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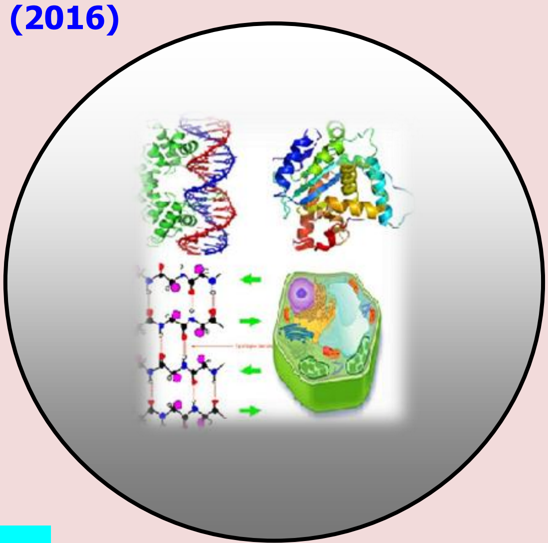

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RESEARCH PAPER

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Green Synthesis and Characterization of Iron Oxide Nanoparticles Using *Clausena indica* Leaf Extract and Evaluation of Antibacterial Activity

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ABSTRACT

A Clausena indica leaf extract mediated process was developed for the synthesis of iron oxide nanoparticles. The green synthesized iron nanoparticles were characterized using UV-visible absorption spectrophotometry (UV-visible), Dynamic light scattering (DLS), X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM) and Transmission electron microscopy (TEM). The results exposed that iron oxide nanoparticles has irregular spherical shape and the size of the iron nanoparticles was in the range of 47.5 nm to 59.5 nm. The antibacterial activity of green synthesized iron oxide nanoparticles were evaluated against eleven bacterial human pathogens. Green synthesized iron oxide nanoparticles exhibited maximum antibacterial activity at a concentration of 30 µg/disc and minimum activity at 10µg/disc concentrations. The present investigation highlights the green synthesized iron oxide nanoparticles can be a potential source for antibacterial agents.
Key words: Clausena indica, Iron Oxide Nanoparticles, Antibacterial Activity and Human Pathogens.

INTRODUCTION

Nanoparticles are clusters of atoms, with sizes ranging between 1 and 100 nm, whereas, a “nano” is used to indicate one billionth of a meter (Sudarenkov, 2013). Synthesis of nanoparticles by various physical (Tosco *et al*., 2014) and chemical (Kassaee *et al*., 2011) approaches end up with several limitations such as toxic chemicals, formation of hazardous by products, large energy requirement, and also expensive (Ali *et al*., 2016). Plant-mediated green synthesis of nanoparticles has attracted intensive research interest because of their eco-friendliness and biocompatibility for various applications like drug delivery, medical diagnostic tools, cancer treatments, agriculture and environment. Plant extracts reduce the metal ions in a shorter time as compared to other green methods. Depending upon plant type and concentration of phytochemicals, nanoparticles are synthesized within a few minutes or hours (Herlekar *et al*., 2014).

Clausena is a genus of the family Rutaceae comprising 15 species. They are shrubs or small trees mainly distributed in South and Southeast Asia. *Clausena indica* (Dalz.) Oliv. is a small tree which grows up to 15 m height. Fruits of *C. indica* are edible and its leaves are used for culinary purposes. According to ethno-medicinal uses, its roots and leaves are used to treat cold, flu, head ache, colic and rheumatism. Essential oil of *C. indica* is also used to massages (Diep *et al*., 2009).

A number of coumarins, indole alkaloids, sesquiterpenes, phenethylcinnamide, four new tryptamine derived amides were reported from the roots and leaves of *C. indica* (Riemer *et al*., 1997). Chemical investigation of volatile oil from the fresh leaves of *C. indica* reported that Sabinene, terpinen-4-ol, γ-terpinene and β-phellandrene were the major constituents (Anil John *et al*., 2011). Consequently, the present research was planned to study the green synthesis and characterization of iron oxide nanoparticles using *Clausena indica* leaf extract and evaluate its antibacterial activity against human bacterial pathogens.

MATERIALS AND METHODS

Collection and identification of plant

Fresh healthy leaves of *C. indica* were collected from Thiruvanamalai Local Park (Figure 1) and were authentically identified by Prof. P. Jayaraman, Institute of Herbal Science, Plant Anatomy Research Centre, West Tambaram, Chennai, India as Rutaceae with voucher specimen number PARC/2015/3146.

Figure 1. Aerial view of *C. indica*

Scientific classification of *C. indica*

Class	:	Magnoliopsida – Dicotyledons
Subclass	:	Rosidae
Order	:	Sapindales
Family	:	Rutaceae
Genus	:	*Clausena*
Species	:	*indica*
Synonyms	:	*Bergera nitida*, *Piptostylis indica*

Preparation of *C. indica* aqueous leaves extract

About 100 g of fresh healthy leaves of *C. indica* were shade dried and the leaves were powdered using kitchen blender. The powdered leaves were soaked in the 200 ml of double distilled water for overnight in a fridge for 4°C and then the rinsed mixtures were boiled for 10 minutes. The extracts were cooled to room temperature and then filtered through Whatman filter paper (No.42).

Synthesis of iron oxide nanoparticles using *C. indica* extract

Iron oxide nanoparticles were synthesized by taking $FeCl_3.6H_2O$ and $FeCl_2.4H_2O$ (1:2 molar ratios) and were dissolved in 100 ml of double distilled water in a 250 ml beaker and heated at 80°C with mild stirring using magnetic stirrer under atmospheric pressure. After 10 minutes, 20 ml of the aqueous solutions of *C. indica* extract was added to the mixture, immediately the light green colour of the *C. indica* extract of the mixture changed to dark black colour. After 10 minutes, 20 ml aqueous solution of sodium hydroxide was added to the mixtures with the rate of 3 ml per minutes for allowing the iron oxide precipitations uniformly.

The mixture was allowed to cool down to room temperature and the iron oxide nanoparticles were obtained by decantation to form magnetite. The magnetites formed were washed three times with double distilled water and three times with ethanol and air dried at room temperature.

Characterization of iron oxide nanoparticles

The absorbance spectra of sample were measured in wavelength within the range from 300-700 nm using a UV-Vis double-beam bio-spectrophotometer Elico-Bl-198. Particle size of magnetic iron oxide nanoparticles was measured by laser diffractometry using a Nano Size Particle Analyzer in the range between 0.6 nm to 6.0 µm. Structure and crystalline size of nanoparticles were determined by XRD using SHIMADZU (Model XRD¬6000). The functional group of nanoparticles were recorded by FT-IR spectroscope (Shimadzu, IR Affinity 1, Japan), with a scan range from 4000 to 500 cm^{-1} with a resolution of 4 cm^{-1}. Morphological analysis of magnetic iron oxide nanoparticles was done using Vega 3 Tescan SEM machine and HR-TEM Jeol model 3010 instrument operated at 200 Kv and a beam current of 104. 1µA.

Screening of antibacterial activity

Bacterial strains

The clinical isolates of *Bacillus cereus*, *B. subtilis*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumonia, Micrococcus luteus*, *Proteus mirabilis*, *P. vulgaris, Pseudomonas fluorescence*, *Staphylococcus aureus* and *Vibrio fluvialis* were obtained from Department of Bacteriology, King Institute of Preventive Medicine, Guindy, Chennai.

Antibacterial analysis by disc diffusion method

The antibacterial activity of green synthesized iron oxide nanoparticles were evaluated using disc diffusion method (Bauer *et al*., 1996). A set of sterile discs (6 mm, Hi-media) were impregnated with different concentrations of iron nanoparticles *i.e.* 10 µg/ disc (10µg/µl), 15 µg/ disc (15µg/µl), 20 µg/ disc (20µg/µl), 25 µg/ disc (25µg/µl) and 30 µg/ disc (30µg/µl) respectively. Subsequently, the culture plates were prepared by pouring 20 mL of Mueller-Hinton agar (MHA) (Hi-media) medium and bacterial suspension swabbed on the medium plates using sterile cotton swab and the plates were kept aside for few minutes. The discs were gently pressed and incubated in inverted position for 24 hours at 37°C. The discs with Norfloxacin (20 µg/ disc) were placed on the MHA plates maintained as positive control. After the incubation period, the susceptibility of the test organisms was determined by measuring the diameter of the zone of inhibition using Himedia zone scale and the obtained results were tabulated for evaluation.

RESULTS AND DISCUSSION

Green synthesis of iron oxide nanoparticles using *C. indica* extract

In the present investigation, the iron oxide nanoparticles were synthesized by greener method using *C. indica* aqueous extract. Similarly, the green synthesis of iron oxide nanoparticles of various sizes and shapes has been reported using plant extracts such as *Musa ornate* (Saranya *et al*., 2017), *Averrhoa bilimbi* (Rosli *et al*., 2018), *Juglans regia* (Izadiyan *et al*., 2018), *Platanus orientalis* (Devi *et al*., 2018) and *Asparagus racemosus* (Vijay Kumar *et al*., 2018). The formation of iron oxide nanoparticles was confirmed by carefully observing the color change during the reaction. The *C. indica* aqueous extract was pale green colour, which turned to black colour after the addition of the Fe^{+3} and Fe^{+2} to the plant extract and addition of NaOH solution as a reducing agent.

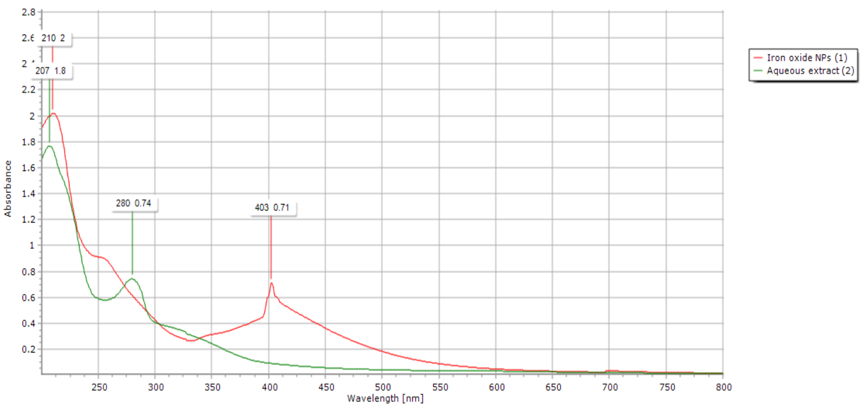

Figure 2. UV-Visible spectrum of green synthesized iron oxide nanoparticles and *C. indica* aqueous extract.

UV-Visible spectroscopy analysis

UV-Visible spectroscopy is a valuable tool for structural characterization of iron oxide nanoparticles. It is well known that the optical absorption spectra of metal nanoparticles are dominated by surface plasmon resonances (Mahdavi *et al*., 2013). From the result in figure 2, it was observed that the two absorption peaks was found at 207 and 280 nm in plant extracts. Peak observed to be at around 210 nm and 403 nm due to surface plasma resonance of synthesized iron oxide nanoparticles. These findings are analogous with the UV – Visible spectrum of iron oxide nanoparticles synthesized by *Averrhoa bilimbi* showed absorption peaks at the range of 440 and 465 nm (Rosli *et al*., 2018). Previous studies suggested iron oxide nanoparticles that synthesized from plant extracts showed absorption peaks between the ranges of 400 nm to 500 nm in UV-visible spectrum (Mittal *et al*., 2013).

DLS analysis

The size distribution and colloidal stability of iron oxide nanoparticles were characterized DLS. The particle size distribution of green synthesized iron oxide nanoparticles are given in figure 3. The average size of iron oxide nanoparticles is found to be below 50 nm. Similarly, Makarov *et al*. (2014) reported the average size of iron oxide nanoparticles synthesized by *Hordeum vulgare* extract observed in DLS is up to 30 nm.

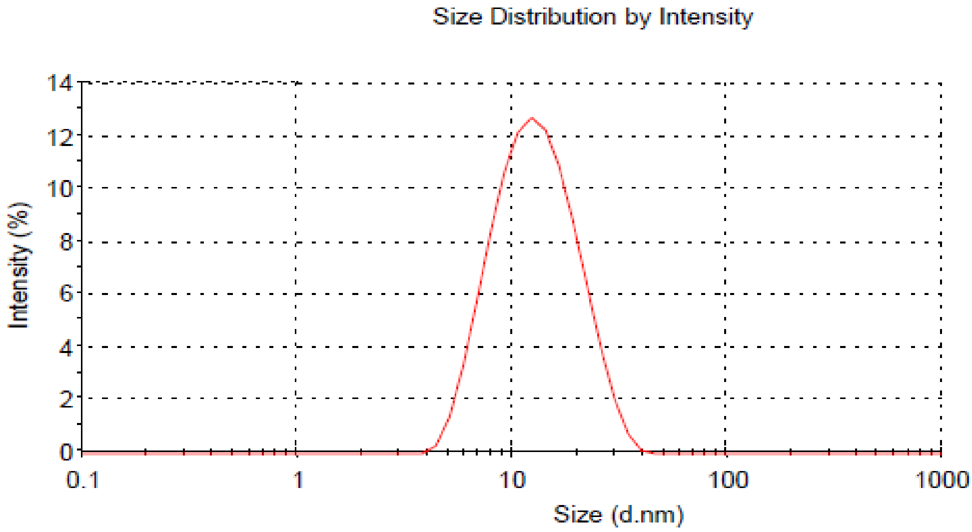

Figure 3. Particle size analysis of iron oxide nanoparticles synthesized by *C. indica* aqueous extract

XRD analysis

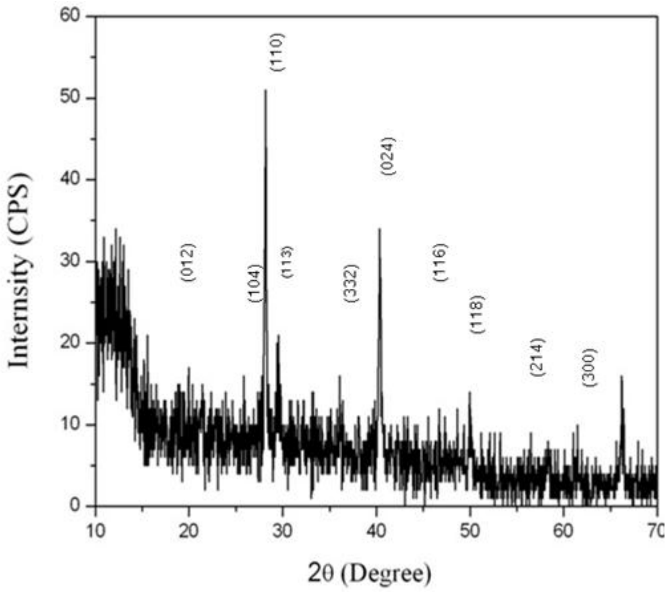

Figure 4. XRD patterns of iron oxide nanoparticles synthesized by *C. indica* aqueous extract FT-IR analysis.

The phase, purity and crystallite size of the iron oxide nanoparticles were studied by XRD. XRD pattern of iron oxide nanoparticles prepared by *C. indica* is shown in Figure 4. The powder XRD pattern of the prepared iron oxide shows that they held a rhombohedral crystal structure. The major strong characteristic peaks of iron oxide nanoparticles were corresponding to crystal faces (012), (104), (110), (113), (332), (024), (116), (118), (214) and (300) of iron oxide (JCPDS.NO.89-8104). Likewise, Malarkodi *et al*. (2018) reported the XRD pattern of the peaks reveals that the iron oxide nanoparticles synthesized by *Emblica officinalis* extract are crystalline in nature and rhombohedral structure.

FT-IR analysis

Figure 5 interpreted the FT-IR spectra of synthesized iron oxide nanoparticles from *C. indica* extract. The absorption peaks in FT-IR spectra of nanoparticles were located mainly at 3320.9 cm^{-1}, 2199.5 cm^{-1}, 2086.5 cm^{-1}, 1992.9 cm^{-1} and 1628.4 cm^{-1}.The peak at 3320.9 cm^{-1} is due to strong –OH bond stretching vibrations, 2199.5 cm^{-1} for C≡N stretching vibrations, 2086.5 cm^{-1} for aliphatic C-H stretching, 1992.9 cm^{-1} for C-C multiple bond stretching and 1628.4 $cm^{-1}$for conjugated carbonyl (–C=O) group stretching vibration and O-H bending. These identified functional groups are reported earlier by FT – IR analysis of various green synthesized iron oxide nanoparticles (Rosli *et al*., 2018; Vijay Kumar *et al*., 2018).

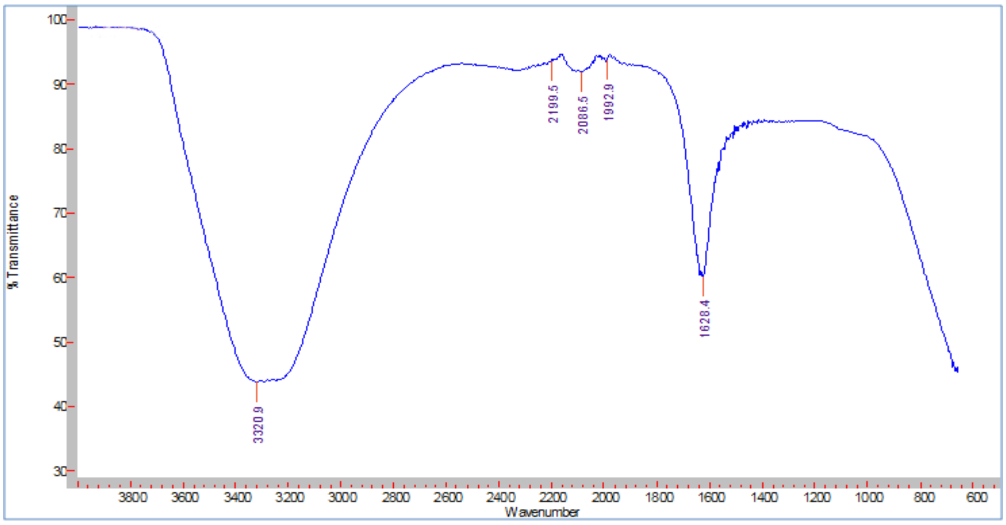

Figure 5. FT-IR spectra of iron oxide nanoparticles synthesized by *C. indica* extract

SEM analysis

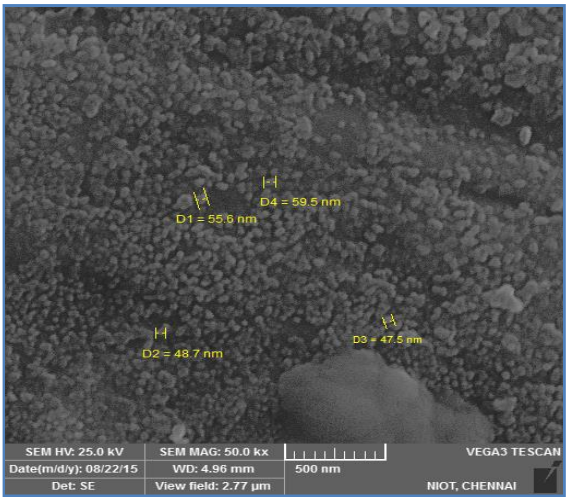

Figure 6. SEM image of the green synthesized iron oxide nanoparticles

The morphology of the green synthesized iron oxide nanoparticles were investigated by SEM. SEM images (Figure 6) revealed that the synthesized iron oxide nanoparticles were aggregated as quasi spherical shapes and range from 47.5 nm to 59.5 mm in size. These findings are in agreement with the results of Vijay Kumar *et al*. (2018) who reported the morphology of the α-Fe_2O_3 has a spherical shape, and the size of the product is rather uniform with diameter of 30-40 nm. Moreover, Veeramanikandan *et al*. (2017) analyzed the morphology of green synthesized iron oxide nanoparticles showed irregular rhombic shapes with panoramic view and range from 117 µm to1.29 mm in size.

HR-TEM analysis

The TEM image of green synthesized iron oxide nanoparticles is shown in figure 7 and revealed the successful synthesis of nanosized particles. It is clearly evident that the particles appear quasi spheroidal with the average core diameter of 50 nm. Similarly, Rengasamy *et al*. (2016) reported that the size of the iron nanoparticles synthesized by *Ricinus communis* leaf extract was in the range of 10 to 35 nm by TEM analysis. In addition, Malarkodi *et al*. (2018) reported TEM image of iron oxide nanoparticles synthesized by *Emblica officinalis* revealed the aggregation of rod shaped of iron oxide crystallite with diameter ranging between 20 nm to 100 nm.

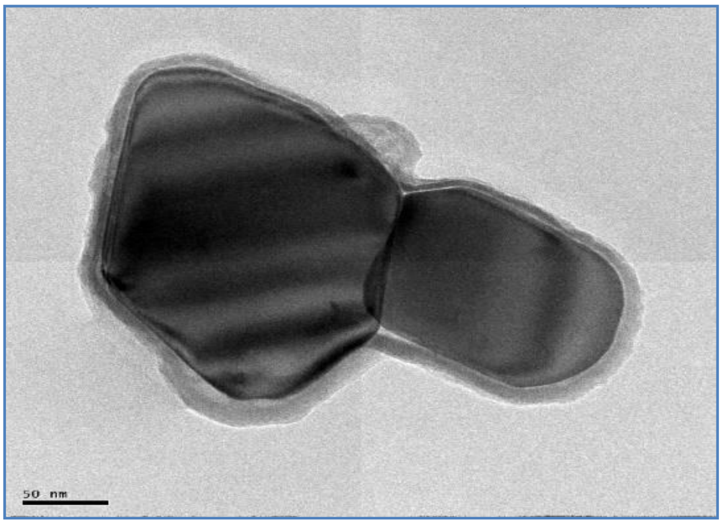

Figure 7. HR - TEM image of the iron oxide nanoparticles synthesized by *C. indica* extract.

Table 1. Antibacterial activity of iron oxide nanoparticles synthesized by *C. indica* aqueous extract

Name of the bacterial pathogens	Green synthesized magnetic iron oxide nanoparticles					Standard antibiotic
	10 µg/disc	15 µg/disc	20 µg/disc	25 µg/disc	30 µg/disc	Norfloxacin 20 µg/ disc
	Zone of inhibition (Diameter in mm)					
Bacillus cereus	12±0.7	13±0.4	15±0.8	18±0.7	20±0.8	12±1.4
Bacillus subtilis	8±1.6	9±1.0	9±1.0	10±1.6	10±1.6	15±0.7
Enterococcus faecalis	13±0.8	15±0.5	17±1.4	18±1.0	19±0.7	20±1.6
Escherichia coli	7±1.0	12±1.0	13±1.0	15±0.9	16±1.0	15±1.6
Klebsiella pneumonia	7±0.7	9±1.0	11±1.6	10±1.6	9±1.0	14±1.6
Micrococcus luteus	10±1.4	13±1.0	15±1.2	16±0.8	18±1.0	12±0.7
Proteus mirabilis	9±1.0	12±0.7	11±1.2	10±2.5	11±0.7	14±0.7
Proteus vulgaris	8±0.7	10±1.0	15±1.0	16±1.6	18±1.0	10±0.7
Pseudomonas fluorescence	13±1.0	14±1.6	16±1.1	17±1.0	18±2.0	23±2.3
Staphylococcus aureus	8±1.6	9±1.0	10±2.1	12±1.4	15±1.6	13±0.8
Vibrio fluvialis	9±1.2	10±1.0	10±1.2	11±2.2	11±1.7	15±1.0

Screening of antibacterial activity of iron oxide nanoparticles synthesized by *C. indica* aqueous extract

The antibacterial properties of green synthesized iron oxide nanoparticles were evaluated against Gram positive and Gram negative bacterial strains using agar well diffusion method. Antibacterial activity results of green synthesized iron oxide nanoparticles were given in Table 1. Of the bacterial stains tested, iron oxide nanoparticles strongly inhibited the growth of *Bacillus cereus* (20 mm), *E. faecalis* (19mm), *M. luteus, P. vulgaris* and *P. fluorescence* (18 mm) at a concentration of 30µg. On the other hand, iron oxide nanoparticles moderately inhibited the growth of *E. coli* (16 mm) and *S. aureus* (15 mm) at a concentration of 30 µg. These nanoparticles showed a low inhibitory effect on the growth of *B. subtilis* (10 mm), *K. pneumonia* (9 mm), *P. mirabilis* and *V. fluvialis* (11 mm). The zone of inhibition produced by the commercial antibiotic, Norfloxacin (20 µg/ disc), was between 10 to 23 mm and was larger than those produced by iron oxide nanoparticles. These results are agreement with of findings of Veeramanikandan *et al*. (2017) who reported green synthesis of iron oxide nanoparticles using *Leucas aspera* leaf extract exhibited considerable antibacterial activity.

The concentration of synthesized iron oxide nanoparticles was a main factor for antibacterial activity of the nanoparticles. Correspondingly, in a study of antibacterial properties of iron oxide nanoparticles synthesized by *Punica granatum* peel extract against *Pseudomonas aeruginosa* reported by Irshad *et al*. (2017) showed concentration dependent bacterial inhibition. In addition Kiruba Daniel *et al*. (2013) used leaf extract of the evergreen shrub *Dodonaea viscosa* to synthesize iron nanoparticles and synthesized nanoparticles showed significant antibacterial activity against human pathogens namely, *E. coli, K. pneumonia, P. fluorescens, S. aureus*, and *B. subtilis*.

CONCLUSION

In this study, iron oxide nanoparticles were successfully synthesized using *C. indica* aqueous extract, the iron oxide nanoparticles possess unique shape and size conformed by XRD, SEM and TEM. The results from the study suggest that the green synthesized iron oxide nanoparticles showed considerable antibacterial activity against different bacterial pathogens. They could be used as alternatives to common antimicrobial agents for treatment of bacterial infections.

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