Green formulation and characterization of Fe nanoparticles containing *Calendula* extract and investigation of the antioxidant, cytotoxic and anti-human cholangiocarcinoma properties

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Abstract

Introduction: Among the simplest nanostructures that are widely used in industry today are metallic nanoparticles. Metallic nanoparticles can bind non-destructively to single-stranded DNA, which is important in medical diagnostics.

Material and methods: In a recent study, the structural and morphological characterization of bio-synthesized FeNPs@*Calendula arvensis* was performed by FT-IR and UV-vis spectroscopy and scanning electron microscopy (SEM) in which SEM images exhibited an equal and uniform spherical morphology in size of 30.13 nm.

Results: In the antioxidant test, the IC_{50} values of FeNPs@*Calendula arvensis* and BHT against DPPH free radicals were 117 and 88 µg/ml, respectively. In the anticancer test, the cells treated with FeNPs@*Calendula arvensis* were assessed by MTT assay for 48 h as regards the anti-human cholangiocarcinoma and cytotoxic properties towards normal (HUVEC) and cholangiocarcinoma carcinoma cell lines, i.e. HCM-CSHL-0174-C22, CCLP-1, and QBC939. The IC_{50} values of FeNPs@*Calendula arvensis* were 196, 237, and 278 µg/ml against HCM-CSHL-0174-C22, CCLP-1, and QBC939 cell lines, respectively. The viability of the cholangiocarcinoma cell line decreased dose-dependently in the presence of FeNPs@*Calendula arvensis*.

Conclusions: It appears that the anti-human cholangiocarcinoma effect of FeNPs@*Calendula arvensis* is due to its antioxidant effects.

Key words: antioxidant, anti-cholangiocarcinoma, *Calendula arvensis*, green formulation, iron nanoparticles.

Introduction

Cancer cells uncontrollably divide to form masses of tissue, which are called tumors. Tumors can grow and interfere with the functions of many bodily systems including the digestive, nervous, and cardiovascular systems. Cancer has been reported to be the first in the rank of causes of death in the Thai population. Liver, colon, and lung cancers are the most prevalent cancers in Thai males, while breast, cervical, and

colon cancers are the most prevalent cancers in Thai females [1].

The modern treatments for cancers are mainly surgery, radiation, and chemotherapy. However, most chemotherapeutic drugs are not specific to only cancer cells, but also cause damage to normal cells, especially bone marrow, mucous glands, mucous membranes, hair, and nails and can lead to the suppression of the immune system [2, 3]. The success of chemotherapy depends on the number of cancer cells, the proliferation rate, the duration of the drug administration, and the therapeutic interval. To avoid drug resistance, polychemotherapy is always used instead of monochemotherapy [4, 5]. Anticancer drugs can also cause some other side effects including nausea, vomiting, agranulocytosis, inhibition of spermatogenesis and ovulation, alopecia, inflammation of mucous membranes, and teratogenesis [3–5]. Some compounds separated from natural products are now being developed as modern medicines for the treatments of cancers, including paclitaxel, *Catharanthus* alkaloids, and derivatives of podophyllotoxin [3, 5].

In recent years, nanotechnology-based therapeutic and diagnostic approaches have shown significant potential to ameliorate cancer therapy [6, 7]. Cancer nanotechnology developed a new area of integrative research in biology, chemistry, engineering, and medicine, and is concerned with major advances in cancer diagnosis, prevention and treatment [7]. In the past few years, nanoparticles (NPs) have become a subject of attraction for scientists due to their maximal efficacy and safety [7, 8]. Due to these applications, recently, the US FDA approved nanotechnology-based anticancer drugs such as, Myocet (Perrigo, Dublin, Ireland), DaunoXome (Gilead Sciences, Foster City, CA, USA), Doxil (Johnson & Johnson, New Brunswick, NJ, USA) and Abraxane (Celgene, Summit, NJ, USA) [7–9].

From all the approaches of NP synthesis, the green synthesis approach is considered the most economic, sustainable, reliable and eco-friendly [6]. This approach of NP synthesis does not require toxic chemicals, high temperature, or high pressure and does not cause harm to human health and the environment [6, 7]. At present, it is also considered a preferred method for NP fabrication because of utilization of low-cost and non-hazardous raw material such as microorganisms fungi, algae, bacteria, plant extracts, natural polymers and proteins [6–9]. These resources contain biomolecules such as proteins including enzymes, polysaccharides, sugars, amides, ketones, aldehydes, and carboxylic acids, but also more importantly various phytochemicals such as terpenes, alkaloids or polyphenols including flavonoids that aid in immediate reduction [6–8]. One of the most important cancers in recent years is cholangiocarcinoma. Many medicinal plants such as *Viola tricolor*, *Zingiber officinale*, *Urtica dioica* L, *Vinca rosea*, *Thymus vulgaris*, *Trigonella foenum-graecum* L, *Taverniera spartea* D, *Rhus coriaria* L, *Taxus baccata* L, *Silybum marianum*, *Thymbra spicata*, and *Polygonum aviculare* are used in traditional medicine to treat cancer [9]. It is predicted that if metal nanoparticles are synthesized and formulated with these plants, their anti-cancer effects against cholangiocarcinoma cells will be much stronger. In the current research, the properties of FeNPs@*Calendula arvensis* formulated by *Calendula arvensis* aqueous extract against common human cholangiocarcinoma cell lines were evaluated.

Material and methods

Preparation of *Calendula arvensis* leaf extract

Fresh *Calendula arvensis* leaf were washed several times with DI-H_2O. Then, 2.0 g of the plant was heated in 100 ml of DI-H_2O for 20 min. Next, the colored mixture was filtered with Whatman filter paper to obtain the aqueous extract. For further use, it was stored in a refrigerator at 4°C.

Green synthesis of FeNPs@*Calendula arvensis* using *Calendula arvensis* leaf extract

A 10 mL of aqueous extract solution (20 mg/ml) was added to 30 ml of $FeCl_3 \times 6\ H_2O$ in the concentration of 0.02 M (deionized water was used for the all steps of this section). The mixture was refluxed for 90 min at 50°C. The color changing from yellow to black indicated the formation of iron nanoparticles. The precipitate was washed three times with water and subsequently centrifuged at 12 000 rpm for 15 min. The obtained black powder was kept in a vial for the chemical characterization and evaluation of its biological activity.

Antioxidant activities of FeNPs@*Calendula arvensis*

The ability of hydrogen atoms or electrons to give off different compounds and nanoparticles in this test is measured by the degree of decolorization of the 2,2-diphenyl-1-picryl-hydrazyl purple solution in methanol.

The DPPH radical ($DPPH^{\bullet}$) is a stable molecule soluble in methanol characterized by its deep-violet color with an absorption maximum at 515 nm. Antioxidants (AH) or other radical species ($R^{\bullet}$) are able to react with this stable radical ($DPPH^{\bullet}$) by providing an electron or hydrogen atom, thus reducing it to 2,2-diphenyl-1-hydrazine (DPPH-H) or a substituted analogous hydrazine (DPPH-R) char-

acterized by colorless appearance or a pale-yellow color which can be easily monitored with a spectrophotometer. This assay is widely used to determine antioxidant activity of antioxidant molecules [10, 11].

In this method, DPPH (Sigma-Aldrich) was used as a stable radical compound. Thus, 100 µl of various dilutions of nanoparticles in methanol was added to 10 ml of 0.005% DPPH solution in methanol. After 1 h of incubation at the absorption room temperature, the samples were read against blank at 518 nm. The DPPH inhibition percentage was computed by the following formula [11]: Inhibition (%) = (Sample A/Control A) × 100.

Anti-human cholangiocarcinoma properties of FeNPs@*Calendula arvensis*

The human cholangiocarcinoma cell lines, i.e. HCM-CSHL-0174-C22, CCLP-1, and QBC939 and the normal cell line (HUVEC), were maintained in a DMEM medium with 10% bovine embryos and 1% penicillin/streptomycin antibiotic (to prevent fungal growth). Prerequisites for cell growth at 37°C are 5% CO_2 with 95% moisture, which was provided by the NÜVE incubator (EC160 model). For MTT assay, when the cells reached at least 70% cell growth, they were separated from the bottom of the flask by trypsin-ethylenediamine tetraacetic acid and centrifuged at 1700 rpm for 1 min. Cell precipitate was prepared in suspension in 1 ml of culture medium. The viability of cells in cell suspension was determined by mixing it with an equal proportion of trypan blue, and counting them with a neobar slide under a light microscope. After confirming that the cells were not infected, cells with a viability of more than 90% were used for testing [12]. To investigate the effect of nanoparticles on cancer cell proliferation, the tetrazolium (MTT) salt colorimetric method was used. For this test, 10^4 cells were added to each 96-well plate well. After 24 h of incubation, concentrations of 1–1000 µg/ml were treated on cancer and normal cells for 24, 48 and 72 h. After these times, 20 µl of MTT solution and 200 µl of base culture medium were added to each well. The plate was placed in a dark CO_2 incubator at 37°C for 4 h in the dark. After this time, 100 µl of DMSO was added to each well. 492 and 630 nm optical readings were placed in the ELISA reader (DANA model DA3200). The cell viability was computed by the following formula [12]: Cell viability (%) = (Sample A/Control A) × 100.

Statistical analysis

To compare the results, in addition to the formula mentioned above, which was calculated as an average of 5 repetitions of experiments, the results were analyzed using SPSS software version 22 and the statistical differences between the treatments were examined by *t*-test and $p < 0.05$ was considered significant.

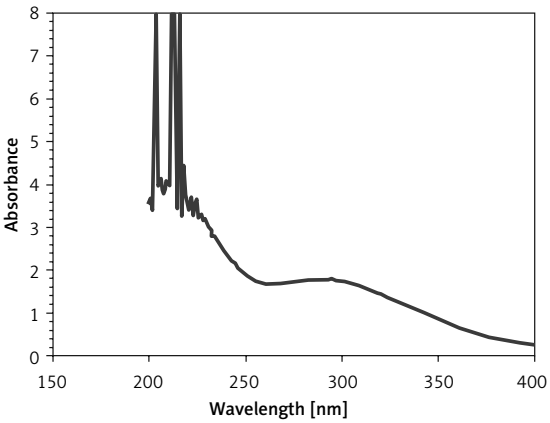

Figure 1. UV-Vis spectrum of biosynthesized FeNPs

Results and discussion

Chemical characterization of FeNPs

UV-visible spectroscopy analysis

UV-Vis spectroscopy, like FTIR, is a technique which is useful in the identification of pure drug compounds. Many molecules contain chromophores which will absorb specific wavelengths of ultraviolet or visible light [13].

Figure 1 presents the UV-Vis spectrum of biosynthesized FeNPs using *Calendula arvensis* extract. The result of UV-Vis. spectroscopy confirms the formation of FeNPs. The peak at 289 nm belong to the biosynthetic FeNPs. This observation is in a good agreement with the previous studies on biosynthesized FeNP nanoparticles [13].

FT-IR analysis

FTIR offers quantitative and qualitative analysis for organic and inorganic samples. Fourier transform infrared spectroscopy (FTIR) identifies chemical bonds in a molecule by producing an infrared absorption spectrum. The spectra produce a profile of the sample, a distinctive molecular fingerprint that can be used to screen and scan samples for many different components. FTIR is an effective analytical instrument for detecting functional groups and characterizing covalent bonding information [13].

Figure 2 presents the FT-IR spectrum of FeNPs. The peak at 582 cm^{-1} relates to bending vibration of Fe-O. These peaks have been previously reported for iron oxide nanoparticles with a small difference in the wavenumber [13]. FT-IR analysis is a suitable method to screen plant secondary metabolites as the capping and reducing agents of

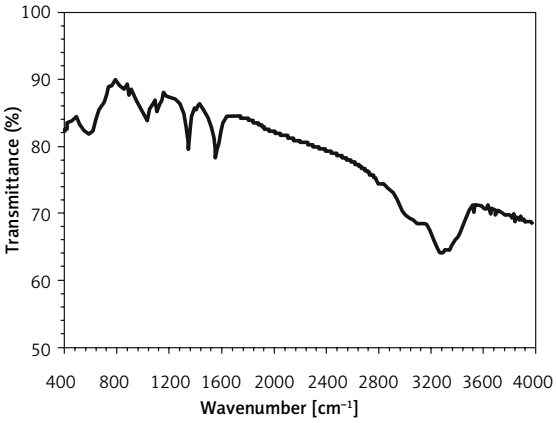

Figure 2. FT-IR spectrum of biosynthesized FeNPs

ferric chloride, precursor to FeNPs. The presence of different IR bands correlates with the presence of various functional groups in *Calendula arvensis* extract. For example, peaks at 3347 and 2974 cm^{-1} relate to O-H and aliphatic C-H stretching; the peaks in the range of 1341 to 1601 cm^{-1} correspond to C=C and C=O stretching, and the peak at 1028 cm^{-1} could be ascribed to -C-O stretching. These peaks can be considered for the presence of various compounds in the plant extract such as phenolic, flavonoid, saponins, quinones, and terpenoids, which have been reported previously [13]. In biosynthesis of metallic nanoparticles, the secondary metabolites of plant extracts, as reducing, stabilizing and dispersing agents, usually bind to NPs over their functional groups of hydroxyl and carbonyl [13].

SEM analysis

The scanning electron microscope (SEM) uses a focused beam of high-energy electrons to generate a variety of signals at the surface of solid specimens. The signals that derive from electron-sample interactions reveal information about the sample including external morphology (texture), chemical composition, and crystalline structure and orientation of materials making up the sample. In most applications, data are collected over a selected area of the surface of the sample, and a 2-dimensional image is generated that displays spatial variations in these properties. Areas ranging from approximately 1 cm to 5 microns in width can be imaged in a scanning mode using conventional SEM techniques (magnification ranging from 20× to approximately 30,000×, spatial resolution of 50 to 100 nm). The SEM is also capable of performing analyses of selected point locations on the sample; this approach is especially useful in qualitatively or semi-quantitatively determining chemical compositions (using EDS), crystalline structure, and crystal orientations (using Electron Backscatter Diffraction (EBSD)). The design and function of the SEM is very similar to the EPMA and considerable overlap in capabilities exists between the two instruments [13].

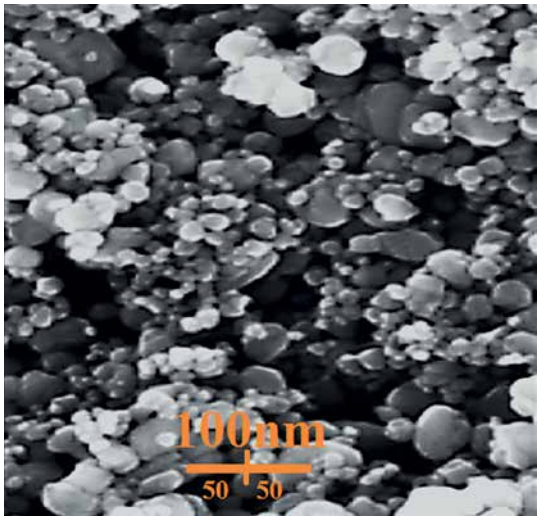

Figure 3. SEM image of FeNPs

An SEM image of FeNPs is shown in Figure 3. The image depicts the spherical morphology for the synthesized FeNPs, which has been reported previously [13]. The uniformity and homogeneity of the FeNPs is confirmed in the SEM images. The FeNPs show a tendency to aggregate; this property for metallic nanoparticles such as FeNPs, CdNPs, CuNPs, AgNPs, TiNPs, and NiNPs has been reported previously [13]. The average size of 30.13 nm was obtained for FeNPs. In our literature review, 10.7 to 96.34 nm was reported for iron oxide biosynthesized using plant extracts as the capping agent for NPs [13].

Cytotoxicity, anti-human cholangiocarcinoma, and antioxidant activities of FeNPs@*Calendula arvensis*

Cancers are caused by a series of mutations in human genes and each mutation causes some new changes in the cell. Chemicals called carcinogens cause cancer in cells. There are more than 100,000 types of chemicals in nature that directly or indirectly affect the cytoplasm and the nucleus of cells and lead to (genetic disorders that cause mutant cup heads) [14–17]. Various viruses, bacteria, and radiation, in turn, produce inherited cancers, which account for about 7% of all cancerous tissue: blood, lymph nodes, sarcoma, carcinoma, embryonic cells, and germ cells. Cancer is a disease that disrupts intercellular relationships and disrupts vital and key genes [17, 18]. These molecular irregularities affect the cell division cycle and lead to a lack of cell differentiation. Cancer can be treated in several ways: surgery, chemotherapy, radiation therapy, immunotherapy, gene therapy,

or a combination of these. Due to the relative inefficiency and very severe side effects of chemotherapeutic drugs, researchers and scientists have been looking for new formulations of various compounds, especially metallic nanoparticles [16–18].

In this investigation, the cells treated with different concentrations of the present FeNPs@*Calendula arvensis* were assessed by MTT assay for 48 h as regards the cytotoxic properties in normal (HUVEC) and malignant cell lines, i.e. HCM-CSHL-0174-C22, CCLP-1, and QBC939.

The absorbance rate was evaluated at 570 nm, which represented viability in the normal cell line (HUVEC) even up to 1000 µg/ml for FeNPs@*Calendula arvensis* (Table I, Figure 4).

In this study, we assessed the antioxidant properties of *Calendula arvensis* aqueous extract green-synthesized FeNPs@*Calendula arvensis* using the DPPH test as a common free radical. Antioxidants are compounds that eliminate the threat of free radicals (FRs) to cell life by preventing the production of FRs or converting them into less active forms. In inflammatory processes in the body, large amounts of superoxide anion radicals are produced by phagocytes. Macrophages and neutrophils produce superoxide and H_2O_2 radicals to defend

Table I. IC_{50} of FeNPs@*Calendula arvensis* in anti-human cholangiocarcinoma tests

Cells		FeNPs@*Calendula arvensis* [µg/ml]
Human cholangiocarcinoma cells	HCM-CSHL-0174-C22	196
	CCLP-1	237
	QBC939	278

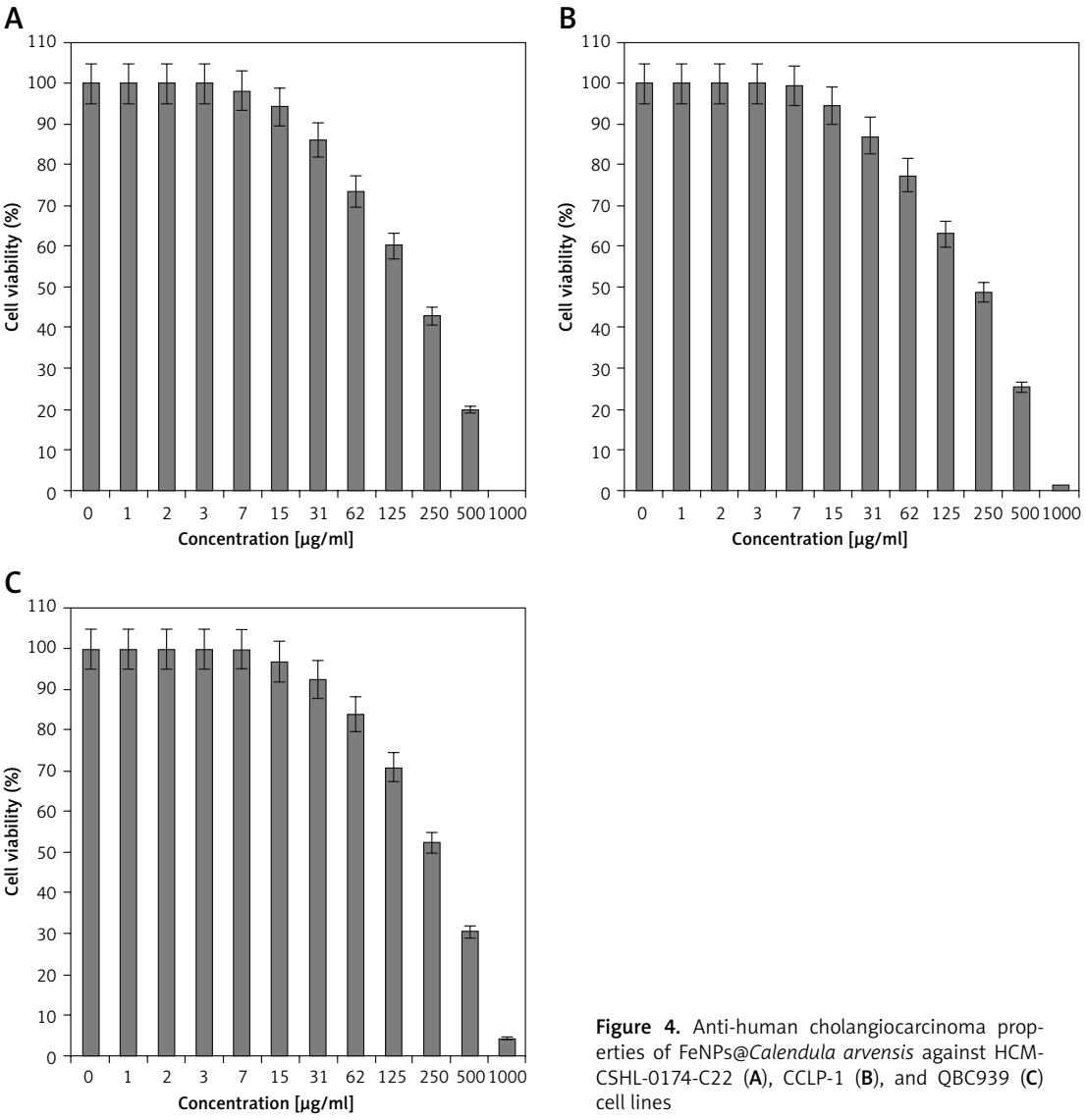

Figure 4. Anti-human cholangiocarcinoma properties of FeNPs@*Calendula arvensis* against HCM-CSHL-0174-C22 (**A**), CCLP-1 (**B**), and QBC939 (**C**) cell lines

Table II. IC_{50} of FeNPs@*Calendula arvensis* and BHT in antioxidant test

	FeNPs@*Calendula arvensis* [μg/ml]	BHT [μg/ml]
IC_{50} against DPPH	117	88

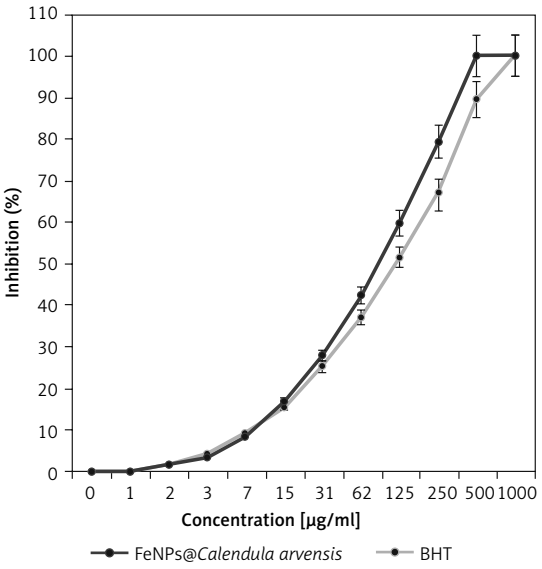

Figure 5. Antioxidant properties of FeNPs@*Calendula arvensis* and BHT against DPPH

against microorganisms [7, 8, 11]. In such cases, the presence of antioxidants is necessary to modify reactions in which FRs are produced and to prevent the harmful effects of reactive oxygen species and to prevent damage to immune cells. Antioxidants are used as anti-aging and anti-cancer agents and against cardiovascular, mitochondrial, Huntington's and nerve-destroying diseases such as Parkinson's. In addition, oral administration of some antioxidants is a supplement to increase energy and strengthen the immune system. Primary sources of natural antioxidants are legumes, fruits and vegetables, identified as dietary antioxidants and potentially reducing disease. Given that the synthetic antioxidants used, such as BHT, can be carcinogenic as well as hepatotoxic, over the last two decades, the tendency of consumers to use natural resources to produce antioxidants has increased and attracted a great deal of attention [8, 11].

The scavenging capacity of *Calendula arvensis* aqueous extract green-synthesized FeNPs@*Calendula arvensis* and BHT at different concentrations expressed as percentage inhibition is presented in Table II and Figure 5.

In conclusion, we have described the fabrication of FeNPs@*Calendula arvensis* based on a green method mediated by *Calendula arvensis* extract. After clinical study, FeNPs@*Calendula arvensis* containing *Calendula arvensis* leaf aqueous extract can be utilized as an efficient drug in the treatment of cholangiocarcinoma in humans. The FeNPs@*Calendula arvensis* showed the best antioxidant activities against DPPH. The IC_{50} values of FeNPs@*Calendula arvensis* and BHT against DPPH free radicals were 117 and 88 μg/ml, respectively. The viability of malignant cell line decreased dose-dependently in the presence of FeNPs@*Calendula arvensis*. The IC_{50} values of FeNPs@*Calendula arvensis* were 196, 237, and 278 μg/ml against HCM-CSHL-0174-C22, CCLP-1, and QBC939 cell lines, respectively.

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Jisen Zhao and Yang Yu are 1st author and co-1st author, respectively.

Conflict of interest

The authors declare no conflict of interest.

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