

Research Article

Evaluation, Antioxidant, Antimitotic and Anticancer Activity of Iron Nanoparticles Prepared by Using Water Extract of *Vitis vinifera* L. Leaves

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Abstract: The water extract of *Vitis vinifera* was used for the rapid synthesis of the iron nanoparticle, which is very simple and eco-friendly in nature. The UV-visible spectroscopy technique was employed to establish the formation of which inhibition onion root growth in a dose-dependent manner. A decrease in the mitotic index may indicate its genotoxic effect on *Allium cepa* root assay. Nanoparticle was studied for its *in vitro* anti-inflammatory and antioxidant activity using different models, all the testing, gave significant correlation existed between concentrations of the nanoparticle and percentage inhibition of protein denaturation and scavenging free radicals. These results clearly indicate that iron nanoparticle is effective against inflammation and free radical-mediated disease.

Keywords: Fe₃O₄, Nanoparticles, *Allium cepa* assay, Anti-inflammatory and Antioxidant.

1. Introduction

Nanoparticles have significant advances owing to a wide range of applications in the field of biomedical, sensors, etc. The biosynthesis of green nanoparticles as an eco-friendly alternative to chemical and physical methods, low costs and formed at ambient temperatures. At the last years, researchers used plants extract instead of dangerous and expensive reagents (1). The extracts of these plants and fruits contain molecules such as phenolic compound. These molecules can be reduction and formation of stable complexes with iron (2). *Vitis vinifera* (Grape) are rich sources of a wide variety of polyphenols (3). Grapes, one of the most popular fruits and the most widely cultivated throughout the world, contain large amounts of phytochemicals including anthocyanins and resveratrol, which offer health benefits (4). Currently, there is no published data on the anti-inflammatory, cytotoxicity and anticancer activity of *Vitis vinifera* leaf extract iron nanoparticles. The purpose of this study is to investigate anti-inflammatory, cytotoxicity and anticancer activity of *Vitis vinifera* leaf extract iron nanoparticles using the Allium Test and anticancer activity by using L20B cell line.

2. Material and Methods

2.1 Water Extract

The *Vitis vinifera* leaves were rinsed with water, dried in a ventilated oven at 55°C for 24 h and subsequently milled and into fine powder using a kitchen blender. The powder was placed in small plastic bags (100 g each) and stored at 4°C until use. The extract was prepared by boiling 20 g powdered plant material mixed with 200 ml distilled water, cooled to room temperature for 10 min and stored at 4°C till used (2).

2.2 Extract phytochemical analysis

2.2.1 Total phenolic content

Add 2 ml of iron nanoparticle to a solution contains (600 µL of 10% Folin-Ciocalteu reagent + 500 µL (2%) of sodium carbonate). Incubated at 45°C for 15 minutes, measured absorbance at 765 nm (5).

2.3 Total flavonoid

Two hundred µL of iron nanoparticle added to 600 µL of methanol, 40 µL of 1 M potassium acetate, 10% aluminum chloride (40 µL) and 1.12 mL of deionized water. Incubated at room temperature for 30 minutes, absorbance was measured at 420 nm (5).

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2.4 Total proanthocyanidins

Add 100 μL of iron nanoparticle to a solution [600 μL of vanillin methanol (4% v/v), 300 μL of hydrochloric acid]. Incubated at room temperature for 15 minutes, measured absorbance at 500 nm (6).

2.5 Preparation of Iron Nanoparticles

Take 40 ml *Vitis* leaves extract, add 40 ml FeSO_4 (1M) dropwise with continuous stirring, till changing the color of solution from pale yellow to black. Centrifuged at 10,000 rpm, at 4°C for 15 minutes, the pellet was dissolved in distilled water for periodic probe sonication for 5 min at $30 \pm 0.5^\circ\text{C}$. Nanosuspension thus obtained was dried in an oven. DLS measurement used to identify iron nanoparticle size, recorded in the range 500–4000 nm. UV-Vis spectral analysis was done at the range of 200–600 nm. FTIR spectra of the Iron nanoparticle were recorded in the range 500–4000 nm by (Shimadzu, Tokyo, Japan).

2.6 Scavenging superoxide activity

Add 100 μL of the water extract to the solution contained (100 μL of ferricyanide and 100 μL of NADH, incubated for 10 minutes at 25°C , absorbance measured at 230 nm then calculated inhibition percentage of scavenging activity for superoxide (7).

$$\% \text{ Inhibition} = \frac{(A_0 - A_1)}{A_0} \times 100 \quad (1)$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

2.7 Hydrogen peroxide scavenging activity

Add 100 μL of 5 mM hydrogen peroxide solution prepared in phosphate buffer (0.1 M (pH 7.4) to 500 μL , incubated for 10 minutes in dark place and absorbance was measured at 230 nm. Percentage of Inhibition is calculated by the equation (7).

$$\% \text{ Inhibition} = 100 - \frac{(A_0 - A_1)}{A_0} \times 100 \quad (2)$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

2.8 DPPH radical scavenging assay

0.1 mM DPPH prepared in methanol take about 200 μL , added to 100 μL of iron nanoparticle, incubated at room temperature in the dark for 15 minutes. Absorbance was measured at 517 nm. Percentage of inhibition is calculated by this equation (8).

$$\% \text{ Inhibition} = \frac{(A_0 - A_1)}{A_0} \times 100 \quad (3)$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

2.9 Nitric oxide (NO) scavenging activity

Add 400 μL of 100 mM sodium nitroprusside, 100 μL of PBS (pH - 7.4) and 100 μL of different concentration of nanoparticle. This reaction mixture incubated at 25°C for 1 h. Add 0.5 mL of Griess reagent (0.1 mL of sulfanilic acid and 200 μL naphthyl ethylenediamine dihydrochloride (0.1%) w/v to 0.5 ml of the mixture, incubated at room temperature for 30 minutes, and finally absorbance is measured at 540 nm (9). Percentage of Inhibition was calculated by the following formula:

$$\% \text{ Inhibition} = \frac{(A_0 - A_1)}{A_0} \times 100 \quad (4)$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

2.10 Inhibition of protein denaturation

Take 500 μL of 1% bovine serum albumin added to 100 μL of the nanoparticle. The mixture was kept at room temperature for 10 minutes, then heating at 51°C for 20 minutes. Solution cooled and absorbance was measured at 660 nm. Acetylsalicylic acid was taken as standard control (10). Percent inhibition for protein denaturation was calculated by:

$$\% \text{ Inhibition} = 100 - \frac{(A_0 - A_1)}{A_0} \times 100 \quad (5)$$

2.11 Anti-inflammatory activity

2.11.1 Inhibition of protein denaturation

500 μL of bovine serum albumin 1% was added to 100 μL of plant nanoparticle. This mixture was kept at room temperature for 10 minutes, followed by heating at 51°C for 20 minutes. The resulting solution was cooled down to room temperature and absorbance was recorded at 660 nm. Acetylsalicylic acid was taken as a positive control (10). The experiment was carried out in triplicates and percent inhibition for protein denaturation was calculated by:

$$\% \text{ Inhibition} = 100 - \frac{(A_0 - A_1)}{A_0} \times 100 \quad (6)$$

Where A_1 is the absorbance of the sample, A_2 is the absorbance of the product control and A_0 is the absorbance of the positive control.

2.12 Heat-induced hemolysis

A volume of 100 μL of 10% RBC was added to 100 μL of the extract. The resulting solution was heated at 56°C for 30 minutes, followed by centrifugation at 2500 rpm for 10 minutes at room temperature. The supernatant was collected, and absorbance was read at 560 nm. Acetylsalicylic acid was used as a positive control. Percent membrane stabilization was calculated by:

$$\% \text{ Inhibition} = 100 - \frac{(A_0 - A_1)}{A_0} \times 100 \quad (7)$$

Where A_1 is the absorbance of the sample, A_2 is the absorbance of the product control and A_0 is the absorbance of the positive control.

2.13 Inhibition of onion root tip mitosis

Take *Allium cepa* (onion) roots as a model for detection the ability of nanoparticle in inhibition onion root mitosis. Onion bulbs rinsed in water at room temperature for 48 h. Bulbs dipped in different concentration of the nanoparticle for 3 h. Distilled water was used as the control and the drug doxorubicin as the standard. After 3 h root tips were transferred to a fixing solution (acetic acid: ethanol in the ratio of 1:3 V/V) for 10-12 h. The roots were then stained using acetocarmine stain and observed under a microscope (4). Mitotic index and inhibition of mitosis were calculated as follows:

$$\text{Mitotic index (MI)} = \frac{\text{Number of dividing cells}}{\text{Number of total cells}} \times 100 \quad (8)$$

$$\% \text{ of Mitotic inhibition} = \frac{\text{MI control} - \text{MI}}{\text{MI test control}} \quad (9)$$

2.14 In vitro Anticancer Activity

The anticancer efficacy of nanoparticle against L20B cell line was evaluated. The colorimetric cell viability MTT assay was used (8). At first, 100 μL /well of L20B cells (106 cell/mL) were cultured in 96-well tissue culture plate. Different concentrations of nanoparticle test solution were prepared by dissolving (10 mg/mL) in water. Then, 100 μL of various concentrations was added to each well and incubated at 37°C for 24h. After the incubation, 10 μL of MTT solution (5 mg/mL) was added to each well and incubated at 37°C for 4 h. Finally, 50 μL of DMSO (dimethyl sulfoxide) was added to each well and incubated for 10 min. L20B cells were cultured in complete medium without an xxx solution as a control. The absorbance was measured for each well at 620 nm using an ELISA reader. The live cells, percentage of viability and inhibition ratio were calculated according to the formula:

$$\text{GI \%} = \frac{(\text{OD of control wells} - \text{OD of test wells})}{(\text{OD of control wells})} \times 100 \quad (10)$$

3. Results and discussion

Table 1 showed phytochemical constituents of *Vitis vinifera* leaves water extract. It was reported that phenolic compounds were associated with antioxidant activity. The total phenolic content of water extract (GAE)/g and 22 mg, total flavonoid 35 mg GAE/g and proanthocyanidin content 28 mg GAE/g respectively.

Which also supports the possible bioactivity of these plant extracts (7).

Table 1. Phytochemical constituents of *Vitis vinifera* leaves.

Phytochemical constituent	Amount
Total Phenol	22 mg GAE/g
Total Flavonoid	35 mg GAE/g
Total Proanthocyanidin	28 mg GAE/g

Fig. (1,2) showed the synthesis of the nanoparticle. Changing the color of extract from pale yellow to black color indicated synthesized iron oxide nanoparticles by the reduction of Fe^{+2} to Fe^0 ions, The UV Visible spectrum of nano iron particle is shown in Fig. (2). The peak at 350 wavelengths indicates the formation of nano iron particles.



Fig. 1. Showed that the changing of extract color from pale brown to black color indicated to Synthesized Iron Oxide nanoparticles: (A) Powder B) nanoparticle colloidal.

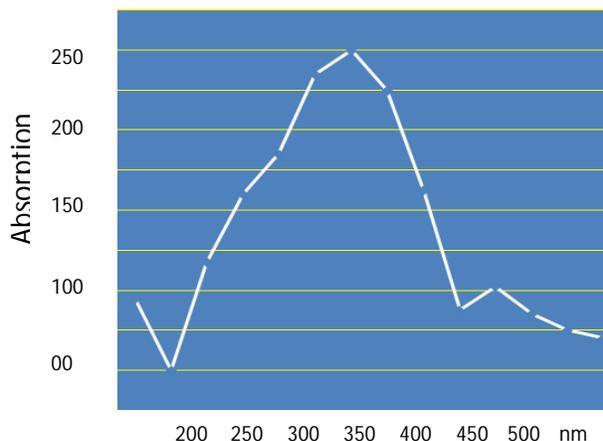


Fig. 2. UV-Vis Absorption Spectra of iron oxide NPs.

The size distribution by using DLS was shown in Fig. (3). The particle size measurement and size distribution with the help of DLS for iron oxide NPs. A colloidal solution of iron oxide NPs, DLS analysis of colloidal solution showed that for iron distributed in the range of 10 to 75 nm. The particles formed were in the range of nanoscale. The functional groups of *Vitis*

vinifera fruit extract which had bioreduction activity in synthesis NPs were studied by FTIR analysis as shown in Fig. (3). Absorption spectrum showed peaks at (540 and 434 cm^{-1} were of Fe-O stretch of iron oxide), 1620 cm^{-1} (C=C stretch), 1031 cm^{-1} (C-N stretch) for iron nanoparticle absorption peaks, at 3440 cm^{-1} (O-H stretch), 2924 cm^{-1} (C-H stretch which proved the synthesis of iron nanoparticles. This FTIR indicated the capping of different polyols on iron NPs and responsible for stabilization of iron nanoparticle (11).

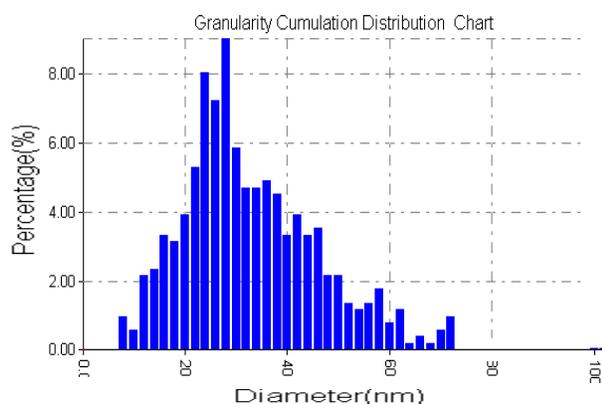


Fig. 3. Showed the size distribution of iron nanoparticle by using DLS.

3.1 Antioxidant activity

3.1.1 Superoxide ion scavenging activity

Fig. (4) showed superoxide anion radical scavenging activity by iron nanoparticle. The scavenging activity of superoxide anion radical by the iron nanoparticles is compared with gallic acid as standard reagents such as to improve iron nanoparticle also a potent scavenger of superoxide radical. As we know superoxide anion radical is one of the strongest reactive oxygen species among the free radicals that are generated (12). The extract was capable of scavenging superoxide anion radical in a dose-dependent manner. IC_{50} of nanoparticle was observed as 28.59 $\mu\text{g}/\text{ml}$.

3.2 Hydrogen peroxide radical

Fig. (5) showed hydrogen peroxide radical scavenging activity by iron nanoparticle. Scavenging of H_2O_2 by nanoparticle may be attributed to the presence of phenolics, which donate an electron to H_2O_2 , thus reducing it to water (13). The iron nanoparticles were capable of scavenging hydrogen peroxide in a dose-dependent manner. IC_{50} of nanoparticle was observed as 21.19 $\mu\text{g}/\text{ml}$.

3.3 Nitric oxide (NO) scavenging activity

Fig. (6) showed nitric oxide scavenging activity by iron nanoparticle. Iron nanoparticle had a potent scavenging activity for nitric oxide radicals. IC_{50} of nanoparticle was observed as 15.30 $\mu\text{g}/\text{ml}$. Nitric oxide plays a crucial role in the pathogenesis of inflammation,

this may explain the use of iron nanoparticle. Free radicals were responsible for several clinical disorders such as diabetes mellitus, cancer, liver diseases, renal failure and degenerative diseases, as a result, of deficient natural antioxidant defense mechanism (14).

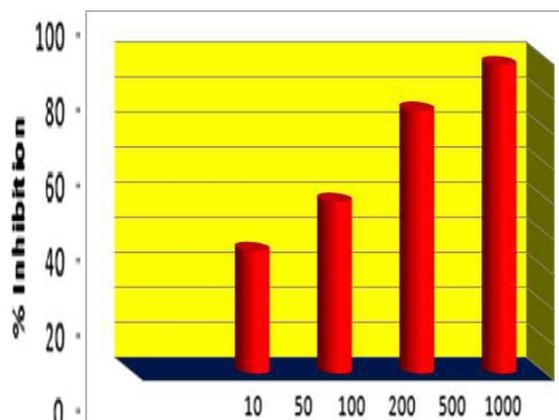


Fig. 4. Showed Superoxide anion radical scavenging activity by iron nanoparticle.

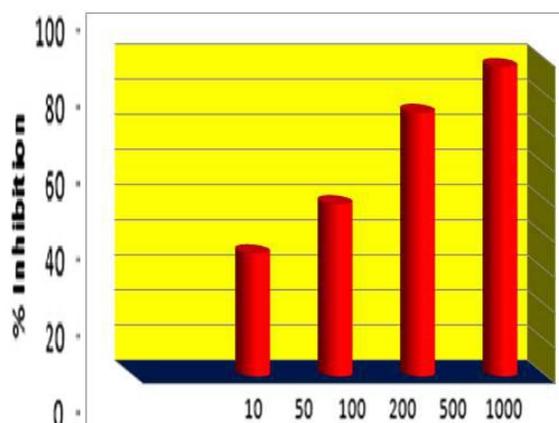


Fig. 5. Showed Hydrogen peroxide radical scavenging activity by iron nanoparticle.

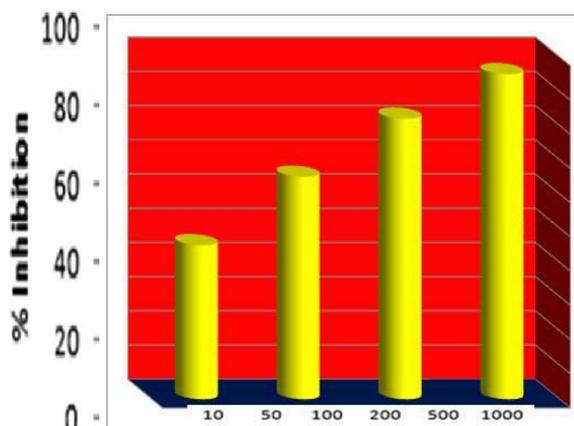


Fig. 6. Showed NO scavenging activity by iron nanoparticle.

3.4 Anti-inflammatory studies

3.4.1 Inhibition of albumin denaturation

Table (2) showed inhibition of albumin denaturation by iron nanoparticles. Denaturation of proteins is the main cause of inflammation (15). Our findings showed that iron nanoparticles had a good ability to inhibit protein denaturation. Maximum inhibition of albumin was 75.8% observed at 500 µg/ml of nanoparticle compared with aspirin at the same concentration Table (2). All concentrations showed significant results when it compares with control $p < 0.01$. This result investigated that nanoparticle was effective in inhibiting protein denaturation of albumin.

Table 2. Showed Inhibition of albumin denaturation by Iron nanoparticles.

Concentration (µg/ml)	Absorbance (nm)	% inhibition of hemolysis
- Control	0.42 ± 0.03	-
50	0.38 ± 0.02*	20
100	0.27 ± 0.05*	39
200	0.20 ± 0.01*	54
300	0.15 ± 0.03*	78
300 Diclofenac sodium	0.13 ± 0.07*	85

* $P < 0.05$ in one way ANOVA.

3.5 Membrane stabilization effect

Table (3) showed the results activity of inhibiting hemolysis at different concentrations of iron nanoparticles. Stabilization of RBCs membrane was studied to improve the mechanism of anti-inflammatory action of iron nanoparticles. The effective concentration was 300 µg/ml in comparing with standard drug diclofenac sodium 300 µg/ml gave good protection against the damaging effect of heat solution. The results provide evidence for membrane stabilization effect of the selected nanoparticles, as an additional mechanism for their anti-inflammatory effect. Due to the relation between RBC membrane and lysosomal membrane, this effect may possibly inhibit the release of the lysosomal content of neutrophils at the site of inflammation (16).

Table 3. Showed the inhibition of protein denaturation.

Concentration (µg/ml)	Absorbance (nm)	% inhibition of hemolysis
- CONTROL	0.57 ± 0.01*	-
200	0.46 ± 0.0*	26.8
300	0.35 ± 0.04*	31.7
400	0.27 ± 0.02*	50.7
500	0.09 ± 0.01*	79.5
500 Aspirin	0.07 ± 0.03*	85.6

* $P < 0.05$ in one way ANOVA.

3.6 Onion root tips mitosis Inhibition

Table (4) showed the average root numbers and root lengths of onion in controls and treatment concentrations of iron nanoparticles. Our results showed that all concentrations of iron nanoparticles caused significant inhibition in the growth and length of onion roots. Measured average root length and the number was 46.15 cm and 26.6 in negative control while 5.72 cm and 21.6 in positive control. However, average root length and number in 12.5 and 25 mg/ml treatment group was decreased significantly in compared with negative control 4.22 cm and 18.6, while 25 mg/ml were 2.88 cm and 21.6 respectively. Polyphenols and flavonoids acting as anti-inflammation and anticancer activity by regulation of cell growth and proliferation, suppression of oncogenes induction of apoptosis, modulation of enzyme activity related to detoxification and DNA repair (17).

Table (5) showed the dividing and total cells counted and mitotic values. We found that significant inhibition in the onion roots treated with the nanoparticles (2.04%, 0.85%, and 0.164%, compared to the negative control). A positive correlation was found between inhibition of root growth and a decrease of MI. The decrease of MI below 22% in comparison to negative control can have a lethal impact on the organism. Decrease the MI may be due to the inhibition of DNA synthesis (18).

Table 4. The average root numbers and root lengths in controls and treatment concentrations.

Treatment groups	Concentrations	Average root number ± SD	Average root lengths (cm) ± SD
Negative control	Tap water	26.6 ± 0.03	46.15 ± 0.41
Positive Control Doxorubicin	1 × 10 ⁻² M	21.6 ± 0.72*	5.72 ± 0.26*
Nanoparticle	12.5 mg/ml	18.6 ± 0.72*	4.22 ± 0.17*
Nanoparticle	25 mg/ml	12.6 ± 0.35*	2.88 ± 0.63*

* $P < 0.05$ in one way ANOVA.

Table 5. The dividing and total cells counted in microscopic observations and mitotic values in control and in treatment concentrations.

Treatment groups	Cells	Concentrations	Total cells	Dividing	MI (%) ± SD
Negative control		Tap water	25000	1530	6.120 ± 0.12
Positive control Doxorubicin		1 × 10 ⁻² M	25000	630	2.52 ± 0.14*
Nanoparticle		5 mg/ml	25000	510	2.04 ± 0.60*
Nanoparticle		10 mg/ml	25000	214	0.85 ± 0.75*
Nanoparticle		20 mg/ml	25000	41	0.164 ± 0.75*

* $P < 0.05$ in one way ANOVA

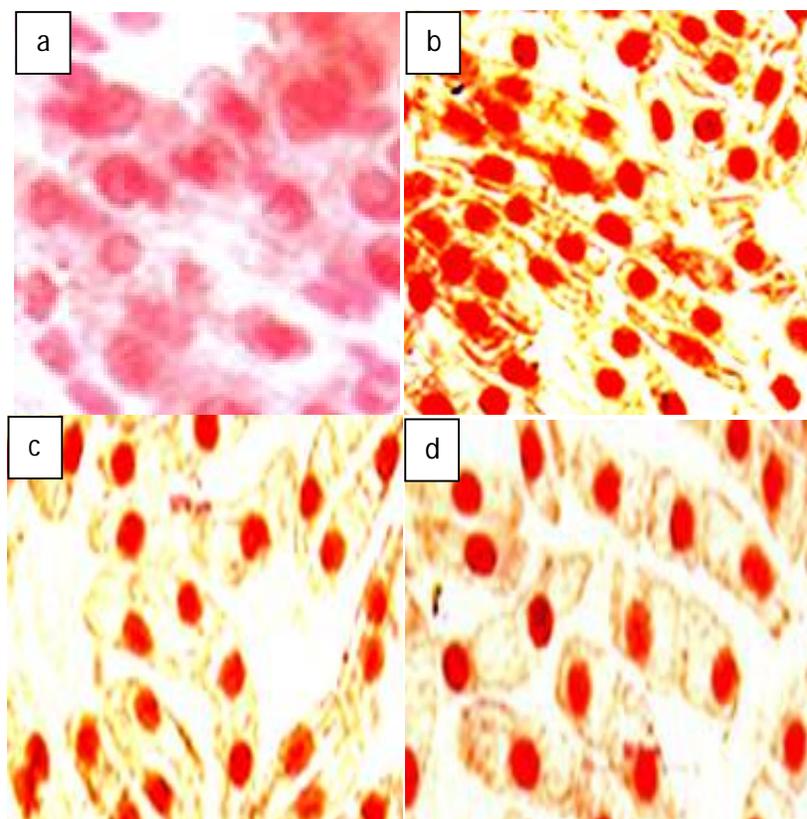


Fig. 7. Showed photomicrographs of root tip cells of *Allium cepa* showing normal cells and treated cell: a) Control b) Doxorubicin c) 5mg/ml d) 10mg/ml.

Root meristem model of *Allium cepa* used for screening of drugs with antimitotic activity (18). In *Allium cepa* root meristem model, root meristematic cells are used for screening of drugs with antimitotic activity (19). In the meristematic region, the cell division is similar to cancer cell division in humans. Therefore, these meristematic cells can be evaluated for the screening of drugs with potential antimitotic activity. *Allium* assay is a bioassay for detecting cytotoxicity and genotoxicity. The decrease in the mitotic index of *Allium cepa* meristematic cells was referred to cellular death and it is evident from shrunken and dark brownish color of the rootlets (20).

Therefore, in this study, *Vitis vinifera* iron nanoparticles were evaluated as a new anticancer agent by using MTT assays. Table (6) showed the anticancer efficacy of nanoparticle against L20B cell line was evaluated. The results of the present study showed potent cytotoxic effects against L20B cell line in concentration 10 mg/ml it gave 70.8% and at 5 mg/ml was 57.5% inhibition, which may be due to existing phytochemicals such as flavonoids and alkaloids (2).

Anticancer drugs are known to kill cancer cells by inhibiting cell cycle. Many phytochemicals of plant damaging DNA during the S-phase of the cell cycle or by blocking the formation of the mitotic spindle in M-phase (22).

Table 6. Showed potent cytotoxic effects against L20B cell line by iron nanoparticles.

Extract No.mg/ml	Mean	GI %
10	0.063	70.8 ± 0.003*
5	0.092	57.5 ± 0.028*
Control	0.218	

* $P < 0.05$ in one way ANOVA

4. Conclusion

In conclusion, the preliminary investigations on the *Vitis vinifera* iron nanoparticle showed anti-inflammatory, antimitotic and antiproliferative assays and suggest that the iron nanoparticles may exhibit inhibitory influence on abnormal cell growth as like in cancer. Though, the present study validates the traditional use of extract nanoparticles in the treatment of cancer.

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