Biosynthesis of Silver Nanoparticles Using *Calendula officinalis* (L.) Extract and Evaluating their Antioxidant Activity

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**Abstract**

The study was conducted in the Faculty of Sciences / University of Kufa from 2/2/2018 to 1/5/2019. Biosynthesis of silver nanoparticles using aqueous, flower extracts of *Calendula officinalis* (L.) was firstly indicated by the color change of reaction mixture from yellow into reddish brown. Concentration 10mM of AgNO₃ and time of incubation 24 hours was done. The characterization of biosynthesized nanoparticles was achieved using UV-Spectrophotometer is a proven technique for the analysis of the nanoparticles. In Scanning electron microscope (SEM) (The shape was spherical and homogenous and the size was ranged between (63-100nm) of silver nanoparticles synthesized by plants *C. officinalis*. X-ray diffraction (the determination of average size of biogenic silver nanoparticles for *C. officinalis* was (16.73)nm, while for the commercial AgNO₃ was (63)nm. FTIR spectroscopy analysis showed the presence of flavonoids, polyphenols and amide groups likely to be responsible for the green synthesis of silver nanoparticle. Antioxidant activity of silver nanoparticle of extract were investigated at 330, 230 and 55 µg/ml that have efficient to reduction absorbance of free radicals. The largest inhibition titer was found in the mixture of DPPH with AgNPs biosynthesis from *C. officinalis* at the concentration 1.5mg/ml(91%) ; while the smallest inhibition titer was found in the mixture of DPPH with AgNPs biosynthesis from *C. officinalis* at the concentration 0.5mg/ml(65%)

1. **Introduction**

For a long time medicinal plants were considered an important source of medicinal value. There are 25% of drugs are derived from plants as a natural sources. They are economically important to human due to their multiple applications, such as Pharmaceuticals, flavors, fragrance, insecticides, dyes, herbicides, food additives, and toxins [1]. The wild populations, belong to the typical subspecies *C. officinalis* flowers, are well established as protection effect against the development of human diseases such as, diabetes and cancer [2]. Plant extracts contain phenolics, terpenoids, polysaccharides and flavones compound that contribute in reduction and stabilizing of silver nanoparticles [3]. *C. officinalis* comprehending few phytochemicals sterols, Carotenoids, terpenoids, flavonoids, α-thujene, γ-terpenene, 1,8-cinol [4], can be used for biotic actions approximating antimicrobial, antioxidant, immunostimulant, spasmogenic and spasmolytic, hepatoprotective, anti-inflammatory. The Nanotechnology provides the ability to engineer the properties of materials by controlling their size, and this has been driven research towards a multitude of potential uses for nanomaterial. Nanotechnology has been spread to number of areas including biomedical services, cosmetics, drug gene delivery, environmental health, food, health care, catalysis, mechanics [5]. The aims of study :- Synthesize of silver nanoparticles by aqueous fruitextracts of *C. officinalis* and Study characterization of AgNPs using (SEM), (AFM)and (EDS). Also, Study the Antioxidant activity.

2. **Materials and methods**

2.1. **Preparation of plant extracts**

Twenty five grams of the fruit *C. officinalis* flowers were cut into small pieces and were placed in 250 ml Erlenmeyer flask containing sterile distilled water and incubated in a shaking incubator at 37°C for 24h and 200 rpm., The extract was filtered using Whatman filter paper No.1 to obtain the aqueous plant extracts [6].

2.2. **Preparation of Silver Nitrate Solution**

The silver nitrate (AgNO₃) was purchased from Hi-Media Laboratories Pvt. Ltd. was used in the present study. A concentration of 10mM AgNO₃ solution was prepared by dissolving 0.169g AgNO₃ in 100 ml double distilled water and used for the green synthesis of silver nanoparticles (AgNPs).
2.3. Synthesis of AgNPs of extracts

The above filtered aqueous extracts of 25 ml was added individually to 100 ml of 0.01M of AgNO3 in a 250 ml Erlenmeyer flask for 10-15 min and was stirred at 150 rpm at 30C using magnetic stirrer. The process continued till a change of colour occurred from yellowish to dark brown due to the surface Plasmon resonance phenomenon give a peak at 450-550 nm in UV-Visible spectrophotometer indicating the completion process of AgNPs synthesis. Then, the extract was centrifuged at 6000 rpm for 15 min and the resultant pellet was kept in hot air oven overnight at 60C to make a fine powder of nanoparticles [7].

2.4. Characterization of Silver Nanoparticles

The initial characterizations of silver nanoparticles were monitored by crude flowers of C.officinalis aqueous extract were utilized as the blank. Centrifuged at 12000 rpm for 30 minutes and wash three times with de-ionized water. The pellet of silver nanoparticles dried in oven at 40 ºC, grinding and kept in dark ambient. X-ray diffraction, UV-Visible spectrophotometric is proven technique for the synthesis the nanoparticles. Scanning electron microscope(SEM)was used for characterization the morphology and size of nanoparticles in electron microscope unit. The microscope was operated at an accelerated voltage at 5-10 KV and different magnification, low vacuum, a spot size 4 and working distances5-10mm [7]. The surface morphology of sample was observed by Atomic Force Microscope (AFM) model.

2.5. DPPH solution (0.1mM)

The solution of 1,1-diphenyl-2-picrylhydrazyl was prepared by dissolving 0.00394 g in 100ml ethanol (99.8%). The solution was used for detection of antioxisant activity of C.officinalis silver nanoparticles [8].

3. Results and Discussion

3.1. Qualitative phytochemicals Analysis

In the estimation of some bioactive components of C.officinalis, Phytochemical analysis using some reagents as in the results shown in table (1) indicate that all reagents applied gave a positive results for all constituents analyzed,. This result is agreement with description of [9]. The results shown in table (1) indicated the presence of Essential oil , flavonoids ,Terpenoids, and Tannins.

| Plant         | Phytochemical Constituents | Eaqueous Extract Color |
|---------------|---------------------------|------------------------|
| C.officinalis | Essential oil ++          | Blue                   |
|               | Flavonoids ++             | Yellow                 |
|               | Glycosides ++             | Brown Ring             |
|               | Terpenoids ++             | Reddish Brown          |

Whereas :- Strongly Present (++), Present (+) , Absent (-)

The flowers extracts contain important compounds are free radical scavenger and strong water soluble, that are necessary in antioxidant and chelating properties. The antioxidant activity of flavonoids depends on the frame and substitution type of hydroxyl groups [10,11]. All these phytochemicals possess better antioxidant activities.

3.2. Biosynthesis of Silver Nanoparticles Using Extracts Flowers of C.officinalis

The efficient concentration C.officinalis, was (10mM) and the efficient time of incubation for production was 24 hours. When the reaction mixture containing the medical plant extracts and the different concentration of AgNO3 were added under dark conditions, the medium color converted from yellow to reddish brown. The appearance of color is a clear indication to formation of silver nanoparticles in the reaction mixture due to reduction of Ag+ ions to Ag metal by the reducing agents (amino acid, polysaccharides, proteins, enzymes etc. ) in the medical plants extracts which were harmless environmentally and complex chemically [12]. It has been newly established that crude extracts have been explored as potential bio-factories for synthesis of metallic NPs [13]. The aqueous fruit extracts of C.officinalis showed the ability for biosynthesis of silver nanoparticles after adding silver nitrate (AgNO3) in a concentration 10mM to extracts, Figure (1 A,B) . Previous studies believed that protein molecules and enzyme, including nitrate reductase, act as good regulating agents for the biosynthesis of AgNPs using the extracts types [14,15]. The morphology and particle size are dependent on several chemical and physical parameters, e.g., incubation time, pH, composition of the culture medium, and growth in light or dark conditions [16,17]. The conversion of extracellular medium color clearly designates that the process of formation of AgNPs is extracellular in nature [17].
3.3. Characterization of Silver Nanoparticles of plant extracts

3.3.1. UV-Visible Spectrophotometric Analysis

UV-visible spectrophotometric analysis is a proven technique for the analysis of nanoparticles. The biosynthesis of nanoparticles can be confirmed by visual observation and measuring the surface plasmon resonance (SPR) band using the absorption spectrum of nanoparticles formed in the reaction mixture of *C. officinalis* extracts has an absorption peak at 410 nm for AgNPs (Figure 2).

This is the evidence of the presence of surface plasmon resonance (SPR) of nanoparticles and a single SPR band, indicating that nanoparticles have a spherical shape. Color change into brown in the reaction due to plasmon resonance peak was observed for the silver nanoparticles maximum located between 400-550 nm. The appearance of a strong band in the spectral pattern is due to the excitation of the localized surface plasmons which cause strong light scattering by an electric field at a wavelength where resonance occurs, as mentioned by [18]. The plasmon resonance band shows the sharp absorbance and indicates little aggregation of the particles in solution. The absorption of brown color due to the excitation of surface plasmon vibration in particles, surface plasmon absorption strongly depends on the particle size, shape dielectric medium and chemical surrounding the UV-Vis absorption spectra of nanoparticles dispersed in water [19].

3.3.2. SEM Analysis

According to the results of the characterization of nanoparticles by SEM, difference was observed in the ability of each medical plants for the biosynthesis of silver nanoparticles. The AgNPs were synthesized by *C. officinalis* had the size (63-100nm) with spherical shape. The concentration of AgNO3 which was added to the extracts also had an effect on the characterization of nanoparticles, the most of them present in spherical form Figures(3). The best concentration in the biosynthesis of AgNPs was 10mM in comparison with other concentrations with variable shapes, most of them present in spherical form Figures(4). The previous studies on the biosynthesized silver nanoparticle from produced nanoparticle showed that the size (30-100 nm) related with [20,21].
3.3.3. X-ray Diffraction analysis

XRD analysis of synthesized AgNPs by *C. officinalis* extracts were done to find the crystalline nature of silver nanoparticles. XRD spectra showed a distinct variation between the silver nanoparticles synthesis from *C. officinalis* in Table (2). Figure (4) showed X-ray of AgNPs depending on size detection of nanoparticale by XRD. The average size of biogenic silver nanoparticale was determined by X-ray for *C. officinalis* was (39.235 nm) where the nanoparticale size of commercial silver was 55.378 nm. The above results confirmed the crystalline nature of AgNPs synthesized in this study. The AgNPs formed from *C. officinalis* was 45.692 nm. The other result showed an average diameter of AgNPs from 20-100 nm [22].

![Figure 3. SEM Micrograph of silver nanoparticle synthesis by *C. officinalis* extracts.](image)

![Figure 4. X-ray diffraction showing peak for silver nanoparticles synthesized by aqueous flower extract of *C. officinalis*.](image)

3.3.4. EDS Analysis

The occurrence of silver was quantified by Energy Dispersive-x-ray Spectroscopy (EDS) analysis through observing the optical absorption peaks of silver elements. The energy dispersive spectroscopy analysis detected the presence of elemental silver which indicated the reduction of silver ions to silver metals in the reaction mixture. The weight percentage of AgNPs formed by *C. officinalis* (76.93%) and 23.07% oxygen Figure (5). The optical absorption peak was observed at 3keV which is a typical absorption of metallic AgNPs as shown in Figure (6). The work by [23,24], reported that producing AgNPs with the weight percentage 67.35% similar with the presence of elemental silver indicating the reduction of silver ions in the reaction of the two types of extracts into silver metals. The EDX spectrum was recorded in the spot-profile mode. Strong signals from the Ag atoms are observed while medium signals were from oxygen and weaker signals were from other atoms.
3.3.5. Atomic Force Microscope (AFM)

Atomic force microscope analysis detected the three-dimensional shape of silver nanoparticle and the average diameter of the nanoparticles. The current density and time can be used to control the size and shape of the final structure. The average diameter of silver nanoparticle biosynthesized from *C. officinalis* 70 nm as shown in Figure (7) indicating three-dimensional images and granularity accumulation distribution charts of the silver nanoparticle. This result may be attributed to the differences in the bio-reduction that may be return to the qualitative and quantitative of extracellular protein/enzyme and other biomolecules that presented in the plant extracts, in addition to their ability of interaction with AgNO₃ [25]. The figures (7-b) showed three-dimensional image of silver nanoparticles revealed a population of homogeneous particles with a regular surface. Table (2) show silver nanoparticles the size of particles obtained ranged from 65.00-80.00 nm, in aqueous fruit extract of *C. officinalis*.

![Figure 6. EDS mapping of *C. officinalis* showing distribution of AgNPs. Red represent Ag, green represents oxygen.](image)

![Figure 7. Two-dimensional image (a), three-dimensional image (b), details (c) for silver nanoparticles was synthesized by aqueous extract of *C. officinalis*.](image)
3.3.6. Antioxidant Activity of Biogenic AgNPs Nanoparticles

Different free radical kinds of various reactivity are formed through a lipid oxidation (OH-, O₂-, L., LOO., LO, etc.), using stable organic radical 1-Diphenyl-2-picrylhydrazyl (DPPH) in the determination of the antioxidant activity of compounds as well as the various plant extract [25]. DPPH method is accurate, easy, and reproducible and it is the best to other free radical scavenging systems [26]. This method is based on the reduction of alcoholic DPPH solutions in the presence of a hydrogen donating anti-oxidant and is a stable nitrogen-centered free radical the color of which changes from violet to yellow upon reduction by either the process of hydrogen or electron-donation. Substances which are able to perform this reaction can be considered antioxidants and, therefore, radical scavengers [27-29]. (DPPH) free radicals scavenging assay were used to detect the antioxidant ability of the AgNPs biosynthesized from *C. officinalis* in vitro by reducing DPPH free radicals which will be evaluated according to the method of [30]. After adding the nanoparticles of *C. officinalis* to (0.1m) DPPH solution, the absorbance (A) was measured at 517 nm after 30 minutes in dark conditions. The results revealed the ability of nanoparticles to scavenging DPPH free radicals indicated by observing the color change from the original color of DPPH purple into yellow color as in Figure (8). These results demonstrated the antioxidant activity of biosynthesized AgNPs from *C. officinalis* in vitro leading evaluating the competency of nanoparticles for antioxidant activity in vitro. The largest inhibition titer was found in the mixture of DPPH with AgNPs biosynthesized from *C. officinalis* at the concentration 1mg/ml (95%); while the smallest inhibition titer was found in the mixture of DPPH with AgNPs biosynthesized from *C. officinalis* at the concentration 0.5mg/ml (65%).

![Figure 8. Antioxidant of Silver nanoparticles synthesis by aqueous extract fruit of *C. officinalis*.](image)

[31], suggested the peroxidation of lipids with free radicals, to form lipid peroxides, which then decompose to form many products inclusive malondialdehyde (MDA). MDA was produced when highly reactive oxygen metabolites, particularly hydroxyl radicals, act on unsaturated fatty acids of phospholipids components of membranes [32]. The results in figure (8) show that AgNPs extracts of *C. officinalis* give more values of MDA in comparison depending on the secondary chemicals content.

| Concentration | Inhibition of DPPH % | Concentration Equivalents for Ascorbic Acid ± S.D |
|---------------|----------------------|-----------------------------------------------|
| AgNPs of *C. officinalis* extracts | | |
| 0.5mg/ml | 66% | 172.00 ± 0.0112 |
| 1mg/ml | 86% | 161.75 ± 0.0331 |
| 1.5mg/ml | 91.00% | 181.50 ± 0.0510 |

Values were Expressed as Mean ± S.D for 3 Different Concentrations

The Studies of [33,34], suggested that AgNPs extract using *C. officinalis* can release more lipid compounds because the phytochemicals (anthocyanins and carbohydrates) and essential oil substances react with (DPPH) to form a pink pigment.
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