organic-inorganic hybrid nanoflowers were synthesized using methanolic extracts of the medicinal plants' *Rosa canina* L. and *Rubus sanctus* Schreber together with copper ions (Cu^{2+}).

Materials and Methods: The synthesized plant extract based–inorganic hybrid nanoflowers (PE-ihNFs) of *R. canina* (Rc-ihNFs) and *R. sanctus* (Rs-ihNFs) were characterized by energy-dispersive X-ray (EDX), Fourier transform infrared spectrometry (FTIR), scanning electron microscopy (SEM) and X-ray diffraction (XRD). Also, several enzymes were selected to determine the enzyme inhibitionactivities of the synthesized PE-ihNFs. For the first-time, enzymes (tyrosinase, α-amylase and α-glucosidase, acetyl and butyryl cholinesterase) inhibition activities of the PE-ihNFs and their plain plant extracts were evaluated *in vitro* assays.

Results: Results showed that the PE-ihNFs demonstrated better α-glucosidase & α-amylase enzyme inhibition activity compared to the plain extracts.

Conclusions: These initial studies are promising for the synthesis of these hybrid nanoflowers containing plant extracts, which might have commercial applications in the pharmaceutical and dermo-cosmetics industries.

Keywords: Hybrid nanoflowers, Enzyme inhibitions, Plants extract, Phytocosmetics

Background

In the last decade, organic/inorganic hybrid nanoflowers have received much attention due to their mesmerizing features such as simple, rapid and green synthesis, high surface roughness, large surface-to-volume ratio, high efficiency, and enzyme stabilizing ability.[1-3] Primarily, Ge et al reported protein-inorganic hybrid nanoflowers that were synthesized with selected proteins (BSA, laccase etc.) and $CuSO_4$.[1] Further, several proteins (enzyme)-inorganic hybrid nanoflowers containing different kinds of organic molecules (macromolecules such as protein, DNA, alginate etc. or small molecules like aminoacids) and ions (Cu^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, etc.,) have been subsequently reported.[4-13] Nevertheless, there are just a few studies reported about synthesis of hybrid nanoflowers exploiting plant extracts in the literature. Baldemir et al reported the construction of snowball like novel organic-inorganic hybrid structure that were established by the extract of *Viburnum opulus* plant and copper (II) ions. It was shown that this hybrid structure presented effective antimicrobial activity against bacterial and fungal pathogens more than the *V. opulus* plain extract.[14] In addition, Ildiz et al established the green method for the synthesis of organic-inorganic hybrid nanoflowers based on fenugreek seeds. The hybrid nanoflowers exhibited higher antimicrobial activity than free extract against some gram-positive (+) and gram-negative (-) bacteria. Both hybrid nanoflowers and free extract did not showeded any anticandidal activities against *C. albicans* or *C. glabrata*.[15] Subsequently, Altınkaynak et al reported the production of an organic-inorganic hybrid structure that was synthesized by using *Camellia sinensis* (L.) Kuntze plant extract and its main components. Synthesized *C. sinensis* extract with the united Cu^{2+} ions' nanoflowers (Nfs) showed high antimicrobial and catalytic effects.[16] Unlike these studies, for the first time, selected medicinal plant extract-copper

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hybrid nanoflowers (PE-ihNFs)'s characterisations and enzyme inhibition activities were studied in this study. Two selected medicinal plants, naturally grown in Turkey, which have traditional applications against different disease symptoms including majorly wound and skin problems, were extracted and they are utilized for the synthesis of hybrid nanoflowers, which were characterized and their activities evaluated in this study.

Rubus sanctus Schreber (Rs) has an antioxidant, hepatoprotective, wound-healing and antiinflammatory activities.[17-20] It is rich in tannins, flavonoids and anthocyanins.[18,21] *Rosa canina* L. fruit is known as rosehip, which is used for the treatment of various illnesses including renal and liver disorders, metabolic disorders, arthritis, diarrhea, and particularly, inflammatory and skin disturbances.[22] Moreover, the fruits contain significant amounts of vitamins and minerals.[23]

Enzymes that have important roles in different pathways in the formation of many physiological and biological activities were selected to evaluate the activity of these synthesized PE-ihNFs. Tyrosinase enzyme, contains copper and catalyzes the hydroxylation of L-tyrosine to the 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to dopaquinone plays a significant role in the biosynthesis of melanin.[24] Overprod and accumulation of melanin occur in varied skin illness such as melasma, senile lentigos, solar melanosis, post inflammatory hyper pigmentation, and ephelides. Inhibition of this enzyme is very critical because of the skin whitening effect in cosmetics as well as the improvement of skin disorders in medicine.[25] The enzymes alpha glucosidase & alpha amylase involved in the breakdown of carbohydrates to glucose that play a significant role in in the mechanism of diabetes. Inhibitions of these digestive enzymes are the potential goals for the therapy of diabetes mellitus.[26] Alzheimer's, which is generally a familial disease, causing dementia is commonly observed among older people. It was based on the cholinergic theory that cholinesterases have a crucial role in neural transmission. Therefore, inhibition of these enzymes is very important in maintaining and increasing choline levels. It appears as target enzymes in the treatment of neurological diseases.[27]

The synthesized plant extract based–inorganic hybrid nanoflowers (PE-ihNFs) of *Rosa canina* (Rc-ihNFs) and *Rubus sanctus* (Rs-ihNFs), were identified with techniques including energy-dispersive X-ray (EDX), Fourier transform infrared spectrometry (FTIR), scanning electron microscopy (SEM) and X-ray diffraction (XRD). Extracts of these plants are mostly rich in phenolic compounds and their inorganic hybrid nanoflowers were examined in terms of tyrosinase enzyme inhibition.

Materials and Methods

Chemical substances and reagents

$CuSO_4.5H_2O$, NaCl, KCl, Na_2HPO_4, KH_2PO_4, HCl, NaOH, other chemicals and solvents were obtained from Sigma Aldrich. Methanol (MeOH) was purchased from Merck.

Preparation of the plant extracts

The whole flowering herb materials were previously collected during Summer 2016-2017 at Central Anatolia region of Turkey, dried in the shade, then ground to powder. All powdered plant samples (0.5 kg) were extracted with MeOH (1.5 L) for 6 days at room temperature. Filtered methanolic extracts were gathered and evaporated until dry. Yields of *R. sanctus* and *R. canina* extracts were calculated as 13.62% and 26.72%, respectively.

Synthesis of plant extract based-Copper hybrid nanoflowers (PE-ihNFs)

Hybrid nanoflowers (PE-ihNFs) were formed in accordance with previous methods with some modifications.[1,14] Prepared copper sulfate pentahydrate solution (333 µL) was added to the 50 mL of phosphate buffered solution (PBS, 10 mM, pH 7.0), which contains plant extracts at different concentrations from 0.02 to 0.2 mg mL^{-1} in 20 different test tubes. Then, each mixture was swirled with vortex device for 30s, and incubated in the dark at +4°C for 3 days. Finally, the coloured precipitates were collected with the centrifugation (5000 rpm, 10 min) and washed with aqua for at least 3 times to remove any boundless molecules. Then, the final specimen was dried at 40°C for further characterization and application.

Characterization of plant extract based-Copper hybrid nanoflowers (PE-ihNFs)

The morphologies of the synthesized PE-ihNFs were examined by using SEM (ZEISS EVO-LS10). The chemical and crystal structures of the PE-ihNFs were characterized using FTIR (Perkin Elmer 400, Imaging System of Spectrometer Spotlight 400) and XRD (Bruker, AXS D8 Advance Model) analysis, respectively. The elemental analysis of the PE-ihNFs was performed by EDX (ZEISS EVO-LS10) analysis.

Tyrosinase inhibition activity assay

Tyrosinase enzyme inhibition method was followed by Jeong et al. In a 96 well plate, 20 µL of sample solution diluted with phosphate buffer, 100 µL of phosphate buffer were mixed with 20 µL of tyrosinase (250 U/mL) in each well and incubate for about 10 min at 25°C. Then 20 µL of 3mML-tyrosine was added as substrate and incubated further 30 min at 25°C. After incubation period, the absorbance was read at 492 nm. Kojic acid (200 µg mL^{-1}) was used as a positive control, while phosphate buffer (100 mM PBS, pH=6.8) was used as negative control in place of sample. Each sample was carried out in triplicate with different concentrations.[28]

Alpha-glucosidase enzymeinhibition activity assay

The inhibition method of α-glucosidase was followed according to Kumar et al.[29] While acarbose (200 µg mL-

[1]) was a positive control substance, phosphate buffer solution was a negative control in place of the sample. The sample solution (25 µL) was diluted with a buffer that was mixed with of *a*-glucosidase (25 µL, 0.5 U/mL), following 10 minutes incubation at 25°C, 25 µL of 0.5 mM PNPG was added to each bore as a substrate then the mixture was further incubated (30 minutes, 37°C). At the end of the incubation phase, 100 µL of sodium carbonate (0.2 M) was included in well to put an end to the reaction and the absorbance was read at 405 nm. The all concentrations were carried out in triplicate to obtain an accurate statistical analysis.

Alfa-amylase enzyme inhibition activity assay

The inhibition method of *α*-amylase was followed by Kumar et al.[29] While acarbose (200 µg mL^{-1}) was a positive control, phosphate buffer solution (pH 6.9, 0.02 M, PBS) was a negative control in place of the specimen. Each specimen was conducted in triplicate with diversed concentrations. The reaction mix containing 50 µL of test solution was diluted with buffer, 25 µL of enzyme (5000 µg/mL, *a*-amylase) and incubated for about 10 minutes at 25°C. Then 50 µL of fresh prepared 0.5 % amyl solution (w/v) was annexed to each well as substrate and incubated for a further 10 minutes at 25°C. After the incubation period, 1 % 3,5-dinitrosalicylic acid (100 µL, DNS) coloring reagent was added and warmed for 10 minutes. The absorbances were read at 540 nm.

Acetylcholinesterase enzyme inhibition activity assay

The cholinesterase inhibition assays were evaluated by Ellman colorimetric method as described by Ozturk.[30,31] 150 µL of 0.1 M phosphate buffer solution (pH=8.0), 10 µL of test solutions dissolving in methanol: DMSO (v/v - 80 %) with diversed concentrations and 20 µL of 0.22 U/mL enzyme acetylcholinesterase solution were incubate for 15 minutes at 25°C. 10 µL of a solution of 0.71 mM AChI (Acetyl-thiocholine) and 10 µL of 0.5 mM DTNB (5, 5-dithiobis-2-nitrobenzoic acid) were put and the mixture absorbance was read at 412 nm. Galanthaminehydrobromide (200 µg mL^{-1}) (Sigma-Aldrich, Germany) was employed as a positive control. The formula of enzyme inhibition capacity's calculation:

$$I\ \% = (A_{control} - A_{test})/A_{control} \times 100$$

Butyrylcholinesterase enzyme inhibition activity assay

The acetyl/butyrylcholinesterase inhibition assessment was evaluated by Ellman et al colorimetric method as described by Öztürk et al.[30,31] 150 µL of 0.1 M phosphate buffer (pH=8.0), 10 µL of test solutions dissolving in methanol: DMSO (v/v - 80 %) with different concentrations and 20 µL of 0.1 U/mL butyryl cholinesterase enzyme solution (butyryl cholinesterase was obtained from equine serum, lyophilized powder, Sigma Aldrich, St. Louis, USA) were incubate for 15 minutes at 25°C. 10 µL of a solution of 0.2 mM (Butyrylthiocholine) and 10 µL of 0.5 mM DTNB was mixed and the absorbances of the mix were measured at 412 nm. Galanthaminehydrobromide (200 µg mL^{-1}) (Sigma-Aldrich, Germany) was utilized as a positive control. The formula of enzyme inhibition capacity's calculation:

$$I\ \% = (A_{control} - A_{test})/A_{control} \times 100$$

Data processing for enzyme inhibition assay

The evaluation of measurements and calculations were conducted by microplate reader control and data analysis software. The percentage inhibition of enzyme was detected by comparison of test samples which react of relative to blank sample. The calculation of the enzymatic reaction measurement was performed by the following equation:

$$E = (C-T)/C \times 100$$

"E": The activity of the enzyme.
"C": The absorbance of the control/blank solvent (the existence of enzyme)
"T": The absorbance of the tested sample

Results and Discussion

Characterization of plant extract based-Copper hybrid nanoflowers (PE-ihNFs)

In this study, PE-ihNFs were synthesized using two different medicinal plant extracts (*R. canina, R. sanctus)* as organic components and Cu(II) ions as an inorganic component in phosphate buffer solution (PBS, pH 7) at specific temperatures for 3 days of incubation. Since plant extracts contain different types of phytochemicals that might contain some important elements such as N, O and S atoms, they can form complexes with Cu(II) ions because of their strong affinity. These three atoms have a good ability to coordinate with metal ions. The interaction among the molecules and metal ions is based on the coordination between metal ions and electron donor groups from the molecules. Herein, in the PE-ihNFs synthesis, the most important interaction is the coordination chemistry between Cu(II) ions and some molecules containing mostly the N atom. Such interactions between some biomolecules and metal ions provide the formation of the hybrid structures with snowball or flower-like figures under certain conditions.

As described in the literature, hybrid nanoflower formation occurs with three successive steps[1,8,12]:

1) *Nucleation step*: primary nanocrystals of copper phosphate occur from Cu(II) and phosphate ions interactions in a phosphate buffer.

2) *Growth step:* The principle nanocrystals act with the organic molecules. Then petals like shapes appear.

3) *Formation of flower step:* In the last step, petal like structures stick to each other and formation of hybrid nanoflowers is completed.

Chemical and crystal structures, morphology and enzymeinhibition activities of the synthesized PE-ihNFs were systematically investigated and characterized using different techniques (SEM, EDX, FTIR, and XRD).

It is confirmed that concentration and chemical content of organic component are the most important parameters that influence the morphology, also the structure of PE-ihNFs. SEM analyzes were conducted to investigate the effect of concentrations of the plant extracts on the accidence of synthesized PE-ihNFs. In Figure 1, the best SEM images were selected among the images of different concentration of the plant extracts in the synthesized nanoflowers (Rc-ihNFs and Rs-ihNFs).

As can be seen from the Figure 1, the PE-ihNFs have spherical morphologies with limited size dispersion. The most ideal blooming structured nanoflower morphologies were obtained for Rc-ihNFs at 0.1 mg mL^{-1}, and for Rs-ihNFs at 0.1 mg mL^{-1}concentrations of the plant extracts in the hybrid system.

The chemical structures of *R. canina* extracts furthermore, Rc-ihNFs were characterized using FTIR for identification functional groups. The important characteristic peaks indicating the formation of hybrid nanoflowers were obtained. In all FT-IR spectra, the absorption bands were showed up at the PE-ihNFs (~557 cm^{-1}, ~624 cm^{-1}, ~960 cm^{-1} and ~1045 cm^{-1}, ~1155 cm^{-1}, ~1160 cm^{-1}, in the existence of phosphate groups (PO_4^{3-})).

The important characteristic peaks indicating the formation of hybrid nanoflowers were obtained. Crystal structure, phase equilibria and the measurement of particle sizes of the NFs were analyzed by XRD.

The plant extract concentrations, which were used for the enzyme analyzes, were determined through SEM analyzes of the hybrid nanoflowers. The concentrations given the best blooming nanoflower structures were selected by analyzing the SEM images.

Figure 1. The Plant Pictures and the Best Selected SEM Images of the PE-ihNFs (Rc-ihNFs and Rs-ihNFs).

Tyrosinase inhibition

The mean inhibition ± standard error was indicated by the calculation of absorbance of the plain extracts and their hybrid nanoflowers against blank sample. Inhibition values are given in Table 1. Results showed that the enzyme inhibition potentials of the plain plant extracts samples were lower compared with hybrid nanoflowers. Moreover, PE-ihNfs of *R. canina* showed inhibition close to 50%, when they were compared to the reference kojic acid, which is a reference plant derived compound.

Alpha-glucosidase and α-amylase enzyme inhibition results

The mean inhibition ± standard error was indicated by the calculation of absorbance of the plain extracts and their hybrid nanoflowers against blank sample. Inhibition values are given in Table 2. All the studied PE-ihNfs showed significant inhibition over 50%, while the majority of the plain plant extracts demonstrated enzyme inhibition results less than 50%, additionally, all the hybrid nanoflowers presented enzyme inhibition over the reference drug acarbose.

Anticholinesterase enzymes inhibition results

The mean inhibition ± standard error was indicated by the calculation of absorbance of the plain extracts and their hybrid nanoflowers against blank sample. Inhibition values are given in Table 3. *R. canina* demonstrated more AChE enzyme inhibitory effect. *R. sanctus* showed both AChE and BChE enzymes inhibitory effect.

Conclusions

The present study demonstrated the green synthesis, characterization and activity of the organic-inorganic hybrid PE-ihNFs enzymes inhibitors. The results were promising that the hybrid nanostructures were synthesized demonstrated appealing blooming structures. Likewise, characterization of the PE-ihNFs was successfully completed by using different techniques (SEM, EDX, FTIR, and XRD). The effect of the plant extracts concentration on the accidence of PE-ihNFs was orderly examined by SEM. These results confirmed that the extract concentrations, moreover, chemical content of the extracts, which have different capacity of amine group, can affect the flower-alike morphology. The basic composition of each ihNFs was analysed by EDX. The chemical and crystal structures of nanoflowers were identified by XRD and FTIR spectroscopy, respectively.

Tyrosinase, which has attracted much attention from the cosmetics and pharmaceutical industry, has an important role in the melanogenesis and enzymatic browning.[32] It is thought that the studied plants and nanoflowers are not suitable for this purpose in cosmetic preparations.

The progress of cholinesterase inhibitors is the most

Table 1. Tyrosinase Enzyme Inhibition (Inhibition% ± SEM) 200 µg mL^{-1} of plant samples

Sample	Tyrosinase Enzyme Inhibition	
	Plain Extract	Nanoflower
Rosa canina	42.96 ± 0.87	46.31 ± 3.74
Rubus sanctus	24.29 ± 4.73	38.63 ± 8.95
Kojic acid	79.51 ± 0.60	

Data represented as mean values ± standard deviation (n=3).

pop clinical strategy for the management of Alzheimer's disease (AD). In fact, in the brain of AD patients, the abnormally nominal grade of acetylcholine has been related to pathological features of AD, especially cognitive decline.[33] *R. canina* demonstrated the highest AChE enzyme inhibitory effect. *R. sanctus* showed not only AChE but also BChE enzymes inhibitory effect. When we studied the inhibitory activities of the plants and their nanoflowers against enzymes related to AD, *R. sanctus* inhibited both acetyl and butyryl cholinesterase. It was found that the nanoflower form of the plants evaluated increased the inhibition of at least one cholinesterase. Therefore, it is thought that cupric chelate inhibits the acetylcholinesterase system and free cupric ion may inhibit the enzyme system depending on concentration.[34]

In this study, we evaluated the ability of plant extracts and their nanoflowers to inhibit the activity of α-glucosidase and α-amylase. The inhibitions of carbohydrate hydrolyzing enzymes are considered as an interesting curative approach to control glycaemic index.[35] α-glucosidase inhibitory action and potent α-amylase inhibition is thought one of the ideal therapeutical strategy to the management of diabetes type 2 was enzyme inhibition. In this study, all the extracts displayed elevated inhibition against α-amylase and important inhibition against α-glucosidase.[36] The hybrid nanoflower exhibited higher effective α-glucosidase, α-amylase enzymes inhibitor activities than the plain plant extract.

In the present study, PE-ihNFs were formed using copper ions. In another study that was completed in our laboratory, *in vitro* antioxidant, and some enzyme activities of copper salts that were used to synthesize hybrid nanoflowers were analyzed. It was found that only copper sulfate had antioxidant activity but lower than the hybrid plant extracts and the reference compound ascorbic acid.[37] The result informed us that increase in enzyme activity is not just coming from the copper but also from the hybrid plant-inorganic structure that formed in the phosphate buffer. This initial study is promising for the synthesis of hybrid nanoflowers containing plant extracts that might potentially have commercial practice in pharmacy especially treatment of metabolic diseases and dermo-cosmetics. The present findings indicated that nanoflowers can be considered as new sources for enzyme inhibitors for the treatment of various chronic illnesses and for applications in the dermo-cosmetic industry.

Availability of data and materials

The medicinal plants were collected from nature. Chemicals were obtained from Sigma® and Merck®. The initial study is promising for the synthesis of these hybrid nanoflowers containing plant extract that might has commercial practice in the pharmaceutical and dermo-cosmetics industries.

Competing interests

The all authors declare that they have no competing interests.

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Table 2. α-glucosidase and α-amylase Enzyme Inhibition (Inhibition % ± SEM) 200 µg mL^{-1} of Plant Samples

Sample/Enzyme	Antidiabetic Activity			
	α-Glucosidase Enzyme İnhibition		α-Amylase Enzyme İnhibition	
	Plain Extract	Nanoflower	Plain Extract	Nanoflower
Rosa canina	40.33 ± 2.36	51.90 ± 0.52	45.20 ± 5.38	83.45 ± 1.07
Rubus sanctus	21.83 ± 2.25	92.27 ± 0.32	30.85 ± 0.19	80.87 ± 0.12
Acarbose	57.95 ± 1.25		56.30 ± 0.87	

Data represented as mean values ± standard deviation (n=3).

Table 3. Anticholinesterase Enzymes Inhibition (Inhibition% ± SEM) 200 µg mL^{-1} of Plant Samples

Sample/Enzyme	Anticholinesterase activity			
	AChE Enzyme İnhibition		BChE Enzyme İnhibition	
	Plain Extract	Nanoflower	Plain Extract	Nanoflower
Rosa canina	64.28 ± 0.57	83.45 ± 1.07	70.59 ± 0.82	51.90 ± 0.52
Rubus sanctus	66.67 ± 0.16	80.87 ± 0.12	77.27 ± 0.12	92.27 ± 0.32
Galanthamine hydrobromide	92.49 ± 1.25		88.92 ± 1.52	

Data represented as mean values ± standard deviation (n=3).

authorship, and/or publication of this study.

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