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ORIGINAL ARTICLE **OPEN ACCESS**

Biocidal activity of Co_3O_4 nanoparticles against *S. oralis, E. faecalis* and *P. aeruginosa* pathogens

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ABSTRACT
Leaf extract-induced bio-synthesis of nanoparticles (NPs) acquired remarkable attention due to fascinating features like low cost, feasibility and lack of harmful solvents or toxic inorganic reducing agents. In this work, we have depicted a simple and cost-effective way to synthesize Co_3O_4 NPs using Aeschynomene Indica leaf extract. Co_3O_4 NPs exhibited a crystallite size of 17nm and hexagonal phased wurtzite structure Predominant enhancement in the physical property of Co_3O_4 NPs has driven them with excellent biomedical properties. Functional groups, especially Co-O bond vibrations 880 and 432 cm^{-1} are identified with the aid of Fourier Transform Infrared spectroscopy (FT-IR. Results of SEM with EDX spectrum convey atomic percentage of Co as 67 % and O as 30.42 %. Elemental mapping micrographs display that Co_3O_4 NPs have distributed evenly without aggregations. The results showed A. Indica leaf extract mediated Co_3O_4 NPs deliver prominent antibacterial activities against S. oralis, E. faecalis and P. aeruginosa. In brief, a very simple and low-cost synthesis strategy for Co_3O_4 nanoparticles has been demonstrated along with the evaluation of antimicrobial activities.
Keywords: *Aeschynomene Indica; Co_3O_4 NPs; Wurtzite structure; Biomedical-field*

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INTRODUCTION

Nanotechnology is a rapidly growing technology that deals with the creation of new materials on a microscopic scale. In other terms, nanotechnology aims to synthesize, characterize, and manipulate matter in size of 1-100 nm. Nanotechnology is the result of tiny reactants being used in science, engineering, and technology [1]. Given their major molecular and atomic characteristics over bulk products, nanoscale materials had distinct and essential properties. Synthesis of Nanoparticles (NPs) is broadly classified into two approaches. 1. Top-to-bottom approach, 2. Bottom-to-top approach. Moreover, top to bottom approach is preferred in which researcher can control their desired size and structural morphology of NPs as a sculptor [2]. Different Top to bottom approaches includes hydrothermal, combustion, sol-gel, sonochemical and co-precipitation method [3–5]. All reported methods pullback due to limitations like high cost, time-consuming, inevitable growth kinetics and hazardous by-products. A facile wet chemical synthesis using plant extract is preferred in this research work due to their low activation energy and controlled nucleation growth [6]. Nanoparticles (NPs) urge applications in the field of chemistry, physics, biology, material science and engineering due to their distinct physical properties like optical properties, conductance and uniformity [7,8]. Generally metal and its oxide NPs are synthesized due to its tremendous efficiency comparable to bulk material. Metal oxide NPs such as FeO [9], NiO [10], CuO [11], Mn_2O_3 [12] and ZnO are utilized as viable agents in biomedical applications [13].

Among them Co_3O_4 NPs shows diverse application, owing to it small ionic radius and high electronegativity. Co_3O_4 possess diverse applications such as catalyst, solar cell, luminescent studies and gas sensor due to their wide band gap energy (3.37 eV) [14]. Eventually, Co_3O_4 also found potential applications in optoelectronics, sensors and spintronics. Moreover, Co_3O_4 NPs are considered to be non-toxic, environmentally friendly photocatalysts and antimicrobial agents, to remove organic pollutants from the environment due to efficiency as a photocatalyst [15–17]. Comparable to noble metals Co_3O_4 is considered to be a low-cost, highly efficient material with good biocompatible material [18]. Hence, Co_3O_4 NPs may be

synthesized and used as an efficient probe to develop low-cost and safe drugs against pathogenic bacteria and fungi. Various chemical methods are employed for the synthesis of Co_3O_4 NPs. All those methods generate or use toxic solvents. To avoid the generation of harmful by-products, using a greener way to synthesize NPs is a bottleneck approach. Green synthesis a wet chemical method is an eco-friendly way at subsequent low temperature [19]. The main advantage of green synthesis is that NPs are synthesized in different shapes based on the polyphenolic compound present, with a large surface area and high porosity. In this research work, we used *Aeschynomene Indica* (*A. Indica*) leaf extract for the synthesis of Co_3O_4 NPs. *A. Indica* is inexpensive and abundantly found all around Asia. *A. Indica* plants are rich in phytochemicals like alkaloids, polyphenolic compounds, terpenoids and flavonoids which serves as stabilizing and capping agent. In addition, leaves of *A. Indica* are reported to contain excess carboxylic and phenolic functional groups, which make them a viable reducing agent to synthesize Co_3O_4 NPs [20,21]. To the best of our knowledge, no reports have been published on using *A. Indica* leaf extract as a bio-reducing agent and evaluating their biocidal activity against pathogens like antibacterial and antifungal effects.

In this manuscript, the synthesis of Co_3O_4 NPs through bioactive substances- beta linalool and citronellol present in *A. Indica* leaves a bio-reduction approach. The wet chemical method was preferred to eliminate the high-cost autoclave. The bioactive substance within leaf extract delivers the hydroxyl group necessary to initiate collision, as a capping and stabilizing agent. This approach effectively decreased NPs size and altered physicochemical properties such as high crystallinity, high purity, uniformity, large specific surface area (SSA) and low cost. The bio-synthesized Co_3O_4 NPs structural defect, morphology, elemental composition and efficacy towards biological application were analyzed through X-ray diffraction analysis (XRD), UV- Diffuse Reflectance Spectroscopy (UV-DRS), Fourier Transform- Infrared Spectroscopy (FT-IR), Scanning Electron Microscopy with EDX colour mapping (SEM with EDX). Additionally, Co_3O_4 NPs are tested against two different microbes namely (*S. oralis, E. faecalis* and *P. aeruginosa*) which revealed excellent efficacy. Based on the above investigation our research crew highly suggests *A. Indica* leaves extract mediated Co_3O_4 NPs can be a probe in the manufacture of antifungal and antibacterial drugs.

MATERIAL AND METHODS

All the chemicals and reagents were of analytical grade. Cobalt Chloride ($CoCl_2$) were purchased from Merck. Laboratory-grade ethanol was used for extraction via the cold percolation method. All the studies were conducted utilizing double distilled water.

Preparation of *Aeschynomene indica* Leaf extract

The leaves of *Aeschynomene indica* were collected from the Devakottai, Tamilnadu, India. The collected plant leaves were washed with distilled water several times and dried without sunlight. Then the dried leaves, are crushed and converted into fine powder which was used for the extraction process. The ethanolic leaf extract process is as follows [30].100g of leaf powder was mixed with 100 mL of ethanol in a 250 ml beaker. The mixed solution was kept undisturbed for 5 days and the solution was filtered using Whatman filter paper. The filtrate was named as *Aeschynomene indica* extract which was used for the synthesis of Co_3O_4 NPs.

Synthesis of Cobalt Oxide Nanoparticles

About 3 gm of $CoCl_2.2H_2O$ was taken and made into 100 Standard measuring flask. Nickel solution was stirred for 2 hr with magnetic stirring with 400 rpm speed and maintained 80^0C throughout the experiment. The ethanolic *Aeschynomene indica* extract was added slowly into the Nickel solution during the process of precipitated obtained. The Precipitate was dry and converted into fine powder which was transferred into a china crucible and kept in a muffle furnace for incineration for about 6 hours at 450^oC. The metal oxide salt thus, formed was ground wellto make it into Co_3O_4 NPs [31],

Characterization

Characterization of Nickel oxide nanoparticles was carried out using various techniques. Fourier transforms infrared spectroscopy (FT-IR PerkinElmer) with a range from 4000 cm-1 to 400 cm^{-1}. The absorbance value was recorded using a UV-visible spectrophotometer with a range of 200-800 nm. The surface morphology and shape of the nanoparticles were determined by scanning electron microscopy (SEM). Nanoparticle composition was analysed using Energy Dispersive X-Ray Analysis (EDAX). X-ray diffractometer, using Cu Kα radiation (λ = 0.1546 nm), with a diffraction angle between 10 and 90° was used to predict the crystalline nature of nanoparticles.

Antibacterial Activity

The antibacterial activity of synthesized Co_3O_4 NPs was tested against *S. oralis, E. faecalis* and *P. aeruginosa* bacteria by Muller Hinton agar well diffusion method. New bacterial strains were cultured by nutrient broth as a cultured medium. In brief, an adequate amount of 2.8-3.0 g of nutrient agar medium was dissolved in DI water. The dissolved agar medium was autoclaved at 121^oC for 15 minutes with pressure of 15lbs. The

agar medium was mixed well under molten conditions and fed onto 100mm petri plates. The warm agar medium was mixed with bacterial inoculums (100 µL). Petri plates containing 20 ml of nutrient-agar medium were allowed to solidify for 24h and adjusted to 0.5 OD value. At this stage, Bio-synthesized Co_3O_4 NPs were placed onto the disc at four distinct concentrations (50, 100, 250 and 500 µg/ml) using sterilized forceps. A well-known antibiotic gentamicin was fixed as positive control (PC) throughout this study. The petri plates were incubated for 24h at 37°C and the experiment was evaluated by measuring the zone inhibitory diameter in millimetres (mm). All the experiments were assessed in three separate plates and summed to retrieve concordant data.

RESULT AND DISCUSSION

Analysis of UV-Visible absorption spectra

The synthesized metal nanoparticles CoO-NPs from the ethanolic extract of *Aeschynomene indica* are monitored by UV-visible spectrometer For cobalt nanoparticles, the absorbance peak appears between the ranges of 350-850 nm. Figure. 1 indicates that the peak is observed at 552.85 nm (0.2091 AU)suggesting the reduction of cobalt and the formation of cobalt oxide nanoparticles. Co_3O_4 NPs are considered stable, and this fact can be the result of a symmetrical polarity structure which depends on the weak interaction of Van der Waals forces within the particle regime

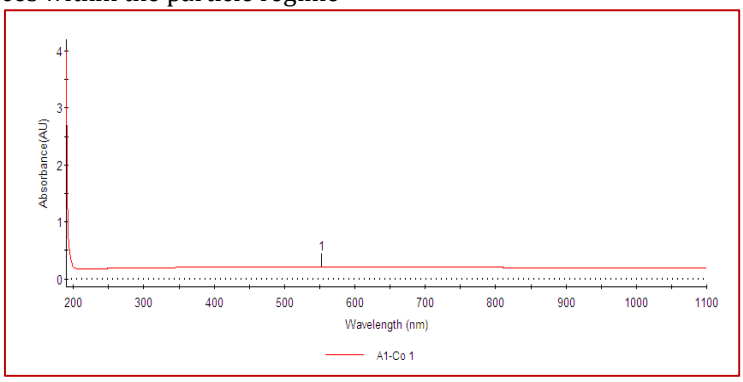

Fig.1 UV-Visible Spectra of Cobalt Oxide Nanoparticles

Analysis of FT-IR

FT-IR analysis was performed to find out the different functional groups that are present in the leaf extract of *Aeschynomene indica* and are helpful for the synthesized nanoparticles and act as capping and stabilizing agents. IR spectrum of plant extract as well as of synthesized NPs is shown in Figure 2. Both types of spectra have almost similar peaks except that in the case of plant extract containing NPS, there is slight shifting and broadening of peaks. A peak obtained at 3415 cm^{-1} is the characteristic peak of the hydroxyl group of phenolic compounds. Some other major peaks were obtained at 1619 cm^{-1}, 1386 cm^{-1}, 1114 cm^{-1} 665 cm^{-1} 579 cm^{-1} and 543 cm^{-1} can be attributed to the carbonyl group, amide group, C–O of alcohols or phenols and CoO, respectively.

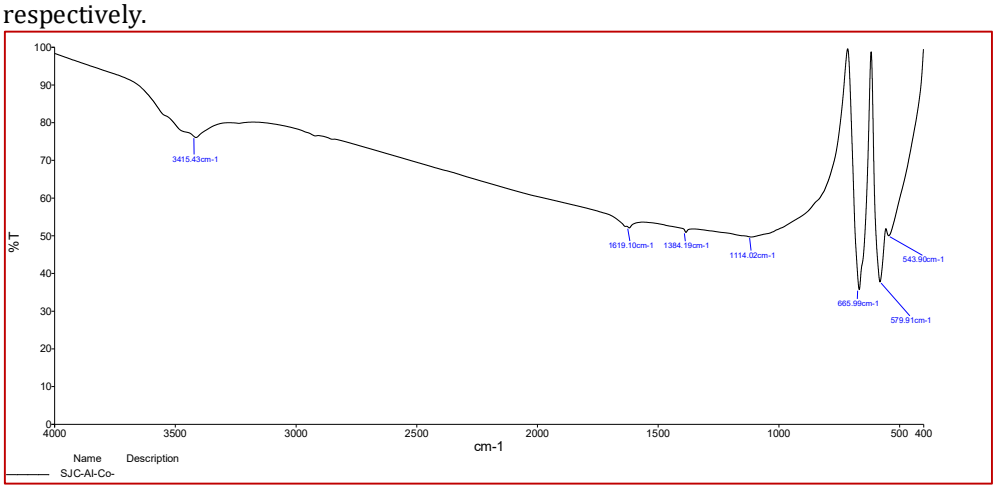

Figure. 2. FT- IR spectrum of Copper Oxide nanoparticles

X-ray Diffraction Pattern

X-ray diffraction (XRD) spectra are used to analyze the crystalline structure of the synthesized Co_3O_4 NPs

with *A. Indica* leaf extract. XRD pattern of green synthesized Co_3O_4 nanoparticles was recorded in the range of 10° to 80°. The spectrum revealed sharp and intense peaks indicating phase purity and the crystalline nature of synthesized Co_3O_4 NPs with the aid of *A. Indica* leaf extract. Co_3O_4 NPs with (2θ) value about 31.75°, 34.40°, 36.25°, 47.63°, 56.61°, 62.89°, 66.49°,67.99°,69.14°corresponding to (h, k, l) plane of (220), (311), (222), (400), (422), (511), (442), (620) and (533) which well matched with standard JCPDS card-no: 01-074-2120 stimulation as shown in Fig. 3 a [22,23]. The high intense diffraction peak is located at 36.25° with a hexagonal phase wurtzite structure, respectively.

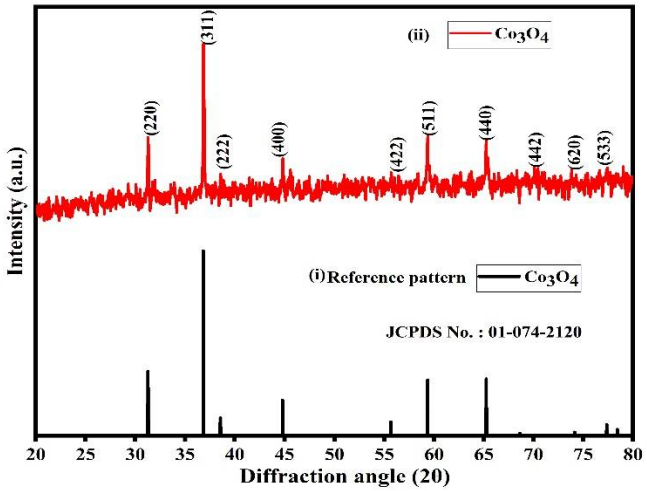

Figure 3. X-ray diffraction spectra of Copper Oxide nanoparticles

SEM Analysis

Figures 4 (a),(b) show the SEM images of CoO nanoparticles at different magnification, which clearly exhibit the nanoparticles like morphology indicate well uniform particles with narrow size distribution lies in the range of 40–60 nm. The surface of as synthesized nanoparticles is very smooth, which facilitates the better contact with the bacterial cell wall and hence increases bacterial killing ability of NPs. Such a behaviour of smooth surfaced NPs has already been established in the literature [17].

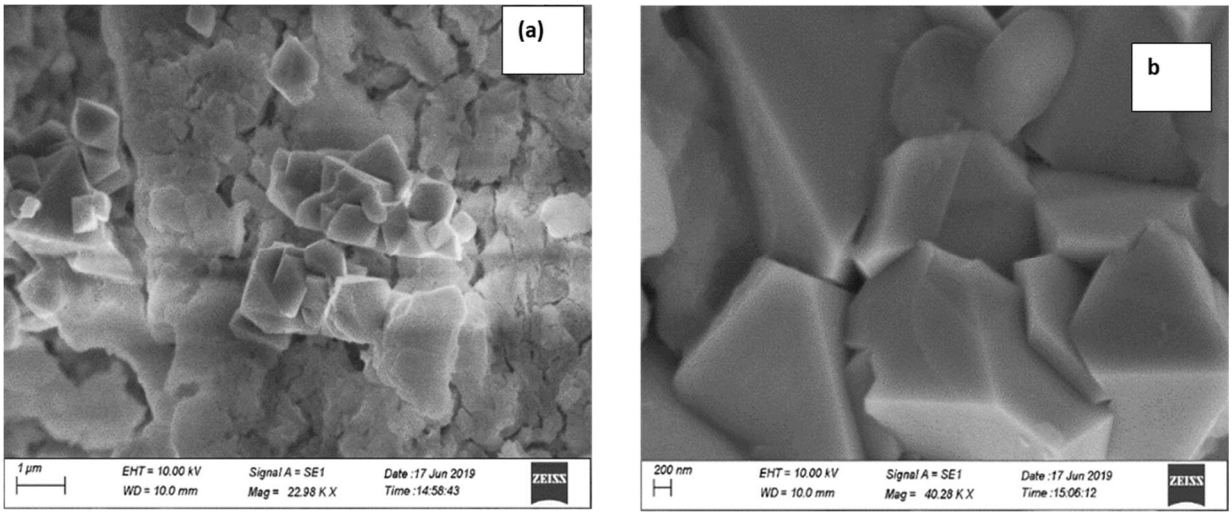

Fig. 4 SEM micrographs of Co_3O_4 NPs with different Magnification (a) 22.98 KX (b) 40.28 KX

Energy Dispersive X-Ray Analysis (EDAX)

The elemental composition of the synthesized Co_3O_4 NPs was evaluated from EDX analysis Figure 5. In this figure, the major peaks indicate the Co and O of the synthesized NPs. However, some minor peaks of carbon, calcium, sodium, sulphur and silicon are also present which are attributed to the plant extract used. The elemental composition of the nanoparticles shows 26 weight percent cobalt and 68 weight percent oxygen corresponding to cobalt oxide (CoO). The compositional data from the EDAX analysis agree well with theoretically calculated values, indicating a good compositional homogeneity across the nanoparticles. The

EDAX spectrum shows sharp peaks between 0 and 2 KeV and between 6 and 8 KeV corresponding to crystalline Co_3O_4 NPs.

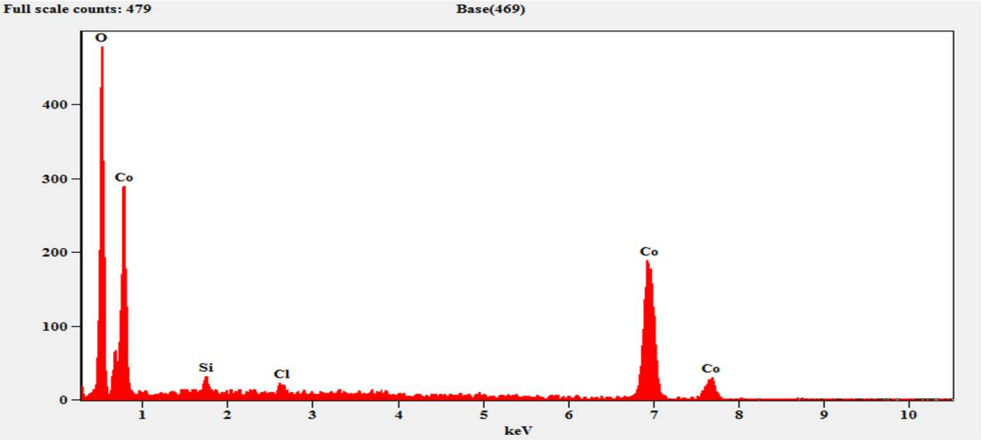

Figure 5. Energy Dispersive X-Ray Analysis (EDAX) of CoO nanoparticle

Antibacterial assay

Conferring to describe literature of the Co_3O_4-NPs, these NPs have been active in catalysis and their biological applications have been less explored. The researchers have initiate that anti-bacterial activity of the NPs increased with increasing their concentration [20]. Also, the researchers have used metal oxide nanoparticles for cytotoxic, antioxidant and enzyme inhibition assays. Marcella Mauro *et al* have studied the use of impaired and healthy human skin cells for Keratinocytes Toxicity studies [21][22]. Elena Boss *et al* have studied that CoO-NPs cross the plasma membrane by a non-endocytosis pathway and thus gain access to the cytoplasm [22, 23].

Results of Co_3O_4-NPs antibacterial activities are presented in Table 1. Cobalt oxide nanoparticles produced good antibacterial activities against both gram positive and gram-negative bacteria. The antibacterial activities of CoO-NPs were higher against gram positive bacteria as compared to gram negative bacteria. Among gram positive bacteria, maximum inhibition zone (17.75±1.06) against was *Pseudomonasa aeruginosa* measured while for gram negative bacteria *Strepcoccus oralis* , maximum inhibition zone (11 ± 2.5) was observed. It is important to note that activity of cobalt oxide NPs against *Pseudomonasa aeruginosa* was even higher than Bacitracin. Such results indicate the potential of these NPs against human pathogens. Among both classes of bacteria, minimum zone of inhibition (16.5±0.7) was found for Pseudomonasa. The gram-positive bacteria have thick peptidoglycan cell wall but porous in nature with higher permeability as compared to gram negative bacteria and such a structure of cell wall facilitates the maximum absorption of these NPs [20–22, 24]. Also, the size of nanoparticles is small which ensures the maximum absorption of NPs by bacterial species and hence resulting into their death. Gram negative bacteria have thin peptidoglycan wall with less permeability [20–22, 24]. So based upon the structural characteristics of cell walls of both type of bacteria, we accept an enhanced permeability and hence greater activity of Co_3O_4 NPs for gram positive bacteria. The cobalt ions interact with thiol groups of bacterial enzymes, because such results indicate the potential of these NPs against human pathogens showed in Figure 7.

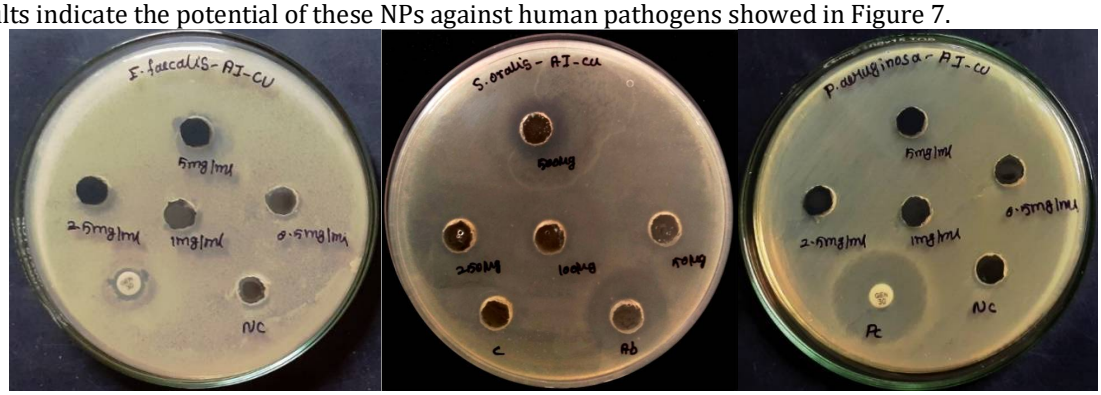

Figure 7. Co_3O_4 NPs against *Streptococcus oralis, Pseudomonasa, Aeruginosa and Enterococcus faecalis*

Table 1: Antibacterial assay

S.NO	Name of the test Sample	Name of the test organism	Zone of inhibition (mm)				
			500 µg/ml	250µg/ml	100 µg/ml	50µg/ml	AB
1	Co_3O_4 NPs	*Enterococcus faecalis*	5.5±0.7	4.25±0.35	3.2±0.28	3.2±0.28	17.75±1.06
2		*Pseudomonasa Aeruginosa*	6.5±0.7	4.25±0.35	3.2±0.28	0	16.5±0.7
3		*Streptococcus oralis*	7.5 ± 2.8	7.5 ± 1.5	0	0	11 ± 2.5

CONCLUSION

Several conclusions have been made with synthesized cobalt oxide nanoparticles. Firstly, cobalt oxide nanoparticles were synthesized by cost effective and eco-friendly green method by using *Aeschynomene indica* leaf extract. The prepared nanoparticles, were analysed by various techniques such as UV, FTIR, XRD, EDX, DLS and SEM. These techniques revealed the successful synthesis of cobalt oxide nanoparticles. Antibacterial activities of synthesized cobalt oxide nanoparticles were analysed against gram positive and gram-negative bacteria and it was found that by increasing concentration of cobalt oxide nanoparticles, antibacterial activity was increased.

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