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Exploration of Herbal Bismuth Nanoparticles Using *Eclipta alba* and *In Vitro* Antimicrobial Activity against Pathogenic Bacterial Strains

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ABSTRACT

In current days, Nanotechnological applications in medicine are an important and attractive field of interest in developing newer and useful therapeutic molecules. In this study, the spherical herbal bismuth nanoparticles using *Eclipta alba* (Ea-BiNP) with average size 40nm have been synthesized by the colloidal-chemical method in water medium. The bi-content has been exposed to the nanoparticles by the method of energy-dispersive X-ray spectroscopy. Further, the synthesized Ea-BiNP has been characterized as biosafe and biocompatible according to the regulations of safety assessment of medical nanopreparations. The *in vitro* antimicrobial activity of EaBiNP has been determined using wide spectra of high pathogenic bacterial members. EaBiNP has high bactericidal action against all investigated test strains: MRSA, *Staphylococcus aureus*, *Vibrio cholerae, Salmonella typhi, S. paratyphi* A, *S. paratyphi B, Acinetobacter baumanii,* ETEC, *Enterobacter* sp., *Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus vulgaris, P. mirabilis, Shigella dysentriae* and *Providentia* sp. has been revealed.

INTRODUCTION

In recent days, many experimental researchers put efforts to construct and synthesize green and environmentally friendly methods for the synthesis of nano size materials that involve the use of plants as surfactants (Iravani, 2011). Plant parts including leaf, root, latex, seed and stem are being used for metal nanoparticles synthesis. The green eco friendly processes in pharmaceutical chemistry and its technology are becoming increasingly popular (Bai and Liu, 2010; Yang et al., 2010). The techniques for obtaining nanoparticles using naturally occurring reagents such as plant extracts, could be considered attractive for medical nanotechnology (Yang et al., 2010; Iravani, 2011).

In the medical industry, the important problem in the struggle against multi drug resistant bacterial pathogens and its treatment modalities is a biggest question mark to retain the health of population (Rieznichenko et al., 2015). Even the modern antimicrobial preparations and vaccines did not possess by a wide range of activity against different classes of pathogens. Thus searching and development of new highly effective antimicrobial substances with high effectiveness against a wide spectra of high pathogen microorganisms that cause most dangerous human and animal diseases is required today. Metal nanoparticles are possessed by high potential in this area. Moreover herbal based metal nanoparticles synthesis and its effective antibiogram analysis are placed in the top priority in pharmaceutical industries due to its less toxic expulsion toward environment.

Considerable efforts are being made on the global level to develop and implement eco-friendly technologies for production of herbal based consumer products for providing better healthcare solutions (Ravindra et al., 2012). In the recent years, there has been much concern about the synthesis of eco-friendly nanoparticles that do not produce toxic wastes in their process of synthesis. This can only be achieved through benign synthesis processes of biological nature which are considered safe and ecologically sound for nanomaterial fabrication as an alternative to conventional physical and chemical methods (Singh et al., 2011; Annamalai et al., 2011).

In several studies, the biological routes to the synthesis of nanoparticles have been proposed by exploiting microorganisms and vascular plants (Prashanth et al., 2011). However, the utilization of herbal and medicinal plant extracts for the synthesis of nanoparticles is a relatively recent activity (Thirumurugan et al., 2010). Various studies suggested that metals including gold and silver have been used mostly for the synthesis of stable dispersions of nanoparticles using the extracts of herbal and medicinal plants. Although several choices of nanoparticles exhibit medicinal properties, this study has its own novelty to determine the usefulness of herbal mediated bismuth nanoparticles as an alternative broad spectrum antimicrobial agent. One of the most promising strategies for overcoming microbial resistance is the use of nanoparticles of herbal origin also better than synthetic and microbial sources.

The major objective of the present study was to determine *in vitro* antimicrobial activity of the synthesized herbal bismuth nanoparticles using *Eclipta alba* against wide spectra of pathogenic bacteria and also characterize the synthesized nanoparticles**.**

MATERIALS AND METHODS

Chemistry of $Bi_2O_{12}S_3$

Bismuth (III) sulphate is an inorganic compound which is available in powdered form. Bi_2O_3 is an alkaline oxide reacts with concentrated sulphuric acid solution to form bismuth (III) sulphate and also referred as dibismuth tris (sulphate) and Dibismuth trisulfate (Abe and Ito, 1978; Chen et al., 2001). The detailed description about bismuth sulphite is depicted in table 1. Bismuth sulphate is isomorphous with the sulphates of yttrium, lanthanum and didymium and it is able to confer upon certain other substances the property of phosphorescence

Table 1: Detailed description about Bismuth sulphate

Chemical Name	Bismuth (III) sulphate
Molecular formula	$Bi_2O_{12}S_3$
Appearance	Brown powder
Molecular weight	706.15 g/mol
H_2 bond acceptor count	12
Monoisotopic mass	705.815986
Complexity	62.2
Covalently bonded unit count	5
Solubility	Soluble in acid; insoluble in water and ethanol
Melting point	405^{o}Dec
Density	5.08

Ea-Bi nanoparticle synthesis

Bismuth sulphate was prepared in the laboratory by sonochemical and chemical condensation method (Prabhu et al., 2010a). Here the aqueous solution of bismuth nitrate and sodium thiosulphate was added in the presence of complexing agents including ethelenediaminetetraacetic acid, triethanolamine and sodium tartarate (Wang et al., 2002). All these mixtures were loaded into a 500mL three neck flasks which were continuously stirred with magnets. After 30 minutes of stirring, the crude water extract of *Eclipta alba* (EA) was added with the chemo mixture (BiS) in the concentration of 1:20 ratio. Further the Ea-Bi mixture was stirred and heated at 60^{o}C for 15 minutes under ambient conditions.

The 10ml 1M sodium hydroxide solution was added into the reactor. After the reaction mixture was adequately mixed under magnetic stirring for 45 minutes, the brown solution was cooled to room temperature (Prabhu et al., 2010a; Iravani, 2011). Here the color change, pH and initial OD values using calorimeter were analyzed. Bismuth nanoparticles using *E. alba* (EaBiNP) were washed with water and ethanol for many times, dried in a desiccator and stored at low temperature until further use.

Caution

1. Synthesized nanoparticles should be stored in a cool place.
2. Keep the container tightly closed in a dry and well-ventilated place.
3. Store away from strong oxidizing agents.

Characterization

The synthesized Ea-BiNP nanoparticles were characterized to analyze the formation and confirmation of its synthesis. Initially the particles were characterized by X-ray diffraction (XRD) patterns where the particles were determined the particle planes where the average particle sizes are calculated using Scherrer's equation (CD=Kλ/B cos Ө).

The surface chemical structure of the synthesized nanocrystals was characterized by fourier transform infrared (FTIR) spectroscopy where the broad bands are due to the O–H stretching vibration attributed for water molecules adsorbed to the nanoparticles surface. Here the peak can be ascribed to the CO_2 from the air. The strong absorption band was attributed due to Bi–O stretching vibration for the EA-BiNP nanoparticles.

The morphology of bismuth herbal nanoparticles was revealed by scanning electron microscopy (SEM) technique revealed the pseudo-spherical morphology for the synthesized nanoparticles.

The particle detection curve was also measured for the product. Zero coercivity and zero remanence on the curve indicate superparamagnetic behavior and the saturation magnetization of the bismuth nanoparticles. The shape and size of the Ea-BiNP have been determined by Transmission electron microscopy (TEM) (JEM-1234, JEOL Ltd, Japan).

The biosafety levels of the synthesized Ea-BiNP has been estimated by in vitro methods using various parameters including cytotoxicity, genotoxicity, mutagenicity and biochemical markers according to the guidelines of safety assessment of medical nanoprearations (Guidelines, 2013).

Antibiogram

The *in vitro* antibacterial activity of EaBiNP has been revealed against all investigated test strains including Methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus, Vibrio cholerae, Salmonella typhi, S. paratyphi* A, *S. paratyphi B, Acinetobacter baumanii,* Enterotoxigenic *Escherichia coli* (ETEC), *Enterobacter* sp., *Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus vulgaris, P. mirabilis, Shigella dysentriae* and *Providentia* sp. All the cultures were aseptically obtained from the Clinical laboratory of Microbiology, Chennai Medical College Hospital and Research Centre, Tiruchirapalli, India.

In this study, the broth microdilution method is used to determine the antimicrobial activity of the synthesized EA-BINP.

In general, the broth dilution testing includes both macrodilution, wherein volumes of broth in test tubes for each dilution typically equal or exceed 1mL and broth microdilution (BMD), in which antimicrobial concentrations are most frequently of smaller volumes in 96-well microtiter plates. Due the reliable and well-standardized method, the broth microdilution technique is implicated in this study and is of particular utility in research studies and in testing of a single antimicrobial agent for 1 bacterial isolate. The method is, however, both laborious and time intensive and because of the ready commercial availability of convenient microdilution systems is not generally considered practical for routine use in clinical microbiology laboratories.

RESULTS AND DISCUSSION

The initial observation of color change from pale brown to dark brown and black threads was determined in various concentrations of crude aqueous extract of *E. alba*. In this investigation, 1: 40 concentrated Ea-Bi solution (minimum concentration that showed maximum color change) showed earliest color change and reached to black threats within 8 hours (Table 2). It is already stated that the pale color is converted to dark threats indicated the nanoparticle synthesis (Sastry et al., 2004; Prabhu et al., 2010a).

Table 2: Color change of synthesized nanoparticles (Ea-BiNP)

Concentration of crude extract of *E. alba**	Time in hours versus color change								
	0	1	2	4	8	16	24	28	32
1:10	1+	3+	4+	5+	6+	6+	6+	6+	6+
1:20	1+	3+	4+	5+	6+	6+	6+	6+	6+
1:30	1+	3+	4+	5+	6+	6+	6+	6+	6+
1:40	1+	3+	4+	5+	6+	6+	6+	6+	6+
1:50	-	1+	1+	1+	1+	1+	3+	-	-
1:60	-	-	-	-	-	1+	-	-	-

[*one part of crude extract of *E. alba* in various parts of sterile double distilled water; - (no color); 1+ (pale brown); 2+ (intermittent brown); 3+ (dark brown); 4+ (blacky brown); 5+ (black solution) and 6+ (black threads)]

The pH (negative logarithm of hydrogen ion concentration) of the various concentrations of crude extract of *E. alba* of various time duration is also well determined. As like table 2, the pH also supported well at 8 hours of herb-metal interactions. The detailed analysis of an increase in pH from 6 to 11 at various concentrations of *E. alba* was impregnated in figure 1. The previous report also suggested with *Aspergillus niger* mediated silver nanoparticles increased the pH levels from 6 to 8.3 and this periodical increase in pH is an indicator of silver nanoparticle production (Prabhu and Revathi, 2009).

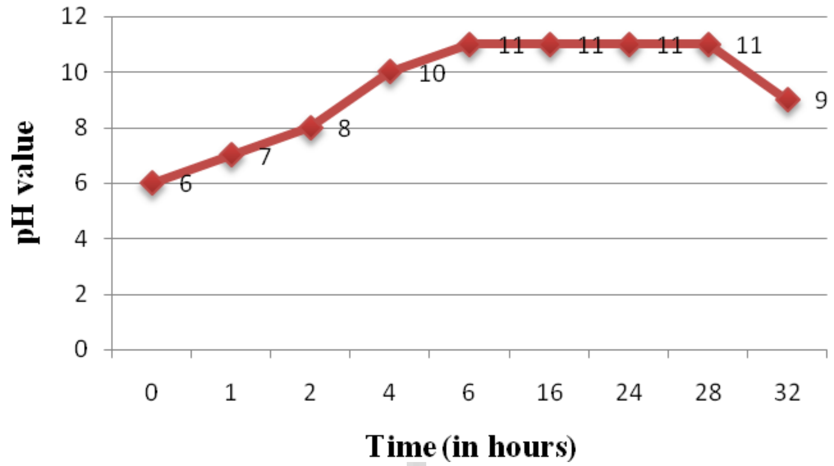

Figure 1: pH variations of interactions of *E. alba* versus $Bi_2O_{12}S_3$

The optical density of the nano solute also increased as the time increases. The steady increase in the OD value of fungal mediated silver nanoparticles also determined and documented (Prabhu et al., 2010b). The XRD patterns of the EA-BiNPs are depicted in table 3 and figure 2. Six notable characteristic peaks can be indexed corresponds to the crystal planes. The average particle sizes were calculated as 9.8nm using Scherrer's equation as mentioned in materials and methods.

Table 3: X-ray diffraction patterns of Ea-BiNP

hkl	**Plane defined by indices (hkl) that pass through the origin**					
	220	**311**	**400**	**422**	**511**	**440**
2Ѳ (deg)	30.086	35.392	42.845	52.964	56.112	62.093
d (A^o)	2.2354	2.5305	2.1254	1.7132	1.45791	1.18347

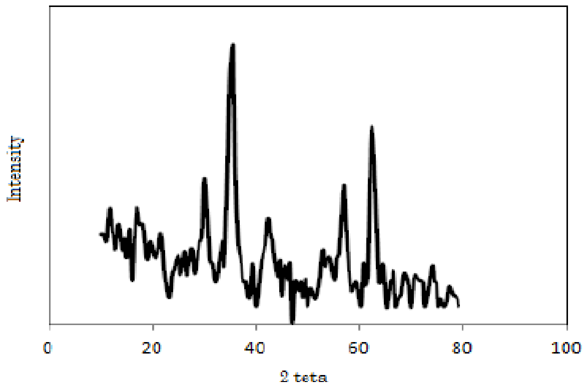

Figure 2: X-ray diffraction pattern of Ea-BiNP

The surface chemistry and its structure of the bismuth nanoparticles were characterized by fourier transform infrared spectroscopy (FTIR) where the peak values suggested the formation of the nanoparticles (Figure 3). The board band of 3410 cm^{-1} is experiential mainly due to the O-H stretching vibration ascribed for water molecules adsorbed to the surface of nanoparticles. The peak of 1615 cm^{-1} can be attributed to the CO_2 from the air surface and the strong absorption band at 580 cm^{-1} is mainly due to Bi-O stretching vibration for the $Bi_2O_{12}S_3$ nanoparticles.

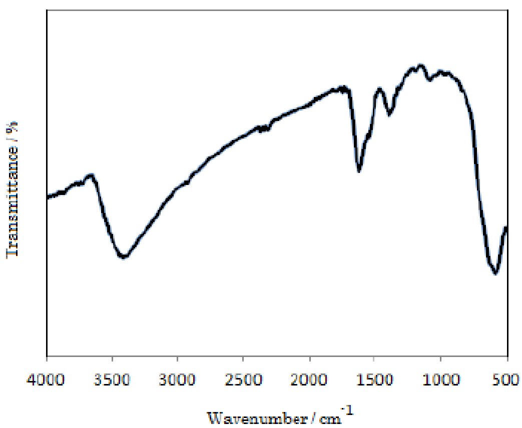

Figure 3: FTIR spectra pattern of Ea-BiNP

The morphological analysis of *E. alba* bismuth nanoparticles was revealed by scanning electron microscopic (SEM) technique that showed the pseudospherical shape for the Ea-BiNP. At 30KV, scanning electron imaging of EA-BiNP showed different sizes ranged from 31 to 43nm (Figure 4).

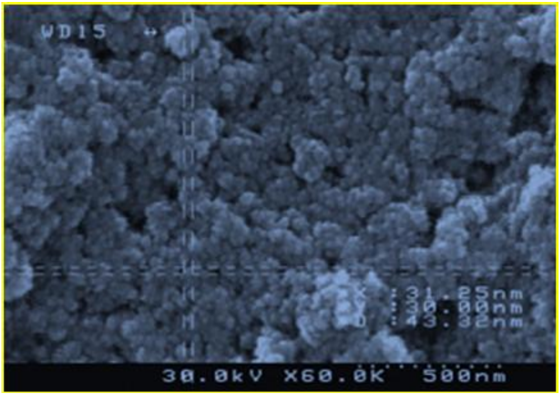

Figure 4: Scanning electron imaging of determining EA-BiNP morphology

Transmission electron imaging showed clear picture that the water dispersion of Ea-BiNP has been synthesized to determine the shape and size that are the major phenomena. By this method, it has been determined that synthesized nanoparticles have spherical form and the particles size is approximately of 40nm (Figure 5). Thus the concentration of obtained Ea-BiNP was 76.4 mg/ml by metal but in other study it was observed as 77.5 mg/ml (Reiznichenko et al.,2015).

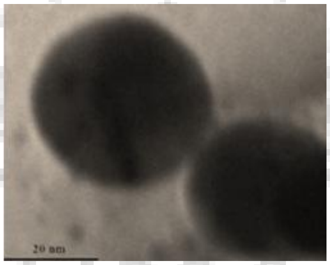

Figure 5: Imaging from TEM showed spherical Ea-BiNP with 40 nm size

In some studies, by chemical method of synthesizing highly monodisperse and ligand-protected bismuth nanoparticles (Bi-NPs) have characterized and reported. The size of the single-crystalline and spherically shaped NPs is controlled between 11 and 22nm mainly by the reaction temperature. The high uniformity of the NPs allows their self-assembly into long-range-ordered two and three dimensional superstructures (Yarema et al., 2010).

The method of preparation presented in this study implies greener environment and conditions than other studies reported earlier (Briand and Burford, 1999; Robles et al., 2016). To the best of our knowledge, this is the first report of *Eclipta alba* -bismuth nanoparticles synthesized by the chemical method and incorporation of bactericidal activity. In the modern medicine, emergence and increase in the antimicrobial resistance characters of the bacterial pathogens and most of the antibiotics losing its effect due to indiscriminate clinical applications. Thus, the necessary state in the development of newer antimicrobials may help in the future infection management.

The observation of antibacterial activity of 40nm Ea-BiNP against the test bacterial pathogens has shown high bactericidal action of the nanoparticles against all bacterial pathogenic strains (Table 4). Total bactericidal nature has been determined in all the concentrations of the Ea-BiNP but in lower concentration and even in high bacterial strength the inhibition rate was low. Such results indicate high potential of Ea-BiNP application in the development of newer molecules for effective prophylactic and therapeutic management of bacterial infections. The comparativeness with the control of the test strain growth indicated the inhibitory action effectively. Separately the bismuth sulphate also interpreted for the determination of bactericidal nature. Effectively most of gram negative bacterial pathogens utilizing the $Bi_2O_{12}S_3$ as a growth promoter.

Table 4: Antibacterial effect of Ea-BiNP against test bacterial pathogens

Bacterial pathogens	**Seed dose of bacteria, CFU/ cm^3**	**Inhibition of bacteria by various concentrations of Ea-BiNP in mg/ml by metal)**				**Control of the test strain growth**
		5	***10***	***25***	***50***	
Acinetobacter baumanii	10^3	+	-	-	-	++++
	10^4	+	-	-	-	
	10^5	++	+	-	-	
	10^6	++	+	-	-	
Enterobacter sp	10^3	+	+	-	-	++++
	10^4	+	+	-	-	
	10^5	++	++	+	-	
	10^6	++	++	+	-	
Enterotoxigenic *E. coli*	10^3	-	-	-	-	++++
	10^4	-	-	-	-	
	10^5	+	+	-	-	
	10^6	++	+	-	-	

Klebsiella pneumoniae	10^3	+	-	-	-	++++
	10^4	+	-	-	-	
	10^5	++	+	-	-	
	10^6	++	+	-	-	
Methicillin resistant *Staphylococcus aureus*	10^3	-	-	-	-	++++
	10^4	-	-	-	-	
	10^5	+	+	-	-	
	10^6	++	+	-	-	
Proteus mirabilis	10^3	++	+	-	-	++++
	10^4	++	+	-	-	
	10^5	++	+	-	-	
	10^6	++	+	+	-	
Proteus vulgaris	10^3	++	+	-	-	++++
	10^4	++	+	-	-	
	10^5	++	+	-	-	
	10^6	++	+	+	-	
Providentia sp	10^3	+	-	-	-	++++
	10^4	+	-	-	-	
	10^5	++	+	-	-	
	10^6	++	+	-	-	
Pseudomonas aeruginosa	10^3	+	+	-	-	++++
	10^4	+	+	-	-	
	10^5	++	++	+	-	
	10^6	++	++	+	-	
Salmonella typhi	10^3	+	+	-	-	++++
	10^4	+	+	-	-	
	10^5	++	+	-	-	
	10^6	++	++	-	-	
Salmonella paratyphi A	10^3	+	+	-	-	++++
	10^4	+	+	-	-	

	10^5	++	+	-	-	
	10^6	++	++	-	-	
Salmonella paratyphi B	10^3	+	+	-	-	++++
	10^4	+	+	-	-	
	10^5	++	+	-	-	
	10^6	++	++	-	-	
Shigella dysentriae	10^3	+	-	-	-	++++
	10^4	+	-	-	-	
	10^5	++	+	-	-	
	10^6	++	+	-	-	
Staphylococcus aureus	10^3	++	+	-	-	++++
	10^4	++	+	-	-	
	10^5	++	+	+	-	
	10^6	++	+	+	-	

[Ea-BiNP (*Eclipta alba* bismuth nanoparticles); CFU (colony forming units); + (growth in scanty); ++ (moderate growth); ++++ (intensive growth) and - (total inhibition of bacterial growth)]

As described above, in this study spherical shaped bismuth based nanoparticles mediated by *E. alba* with an average size of 40nm has been synthesized by the colloidal chemical method in a aqueous medium. The synthesized nanoparticles has been characterized by XRD, FTIR, SEM and TEM and also proved the non cytotoxic, non genotoxic, non mutagenic and biosafety analysis. Also the synthesized Ea-BiNP exhibited widely in vitro bactericidal activity against the test bacterial pathogens obtained from Clinical Microbiology laboratory.

For particle toxicity, the three crucial factors are size, shape, and chemical composition. A reduction in the size of a nanosized particle results in an increase in the specific surface area of the nanostructured powder. Therefore more chemical species may attach to its surface, which enhances its reactivity and results in an increase in its toxic effects (Delgadillo et al., 2012).

The mechanism of antimicrobial activity for nanoparticles is not completely studied and their precise mechanism of action against bacterial pathogens remains to be fully elucidated. More studies have shown that a positive charge on the metal ion is critical for antimicrobial activity, allowing for electrostatic attraction between the negative charge on the bacterial cell membrane and the positive charge on the nanoparticle (Delgadillo et al., 2012; Kim et al., 2007). It has been reported that silver nanoparticles can damage DNA, alter gene expression, and affect membrane-bound respiratory enzymes (Feng et al., 2000; Yamanaka et al., 2005). Here we present early evidence of the antimicrobial activity of *E. alba* bismuth nanoparticles with good evidence of characterization and in vitro antibacterial action.

CONCLUSION

The results observed while performing these experiments suggested being excited about a new kind of antimicrobial of broad spectrum, with effective and low cost. This herbal based Ea-BiNps could be used as surface disinfectant; hospitals, pediatric and geriatric clinics, food and pharmaceutical industries, laboratories and in general all locations where microbial contamination needs to be counteracted. The interesting alternative is to adhere as a film within valves, catheter, prosthesis and dental implants. In several studies and also in pharmaceutical market, silver nanoparticles have been incorporated to several items; however Ea-BiNPs would have the advantage of being more effective and lower toxic side effects.

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