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Evaluation of Antibacterial and Cytotoxic activity of Green Synthesized Cobalt Nanoparticles using *Raphanus sativus var. longipinnatus* Leaf Extract

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Abstract: In the present study cobalt nanoparticles were synthesized by an ecofriendly and cost effective method using *Raphanus sativus var. longipinnatus* leaf extract and characterized using various techniques such as UV-visible spectrophotometry, Fourier transform infrared spectrometry and Scanning electron microscopy coupled with Energy dispersive micro analysis. The spectroscopic methods confirmed the formation of cobalt nanoparticles and the microscopic technique confirmed the shape and size of the cobalt nanoparticles as spherical with an average particle size of 80nm. Antibacterial activity of the synthesized nanoparticles was measured by disc diffusion method. The cobalt nanoparticles showed effective antibacterial activity against Gram negative bacteria.
Keywords: Cobalt nanoparticles, antibacterial activity, Gram negative bacteria.

1. Introduction:

Nanoparticles are the nano-sized particles[1,2] which have found various applications in the fields of medicine [3,4,5,6], biology [7,8,9,10], catalysis [11,12,13]etc. The nanoparticles can be synthesized by physical, chemical or biological method. Cobalt nanoparticles (CoNPs) can be synthesized by various approaches like ultrasonic spray pyrolysis, DC magnetron sputtering [14], thermal decomposition [15], electrochemical [16] and Liquid-Phase Reduction[17] process and also by biological methods such as microbial synthesis [18] of nanoparticles.

Plant mediated biological synthesis of nanoparticles is gaining importance due to its simplicity, cost effective and eco-friendliness [19,20]. CoNPs could be efficient nanoparticles as they possess good catalytic [21,22] and high performance permanent magnetic properties [23,24] and also possess biomedical [25] and cytotoxic [26] activity.

Raphanus sativus var. longipinnatus (Radish) is a common vegetable crop in Asia. It has large amounts of vitamin B and C as well as pectin, phytin, manganese, iron and copper. Leaves are used to treat dysentery, asthma, cough, diarrhea and malnutrition [27]. It contains ferulic acid, gentisic acid, raphanusin, erucic acid, sinapate, raphanin and sulforaphen. The seeds are carminative, diuretic and laxative. Roots have been used for treating syphilis, haemorrhoids, gonorrhea, cancer and urinary complaints[28]. In this investigation, plant mediated synthesis of CoNPs were carried out using aqueous leaf extract of *Raphanus sativus var. longipinnatus* and characterized using UV-visible spectroscopy, Fourier transform infrared spectroscopy and scanning electron microscopy, and Energy Dispersive X- RayMicroanalysis.The antibacterial activities of the synthesized CoNPs have been investigated against Gram negative bacteria and also evaluated

for their cytotoxic activity on HeLa cell lines. Results show that the reported CoNPs are having bactericidal and cytotoxic activities. Plant mediated synthesis of cobalt nanoparticles has not been reported so far.

2. Material and Methods:

2.1 Plant Collection and preparation of leaf extract and 10mM Cobalt acetate solution:

Raphanus sativus var. longipinnatus leaves were collected from the local market, Telangana, India (Fig 1A). The leaves were rinsed profusely with distilled water followed by Milli Q water to remove the dust and other contaminants, then dried at room temperature (37°C) in shade to remove the moisture. To prepare a leaf extract, 15gms of green leaves was added to 90ml of Milli Q water and incubated on hot plate at 60°C for 10min. After cooling the extract was filtered using Whatman No.1 filter paper and the filtrate was stored at 4°C for further use (Fig 1B).To prepare 10mM cobalt solution, 0.291 gms of cobalt acetate (AR grade) was mixed in 100ml of Milli Q water and stored in a bottle.

2.2 Synthesis of Cobalt Nanoparticles using the Radish Leaf Extract:

The aqueous leaf extract of *Raphanus sativus var. longipinnatus* and 10 mM Cobalt acetate solution were mixed in the ratio of 1:5 and incubated on hot plate at 40°C for 90 min until change in colour was observed.

2.3 Characterization of Cobalt Nanoparticles:

2.3.1 UV-vis spectrophotometry:

An Elico 159 UV–visible spectrophotometer was employed for the spectrometric analysis of biosynthesized cobalt nanoparticles. The reduction of cobalt was measured periodically at 200–700 nm. A spectrum of nanoparticles was plotted with wave length on x-axis and absorbance on y-axis.

2.3.2 Fourier transform infrared (FTIR) Spectroscopy:

For removing the biochemical compounds or uncapping ligands of the nanoparticles, the 500 mL residual solution of reaction mixture was centrifuged at 10,000 rpm for 30 min and the precipitate was resuspended in 10 mL Millipore water. The centrifugation and resuspension processes were repeated for 3–5 times. The purified suspension was dried in an oven at 50°C to obtain the stable powder and analysed by Fourier transform infrared spectrum (FTIR), Perkin Elmer-RX1 spectrophotometer.

2.3.3 Scanning electron microscopy–energy dispersive X-ray (SEM–EDX) microanalysis:

Scanning electron microscope (SEM) analysis was carried out using Ziess 700 scanning electron microscope machine compatible with EDX machine. The reaction mixture was centrifuged at 10,000 rpm for 30 min and the pellet was re-dispersed in 10 mL ethanol and washed 3 times with Millipore water to obtain the pellet. The pellet was resuspended in Millipore water, ultrasonicated and thin films of sample were prepared on carbon coated copper grid and analysed for size and shape determination. The particle size and shape of nanoparticles can be analysed by using image magnification software compatible with SEM and helps in determining the presence and formation of silver nanoparticles. The Electron Dispersive X-ray Microanalysis which confirm the presence of elemental cobalt signal.

2.4 Antibacterial activity:

The antibacterial assays were done on human pathogenic strains like Pseudomonas putida and Klebsiella pneumoniea by disc diffusion method. Luria Bertani (LB) broth/agar medium was used to cultivate bacterial strains. Fresh overnight inoculum (100 µL) of each culture was spread on to Luria Bertani agar plates. Sterile Whatman No.1 paper discs of 5mm diameter containing 10 µL of *Raphanus sativus var. longipinnatus* leaf extract (5 µg), 10 µ L of RsCoNPs (1mg/mL), 10 µL of Cobalt solution (10mM) and 10 µL of ampicillin (1mg/mL) were placed in each plate in serial order. After overnight incubation at 37 ^{o}C, zone of inhibition was measured (diameter in mm). The bactericidal activity is evaluated by the size of clear zone and greater the zone of inhibition greater the bactericidal activity.

2.5 Cytotoxic activity:

a) Hela Cell line maintenance and growth

The cytotoxicity potential of CoNPs was studied against HeLa cell lines (human epitheloid cervix carcinoma} which was purchased from NCCS (National centre for cell sciences), Pune, India. Hela cell lines were subcultured and were maintained at 37^0C at 5% CO2 in CO2 incubator. Cultures were examined for every 24hr under an inverted microscope to assess the degree of confluency and to confirm the absence of any microbial contamination.

b) Evaluation of cytotoxic activity of the Cobalt nanoparticles on (MTT) test

In-vitro study of cytotoxicity effect of CoNPs was assessed by MTT (3- (4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay. Cell lines were subcultured and 250µl of media (containing 10000cells) were transferred into 96 well plates and incubated for 24 hrs. Synthesized CoNPs were added at different dilutions (10, 5,1.25,0.312µg) and then final volume was made to 200µl with the media and incubated for 4 hrs. After incubation 20µl of MTT reagent (6mg/ml in PBS) was added to each well containing media and incubated for 3 hrs at 37 °C under an atmosphere of 5% CO_2 until a purple precipitate was observed. Media was removed without disturbing the cells and 200µl DMSO (MTT solvent) was added to dissolve the purple precipitate. Absorbance was read at 570 nm with a reference filter of 630 nm. Percentage cytotoxicity was calculated and used for finding the IC_{50} value of the concentration required for 50% cell death by synthesized CoNPs.

3. Results and Discussion:

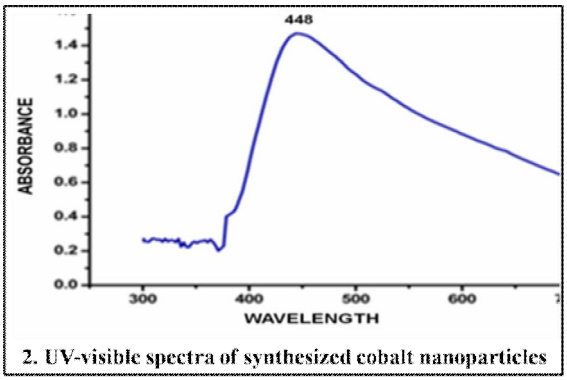
3.1 Synthesis of Cobalt Nanoparticles using Rs Leaf Extract:

This study deals with the synthesis, characterization and exploring biomedical applications of Cobalt nanoparticles, synthesized by using leaf extract of *Raphanus sativus var. longipinnatus*. The synthesized Cobalt nanoparticles were reddish brown in color. The color of the extract changed from light greenish yellow to reddish brown after addition of cobalt nitrate and on incubation for 90min at 40ºC.The colouration was due to the excitation of the surface Plasmon vibration of the CoNPs. Change in colour after the reduction of cobalt ions to cobalt nanoparticles is shown in (Fig.1C).

1 . A. *Raphanus sativus var. longipinnatus* B. Rs Leaf Extract C.RsCoNPs

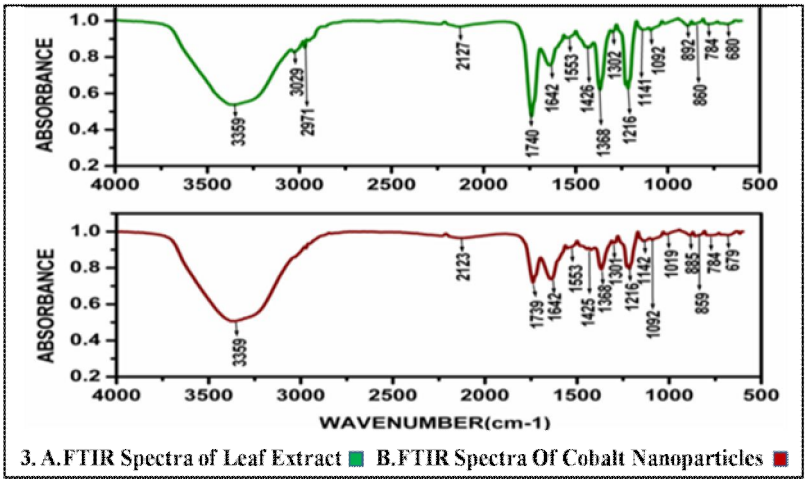
3.2 Characterization of Cobalt Nanoparticles:

The UV visible spectrum for the cobalt nanoparticles in aqueous solution showed the absorption peak at 448nm (Fig.2) which is due to the surface Plasmon vibration.



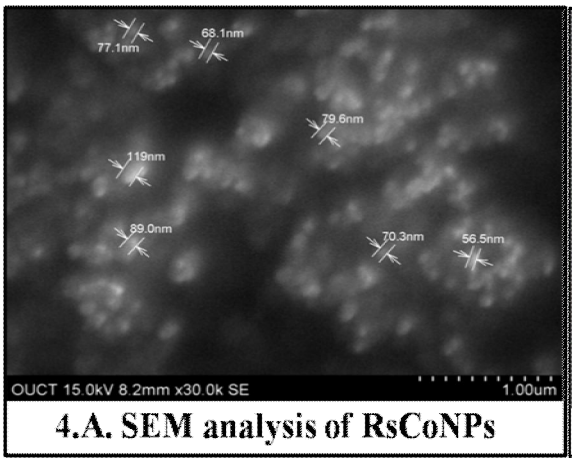
2. UV-visible spectra of synthesized cobalt nanoparticles

The FTIR spectrum showed absorption peaks at 3359, 2123, 1739, 1642, 1553, 1425, 1368, 1301, 1216, 1142, 1092, 1019, 885, 859, 784, 679 cm $^{-1}$ are due to the presence of phytochemicals (Fig. 3A & B) and the peaks observed at 3029 and 2971cm^{-1} are due to binding of cobalt to the phytochemicals present in the leaf extract.

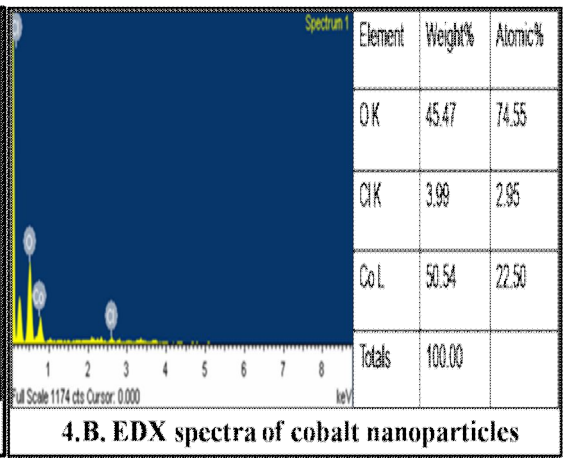


3. A.FTIR Spectra of Leaf Extract B.FTIR Spectra Of Cobalt Nanoparticles

The SEM analysis (Fig.4A) showed the spherical shaped cobalt nanoparticles with an average particle size of 80nm and EDX (Fig.4B) spectra showed the elemental signal for the presence of cobalt.



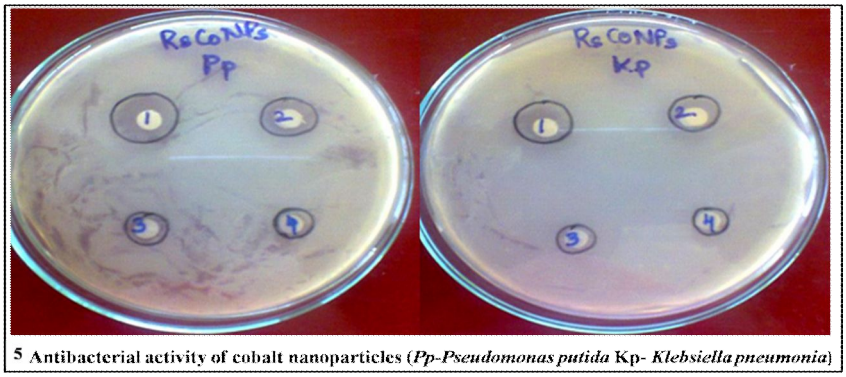
4.A. SEM analysis of RsCoNPs



4.B. EDX spectra of cobalt nanoparticles

3.3 Antibacterial activity of Cobalt nanoparticles by Disc diffusion method:

Antibacterial activity of synthesized cobalt nanoparticles against Gram negative organisms (*Pseudomonas putida and Klebsiella pneumonia)* was observed and zone of inhibition was measured (Fig.5). The results indicated that cobalt nanoparticles synthesized from *Raphanus sativus var. longipinnatus* leaf extract showed effective antibacterial activity in Gram negative bacteria.



5 Antibacterial activity of cobalt nanoparticles (*Pp-Pseudomonas putida* Kp- *Klebsiella pneumonia*)

Table: 1 showing the antibacterial activity by zone of inhibition

Bacteria	1.Ampicillin (1mg/ml) 10µl	2.RsCONPs (1mg/ml) 10µl	3. Leaf extract 10µl	4. 10 mM Cobalt solution 10µl
Pseudomonas putida	12mm	10mm	7mm	5mm
Klebsiella pneumonia	10mm	8.5mm	7mm	5mm

3.4 Cytotoxic activity of Cobalt nanoparticles:

In this study, a dose dependent approach was employed to evaluate the cytotoxicity of the nanoparticles on humans. The in vitro screening of the CoNPs showed potential cytotoxic activity against the Hela cancer cell lines. The results are shown in Table 1. Complete mortality rate that is 61.3 % cell death was observed in 10µg/µl concentration of CoNPs. Hence, the inhibitory concentration at 50% (IC50) was fixed at 5µg/µl of CoNPs for HeLa cells and cytotoxicity 52.5 %. At a 1.25 µg/µl concentration 31.3 % of cytotoxicity and at a concentration 0.312µg/µl, 7.7 % cytotoxicity was recorded. Further experiments were carried out with the standard anticancer drug Doxorubicin (2µg/2µl) in this study to confirm and correlate the anticancer activity of CoNPs.

Table: 2 showing the cytotoxic activity of Cobalt nanoparticles on HeLa cell lines

S.No	Concentration of CoNPs(µg/µl)	Dilution	Cell viability (%)
1	10	Neat	38.73±0.98
2	5	1:1	52.48±0.47
3	1.25	1:4	68.73±1.53
4	0.312	1:16	92.32±0.38
5	Cell Control	-	100
6	Doxorubicin(2µl)	2µl	36.836±0.25

Conclusion

A most important need in the field of nanotechnology is the development of reliable and eco-friendly processes for synthesis of metallic nanoparticles. Here, we have reported a simple biological and cost effective

approach for preparation of stable cobalt nanoparticles by reduction of cobalt nitrate solution with a reduction method using leaves of *Raphanus sativus var. longipinnatus aqueous* extract as the reducing agent. Biologically synthesized cobalt nanoparticles could be of immense use in medicine for their efficient antibacterial and cytotoxic properties. The characteristics of the obtained cobalt nanoparticles were studied using UV-Vis, FTIR and SEM-EDX techniques and confirmed the formation of cobalt nanoparticles. The experimental results showed that the average size of synthesized cobalt nanoparticles was about 80 nm.

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