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Original Article

Free Radical Scavenging Activity and its Embryonic Toxicology Effect of *Justicia betonica* Leaves Mediated Iron Oxide Nanoparticles

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ABSTRACT

Background: An important milestone in the use of nanoscience and nanotechnology is the development of a dependable and environmentally acceptable green method for the manufacture of magnetic nanoparticles. Numerous biomedical applications of iron oxide nanoparticles have been verified, including drug transport, magnetic resonance imaging, the identification, diagnosis, and management of diseases like cancer and neurological disorders, as well as low toxicity and biological compatibility. **Aim and Objectives:** The aim of the current study was determining the free radicals and embryonic toxicology effects of the *Justicia betonica* mediated iron oxide nanoparticles. **Materials and Methods:** To determine the free radical scavenging assay and their zebra fish embryonic toxicology of *Justicia betonica* leaves mediated iron oxide nanoparticles. **Results:** Concentration based free radicals suppression iron oxide nanoparticles is significant for oxidative stress-related concerns. Zebrafish embryonic toxicology experiments indicated safe concentration range in iron oxide nanoparticles. **Conclusion:** According to the results of this study, green produced iron oxide nanoparticles may be a promising source of antioxidant agents with lower toxicity.

Keywords: Antioxidant Activity, Green Synthesis, Iron Oxide Nanoparticles, Metal Oxide Nanoparticles, Zebrafish Embryonic Toxicology.

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INTRODUCTION

In ancient times, all herbal plants were utilized for medicinal preparations, whether it was taken from raw plant materials or extracts, dried powder, a mixture of plants, oils, etc. The application of medicinal herbs from different cultures and several thousands of herbs were isolated (Ekpo and Etim 2009). Classical medicines were used from several herbs, and they treated infectious and chronic disorders. Nowadays, herbs are also used as antibiotics against microbial pathogens, creating a major problem in developing drug resistance (Bhatia and Narain, 2010). In the *Acanthaceae* family, *Justicia* is the best and most important genus around 700 species. Justicia was grown in warm temperatures and occurred in south Asia. In India, this herb was used in the treatment of vomiting, constipation, pain reliever, swelling, malaria, paralysis, etc. The properties of *Justicia* are anti-inflammatory, antimicrobial, antioxidant, antimalarial, and analgesic (Awan *et al*., 2014). The herbs were used to prepare the nanoparticles and the green synthesis of nanoparticles was cost-effective, eco-friendly, biocompatible, and biodegradable. Nanotechnology is a method to synthesize nanoparticles as different structures, chemical composition, and morphology (Shah *et al*., 2015; Pauline *et al*., 2025). Nanoparticles' high surface-area-to-volume ratio makes them useful in various fields, including medical treatment, ecology, biosensors, catalysis, technology, and food production (Rajesh *et al*., 2024; Khandel and Shahi, 2016). Iron oxide nanoparticles are magnetic nanoparticles, Fe including a nano zero-valent iron, oxyhydroxide, oxide, and hydroxide groups are attached and form Fe (II) and Fe (III) ions (Flieger *et al*., 2024). The iron nanoparticle was present in nanomaterial in the form of core-shell nanoparticles or Nano alloys (Kharisov *et al*., 2016). They have a wide range of applications such as pharmaceutical applications, MRI, particle size analysis, stem cell tracking, food preservation or processing, dye degradation, treatment of cancer, and antimicrobial activity against various pathogens (Negrescu *et al*., 2022). The aim of the current study was to determine the free

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radicals and embryonic toxicology effects of the *Justicia betonica* mediated iron oxide nanoparticles.

MATERIALS AND METHODS

Preparation of plant extract

J. betonica was harvested from the Saveetha Dental College Nanoherbal Garden. To obtain the elimination of the dust particles, 2 g of fresh leaves were collected and cleaned four times under the running water. Following the fine-cutting and crushing of the leaves with a mortar and pestle, 100 mL of water were added to the leaf paste. For 15 to 20 min, the mixture of *J. betonica* solutions was heated at fifty degrees Celsius using a heating mantle. Following the boiling process, the solution was filtered via muslin cloth, and the nanoparticle solution and biomedical application were generated using the filtrate extract.

Preparation of nanoparticles

Iron oxide nanoparticles were prepared using *Justicia betonica* leaves as a reducing and stabilizing agent. 1 g of *J. betonica* leaves were dissolved in 100 mL distilled water and the extract was heated at 40-50°C for 10 min and filtered using muslin cloth. 20 mM of iron chloride was used for the preparation of iron chloride solution. 50 mL of *J. betonica* leaf extract and 50 mL of 20 mM of iron chloride to make it a 100 mL of nanoparticle solution. The prepared solution was kept in an orbital shaker and after 48 hr the solution was centrifuged at 8000 RPM for 10 min. After centrifugation, the pellet was collected and stored for further use.

Antioxidant activity

The antioxidant activity of the prepared nanoparticles was checked using the following three assays.

DPPH assay

A stock solution of 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) at 0.1 mM in methanol was made up. For each experiment, the stock solution was reduced in volume until it reached an ideal concentration of 20 µM in methanol to make a new working solution. In a 96-well plate, 200 µL of the DPPH working solution was incorporated to simulate various concentrations of the *Justicia betonica*-mediated iron oxide nanoparticles (10, 20, 30, 40, and 50 µg/mL). The plate was left to incubate in the dark at room temperature for 30 min. Ascorbic acid was used as the standard, and the absorbance was measured at 517 nm using a tiny plate reader. Methanol served as a blank.

H_2O_2 assay

In the current investigation, the antioxidant activity was examined using the hydroxyl radical scavenging assay developed by Halliwell *et al.*, 100 µL of 28 mM 2-deoxy-2-ribose was added to 1 mL of the reaction mixture. To that, various quantities of iron oxide nanoparticles (10 to 50 µg/mL) mediated by *Justicia betonica* were added. Additionally, 200 µL of EDTA, 200 µL of 200 µm ferric chloride, and 100 µL of ascorbic acid were added. The optical density at 532 nm was acquired after an hour of incubation at 37°C and contrasted to the blank solution. Vitamin E is a representation of a positive control.

FRAP Assay

Reagents for Frap Assay

a) Acetate buffer 300 mM pH 3.6: 1 L of distilled water is added to 3.1 g of sodium acetate trihydrate and 16 mL of glacial acetic acid.

b) 10 mM in 40 mM HCl of TPTZ (2, 4, 6-tripyridyl-s-triazine) (M.W. 312.34) (M.W. 36.46).

c) $FeCl_3$. 6 H_2O: 20 mM (M.W. 270.30). Just prior to testing, a, b, and c were combined in a 10:1:1 ratio to create the functioning FRAP reagent. $FeSO_4$ H_2O: 0.1-1.5 mM in methanol was the standard. The German manufacturer Merck prepared all the laboratory reagents.

Procedure

3.6 mL of FRAP solution is mixed with 0.4 mL of distilled water, and the mixture gets incubated at 37°C for 5 min. Following that, 80 mL of *Justicia betonica* at a certain concentration was added to this solution, and it was incubated at 37°C for 10 min. The absorbance of the reaction mixture was measured at 593 nm. The calibration curve was created using five different concentrations of $FeSO_4$, $7H_2O$ (0.1, 0.4, 0.8, 1, 1.12, and 1.5 mM), and the absorbance values were calculated in a manner identical to that of the sample solutions.

Zebrafish toxicology

Zebrafish embryonic toxicology evaluation of Iron Oxide nanoparticles

Fish maintenance and Fe_2O_3NP exposure

Danio rerio, or wild-type zebrafish, were purchased from nearby Indian vendors and kept in separate tanks with carefully regulated pH (6.8 from 8.5), light/dark cycle (14:10 hr), and temperature (280±20°C). The fish were fed optimal food two times a day or commercially available dry blood worms. To create zebrafish embryos, three males and one female were crossed in each breeding tank. Viable eggs were then gathered and successively rinsed with freshly made E3 media devoid of methylene blue. Fertilized eggs were deposited with 20 embryos per 2 mL solution in culture plates of six, twelve, or 20 wells, depending on the well size. A triplicate of the control and experimental treatment groups was performed. A freshly prepared stock suspension of TCF-Fe_2O_3NPs at five different concentrations was introduced straight to the E3 medium to set up the experimental treatment. For 15 min, the solution was sonicated to scatter the nanoparticles

while preserving a pH range of 7.2-7.3. For 24 to 96 hr after fertilization, healthy fertilized embryos were subjected to various doses of Fe_2O_3NPs, which ranged from 0 to 1000 µg/L. The E3 medium in which the embryos were cultured was supplemented with the Fe_2O_3NPs. The experiment also included control groups. Every 12 hr, dead embryos were taken out of the groups exposed to nanoparticles. To keep out light, all experimental plates were covered with foil and kept at a temperature of 28°C.

Zebrafish embryo evaluation

Using a stereo microscope, the various stages of development of embryos of zebrafish were observed during the exposure time that followed fertilization. For 24-78 hr, the embryos were exposed to different concentrations of iron oxide nanoparticles (5, 10, 20, 40, and 80 µg/mL). The percentages of hatching and embryonic death were measured every 24 hr. The investigation's outcomes included death rates of the embryos and young, the proportion of hatching, and detecting and recording any abnormalities in the larvae and embryos in the treatment and control groups. A COSLAB - Model: HL-10A light microscope was used to take visualizations of embryos with abnormalities, and every 24 hr, the percentage of faulty embryos was noted.

RESULTS

Visual observation

J. betonica was used to synthesize green iron oxide nanoparticles, and then iron chloride solution and *J. betonica* extract were added. The final color of the synthesized iron oxide nanoparticles was noted to be dark blackish, while the early stage of the iron chloride solution with the *J. betonica* extract was pale brown in color (Figure 1). The synthesized nanoparticles correlated with color.

UV-visible Spectroscopy

The first characterization of the nanoparticles was done using UV-visible spectroscopy, which confirmed the optical determination. The nanoparticle size range is (250 - 650 nm). At 24 and 36 hr, the greatest peak of the *J. betonica*-mediated iron oxide nanoparticles was detected at 435 nm (Figure 2). After centrifuging the produced nanoparticles, the pellet was obtained. Pellets containing iron oxide nanoparticles have been utilized in the following biomedical applications.

Antioxidant activity

In this current research, the free radical scavenging activity of *J. betonica*-mediated iron oxide nanoparticles was determined using various assays, such as FRAP assay, H_2O_2 assay, and DPPH assay. In comparison to a standard antioxidant, the results indicated that the iron NPs revealed unique antioxidant qualities in all three tests.

In the DPPH radical scavenging assay, the iron oxide nanoparticles also evaluated concentration based on the percentage of inhibition of DPPH assay, with the concentration range from (10-50 µg/mL) and the % of inhibition range from 63.92%, 74.24%, 81.73%, 83.92%, and 89.39%. In comparison of standard antioxidants, the percentage of inhibition ranges from 66.25%, 78.52%, 85.63%, 88.68%, and 93.15% (Figure 3A) respectively. This reveals more evidence of the iron NPs' more potent antioxidant qualities compared with the standard antioxidant at all assay concentrations evaluated.

Similarly, in the hydroxyl radical scavenging assay, both standard antioxidant and iron oxide nanoparticles were evaluated for concentration-based scavenging activity. Figure 3B, the various concentrations of the nanoparticles and standard antioxidants (10, 20, 30, 40, and 50 µg/mL) and the inhibitory percentage range from (50.5 - 87.3%), and the standard (51.1 - 89.9%). This suggests that, at all tested concentrations, iron nanoparticles were more effective than the standard antioxidant at scavenging hydroxyl radicals.

Figure 3C, in the FRAP radical scavenging assay, the iron oxide nanoparticles noted the concentration based on the inhibitory percentage of the assay, with the inhibitory percentage of the nanoparticles, ranging from 70.46%, 74.92%, 78.38%, 82.93%, 87.35%, and the inhibitory percentage of the ascorbic acid range from 72.98%, 76.84%, 81.31%, 85.84%, and 90.89%. In addition, at all quantities, the iron NPs revealed enhanced restriction of FRAP radicals that the standard antioxidant.

Antioxidant iron oxide nanoparticles are expected for applications such as targeted therapy, cancer treatment, drug delivery, and the management of oxidative stress, among other areas. These nanoparticles' antioxidant qualities make them an excellent possibility for targeted propagation, which could develop novel possibilities for reducing oxidative stress in *in vivo* conditions.

Embryonic toxicology

The zebrafish embryonic toxicology has been determining the hatching rate of the embryos in 60% at 80 µg/mL of iron oxide nanoparticles, 65% at 40 µg/mL, 70% at 20 µg/mL, 75% at 10 µg/mL, and 90% at 5 µg/mL and compared with the control in 100% of hatching rate (Figure 4A). It has been demonstrated the viability rate of iron oxide nanoparticles 5, 10, 20, 40, and 80 µg/mL and the % of viability rate was revealed to the 90, 75, 70, 65, and 60 and the control viability rate was 100% for the embryos in Figure 4B respectively. Figure 4C, On the first day, the morphological imaging showed that there were partially developed viable embryos through the egg; on the second day, there were fully developed viable embryos through the egg; and on the third day, healthy zebrafish developed. There were no visible somatic irregularities such as curved tail or spine, and there were no symptoms of inflammation.

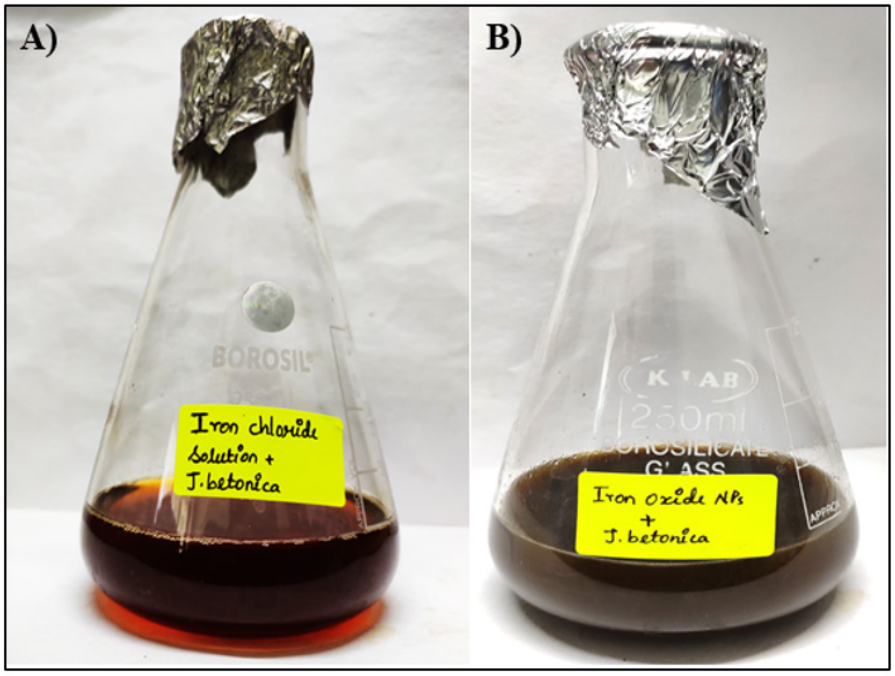

Figure 1: Green synthesis of iron oxide nanoparticles using *J. betonica*.

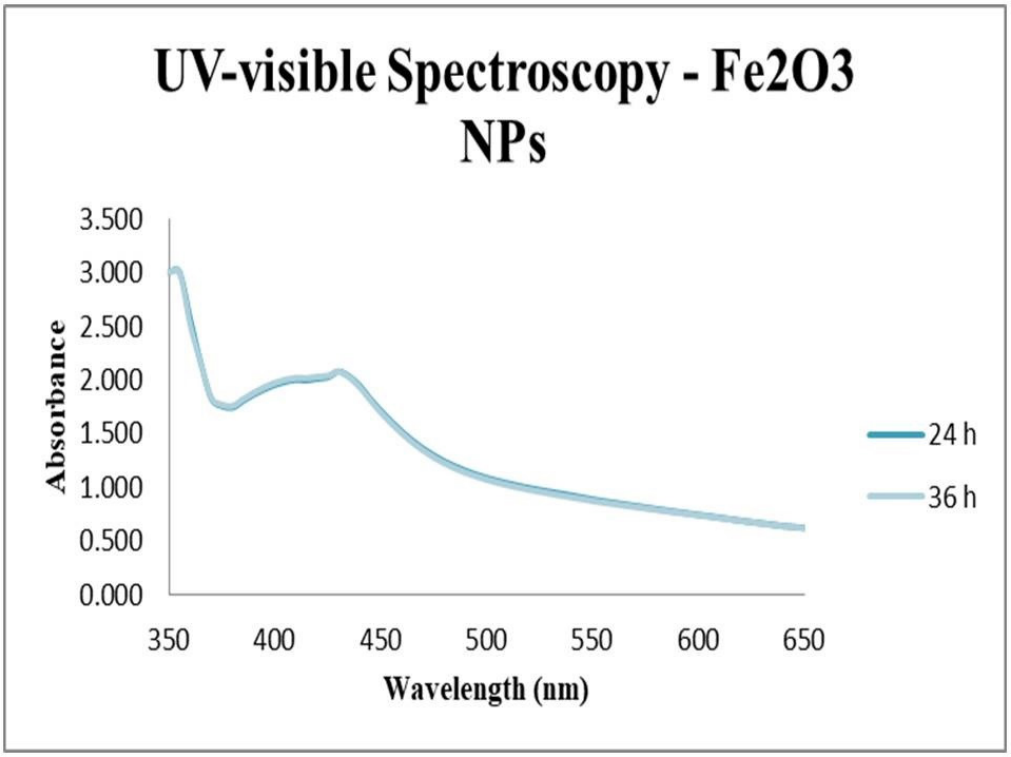

Figure 2: The image represents the UV-visible Spectroscopy of Iron oxide nanoparticles.

DISCUSSION

The *Justica betonica* leaf contains alkaloids, phenols, steroids, terpenoids, and flavonoids are present, the *J. betonica* phytochemical and bioactive compounds are present (Huston *et al.*, 2021), both react as reducing properties of iron chloride compound converted into iron ions and its prevent and the reduction efficacy of iron oxide nanoparticles. In a previous study, *Musa paradisiaca* peel extract mediated iron oxide nanoparticles, its eco-friendly synthesized nanoparticles (Al-darwesh *et al.*, 2024). In the present research, the iron oxide nanoparticle changed color gradually from brown to black; this color indicates the presence of nanoparticles. In a similar study, *Moringa oleifera*-mediated iron oxide nanoparticles

were observed in the color change reaction; it turned brown to black (Das *et al.*, 2017). The green synthesized iron oxide nanoparticle confirmed the broad absorption band formed in UV-visible spectroscopy at 435 nm in 24 and 36 hr. In the previous research, *Piper chaba*-mediated iron oxide nanoparticles were shown the maximum absorbance peak at 390 nm, it was confirmed the synthesized iron oxide nanoparticle (Reitz *et al.*, 2012). Similarly, the green synthesis of iron oxide nanoparticles using *Oscillatoria limnetica* was observed at a maximum peak of 471 nm. The synthesized nanoparticles were revealed in the absorbance peak at 471 nm (Yusuf *et al.*, 2023). On the other hand, the iron oxide nanoparticles using *Prosopis africana* leaf extract showed a peak at 400 nm for the range between 200 to 800 nm (Osazuwa *et al.*, 2017). *Borassus flabellifer* tender seeds peal-mediated iron oxide nanoparticles showed antioxidant properties against DPPH and H_2O_2 assay. In the DPPH assay, the iron oxide nanoparticles were added in various concentrations (20 to 100 µg/mL) and the inhibitory percentage was 43.04% to 85.53%. Similarly, in the H_2O_2 assay, the % of inhibition revealed 44.12%-91.79% respectively (Sandhya and Kalaiselvam, 2020). In the previous research, free radical scavenging activity of various fixation concentrations of Fe_2O_3 NPs (50 to 250 µg). In the DPPH assay, the % of the scavenging ranges from 32.54 to 84.28%, and the standard (ascorbic acid) is 28.25 to 81.41% of scavenging. The iron oxide nanoparticles had shown higher antioxidant properties compared with the standard (Singh *et al.*, 2020). In a similar study, the biosynthesis of IONP using aqueous extract of *Penicillium* spp. evaluated the antioxidant activity using the DPPH method. Various fixations of IONPs (0.625 to 160 µg/mL) and the range of percentage of scavenging in (47 to 63%), and the ascorbic acid revealed the percentage of scavenging in (50 to 94%) (Zakariya *et al.*, 2022). A previous study, *Rhamnus virgata* using Fe_2O_3NPs showed antioxidant activity against DPPH assay, with the fixed concentration range from (1 - 200 µg/mL) and the higher concentration (200 µg/mL) showed 79.4% scavenging (Abbasi *et al.*, 2019). In a similar study, the green synthesis of iron oxide nanoparticles using *Hydrocotyle umbellate* was determined in the embryonic toxicology using zebrafish embryos. The hatching % of 100% in the least fixation 80% in higher fixation and the viable % of the least concentration is 100% and the higher concentration is shown in 60% of the viability rate. It was revealed that less toxicity in iron oxide nanoparticles mediated *H. umbellate* (Prabhu, *et al.*, 2024). In the previous research, iron oxide nanoparticles revealed the toxicity range from (10 to 38%) delayed hatching in the fixation range from (40 to 60 ppm), it has a dose-dependent manner (Santhosh *et al.*, 2024; Thirumurthi *et al.*, 2022). In similar research, the metal oxide nanoparticles showed the hatching rate of the zebrafish embryos and the fixation of concentrations in (0.001 to 0.01 ppm). The hatching % of embryos is 100% in 0.001 and 88.3% in 0.01 ppm and the lower hatching rate of the embryos in higher concentrations (Stevens *et al.*, 2024). In the previous study, the *C. sinensis*-mediated metal oxide revealed the toxicology results in a range from (50 to 75% viability rate) and the fixation of metal oxide nanoparticles in (1 to 8 µL). There were no malabsorptions and no somatic formation, it revealed less toxicity (Aardra *et al.*, 2023).

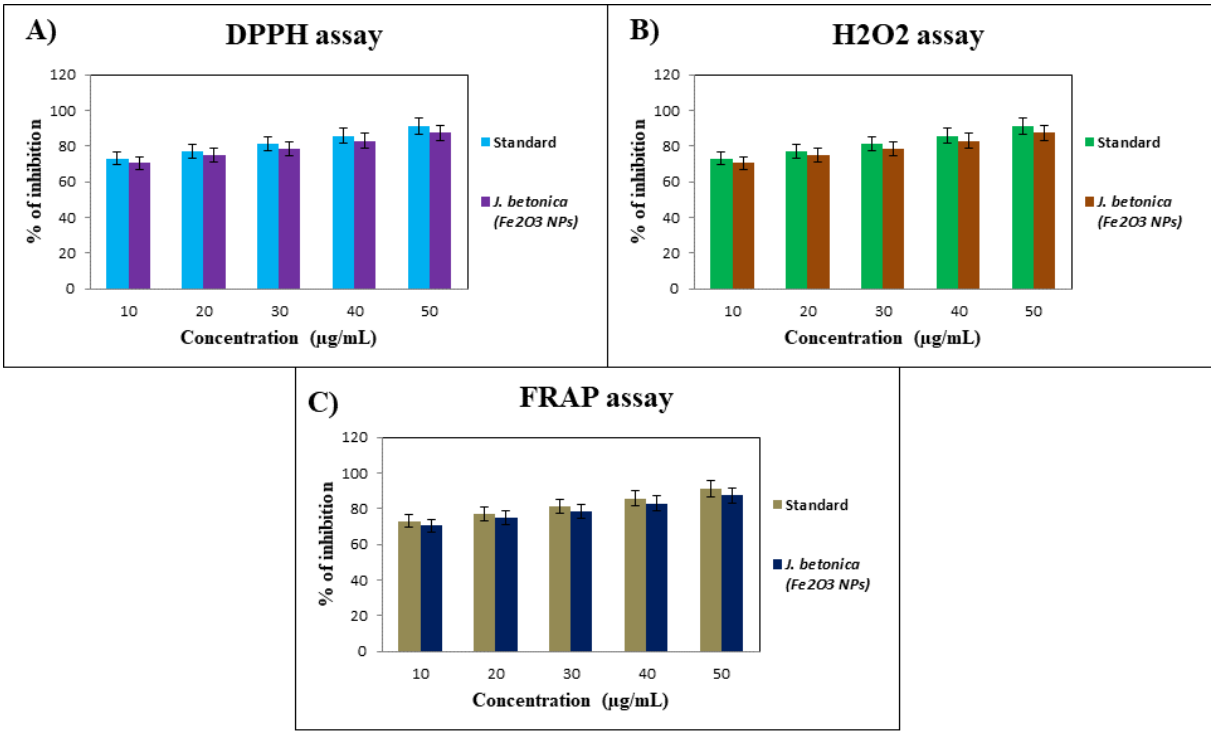

Figure 3: The graphical image represented the antioxidant activity against iron oxide nanoparticles. A) DPPH radical scavenging assay, B) H_2O_2 assay, and C) FRAP assay.

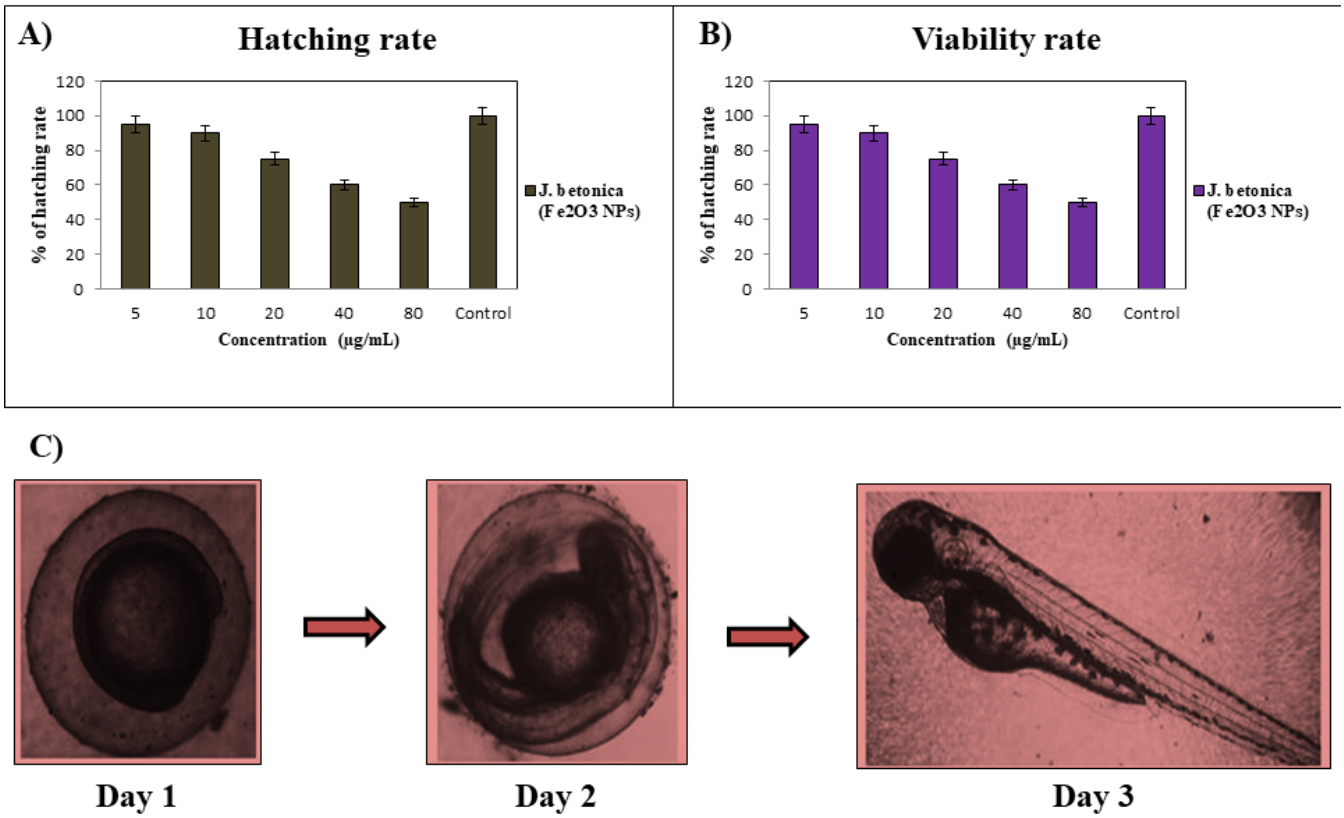

Figure 4: The graphical image was represented the Embryonic toxicology against iron oxide nanoparticles. A) Hatching rate of embryos, B) Viability rate of embryos, and C) Development stage of Zebrafish embryos, Embryonic Toxicology.

CONCLUSION

The green synthesis of iron oxide nanoparticles from *Justicia betonica* is both cost-effective and environmentally friendly, and the resulting nanoparticles are biocompatible in biomedical applications. The herbal substance was utilized to reduce metal ions for nanoparticle synthesis. Iron oxide nanoparticles containing *J. betonica* are another safe drug for antioxidant agents with fewer side effects. As a result, green synthesised iron oxide nanoparticles deliver an exciting potential for remedy in pharmaceutics and therapeutic fields.

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ABBREVIATIONS

Fe (II): Ferric; **Fe (III):** Ferrous; **MRI:** Magnetic Resonance Image; **J. betonica:** *Justicia betonica*; **mM:** Milli Molar; **RPM:** Rotation Per Minutes; **DPPH:** 2,2-Diphenyl-1-Picrylhydrazyl; **EDTA:** Ethylenediamine Tetraacetic Acid; **$FeSO_4$:** Ferrous Sulphate; **Fe_2O_3NP or IONP:** Iron Oxide Nanoparticles; **FRAP:** Ferric Reducing Antioxidant Power; **H_2O_2:** Hydrogen Peroxide; **µg/mL:** Microgram Per Microliter; **µL:** Microliter; **SIMATS:** Saveetha Institute of Medical and Technical Science; **HCl:** Hydrochloric Acid; **M.W.:** Molecular Weight; **hr:** Hour; **ppm:** Parts Per Million.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTIONS

The authors confirm contribution to the paper as follows: Study design and conception was created by Rajeshkumar Shanmugam, Sulochana Govindharaj, Kaavya, Yuvabalaji. Data Collection was carried out by the Sulochana Govindharaj and Yuvabalaji. Analysis and interpretation of results was performed by the Rajeshkumar Shanmugam, Sulochana Govindharaj, Kaavya, and Yuvabalaji. Draft manuscript preparation was executed the Rajeshkumar Shanmugam, Sulochana Govindharaj, and Yuvabalaji. All authors reviewed the results and approved the final version of the manuscript.

SUMMARY

The green synthesis of iron oxide nanoparticles based *Justica betonica,* the iron oxide nanoparticles showed an antioxidant potential and less toxicity in embryonic toxicology. It has biological safety and dose dependent potential for toxicology evaluation. It promising the free radical properties while required the careful evaluation of their development products.

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