Calendula officinalis green-mediated silver nanoparticles: Formulation, characterization and assessment of colorectal cancer activities green-mediated silver nanoparticles: Formulation, characterization and assessment of colorectal cancer activities

Type
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Keywords
Green synthesis, Antioxidant, Silver nanoparticles, Cytotoxicity, Calendula officinalis, Anti-human colorectal cancer

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Introduction
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Material and methods
The synthesized AgNPs@Calendula officinalis were characterized by analytical techniques including EDX, FE-SEM, XRD, UV-Vis., and FT-IR. The anti-human colorectal cancer activities of AgNPs@Calendula officinalis were evaluated using MTT assay.

Results
The nanoparticles were formed in a spherical shape in the range of 38.05 to 75.41 nm for the particle size. On the other hand, the MTT assay was run to evaluate anti colorectal cancer activity of AgNPs@Calendula officinalis. In the cellular and molecular part of the recent study, the treated cells with AgNPs@Calendula officinalis were assessed by MTT assay for 48 h about the cytotoxicity and anti-human colorectal carcinoma properties on normal (HUVEC) and colorectal carcinoma cell lines i.e. WiDr, SW1417 [SW-1417], and DLD-1. In the antioxidant test, the IC50 of AgNPs@Calendula officinalis and BHT against DPPH free radicals were 222 and 124 µg/mL, respectively. The viability of malignant colorectal cell line reduced dose-dependently in the presence of AgNPs@Calendula officinalis. The IC50 of AgNPs@Calendula officinalis were 430, 326, and 392 µg/mL against WiDr, SW1417 [SW-1417], and DLD-1 cell lines, respectively.

Conclusions
After the clinical study, silver nanoparticles containing Calendula officinalis leaf aqueous extract may be used to formulate a new chemotherapeutic drug or supplement to treat the several types of human colorectal carcinoma.
**Calendula officinalis** green-mediated silver nanoparticles: Formulation, characterization and assessment of colorectal cancer activities

Abstract

The biosynthesis of metal nanoparticles using medicinal plants is not only economical but also environmentally friendly as well as having miscellaneous biomedical applications. In the present study, silver nanoparticles were green-synthesized using the aqueous extract of *Calendula officinalis*. The synthesized AgNPs@*C. officinalis* were characterized by analytical techniques including EDX, FE-SEM, XRD, UV-Vis., and FT-IR. The anti-human colorectal cancer activities of AgNPs@*C. officinalis* were evaluated using MTT assay. The nanoparticles were formed in a spherical shape in the range of 38.05 to 75.41 nm for the particle size. On the other hand, the MTT assay was run to evaluate anti colorectal cancer activity of AgNPs@*C. officinalis*. In the cellular and molecular part of the recent study, the treated cells with AgNPs@*C. officinalis* were assessed by MTT assay for 48 h about the cytotoxicity and anti-human colorectal carcinoma properties on normal (HUVEC) and colorectal carcinoma cell lines i.e. WiDr, SW1417 [SW-1417], and DLD-1. In the antioxidant test, the IC50 of AgNPs@*C. officinalis* and BHT against DPPH free radicals were 222 and 124 µg/mL, respectively. The viability of malignant colorectal cell line reduced dose-dependently in the presence of AgNPs@*C. officinalis*. The IC50 of AgNPs@*C. officinalis* were 430, 326, and 392 µg/mL against WiDr, SW1417 [SW-1417], and DLD-1 cell lines, respectively. After the clinical study, silver nanoparticles containing *C. officinalis* leaf aqueous extract may be used to formulate a new chemotherapeutic drug or supplement to treat the several types of human colorectal carcinoma.

**Keywords:** *Calendula officinalis*; Silver nanoparticles; Green synthesis; Antioxidant; Anti-human colorectal cancer; Cytotoxicity.

1. Introduction

*Calendula officinalis* is well known as a medicinal plant. The plant belongs to Asteraceae family [1]. So far, various usages have been reported for *C. officinalis* [2]. The plant is used for jaundice, blood purification. The plant is an effective agent for sunburn, burns, and dry dermatosis and is also known as an anti-inflammatory and wound healing drug [3,4]. *C. officinalis* has antifungal, hypoglycemic, anti-inflammatory, and hypolipidemic properties [2]. The extracts of *C. officinalis* are dominated by various classes of secondary metabolites. The plant
is rich in terpenoids, flavonoids, coumarins, saponins, phenolic acids, lipids, and glucosides. The presence of these compounds is the major reason for *C. officinalis* ability to cure different diseases [5-7].

The previous studies have been indicating when metallic nanoparticles are green-synthesized by ethnomedicinal plants rich in antioxidant molecules, their therapeutic properties such as anti-human cancer effects significantly increase. Many researchers use chemotherapy to treat several types of cancers [8-11]. Chemotherapeutic supplements make several side effects on the many organs, so today the effective chemotherapeutic drug formulation from nanoparticles is valuable [10-12]. 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. Nanoparticles also can pass through the vessel and position the target organ in the body, which is used in biomedicine, imaging and therapy [8-10]. Biomedical applications of nanoparticles include drug carriers, tracking or labeling materials, carriers for gene therapy, hyperthermia, and materials for magnetic resonance imaging. To use nanoparticles to deliver a drug molecule or DNA or a gene in gene therapy, chemical changes at the nanoparticle surface are always required for specific interactions with the desired biomolecule. Nanoparticles are used for imaging for medical purposes or *in vitro* and *in vivo* chemical processes. Metallic nanoparticles have received a lot of attention because of their antifungal, photocatalytic and UV absorbing properties [9,10]. Due to the antibacterial properties of these metal oxide nanoparticles, they can be used in the food industry and active food packaging. Also, metallic nanoparticles are potentially used in hyperthermia, magnetic resonance imaging (MRI), diagnosis and treatment of tumors or cancer, biomarkers, biodegradation, biotechnology and the removal of important organic, inorganic and radioactive contaminants due to their high biocompatibility [8-14]. Metallic nanoparticles have many applications in various fields such as fuel cells (hydrogen, methanol), glucose detection, drug delivery, toxicology, and biological interactions [10,11]. Metallic nanoparticles as a strong antioxidant resource are much less toxic than metals and also these nanoparticles have high power in scavenging free radicals (FR), so, it can be used as a natural antioxidant. Studies show that these nanoparticles detoxify hydroperoxidases and lipoxydeperoxidases at the cytoplasmic and mitochondrial matrix levels. Metallic nanoparticles such as copper, silver and titanium have very high antimicrobial properties that can be used in various industrial and biomedical sectors [9,11]. Nanoparticles can also be used as coatings on molecules to bind or interact with biological targets, to ensure the presence of these nanoparticles in the target part of the body, carriers are used to accurately deliver these nanoparticles, in which peptides have been introduced as one of the best carriers [12]. Metallic nanoparticles containing medicinal plants have very significant anti-cancer effects. In recent years, these metal nanoparticles containing herbs have been used to treat various cancers of the ovaries, prostate, esophagus, stomach, lungs, and various leukemias [8-12].

It is predicted that if metal nanoparticles are synthesized and formulated with the plants, their anti-cancer effects against colorectal cancer cells will be much stronger. In the current research, the properties of silver nanoparticles
formulated by *Calendula officinalis* leaf aqueous extract against common colorectal adenocarcinoma cell lines i.e. WiDr, SW1417 [SW-1417], and DLD-1 were evaluated.

2. Material and Methods

2.1 Materials

Phosphate buffer solution (PBS), Sabouraud Dextrose Agar, Sabouraud Dextrose Medium, Muller Hinton Agar, Mueller Hinton Medium, carbazole reagent, 4-(Dimethylamino)benzaldehyde, Dulbecco's Modified Eagle Medium (DMEM), Ehrlich solution, dimethyl sulfoxide (DMSO), hydrolysate, decamplmaneh fetal bovine serum, borax-sulphuric acid mixture, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and antimycotic antibiotic solution all were achieved from Sigma-Aldrich company of USA.

2.2. Preparation and extraction of aqueous extract

First, the dried leaves of *Calendula officinalis* were grounded. Then, 80 g of the sample was macerated in 500 mL of boiling water for 3 h. Next, the filtration and evaporation were applied to obtain the concerted extract. Finally, the extract was put in a freeze drier for 72 h to produce the powder extract of *Calendula officinalis*.

2.3. Green synthesis and chemical characterization of AgNPs@*C. officinalis*

A reported procedure (with some modifications) was used to green-synthesis of AgNPs@. *officinalis* [8]. First, 25 mL of the plant extract (0.2 g in 25 mL of water) was added to 50 mL of 0.1 M AgNO₃. Then, the mixture was stirred for 24 h at 30 °C. After the time, the silver nanoparticle was formed. The obtained AgNPs@*C. officinalis* was washed three times with water:ethanol and centrifuged at 10000 rpm for 15 min. Finally, the precipitate was dried at room temperature. The synthesized nanoparticles as a dark brown powder were kept in a vial for chemical characterization and biological activity evaluation.

2.4. Chemical Characterization techniques

Different factors of the nanoparticles like shape, particle size, fractal dimensions, crystallinity, and surface area are characterized by FT-IR spectroscopy, XRD, SEM, and EDS. In the present study, the FT-IR spectra of the synthetic nanoparticles were recorded by a Shimadzu FT-IR 8400 ranging from 400 to 4000 cm⁻¹ (KBr disc); The FE-SEM Images and EDS result were reported using MIRA3TESCAN-XMU. The AgNPs@*C. officinalis* XRD pattern was recorded in the 2θ which ranged from 20 to 80° by a GNR EXPLORER instrument at a 40 KV voltage, a current of 30 mA, and Cu-Kα radiation (1.5406 Å).

2.5. Antioxidant activities of AgNPs@*C. officinalis*

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.
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 [15]:

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

In this formula, “Control A” shows the negative control of light absorption that lacks nanoparticles, and “Sample A” expresses the amount of light absorption of different concentrations of nanoparticles [15].

### 2.6. Anti-human colorectal cancer properties of AgNPs@C. officinalis

In this research, the following cell lines were used to assess the anti-human colorectal carcinoma properties of silver nitrate, C. officinalis leaf aqueous extract and AgNPs@C. officinalis using an MTT method.

a) Human colorectal cancer cell lines
   - WiDr, SW1417 [SW-1417], and DLD-1
b) Normal cell line
   - HUVEC

These cells 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 [16]. To investigate the effect of nanoparticles on cancer cell proliferation, tetrazolium (MTT) salt colorimetric method was used. For this test, \(10^5\) 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 [16]:

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

3. Results and Discussion

3.1. Chemical characterization of AgNPs@C. officinalis

**XRD analysis:** The XRD diffraction patterns of AgNPs@C. officinalis evaluated its crystallinity. The pattern of the diffractogram is shown in Figure 1. The formation of nanoparticles was approved to this result. Despite the small size of AgNPs@C. officinalis, the pattern of XRD indicated well crystallizing. The achieved data were compared with the standard database of JCPD card 04–0783. The signals with 2θ values of 38.325, 44.915, 64.655, and 77.735 are indexed as (111), (200), (220), and (311) planes. A 43.49 nm was measured for the crystal size of AgNPs@C. officinalis that was calculated using X-ray diffraction and according to Scherer’s equation. Various crystalline sizes according to XRD analysis for the biosynthesized AgNPs using plants extracts. Instead of, 27.18 nm for *Salvia leriifolia* leaf extract [17]; 18 nm for Caesalpinia pulcherrima extract [18]; 45 nm for *phanthalus emblica* extract [19]; 50 nm for *Berberis vulgaris* extract [20]; and 5 nm has reported for Selaginella bryopteris extract [21].

![Figure 1. XRD Pattern of AgNPs@C. officinalis](image-url)
**SEM analysis:** The morphology of AgNPs@*C. officinalis* was assessed by the FE-SEM technique. Figure 2 presents the FE-SEM of AgNPs@*C. officinalis*. The images show the spherical shape for the nanoparticles with particle size in the range of 22.43 to 57.57 nm. Furthermore, the nanoparticles are aggregated. This is a general property of the green synthesized metallic nanoparticles, that was found in our literature review [17,22-24]. In our review of literature, the size of silver nanoparticles, which were synthesized using plant extract was in the range of 5 to 251.1 nm [9-21].

![SEM Images of ZnNPs@C. officinalis](image)

**Figure 2. SEM Images of ZnNPs@C. officinalis**

**EDS analysis:** The qualitative analysis of EDS was run to screen the elemental analysis of AgNPs@*C. officinalis*. The EDS diagram of AgNPs is shown in Figure 3. The findings approved the appearance of silver (by the peaks at 3.02 keV for AgLα and peak at 3.19 keV for AgLβ), oxygen (by the peak around 0.5 keV for OLα), and carbon (by the peak around 0.3 keV for CLα) in AgNPs@*C. officinalis*. The signal for silver has been reported by other research groups [21]. The presence of oxygen and carbon approved the linkage between silver nanoparticles and organic compounds of the plant extract.
UV-Vis. analysis: The UV–Vis. spectra of the green-synthetic nanoparticles of AgNPs@C. officinalis is presented in figure 4. The surface plasmon resonance (SPR) of AgNPs@C. officinalis was completed using UV–Vis. spectroscopy. The produce of the biosynthetic AgNPs@C. officinalis was observed. The advanced SPR bands at the wavelength of 452 nm approved the formation of the silver nanoparticles. The bands are very close to a previously reported on the green synthesized of silver nanoparticles using Fritillaria extract [25].
Figure 4. UV–Vis. spectrum of biosynthesized AgNPs@C. officinalis

**FT-IR analysis:** The FT-IR spectrum of silver nanoparticles is shown in Figure 5. The formation of AgNPs@C. officinalis is approved by the presence of the peaks at wavenumbers of 513, 582 and 640 cm\(^{-1}\). Similar peaks with some differences in the wavenumber have been reported for green-synthetic AgNPs by other research groups [21]. The other peaks in the spectrum are attributed to the functional groups of different organic compounds in C. officinalis extract, which are linked to the surface of AgNPs@C. officinalis. The presence of secondary metabolites such as phenolic, flavonoid, saponins, Quinones, Terpenoids in C. officinalis extract has been reported previously [2,4,7,26]. The peaks in 3423 and 2921 cm\(^{-1}\) are related to O-H and aliphatic C-H stretching; the peaks from 1550 to 1683 cm\(^{-1}\) are corresponded to C=C and C=O stretching, and the peaks at 1010 cm\(^{-1}\) could be ascribed to -C-O and C-O-C stretching.
3.2. Cytotoxicity, anti-human colorectal cancer, and antioxidant activities of AgNPs@Calendula officinalis

With the advancement of life sciences, measuring the rate of proliferation, survival and cell mortality under different conditions has become very important. In this regard, MTT analysis has greatly contributed to the study of biocompatibility of various materials by providing a highly safe non-radioactive colorimetric system. Cytotoxicity tests are tests that examine the side effects of various compounds on the cell. These processes take place in the environment outside the human body or the so-called extraterrestrial. Most of these processes also use cell culture. In MTT analysis according to ISO 10993-5 international standard, different equipments are tested for cytotoxicity, if they do not have toxic effects, they will obtain the necessary standards and licenses and enter the buying and selling market. The MTT set is the best-known test for cell viability. The main purpose of this test is to evaluate the toxicity of compounds, drugs or other supplements on the cell. Of course, it may also be mentioned in articles as a process for examining cell proliferation or counting [27-29]. MTT analysis can differentiate between living and dead cells by affecting intracellular organs. In this method, the cells, after being cultured in the laboratory, are "treated" with the desired substances to evaluate their toxicity. At the end of this
test, for each concentration of the substance, the cell viability is determined. Although this method is primarily for water-soluble solutions and compounds, it is currently used for other compounds soluble in organic solvents and nanoparticles. The behavior and rate of cell proliferation may increase or not change at all under the influence of hormones, growth factors, cytokines and mitogens. Also, some drugs and cytotoxic (toxic) substances, such as anticancer drugs, may cause necrosis or apoptosis (death) of cells or slow down the rate of proliferation and growth or even loss of cell structure [28-31]. Proper analysis of the MTT test can evaluate many of these behaviors. The MTT analysis basis is based on mitochondrial activity. This activity is usually stable in living cells. Hence, any change in several active and living cells is linked to mitochondrial property. This examination is a colorimetric way based on the breakdown and reduction of yellow tetrazolium crystals by the succinate dehydrogenase, and the formation of insoluble purple crystals performs the final analysis. Unlike other methods, MTT analysis eliminates the cells washing and shrinking steps, which usually causes the loss of cells part and increases the work error. That is, all the steps of the experiment, from the cell culture beginning to reading and analyzing the findings with a photometer, are done in a completely compact way and in a "micro plate". Hence the sensitivity, accuracy, and repeatability of the test is high [30-32].

In this study, the treated cells with different concentrations of the present silver nitrate, *C. officinalis* leaf aqueous extract, and AgNPs@*C. officinalis* were assessed by MTT assay for 48h about the cytotoxicity properties on normal (HUVEC) and colorectal malignancy cell lines i.e. WiDr, SW1417 [SW-1417], and DLD-1.

The viability of malignant colorectal cell line reduced dose-dependently in the presence of silver nitrate, *Calendula officinalis* leaf aqueous extract, and AgNPs@*C. officinalis*. The IC50 of AgNPs@*C. officinalis* were 430, 326, and 392 μg/mL against WiDr, SW1417 [SW-1417], and DLD-1 cell lines, respectively (Tables 1,2). The IC50 of *C. officinalis* leaf aqueous extract were 911, 673, and 713 μg/mL against WiDr, SW1417 [SW-1417], and DLD-1 cell lines, respectively (Tables 1,2).

The absorbance rate was evaluated at 570 nm, which represented viability on normal cell line (HUVEC) even up to 1000μg/mL for silver nitrate, *C. officinalis* leaf aqueous extract, and AgNPs@*C. officinalis* (Tables 1,2).

**Table 1.** The anti-colorectal cancer properties of AgNO3, AgNPs@*C. officinalis*, and *C. officinalis* leaf aqueous extract against human colorectal cancer cell lines.

| Concentration (μg/mL) | Cell Viability (%) |
|-----------------------|--------------------|
|                       | WiDr   | SW1417 [SW-1417] | DLD-1 | HUVEC |
| AgNO3 (0)             | 100±0<sup>a</sup> | 100±0<sup>a</sup> | 100±0<sup>a</sup> | 100±0<sup>a</sup> |
| AgNO3 (1)             | 100±0<sup>a</sup> | 100±0<sup>a</sup> | 100±0<sup>a</sup> | 100±0<sup>a</sup> |
| AgNO3 (2)             | 100±0<sup>a</sup> | 100±0<sup>a</sup> | 100±0<sup>a</sup> | 100±0<sup>a</sup> |
|                | 100±0    | 100±0    | 100±0    | 100±0    |
|----------------|----------|----------|----------|----------|
| AgNO3 (3)      |          |          |          |          |
| AgNO3 (7)      |          |          |          |          |
| AgNO3 (15)     |          |          |          |          |
| AgNO3 (31)     |          |          |          |          |
| AgNO3 (62)     | 98.4±1.34| 95.2±0.83| 100±0a   | 95±1a    |
| AgNO3 (125)    | 94.6±0.89| 88.4±1.34| 97.2±0.83| 91.6±0.89|
| AgNO3 (250)    | 89±1.22  | 79.4±0.54| 90±0.7a  | 85.6±0.89|
| AgNO3 (500)    | 81±1ab   | 66.4±0.54| 83.8±0.44| 77±1.22ab|
| AgNO3 (1000)   | 68.2±1.3ab| 53.6±0.89b| 70.4±1.34ab| 62.2±0.83ab|
| C. officinalis (0) | 100±0a   | 100±0a   | 100±0a   | 100±0a   |
| C. officinalis (1) | 100±0a   | 100±0a   | 100±0a   | 100±0a   |
| C. officinalis (2) | 100±0a   | 100±0a   | 100±0a   | 100±0a   |
| C. officinalis (3) | 100±0a   | 100±0a   | 100±0a   | 100±0a   |
| C. officinalis (7) | 100±0a   | 100±0a   | 100±0a   | 100±0a   |
| C. officinalis (15) | 100±0a  | 100±0a   | 100±0a   | 100±0a   |
| C. officinalis (31) | 100±0a   | 100±0a   | 100±0a   | 100±0a   |
| C. officinalis (62) | 97.6±0.89a| 96.4±1.34a| 97.4±0.89a| 100±0a   |
| C. officinalis (125) | 92.4±0.54a| 90±1a    | 92±1.22a  | 99.2±0.44a|
| C. officinalis (250) | 80.6±0.89ab| 74±0.7ab | 79.2±1.3ab | 98±0.7a  |
| C. officinalis (500) | 64±1ab   | 58.4±0.89b| 60.6±0.89bc| 96±1a   |
| C. officinalis (1000) | 47±1.22b | 34.2±1.3bc| 35.8±0.44bc | 91.8±0.44a|
| AgNPs® C. officinalis (0) | 100±0a   | 100±0a   | 100±0a   | 100±0a   |
| AgNPs® C. officinalis (1) | 100±0a   | 100±0a   | 100±0a   | 100±0a   |
| AgNPs® C. officinalis (2) | 100±0a   | 100±0a   | 100±0a   | 100±0a   |
| AgNPs® C. officinalis (3) | 100±0a   | 100±0a   | 100±0a   | 100±0a   |
| AgNPs® C. officinalis (7) | 100±0a   | 100±0a   | 100±0a   | 100±0a   |
| AgNPs® C. officinalis (15) | 99.6±0.89a| 98±0.7a  | 100±0a   | 100±0a   |
| AgNPs® C. officinalis (31) | 95.4±0.54a| 92.2±0.44a| 97±1a    | 99.8±0.44a|
| AgNPs® C. officinalis (62) | 88.4±0.54a| 84.4±1.34a| 90.4±1.34a| 98.6±0.89a|
| AgNPs® C. officinalis (125) | 77.6±0.89ab| 70±1.22ab| 77.2±1.3ab | 95±0.7a  |
| AgNPs® C. officinalis (250) | 61.4±1.34b| 54.8±0.44b| 60±1b    | 90.4±0.89a|
| AgNPs® C. officinalis (500) | 45.6±0.89b| 39.2±0.44bc| 42.4±1.34bc | 84±1.22a |
| AgNPs® C. officinalis (1000) | 22±1c    | 11.2±1.3c | 14.4±0.54c | 77±1ab   |

The several words present significant differences between experimented groups ($P≤0.01$).
Table 2. The IC50 of AgNO3, AgNPs@C. officinalis, and C. officinalis leaf aqueous extract in cytotoxicity and anti-colorectal cancer tests.

|                          | AgNO3 (µg/mL) | AgNPs@C. officinalis (µg/mL) | C. officinalis (µg/mL) |
|--------------------------|---------------|-------------------------------|------------------------|
| IC50 against WiDr        | -             | 430±0c                        | 911±0a                 |
| IC50 against SW1417 [SW-1417] | -             | 326±0d                        | 673±0b                 |
| IC50 against DLD-1       | -             | 392±0cd                       | 713±0b                 |
| IC50 against HUVEC       | -             | -                             | -                      |

The several words present significant differences between experimented groups (P≤0.01).

In this study, we determined the AgNPs@C. officinalis antioxidant properties by the free radical (DPPH) test. Free radicals (FRs) are unstable molecules or atoms that have an unpaired electron. FRs are formed by breaking a bond of a stable molecule. This property increases their chemical reactions. The main important FR in humans is O2. Oxygen molecules are exposed to various radiations, stress, and smoke from smoking, etc. By taking an electron from other molecules, it destroys other molecules, cells and DNA. Wherever FRs are mentioned, antioxidants are the main way to fight them and regenerate damaged cells [9-11]. Because antioxidants destroy FRs and increase the body's immunity against a variety of diseases. 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 [8,9]. 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 [11,15,33,34].

The scavenging capacity of C. officinalis leaf aqueous extract green-synthesized AgNPs@C. officinalis and BHT at different concentrations expressed as percentage inhibition has been indicated in Tables 3,4.
In the antioxidant test, the IC50 of *C. officinalis* leaf aqueous extract, AgNPs@*C. officinalis*, and BHT against DPPH free radicals were 447, 222, and 124 µg/mL, respectively (Tables 3,4).

**Table 3.** The antioxidant activities of AgNO₃, AgNPs@*C. officinalis*, *C. officinalis* leaf aqueous extract, and butylated hydroxyl toluene against DPPH.

| Concentration (µg/mL) | DPPH inhibition (%) |
|-----------------------|---------------------|
| **AgNO₃ (0)**         | 0±0a                |
| **AgNO₃ (1)**         | 0±0a                |
| **AgNO₃ (2)**         | 0±0a                |
| **AgNO₃ (3)**         | 1.2±0.83a           |
| **AgNO₃ (7)**         | 2±1a                |
| **AgNO₃ (15)**        | 4.2±0.83a           |
| **AgNO₃ (31)**        | 8.2±0.83a           |
| **AgNO₃ (62)**        | 12.2±1.3a           |
| **AgNO₃ (125)**       | 18.2±0.83a          |
| **AgNO₃ (250)**       | 25±1ab              |
| **AgNO₃ (500)**       | 35±1.22ab           |
| **AgNO₃ (1000)**      | 48.8±0.44b          |
| **AgNPs@*C. officinalis* (0)** | 0±0a                |
| **AgNPs@*C. officinalis* (1)** | 0±0a                |
| **AgNPs@*C. officinalis* (2)** | 2.4±0.89a           |
| **AgNPs@*C. officinalis* (3)** | 4.2±0.44a           |
| **AgNPs@*C. officinalis* (7)** | 7.4±1.34a           |
| **AgNPs@*C. officinalis* (15)** | 12.4±1.34a          |
| **AgNPs@*C. officinalis* (31)** | 18±1a               |
| **AgNPs@*C. officinalis* (62)** | 27±1ab              |
| **AgNPs@*C. officinalis* (125)** | 39.2±0.83ab         |
| **AgNPs@*C. officinalis* (250)** | 53.2±1.3b           |
| **AgNPs@*C. officinalis* (500)** | 70.2±0.83bc         |
| **AgNPs@*C. officinalis* (1000)** | 100±0c              |
| ***C. officinalis* (0)**  | 0±0a                |
| ***C. officinalis* (1)** | 0±0a                |
| ***C. officinalis* (2)** | 1.2±0.83a           |
The several words present significant differences between experimented groups ($P \leq 0.01$).

### Table 4. The IC50 of AgNO3, AgNPs@C. officinalis, C. officinalis leaf aqueous extract, and butylated hydroxyl toluene in the antioxidant test.

| Concentration (µg/mL) | AgNO3 | AgNPs@C. officinalis | C. officinalis | BHT |
|-----------------------|-------|---------------------|---------------|-----|
| IC50 (µg/mL)          |       | 222±0b              | 447±0a        | 124±0c |

The several words present significant differences between experimented groups ($P \leq 0.01$).

### 4. Conclusion
In conclusion, we like to introduce a green method to synthesis silver nanoparticles for the first time using the extract of *C. officinalis*. The structural features of nanoparticles were evaluated through various analytical techniques such as FT-IR, XRD, FE-SEM, and EDS analyses. The techniques approved that AgNPs@*C. officinalis* had been synthesized in the best possible condition. The viability of malignant colorectal cell line reduced dose-dependently in the presence of AgNPs@*C. officinalis*. The IC50 of AgNPs@*C. officinalis* were 430, 326, and 392 µg/mL against WiDr, SW1417 [SW-1417], and DLD-1 cell lines, respectively. The AgNPs@*C. officinalis* showed the best antioxidant activities against DPPH. The IC50 of AgNPs@*C. officinalis* and BHT against DPPH free radicals were 222 and 124 µg/mL, respectively. After the clinical study, AgNPs@*C. officinalis* containing *C. officinalis* leaf aqueous extract can be utilized as an efficient drug to treat the colorectal cancer in humans.

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