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Exploiting Rhizobium for Cadmium Sulphide Nanoparticle Synthesis: Heterologous Expression of an *Escherichia coli* DH10B Enzyme, YbdK [EC: 6.3.2.2] in *Sinorhizobium fredii* NGR234

Akash Dave , Tanvi Khanna  and Pushpa Robin* 

Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara - 390 002, Gujarat, India.

Abstract

Escherichia coli DH10B has 1.1 kb ybdK gene which is responsible for encoding YbdK enzyme that possess a Gamma glutamyl cysteine synthetase activity. ybdK gene was ligated downstream of a constitutive derepressed lac promoter of a low copy number plasmid vector pBBR1MCS-2, giving rise to a recombinant plasmid pPAT. *Sinorhizobium fredii* NGR234 transformed with pPAT showed an augmented production of glutathione which in turn increased the production of cadmium sulphide nanoparticles to some extent. Also, a heterologous expression of YbdK in *Sinorhizobium fredii* NGR234 improved the oxidation status of bacterial cells which is confirmed by fluorescence microscopy images and fluorometry. Genetically modified (GM) cells stained by DCFDA showed a significant decrease in fluorescence compared to wild type (WT) cells. Physical and chemical properties of the nanoparticles produced by the pPAT transformed *Sinorhizobium fredii* NGR234 differed significantly compared to wild type (WT) *Sinorhizobium fredii* NGR234. Comparative analysis of the nanoparticles by FTIR and SEM analysis revealed the functional groups attached to nanoparticles and average nanoparticle size respectively. Nanoparticles synthesized by genetically modified (GM) bacteria were about 3 times smaller in size compared to those produced by wild type (WT) rhizobium. FTIR analysis revealed an augmented presence of peptide with the nanoparticles produced by GM bacteria compared to those produced by the WT bacteria. XRD data revealed that biosynthesized CdS nanoparticles are face centered crystalline particles which was confirmed by comparing the peaks to standard JCPDS data (JCPDS card no. 10-454).

Keywords: *Sinorhizobium fredii* NGR234, YbdK, Glutathione, Heavy metals, Cadmium sulphide, Nano particles, Green synthesis

*Correspondence: pushparobin@gmail.com; +91 9879120161

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INTRODUCTION

Heavy metal pollution is one of the major environmental issues faced by the world. This problem is predominantly due to uncontrolled anthropogenic activities which has reached an alarming levels and it requires an immediate and drastic efforts to mitigate. Metals whose density is greater than 5g/cm^3 are considered as heavy metals.¹ Heavy metals can cause range of biological deformities and instabilities at molecular level including DNA damage, cell membrane damage and protein misfolding which can cause serious problems within the biological system by hampering the normal function of macromolecules.¹ Research around the world is focused on developing new technologies and methods to tackle the problem of heavy metal pollution by green methods which can be broadly classified as Bioremediation.² Microorganisms like fungi, bacteria and viruses plays a major role in bioremediation of heavy metals from the soil as well as water.² Bacteria possess varieties of mechanisms such as extracellular barrier, efflux pump, intracellular /extracellular sequestration and reduction of metal ions to resist the presence of heavy metal in their surroundings.³ Many genus of soil bacteria and Plant growth promoting bacteria (PGPB) such as *Mesorhizobium*, *Sinorhizobium* and *Bradyrhizobium* have shown their ability to tolerate the presence of heavy metals like Cd, Co, Fe, Ni, Zn and Cu⁴ in their environment.

The remarkable ability of bacteria to reduce the metal ions thus converting them into less toxic forms is the best candidate for metal nanoparticle synthesis.⁵ Wild type as well as genetically modified bacteria have a potential to produce varieties of metal nanoparticles in *in vitro* conditions.^{5,6} Also *Escherichia coli* (MTCC10312) showed extracellular⁷ production of cadmium sulphide quantum dots. And a cyanobacteria *Oscillatoria limnetica* has shown its ability to produce silver nanoparticles.⁸ Not only wild type but genetically modified bacteria could be a good tool to manufacture nanoparticles in *in vitro* conditions. It was reported that genetically modified *Escherichia coli* over expressing glutathione synthetase showed augmented biosynthesis of cadmium sulphide nanoparticles.⁹ Also *Escherichia coli* expressing *Candida albicans* Metallothionine gene

and *Rhizobium tropici* Phytochelatin synthetase gene showed enhanced production of silver nanoparticles¹⁰ and selenium nanoparticles¹¹ respectively. Biogenic nanomaterial has unique physical and chemical properties and they are used in varieties of industries such as electronic, chemical, photonics, energy and medical.¹² It is clear that microbe based nanoparticles production could be cheaper and environment friendly. Also, their unique properties allows its traditional as well as novel application in a wide spectrum of Industries.¹³ Genetically modified *Escherichia coli* strain coexpressing Metallothionine and Phytochelatin synthetase has been reported to produce 33 inorganic nanoparticles (20 single element and 13 multi-element),¹⁴ which is way more than capacity of a synthetic reaction to produce that many inorganic nanoparticles in a single reaction. Additionally, the ease to genetically manipulate the microbes makes it a best candidate for production of nanoparticles in a way which consumes very less amount of energy thus contributing less in carbon foot print addition to our environment. Phytochelatin as well as Metallothionine have been extensively expressed/coexpressed/overexpressed for biosynthesis of nanoparticles for example PCS and ghs1 genes of *Schizosaccharomyces pombe* overexpressed in *E.coli* for CdS nanoparticles synthesis.¹⁵ Also an enhanced production of glutathione by *Escherichia coli* showed intracellular production of CdTe quantum dots.¹⁶ The probable mechanism of thiol containing proteins, amino acids and short peptides is to form metal thiolate conjugate with the heavy metals, which can be explained by $\text{nRSH} + \text{Mn}^+ \leftrightarrow (\text{RS})_n\text{-M} + \text{nH}^+$ equation.¹⁷

Glutathione synthesis in prokaryotes is governed by two enzymes, Gamma glutamyl cysteine synthetase (γ -GCS) that catalyzes the ligation of glutamate and cysteine to form gamma glutamyl cysteine while the second enzyme glutathione synthetase catalyzes the ligation of gamma glutamyl cysteine and glycine to form a unique tripeptide glutathione.⁹ Glutathione has numerous functions such as metal chelation and alleviation of ROS inside the cells. Gamma glutamyl cysteine synthetase is a rate limiting enzyme of glutathione synthesis pathway, which binds to glutathione and controls its production by feedback mechanism whenever necessary.

Table1. Bacterial strains used in this study

Bacterial strain (Wild type /GMOs) and antibiotic selection	Growth medium	Optimum Temp.	Source and description
Wild type bacteria			
<i>Escherichia coli</i> DH10B	Luria Bertani broth/agar (LB)	37 °C	The department of Biochemistry, MSU Baroda
Streptomycin (50ug/ml)	Yeast extract Mannitol broth /agar (YEM)	28 °C	Generous gift by Dr. Jose M ^a Vinardell Gonzalez, Facultad de Biología, Universidad de Sevilla, Spain
<i>Sinorhizobium fredii</i> NGR 234			
Rifampicin (18ug/ml)	Luria Bertani broth/agar (LB)	37 °C	<i>Escherichia coli</i> DH10B transformed with the recombinant plasmid (pPAT)
Transformants			
<i>Escherichia coli</i> DH10B (pPAT)	Yeast extract Mannitol broth /agar (YEM)	28 °C	<i>Sinorhizobium fredii</i> NGR 234 transformed with the recombinant plasmid (pPAT)
Kanamycin (25ug/ml)			
<i>Sinorhizobium fredii</i> NGR 234 (pPAT)			
Rifampicin(18ug/ml) and Kanamycin (25ug/ml)			

[pPAT plasmid abbreviation- p(plasmid), P(Pushpa), A(Akash), T(Tanvi)].

Escherichia coli DH10B YbdK, a carboxylate amine ligase also exhibits gamma glutamyl cysteine synthetase activity. It is a smaller protein with only 372 amino acids which possesses a different overall fold. Tertiary protein structure of YbdK is more similar to Glutamine synthase than bacterial γ-GCS.¹⁸ So this study hypothesizes the use of structural dissimilarity of YbdK to hinder the feedback mechanism of glutathione and use a *Sinorhizobium fredii* NGR234 as a chassis¹⁹ for efficient secretion of glutathione. Also, *Sinorhizobium fredii* NGR234 lacks γ-GCS as well as YbdK enzymes which makes it a good candidate for an introduction of glutamyl-cysteine ligase activity in its system.

MATERIALS AND METHODS

Microorganisms, plasmids and primers used in this study

Bacteria and their growth conditions²⁰ for this study are mentioned in Table 1. Plasmids and Primers used in this study are described in detail in Table 2.

Genomic DNA extraction, plasmid purification and Transformation of bacteria

Genomic DNA from *Escherichia coli* DH10B was extracted by using a standard CTAB method.²¹ Plasmid purification was done by using a modified method involving silicone dioxide.²² *Escherichia coli* DH10B was transformed by a standard calcium chloride transformation method²³ while *Sinorhizobium fredii* NGR234 was transformed by electroporation.²⁴

Glutathione estimation

Intracellular and extracellular glutathione was estimated by the method based on reduction of GSSG by NaBH₄. This method depends on two measurements of GSH before and after reduction of GSSG in bacterial supernatant.²⁵ Cell free lysate was used to estimate extracellular glutathione.

Detection of total intracellular ROS in bacteria by fluorescence microscopy and fluorometer

Sinorhizobium fredii NGR234 (pPAT) and *Sinorhizobium fredii* NGR234 cells were grown in YEM broth with the appropriate conditions mentioned above till their OD reaches 0.8. Approximately 1 ml cells were pelleted down at 8000 rpm for 2 minutes, washed with PBS twice and again resuspended in PBS for ROS determination. ROS was estimated by DCFDA which

enters the cells and reacts with reactive oxygen to form green fluorescent colored compound dichlorofluorescein (DCF).²⁶ The estimation of ROS was done by both fluorometer and microscopy. A stock solution of DCF-DA (1mM) was prepared in PBS and was further diluted to 10 μ M working concentration in the cells dispersed in PBS. Cells were then incubated for 30 minutes at 30°C in total dark condition. After that cells were again washed twice with PBS before fluorometry and microscopy experiment. Fluorescence intensity was measured at excitation of 485 nm and emission at 520 nm (Biotek). For both experiments unstained *Sinorhizobium fredii* NGR234 cells suspended in PBS were used as control. Microscopy was performed by fluorescent Nikon-Ti2E live imaging system at 60X magnification.

In vitro synthesis of Cadmium sulphide nanoparticles by wild type and genetically modified rhizobium

Bacterial cells were allowed to grow in YEM medium for 48 hr as mentioned in Table 1 and their cell free lysate was collected by centrifugation

at 8000 RPM for 5 minutes, which was used for synthesis of cadmium sulphide nanoparticles. Cadmium chloride (0.25M) and sodium sulphide (0.25M) (1:1) were allowed to react with equal volume of cell free lysate at proper conditions to initiate the synthesis of cadmium sulphide nanoparticles.⁷

Characterization of Cadmium sulphide nanoparticles

First step in characterization of nanoparticles was to have a clearer look into its morphology by observing the biosynthetic nanoparticles under SEM (Sigma VP SEM/Carl Zeiss NTS) and recording their micrographs.^{7,27} FTIR spectrophotometry was performed by Bruker Alpha 2 spectrophotometer within the infrared range of 500-3500 cm^{-1} , to have a better understanding of the functional groups attached with nanoparticles.²⁸ Finally XRD was performed by XPERT PRO XRD machine to get a clear cut understanding about its crystalline nature. The intensities were recorded in a range of 10- 80° 2 θ angle.⁷

Table 2. Plasmids and primers used in this study

Plasmid/ Primers	Size in base pairs (bp)	Description	Antibiotic selection	Source
pBBR1MCS2	5148 bp	Low copy number cloning and expression vector having a derepressed lac promoter.	Kanamycin 25 μ g/ml	The M.S. University of Baroda.
pPAT	6264 bp	1119 bp ybdK gene from <i>Escherichia coli</i> DH10B ligated in pBBR1MCS2 plasmid	Kanamycin 25 μ g/ml	ybdK gene was amplified by PCR using forward and reverse primers from <i>Escherichia coli</i> DH10B genome and then ligated in pBBR1MCS2 plasmid after a restriction digestion of vector and PCR amplified gene
Forward primer	34 bp	CGCTCGAGAGGAGGATAG TCATGCCATTACCCGA 34 base pairs primer flanking Xho1(CTCGAG) restriction enzyme site at 5' end	NA	NA
Reverse primer	28 bp	TTAAGCTTTAGTACCCGG CCCAGATCT 28 base pairs primer flanking Hind3 (AAGCTT) restriction enzyme site at 5'end	NA	NA

RESULTS AND DISCUSSION

Construction of a recombinant pPAT plasmid and transformation of bacteria

1119 bp ybdK gene was amplified from the genomic DNA of *Escherichia coli DH10B* by using forward and reverse primers flanked by Xho1 and Hind3 restriction sites respectively at their 5' end (Table 3). Plasmid vector pBBR1MCS2 and ybdK gene (amplified from the genomic DNA) were sequentially digested with Xho1 and Hind3 restriction enzymes (thermofisher scientific)²⁹ (Fig. 1a), followed by ligation using DNA ligase enzyme (thermofisher scientific). After ligation, *Escherichia coli DH10B* was transformed with ligation mixture and cells were spreaded on LB agar medium containing kanamycin 25µg/ml and allowed to grow overnight at 37°C. Single colony was selected from the transformation plate and

allowed to grow in LB medium supplemented with kanamycin 25µg/ml till 0.8 OD. Cells were pelleted down and plasmid was isolated from them followed by its digestion with enzymes Xho1 and Hind3 giving rise to two distinct bands of 5.1 kb and 1.1 kb on 1% agarose gel, thus confirming the presence of recombinant plasmid pPAT in *E.coli DH10B* cells (Fig. 1b). Recombinant plasmid pPAT was purified from *Escherichia coli DH10B* and electroporated in *Sinorhizobium fredii NGR234*. 12 kv/cm at a fixed time of 5 ms gave maximum transformation efficiency. Recombinant plasmid was again isolated from *Sinorhizobium fredii NGR234* and was confirmed by a PCR amplification of ybdK gene from the purified pPAT plasmid. PCR amplification gave a clear band of 1.1kb, which confirmed the transformation of *Sinorhizobium fredii NGR234* by pPAT plasmid (Fig. 1c).

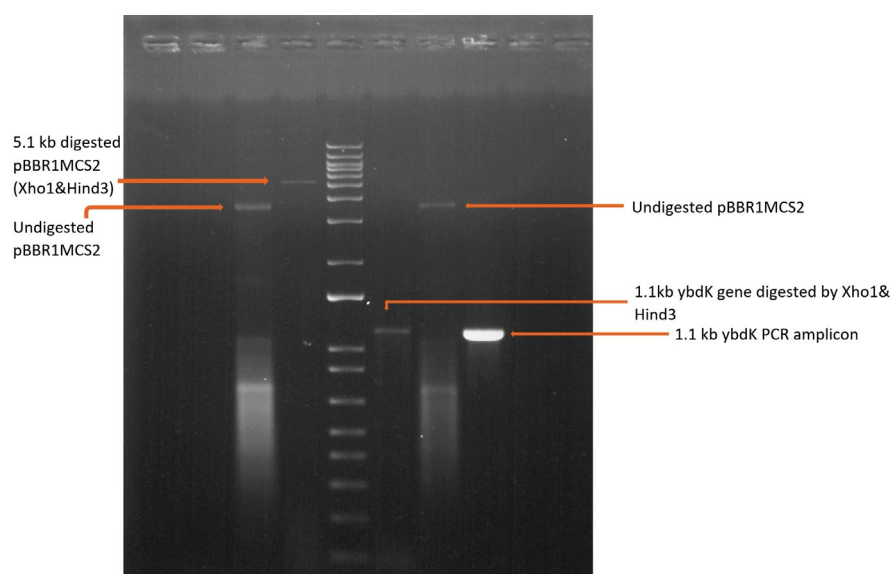


Fig. 1a. Sequential digestion of vector pBBR1MCS2 and insert ybdK gene before ligation.

Table 3. Extracellular and intracellular levels of glutathione produced by WT and GMO *S.fredii NGR 234*. All the values are represented in mean $\pm$ SD format

	Organism	GSH mM	GSSG mM	Total Glutathione mM	GSH/ GSSG ratio of mean
Extracellular	<i>Sinorhizobium fredii NGR 234</i>	0.738 $\pm$ 0.004	3.365 $\pm$ 0.035	4.07 $\pm$ 0.001	0.21
Extracellular	<i>Sinorhizobium fredii NGR 234</i> (pPAT)	0.375 $\pm$ 0.021	93.835 $\pm$ 0.035	94.275 $\pm$ 0.035	0.004
Intracellular	<i>Sinorhizobium fredii NGR 234</i>	0.650 $\pm$ 0.001	8.875 $\pm$ 0.021	9.520 $\pm$ 0.014	0.07
Intracellular	<i>Sinorhizobium fredii NGR 234</i> (pPAT)	0.999 $\pm$ 0.002	176.44 $\pm$ 0.015	177.445 $\pm$ 0.008	0.005

[GSH- Reduced glutathione, GSSG- Oxidized glutathione].

Detection of total intracellular ROS in bacteria by fluorescence microscopy and fluorometer

Fig. 2a and 2b (images taken by fluorescent microscope) shows green coloured fluorescence of DCF inside the bacterial cells. Equal number of *Sinorhizobium fredii* NGR234 and *Sinorhizobium*

fredii NGR234 (pPAT) cells were taken and stained which showed that cloning *Escherichia coli* DH10B ybdK gene in *Sinorhizobium fredii* NGR234 significantly improved its oxidative status. *Sinorhizobium fredii* NGR234 (pPAT) showed less fluorescence compared to *Sinorhizobium fredii*

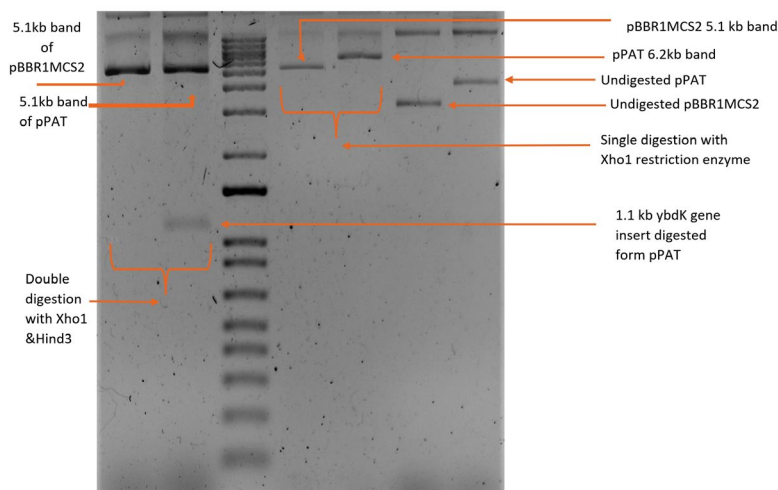


Fig. 1b. Sequential digestion of recombinant plasmid pPAT by Xho1 and Hind3 enzymes.

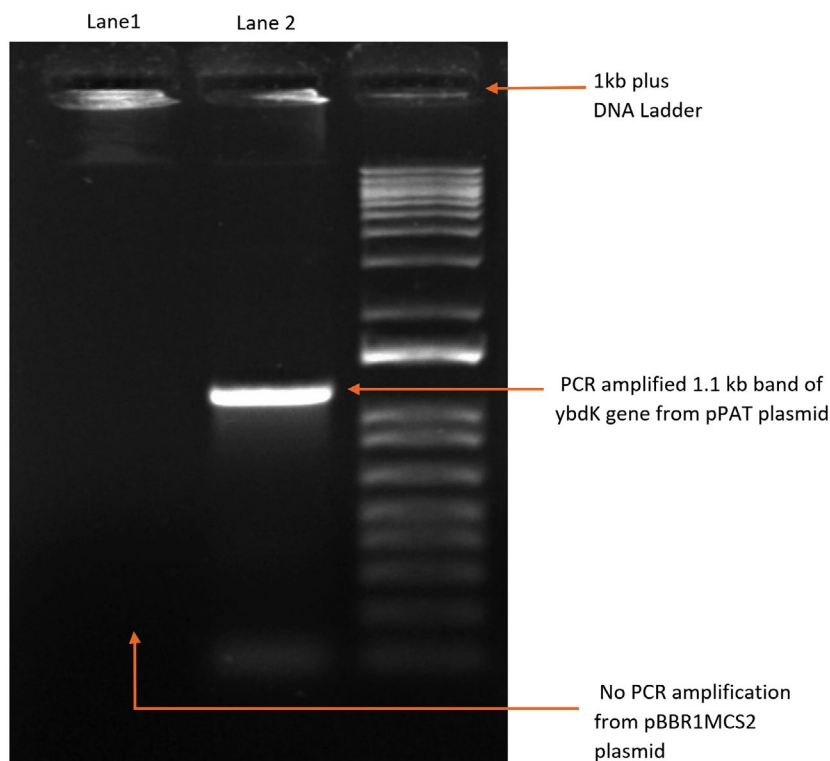


Fig. 1c. PCR amplification of ybdK gene form pPAT (lane2) and pBBR1MCS2 (Lane1).

NGR234. For further confirmation fluorescence intensity was measured by fluorometer which showed a significant decrease in fluorescence emitted by GM bacteria compared to WT bacteria as shown in Fig. 2c. This is an indication of a proper heterologous expression of YbdK in *Sinorhizobium fredii* *NGR234*.

Bacterial growth curve

We examined whether an overexpression of YbdK enzyme has any effect on the growth of *S. fredii* *NGR 234* or not. *Sinorhizobium fredii* *NGR234* and *Sinorhizobium fredii* *NGR234* (pPAT) were allowed to grow in YEM medium for 70 hours with proper conditions mentioned in Table 1 and

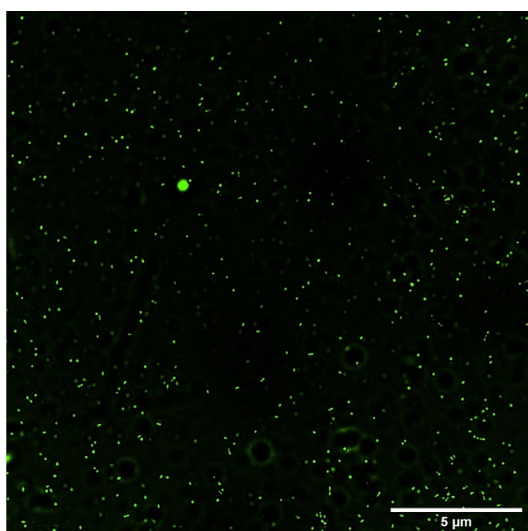


Fig. 2a. ROS visualization by DCFDA staining of *S.fredii* *NGR 234*.

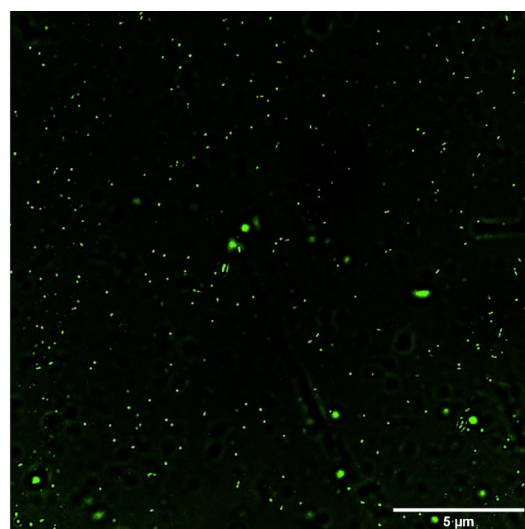


Fig. 2b. ROS visualization by DCFDA staining of *S.fredii* *NGR 234* (pPAT).

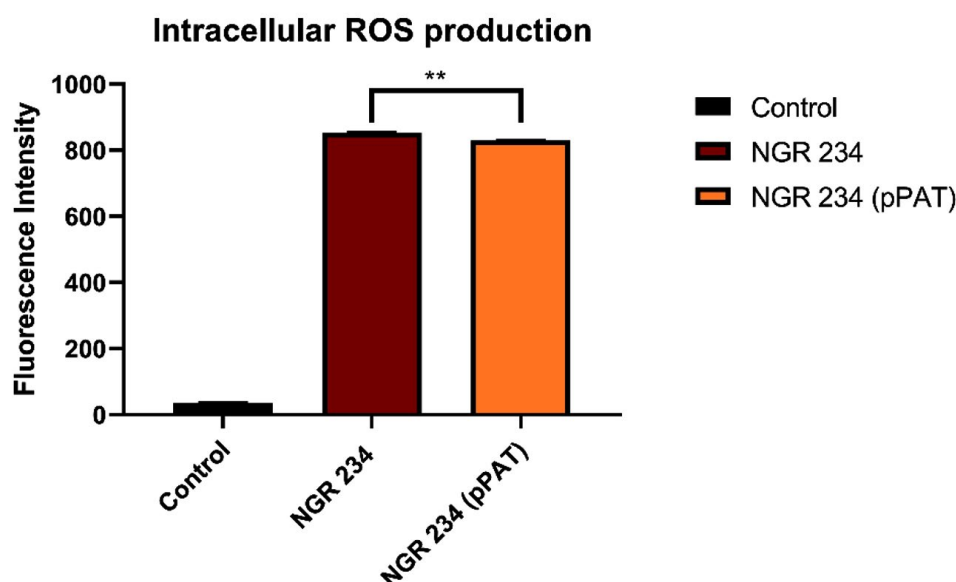


Fig. 2c. Intracellular ROS production by WT and GM bacteria. Data expressed as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$ (n=3).

OD at 600 nm was recorded every 4 hours. It was observed that *S. fredii* NGR 234 (pPAT) showed increased OD between 25th - 40th hour as compared to *Sinorhizobium fredii* NGR234 cells (Data not shown). This is due to increased production of glutathione which helps to reduce the total oxidative stress of the growth media.

Glutathione estimation

Intracellular and extracellular glutathione was estimated from *Sinorhizobium fredii* NGR234 and *Sinorhizobium fredii* NGR234 (pPAT). To

estimate extracellular glutathione, cell free lysate was used. Cells and lysate were separately analyzed for glutathione after 48 hours incubation in YEM medium. 23 fold increase in extracellular levels of total glutathione (GSH+GSSG) was observed in *Sinorhizobium fredii* NGR234 (pPAT) compared to *Sinorhizobium fredii* NGR234 while intracellular total glutathione in *Sinorhizobium fredii* NGR234 (pPAT) increased upto 19 folds approximately compared to *Sinorhizobium fredii* NGR234 cells. Also *Sinorhizobium fredii* NGR234

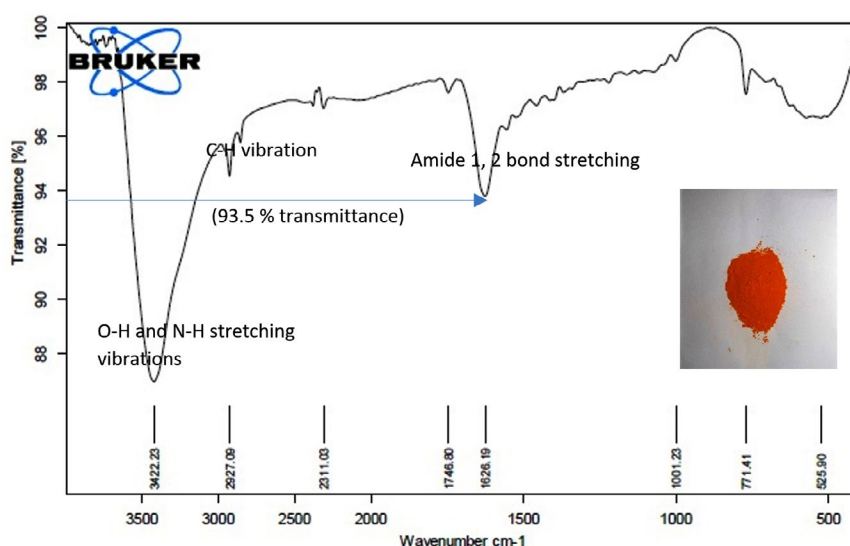


Fig. 3a. FTIR of CdS nanoparticles from *S. fredii* NGR234.

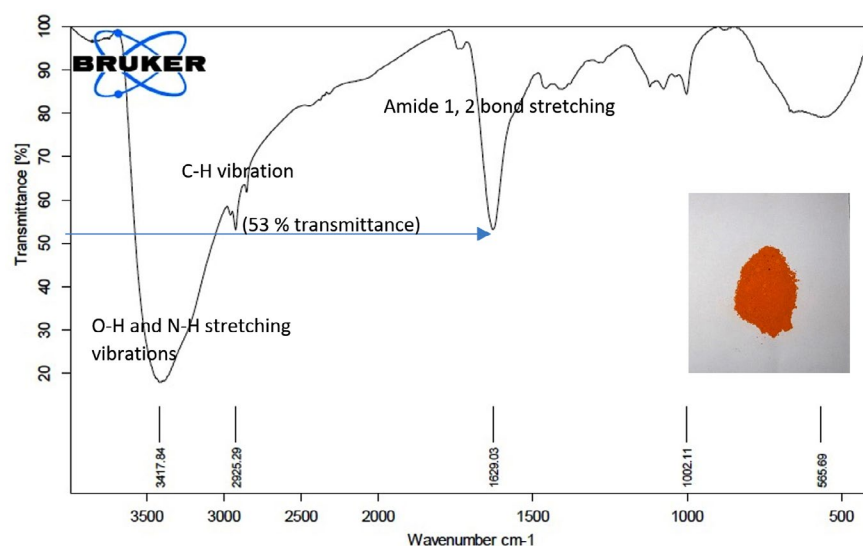


Fig. 3b. FTIR of CdS nanoparticles from *S. fredii* NGR234 (pPAT).

(pPAT) showed 1.5 folds increase in the production of reduced GSH (Intracellular) as compared to *Sinorhizobium fredii* NGR234. Extracellular and intracellular GSH/GSSG ratio for *Sinorhizobium fredii* NGR234 was 0.21 and 0.07 respectively while the extracellular and intracellular GSH/GSSG ratio for *Sinorhizobium fredii* NGR234 (pPAT) was 0.004 and 0.005 respectively, mentioned in Table 3. Both Intracellular and extracellular total glutathione showed a significant increase in GMO bacteria

compared to its wild type counterpart which clearly indicates the steady state constitutive expression of YbdK enzyme and in turn more production of glutathione. The drastic decrease in GSH/GSSG ratio of GMO bacteria compared to wild type bacteria could indicate more transport of GSH outside the bacterial cells, thus it has become clear that constitutive over expression of YbdK enzyme in *Sinorhizobium fredii* NGR234 can increase total glutathione levels inside and outside

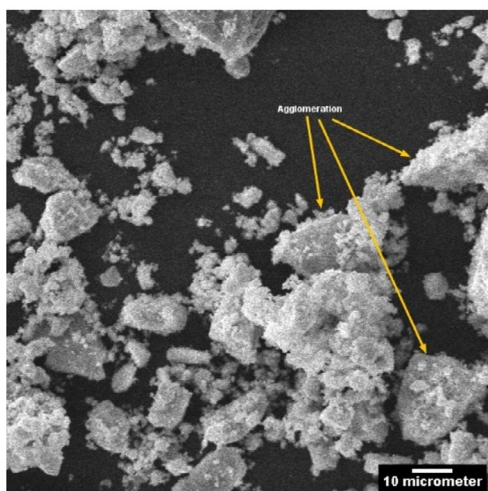


Fig. 4a. SEM of CdS nanoparticles from *S. fredii* NGR 234.

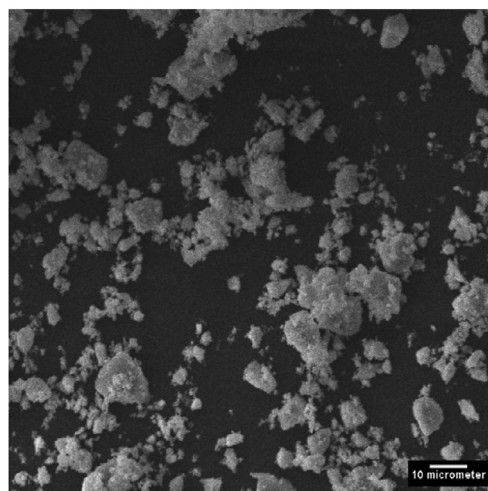


Fig. 4b. SEM of CdS nanoparticles from *S. fredii* NGR 234-(pPAT)

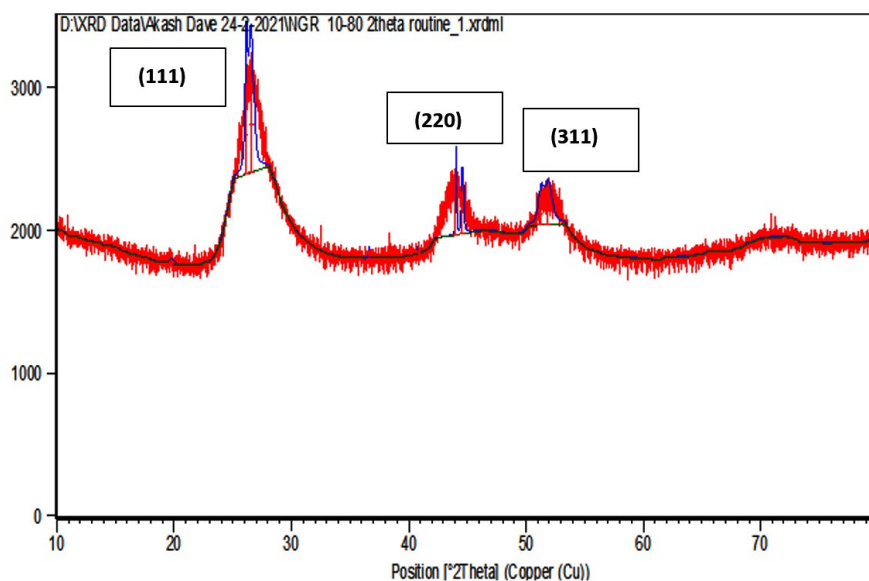


Fig. 5a. XRD of CdS nanoparticles from *S. fredii* NGR 234.

the cells. Moreover, it is a fact that glutathione reductase enzyme is only present inside the cell cytoplasm so the intracellular levels of GSH is higher in GMO bacteria compared to wild type. Higher levels of GSSG inside and outside the cells indicates an elevated production of GSH molecules which were later oxidized to GSSG and started accumulating. This is because the cells have basal levels of glutathione reductase enzymes while only YbdK enzyme was overexpressed, so this imbalance of alleviated glutamyl-cysteine ligase activity compared to glutathione reductase activity might be the reason for increased GSSG accumulation which is also an indirect sign for the proper overexpression of *Escherichia coli* DH10B YbdK enzyme in *Sinorhizobium fredii* NGR234. Due to lack of cognate antibodies for YbdK, western blot analysis was not performed.

Cadmium sulphide nano particle synthesis and characterization

We tested the ability of *Sinorhizobium fredii* NGR234 (pPAT) cells to produce the nanoparticles. 50 ml cell free lysate of *Sinorhizobium fredii* NGR234 (pPAT) cells produced 2.059 ± 0.001 g cadmium sulphide bionanocrystals, while the cell free lysate of *Sinorhizobium fredii* NGR234 produced 2.009 ± 0.001 g cadmium sulphide bionanocrystals. We have seen that cell

free lysate of *S. fredii* NGR 234 (pPAT) had a very high amount of GSSG (Table 3). Reaction between sodium sulphide and cell free lysate could have initiated a cascade of electron donation to GSSG which generated CdS nanoparticles.³¹ We also observed that the dry weight of the nanoparticles obtained from GMO (genetically modified) cells supernatant was about 20.28% more in comparison to the nanoparticles obtained from wild type cells supernatant. FTIR was performed further to get a clear idea about the functional groups attached with the nanoparticles. FTIR analysis showed various transmittance peaks between 3500cm^{-1} to 500cm^{-1} (Fig. 3a and b). The peaks at 1629.03 cm^{-1} and 3417.84 cm^{-1} for *Sinorhizobium fredii* NGR234 (pPAT) gave transmittance of about 51% and 19% respectively, while the similar ranged peaks at 1626.19 cm^{-1} and 3422.23 cm^{-1} from *Sinorhizobium fredii* NGR234 gave transmittance of 87% and 93.5% respectively. The decrease in transmittance of both peaks for *Sinorhizobium fredii* NGR234 (pPAT) suggests that it has successfully overexpressed glutathione. The peak around 1629 cm^{-1} in the Fig. 3a and 3b represents an amide 1 and 2 bonds stretching from the conversion of C-OH biomolecules. The amide bonds of protein and especially glutathione may interact with nanoparticles to stabilize it.³⁰

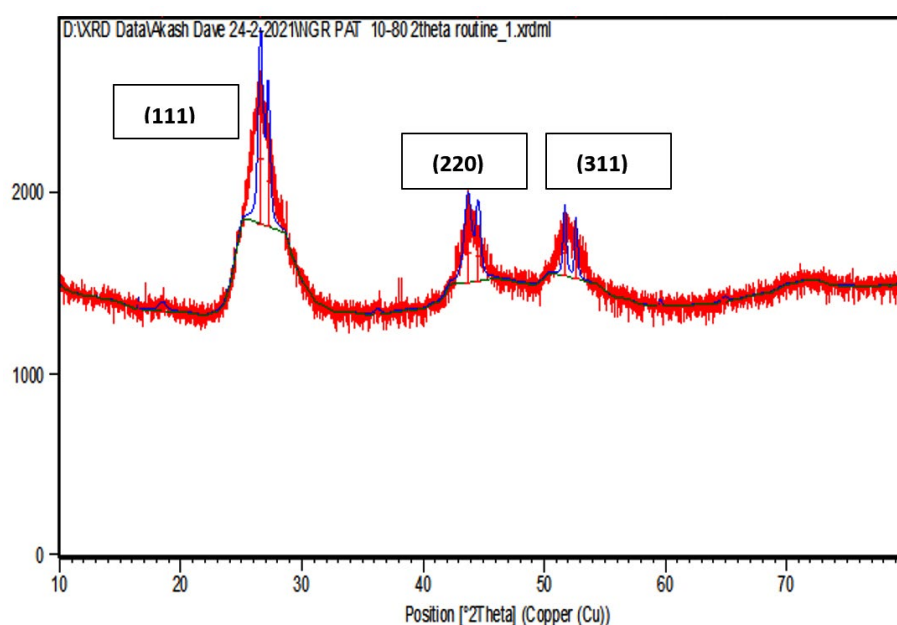


Fig. 5b. XRD of CdS nanoparticles from *S.fredii* NGR 234-pPAT

Also the broad and strong peak between 3200 cm^{-1} - 3400 cm^{-1} indicates the stretching vibrations of O-H (phenols) and N-H (amines) bonds due to primary and secondary amine linkages of proteins and amino acids. Small peaks around 2927 cm^{-1} (Fig. 3a) and 2925 cm^{-1} (Fig. 3b) corresponds to the C-H vibration of alkanes. The data showed decrease in transmittance of the peak around 1629 cm^{-1} for *Sinorhizobium fredii* NGR234 (pPAT) compared to *Sinorhizobium fredii* NGR234. This corresponds to amide 1 and 2 stretching representing the increase in peptide bonds in the nanoparticles which indirectly confirms the elevated levels of glutathione in nanoparticles formed by *Sinorhizobium fredii* NGR234 (pPAT).

SEM observation revealed the capability of the organisms to produce variety of nanoparticles. The particles displayed vivid shapes ranging from a simple triangular shape to a complex heptahedron shapes for both organisms. Less aggregation and smaller size of nanoparticles were obtained by *Sinorhizobium fredii* NGR234 (pPAT) as compared to *Sinorhizobium fredii* NGR234 (Fig. 4a and 4b). It is already known that the organism which overexpresses glutathione tends to produce smaller and identical nanoparticles with very less aggregates and internal agglomeration.⁹ Average size of nanoparticles produced by *Sinorhizobium fredii* NGR234 was 909 nm while *Sinorhizobium fredii* NGR234 (pPAT) produced nanoparticles of size 343 nm suggesting the proper overexpression and secretion of glutathione outside the cells by GM bacteria. SEM images revealed that the nanoparticles formed by *Sinorhizobium fredii* NGR234 (pPAT) were comparatively smaller and showed less aggregation and showed better dispersion compared to the nanoparticles formed by *Sinorhizobium fredii* NGR234. Previously it has been reported that *Escherichia coli* ABLE C cells overexpressing glutathione produced more dispersed nanoparticles and showed less aggregation.⁹ It is possible that more number of glutathione molecules will bind more CDS particles thus sequestering smaller particles resulting in formation of dispersed nanoparticles. Such explanation has been previously stated by many researchers for the production of smaller nanoparticles by the microorganisms either overexpressing Phytochelatin or glutathione.¹¹

XRD data pattern of CdS nanoparticles from *Sinorhizobium fredii* NGR234 gave major peaks for 2 theta at 26.54, 44.56 and 51.86 angles which corresponds to (111), (220) and (311) planes (Fig. 5a). And the CdS nanoparticles from *Sinorhizobium fredii* NGR234 (pPAT) gave major peaks for 2 theta at 26.59, 43.67 and 51.65 angles which corresponds to (111), (220) and (311) planes (Fig. 5b). The lattice plane of CdS nanoparticles showed d spacing of 3.2 Å to the strongest peak (111) for the nanoparticles produced by both GMO and WT which indicates a face centered crystalline nanoparticle structure. The peaks obtained corresponds to the JCPDS card no. 10-454 for CdS nanoparticles. The main difference between the XRD peaks of nanoparticles produced by WT and GMO is the decrease in intensity of the peaks for *S. fredii* NGR 234 (pPAT) which indicates the smaller crystalline size compared to *S. fredii* NGR 2347. This results correlates with the SEM results where it was seen that *S. fredii* NGR 234 (pPAT) produces significantly smaller nanoparticles.

CONCLUSION

S. fredii NGR 234 is an agriculturally important rhizobium which exhibits a PGPR activity and can promote nitrogen fixation in multiple legumes. The aim behind this work is establishing *S. fredii* NGR 234 as a standard chassis for simple and complex genetic modification. Many rhizobium possesses multiple plasmids as large as 2-3 Mb in size which indicates that they can tackle plasmid load very easily. This ability should be put to use by introducing foreign genes which are industrially and agriculturally important to us. So we did this by introducing a plasmid containing ybdK gene in *S. fredii* NGR 234. It is evident from the above results that *S. fredii* NGR 234 is capable of synthesizing CdS nanoparticles. Also, it has become clear that overexpressing YbdK enzyme elevates glutathione secretion by *S. fredii* NGR 234 cells which in turn increase the synthesis of nanoparticles as well as change the physical and chemical properties of nanoparticles. Extracellular GSSG production by the genetic modified rhizobium was about 1.88 folds higher as compared to the wild type rhizobium. Freely available GSSG in cell free lysate would have acted as the precursor for the cascade reaction

which starts as soon as sodium sulphide reacts with the cell free lysates. The sulphide would have acted as a nucleophile and donated its electrons to bisdisulphide of GSSG, similar to the transsulfuration reactions thus generating CdS and lanthi-MT type of molecules. So we conclude that more amount of GSSG in lysate can generate more thiolated molecules after a reaction with sodium sulphide thus decreasing the Cd/thiol ratio and generating smaller nanoparticles.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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Phytochemical evaluation and Chemical characterization of *Bauhinia variegata* L. bark extract by TGA/DSC, FT-IR and GC-MS analytical techniques: Pharmaceutical Aspects

Khanna Tanvi, Dave Akash and Robin Pushpa*

Department of Biochemistry, The Maharaja Sayajirao University of Baroda, Vadodra, Gujarat, 390002, INDIA

*pushparobin@gmail.com

Abstract

Bauhinia variegata (Linn.), *Caesalpiniaceae*, known as *Kachnar* in Ayurvedic texts has been shown to have promising anticancer potential. The presence of bioactive molecules in plants which can be commercialized has led to research in knowing their chemical properties. Previous studies from our laboratory have validated the claim of anticancer property of *Bauhinia variegata* by providing biochemical evidence for the effect of petroleum ether bark extract (PEBE) and chloroform bark extract (CBE) on A549 and H460 lung cancer cell lines respectively. This work aimed at the application of various analytical techniques (thermal analysis, Fourier infrared transform spectroscopy and GC-MS) in the characterization of dried extracts of *Bauhinia variegata* with medicinal properties. TG-DSC-FTIR-GC-MS was applied to monitor the thermal stability and chemical properties of *Bauhinia variegata* bark extracts.

The TG analysis of PEBE and CBE showed mass loss within three steps in different temperature ranges. The DSC curves for the dried extracts of *Bauhinia variegata* showed that thermal processes occur between 50 – 100°C for PEBE and 50-70°C for CBE. The FTIR spectroscopic studies of petroleum ether and chloroform bark extracts of *Bauhinia variegata* revealed different characteristic peak values with various functional groups in the extracts. The GC-MS study of PEBE revealed 18 compounds and CBE revealed 8 compounds. Thus, we have been successful in narrowing down to very few probable anticancer compounds from *Bauhinia variegata* bark extracts and also through this study, it was possible to substantiate the possibility of applying these techniques for use in the herbal medicines production from medicinal plants.

Keywords: Dried *Bauhinia variegata* bark extract, Thermal analysis, FTIR, GC-MS.

Introduction

Despite advancements in medicine, a treatment for cancer eludes mankind. Though a plethora of drugs that will help

fight against cancer exist, problems of side effects and multi-drug resistance limit their use. Natural products from plants can help overcome this limitation. *Bauhinia variegata*, known as *Kachnar* in Indian Ayurvedic texts, is a tree found in northern parts of India. In traditional system of medicines like Ayurveda, Unnani, it is used as medicine for basic health care⁸. *Bauhinia variegata* is used as traditional medicine in the treatment of liver disorders, cancer, diabetes^{3,4,10,11}.

Previous reports have shown various pharmacological activities like antidiabetic, antitumor, antimicrobial, anti-inflammatory etc^{5,9,11}. Literature also showed that *Bauhinia variegata* leaf extracts exhibit antibacterial activity and possessed better antioxidant potential².

The presence of varied compounds in *Bauhinia variegata* flower as well as bark extracts showing different pharmacological activities has been reported^{3,11}. There are only a couple of preliminary studies reporting anticancer potential for this plant but ayurvedic texts have mentioned of it. Our laboratory has tried to validate the claim of anticancer property of *Bauhinia variegata* by providing biochemical evidence for the effect of petroleum ether bark extract (PEBE) and chloroform bark extract (CBE) on A549 and H460 lung cancer cell lines respectively. So, it interested us to look at these extracts in modern science perspective.

Thermal analysis has become a significant tool for characterization of dry extracts and plant products. TG alongside other techniques helps in better understanding of the organic molecules especially if the compounds are intended for pharmaceutical use². FTIR analysis will help reveal the chemical composition. Many authors have shown applications of different thermal analysis techniques in pharmaceutical technology and the standardization and characterization of dried extracts of medicinal plants^{5,12}.

The method of drug treatment aims at finding a product which attends market necessities as well as provides efficacy, safety and quality. This thermal analysis is going to be useful in studying the behavior of drugs degradation during its dumping process after its expiration since it may also release some toxic by products which may be harmful for the society. The objective of this study was to characterize the dried extracts of *bauhinia variegata* by TG/DSC analysis and FTIR coupled with GC-MS (TG-DSC-FTIR-GC-MS).



Bauhinia variegata Bark Extract: Assessment of its Anti-proliferative and Apoptotic Activities on A549 and H460 Lung Cancer Cell Lines

Tanvi Khanna, Akash Dave, Sejal Purani, Jagath Vedamurthy, Dhaval Jivani and Pushpa Robin*

Department of Biochemistry, The Maharaja Sayajirao University of Baroda, Vadodara - 390002, Gujarat, India; pushparobin@gmail.com

Abstract

The hunt for novel anticancer drugs with minimal side effects continues. This study strengthens the claim by providing biochemical evidences of anticancer activities of *Bauhinia variegata* bark extracts on lung carcinoma cells (A549 and H460). Bark extracts of *Bauhinia variegata* were prepared by different solvents using Soxhlet apparatus and tested for their antioxidant potential by DPPH assay. The lung cancer cell lines were treated with *Bauhinia variegata* bark extracts and viability of cells was measured by MTT assay; metastatic ability was determined through Scratch assay and effect on DNA integrity was shown by gel electrophoresis. The Petroleum Ether Bark Extract (PEBE) inhibits proliferation (A549, IC₅₀ = 1.5 mg/ml) at 48 h treatment. DNA damage was observed in A549 cells by agarose gel electrophoresis. The Chloroform Bark Extract (CBE) inhibited proliferation of H460 (IC₅₀ = 1 mg/ml) with DNA damage after 24 h treatment. Soft agar assay indicated decreased ability to form colonies and scratch test showed impaired migration of A549 and H460 to PEBE and CBE treatment respectively. Apoptosis was detected using fluorescent dye staining in A549 and H460 cells. Caspase 3 activity was increased significantly in A549 and H460 cells. PEBE and CBE decrease the mitochondrial membrane potential gradient ($\Delta\Psi_m$) of A549 and H460 cells respectively. This study categorically proves the cytotoxic activity of *Bauhinia variegata* bark extracts on A549 and H460 cells.

Keywords: Anticancer Effect, Caspase, DNA Damage, Metastasis, Non-small Cell Lung Cancer Cell Lines

Abbreviations: Non-Small Cell Lung Cancer (NSCLC), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Petroleum Ether Bark Extract (PEBE), Chloroform Bark Extract (CBE), Petroleum Ether (PE), N-hexane (HX), Chloroform (CHL), Ethyl Acetate (EA), Methanol (MET), Water (AQ), Dimethyl sulfoxide (DMSO), 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), Standard Deviation (SD), Standard Error of Mean (SEM), Dulbecco's Modified Eagle's Medium (DMEM), 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA), Phosphate Buffer Saline (PBS), Hydrogen peroxide (H2O2), TAE (Triacetate- EDTA), Sodium Chloride (NaCl), Half Maximal Inhibitory Concentration (IC₅₀), Tetramethyl Rhodamine, Methyl Ester (TMRM),

4',6-diamidino-2-phenylindole (DAPI), Analysis Of Variance (ANOVA), Acridine Orange (AO), Ethidium bromide (EtBr)

1. Introduction

Exponential increase in cancer incidences is a global burden. Every year India reports about 70,275 lung cancer cases (fourth among all cancers) with 50 % mortality within a year and 5-year survival has remained at 11-17 % for these lung cancer patients^{1,2}. Non-small cell lung cancer (NSCLC) contributes for about 85 % of the lung cancer cases while 15 % cases are SCLC².

*Author for correspondence

Correlation of Autoimmune Thyroid Disease with Subclinical Hypothyroidism in Primarily Infertile Female Population

Tabassum Mansuri, Bhanu Pratap Singh Shekhawat, Akash Dave, Richa Chaturvedi, Sadiq Khalifa, Chitra Nair, Malvika Chaudhari and Pushpa Robin*

Department of Biochemistry, Faculty of Science, The M.S. University of Baroda, Vadodara, Gujarat, India

***Corresponding Author:** Pushpa Robin, Department of Biochemistry, Faculty of Science, The M.S. University of Baroda, Vadodara, Gujarat, India.

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Abstract

Infertility is a serious health concern affecting 15% of couples of reproductive ages worldwide and female infertility has been recognized as a global public health issue by WHO. Reports on the prevalence of infertility in women with chronic endocrine disorders are abundant but 'subclinical' aberrations in hormone levels affecting fertility are difficult to discern and hence often go undiagnosed, subclinical hypothyroidism (SCH) being the case in point. SCH acts as a silent perpetrator of infertility and by and large goes unnoticed owing to asymptomatic condition. Autoimmune thyroid disease (AITD) is one of the most common etiological factors responsible for hypothyroidism. A correlation between the prevalence of SCH and AITD in population, if any, will help in counseling of infertile females. This study was undertaken to estimate the prevalence of SCH in infertile females and to find out the prevalence and involvement of AITD amongst them so as to help in making decisions to decide course of treatment.

Keywords: TSH; Subclinical Hypothyroidism; Infertility; Prevalence; Autoimmune Thyroid Disease; Correlation

Abbreviations

SCH: Subclinical Hypothyroidism; TSH: Thyroid Stimulating Hormone; fT_3 and fT_4 : Free Thyroid Hormone Level; AITD: Autoimmune Thyroid Disease; ET: Euthyroid; IF: Infertile Female; Ab: Antibody

Introduction

Female infertility occurs in about 37% of all infertile couples. A continuous interaction with multiple endocrine organs is responsible for successful reproduction. An aberration in any of the endocrine pathway can result in infertility and this makes the diagnosis of infertility a difficult exercise for the physician. Thyroid disorders, both hyperthyroidism and hypothyroidism, can influence the functionality of the ovaries. Reproduction can be jeopardized by alterations in the hypothalamus-pituitary-thyroid axis, a direct effect or autoimmunity affecting thyroid hormone levels. Overt Hypothyroidism affecting fertility is easy to diagnose and treat but diagnosing subclinical hypothyroidism (SCH) (elevated TSH and normal thyroid hormone levels) and deciding how to treat it is tricky. Studies have reported the association of overt hypothyroidism with infertility, miscarriage, and adverse pregnancy outcomes in female populations [1,2]. Subclinical hy-

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