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RESEARCH ARTICLE

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Synthesis and Characterization of Copper-Nanoflowers with the Utilization of Medicinal Plant Extracts for Enhanced Various Enzyme Inhibitory Activities

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In this study, organic-inorganic hybrid nanoflowers were synthesized using methanolic extracts of the medicinal plants *Ajuga chamaepitys* subsp. *chia* var. *chia*, *Achillea wilhelmsii*, *Bongardia chrysogonum*, *Malva sylvestris*, *Phlomis grandiflora* var. *grandiflora*, *Verbascum* sp. together with copper ions (Cu^{2+}). The synthesized plant extract based-inorganic hybrid nanoflowers (PE-ihNFs) of *A. chamaepitys* subsp. *chia* var. *chia* (Ac-ihNFs), *A. wilhelmsii* (Aw-ihNfs), *B. chrysogonum* (Bc-ihNFs), *M. sylvestris* (Ms-ihNFs) *P. grandiflora* var. *grandiflora* (Pg-ihNFs), and *Verbascum* sp. (Vs-ihNFs) were characterized by Scanning Electron Microscopy (SEM), Energy-Dispersive X-ray (EDX), Fourier transform infrared spectrometry (FTIR), and X-Ray Diffraction (XRD). Also, several enzymes were selected to evaluate the enzyme inhibition activities 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 with comparison to their plain plant extracts were evaluated *in vitro*. Results show that the among all the analyzed PE-ihNFs, demonstrated better α-glucosidase & α-amylase enzyme inhibition activity compared to the plain extracts. These initial studies are promising for the synthesis of these hybrid nanoflowers containing medicinal plant extracts, which might have commercial applications in the pharmaceutical and dermo-cosmetics industries.

Keywords: enzyme inhibitions, hybrid nanoflowers, medicinal plants, plants extract.

Introduction

Plants which have a rich phytochemical and biomolecule contents are selectively used in green nanotechnology due to several advantages such as cheap, no risk of contamination, readily available, no expertise and no need complicated instrumentation.[1,2] The active components of plant generally composed of proteins, quinones, flavonoids, catechins, amino acids, vitamins, polysaccharides, poly-phenols, terpenoids and organic acids. Besides the therapeutic commercial uses of plants, biomaterials obtained using their active ingredients are preferred over plain plant extracts with their effective characteristics.[3] Different type of medicinal plants has been extensively used since ancient times with their biologic activities. The medicinal plants *Ajuga chamaepitys* (L.) SCHREBER subsp.

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chia (SCHREBER) ARCHANGELI var. *chia* (SCHREBER) ARCHANGELI, *Achillea wilhelmsii* C. Koch, *Bongardia chrysogonum* L. Spach., *Malva sylvestris* L., *Phlomis grandiflora* HS Thompson var. *grandiflora*, *Verbascum* sp. L. can also be used in green nanotechnology with its remarkable methanolic ingredients.

Ajuga chamaepitys (*L.*) Schreber subsp. *chia* (Schreber) Archangeli *var. chia* (Schreber) *Archangeli* (Ac) commonly known as yellow bugle is widely used for the treatment of hemorrhoids in Anatolia.[3] Moreover, it has emmenagogue, wound healing, diuretic and tonic properties. The plant is rich in phenolic compounds such as iridoid glycosides, and flavonoid derivatives.[4] *Achillea wilhelmsii C. Koch* (Aw) is called following the mythological Greek fighter Achilles, who utilized *Achillea* species for treatment wounded-warriors during the Trojan War.[5] Herbal preparation of *Achillea* species are very often employed as an emmenagogue and diuretic, painkiller for abdomen, antidiarrheal, spasmolitic and, moreover for healing hemorrhoids and wounds.[3,6] *A. wilhemsii* has significant antioxidant activity that might be due mainly to its flavonoids and terpenoid compounds.[7,8] Several *Verbascum* sp. L., were evaluated for their immunostimulant, antidiabetic, anticancer, antiulcer, anti-inflammatory and antinociceptive activities in a variety of scientific studies.[9–14] This plant contains iridoid glycosides (ocubine and derivatives, catalpol), saponoside (ilwensisaponin A), phenylethanoids and terpenes.[13,15–16] The aqueous root extract of *Bongardia chrysogonum* L. Spach. (Bc) has been used as a folk medicine to heal hemorrhoids in southeast Anatolia.[3] Moreover, the *in vitro* data demonstrated that the plant extract has anticancer and anti-inflammatory effects.[3,17] Moreover, an *in vivo* study showed a positive effect in the treatment of diabetes-induced benign prostatic hyperplasia.[18] In a scientific study, the effects on carbohydrate and fat metabolism of the decoction of the plant were evaluated. Results revealed that the decoction of the plant decreased the glucose, triglycerides, urea, total protein and cholesterol, liver enzymes and minerals levels in serum. The serum calcium, albumin and globulin increased in the long period (>30 days) after a significant reducing in the short period (<30 days).[19] A phytochemical search on the plant revealed saponosides (hederacoside A, leontoside A and D, symphyteoxide B) and alkaloids (bongardamine, bongardine, N (acetyl bongardine).[20–22] *Malva sylvestris* L. (Ms), which is known as mallow in English, has potent anti-inflammatory, antioxidant, anticancer and skin tissue integrity activities.[23] *Phlomis grandiflora* HS Thompson var. *grandiflora* (Pg) have been applied to cure ailments of the respiratory tract and wounds in folk medicine and contains mostly essential oils.[24–26]

In recent times, a novel method of producing biomaterial was accidentally found by Zare and coworkers that called organic-inorganic hybrid nanoflower. Zare and his colleagues firstly reported that organic-inorganic hybrid nanocompounds synthesized with selected proteins (BSA, laccase etc.) and $CuSO_4$.[27] Further, several enzyme-inorganic hybrid nanoflowers containing different kinds of organic molecules (macromolecules such as protein, DNA, alginate etc. or amino acids) and ions (Cu^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, etc.) have been subsequently reported.[28–37] Organic/inorganic hybrid nanoflowers took the attention of the researchers owing to their simple, rapid and green synthesis, extraordinary surface roughness, great surface-to-volume ratio, high efficiency, and enzyme stabilizing ability.[27,38,39] However, just a few studies were reported about synthesis of hybrid nanoflowers utilizing plant extracts. Ildiz et al. stated the construction of novel snowball like organic-inorganic hybrid structure (NSBs) that were established by the extract of *Viburnum opulus* (VO) plant and copper (II) ions. The hybrid structure presented effective antimicrobial activity against bacterial and fungal pathogens compared to the *V. opulus* extract.[40] Baldemir et al. reported the production of an organic-inorganic hybrid structure that was synthesized by using *Camellia sinensis* (L.) (tea) Kuntze extract and its main compounds. Synthesized tea extract with the incorporated Cu^{2+} ions' nanoflowers (Nfs) showed significant antimicrobial and catalytic effect.[1] Furthermore, Altinkaynak et al., established the green method for the synthesis of *Trigonella foenum-graecum* L. seed extract-inorganic hybrid nanoflowers. The hybrid nanoflowers exhibited higher antimicrobial activity than free extract against some Gram-positive (+) and Gram-negative (−) bacteria.[41]

In present study, we aimed to synthesize and characterization of medicinal plant extract-copper hybrid nanoflowers (PE-ihNFs) using *A. chamaepitys* subsp. *chia* var. *chia*, *A. wilhelmsii*, *B. chrysogonum*, *M. sylvestris*, *P. grandiflora* var. *grandiflora*, and *Verbascum* sp. extracts and show their enzyme inhibition activities. 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 (the formation of 3,4-dihydroxyphenylalanine (DOPA)) and the oxidation (to form dopaquinone),

plays a significant role in the biosynthesis of melanin.[42] Over manufacture and aggregation of melanin occurs in varied skin problems 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.[43] The enzymes alpha glucosidase & alpha amylase involved in the breakdown of carbohydrates to glucose that act a significant role in in the mechanism of diabetes mellitus (DM). Inhibitions of these digestive enzymes are the potential goals for the treatment of DM.[44] 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.[45]

Subsequently, these medicinal plants, naturally grown in Turkey, which have traditional applications against different disease symptoms including wound and skin problems were utilized for the extraction and synthesis and characterization of nanoflowers with their uses of enzyme inhibition activities. The synthesized plant extract based-inorganic hybrid nanoflowers (PE-ihNFs) of *A. chamaepitys* subsp. *chia* var. *chia* (Ac-ihNFs), *A. wilhelmsii* (Aw-ihNfs), *B. chrysogonum* (Bc-ihNFs), *M. sylvestris* (Ms-ihNFs) *P. grandiflora* var. *grandiflora* (Pg-ihNFs), *Verbascum* sp. (Vs-ihNFs) were characterized by some techniques (EDX, SEM, FTIR and 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

Chemicals and Reagents

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

Preparation of the Plant Extracts

The whole flowering plant materials were previously collected during Summer 2016–2017 at the Central Anatolia of Turkey, dried in the shade, then ground to powder. Herbarium samples of plants are kept in Gazi University Faculty of Pharmacy (GUE). The scientific name of the plants, the used part and the herbarium number are given below in order:

Ajuga chamaepitys (L.) SCHREBER subsp. *chia* (SCHREBER) ARCHANGELI var. *chia* (SCHREBER) ARCHANGELI ; herb; GUE3492
Achillea wilhelmsii C. Koch; herb; GUE3490
Bongardia chrysogonum L. Spach.; tuber; GUE3495
Malva sylvestris L.; leaf; GUE3489
Phlomis grandiflora HS Thompson var. *grandiflora*; leaf; GUE3491
Verbascum sp.; leaf; species not detected

All plant samples (500 g) were extracted with 3X500 mL MeOH for 6 days at room temperature. Filtered methanolic extracts were collected and dried. Then the extracts were utilized for further *in vitro* evaluation.

Synthesis of Plant Extract – Copper Hybrid Nanoflowers (PE-ihNFs)

Plant extract-copper hybrid nanoflowers (PE-ihNFs) were synthesized according to formerly reported methods with some modifications.[27,40] Prepared copper sulfate pentahydrate solution (333 μL) was added to the phosphate buffered solution (PBS) (10 mM, 50 mL, pH 7.0), which contains plant extracts at different concentrations range from 0.02 to 0.2 mg mL^{-1} in 20 different test tubes. Final concentrations of copper ions were 0.8 mM. Then, each mixture was swirled with vortex device for 30s, and waited in the darkness at $+4\,^{\circ}C$ for 3 days. Eventually, the colored precipitates were collected with centrifugation (5000 rpm, 10 min) and washed with water for at least 3 times. The final specimen was vaporized until dry at 40 °C for further characterization and enzyme inhibition assays.

Characterization of Herbal Extract – Copper Hybrid Nanoflowers (PE-ihNFs)

The morphologies of the synthesized PE-ihNFs were examined by using Scanning Electron Microscopy (ZEISS EVO-LS10). The chemical and crystal structures of the PE-ihNFs were characterized using Fourier Transform Infrared spectroscopy (Perkin Elmer 400, Spectrometer Spotlight 400 Imaging System) and X-Ray Diffraction (Bruker, AXS D8 Advance Model) analysis, respectively. The elemental analysis of the PE-ihNFs was performed by energy dispersive X-ray (ZEISS EVO-LS10) analysis.

Tyrosinase Inhibition Activity Assay

Tyrosinase inhibition was detected by the improved dopa chrome method. L–DOPA was used as substrate. A part of the plant extracts and their nanoflowers, under the optimal conditions, diffused in DMSO (PBS, 6.8 pH, 80 μL), 40 μL of L–DOPA, and 40 μL tyrosinase enzymes were added into each well. Analysis was performed in a microplate by using ELISA plate reader and absorbances were measured at 475 nm. Results were evaluated by comparing with the DMSO and α-kojic acid.[46]

α-Glucosidase Enzyme Inhibition Activity Assay

The inhibition method of α-glucosidase was followed according to Kumar et al.[47] Acarbose was utilized as reference. The test solution (25 μL) was diluted with a PBS which added to α-glucosidase (25 μL, 0.5 U/mL). After the 10 min incubation at 25 °C, 25 μL of 0.5 mM PNPG was added to each well then the mixture was further waited for 30 min at 37 °C. At the end of the incubation term, sodium carbonate (0.2 M, 100 μL) was joined to put an end to the reaction and the absorbances were read at 405 nm. All concentrations were carried out in triplicate to obtain an accurate statistical analysis.

α-Amylase Enzyme Inhibition Activity Assay

The inhibition method of α- amylase was followed by Kumar et al.[47] While acarbose was a positive control, PBS (pH 6.9, 0.02 M, PBS) was a negative control in place of the specimen. Each specimen was conducted in triplicate with diverse concentrations. The reaction mix containing 50 μL of test solution was diluted with buffer, 25 μL of enzyme (5000 μg/mL, α-amylase) and incubated for about 10 min at 25 °C. Then 50 μL of freshly prepared 0.5 % starch solution (w/v) was annexed to each well as a substrate and incubated for a further 10 min at 25 °C. Incubation period was followed by addition of 1 % 3,5-dinitrosalicylic acid (DNS, 100 μL) coloring reagent and heated in a water bath for 10 min. 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 Öztürk.[48,49] 150 μL of 0.1 M PBS (pH = 8.0), 10 μL of sample solutions in MeOH/DMSO (4k:1k, v/v) with diverse concentrations and 20 μL of 0.22 U/mL acetylcholinesterase enzyme solution (type-VI–S, EC 3.1.1.7) were incubated for 15 min 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 mixed and the absorbances of the mixture were read at 412 nm (Epoch, Biotek, USA). Galantamine hydrobromide (Sigma-Aldrich, Germany) was exploited as a positive control.

Butyrylcholinesterase Enzyme Inhibition Activity Assay

The acetylcholinesterase/butyrylcholinesterase inhibition assay was evaluated by Ellman colorimetric method as described by Öztürk.[48,49] 150 μL of 0.1 M PBS (pH = 8.0), 10 μL of test solutions in MeOH/DMSO (4k:1k, v/v) with different concentrations and 20 μL of 0.1 U/mL enzyme solution (butyrylcholinesterase was obtained from equine serum) were incubate for 15 min at 25 °C. 10 μL of a solution of 0.2 mM (Butyrylthiocholine) and 10 μL of 0.5 mM DTNB were mixed and the absorbances of the mix were measured at 412 nm. Galantamine hydrobromide (Sigma-Aldrich, Germany) was used as a positive control.

Results and Discussion

Characterization of Herbal Extract Based-Copper Hybrid Nanoflowers (PE-ihNFs)

In this study, PE-ihNFs were synthesized using five different medicinal plant extracts (*A. chamaepitys* subsp. *chia* var. *chia*, *A. wilhelmsii*, *B. chrysogonum*, *M. sylvestris*, *P. grandiflora* var. *grandiflora*, *Verbascum* sp.) as organic components and Cu (II) ions as an inorganic component in PBS, 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.[27,40]

As described in the literature, self assembled hybrid nanoflower formation occurs with three successive steps: nucleation, growth, and formation of flower step. In the last step, petal like structures stick to each other and formation of hybrid nanoflowers is completed.[27,32,36] Chemical and crystal structures, morphology and enzyme inhibition 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 herbal extracts on the morphology of synthesized PE-ihNFs. SEM images of Ac-ihNFs (*Figure 1*) are given as an example of the effect of different concentrations on the morphology.

In *Figure 2*, the best SEM images were selected among the images of different concentration of the plant extracts in the synthesized nanoflowers (Aw-ihNfs, Bc-ihNFs, Ms-ihNFs, Pg-ihNFs and Vs-ihNFs). As can be seen from the *Figure 2*, all of the PE-ihNFs have globular accidence with a snowball like dimensions. The most ideal blooming structured nanoflower morphologies were obtained at various concentrations: for Ac-ihNFs at 0.1 mg mL^{-1} concentration; for

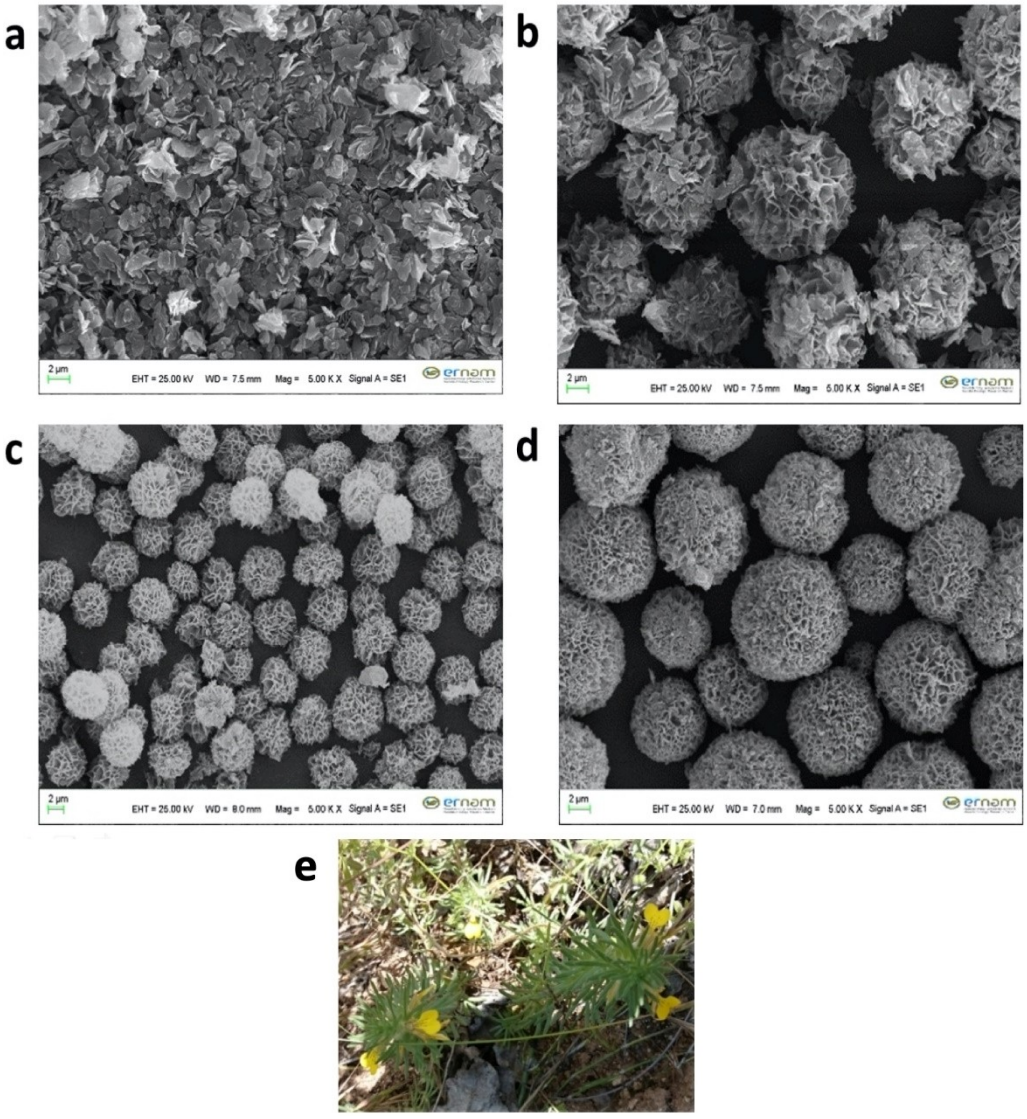

Figure 1. SEM images of Ac-ihNFs containing (a) 0.02 mg mL^{-1} (b) 0.05 mg mL^{-1} (c) 0.1 mg mL^{-1} and (d) 0.2 mg mL^{-1}of the plant extract, (e) ***A. chamaepitys*** subsp. *chia* var. *chia*.

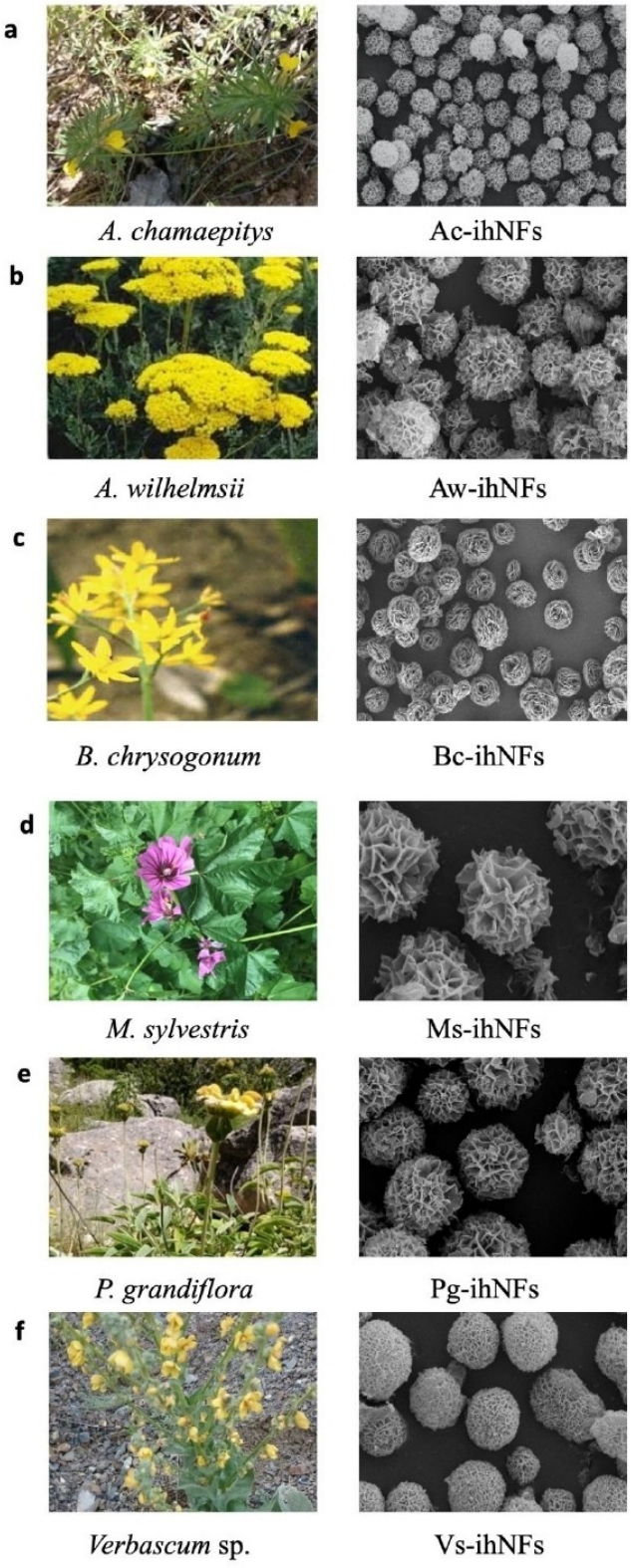

Figure 2. All the plant pictures and the best selected SEM images of the PE-ihNFs (Aw-ihNfs, Bc-ihNFs, Ms-ihNFs, Pg-ihNFs and Vs-ihNFs).

Aw-ihNFs at 0.02 mg mL^{-1}; for Bc-ihNFs at 0.02 mg mL^{-1}; for Ms-ihNFs at 0.1 mg mL^{-1}; for Pg-ihNFs at 0.02 mg mL^{-1} and for Vs-ihNFs at 0.1 mg mL^{-1} concentrations of the plant extracts that added into the hybrid system. In formation mechanism; there are three stages. The first step is defined as the nucleation step. Phosphate ions react with copper ions to obtain primary copper phosphate nanocrystals. In second stage, Nanocrystals coordinate primary amine groups to initiate the nucleation process. In the last step is defined as growth stage. The precursor nanocrystals formed spontaneously form leaves originating from the large center with the incubation period. At this stage, anisotropic growth is supported by copper ions and organic molecules.

The chemical structures of *A. chamaepitys* subsp. *chia* var. *chia*, *A. wilhelmsii*, *B. chrysogonum*, and *P. grandiflora* var. *grandiflora*, extracts furthermore, Ac-ihNFs, Aw-ihNfs, Bc-ihNFs, and Pg-ihNFs, Rc-ihNFs were characterized using FTIR for identification of functional groups (*Figure 3*).

The important characteristics peaks indicating the formation of hybrid nanoflowers were obtained for each well shaped nanoflowers. In all FTIR spectra of PE-ihNFs, the absorption bands were displayed at ~557 cm^{-1}, ~624 cm^{-1}, ~960 cm^{-1} and ~1045 cm^{-1}, ~1155 cm^{-1} and ~1160 cm^{-1} in the PE-NFs. They were bound to P=O and P–O vibrations, by displaying the existence of phosphate bands for PE-ihNFs by FTIR.

The important characteristic peaks indicating the formation of hybrid nanoflower were obtained, for *A. chamaepitys* subsp. *chia* var. *chia* plant extracts (*Figure 4*). Crystal structure, phase equilibria and the measurement of particle sizes of the NFs were analyzed by XRD. The report of the FTIR, EDX and XRD results are offered as *Supporting Information* due the high volume of data in this manuscript. The plant extract concentrations, which were used for the enzyme analyzes, were determined through SEM analyzes of the hybrid nanoflowers. The concentrations that gave the best blooming nanoflower structures were selected by analyzing the SEM images.

Tyrosinase Inhibition

Tyrosinase, which has attracted much attention compared to the cosmetics and pharmaceutical industry, has an important role in the enzymatic browning and melanogenesis.[50] In this study, we evaluated the tyrosinase activity of hybrid nanostructures with compare the plain plant extracts. The mean inhib-

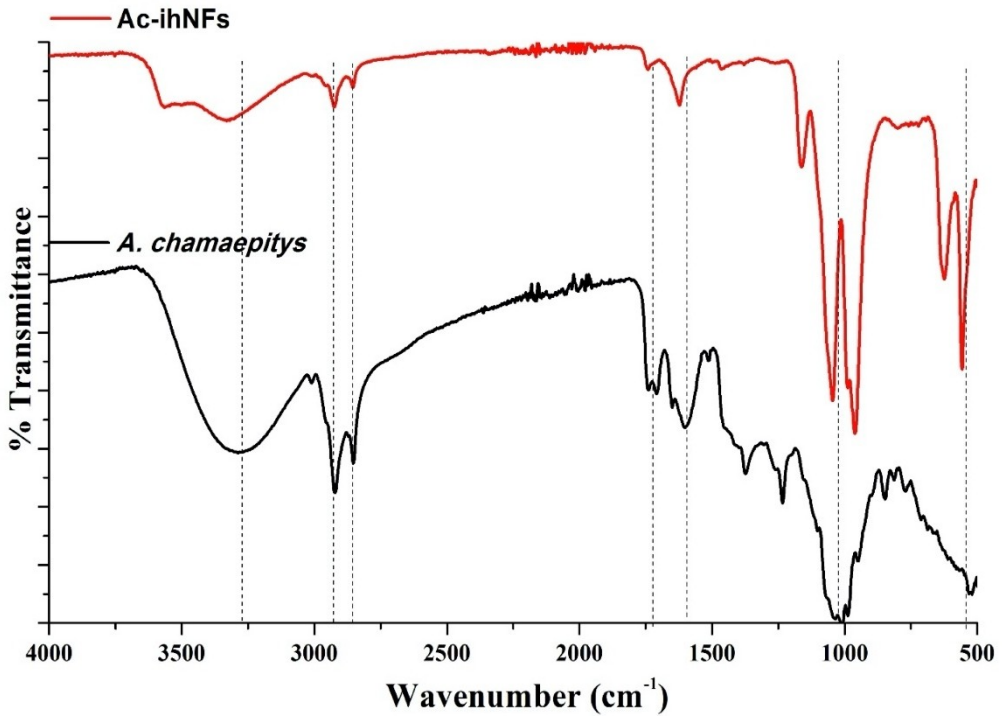

Figure 3. Selected FTIR analyzes result of (upper-red line) Ac-ihNFs and (bottom-black line) *A. chamaepitys* subsp. *chia* var. *chia* extract.

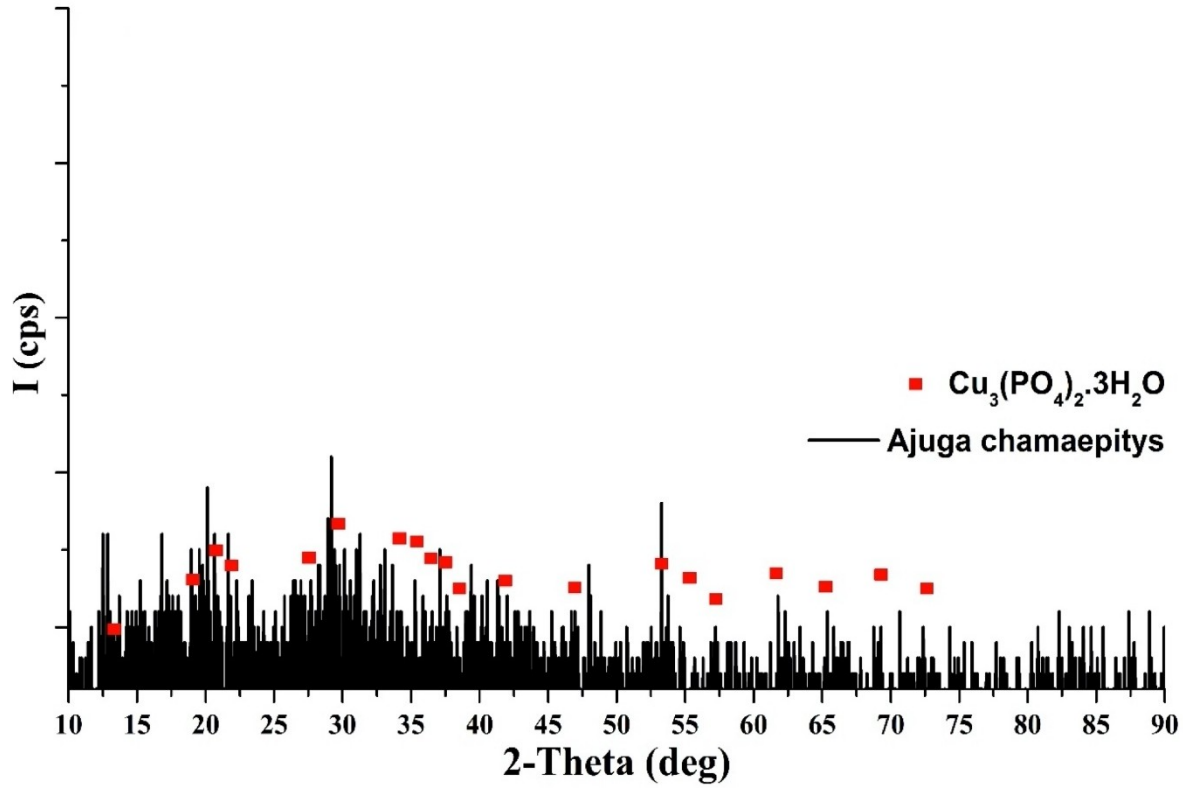

Figure 4. XRD patterns of Ac-ihNFs in accord the peak position of the $Cu_3(PO_4)_2 \cdot 3H_2O$ (JCPDS) card (00–0220548).

ition ± standard error was indicated by the calculation of absorbance of the plain extracts and their hybrid nanoflowers compared to blank sample. Inhibition values were given in *Table 1*.

The highest tyrosinase inhibition was displayed by the nanoflowers of *A. chamaepitys subsp. chia var. chia* and *B. chrysogonum*. The hybrid nanostructures were verified to increase tyrosinase inhibitory effects more than the plain plant extracts: *A. chamaepitys* subsp. *chia* var. *chia* and *B. chrysogonum* exhibited tyrosinase inhibition 51.04 ± 6.56 %, 60.08 ± 7.94 %, respectively. *B. chrysogonum* containing pyridine-derived alkaloid compounds with anti-tyrosinase effect was the most effective plant-inorganic hybrid nanoflower.[51] Whilst the plain plant extract enzyme inhibition was 46.85 ± 2.74 %, the hybrid nanoflower form of the plant extract enzyme inhibition increased to 60.08 ± 7.94 % at the same concentration. Approximately a 28 % enzyme inhibition increase was achieved with the hybrid nanoflower. The nanoflower form obtained from *A. chamaepitys subsp. chia var. chia*, which is rich in phenolic compounds such as flavonoids and anthocyanins and terpenes and has a significant antityrosinase effect, was also found to be slightly more effective than the plain plant extract.[52] Previous studies by Llorent-Martínez et al. also support the high antityrosinase potential of methanolic *A. chamaepitys* subsp. *chia* var. *chia* extract.[53] It might be possible to attribute the increased activity in the form of *B. chrysogonum* and nanoflowers to the nitrogen content in the plant extract, resulting in better and more stable nano-flower formation.

α-Glucosidase and α-Amylase Enzyme Inhibition Results

In the present study, we evaluated the ability of plant extracts and their nanoflowers to obstruct the activity of α-glucosidase and α-amylase. The inhibitions of carbohydrate hydrolyzing enzymes are thought as an amazing curative approach to balance glycaemic index.[54] α-glucosidase inhibition and potent α-amylase inhibitory action is considered as the ideal therapeutical strategy to the control of DM (Type 2). All the extracts displayed elevated inhibition against α-amylase and important inhibition activity against α-glucosidase. 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. The hybrid nanoflower exhibited higher effective α-glucosidase, α-amylase enzymes inhibitor activities than the plain plant extract. Our results support the study of Dokuyucu et al., and the highest antidiabetic activity was found in *Bongardia chrysogonum*.[18]

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.[55] 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.

Anticholinesterase Enzymes Inhibition Results

The mean inhibition ± standard error was indicated by the calculation of absorbance of the plain extracts and

Table 1. Tyrosinase enzyme inhibition (Inhibition % ± S.E.M.) 200 μg mL^{-1} of plant samples.

Sample/Reference	*Tyrosinase Enzyme Inhibition*	
	Plain extract	*Nanoflower*
Ajuga chamaepitys subsp. *chia* var. *chia*	$44.42 \pm 2.61^{c,d,e}$	$\mathbf{51.04 \pm 6.56^{b,c}}$
Achillea wilhelmsii	$36.63 \pm 0.58^{e,f}$	$\mathbf{48.24 \pm 8.78^{c}}$
Bongardia chrysogonum	$46.85 \pm 2.74^{c,d}$	$\mathbf{60.08 \pm 7.94^{b}}$
Malva sylvestris	18.10 ± 1.43^{g}	$\mathbf{38.16 \pm 3.88^{d,e,f}}$
Phlomis grandiflora var. *grandiflora*	28.89 ± 1.96^{f}	$\mathbf{36.86 \pm 7.77^{e,f}}$
Verbascum sp.	32.58 ± 5.23^{f}	$\mathbf{49.60 \pm 8.35^{c}}$
Kojic acid	$\mathbf{80.01 \pm 1.49^{a}}$	

Data represented as mean values ± standard deviation (n = 3). ***One-Way-ANOVA Duncan test.

Table 2. α-glucosidase and α-amylase enzyme inhibition (Inhibition % ± S.E.M.) 200 μg mL^{-1} of plant samples.

Sample/Reference	Antidiabetic activity α-glucosidase enzyme inhibition		α-amylase enzyme inhibition	
	Plain extract	*Nanoflower*	*Plain extract*	*Nanoflower*
Ajuga chamaepitys subsp. *chia* var. *chia*	40.66 ± 1.75^{f}	**72.54 ± 2.46^{b}**	36.35 ± 2.72^{e}	**82.97 ± 2.26^{b,c}**
Achillea wilhelmsii	39.83 ± 2.02^{f}	**73.29 ± 4.39^{b}**	37.33 ± 2.89^{e}	**81.08 ± 0.82^{c}**
Bongardia chrysogonum	42.00 ± 0.86^{f}	**55.23 ± 3.05^{d,e}**	79.32 ± 0.17^{c}	**85.85 ± 0.29^{a,b}**
Malva sylvestris	44.67 ± 4.01^{f}	**50.89 ± 0.51^{e}**	17.76 ± 1.67^{g}	**87.58 ± 0.42^{a}**
Phlomis grandiflora var. *grandiflora*	32.33 ± 3.81^{g}	**87.42 ± 2.90^{a}**	32.13 ± 2.36^{f}	**79.73 ± 0.48^{c}**
Verbascum sp.	30.88 ± 2.08^{g}	**63.08 ± 4.34^{c}**	36.61 ± 5.71^{e}	**80.82 ± 0.70^{c}**
Acarbose	**57.56 ± 0.52^{d}**		**56.49 ± 1.52^{d}**	

Data represented as mean values ± standard deviation (n = 3). ***One-Way-ANOVA Duncan test.

their hybrid nanoflowers against blank sample. Inhibition values are given in *Table 3*. However, *A. wilhelmsii* and *P. grandiflora* var. *grandiflora* demonstrated more BChE enzyme inhibitory effect, *Verbascum* sp. showed both AChE and BChE enzymes inhibitory effect.

The improvement of cholinesterase inhibitors is the most pop clinical strategy aimed for the treatment of Alzheimer's disease (AD) yet. Actually, in the brain of AD patients, the abnormally nominal grade of acetylcholine has been about to pathological properties of AD.[56] *A. wilhelmsii* and *P. grandiflora* var. *grandiflora* demonstrated the highest BChE enzyme inhibitory effect. *Verbascum* sp. showed both AChE and BChE enzymes inhibitory effect. It has also been demonstrated by previous studies that verbascoside, a phenylethanoid glycoside derivative found in many verbascum species, is a moderate enzyme inhibitor.[57] When we investigated the inhibitory activity of the plant and their nanoflowers against enzymes related to AD, *Verbascum* sp. extract inhibited both acetyl and butyryl cholinesterase. Therefore, it is thought that copper chelate inhibits the acetylcholinesterase system and free cupric ion may inhibit the enzyme system depending on concentration.[58]

Conclusion

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 influences of the herbal extracts' concentration on the morphology of PE-ihNFs were orderly investigated by SEM. These results confirm that the extract concentrations, moreover, chemical content of the extracts, which have different capacity of amine group, can affect the blossom-like morphology. The basic structure of each ihNFs was analysed by EDX. The chemical and crystal structures of nanoflowers were identified by making use of XRD

Table 3. Anticholinesterase enzymes inhibition (Inhibition % ± S.E.M.) 200 μg mL^{-1} of plant samples.

Sample/Reference	Anticholinesterase activity			
	AChE enzyme inhibition		BChE enzyme inhibition	
	Plain extract	*Nanoflower*	*Plain extract*	*Nanoflower*
Ajuga chamaepitys subsp. *chia* var. *chia*	85.71 ± 0.12^{b,c,d}	82.99 ± 2.26^{d,e}	66.67 ± 0.09^{d}	72.54 ± 2.46^{c}
Achillea wilhelmsii	86.53 ± 0.13^{c}	81.08 ± 0.82^{e}	53.84 ± 0.13^{f}	**73.29 ± 4.39^{c}**
Bongardia chrysogonum	12.86 ± 4.87^{h}	23.12 ± 3.77^{g}	23.34 ± 0.25^{g}	9.50 ± 0.81^{h}
Malva sylvestris	72.41 ± 0.09^{f}	**87.58 ± 0.42^{b,c}**	63.64 ± 0.08^{d,e}	50.89 ± 0.51^{f}
Phlomis grandiflora var. *grandiflora*	89.26 ± 0.67^{b}	79.73 ± 0.45^{e}	77.27 ± 0.90^{b}	**87.43 ± 2.90^{a}**
Verbascum sp.	72.72 ± 0.82^{f}	**80.82 ± 0.71^{e}**	60.53 ± 0.63^{e}	**63.08 ± 4.34^{e}**
Galantamine hydrobromide	**93.87 ± 0.56^{a}**		**89.89 ± 0.01^{a}**	

Data represented as mean values ± standard deviation (n = 3). ***One-Way-ANOVA Duncan test.

and FTIR methods, respectively. Results showed that the enzyme inhibition potentials of the plain plant extracts samples were lower compared with hybrid nanoflowers. Moreover, PE-ihNfs of *A. chamaepitys* subsp. *chia* var. *chia* and *B. chrysogonum* showed inhibition above 50%, when they were compared to the reference kojic acid, a plant derived compound.

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 Alzheimer and Diabetes Mellitus, and for skin lightening and whitening purposes in the dermocosmetic industry.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contribution Statement

This study was carried out in collaboration of all the authors. The idea and experiments were conceived and designed by U. Koca-Caliskan and N. Ozdemir. The plants were collected and extracted by C. Dönmez and U. Koca-Caliskan. The synthesis and characterizations of plant extract-copper hybrid nanoflowers (PE-ihNFs) were performed by C. Altinkaynak and N. Ozdemir. *In vitro* enzyme inhibition activities were performed by C. Donmez, F. Ayaz and N. Eruygur. Data analysis and writing the article were performed by all authors.

References

[1] N. Baldemir, B. Köse, N. Ildız, S. İlgün, S. Yusufbeyoğlu, V. Yilmaz, I. Ocsoy, 'Synthesis and characterization of green tea (*Camellia sinensis* (*L.*) *Kuntze*) extract and its major components-based nanoflowers: a new strategy to enhance antimicrobial activity', *RSC Adv.* **2017**, *7*, 44303–44308.

[2] R. Mohammadinejad, S. Karimi, S. Iravani, R. S. Varma, 'Plant-derived nanostructures: types and applications', *Green Chem.* **2016**, *18*, 20–52.

[3] U. Koca-Caliskan, C. Aka, M. G. Oz, 'Plants Used in Anatolian Traditional Medicine for the Treatment of Hemorrhoid', *Rec. Nat. Prod.* **2017**, *11*, 235–250.

[4] A. Venditti, C. Frezza, F. Maggi, G. Lupidi, M. Bramucci, L. Quassinti, A. Bianco, 'Phytochemistry, micromorphology and bioactivities of *Ajuga chamaepitys* (L.) Schreb. (Lamiaceae, Ajugoideae): Two new harpagide derivatives and an unusual iridoid glycosides pattern', *Fitoterapia* **2016**, *113*, 35–43.

[5] E. Küpeli-Akkol, U. Koca, I. Pesin, D. Yilmazer, 'Evaluation of the wound healing potential of *Achillea biebersteinii* Afan.(Asteraceae) by in vivo excision and incision models', *Evid. Based Complement. Alternat. Med.* **2011**, *2011*, 7.

[6] T. Baytop, 1999. Therapy with Medicinal Plants in Turkey, Past and Present, *2*nd edition, Nobel Tıp Istanbul, Turkey.

[7] S. Afsharypuor, S. Asgary, G. B. Lockwood, 'Constituents of the essential oil of *Achillea wilhelmsii* from Iran', *Planta Med.* **1996**, *62*, 77–78.

[8] M. Mahmoudabady, M. Lashkari, S. Niazmand, M. Soukhtanloo, 'Cardioprotective effects of *Achillea wilhelmsii* on the isolated rat heart in ischemia-reperfusion', *J. Tradit. Complement. Med.* **2017**, *7*, 501–507.

[9] V. Mathad, 'Studies on the profile of immunostimulant activities of modified iridoid glycosides', *Bioorg. Med. Chem.* **1998**, 6, 605–611.

[10] T. Konoshima, M. Takasaki, H. Tokuda, H. Nishino, 'Cancer chemopreventive activity of an iridoid glycoside, 8-acetylharpagide, from *Ajuga decumbens*', *Cancer Lett.* **2000**, *157*, 87–92.

[11] B. Ahmed, A. J. Al-Rehaily, T. A. Al-Howiriny, K. A. El-Sayed, M. S. Ahmad, 'Scropolioside-D2 and harpagoside-B: two new iridoid glycosides from *Scrophularia deserti* and their antidiabetic and antiinflammatory activity', *Biol. Pharm. Bull.* **2003**, *26*, 462–467.

[12] I. Gürbüz, A. M. Özkan, E. Yesilada, O. Kutsal, 'Anti-ulcerogenic activity of some plants used in folk medicine of Pinarbasi (Kayseri, Turkey)', *J. Ethnopharmacol.* **2005**, *101*, 313–318.

[13] E. Küpeli, I. I. Tatli, Z. S. Akdemir, E. Yesilada, 'Bioassay-guided isolation of anti-inflammatory and antinociceptive glycoterpenoids from the flowers of *Verbascum lasianthum* Boiss. ex Bentham', *J. Ethnopharmacol.* **2007**, *110*, 444–450.
[14] P. Dimitrova, E. Kostadinova, V. Milanova, K. Alipieva, M. Georgiev, N. Ivanovska, 'Antiinflammatory properties of extracts and compounds isolated from *Verbascum xanthophoeniceum* Griseb', *Phytother. Res.* **2012**, *26*, 1681–1687.
[15] I. I. Tatli, Z. S. Akdemir, 'Chemical constituents of *Verbascum* L. species', *J. Pharm. Sci. (FABAD)* **2004**, *29*, 93–107.
[16] I. I. Tatli, Z. S. Akdemir, E. Bedir, I. A. Khan, 'Saponin, iridoid, phenylethanoid and monoterpene glycosides from *Verbascum pterocalycinum* var. *mutense*', *Turk. J. Chem.* **2004**, *28*, 111–122.
[17] M. Assaf, R. N. Haddadin, N. A. Aldouri, R. Alabbassi, S. Mashallah, M. Mohammad, Y. Bustanji, 'Anti-cancer, anti-inflammatory and anti-microbial activities of plant extracts used against hematological tumors in traditional medicine of Jordan', *J. Ethnopharmacol.* **2013**, *145*, 728–736.
[18] R. Dokuyucu, K. H. Gozukara, O. Ozcan, N. K. Sefil, A. Nacar, A. Dokuyucu, M. Inci, 'The Effect of *Bongardia chrysogonum* on Prostate Tissue in a Rat Model of Streptozotocin-Induced Diabetes', *Turkish Society of Physiological Sciences 41st National Physiology Congress* **2015**, *215*, 50, TURKEY.
[19] A. Arslan, E. A. Cakmak, M. Ozaslan, B. Cengiz, C. Bagci, M. Tarakcioglu, R. Kocabas, 'The effects of *Bongardia chrysogonum* (L) spach extract on the serum parameters and liver, kidney, and spleen tissues in rats', *Biotechnol. Biotechnol. Equip.* **2005**, *19*, 170–179.
[20] Atta-ur-Rahman, D. Shahwar, I. M. Choudhary, B. Sener, G. Toker, K. H. C. Baser, 'Alkaloids of *Bongardia chrysogonum*', *Phytochemistry* **1999**, *50*, 333–336.
[21] Atta-ur-Rahman, D. Shahwar, I. M. Choudhary, B. Sener, G. Toker, K. H. C. Baser, 'Triterpenoid saponins from *Bongardia chrysogonum*', *J. Nat. Prod.* **2000**, *63*, 251–253.
[22] Atta-ur-Rahman, D. Shahwar, Z. Parween, I. M. Choudhary, B. Sener, G. Toker, K. H. C. Baser, 'New alkaloids from *Bongardia chrysogonum*', *Nat. Prod. Lett.* **1998**,*12*, 161–173.
[23] C. Gasparetto, C. A. F. Martins, S. S. Hayashi, M. F. Otuky, R. Pontarolo, 'Ethnobotanical and scientific aspects of *Malva sylvestris* L.: a millennial herbal medicine', *J. Pharm. Pharmacol.* **2011**, *64*, 172–189.
[24] Liolios, H. Laouer, N. Boulaacheb, O. Gortzi, I. Chinou, 'Chemical composition and antimicrobial activity of the essential oil of Algerian *Phlomis bovei* De Noé subsp. *bovei*', *Molecules* **2007**, *12*, 772–781.
[25] M. M. Özcan, J. C. Chalchat, Y. Bagci, H. Dural, G. Figueredo, A. Savran, 'Chemical composition of essential oils of *Phlomis grandiflora* HS Thompson var. *grandiflora* flowers and leaves Of Turkish origin', *J. Food Biochem.* **2011**, *35*, 125–132.
[26] C. Dönmez, U. Koca-Çalışkan, 'Ethnopharmacological survey on *Phlomis grandiflora*: in vivo antihemorrhoidal model', *Current Perspectives on Medicinal and Aromatic Plants (CUPMAP)* **2019**, *2*, 40–46.
[27] J. Ge, D. Lei, R. N. Zare, 'Functional Protein-Organic/Inorganic Hybrid Nanomaterials', *Nat. Nanotechnol.* **2012**, *7*, 428–432.
[28] J. Sun, J. Ge, W. Liu, M. Lan, H. Zhang, P. Wang, Y. Wanga, Z. Niu, 'Multi-enzyme co-embedded organic-inorganic hybrid nanoflowers: synthesis and application as a colorimetric sensor', *Nanoscale* **2014**, *6*, 255–262.
[29] X. Wang, J. Shi, Z. Li, S. Zhang, H. Wu, Z. Jiang, C. Tian, 'Facile one-pot preparation of chitosan/calcium pyrophosphate hybrid microflowers', *ACS Appl. Mater. Interfaces* **2014**, *6*, 14522–14532.
[30] Z. Zhang, Y. Zhang, R. Song, M. Wang, F. Yan, L. He, H. Zhang, 'Manganese (II) phosphate nanoflowers as electrochemical biosensors for the high-sensitivity detection of ractopamine', *Sens. Actuat. B Chem.* **2015**, *211*, 310–317.
[31] B. Somturk, I. Yilmaz, C. Altinkaynak, A. Karatepe, N. Özdemir, I. Ocsoy, 'Synthesis of urease hybrid nanoflowers and their enhanced catalytic properties', *Enzyme Microb. Technol.* **2016**, 86, 134–142.
[32] H. Kim, J. M. Jeong, S. J. Lee, B. G. Choi, K. G. Lee, 'Protein-directed assembly of cobalt phosphate hybrid nanoflowers', *J. Colloid Interface Sci.* **2016**, 484, 44–50.
[33] B. Zhang, P. Li, H. Zhang, H. Wang, X. Li, L. Tian, Q. Zhang, 'Preparation of lipase/Zn3 (PO4) 2 hybrid nanoflower and its catalytic performance as an immobilized enzyme', *Chem. Eng.* **2016**, *291*, 287–297.
[34] S. Park, B. S. Batule, M. Chung, K. S. Kang, T. J. Park, M. I. Kim, H. G. Park, 'A simple and eco-friendly one-pot synthesis of nuclease-resistant DNA-inorganic hybrid nanoflowers', *J. Mater. Chem. B.* **2017**, *5*, 2231–2234.
[35] C. Altinkaynak, E. Kocazorbaz, N. Özdemir, F. Zihnioglu, 'Egg white hybrid nanoflower (EW-hNF) with biomimetic polyphenol oxidase reactivity: synthesis, characterization and potential use in decolorization of synthetic dyes', *Int. J. Biol. Macromol.* **2018**, *109*, 205–211.
[36] C. Gulmez, C. Altinkaynak, N. Özdemir, O. Atakisi, 'Proteinase K hybrid nanoflowers (P-hNFs) as a novel nano-biocatalytic detergent additive', *Int. J. Biol. Macromol.*. **2018**, *119*, 803–810.
[37] D. Shcharbin, I. Halets-Bui, V. Abashkin, V. Dzmitruk, S. Loznikova, M. Odabaşı, M. Bryszewska, 'Hybrid metal-organic nanoflowers and their application in biotechnology and medicine', *Colloids Surf. B* **2019**, *182*, 110354.
[38] J. Cui, S. Jia, 'Organic-inorganic hybrid nanoflowers: A novel host platform for immobilizing biomolecules', *Coord. Chem. Rev.* **2017**, *352*, 249–263.
[39] C. Wang, R. Tan, Q. Wang, 'One-step synthesized flower-like materials used for sensitively detecting amyloid precursor protein', *Anal. Bioanal. Chem.* **2018**, *410*, 6901–6909.
[40] N. Ildiz, A. Baldemir, C. Altinkaynak, N. Özdemir, V. Yilmaz, I. Ocsoy, 'Self assembled snowball-like hybrid nanostructures comprising *Viburnum opulus* L. extract and metal ions for antimicrobial and catalytic applications', *Enzyme Microb. Technol.* **2017**, *102*, 60–66.
[41] C. Altinkaynak, N. Ildız, A. Baldemir, N. Özdemir, V. Yılmaz, I. Ocsoy, 'Synthesis of organic-inorganic hybrid nanoflowers using *Trigonella foenum-graecum* seed extract and investigation of their anti-microbial activity', *Derim* **2019**, *36*, 159–167.
[42] S. Ates, E. Cortenlioglu, E. Bayraktar, U. Mehmetoglu, 'Production of L–DOPA using Cu-alginate gel immobilized tyrosinase in a batch and packed bed reactor', *Enzyme Microb. Technol.* **2007**, *40*, 683–687.
[43] T. S. Chang, 'An updated review of tyrosinase inhibitors', *Int. J. Mol. Sci.* **2009**, *10*, 2440–2475.

[44] R. Subramanian, M. Z. Asmawi, A. Sadikun, '*In vitro* α-glucosidase and α-amylase enzyme inhibitory effects of *Andrographis paniculata* extract and andrographolide', *Acta Biochim. Pol.* **2008**, *55*, 391–398.

[45] M. Fadaeinasab, A. Hadi, Y. Kia, A. Basiri, V. Murugaiyah, 'Cholinesterase enzymes inhibitors from the leaves of *Rauvolfia reflexa* and their molecular docking study', *Molecules* **2013**, *18*, 3779–3788.

[46] S. H. Jeong, Y. B. Ryu, M. J. Curtis-Long, H. W. Ryu, Y. S. Baek, J. E. Kang, W. S. Lee, K. H. Park, 'Tyrosinase inhibitory polyphenols from roots of *Morus ihou*', *J. Agric. Food Chem.* **2009**, *57*, 1195–1203.

[47] D. Kumar, N. Gupta, R. Ghosh, R. H. Gaonkar, B. C. Pal, 'α-Glucosidase and α-amylase inhibitory constituent of *Carex baccans*: Bio-assay guided isolation and quantification by validated RP-HPLC-DAD', *J. Funct. Foods* **2013**, *5*, 211–218.

[48] L. Ellman, K. D. Courtney, V. Andres Jr, R. M. Featherstone, 'A new and rapid colorimetric determination of acetylcholinesterase activity', *Biochem. Pharmacol.* **1961**, *7*, 88–95.

[49] M. Öztürk, M. E. Duru, Ş. Kivrak, N. Mercan-Doğan, A. Türkoglu, M. A. Özler, '*In vitro* antioxidant, anticholinesterase and antimicrobial activity studies on three Agaricus species with fatty acid compositions and iron contents: A comparative study on the three most edible mushrooms', *Food Chem. Toxicol.* **2011**, *49*, 1353–1360.

[50] S. Zolghadri, A. Bahrami, M. T. Hassan Khan, J. Munoz-Munoz, F. Garcia-Molina, F. Garcia-Canovas, A. A. Saboury, 'A comprehensive review on tyrosinase inhibitors', *J. Enzyme Inhib. Med. Chem.* **2019**, *34*, 279–309.

[51] Atta-ur-Rahman, D. Shahwar, I. M. Choudhary, B. Sener, G. Toker, K. H. C. Baser, 'Alkaloids of *Bongardia chrysogonum*', *Chem. Inform.* **2010**, *30*, 0–0.

[52] Z. H. Israili, B. Lyoussi, 'Ethnopharmacology of the plants of genus *Ajuga*', *Pak. J. Pharm. Sci.* **2009**, *22*, 425–462.

[53] E. J. Llorent-Martínez, G. Zengin, P. Ortega-Barrales, M. Zakariyyah Aumeeruddy, M. Locatelli, A. Mollica, M. F. Mahomoodally, 'Characterization of the Phytochemical Profiles and Biological Activities of *Ajuga chamaepitys* subsp. *chia* var. *chia* and *Ajuga bombycina* by High-Performance Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry (HPLC-ESI-MSn)', *Anal. Lett.* **2019**, *52*, 852–868.

[54] G. Zengin, M. J. Rodrigues, H. H. Abdallah, L. Custodio, A. Stefanucci, M. Z. Aumeeruddy, M. F. Mahomoodally, 'Combination of phenolic profiles, pharmacological properties and in silico studies to provide new insights on *Silene salsuginea* from Turkey', *Comput. Biol. Chem.* **2018**, *77*, 178–186.

[55] G. D. Durbilmez, E. Bor, C. Dönmez, F. Geçili, N. Özdemir, U. Koca-Çalışkan, 'Synthesis of Hybrid Nanoflowers with Plant Extracts Traditionally Utilized In Skin Problems and Their Activity Profile', In I. International Aromatic Plants and Cosmetics Symposium-AROPCOS, **2019**, 41–45, IĞDIR-TURKEY.

[56] Q. Li, S. He, Y. Chen, F. Feng, W. Qu, H. Sun, 'Donepezil-based multi-functional cholinesterase inhibitors for treatment of Alzheimer's disease', *Eur. J. Med. Chem.* **2018**, *158*, 463–477.

[57] C. Kahraman, I. I Tatlı, I. E. Orhan, Z. S. Akdemir, 'Cholinesterase inhibitory and antioxidant properties of *Verbascum mucronatum* Lam. and its secondary metabolites', *Z. Naturforsch. C* **2010**, *65*(11–12), 667–674.

[58] E. Mario, S. Bolton, 'Inhibition of Acetylcholinesterase by Chelates III', *J. Pharm. Sci.* **1968**, *57*, 418–422.

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