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PREPARATION AND EVALUATION OF ISONIAZIDE NANO-CONJUGATES FOR IMPROVING THE THERAPEUTIC EFFICIENCY

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ABSTRACT: Raise in the multidrug resistance gave challenge to the treatment of tuberculosis with first line medication due to less response. Nanoparticle drug delivery has proven efficiency in improving the therapeutic efficacy of many drugs. Reduction of dosing frequency was achieved by applying drug delivery using Nano medicine and Green Nanotechnology. The present study aimed in the production of such drug conjugates of Isoniazid using interdisciplinary technology of nano medicine and green nanotechnology. Isoniazid Nano-conjugates were produced by simple incubation and formed conjugates were analyzed for the efficiency of the plant extract to conjugate the drug. The therapeutic efficacy of the conjugated drug was evaluated using luciferase Reporter phage assay against *M.tuberculosis* H_{37}RV. High drug entrapment efficiency of plant extract was achieved, ranging from 85-95% and effective particle sized drug-conjugates, ranging from 246-356nm were formed. The relative light units were also less indicating the persistence of activity of the Isoniazid and the inhibition was 93%. Hence Isoniazid Nano-conjugates have the potential for TB Chemotherapy which could be a cost-effective method for the production of Nanodrugs.

INTRODUCTION: Major health concern of TB control is the emergence of Drug resistance and TB co infection with HIV. Globally, high incidence rate of 3.7% MDR-TB cases and 20% of TB-prevalent cases were recorded and among them 9% are estimated to be XDR-TB [1, 2]. Drug resistance reflects the profound active transmission of MDR-TB Strains.

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Though there are many modern interdisciplinary approaches of drug discovery [3] such as 1) Genomics & proteomics for identification of new molecular targets of bacterial metabolic pathways, 2) Molecular biology approaches which focus on validation of molecular models and moreover 3) Using Databases like GenoMycDB, which is a system Biology approach, still the drug regimen of TB chemotherapy i.e. “short course” therapy initiated under DOTS strategy by WHO (1993) is the only recommended treatment till today.

Why still Short course therapy is the recommended TB treatment? Is it due to effective way of action of these short course drugs i.e. streptomycin, isoniazid, rifampicin, ethambutol and sometimes

pyrazinamide on *Mycobacterium.* If the treatment is effective then what is the reason for drug resistence? Is Dosing frequency & dosage of antibiotic is a major concern for emergence of Drug resistance. Or due to the Mycobacterial complex structure. If we look for a reason, drug resistence is due to both High dosage and adaption of Mycobactrium towards antibiotics.

Inspite of many new advances of Drug discovery, due to the Complex structure of *tuberculosis* bacteria they fail to treat the bacteria at particular target. So, no choice left other than "Short-course therapy", because these TB Drugs can cross the lipid rich barrier of Mtb and show effective response. But comparing the present dosage standard of drugs and ADMEK properties of these drugs, most of the drug taken into the body is being metabolized and also causative of severe side effects [4]. Number of efforts have been made to overcome this problem, the strategy [5-7] would certainly aid in overcoming these drawbacks is only through drug encapsulation (Micro or Nano-encapsulation) i.e. 'Nanomedicine'.

Nanomedicine, an innovation of Nanotechnology material sciences and also capable of employing molecular genomics and proteomics based for treatment of wide range of diseases especially pulmonary diseases. This multidisciplinary approach [5] links material science has made possible of targeted drug delivery in chronic pulmonary diseases. Material science i.e. use of nanoscale materials that are about same size as biologic substitutes easily react with natural biologic substances on cell surface or intracellular. There are wide varieties of Nanoparticles, one such advance in material science and nanomedicine is use of polymers [8] (PA-824 a nitroimidazopyran) as encapsulating material.

These polymers may be natural extracted [9] from different origin such as algal origin-alginate, from plant-cellulose, pectin. etc., from animal- chitosin, chondrotin.etc or may be synthetic [10-13] such as PLGA, polyhydroxy butyric acid.etc. Briefly, use of natural polymers can improve tolerability of toxic chemotherapeutics, eventually bioavailability and therapeutic index. But due to the development of toxicological hazards such as cytotoxicity, genotoxicity, lungtoxicity due to the nanoparticle drug delivery formulations [14], it still remain as a scientific research interest but failed to develop as a diagnosis.

So there raised a question if polymers derived from natural origin can efficiently act as carriers why cannot the natural source directly be used? This was also made possible by our researchers i.e. through "Green Nanotechnology" [15]. This is also an interdisciplinary approach that involves green chemistry methods i.e. by employing natural extracts as reducing, capping and stabilizing agents to attain micro or nano encapsulated particles with desired morphology and size. Attaining encapsulation through natural extracts is a novel approach to the drug discovery. And this marvellous approach has shown success in formation of biosynthesis of nanoparticles by microorganisms, "Silver Nanoparticles" [16] from a wide range of extracts originated from bacteria to plant origin were reported.

"Gold Nanoparticles" using Mentha arvensis leaf extract [17] was also made possible. But the mechanism of encapsulation is a simple bioreduction by combinations of biomolecules found in the extracts of certain organisms is environmentally benign but chemically complex. The sources of these bio molecules can be bacteria [18], dinoflagellates, algae [19], plant extracts [20, 21] and also from animals, that are efficient in nanoparticle production and proven anti-tubercular activity.

Thus the present study is a humble attempt to develop a cost effective, eco-friendly method for the production of tuberculosis drug conjugates using green plant extracts. Pure natural constituents which can bioreduce/ entrap molecules. The extract of the plant contains biomolecules, alkaloids and moreover "Polysaccharides" (whose importance discussed in 3rd paragraph) are the bio molecules used for entrapping tubercular drugs. The effective first-line drug against MTb chosen here is Isoniazid (H) because of its importance in initial administration upon detection of Tb and moreover due to its effective mechanism of activity.

Here, in this study we report production of Isoniazid Nano-conjugates and their entrapment

efficiency. Further, characterization of formed conjugates was done by using FESEM. Finally, the anti tubercular activity of solidified drug conjugates against *Mycobacterium*.

MATERIALS & METHODS:

Isoniazid procured from M/s. Yerrow chemicals, Plant extract**,** weed plants with medicinal value which has profusely grown in and around Visakhapatnam collected locally and processed, dialysed using membrane (25.4mm diameter of capacity 5.07ml/cm). All the other chemicals used were procured from Himedia and, all other solvents used are of reagent grade.

Laboratory strains, Two strains of ***M.tuberculosis*** were used in this study.

1. Standard $H_{37}RV$
2. Clinical isolates of H, R, S & E resistant strains. Obtained from National Institute for Research in Tuberculosis (NIRT).

Preparation of Extract:

The leaves of the plant taken, weighed 50gm and surface sterilized with distilled water followed by ethanol. Thus contaminant free leaves are crushed using 200ml distilled water to obtain a fine extract repeatedly filtered using Whattman filter paper no:1. Thus obtained plant extract used for further production of NP's.

Production of INH Nano-conjugates (INH-NCs) and Particle Size Analysis:

Drug-Extract conjugated NCs were produced by simple incubation procedure. Briefly, Isoniazid stock solution was incubated for 2hrs with fine extract in ratio of 4:1 respectively at room temperature. Incubation time directly reflects the constancy in Optical Density of reaction mixture. Initial production of NCs was detected by change in opacity of reaction mixture from more opaque to less opaque. Thus NCs were collected by centrifugation at 4000rpm for 30min. Presence of NCs was analysed by Nanoparticle analysis at 173˚ scattering angle and at 25°c temperature using Nanoparticle analyzer SZ-100.

Drug Entrapment studies:

The amount of isoniazid conjugated to the plant extract was estimated by quantifying the amount of isoniazid released from the extract upon sonication at particular intervals of time. The quantification was done in terms of UV-absorbance recorded using UV-Visible Spectrophotometer UV-1800 SHIMADZU. The percentage drug encapsulation efficiency was determined by formula:

Drug entrapment efficiency =

$$\left(\frac{\text{Practical amount of drug loaded}}{\text{Theoretical amount of drug loaded}} \right) \textbf{ x 100}$$

Characterization of Nanoconjugates (NCs):

Thus produced nanoconjugates having effective size was observed in analysis. So further morphological features of the produced nanoconjugates were characterized using FESEM (ultra 55 from Carl ZEISS). The particles were fixed and images were recorded without coating a metal on specimen but using a field emission filament that allowed getting high resolution imaging.

Solidification:

Nanoconjugates present in (aqueous) liquid dispersion after preparation, were solidified in order to achieve Stability and facilitated handling as well as readiness for further processing (formulation of tablets, capsules, powders). Here the drying was done using both Vacuum evaporation and Freeze drying. Vacuum drying is a time consuming process. Apart from this high temperature required for drying which is a risk of affecting drug molecular properties. We preferred to dry the sample using the freeze drying technique with the Lark pengu Classic plus freeze dryer. Using freeze drying the dried sample was obtained within a day while it took 3-4 days for the vacuum drying. Obtained powdered form of NPs was transferred to vials in contaminant free environment. Further particle size of the powdered form was reanalysed by dissolving in to solvent used and performing nanoparticle analysis. The solvent used here is water.

Anti-*Mycobacterial* sensitivity:

Sensitivity testing is the most important part of the study, this was done against standard $H_{37}R_V$ and also clinical isolates of S, H, R & E resistant strains cultured and maintained by Department of Bacteriology, National Institute for Research in Tuberculosis, Chennai. Luciferase Reporter Phage

(LRP) Assay, a phenotypic method which is less time consuming method and literature comparision [22, 23] with BACTEC system shows 98% accuracy of efficiency and it's a cost-effective method. In this technique, viable mycobacteria are infected with reporter phages expressing firefly luciferase gene. Easily detectable signals are seen in a few minutes after the infection of *M.tuberculosis* with reporter phages.

Light production requires metabolically active *M.tuberculosis* cells, in which reporter phage replicate and luciferase gene is expressed [24]. Both the $St.H_{37}$ RV and clinical isolates of S, H, R & E were incubated with Isoniazid-NCs. Thus the drug-sucessptabilty or drug-resistance was measured in terms of light production after the infection with luciferase reporter phages.

RESULTS & DISCUSSION:

Production & Particle size Analysis of INH-NCs: The incubation time is the time for the drug to be entrapped into the plant extract, specifically to the polymers. Even after attaining the constant OD, further observed has shown retaining constancy for more than an hour. After this particular time the OD value started increasing, this increase is due to the formation of collides and agglomerate. Colloidal form of agglomerates may not be effective drug conjugates. For this reason these mixtures were taken immediately after attaining constancy. Many peaks of Isoniazid particles were observed in particle analysis which shows a broad range of isoniazid particles that differ in their diameter, but the particle size of 246-356nm was found having high cumulative frequency. Z-average of the reaction mixture was positive as shown in the **Fig.1**

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Measurement Results

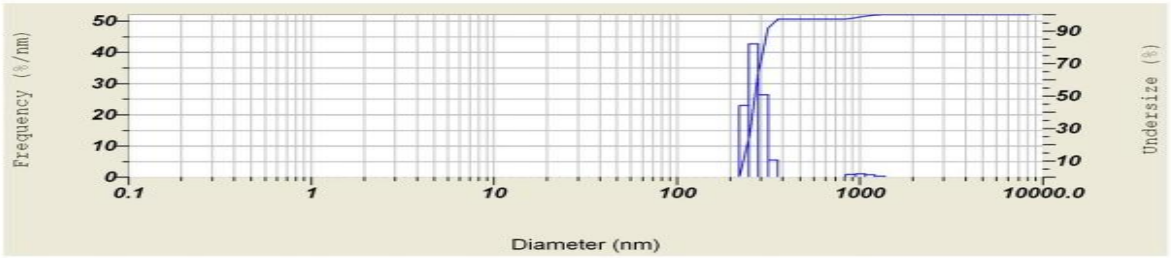
Date	:	Monday, October 22, 2012 3:01:55 PM
Measurement Type	:	Particle Size
Sample Name	:	Nagasejitha_Sample 4
Scattering Angle	:	173
Temperature of the holder	:	25.1 °C
T% before meas.	:	7623
Viscosity of the dispersion medium	:	0.893 mPa·s
Form Of Distribution	:	Standard
Representation of result	:	Volume
Count rate	:	1181 kCPS

Calculation Results

Peak No.	S.P.Area Ratio	Mean	S. D.	Mode
1	0.97	268.7 nm	28.0 nm	263.8 nm
2	0.03	1031.4 nm	121.3 nm	999.9 nm
3	---	--- nm	--- nm	--- nm
Total	1.00	290.5 nm	131.8 nm	263.8 nm

Cumulant Operations

Z-Average	:	1878.3 nm
PI	:	0.824



No.	Diameter	Frequency	Cumulation	No.	Diameter	Frequency	Cumulation	No.	Diameter	Frequency	Cumulation
1	0.34	0.000	0.000	29	10.34	0.000	0.000	57	315.27	26.342	91.757
2	0.38	0.000	0.000	30	11.68	0.000	0.000	58	356.20	5.378	97.135
3	0.43	0.000	0.000	31	13.20	0.000	0.000	59	402.44	0.000	97.135
4	0.49	0.000	0.000	32	14.91	0.000	0.000	60	454.69	0.000	97.135
5	0.55	0.000	0.000	33	16.84	0.000	0.000	61	513.71	0.000	97.135
6	0.62	0.000	0.000	34	19.03	0.000	0.000	62	580.41	0.000	97.135
7	0.70	0.000	0.000	35	21.50	0.000	0.000	63	655.76	0.000	97.135
8	0.80	0.000	0.000	36	24.29	0.000	0.000	64	740.89	0.000	97.135
9	0.90	0.000	0.000	37	27.45	0.000	0.000	65	837.07	0.000	97.135
10	1.02	0.000	0.000	38	31.01	0.000	0.000	66	945.74	0.820	97.955
11	1.15	0.000	0.000	39	35.03	0.000	0.000	67	1068.52	1.047	99.003
12	1.30	0.000	0.000	40	39.58	0.000	0.000	68	1207.24	0.729	99.732
13	1.47	0.000	0.000	41	44.72	0.000	0.000	69	1363.97	0.268	100.000
14	1.66	0.000	0.000	42	50.53	0.000	0.000	70	1541.04	0.000	100.000
15	1.87	0.000	0.000	43	57.09	0.000	0.000	71	1741.10	0.000	100.000
16	2.11	0.000	0.000	44	64.50	0.000	0.000	72	1967.14	0.000	100.000
17	2.39	0.000	0.000	45	72.87	0.000	0.000	73	2222.51	0.000	100.000
18	2.70	0.000	0.000	46	82.33	0.000	0.000	74	2511.05	0.000	100.000
19	3.05	0.000	0.000	47	93.02	0.000	0.000	75	2837.04	0.000	100.000
20	3.45	0.000	0.000	48	105.10	0.000	0.000	76	3205.35	0.000	100.000
21	3.89	0.000	0.000	49	118.74	0.000	0.000	77	3621.48	0.000	100.000
22	4.40	0.000	0.000	50	134.16	0.000	0.000	78	4091.63	0.000	100.000
23	4.97	0.000	0.000	51	151.57	0.000	0.000	79	4622.81	0.000	100.000
24	5.61	0.000	0.000	52	171.25	0.000	0.000	80	5222.96	0.000	100.000
25	6.34	0.000	0.000	53	193.48	0.000	0.000	81	5901.02	0.000	100.000
26	7.17	0.000	0.000	54	218.60	0.000	0.000	82	6667.10	0.000	100.000
27	8.10	0.000	0.000	55	246.98	22.837	22.837	83	7532.65	0.000	100.000
28	9.15	0.000	0.000	56	279.04	42.578	65.415	84	8510.56	0.000	100.000

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FIG 1: PARTICLE ANALYSIS OF INH-NP_S

Drug Entrapment Efficiency:

The drug entrapment efficiency of formed INH-NCs was found to be 85%-95%±SD that was similar to the theoretically expected value of efficiency. High percentage of efficiency reflects that maximum isoniazid initially taken was entrapped in to the plant material.

Characterization of Nanoconjugates (NCs):

Envision of the sample was obtained using FE-Scanning Electron Microscope. The images of the Isoniazid sample obtained are as shown below in **Fig.2** The INH particles when observed under FESEM found as hexagon shaped particles akin to once reported in literature [25] (as shown in the circle in the above figure) here found entrapped by plant material. The possible mechanism behind the binding of drug to the plant material may be due to entrapment or bonding or cross linking. From the literature review, the free functional groups present usually hydrogen or hydroxyl groups in the plant material involves in cross linking with drug molecule which may be covalent or non-covalent [26].

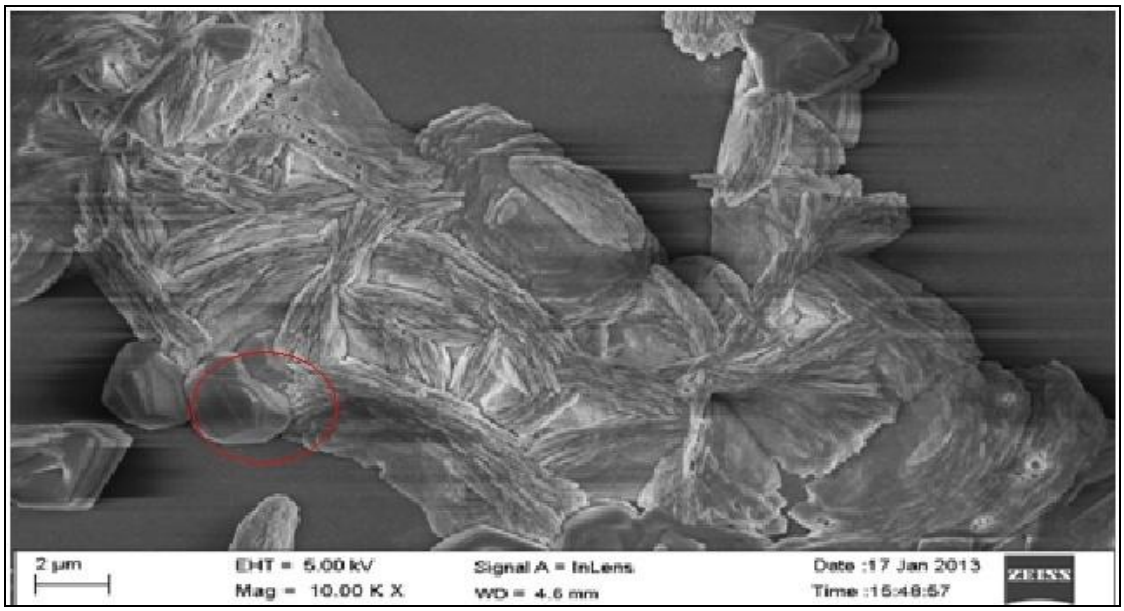

FIG.2: INH-NP$_S$

Anti-*Mycobacterial* sensitivity:

Thus the effective form of the produced particles i.e. powdered form was obtained after freeze drying for 3 days. Luciferase reporter phage assay clinically proven to be recommended method for checking the efficacy of the anti-tubercular drugs. Anti-mycobacterial activity is indicated by fifty percent reduction in relative light units (RLU) in the presence of compound in comparison with compound free control. The inhibitory concentration of the formed conjugates was effective against *M.tuberculosis* was recorded as shown in **Table 1** and **2.**

TABLE 1: ANTI *MTB* TESTING AGAINST H_{37}RV STRAIN

***M.tuberculosis* strain**	**Compound details**	**% Reduction in RLU**	
		5µg/ml	**10µg/ml**
H_{37}RV	INH-NP	78.94	93.41
	Isoniazid(0.2µg/ml)	81.46	

TABLE 2: ANTI *MTB* TESTING AGAINST CLINICAL ISOLATES

***M.tuberculosis* strain**	**Compound details**	**% Reduction in RLU**	
		50µg/ml	**100µg/ml**
S,H,R & E Resistant	INH-NP	67.22	84.30
	Isoniazid(0.2µg/ml)	33.25	

Thus presence of anti-mycobacterial activity of INH-NPs against *Mycobacterium tuberculosis* indicates that the formed conjugates does not mediate functional activity of the drug i.e. isoniazid activity.

CONCLUSIONS: Here we promulgate our report stating that Isoniazid drug conjugates using Green-Nanomedicine can be a remedy for the tuberculosis disease. From the evidence shown by FESEM, the drug-conjugates formed are efficient enough to kill *mycobacterium* and having high drug entrapment efficiency of 85-95%. We can confidently say that with the pure form of this conjugate 100%

inhibition will be possible, while there was 93.41% of inhibition with crude extract. The literature report indicate the usage of 'either' nanotherapeutics i.e. different nano-formulations ranging from liposomes to polysaccharides [27-30] 'or' usage of plant extracts made out of solvents [31-32]. But this is an attempt to produce nano drug conjugate using plant extracts directly without treating them with solvents. Thus production of these drug-conjugate is a cost-effective, eco-friendly method than that of use of nanoscale materials. As it is time for choice of appropriate diagnosis and treatment of MDR-Tubersulodis [33], the adoption of production of Nanoconjugates using Green Nanotechnology could reduce the dosage. Reduction of dosage without affecting bioavailability would definitely be an appropriate diagnosis for TB and can prevent the probability of emergence of MDR-Tuberculosis. Such eco-friendly produced nanoparticles can meet the disadvantage of Bio-hazards and eco-toxicity conventional Nanoparticles.

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