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# Magnetite Nanoparticles Different Sizes Effectiveness On Growth And Secondary Metabolites in *Ginkgo Biloba* L. Callus

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**Abstract**

Nanotechnology is one of the most important recent trends to improve the production of secondary metabolites in tissue culture for wide commercial use. Therefore, in this study, the effects of magnetite nanoparticles (MNPs) of different sizes (10.77, 20.5, and 29.3 nm) and different concentrations (0.5, 1, and 2 ppm) on the growth and production of active constituents in *Ginkgo biloba* L callus were investigated. The best medium for callus growth and proliferation of *Ginkgo biloba* L was MS medium supplemented with 1 ppm naphthalene acetic acid (NAA), 1 ppm kinetin, and 0.5 ppm MNPs with a size of 10 nm, which gave the maximum fresh weight of callus and percentage increase in fresh weight after two subcultures. The maximum accumulation of ginkgolide A and bilobalide was obtained when the callus was stimulated by MNPs 10 nm at 2 ppm. This study also focused on the estimation of phenolic compounds by HPLC and free radical scavenging (antioxidant activity) with 2, 2-diphenyl-1-picrylhydrazyl (DPPH) for their indirect effect on the production of active constituents. In addition, MNPs of different sizes had a significant effect on the number of phenolic compounds, with maximum values recorded at (2 ppm + size 10.77 nm). The major components were quercetin, kaempferol, p-coumaric acid, rutin and Caffeic acid. Moreover, the highest percentage of antioxidant activity (DPPH) was recorded when the callus was treated with (2 ppm + size 10.77 nm), which reached 77.29%. This study highlights the importance of MNPs in the production of ginkgolides A and bilobalides from *Ginkgo biloba* L. and focuses on the relationship between their size and concentration. Moreover, to obtain a good and durable source of active compounds of medicinal and industrial importance at a large scale and at the lowest cost.

Keywords: Ginkgo biloba, Callus growth, Magnetite NPs, Different sizes, Ginkgolide A and Bilobalide, phenolic compounds.

## 1. Introduction

In recent years, significant advances have been made in nanotechnology. This research has come into the limelight and is being talked about in most countries. It enables us to obtain products that are characterized by high quality, purity, and freedom from impurities. Nanoparticles have specific physicochemical properties to enhance plant metabolism such as high reactivity, large surface area, particle morphology, and tunable pores [1]. The activity of nanoparticles depends on their structure, size, and concentration, which vary from plant to plant [2]. Therefore, there is a need to use new technologies that can support agricultural productivity in feeding the explosively growing world population [3]. The use of nanomaterials in agriculture, on the other hand, aims to increase performance and reduce waste than product aspects and approaches [4-6]. Iron is one of the most important elements for plant growth and plays a significant role in photosynthetic reactions. Iron activates various enzymes and contributes to RNA synthesis and increases photosystem efficiency [7]. Many methods are used for the synthesis of metal nanoforms such as physical, chemical and biological measures [8-9]. Magnetite nanoparticles can be prepared by various chemical and green methods for many applications, the most important of which is in the agricultural field [10-12]. In this regard, there are several types of research on plant species using iron nanoparticles [4, 13-18]. Nevertheless, the synthesis

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of active compounds in tissue cultures such as the callus of *Ginkgo biloba* L. using nanotechnology technique is late (or limited) in the list of applications of this science. *Ginkgo biloba* L., which originated in China, is one of the most widely used herbal preparations in the world [19]. Due to its low side effects, the use of herbal supplements has skyrocketed in the last 50 years [20]. The ginkgo tree belongs to the Ginkgoaceae family and is considered one of the endangered plants in Egypt, which needs to be preserved and maintained by conventional methods or by using modern technologies in the field of biotechnology [21]. The ginkgo tree produces diterpenes (ginkgolides A, B, C, and J), sesquiterpenes (bilobalide), ginkgo flavonol glycosides (the glycosides kaempferol, quercetin, and isorhamnetin), triterpenes (sterols), organic acids, polyprenols including ginkgolic acid and tannic acid [22]. Several components present in the leaves have been mainly identified and belong to the four major chemical classes which are ginkgolides, bilobalide, polyphenols, and flavonoids [23]. All of these compounds are effective in the treatment of peripheral and cerebral arterial circulatory disorders, especially in the elderly, also in patients with mild to severe Alzheimer's disease, and in the treatment of neurological, dementia disorders and diabetes [24] and diabetes [25]. Production of secondary metabolites in plant cells and organ cultures using tissue culture technique could be a successful alternative method to extraction from whole plants, thus conserving natural plant resources [26]. Elicitation is a complex process and depends on many factors, such as the type and concentration of elicitors, the growth stage of the culture, and the exposure time to the elicitor [27]. Enhanced callus induction and production of many valuable secondary metabolites using different elicitors and nano elicitors have been reported in different plant species [28-31]. Therefore, the present research work aimed at standardizing parameters for maximum biomass production and enhancement of accumulation of ginkgolide A, bilobalide, and phenolic compounds in callus cultures of *Ginkgo biloba* L. using different sizes and concentrations of magnetite nanoparticles ($Fe_3O_4$NPs) as an elicitor

## 2. Materials and methods

### *2.1. Preparation and characterization of magnetite nanoparticles different sizes*

#### *2.1.1. Preparation of magnetite nanoparticles*

The aim of this study is to prepare magnetite nanoparticles (MNPs) of different sizes. According to [11], magnetite nanoparticles were synthesized in an aqueous solution by a surfactant-free oxidation method with some modifications. Briefly, a sodium hydroxide solution (99.99%, Sigma-Aldrich, USA) (21mM) in deionized water with intense stirring under the atmosphere of $N_2$. Sodium nitrate (99.99%, Sigma-Aldrich, USA) (8.80 mmol) was added as an oxidizing agent. Then, deaerated ferrous chloride aqueous solution (0.1 M) was added to the alkaline solution, which was kept at 37, 45 °C, and 60 °C, respectively, for 24 h. The solution was then added to the alkaline solution. The MNPs were separated and washed by centrifugation with deionized water. The resulting precipitates were dried under vacuum at 60 °C.

#### *2.1.2. Characterization of magnetite nanoparticles*

A High-Resolution Transmission Electron Microscope (HR-TEM) with a 200 kV accelerating voltage was used to image the prepared's actual morphology (Tecnai G2, FEI, Netherlands). To minimize particle aggregation, a diluted nanoparticle solution was ultrasonicated for 5 minutes. Three drops of the sonicated solution were deposited on a carbon-coated copper grid with a micropipette and allowed to dry at room temperature. For morphological evaluation, HR-TEM images of magnetite nanoparticles deposited on the grid were captured. The average particle size distribution determined by the zeta sizer was calculated using the Dynamic Light Scattering (DLS) technique (Malvern, ZS Nano, UK). X-ray Diffraction (XRD) was used to examine the chemical composition of as-prepared magnetite nanoparticles. The corresponding XRD pattern was collected using a scanning mode (X'pert PRO, PAN analytical, Netherlands) with a Cu K radiation tube (= 1.54 A) operating at 40 kV and 30 mA. The standard ICCD library installed in PDF4 software was used to interpret the obtained diffraction pattern. All of the preparation and characterization procedures were carried out at the Agricultural Research Center's Nanotechnology and Advanced Materials Central Lab (NAMCL).

### *2.2. Induction, growth and proliferation of callus cultures*

#### *2.2.1. Plant material collection*

The one-year-old branches were taken from a male *Ginkgo biloba* L. tree that grows to 30 years old and grows in the Orman Botanical Garden in Cairo, Egypt. The leaves are cut from the branches (green and healthy).

#### *2.2.2. Plant material sterilization*

*Ginkgo biloba* L. leaves were washed under running tap water for 30 minutes, followed by 5 minutes of detergent. Under aseptic conditions, the leaf surface was sterilized by immersion in 70% ethanol for 30 seconds and then rinsed three times with sterilized distilled water and transferred for 2 minutes to 0.1% mercuric chloride (w/v), followed by rinsing with sterile distilled water. All leaves are

sterilized in 40% (v/v) industrial bleach (Clorox) 5.25% (w/v) available chlorine, sodium hypochlorite solution with one drop of Tween-20, vigorously shaken for 20 minutes, and rinsed with sterile distilled water three-four times. Consequently, under a laminar flow hood, the leaves exposed to the sterilizing agent were cut to a length of 1 cm.

#### *2.2.3. Culture conditions and callus induction*

The sterilized leaves were cut lengthwise and cultivated on basal medium (MS) to induce callus formation, supplemented with 100 ppm myositol, 30 g / l sucrose, 1.0 ppm naphthalene acetic acid (NAA), 1.0 ppm kinetin, and solidified with 2.5g / l phyta gel [32]. The pH of the medium was adjusted to 5.7 with 0.1N HCl and 0.1 N NaOH, before autoclaving at 121°C for 20 minutes. The culture was incubated in dark at 25± 2°C and harvested after 30 days. To induce callus formation, explants were cultured on MS medium supplemented with 1 mg/l naphthalene acetic acid (NAA), 1 mg/l N-furfuryladenine (kinetin). This medium was the best and optimal medium for callus induction and formation [33]. The cultures were incubated in dark at 25± 2 °C and then subcultured after four weeks. For callus growth, an elicitor, MNPs different sizes at rates of (0.5, 1.0, and 2.0 ppm), were selected to improve the growth of callus cultures. Medium without MNPs serves as a control. Culture medium was supplied by DuchefaBiochemie chemical Co., Netherlands. The pH of the medium was adjusted to 5.7 - 5.8 and solidified with 3% w/v phytagel Media (40-45 ml volumes) were dispensed into glass jars closed with autoclavable caps and autoclaved at 1.1 kg/cm2 for 15 minutes at 121°C. An equal amount of callus (1 g) was cultured on MS medium supplemented with 1 mg/l NAA and 1 mg/l kinetin and augmented with MNPs (as previously mentioned). The cultures were incubated in a growth room under 16 hours photoperiod provided by cool white fluorescent lamps (F 40t9d/38, Toshiba) at a temperature of 25±2°C. The mean fresh weight of callus (g/jar), color, and texture of callus were recorded after four weeks of culture. Callus fresh weight was taken after removing the excess moisture from the surface using blotting paper. For callus proliferation, it was carried out using various sizes of MNPs and different concentrations. The cultures were incubated in a growth room under 16 hours photoperiod provided by cool white fluorescent lamps (F 40t9d/38, Toshiba) at a temperature of 25±2°C. After four weeks of culture, the mean fresh weight of callus (g/jar) and its percentage increased. Every four weeks the callus is transferred to a new medium to evaluate the proliferation.

#### *2.2.4. Active constituents' promotion*

Equal amounts of callus (2 g) were cultivated on the MS basal medium (as previously stated, which gave the best growth parameters) with some chemical inducers to promote active constituents in *Ginkgo biloba* L callus. A piece of subculture callus (100 mg) was put on different concentrations of nanoparticles after six weeks of callus formation. Three sizes of MNPs with three levels compared to control were included in the experiment as follows:

Control (without nano iron oxide).

- $Fe_3O_4$ at "size 10.77 nm" (0.5, 1, and 2 ppm).
- $Fe_3O_4$ at "size 20.50 nm" (0.5, 1, and 2 ppm).
- $Fe_3O_4$ at "size 29.33 nm" (0.5, 1, and 2 ppm).

Nano iron oxides were prepared at different concentrations using distilled water.

### 2.3. *Qualitative* and quantitative identification of active constituents

After 30 days from culture, the samples of fresh *Ginkgo biloba* L. callus were collected to determine some growth parameters and active constituents.

#### *2.3.1. Ginkgolide A and bilobalide*

#### *2.3.1.1. Extraction of ginkgolide A and bilobalide*

Ginkgolide A and bilobalide were extracted by mortar in methanol 10 ml after dilution with an equal volume of water from 6 g fresh callus. In the resulting mixture, ethyl acetate (20 ml) was added and the mixture was shaken on a vortex mixer for 40 minutes and then centrifuged at 3000 rpm; the ethyl acetate stage was transferred to a glass tube. Five times, the extraction process was repeated. The combined ethyl acetate layer was evaporated by nitrogen at 50oC upon extraction. The residue was dissolved in 2 ml of methanol and the samples were held frozen in a sealed vial until separated the active constituents by High-Performance Liquid Chromatography (HPLC) in the central lab of Desert research Center (DRC

#### *2.3.1.2. HPLC conditions*

Active constituents of *Ginkgo biloba* L. callus were determined by High- Performance Liquid Chromatography (HPLC) according to the method of [34]. The HPLC system was a Dionex Ultimate 3000 equipped with an auto-sampler, quaternary pump, and a diode array detector. Samples were chromatographed BDS hypersil C18 (4.6 x 250 mm, particle size 5µm). The separation was performed with methanol and water (23:77v/v) as the elusion solvent rate of 1ml/min and the detection wavelength was 205/220 nm. The peak area and the percentage of ginkgolide A and bilobalide were calculated using an external standard by the computer software of HPLC.

*2.3.2. Phenolic compounds*

*2.3.2.1. Extraction of phenolic compounds*

The G*inkgo biloba* L. callus (0.5 g FW) was extracted by sonification in methanol (5 mL) for 30 min at room temperature.

*2.3.2.2. HPLC conditions*

A validated HPLC method was used to separate the phenolic acids and flavonoids in sample extracts according to [35, 36]. HPLC review, preparations, and conditions were described in detail by [37, 38]. The mobile phase consisted of: A- methanol: 0.5 % acetic acid (1:4 v/v); B-methanol, at gradient program (B from 0 to 100 %), at temp. 25°C. The flow rate was 1 ml/min., injection volume was 10 µl, and detection wavelength was 254 nm. Quantification has been carried out based on the calibration curves for the following phenolic acid standards: Vanillic acid, Gallic acid, Catechin, Resorcinol, Caffeic acid, Narinigen, Rutin, p-coumaric acid, Ferulic acid, Myrecetin, Quercetin, Apigenin, and Kaempferol (Sigma-Aldrich Co.).

*2.3.3. Antioxidant activity by the DPPH Assay*

The method of [39] was used with some modifications for the DPPH (1,1-Diphenyl-2-picrylhydrazyl radical) assay. 1 mL of methanolic extract was combined with 4 mL of a 0.004 % DPPH solution in methanol 80 %. The reaction mixture was vortexed and left at room temperature for 30 minutes in the dark. The mixture's absorbance was calculated spectrophotometrically at 517 nm. The following equation was used to measure the potential to scavenge DPPH radicals:

$$(RSC\ \%) = (A_{Blank} - A_{Sample}) / (A_{Blank}) \times 100$$

Where, (RSC %) = DPPH radical scavenging activity (%), $A_{Blank}$ is the absorbance of DPPH radical +methanol (1 ml DPPH and 3 mL methanol), $A_{Sample}$ is the absorbance of (DPPH radical + sample extract)

## 2.4. Statistical analysis

The experiments were set up in a totally random order. The obtained data were analyzed using analysis of variance (ANOVA) and Duncan's multiple range test [40], as modified by [41], were performed to analyze the obtained data.

# 3. Results and discussion

## 3.1. Characterization of magnetite nanoparticles different sizes.

*3.1.1. HR-TEM analysis result*

The physicochemical characterization of the synthesized MNPs is shown in figure (1A) to investigate their properties using various techniques. The HR-TEM electrograph reveals near spherical form, smooth surface and average size of 10 nm, 20 nm and 29 nm, respectively, in nanoparticle morphology and size determination.

*3.1.2. Dynamic Light Scattering (DLS) Analysis*

Figure (1B) shows the particle size (DLS) used to calculate the nanometer scale of hydrodynamic diameters. The NP magnetite size was 29.3 nm, 20.5 nm and 10.8 nm, respectively.

*3.1.3. X-Ray diffraction (XRD) pattern of magnetite nanoparticles.*

The XRD pattern of the magnetite nanoparticles synthesized is shown in figure (1C). The peaks were assigned to (220), (311), (400), (511), and (440) $Fe_3O_4$ nanoparticles at 2 θ = 30.13 °, 35.49 °, 43.14 °, 57.05 °, and 62.65 °, meaning that the crystalline structure of synthesized $Fe_3O_4$ nanoparticles displayed a wurtzite cubic phase structure (Zincite, JCPDS 04-013-7099).

## 3.2. Effect of MNPs on induction, growth and proliferation of callus cultures

*3.2.1. Effect of MNPs on induction of callus cultures*

The induction and production of callus from the leaves shown in (Figure 2). To induce callus initiation and formation, explants were cultured on MS medium supplemented with 1 ppm NAA and 1 ppm Kin. Callus was produced after four weeks of culture. This medium was the best and optimal medium for callus induction and formation, which gave the best growth parameters.

*3.2.2. Effect of MNPs on callus growth*

Data recorded in Table (1) and Figure (3) showed the effect of MNPs different sizes and concentrations on callus cultures .Callus fresh weights was varied depending on size of MNPs and their concentrations. Comparing the effect of different tested sizes and concentrations of MNPs on callus growth, it could be noticed that callus fresh weights was gradually decreased with increasing the size and concentration of MNPs from 10.77 to 29.3 nm and 0.5 to 2.0 ppm, respectively. Moreover, the maximum callus fresh weight was obtained with 10.77 nm at 0.5 ppm ,which gave 3.68 g of A mix of yellow, brown and little green friable callus (Figure 5) , followed by the same size (10.77 nm) at 1.0 and 2.0 ppm ,which gave 3.17 and 2.88 g callus, respectively. Also, data revealed that, the size of magnetite nanoparticles (10.77 nm) is produced the maximum fresh weight of callus compared with two other sizes. The same result was reported in *Linum usitatissimum* [30]. A previous study showed the effect of magnetite nanoparticles on the growth of

*Stevia rebaudiana* L. callus [16]. They found that the treatment with magnetite NPs had a positive effect on fresh weight when stevia callus treated only with 0.25 and 0.5 ppm compared with the control. Also, they found that the positive role of magnetite NPS on stevia callus may be due to it plays a key role in growth and development [43].

On the other hand, the lowest callus fresh weight (1.54, and 1.51g) were observed in explants cultures in the presence of MNPs with 29.3 nm at 1 and 2 ppm, respectively. Increasing the size to 29.3 nm and with all concentrations of MNPs remarkably lowered weights of callus with color ranging from yellowish- brown to dark brown and friable texture (Figure 4). In this connection, the metal ions at low concentrations and size play an important role in the vital activities of the cell to maintain the growth of organisms, while at high concentrations they cause severe damage to the organisms [44]. Moreover, [45] reported that the grape callus growth was inhibited by the addition of high concentrations of nanoparticles. The high reduction of callus production may be due to the stress in the medium by these particles on cells growth and division. Both positive and negative effects of nanoparticles on plant growth have been reported. The results of some studies have shown inhibitory effects of nano oxide materials such as Cu, Al, Si, Fe and Zn on development of plant growth [23]. Metal ions such as Fe, Ni, Co, Zn, and Mn are essential for regulating enzyme activity although they are highly toxic in high concentrations [46].

### 3.2.3. *Effect of MNPs on callus proliferation*

Concerning callus proliferation, Table (2) shows that the highest percentage of increase in fresh weight (81.52%') was observed on MS medium supplemented with the size of 10.77 nm at 0.5 ppm MNPs reaching 6.68 g / jar (Figure 5). Followed by MS medium supplemented with 1 ppm at the same size which gave 5.27 g/ jar with the percentage of increase in callus fresh weight of 66.25% then MS medium supplemented with 2 ppm MNPs (4.52 g/ jar), that gave 56.94% of the increase in callus fresh weight. Comparing the effect of different tested sizes of iron nanoparticles on the percentage of increase in fresh weight of callus, it could be noticed that fresh weight and percentage of increase in fresh weight were gradually decreased with increasing the size from 10.77 nm to 29.3 nm.

It could be concluded from the obtained data in (Table 1 and 2) that MS medium supplemented with MNPs size 10.77nm at 0.5 mg/l was the best medium for callus growth and proliferation from callus derived from leaf segments of *Ginkgo biloba* L., it gave the maximum mean fresh weight of callus and percentage of increase in fresh weight, comparing to the other tested media. The same observation was reported and broad agreement with [47] who reported that low size (25 nm) MNPs pass into callus cells and induce low toxicity level in the callus cultures of *Linum usitatissimum*. Also, they found that a high concentration of $Fe_3O_4$ NPs (1.5 mg/l) was most strongly reduced callus size. Moreover, [30] showed that three nano elicitors (ZnO, SiO2, and $Al_2O_3$) with different concentrations affected significantly the fresh weight of callus. Although, they reported that the average callus fresh weight decreased with the increase in nano elicitor concentration.

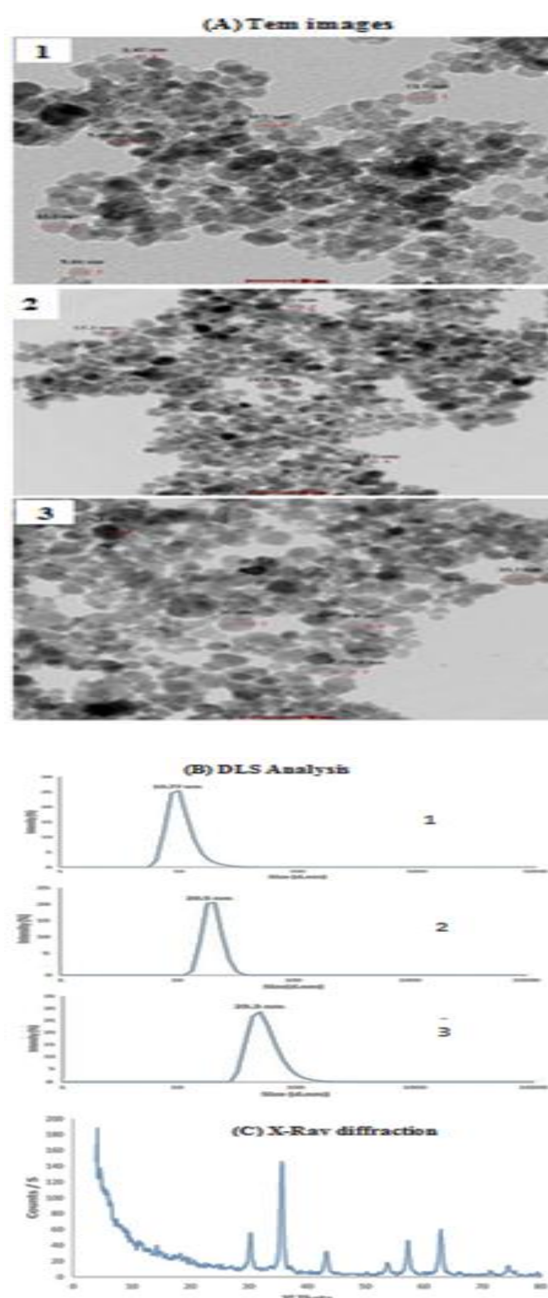


**Fig.1.** Characterization of magnetite nanoparticles different sizes **(A)** TEM images for magnetite nanoparticles synthesized at (1) 60°C, (2) 45 °C, and (3) 37 °C, **(B)** Particle size for magnetite nanoparticles synthesized at (1) 60 °C, (2) 45 °C, and (3) 37 °C, and **(C)** X-ray powder diffraction patterns of magnetite nanoparticles.

Table 1: Effect of magnetite nanoparticles different sizes and concentrations on callus growth of *Ginkgo biloba* L. (Data were taken after 4 weeks of culture)

| Treatments Size (nm) | Concentration (ppm) | Fresh weight (g/jar) | Color and texture (%) |
|---|---|---|---|
| Control | - | 2.43d | Greenish white friable |
| 10.77 | 0.5 | 3.68 a | A mix of yellow, brown and little green friable |
| | 1.0 | 3.17 b | Yellow- brown friable |
| | 2.0 | 2.88 c | Yellow friable |
| 20.50 | 0.5 | 2.31 e | Yellowish brown friable |
| | 1.0 | 1.98 f | Yellowish brown friable |
| | 2.0 | 2.03 f | Yellowish brown friable |
| 29.33 | 0.5 | 1.62 g | Yellowish brown friable |
| | 1.0 | 1.54 h | Brown friable |
| | 2.0 | 1.51 h | Dark brown friable |

Means followed by the same letter within a column are insignificantly different at P≤ 0.05

Table 2: Effect of magnetite nanoparticles different sizes and concentrations on callus proliferation of *Ginko biloba* L (Data were taken after 4 weeks of culture)

| Treatments Size (nm) | Concentration (ppm) | Fresh weight (g/jar) |
|---|---|---|
| Control | - | 2.92e |
| 10.77 | 0.5 | 6.68a |
| | 1.0 | 5.27b |
| | 2.0 | 4.52c |
| 20.5 | 0.5 | 3.18d |
| | 1.0 | 2.57f |
| | 2.0 | 2.53f |
| 29.33 | 0.5 | 1.97g |
| | 1.0 | 1.86h |
| | 2.0 | 1.82h |

Means followed by the same letter within a column are insignificantly different at P≤ 0.05

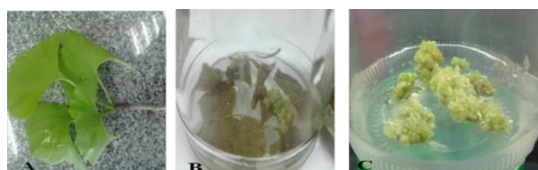


**Fig.2.** Establishment of *in vitro* leaf culture from *Ginkgo biloba* L. (A) Intact leaves (B) and (C) Callus I nitiation after two weeks and callus formation after four weeks.

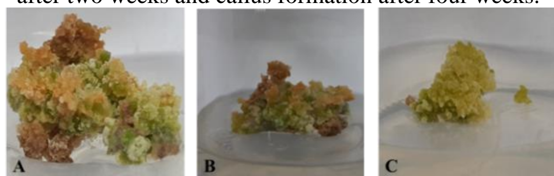


**Fig.3.** The maximum callus growth on MS medium supplemented with 1 ppm NAA+1 ppm Kin, and augmented with 10.77 nm $Fe_3O_4$ NPs at (A) 0.5 ppm,(B) 1 ppm and (C)2 ppm.

Fig.4: The callus growth on MS medium supplemented with 1 ppm NAA+ 1 ppm Kin, and augmented with 29.3 nm Fe3O4 NPs at (A) 0.5 ppm, (B) 1 ppm and (C) 2 ppm.

**Fig. 5:** Proliferated callus of *Ginkgo biloba* L. on MS medium supplemented 1 ppm NAA+ 1 ppm Kin and augmented with size of 10.77 nm at 0.5 ppm Fe3O4 NPs.

## 3.3. Effect of MNPs on active constituents in Ginkgo biloba L. callus

### *3.3.1. Effect of MNPs on ginkgolide A and bilobalide content*

The effect of different sizes of magnetite (10.77, 20.5, and 29.3) nm on bilobalide and ginkgolideA content of *Ginkgo biloba* L. callus is shown in (Table 3 and Figures 6). In light of the obtained results, data from HPLC analysis showed that MNPs different concentrations had a positive effect on bilobalide and ginkgolideA compared with the control. Data showed that after applying all doses of MNPs, callus had a higher bilobalide content (major compound) than ginkgolide A. Concerning, effect of MNPs different sizes and concentrations on bilobalide and ginkgolide A content, data showed that the highest values of bilobalide (3.85 and 3.28 mg/g) and ginkgolide A (1.91 and 1.52 mg/g) were obtained in comparison with other treatments when MNPs was applied at the lowest size 10.77 nm with concentrations (2 and 1 ppm), respectively. In addition, it was observed that MNPs size 20.5 nm (with three concentrations) gave more amounts of bilobalide and ginkgolide A than MNPs size 29.3 nm. The results confirmed that, increasing the concentration of bilobalide and ginkgolideA in *Ginkgo biloba* L. callus is related to the small size of MNPs used. Consequently, the magnetite nanoscale is arranged (descending) according to its effect on the increase in active ingredients (10.77, 20.5, and 29.3), respectively. In this context, iron is needed in very limited amounts for adequate plant growth and development, and its deficit may create major disruptions in the physiological and metabolic processes occurring in the plant. Iron is a cofactor that catalyzes specific biochemical reactions for around 140 enzymes [7]. In this respect, iron plays a crucial role in plant development and growth. [43].

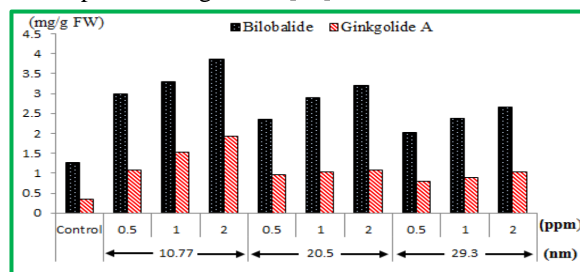


**Fig. 6**: Bilobalide and ginkgolide A (mg/g FW) in *ginkgo biloba* L. callus treated by MNPs different size nanoparticles

Table 3: Effect of magnetite nanoparticles different sizes and concentrations on bilobalide and ginkgolide A (mg/g FW) in *ginkgo biloba* L. callus

| Treatments | | Bilobalide | Ginkgolide A |
|---|---|---|---|
| Size (nm) | Concentration (ppm) | | |
| Control | - | 1.25 | 0.34 |
| 10.77 | 0.5 | 2.97 | 1.06 |
| | 1.0 | 3.28 | 1.52 |
| | 2.0 | 3.85 | 1.91 |
| 20.50 | 0.5 | 2.34 | 0.96 |
| | 1.0 | 2.88 | 1.02 |
| | 2.0 | 3.19 | 1.07 |
| 29.33 | 0.5 | 2.00 | 0.79 |
| | 1.0 | 2.36 | 0.88 |
| | 2.0 | 2.65 | 1.02 |

### 3.3.2. *Effect of MNPs on phenolic compounds*

The results illustrated in (Table 4) showed that thirteen phenolic compounds appeared in the *ginkgo biloba* L. callus, the major components were quercetin, kaempferol, p-coumaric acid, rutin, and caffeic acid whereas, the minor components were resorcinol, catechin, vanillic acid, myrecetin, ferulic acid, naringenin, and gallic acid respectively. Also, the magnetite nanoparticles different sizes increased phenolic compounds compared with the control, where the maximum values were recorded at the smaller size (10.77 nm). The same size (10.77 nm) MNPs treatments were increased all phenolic compounds in callus when applied at 2 ppm compared with other treatments. In contrast, gallic acid recorded the maximum value at (1 and 2 ppm) with sizes (20.5 and 29.3 nm) respectively. Likewise, ferulic acid recorded the highest values at (1 ppm) in sizes 20.5 and 29.3 nm. Concerning resorcinol, it was detected only when MNPs were applied at (1 and 2) ppm in size 10.77 nm and (2 ppm) in size 20.5 and 29.3 nm, respectively. Regarding catechin, it was found in all MNPs treatments and not detected in the control treatment. In this connection, Plant secondary metabolites such as flavonoids, phenolic acids, catechins, tannins, and proanthocyanidins are the most likely to have a health-promoting impact [48, 49]. At present, the focus of scientific research has been on plants as a source of antioxidant-potential phytochemicals. The responsibility for this activity has been established by different classes of plant secondary metabolites. Such natural compounds have numerous structures and have many defensive mechanisms [50]. Several studies have shown that these compounds protect toward diseases caused by excess free radicals, including cancer, atherosclerosis, Alzheimer's disease, Parkinson's disease, ischemic and cardiovascular disease, and so on [51, 52]. Moreover, recent plant biotechnology studies have shown that different in vitro systems of different plant species may be an abundant, alternate source of strong antioxidant polyphenolic compounds, much greater than that of intact plants [53]

### 3.3.3. *Effect of MNPs on antioxidant activity (DPPH)*

Data in Figure (7) showed that the scavenging rates of DPPH free radical by magnetite different size nanoparticles were more than control. The highest percentages of antioxidant activity (DPPH) were recorded when *Ginkgo biloba* L. callus treated with (2 ppm + size 10.77 nm), followed by (1 ppm + size 20.5 nm) and (1 ppm + size 29.3 nm) compared with the control, the percentages reached 77.29, 66.97 and 62.45 %, respectively in descending order.

Table (4): Effect of magnetite nanoparticles different sizes and concentrations on phenolic compounds (mg/ 100g FW) in *Ginkgo biloba* L. callus

| Phenolic compounds | Treatments | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | Size of MNPs (nm) | | | | | | | | |
| | | 10.77 | | | 20.5 | | | 29.3 | | |
| | | Concentration of MNPs (ppm) | | | | | | | | |
| | | 0.5 | 1 | 2 | 0.5 | 1 | 2 | 0.5 | 1 | 2 |
| Vanillic acid | 0.23 | 3.3 | 5.9 | 7.4 | 3.0 | 4.6 | 6.3 | 2.9 | 3.9 | 5.2 |
| Gallic acid | 2.11 | 7.9 | 9.5 | 10 | 6.2 | 8.9 | 8.4 | 5.5 | 8.1 | 8.5 |
| Catechin | ND | 1.8 | 3.6 | 4.6 | 1.5 | 2.3 | 2.8 | 0.22 | 1.4 | 1.7 |
| Resorcinol | ND | ND | 1.3 | 1.9 | ND | ND | 0.8 | ND | ND | 0.8 |
| Caffeic acid | 3.22 | 9.6 | 10 | 15 | 7.8 | 8.8 | 11 | 7.6 | 8.6 | 11 |
| Narinigen | 1.41 | 5.2 | 8.4 | 9.8 | 4.6 | 7.5 | 9.1 | 4.5 | 7.4 | 9.6 |
| Rutin | 3.91 | 29 | 32 | 36 | 27 | 31 | 32 | 25 | 31.3 | 31 |
| p-coumaric acid | 5.02 | 11 | 12 | 18 | 11 | 14 | 15 | 8.1 | 8.9 | 10 |
| Ferulic acid | 0.18 | 6.2 | 9.6 | 10 | 4.9 | 7.5 | 5.5 | 2.2 | 6.5 | 3.3 |
| Myrecetin | 0.29 | 5.6 | 7.8 | 9.1 | 5.2 | 7.4 | 8.7 | 4.4 | 7.2 | 8.7 |
| Quercetin | 41.8 | 109 | 127 | 135 | 103 | 123 | 131 | 95. | 118 | 12 |
| Kaempferol | 39.9 | 78 | 87 | 90 | 75 | 80 | 82 | 73 | 79.5 | 81 |

ND= not detectable

In this respect, a measure of non-enzymatic antioxidant activity is DPPH radical scavenging activity. *Ginkgo biloba* contains a variety of flavonoids and terpenes known to be antioxidant compounds and this is the main explanation for the medicinal effect [54, 55]. The antioxidant function of *Ginkgo biloba* leaves is responsible for this defensive impact [56]. Much attention has recently been given to the production of biocompatible nanoparticles that have antioxidant properties. In this regard, [57] demonstrated that MNPs enhanced scavenging DPPH antioxidant.

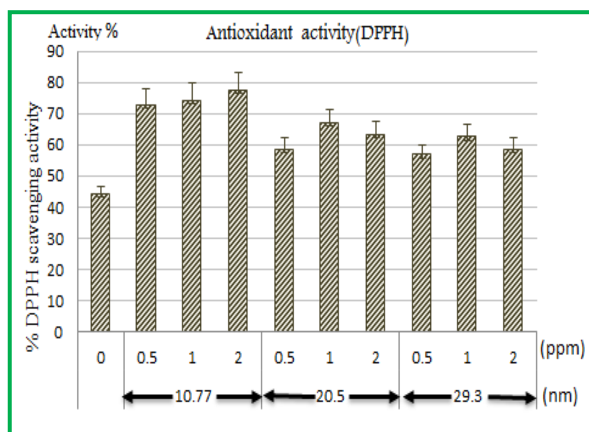


**Fig.7:** Effect of **magnetite nanoparticles** different sizes 10.77, 20.5 and 29.3 nm on antioxidant activity (DPPH %) in *Ginkgo biloba* L. callus

## 4. Conclusion

The present study concludes that treatments with MNPs of different sizes appeared to be effective on callus growth and these treatments had a primitive role in enhancing ginkgolide A, bilobalide, phenolic compounds, and antioxidant activity (scavenging free radical using DPPH) in callus cultures of *Ginkgo biloba*, and this was evident at the low MNPs size (10 nm). The results obtained from this work may have a valuable application for obtaining a high weight of callus, which could be used as a natural source for the commercial production of secondary ingredients.

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## 6. Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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