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

**Type**
Research paper

**Keywords**
Antioxidant, Anti-cholangiocarcinoma, Calendula arvensis, Green formulation, Iron nanoparticles

**Abstract**

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

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

**Results**
In the antioxidant test, the IC50 of FeNPs@Calendula arvensis and BHT against DPPH free radicals were 117 and 88 µg/mL, respectively. In the anticancer test, the treated cells with FeNPs@Calendula arvensis were assessed by MTT assay for 48h about the anti-human cholangiocarcinoma and cytotoxicity properties on normal (HUVEC) and cholangiocarcinoma carcinoma cell lines i.e., HCM-CSHL-0174-C22, CCLP-1, and QBC939. The IC50 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 cholangiocarcinoma cell line reduced 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 their antioxidant effects.
Green formulation and characterization of Fe nanoparticles containing *Calendula* extract and investigation of the antioxidant, cytotoxicity and anti-human cholangiocarcinoma properties

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**Abstract**

One of the simplest nanostructures that is widely used in industry today is metallic nanoparticles. Metallic nanoparticles can bind non-destructively to single-stranded DNA, which are important in medical diagnostics. In the recent study, the structural and morphological characterization of bio-synthesized FeNPs@*Calendula arvensis* was performed by FT-IR and UV-vis spectroscopy, scanning electron microscopy (SEM) that SEM images have exhibited an equal and uniform spherical morphology in size of 30.13 nm. In the antioxidant test, the IC50 of FeNPs@*Calendula arvensis* and BHT against DPPH free radicals were 117 and 88 µg/mL, respectively. In the anticancer test, the treated cells with FeNPs@*Calendula arvensis* were assessed by MTT assay for 48h about the anti-human cholangiocarcinoma and cytotoxicity properties on normal (HUVEC) and cholangiocarcinoma carcinoma cell lines i.e., HCM-CSHL-0174-C22, CCLP-1, and QBC939. The IC50 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 cholangiocarcinoma cell line reduced dose-dependently in the presence of FeNPs@*Calendula arvensis*. It appears that the anti-human cholangiocarcinoma effect of FeNPs@*Calendula arvensis* is due to their antioxidant effects.

**Keywords:** Antioxidant; Anti-cholangiocarcinoma; *Calendula arvensis*; Green formulation; Iron nanoparticles.
1. 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 the 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 mainly are surgery, radiation, and chemotherapy. However, most of 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 monochemotheapy [4,5]. The anticancer drugs can also cause some other side effects including nausea, vomiting, agranulocytosis, inhibition of spermatogenesis and ovulation, alopecia, inflammation of mucous membranes, and terratogenesis [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 the 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 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 has 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 NPs synthesis, green synthesis approach is considered the most economic, sustainable, reliable and eco-friendly [6]. This approach of NPs synthesis does not require toxic chemicals, high temperature, 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 NPs 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.

2. Experimental

2.1. Preparation of Calendula arvensis leaf extract

Fresh Calendula arvensis leaf were washed several times with DI-H2O. Then, 2.0 g of the plant was heated in 100 mL DI-H2O for 20 minutes. Next, the colored mixture was filtered by Whatmann filter paper to obtain the aqueous extract. For further use, it was stored in refrigerator at 4 °C.

2.2. The 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 FeCl3.6H2O in the concentration of 0.02 M (deionized water was used for the all steps of this section). The mixture was refluxed for 90 minutes at 50 °C. The color-changing from yellow to black indicated the formation of iron nanoparticles. The precipitate was triplet washed with water and centrifuged at 12000 rpm for 15 min subsequently. The obtained black powder was kept in a vial for the chemical characterization and evaluation of its biological activity.

2.3. 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 and 2-diphenyl-1-picryl-hydrazyl purple solution in methanol. The DPPH radical (DPPH•) 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•) are able to react with this stable radical (DPPH•) 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) characterized by colorless or pale-yellow color which could be easily monitored with a spectrophotometer. This assay is widely used to determine antioxidant activity of antioxidant molecules [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 hour 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]:

\[
\text{Inhibition (\%)} = \frac{\text{Sample A}}{\text{Control A}} \times 100
\]

2.4. Anti-human cholangiocarcinoma properties of FeNPs@Calendula arvensis

The human cholangiocarcinoma cell lines i.e. HCM-CSHL-0174-C22, CCLP-1, and QBC939 and 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% CO2 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-ethyldiamine tetraacetic acid and centrifuged at 1700 rpm for 6-1 minutes. 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, tetrazolium (MTT) salt colorimetric method was used. For this test, 10^4 cells were added to each 96-well plate well. After 24 hours of incubation, concentrations of 1-1000 μg/ml were treated on cancer and normal cells for 24, 48 and 72 hours. 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 CO2 incubator at 37 °C for 4 hours in the dark. After this time, 100 microliters 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]:

\[
\text{Cell viability (\%)} = \frac{\text{Sample A}}{\text{Control A}} \times 100
\]

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.

3. Results and discussion

3.1. 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 ultra violet or visible light [13].

Fig.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 to the previous studies on biosynthesized of FeNPs nanoparticles [13].

![](image)

Figure 1: UV–Vis. the spectrum of biosynthesized *FeNPs*

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].
Fig. 2 presents the FT-IR spectrum of FeNPs. The peak at 582 cm\(^{-1}\) relate 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 the plant secondary metabolites as the capping and reducing agents of ferric chloride precursor to FeNPs. The presences of different-IR bands correlate to the presences of various functional groups in *Calendula arvensis* extract. For example, peaks in 3347 and 2974 cm\(^{-1}\) related to O-H and aliphatic C-H stretching; the peaks at a range of 1341 to 1601 cm\(^{-1}\) correspond to C=C and C=O stretching, and 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, Terpenoids which have been reported previously [13]. In biosynthetic of metallic nanoparticles, the secondary metabolite of plant extracts, as reducing, stabilizing and dispersing agents usually bind to NPs over their functional groups of hydroxyl and carbonyl [13].

![FT-IR spectrum of biosynthesized FeNPs](image)

**Figure 2:** FT-IR spectra of biosynthesized *FeNPs*

**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 20X to approximately 30,000X, 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 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].

SEM image of FeNPs is shown in Fig 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 shows a tendency to aggregate, this property for metallic nanoparticles such as FeNPs, CdNPs, CuNPs, AgNPs, TiNPs, and NiNPs have 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 biosynthesized of iron oxide using plant extracts as the capping agent for NPs [13].

Figure 3. SEM Images of FeNPs

3.2. 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 cause cancer cells called carcinogens. 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 treated cells with different concentrations of the present FeNPs@*Calendula arvensis* were assessed by MTT assay for 48h about the cytotoxicity properties on normal (HUVEC) and malignancy cell lines i.e. HCM-CSHL-0174-C22, CCLP-1, and QBC939.

The absorbance rate was evaluated at 570 nm, which represented viability on normal cell line (HUVEC) even up to 1000μg/mL for FeNPs@*Calendula arvensis* (Table 1, Fig. 4).
The anti-human cholangiocarcinoma properties of FeNPs@Calendula arvensis against HCM-CSHL-0174-C22 (A), CCLP-1 (B), and QBC939 (C) cell lines.

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

**Table 1.** The IC50 of FeNPs@Calendula arvensis in the anti-human cholangiocarcinoma tests.

<table>
<thead>
<tr>
<th>Cells</th>
<th>FeNPs@Calendula arvensis (µg/mL)</th>
</tr>
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<tbody>
<tr>
<td>HCM-CSHL-0174-C22</td>
<td>196</td>
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In this study, we assessed the antioxidant properties of *Calendula arvensis* aqueous extract green-synthesized FeNPs@*Calendula arvensis* by using the DPPH test as a common free radical. Antioxidants are compounds that eliminate the threat of 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 H₂O₂ radicals to defend 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, anti-cancer, 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 reduce 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 has been indicated in Table 2, Fig. 5.
Fig. 5. The antioxidant properties of FeNPs@*Calendula arvensis* and BHT against DPPH.

<table>
<thead>
<tr>
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<th>FeNPs@<em>Calendula arvensis</em> (µg/mL)</th>
<th>BHT (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 against DPPH</td>
<td>117</td>
<td>88</td>
</tr>
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</table>

4. Conclusion

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* leaves 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 IC50 of FeNPs@*Calendula arvensis* and BHT against DPPH free radicals were 117 and 88 µg/mL, respectively. The
viability of malignant cell line reduced dose-dependently in the presence of FeNPs@*Calendula arvensis*. The IC50 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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**References**


