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Green Synthesis of Iron Oxide Nanoparticles using Hydrocotyle Umbellata L. and its Toxicology Evaluation

Jyotsna Saradha Prabhu[1], Subramanian RM[2], Sulochana Govindharaj[1], Rajeshkumar Shanmugam[1*]

[1]Nanobiomedicine Lab, Centre for Global Health Research, Saveetha Medical College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Chennai, India
[2]Department of Orthopaedics, Saveetha Medical College and Hospital, Saveetha Institute of Medical and Technical Sciences, Chennai, India
Email: rajeshkumars.smc@saveetha.com

In translational research, nanotechnology is one of the most frequently used methods. Significant emphasis has been paid to the environmentally benign process of developing metallic nanoparticles using biological resources.The purpose of this research is to synthesise Fe2O3 nanoparticles by using the plant Hydrocotyle umbellata in an environment friendly manner. This includes making it less toxic towards the Earth.The cytotoxicity was then assessed using the Brine Shrimp Lethality Assay and a UV spectrophotometer was done. An embryological toxicity evaluation was also done using zebrafish. The plant solution was first prepared using the plant product. Followed by the preparation of the nanoparticle solution. A UV spectrophotometer was done to check the absorbance level. While the brine shrimp lethality assay was done to evaluate the embryological cytotoxicity. The results have been graphically represented and it proves that the iron oxide nanoparticles are indeed an approachable option for various reasons. The toxicity levels were low in higher concentration and the mortality rate also, which proves to be of immense advantage. This study concludes that iron oxide nanoparticles that were synthesised using the plant Hydrocotyle umbellata was less toxic and has been proving to be of various advantages.

Keywords: Green synthesis, Iron oxide nanoparticles, nanotechnology, toxicity, Zebrafish embryos.

1. Introduction

The word “Nano” from the word nanoparticles is derived from the Greek word ‘nanos’ meaning ‘dwarf’. The most distinguished nanoparticles are heavy metals such as nickel, cadmium, manganese, zinc, titanium, iron, gold, and silver (Bhardwaj et al.,2023).

Nanoparticles are particles that are unique in size as they range from 1 to 100 nm. Ultra small size and their large volume are the major factors on which the reactivity of these nanoparticles depend on. The transition between bulk and atomic particles or minute size particles shows the sole existence of nanoparticles. The surface, shell and core are the three layers of the nanoparticles (Wang et al., 2009). The major applications of metallic nanoparticles are in the field of biotechnology, electronics, packaging and cosmetics (Sardar et al., 2014). Some microorganisms are responsible for the formation of nanoparticles in extreme conditions. Nanoparticles produce different kinds of minerals to help reduce the toxicity of chemical species (Ameen et al., 2021). In today's modern world various, physico - chemical and environment friendly methods are present to synthesise these nanoparticles. Some of these methods are gel-sol method, sonochemical and hydrothermal methods (Mahesh 2023).

Iron Oxide Nanoparticles have shown and proven promising effects in the field of biomedicals because of their special physico-chemical features. They have proven to be highly biocompatible and their magnetic properties make it possible to manipulate it by an external magnetic field (Laurent et al., 2010). Characterization of these nanoparticles is essential to understand the stability and functionality of these particles. In order to understand the distribution of various sizes and morphology of these nanoparticles, X- ray diffraction along with Transmission Electron Microscopy was used (Manaia et al., 2017). The synthesis of these particles depends on the decomposition of organometallic precursors. It has been proved that decomposition has successfully assisted in the preparation of monodisperse nanoparticles (Cui et al., 2009). Nanoparticles of iron oxide help in the immobilisation of heavy metals, redox potential, and mainly the variation of pH. The procurement of nanoparticles at large quantities are found in places with conditions like low temperature and places where the supersaturation of different elements is at a higher degree. The various kinds of nanoparticles are Ferrihydrite, Goethite, Hematite, Magnetite and Maghemite (Xu et al., 2013).

Hydrocotyle umbellataL, is a perennial water plant, which is commonly known as Pennywort. This plant is mostly found in fresh or brackish water in ponds. The roots are sunken below the water and its petioles or stalks float on the surface and its leaves float above the water level (Rocha et al., 2011). This plant has the phytoremediation potential against various heavy metals because of its filiform roots, fast growth and stoloniferous reproduction. The main advantages of Hydrocotyle Umbellata L, its phytochemical extraction ability which is possible due to its filiform roots and its rapid growth in marshy and marginal areas. It has the ability to metabolise both organic and inorganic substances proving to be beneficial. It has also been used as a waste water purifier in sewage, it helps in the elimination of chromium from textile waste (Saeed et al., 2024). The main purpose of green chemistry is to develop products in a way that prevents the environment from getting polluted and to reduce the amount of waste products. The use of biomaterials is a huge replacement for hazardous substances (Saito et al., 2014). The toxicity of nanostructured materials has created a major concern in all the fields. Iron oxide nanoparticles (IONP) were coated to help reduce the level of cytotoxicity towards the environment (Valdiglesias et al., 2015). Studies prove that green synthesis of nanoparticles is less toxic towards the environment. The cellular extracts from the biological organisms can be used to synthesise nanoparticles of different size and chemical composition (Shah et al., 2015). There are two major ways to synthesise nanoparticles, one is the "Top-Down Approach" and the "Bottom-Up Approach". The Top-Down Approach mainly focuses on

producing nanoparticles by reducing the size of the particles using physico-chemical methods. Bottom-Up Approach focuses on the production of nanoparticles with the main reactions being either oxidation or reduction. The Bottom-Up Approach is considered as a greener way to synthesise nanoparticles (Arole et al., 2014).The main motto of this study is to synthesise iron oxide nanoparticles using the water plant Hydrocotyle umbellata L. This study also studies the cytotoxicity activity of the nanoparticles using zebra fish.

2. Materials and Methods

The preparation of Hydrocotyle umbellata L mediated iron oxide nanoparticles was done in two steps. One, the preparation of the plant extract and two, the preparation of the nanoparticle solution.

2.1 Preparation of Plant Extract

The plant extract was prepared by the addition of 1g plant powder mix to 100 ml of water and the solution boiled at 50 to 60°C for 15 to 20 mins using a heating mantle. The solution was then filtered using a Whatman Filter paper. The filtrate was extracted and then stored in the refrigerator.

2.2 Preparation of Nanoparticle Solution

The preparation of the nanoparticle solution was done by adding 0.486g of iron chloride to 50mL of distilled water along with 50 mL of the prepared plant extract. The final solution was then placed in an Orbital shaker for the following 48 hours. The solution was checked periodically for any obvious colour changes, followed by UV reading to verify the synthesis of nanoparticles in various different intervals of time. The final solution was transferred into the sterile centrifuge tubes, the centrifugation process for 10 mins at 8000 rpm. The formed supernatant was removed and the pellet formed is collected for further biomedical research.

2.3 Cytotoxic Effect

2.3.1 Brine Shrimp Lethality Assay:

To begin with, 2g of iodine free salt was dissolved in 200mL of distilled water to form the salt solution. An ELISA plate with 6 wells was taken and it was filled with 10-12 ml of saline water. Then 10 nauplii were added slowly to each well of various concentrations such as 5 µg/mL, 10 µg/mL, 20 µg/mL, 40 µg/mL, and 80 µg/mL. This was followed by the addition of nanoparticles according to the concentration level of each well. All plates were incubated for the following 24 hrs. Post 24 hrs, the plates were observed and the number of live nauplii was calculated using the given formula, Number of dead nauplii / number of dead nauplii + number of live nauplii x 100. This method was followed by (Chandran et al., 2024, Shanmugam et al., 2024, and Singh et al., 2023).

2.4 Zebrafish embryonic toxicology evaluation of iron oxide nanoparticles

2.4.1 Fish maintenance and IONPs exposure

Danio rerio, a wild type zebrafish was purchased from a local Indian vendor for the purpose of analysing the toxicity of Iron oxide nanoparticles using Hydrocotyle umbellata L. The

zebrafishes were accommodated in individual tanks, with the temperature being controlled at $28^0 \pm 2^0$C, the pH between 6.8-8.5 maintaining a neutral solution and finally, the light-dark cycle was maintained at the ratio of 14:10 hrs. The fishes were fed twice a day with either dry blood worms or optimum food. After a few days, each female was crossed with three males in separate breeding tanks and the embryos were collected. Out of all the embryos collected the most viable ones were collected and rinsed with freshly prepared E3 medium for a minimum of three times without the use of methylene blue. The collected fertilised eggs were placed in a culture plate of sizes 6, 12 and 24 wells. In each well 20 embryos were placed per 2mL solution. This was followed by replicating the experimental treatments and control groups three times. In order to prepare an experimental treatment, a stock suspension of IONPs with concentrations of 5 different types was freshly produced and directly added to the E3 medium. After which for 15 mins the solution was sonicated to disperse the nanoparticles at the same time maintaining the pH at the range of 7.2-7.3. Out of all the embryos collected, the healthy ones were filtered and were then exposed to different concentrations of Iron Oxide Nanoparticles such as 5 µg/mL, 10 µg/mL, 20 µg/mL, 40 µg/mL and 80 µg / mL. The embryos were exposed to the IONPs for the next 24 to 96 hours post fertilisation. Following this, the IONPS was then added to the E3 medium, and incubation of the embryos took place. The control groups for this study were done by removing the dead embryos from the nanoparticles for every 12 hours. The experimental plates were wrapped in foil to prevent the exposure of sunlight and the temperature was constantly maintained at 28°C.

2.4.2 Zebrafish embryo evaluation

A stereo microscope was used to monitor the stages of the zebrafish embryo's development after fertilisation. The embryos were exposed to different concentrations of IONPs. Concentrations were 5 µg/mL, 10 µg/mL, 20 µg/mL, 40 µg/mL and 80 µg/mL. The exposure was done for 24 to 78 hours in a high power field. Rates of mortality and hatching were monitored on a regular basis of 24 hours. The endpoints of the study included the hatching and mortality rate of the embryo. All malformations and identifications were documented from both control and treatment groups. A COSLAB- Model: HL-10A light microscope was used to take photographs of the malformed embryos. All changes were documented in an interval of 24 hrs.

3 Results

3.1 Preparation of iron oxide nanoparticles

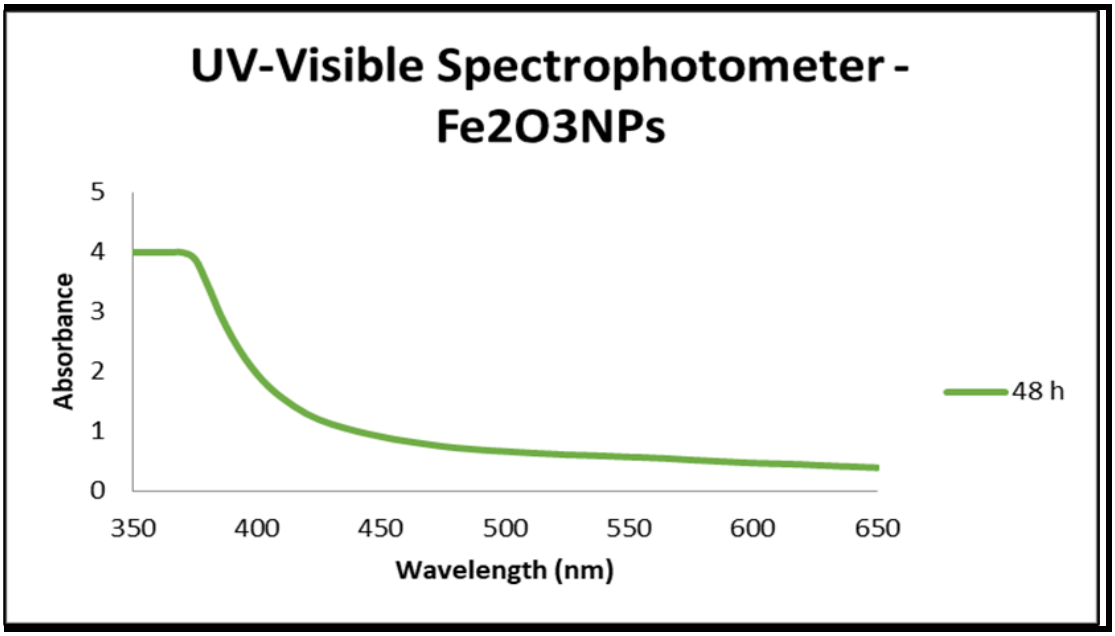
Initially, the color changes in the solution were observed through visual observation of the green synthesis of iron oxide nanoparticles employing H. umbellata (Figure 1). Both the plant extract and the precursor solution for nanoparticles are included in the reaction mixture. Iron (ion) was produced by a reaction between the phytochemical ingredient of the plant extract and iron chloride, which was utilized as a reducing agent for the metal oxide nanoparticles. From brown to dark brown, the iron oxide nanoparticles showed their original shade. The synthesized nanoparticles should be validated.

Figure 1: Green synthesis of iron oxide nanoparticles A) H. umbellata B) Boiling of Extract, C) Reaction mixture (Iron chloride solution + H. umbellata extract), and D) green synthesis of iron oxide nanoparticles.

A B C D

3.2 UV-visible Spectroscopy of Iron oxide nanoparticles.

Figure 2: UV - Visible Spectrophotometer of Iron Oxide Nanoparticles



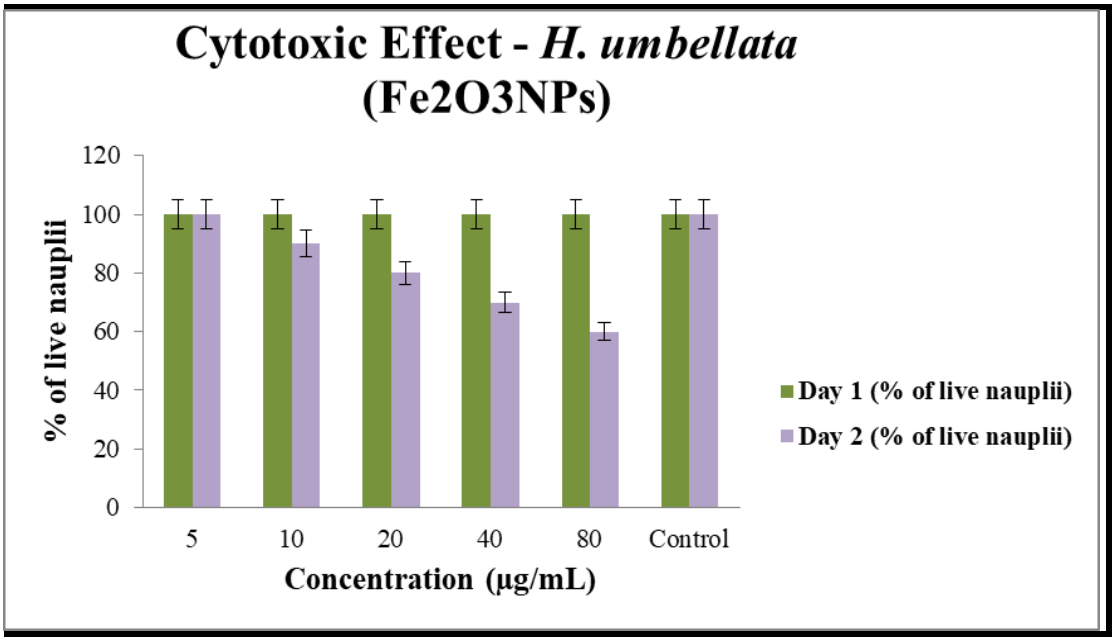
The (Figure 2) given UV - visible Spectrophotometer showed the absorbance level at 48 hrs. It was seen that at the wavelength of 375 (nm) nanometers, the absorbance of Iron oxide Nanoparticles was high. The maximum peak was observed and confirmed the synthesised nanoparticles solution.

3.3 Cytotoxic Effect

The cytotoxic effect of the synthesised Hydrocotyle umbellata mediated iron oxide

nanoparticles was evaluated, live nauplii was used. Various concentrations such as 5 µg/mL, 10 µg/mL, 20 µg/mL, 40 µg/mL, and 80 µg/mL were used to test its toxicity. Graph 1 shows the results of the cytotoxic analysis. Along with the different concentrations a control group was also present. No significant changes were seen when 5% of Hydrocotyle umbellata L. mediated iron oxide nanoparticles were added to the live nauplii. A gradual increase in the death rate of the nauplii was recorded as the concentration of the synthesised iron oxide nanoparticles was increased. No change was seen in the control group. A colour was seen from pale brown to dark brown.

Graph 1: Cytotoxic Effect of Hydrocotyle umbellata L mediated Iron oxide nanoparticles against Brine Shrimp Lethality Assay.



The above graph depicts the cytotoxic effect of Hydrocotyle umbellata L mediated IONPs. Day 1 results were depicted in green colour and the day 2 results were depicted using purple. On day 1, the percentage of nauplii at 5 µg/mL remained at 100% but as the concentration increased there happened to be a slight decrease in the percentage of live nauplii. On day 2, the percentage of live nauplii in 5 µg/mL, showed no significant changes were seen. In 10 µg/mL, 20 µg/mL, 40 µg/mL, 80 µg/mL the percentage of live nauplii is 90%, 80%, 70% and 60% respectively. A control group was also used which showed no change on day 1 and on day 2. This proved that the cytotoxicity that led to the decrease of nauplii was due to the iron oxide nanoparticles. This graph concludes that the nanoparticles have a concentration dependent relation with the nauplii. 5 µg/mL and 10 µg/mL show very less toxic effect, hence it has been proved that iron oxide nanoparticles can be used in various applications.

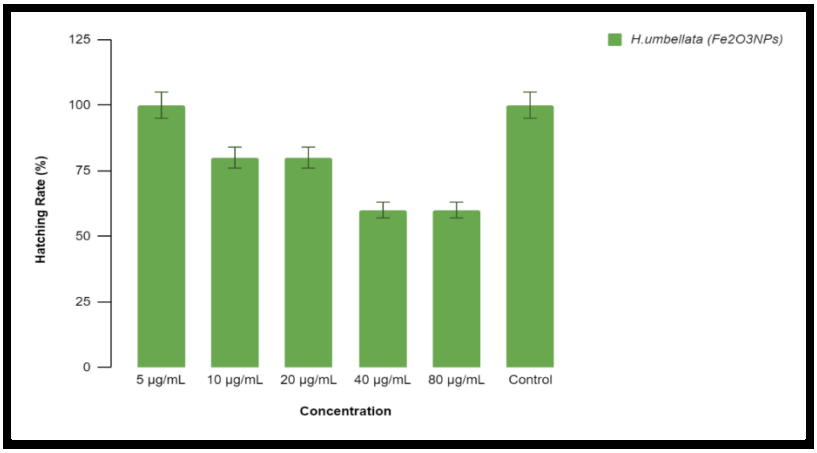
3.4 Embryonic toxicology evaluation.

3.4.1 Hatching rate of the zebra fish embryos

The above graph depicts the hatching rate of zebrafish embryos with respect to the

concentration of iron oxide nanoparticles. At 5µg/mL the 100% of hatching rate was observed and Increased the amounts of concentrations of iron oxide nanoparticles such as 10 µg/mL, and 20 µg/mL have a hatching rate of 80% while 40 µg/mL and 80 µg/mL have a hatching rate of 55% (Graph 2) respectively. The used control group showed no changes (100 % of hatching rate).

Graph 2: It shows that the Hatching rate of zebra fish using iron oxide nanoparticles



3.4.2 Viability rate of the zebra fish embryos.

The above graph depicts the viability rate of zebrafish embryos with respect to the concentration of iron oxide nanoparticles. At 5µg/mL and 10 µg/mL showed the 100% viability rate and the Increased amounts of concentrations such as 20 µg/mL, and 40 µg/mL have a viability rate of 80% while 80 µg/mL have a hatching rate of 60% (Graph 3). The used control group showed no changes (100 % of viable rate). There was not revealed the abnormalities and malnutritions such as tail bend and any edema inflammations (Figure 3).

Graph 3: Itshows the Viability rate of zebra fish using iron oxide nanoparticles

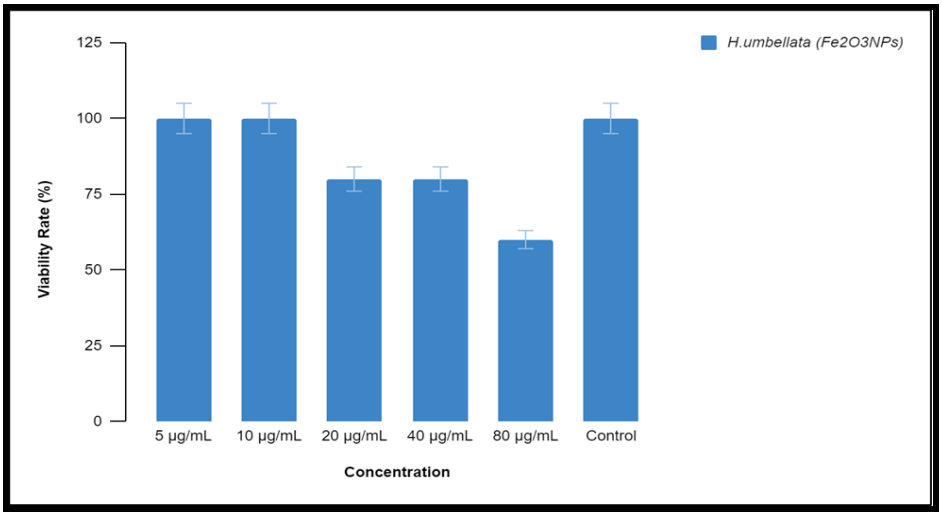
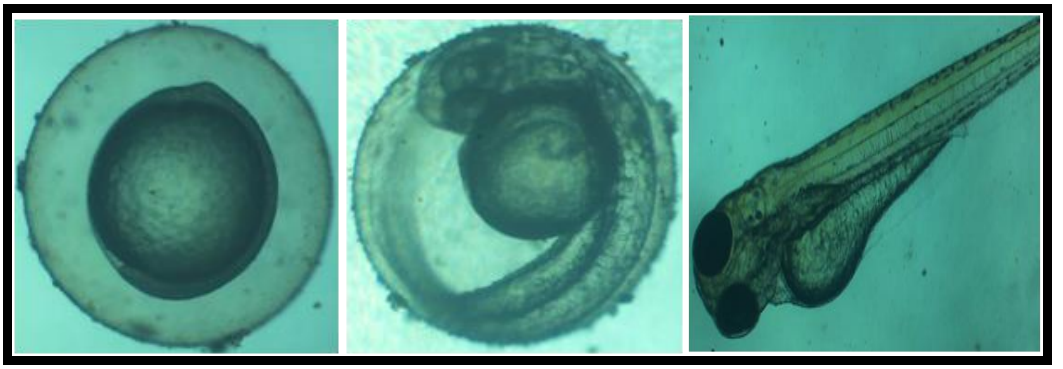


Figure 3: The image represents the zebrafish embryo's development with the loading of iron oxide nanoparticles in the zebrafish embryos, A) Day 1, B) Day 2, and C) Day 3.



A B C

4 Discussion

The present study observed the green synthesised iron oxide nanoparticles in colour change reaction, the pale brown to darkish brown. In a previous study, the green synthesised silver and iron oxide nanoparticles were noted to change from dark black to dark brown (Shejawal et al., 2020). The maximum peak was confirmed by the synthesised nanoparticles, the current research the maximum peak was noted in 375 nm. In previous studies, the synthesis of iron oxide nanoparticles using various plant extracts showed a similar absorption pattern. A strong absorbance was seen at around 370-380 nm by the iron oxide nanoparticles. P. Serrulata leaves indicated absorption peaks at 280 nm and 284 nm, respectively Şengönül, H., & Demircan, O. (2024). Another study reported that the synthesis of iron oxide nanoparticles also displayed a similar high absorbance at 375 nm. A reduction in the absorbance was shown with the increase in wavelength (Singh et al., 2021). The surface plasmon resonance (SPR) band's typical center is approximately 425 nm, as seen by the plot. This band shows how metal nanoparticles are forming. Metal nanoparticle production was additionally assisted by the emergence of a yellowish-brown shade (Alexeree et al 2024).

In cytotoxic effect, green synthesized iron oxide nanoparticles observed the toxicity using brine shrimp lethality assay. It was noted the higher concentration also less toxicity. Similarly, Ocimum tenuiflorum and Ocimum gratissimum-mediated green synthesis processed metal nanoparticles showed the toxicity level in higher concentration 40 and 80 µg/mL had 50 % of live rate (Varghese et al., 2024). In other hands, The effectiveness of ginger and lemongrass extract-infused copper oxide nanoparticles against brine shrimp at various doses over a day. A comparison of 40µl and 80µl with the control reveals that 5µl, 10µl, and 20µl have strong cytotoxic activity. This indicates that when copper nanoparticles mediated by ginger and lemongrass are utilized to verify the fatal assay, there may be a cytotoxic effect present (Mohamed et al., 2023).

The green synthesis of iron oxide nanoparticles was analysed in embryonic toxicology using zebrafish embryos, the toxicity was noted to be dose dependent. In previous work, camellia

sinensis mediated metal oxide nanoparticles was observed the hatching rate of the embryos in the addition of the nanoparticles, 16 µl, 8 µl, 4 µl, 2 µl, and 1 µl and the hatching rate was 25%, 38%, 50%, 60%, and 65%. The metal oxide nanoparticles had reduced the hatching rate (Aardra et al 2023). In other research work, the ethanolic extract of Croton bonplandianum had a less toxicity in higher concentration 80 µg/mL in 50 % of viability rate. There was a lack of morphological observation and abnormalities (Shanmugam et al., 2024).Overall, all the assays done have proved that the iron oxide nanoparticles are less toxic than what has been done in previous studies using various other plants. Thus, proving to be a better advantage than the other studies.

5 Conclusion

The synthesis of nanoparticles using Hydrocotyle umbellata L. was done in an environment-friendly manner. From this study it can be concluded that these nanoparticles possess less toxicity. The nanoparticles were alternated to use the medicine for toxicology. It has been proved that it is indeed a green synthesis.

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