Report for R&D Projects
Section A: Project Details

Section-A: Project Details

A1. Project Title: Polymer supported green nano particles: using plants of north east India; studies on toxicity and anticancer property

A2. DBT Sanction Order No. & Date: BT/473/TBP/2012, dated 13th February, 2014

A3. Name of Principal Investigator: Professor Niranjan Karak (TU) and Professor Ansuman Chattopadhyay (VU)

Name of Co-PI/Co-Investigator: Dr. Satya Sundar Bhattacharya (TU) and Professor Shelley Bhattacharya (VU)

A4. Institute: Tezpur University, Assam and Visva-Bharati University, WB

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A6. Total Cost: 58.81 lakh [33.46 lakh (TU) + 25.35 lakh (VU)]

A7. Duration: 36 months

A8. Approved Objectives of the Project:

1. Synthesis of green silver nanoparticles using extracts of plants of north eastern India.
2. Physical characterization of the synthesized nanoparticles.
3. *In vitro* and *in vivo* toxicity study using animal model of different taxonomic position.
4. Screening of the particles for their anti-carcinogenic activity using cancer cell lines.

A9. Specific Recommendations made by the Task Force (if any):
Not applicable

Section-B: Scientific and Technical Progress

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B1. **Progress made against the Approved Objectives, Targets & Timelines during the Reporting Period** (1000-1500 words for interim reports; 2500-3500 words for final report; data must be included in the form of up to 3 figures and/or tables for interim reports; up to 7 figures and/or tables for final reports):

Please see the details report

B2. **Summary and Conclusions of the Progress** (minimum 100 words, maximum 200 words): *Please see the details report*

B3. **Details of New Leads Obtained, if any:** *Please see the details report*

B4. **Details of Publications & Patents, if any:** Please see the details report

Section-C: Details of Grant Utilization

Section-C: Details of Grant Utilization#

C1. Equipment Acquired or Placed Order with Actual Cost:

At Tezpur University (TU):
UV-visible spectrophotomer (Evolution 300) procured and working satisfactorily. Cost is Rs. 8.02942 lakh
Please see the actual UC and SE of TU.

At Visva-Bharati (VU):
ELISA Reader procured and working satisfactorily. Cost is Rs. 2.62342 lakh
Please see the actual UC and SE of VU.

C2. Manpower Staffing and Expenditure Details:
One RA and one JRF with total expenditure Rs. 7.54103 lakh (TU)
One JRF with total expenditure Rs. 3.10313 lakh (VU)
Please see the actual UC and SE of TU and VU.

C3. Details of Recurring Expenditure:
Total Rs. 10.61551 lakh (TU) and Rs. 7.63 lakh (VU)
Please see the actual UC and SE of TU and VU.

C4. Financial Requirements for the Next Year with Justifications: Not applicable

#Grant utilization details (UC&SE, Assets Certificate & manpower details) also required to be submitted separately as per the prescribed format

Details report

1.1 Introduction

Silver is well known for possessing an inhibitory effect toward many bacterial strains and microorganisms commonly present in medical and industrial processes [1]. In medicines, silver and silver nanoparticles have a wide application including skin ointments and creams containing silver to prevent infection of burns and open wounds [2], medical devices and implants prepared with silver-impregnated polymers [3]. In textile industry, silver-embedded fabrics are now used in sporting equipment [4].

Among the synthetic methods used for the preparation of silver nanoparticles, some toxic chemicals such as $NaBH_4$, citrate, or ascorbate are most commonly used as a reducing agent [5-7]. Considering that such reducing agents may be associated with environmental toxicity or biological hazards, the development of a green synthesis approach for silver nanoparticles is desired [8].

Plant or plant extract have been suggested as possible ecofriendly alternatives to the conventional methods. Using plant for nanoparticles synthesis can be advantageous over other biological processes by eliminating the elaborate process of maintaining cell cultures [9]. It can also be suitably scaled up for large-scale synthesis of nanoparticles. Green synthesis of silver nanoparticles has been reported using extracts of various plants such as *Nelumbo nucifera* [10], *Euphorbia hirta* [8], *Ocimum sanctum* [11], *Desmodium triflorum* [12], *Cinnamonum camphora* [13], *Moringa oleifora* [14], *Eucalyptus hybrida* [15], *Eucalyptus chapmaniana* [16] *Memecylon umbellatum* [17], etc. In this context, Karak and co-workers have reported the utilization of various bio-resources for preparation of polymer stabilized silver nanoparticles including *Thuja occidentalis* [18-20]. The obtained silver nanoparticles were found to be non-hazardous with anticancerous and antibacterial properties. Thus the plants like *Thuja occidentalis*, *Mentha longifolia*, *Dillenia indica*, *Aloe vera* etc. will be chosen to prepare the extracts. Literature reports many chemical components of such extracts contain polyphenolic compounds, which have potential anticancer activity as well as they have the potential to reduce silver ion to silver nanoparticle. Compounds like pulegone (*Mentha longifolia*), Emodin (*Aloe vera*), Thujone (*Thuja occidentalis*), etc. have proven anticancer activity.

1.2 Objectives of the Project

i. Synthesis of green silver nanoparticles using extracts of plants of north eastern India.
ii. Physical characterization of the synthesized nanoparticles.
iii. *In vitro* and *in vivo* toxicity study using animal model of different taxonomic position.
iv. Screening of the particles for their anti-carcinogenic activity using cancer cell lines.

1.3 Work done

Work done in the parent institute (Tezpur University, Tezpur, Assam)

Objective 1

Synthesis of green silver nanoparticles using extracts of plants of north eastern India.

The leaf extracts of different plants such as *Thuja occidentalis*, Indian Aleo vera, Basak, Tulsi, Bhel and *Mentha arvensis* (Linn.) were used for synthesis of silver nanoparticles (AgNP) by a reductive technique. These plants were collected from the North-eastern region of the country as per the mandate of the project. AgNP were synthesized by following the standard procedure as described below.

About 2.0 g of leaves of the plant were washed with tap water to remove soil particles and further with de-ionized water and made into a paste by using domestic kitchen blender. The aqueous extract was prepared by stirring the paste in 50 mL of water at 50 ºC for about 20 min, followed by filtration through a muslin cloth and filter paper. 2 mL of the plant extract was mixed with 0.01 M AgNO3 in 25 mL of 5% (w/v) poly(ethylene glycol) solution. Formation of AgNP was indicated by gradual change in colour from colourless to brown. The same procedure is followed without using poly(ethylene glycol), as the stabilizer. The samples were given for anticancer activity testing to the collaborative institute as and when required.

Based on the primary report obtained from the collaborative institute, the details characterization was done from the *Thuja occidentalis* and *Mentha arvensis* (Linn.) aqueous extracts mediated AgNPs. Apart from doing all these, affords are being devoted to explore the catalytic action of the synthesized AgNPs. The researchers of the parent institute published a paper in **Tetrahedron Letters** (Tetrahedron Letters 57 (2016) 549-553) demonstrating catalytic activity of AgNPs formed from *Aloe vera* in the efficient conversion of aldehydes into nitriles.

Objective 2

Physical characterization of the synthesized nanoparticles.

Aqueous leaf extract mediated synthesised AgNPs were characterized by using UV-visible (Fig. 1), DLS (Fig. 2), FTIR (Fig. 3), XRD (Fig. 4), AFM (Fig.5), SEM and EDX (Fig. 6), and TEM (Fig. 7).

The surface plasmon resonance (SPR) band in UV–visible spectra was observed at 447 nm (Fig. 1) which was due to the reduction of silver ions. The DLS analysis of AgNPs revealed a Zeta average of 159.5 nm and polydispersity index (PDI) of 0.291 (Fig. 2). However, this size was slightly bigger than actual as DLS method measured the hydrodynamic radius [21].

The band intensities of infrared spectrum of AgNPs are depicted in Figure 3. The spectrum showing strong absorption bands at 2871, 1721, 1461, 1351 and 1104 cm^{-1} represents C-H, C=O,O-H, C-N, -C-O-; and weak signals at 3386, 3256, 2152, 1641,1283, and 843 cm^{-1} represent O-H, N-H, C≡C, C=C, C-C, and C-H, respectively. The C=O stretching vibration of amide is characteristic of plant proteins which act as capping agents for nanoparticles. The O-H bending of the phenolic group, O-H stretching of alkenes, and C=O stretching of side chains of proteins are responsible for the reduction of metal ion. It is well known that biological components interact with metal salts and support the reduction process [22]. The change in absorbance had direct association with FTIR spectrum (Fig. 3) clearly supported the existence of aliphatic and aromatic, hydroxyl, and carboxylic groups in the plant extract of Aloe vera which had the ability to reduce Ag^{+} to Ag^{0}. The XRD pattern confirmed crystalline nature of AgNPs (Fig. 4). Intense peaks were observed at 37.97°, 44.21°, 64.32°, and 77.19° corresponding to Bragg's reflections of metallic AgNPs, crystallized in the face centered cubic (fcc) structure with basal (111, 200, 220 and 311) lattice planes, respectively. Some additional as yet-unassigned peaks were also observed in the XRD pattern due to the presence of bioorganic matters and capping agents [23]. This pattern was in concurrence with earlier report [24]. AFM analysis (Fig. 5) confirmed the presence of spherical shaped

particles (approximately 6 nm) of AgNPs-Av. The EDX spectra along with SEM image of AgNPs-Av have been shown in (Fig. 6a and b). The typical characteristic absorption signal for silver nanoparticles was absorbed in EDX profile at approximately 2.5–3.5 keV which was due to surface Plasmon resonance confirming the formation of AgNPs. The elemental composition of synthesized AgNPs was analyzed through EDX (Fig. 6a). In addition to peaks for Ag, the presence of peaks for C and O was due to the capping of AgNPs by bio molecules of Aloe vera extract. A transmission electron microscope (TEM) demonstrated that the AgNPs were largely uniform with narrow size distribution (Fig. 7). TEM images of AgNPs illustrated that the particles were spherical in shape with an average diameter of about 25–28 nm. Presented micrographs also ascertained that the particles were well dispersed and no sign of agglomeration was evidenced. This predicts the stability of the nanoparticles. However, AgNPs obtained by conventional wet chemical method, using sodium borohydride as the reducing agent was not stable even for a day without any stabilizer. For efficient activity at bio-nano interface, stability of the nanomedicine is an essential pre-requisite.

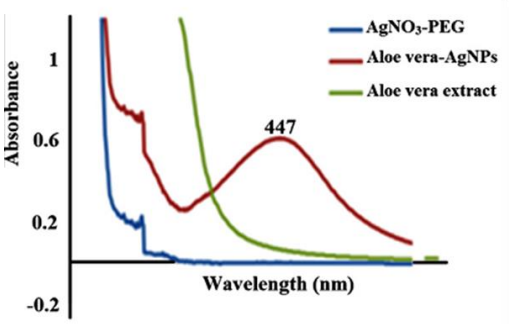

Figure 1. UV–visible spectra of AgNPs-Av.

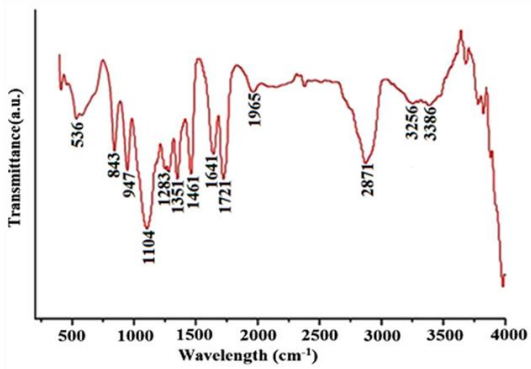

Figure 3. FTIR spectrum of AgNPs-Av.

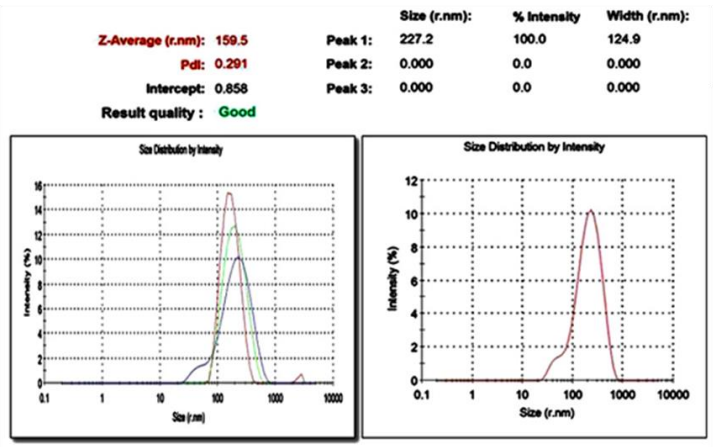

Figure 2. Size distribution of *Aloe vera* mediated AgNPs.

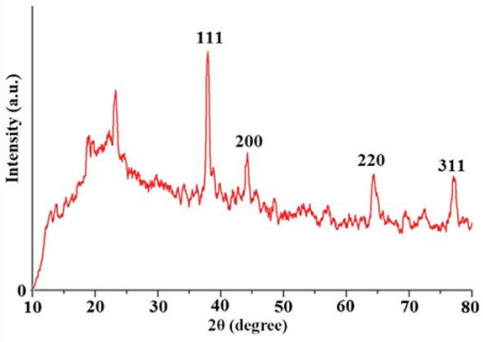

Figure 4. XRD pattern of AgNPs-Av.

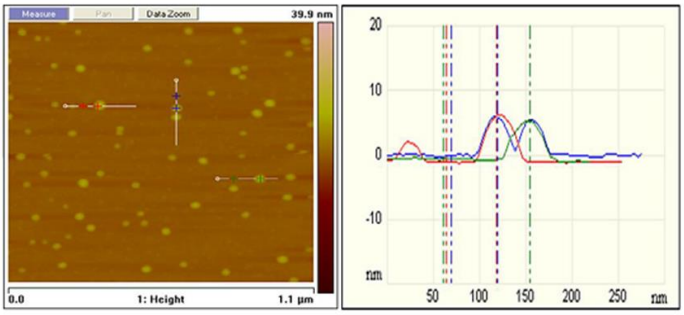

Figure 5. AFM image of AgNPs-Av.

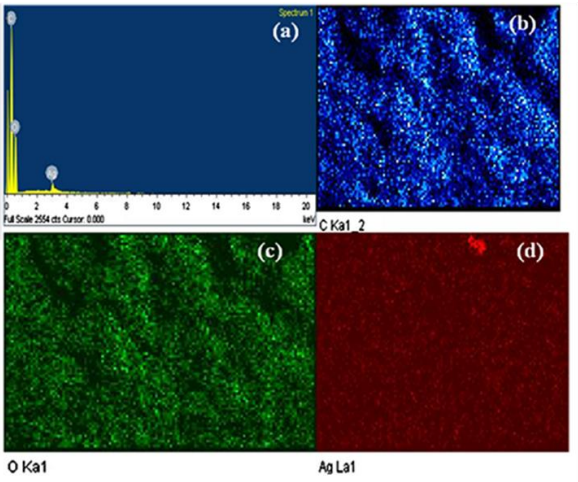

Figure 6. (a) EDX spectrum and (b–d) elemental mapping of synthesized AgNPs-Av.

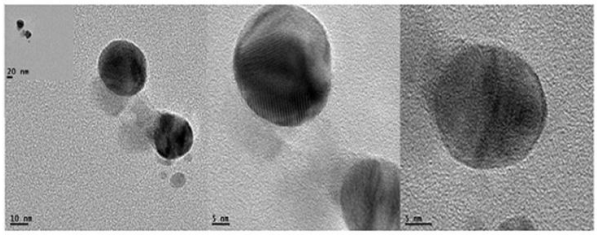

Figure 7. TEM image of AgNPs-Av.

Effect of silver nanoparticle on the soil health and plant growth

The effect of silver nanoparticles synthesized through green route (AgNP) has been experimented on a vegetable crop (common name: tomato, scientific name: *Badshah F1 hybrid*). Tomato has been considered for this experimentation as because it is a highly used Rabi crop in both household and food industrial sector. Here, in this experimental setup $AgNO_3$ has also been taken as a positive control along with the basic control plot. The treatment combinations are as follows: Control, $AgNP_{15}$ kg ha^{-1}, $AgNP_{30}$ kg ha^{-1}, $(AgNO_3)_{15}$ kg ha^{-1} and $(AgNO_3)_{30}$ kg ha^{-1}.

The inherent properties of the soil are as follows: pH=5.11±0.05, Available N=159.6±20.19, Available P= 40.52±0.04, Available K= 113.40±5.96, Total organic carbon= 0.8±0.02, Microbial biomass carbon=188.7±4.3, Urease activity= 12.38±0.05 and Phosphatase activity=4.61±0.04. The data on changes in pH, available K, TOC and MBC of the planted soil under cultivation of tomato is presented in Figure 8. The soil is inherently acidic in nature (basic soil=5.11±0.05). From Figure 8(A) it is easily observed that there is an increase in soil pH after cultivation of tomato crop. Significantly higher pH was observed in case of $AgNP_{15}$ kg ha^{-1} (5.8±0.1) and $AgNP_{30}$ kg ha^{-1} (5.8±0.1). Similarly the K content has also been increased in both the AgNP treated samples compared to control and $AgNO_3$ treatments ($AgNP_{15}$ kg ha^{-1}=144.03±5.1 and $AgNP_{30}$ kg ha^{-1}=148.6±1.15). The status of TOC content in soil does not changed mostly in first 30 days of plantation. However, in the later period of study all most all the samples have showed higher TOC value than 30 days. Most positive result was obtained from $AgNP_{15}$ kg ha^{-1} (1.45±0.03). Microbial biomass carbon mainly dictates the mass of the living components of soil organic matter. The microbial biomass mainly decomposes the animal and plant residue and soil organic matter and enhances the release of plant available nutrients. The MBC of the soil noticeably increased in AgNP

treated samples compared to both the positive control and $AgNO_3$ treatments after 60 days of tomato cultivation (Figure 8B). As compared to the soil inherent MBC content it is almost 4.63 and 3.02 fold increased in case of $AgNP_{15}$ kg $ha^{-1}$and $AgNP_{30}$ kg ha^{-1} treatments respectively (P=0.000).

Figure 9 represents the data on changes in soil easily mineralizable nitrogen, available phosphorus and soil enzyme (urease and phosphatase) activities in the planted soil. The rate of nitrogen mineralization has been enhanced gradually in case of nanoparticle treatments from 30 to 60 days. Highest N mineralization was recorded in $AgNP_{15}$ kg ha^{-1} treatments (354.7±26.57) followed by $AgNP_{30}$ kg ha^{-1} (336±28.49). On the other hand P availability was also found to have increased after cultivation of tomato. Significantly high availability of P was recorded in $AgNP_{30}$ kg ha^{-1} followed by $AgNP_{15}$ kg ha^{-1}. Urease and phosphatase are the important soil enzymes and plays a significant role in mineralization of N and P. The changes in the activities of urease and phosphatase enzymes in the cultivated soil are also shown in figure 2. Urease and phosphates activity in the soil considerably increased over time in tomato cultivation. Maximum activity of urease and phosphatase was observed in case of $AgNP_{15}$ kg ha^{-1} and $AgNP_{30}$ kg ha^{-1} treatments respectively (42.16±0.02 and 17.6±0.1).
Table 1 dictates the results of some of the important fruit and plant parameters: total acidity, pericarp thickness, total chlorophyll content, total carotenoid content, total soluble solid and lycopene content of the harvested samples. The total acidity and pericarp thickness was significantly higher in case of $AgNP_{15}$ kg ha^{-1}. Two important plant pigments (Chlorophyll and carotenoid) were also analyzed to identify their response to the synthesized treatments. Maximum chlorophyll and carotenoid content was also observed in $AgNP_{30}$ and $AgNP_{15}$ kg ha^{-1} (22.38±0.02 and 7.77±0.03). Lycopene content was also found to have highly increased in AgNP treatments as compared to control and $AgNO_3$ treatments.

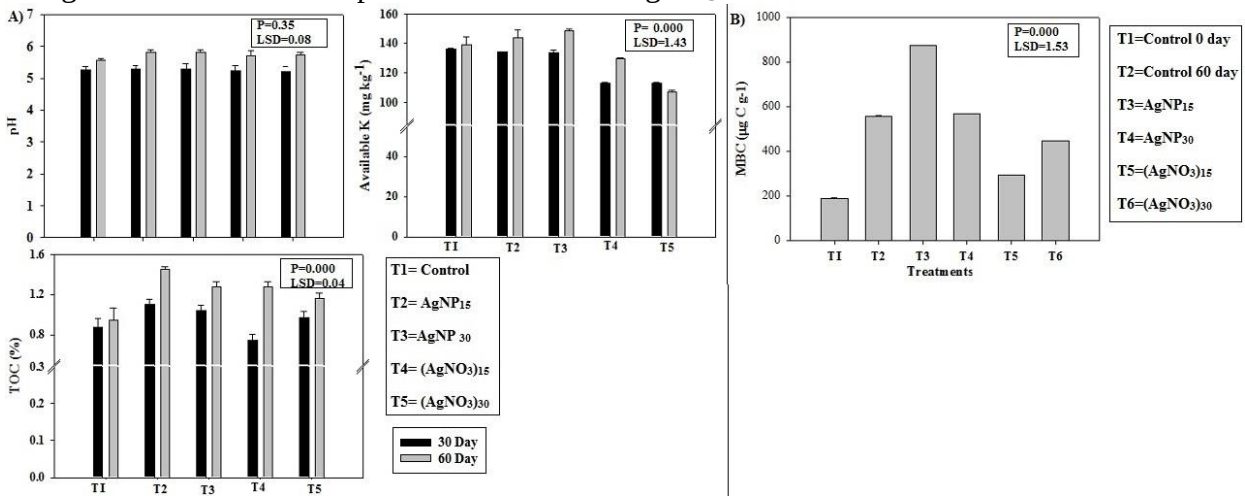

Figure 8: Effect on pH, Available K, TOC and MBC of soil.

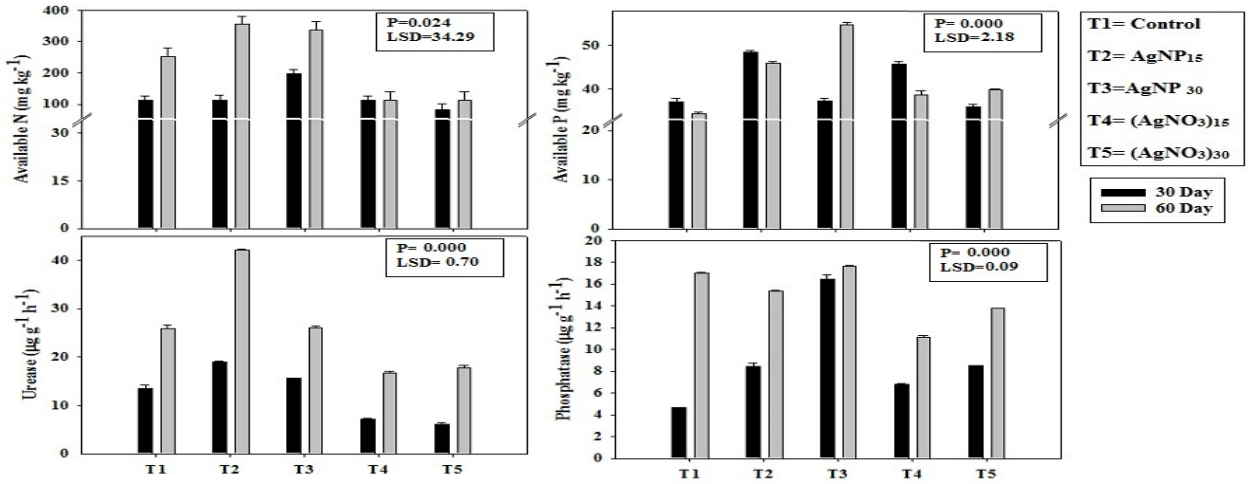

Figure 9: Effect on available N, P, urease and phosphatase activity of soil.

Table 1: Representation of the plant and fruit parameters.

Treatments ($kg\ ha^{-1}$)	Total acidity (%)	Pericarp thichness (cm)	Total chlorophyll (mg chl/gm leaf)	Total carotenoid(mg carotenoid/gm leaf)	Total soluble solid (°Brix)	Lycopene ($mg\ kg^{-1}$)
Control	1.83±0.07	0.57±0.06	16.09±0.01	5.69±0.01	4±0.1	6.29±0.01
$AgNP_{15}$	1.9±0.09	0.6±0.17	18.05±0.03	7.77±0.03	3.8±0.2	28.91±0.02
$AgNP_{30}$	1.54±0.07	0.5±0.10	22.68±0.02	6.08±0.01	4±0.2	27.39±0.03
$(AgNO_3)_{15}$	1.49±0.07	0.4±0.10	5.67±0.01	3.09±0.002	4.2±0.2	8.04±0.01
$(AgNO_3)_{30}$	1.24±0.07	0.3±0.17	8.35±0.02	3.95±0.001	4.4±0.19	9.88±0.02
P value	0.00	0.09	0.00	0.00	0.00	0.00
LSD	0.06	0.11	0.02	0.01	0.14	0.02

An experiment was conducted to identify the efficacy of green silver nanoparticles prepared from *Thuja occidentalis* leaves extracts (GSNP) and conventionally prepared silver nanoparticles (CSNP) on plant growth and metabolism. Moreover we have also conducted qRT-PCR analysis to observe the expression of mRNA of *Fd* (Ferrodoxin) and *NR* (Nitrate reductase) genes in leaves of *Phaseolus vulgaris.*

The leaf number and LAI increased sharply in plants grown in $GSNP_{20}$, $GSNP_{25}$, and $GSNP_{50}$ soils (Figure 10). Higher pod yield was observed under GSNP treatments compared to control conditions and CSNP treatments.

The LAI and leaf number of *P. vulgaris* plants cultivated under various treatment conditions were recorded 15 and 45 days after sowing (DAS) and at physiological maturity (60 DAS). Maximum leaf number was counted under $GSNP_{50}$, followed by $GSNP_{25}$ and $GSNP_{20}$ (P = 0.000; LSD = 1.33). However, $GSNP_{50}$ provided maximum LAI, followed by $GSNP_{25}$, $CSNP_{50}$, and $GSNP_{20}$ (P = 0.000; LSD = 0.245).

Interestingly, N uptake in pods followed the trend $GSNP_{50} > CSNP_{50} = GSNP_{20} > CSNP_{20}$ (P = 0.000; LSD = 0.28). $GSNP_{50}$ and $CSNP_{50}$ yielded the highest P uptakes (P = 0.000; LSD = 1.59). Moreover, $GSNP_{50}$ yielded the lowest pod weight loss, followed by $GSNP_{25}$, $GSNP_{20}$, and $GSNP_{100}$ (P = 0.000; LSD = 1.25) (Figure 10).

Proline content mainly increases in plants under stress. Here in this experiment proline content was quite higher in CSNP treatments compared to GSNP suggesting higher oxidative stress. The effects of the various SNP treatments on chlorophyll and NR activity in

P. vulgaris leaves are presented in Fig. 4 $GSNP_{50}$ and $GSNP_{25}$ conditions, followed by $CSNP_{50}$ and $GSNP_{20}$ depicted higher amount of chlorophyll content (P = 0.000; LSD = 0.04) (Fig. 4). Likewise higher NR activity was observed in case of $GSNP_{50}$, followed by $CSNP_{50}$ (P = 0.000; LSD = 1.28). Considerably higher protein content in *P. vulgaris* pods was recorded under GSNP treatments compared to CSNP treatments; the overall trend in protein content was $GSNP_{50}$ > $GSNP_{25}$ > $GSNP_{100}$ > $CSNP_{50}$ > $GSNP_{20}$ > $CSNP_{25}$ > $CSNP_{100}$ > $CSNP_{20}$ > control (P = 0.000). The expression of NR and Fd genes were 3.58 and 25.46 times higher in GSNP-treated plants than control. Profuse chlorophyll formation, high pod yield, and high protein contents observed indicated that the GSNP treatment greatly stimulated the plant's metabolism. The reason behind these effects was the remarkable expression of NR and Fd genes in GSNP-treated plants (Figure 4). Ferredoxin is an important enzyme that controls electron transportation from photosystem I to photosystem II during photosynthesis; it is the last electron acceptor in non-cyclic photophosphorylation. In addition, the function of nitrate reductase in N assimilation and amino acid synthesis is also well known.

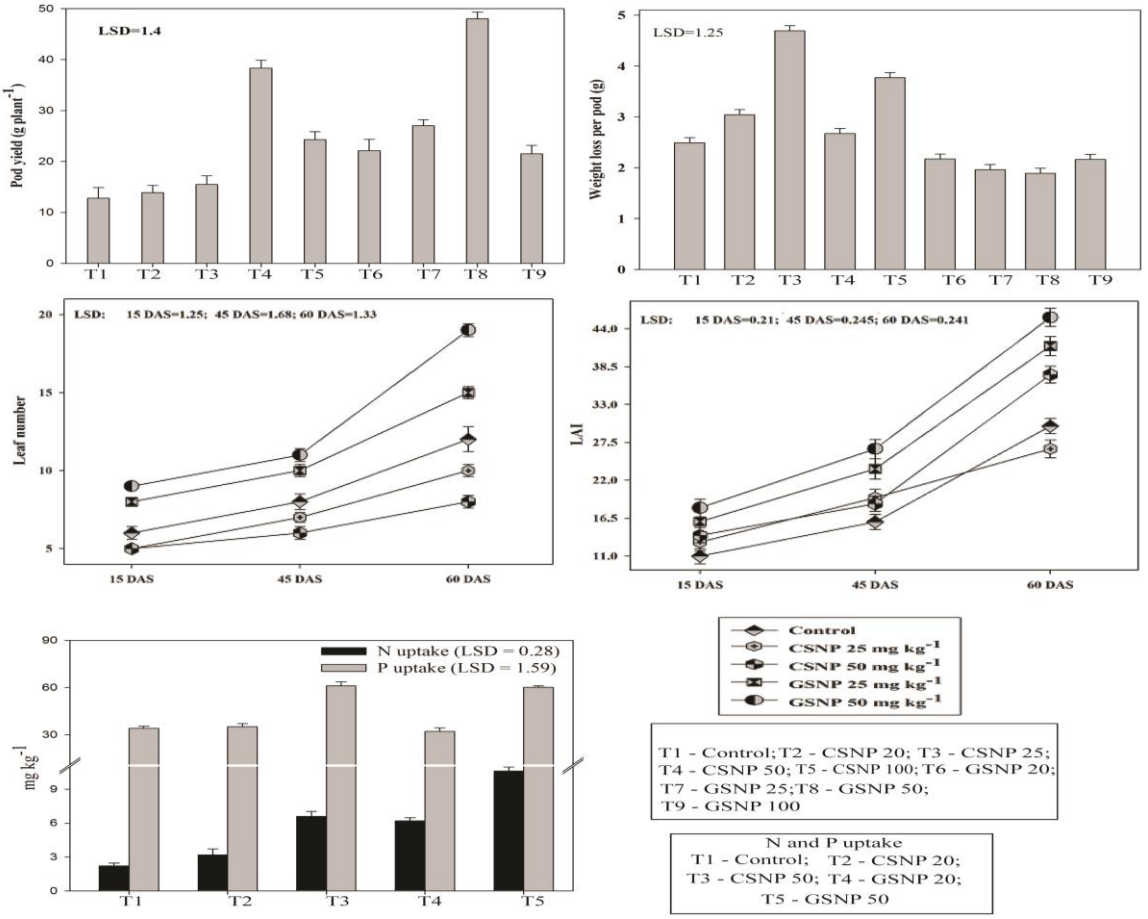

Figure 10: Impacts of GSNPs and CSNPs on leaf number, LAI, pod yield, weight loss per pod, and nutrient uptake (N and P) of *P. vulgaris*. LSD = least significant difference; error bars represent standard deviations.

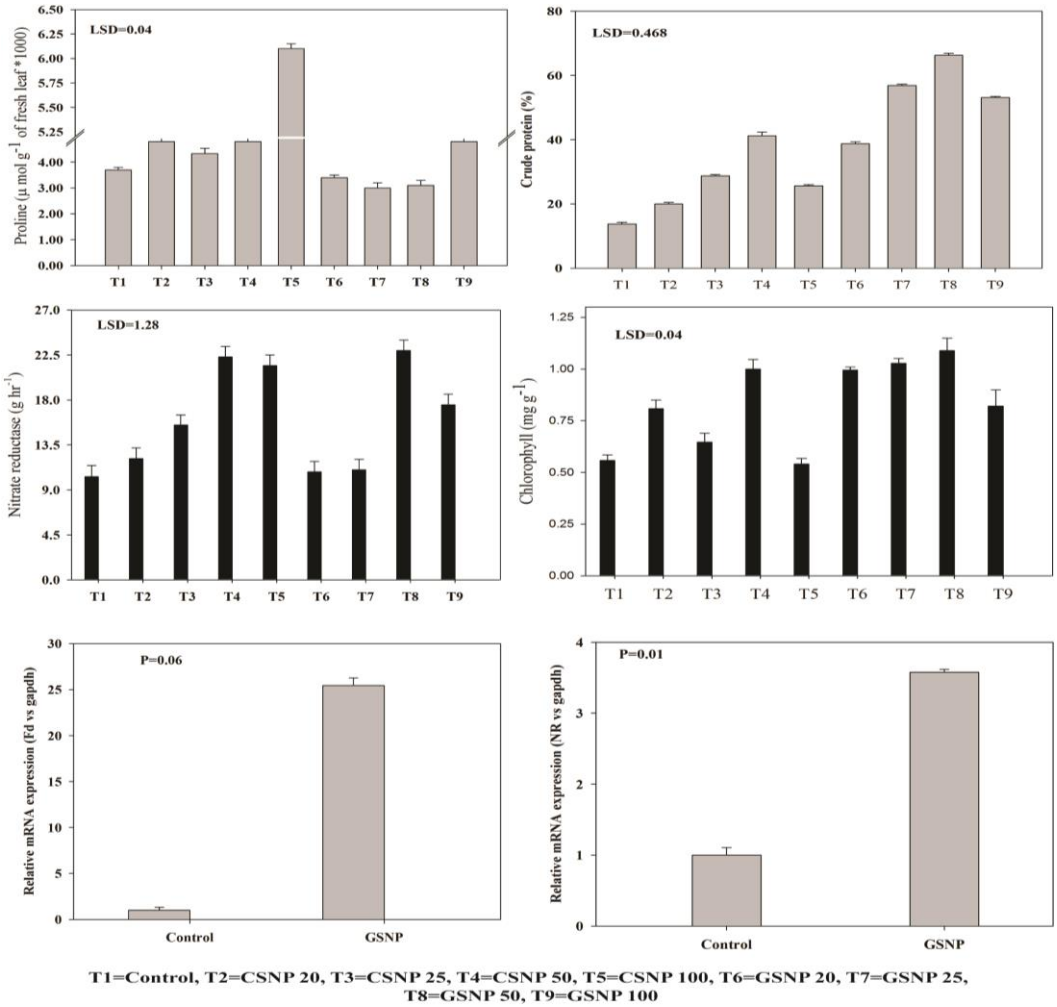

Figure 11: Impacts of SNPs on proline levels, crude protein content, chlorophyll content, activity of nitrate reductase (NR) enzyme, and the expression of mRNA for NR and Fd in leaves of *P. vulgaris* under various treatments. Quantitative RT-PCR was performed using gene-specific primers where GAPDH served as internal control. The results were compared on the basis of ANOVA followed by LSD and t–test analysis. LSD = least significant difference. Error bars represent standard deviations.

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Work done in the collaborating institute (Visva Bharati University, Santiniketan, West Bengal)

Objective 3

In vitro and in vivo toxicity study using animal model of different taxonomic position.

In vitro toxicity study has been conducted with *Thuja occidentalis* mediated 'green' silver nanoparticles (GSNP) in isolated human peripheral blood mononuclear cells (HPBMCs) as well as in rat hepatocytes. It has been observed that *Thuja* extracted GSNPs is moderately toxic (45% cell death at 48h) to HPBMCs at a concentration of 12.5 µg/mL which is highly toxic to the cancer cells (>80% cell death). The same GSNPs were found to be nontoxic to isolated rat hepatocytes even at a concentration of 50 µg/mL treated for 8 h. Interestingly, Study of sister chromatid exchange revealed that GSNP showed significantly higher SCE frequency (10.84±0.57/cell) than that of chemically (NaBH4) synthesized nanoparticle (CSNPs) (3.49±0.24/cell) or that of the untreated control (2.21±0.9/cell).

A second type of silver nanoparticle was synthesized using *Mentha arvensis* (pudina) leaf extract as reducing agent. This nanoparticle (M-AgNP) was also used for cell survival study in human peripheral blood mononuclear cells (HPBMC) with the dose (12.5µg/mL) showing maximum cell death in cancer cells. After 48h treatment with M-AgNPs, 50% of cells died in HPBMCs which was significantly less than that of cancer cells.

In vivo toxicity study in zebrafish was performed using M-AgNPs along with GSNPs and CSNPs. Calculated 96 h-LC50 value was found to be 34 μgL^{-1} for both M-AgNPs and GSNPs whereas, CSNPs were found to be more toxic with the LC50 value of 28 μgL^{-1}. Reduced glutathione (GSH) assay in liver tissue also corroborated the data in which depletion of GSH was more pronounced in CSNP-treated group (with 57.26% GSH level compared to untreated group) than both M-AgNP-treated (85.12%) and GSNP-treated (73.65%) fish population after 24 h of exposure in $1/10^{th}$ of LC50.

In vivo toxicity study was also conducted in Swiss albino mice using M-AgNPs and CSNPs. 48 h-LD50 values were found to be 21.6 mg/kg body weight and 12.96 mg/kg body weight respectively. $1/3^{rd}$ LD50 dose was used to treat the mice intra-peritoneally every alternate day for 7 days. Liver function tests and other biochemical assays for oxidative stress determination were performed. Liver function tests showed that CSNPs induced higher toxicity in mice liver compared to control and M-AGNPs treated groups (Figure 12). Significant increase in MDA production, catalase and reduced glutathione level in the liver of CSNP-treated mice indicates increased lipid peroxidation and generation of oxidative stress (Figure 13). These results pointed out that CSNPs are more toxic to liver cells compared to M-AGNPs treatment *in vivo*.

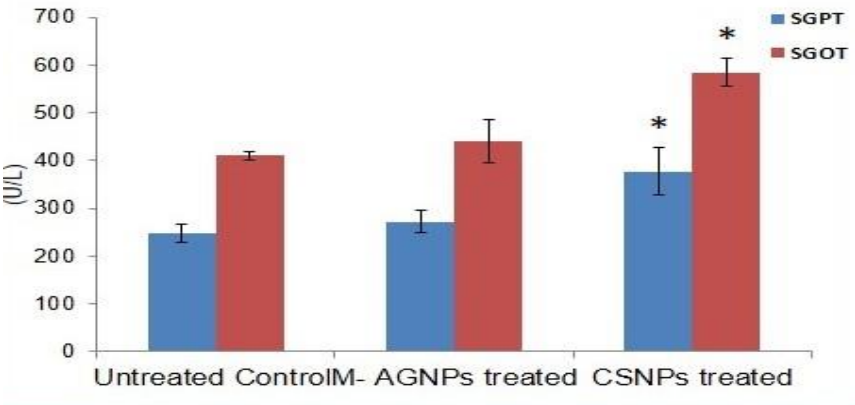

Figure 12: Histograms showing serum glutamic pyruvic transaminase (SGPT) and serum glutamic-oxaloacetic transaminase and (SGOT) level in liver tissue of Swiss albino mice, treated with M-AGNPs and CSNPs for seven days. (*significantly different from control. $p<0.05$).

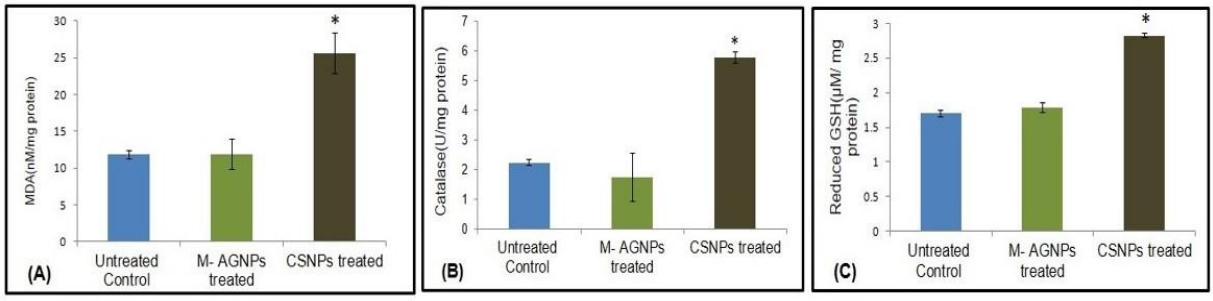

Figure 13: (A) TBARS/MDA production; (B) Catalase enzyme level; (C) Reduced glutathione content in liver tissue of Swiss albino mice, treated with M-AGNPs and CSNPs for seven days. (*significantly different from control. $p<0.05$).

Objective 4

Screening of the particles for their anti-carcinogenic activity using cancer cell lines.

Green silver nanoparticle samples (GSNPs) prepared using *Thuja occidentalis*, Indian *Aleo vera*, Basak, Tulsi, Bhel and *Mentha arvensis* (Linn.) leaf extracts were received from Tezpur University, Assam. All the GSNPs showed inhibitory activity against cancer cells [breast (MCF-7, MDA MB 231), cervical (HeLa) and oral (KB)]. Among these GSNPs, *Thuja* leaf-extract mediated GSNP was taken for further studies as it demonstrated a higher degree of inhibitory activity on cell growth compared to the other samples. This sample exhibited anticancer activity at a concentration range of 6.25-50 μg/mL. GSNP treatment at different doses for 48 h in both the breast cancer cell lines, MCF7 and MDA MB 231 were conducted and more than 50% inhibition of cell proliferation was recorded at a concentration of 6.25 μg/mL. Cell death increased further with increasing doses of GSNP. In HeLa cells 44% death was observed at a concentration of 25 μg/mL of GSNP which reached more than 50% at the highest dose (50 μg/mL). KB cells did not show cytotoxicity at the lower doses; but at a dose of 25 μg/mL, 63% death was recorded which further increased at 50 μg/mL. This assay confirmed that the GSNPs were most effective against human breast cancer, followed by cervical and oral cancer in a dose dependent manner. Considering the response of the breast cancer cells further studies were performed using only these two cell lines.

The treated breast cancer cell lines were critically assessed at different time points for any morphological alteration. Changes in morphology were observed from 2 h onwards post exposure to GSNPs. After 48 h of treatment normal cellular morphology was distorted as compared to the control. Exposure to GSNPs reduced both cell size and number and no recovery was observed until 96 h. Moreover the cells were found to lose their typical morphology and adhesion property while they clumped on treatment with GSNPs.

With Hoechst staining nuclear condensation and fragmentation was evident in GSNPs treated cancer cells.

Treatment with different concentrations of GSNPs did not induce DNA strand scissions either in super coiled plasmid DNA or in the calf thymus DNA. This clearly indicates that GSNPs do not have direct DNA binding/damaging property. Therefore it may be stated that the observed toxicity of these nanoparticles may be due to indirect action which is most probably generated by reactive oxygen species (ROS) leading to nuclear fragmentations and consequently to apoptosis.

Anticancer effect of M-AgNPs on MCF-7, MDA 231 and HeLA cell lines was studied following MTT assay and it was found significaltly cytotoxic to all the three cell lines used in this study.

Colony formation assay or clonogenic assay was done to observe the colony formation of MCF7 cell lines after treatment with GSNP, M-AgNP and CSNP with different doses. The data revealed that the IC50 value of of CSNP was 0.5μg/mL, followed by M-AgNP (0.8μg/mL) and GSNP (1μg/mL).

Both GSNPs and M-AgNPs were used for DNA strand scission assay in pBR322 and calf thymus DNA. Results did not indicate any direct DNA damaging effect of these nanoparticles.

Generation of ROS in M-AgNP-treated MCF7 was studied using fluorescence microscopy. The fluorescence intensity/mg protein was evaluated and found to be increasing in a dose and time dependent manner (Figure 14). The oxidative stress response in MCF7 was further confirmed by studying the expression pattern and distribution of Nrf2 in nuclear and cytosolic fractions.

The fluorescence-activated cell sorting (FACS) analysis of MCF7 cells after treatment with 12.5 μg/mL M-AgNPs showed a significant increase in the fraction of cells present in

the sub-G1 phase and subsequent decline of cells at the G1 phase compared to untreated control cells (Figure 15), indicating apoptosis as possible cell death pathway in them.

Changes in cell morphology were observed after exposure of MCF7 and MDA-MB-231 to M-AgNP (1.56 and 12.5 µg/mL; Figure 16). After 48 h of exposure, normal cellular morphology was distorted significantly in both cases compared to the control. From the microscopic study, it was abundantly clear that synthesized M-AgNP treatment can induce death and severe morphological alterations in human breast cancer cells.

In breast cancer cells, the type of cell death induced by M-AgNPs was monitored under the fluorescent microscope. Both live and early apoptotic cells were seen in the presence of 1.56 µg/mL M-AgNPs, while in the presence of 12.5 µg/mL M-AgNPs, nearly all were late apoptotic cells (Figure 17). Untreated MCF7 and MDA-MB-231 cells showed 94% and 95.6% live cells, respectively. When treated with 1.56 µg/mL M-AgNPs, MCF7 and MDA-MB-231 cells exhibited 74% and 80% early apoptotic cells. Treatment with 12.5 µg/mL M-AgNPs showed 30% early apoptotic cells with 65% late apoptotic cells in MCF7 and 10% early apoptotic cells with 87% late apoptotic cells in case of MDA-MB-231.

M-AgNP-induced nuclear fragmentation was studied through Hoechst staining (Figure 18). Nuclear condensation and fragmentation were evident and prominent in M-AgNP-treated cancer cells. The extensive nuclear fragmentations might have also activated the apoptotic pathways.

Expression patterns of PARP1, P53, P21, Bcl2, Bax, cleaved caspase 9 were observed in controlled and treated cells at various time points (2, 4, 8, 24 and 48 h; Figures 19 and 20). Upregulation of PARP1, P53, P21, Bax and cleaved caspase 9 was observed in MCF7 cells after M-AgNPs treatment, whereas Bcl2 was downregulated. Cleaved bands of PARP1 were prominent at 24 and 48 h after treatment with M-AgNPs. In MDA-MB-231 cells, the mutant P53 protein was downregulated, whereas PARP1, P53, P21, Bax, cleaved caspase 9 and both procaspase 3 and cleaved caspase 3 proteins were upregulated. Cleavage of PARP1 protein was noticed 2 h onward, which was prominent at 8 and 48 h. Expression patterns of the studied genes clearly indicates that *Mentha arvensis*-mediated GSNPs induced apoptosis in MCF7 and MDA-MB-231 breast cancer cells.

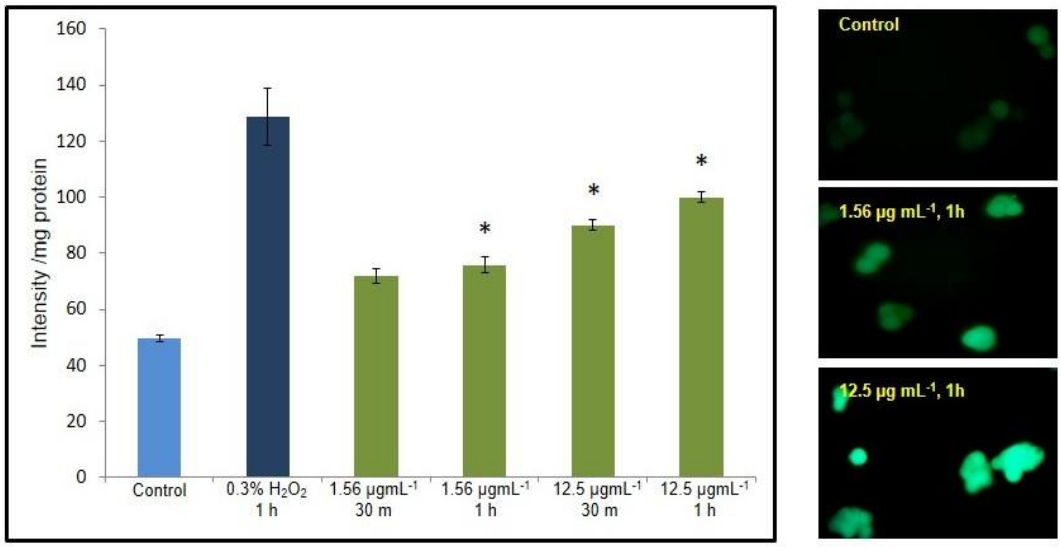

Figure 14: Graph showing fluorescence intensity/mg protein after treatment with M-AGNPs in MCF7 cells, by DCFDA staining method. 0.3% H_2O_2 was taken as positive control for 1 h. (*significantly different from control. $p<0.05$). Pictures are showing increasing fluorescence after treatment with M-AGNPs for 30 min and 1 hour. Magnification 200X.

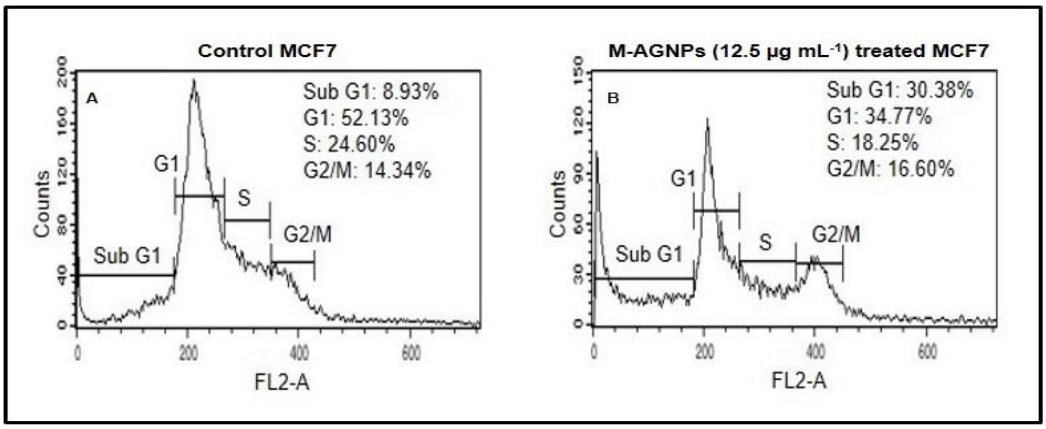

Figure 15: Cell cycle analysis of MCF7 cells without treatment (control) and after treating with M-AGNPs; 12.5 µg mL^{-1} for 48 h.

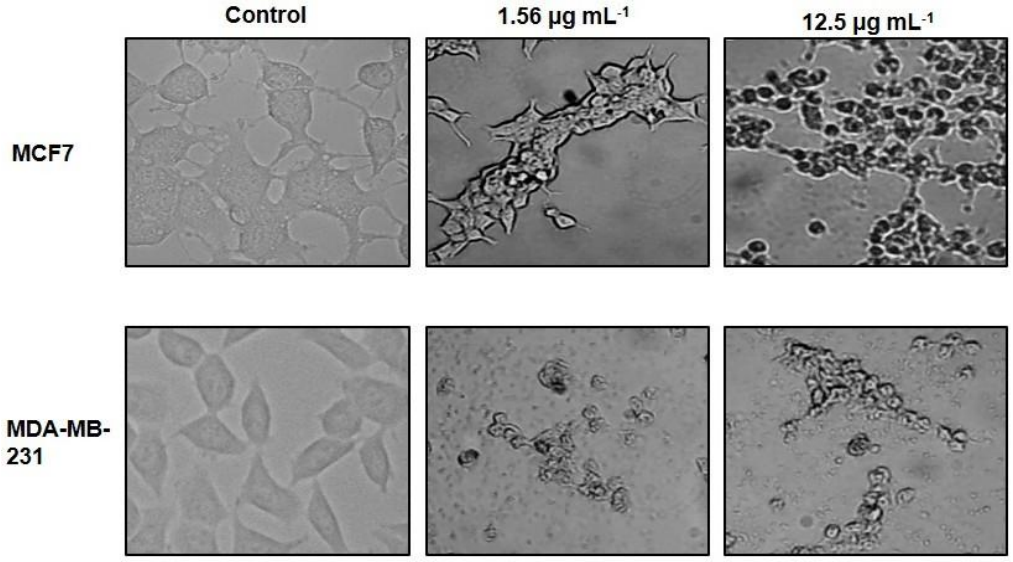

Figure 16: MCF7 and MDA-MB-231 cells showing morphological changes after treatment with M-AGNPs (1.56 µg mL^{-1} and 12.5 µg mL^{-1}) for 48h. Magnification 200X.

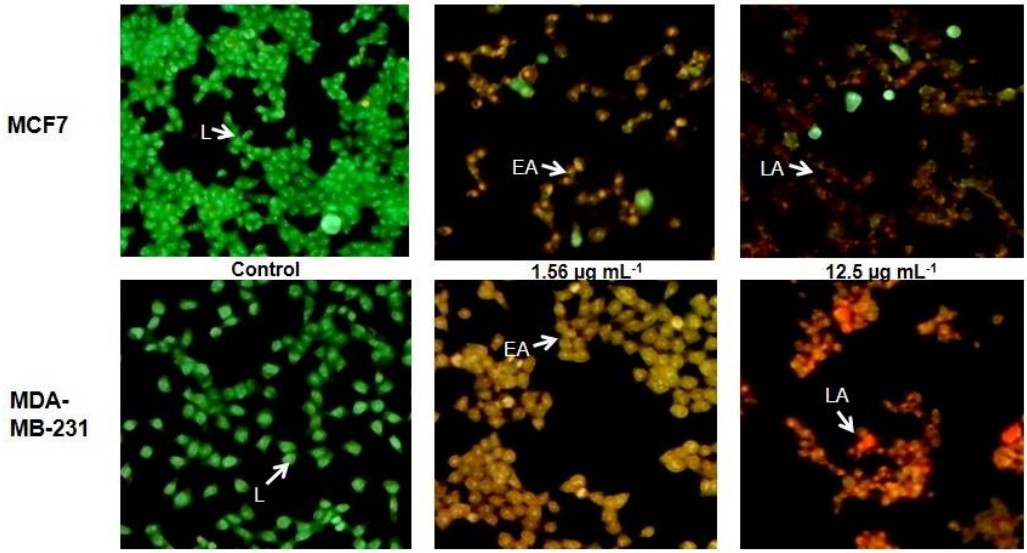

Figure 17: MCF7 and MDA-MB-231 cells showing live cells (L), early apoptosis condition (EA) and late apoptosis condition (LA) after treatment with M-AGNPs (1.56 µg mL^{-1} and

12.5 µg mL^{-1}) for 48 h when stained with acridine orange and ethidium bromide. Magnification 100X.

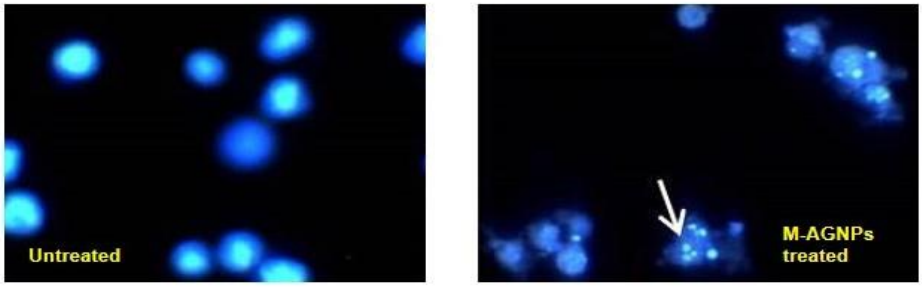

Figure 18: 48h of M-AGNPs treatment is showing clear nucleus breakage in MCF7 cells (white arrows) magnification 400X.

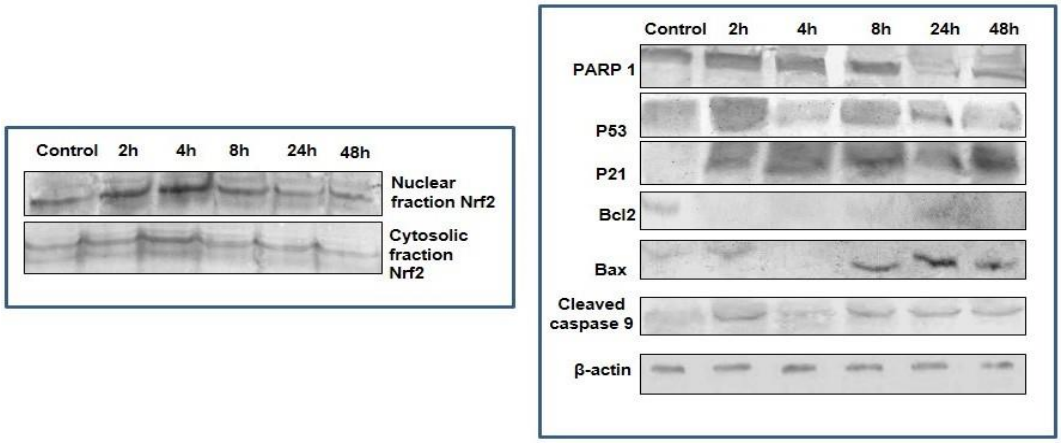

Figure 19: Expression patterns of PARP1, P53, P21, Bcl2, Bax, and cleaved Caspase 9 along with Nrf2 (in nuclear and cytosolic fractions) proteins in MCF7 cells at different time points after treatment with M-AGNPs (1.56 µg mL^{-1}). β-actin was taken as loading control.

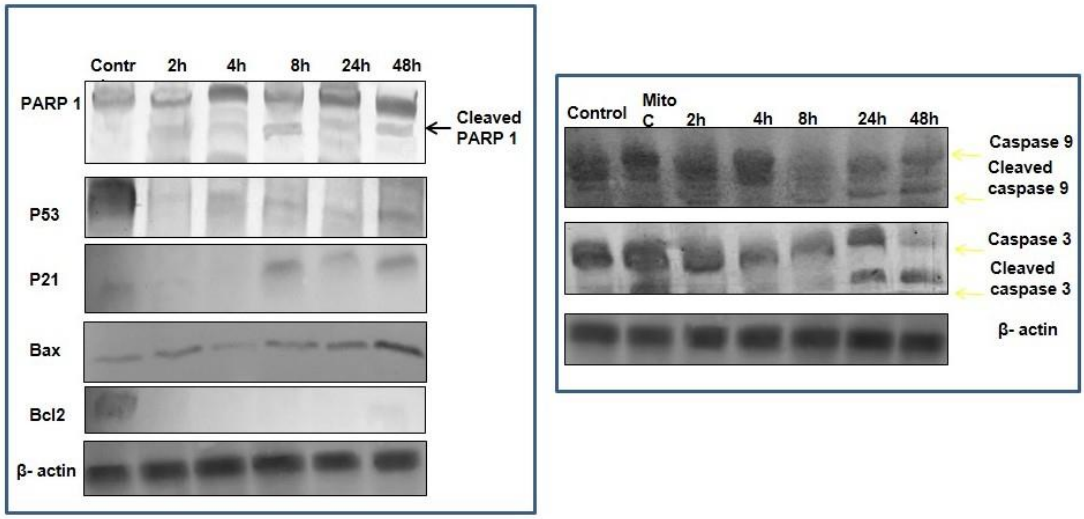

Figure 20: Expression patterns of PARP1, P53, P21, Bcl2, Bax, Caspase 3 and Caspase 9 proteins in MDA -MB-231 cells at different time points after treatment with M-AGNPs (1.56 µg mL^{-1}). β-actin was taken as loading control. Mitomycin C at a dose of 50µM was taken as positive control for Caspase 3 and Caspase 9 expression.

B2. Summary and Conclusions

Silver nanoparticles (AgNPs) are synthesized, characterized and used for biomedical applications such as anti-cancerous material. Different leaves such as *Thuja occidentalis, Aloe vera, Mentha arvensis* (Linn.), etc. leaves extract mediated AgNPs prepared under ambient conditions showed a narrow size distribution with average particle size less than 10 nm. These nanoparticles are characterized by various instrumental techniques. Interestingly, these nanoparticles exhibited anticancer activity against human breast (MCF 7, MDA MB 231) and cervical cancer (HeLa) as well as mouth epidermoid carcinoma (KB) cell lines at the concentration range of 6.25-50 µg/mL. Contrarily they are quite compatible with normal mammalian cells (human peripheral blood mononuclear cells and rat hepatocytes) *in vitro*. Moreover, the nanoparticles exhibited good compatibility to soil health. Thus, the prepared nanoparticles are highly biocompatible and have strong potential in the development of anticancer chemotherapeutics.

B3. Details of new lead

i) Spherical silver nanoparticles are synthesized by different leaves aqueous extract.
ii) These silver nanoparticles showed very efficient anti-cancerous properties.
iii) These nanoparticles are relatively non-toxic to healthy cells.

B4. Details of Publications & Patents, if any

1. V. K. Das, S.N. Harsha and N. Karak, Highly efficient and active silver nanoparticles catalyzed conversion of aldehydes into nitriles: A greener, convenient and versatile "NOSE" approach, Tetrahedron Lett. 57(2016)549–553
2. S. Barua, P. P. Banerjee, A. Sadhu, A. Sengupta, S. Chatterjee, S. Sarkar, S. Barman, A. Chattopadhyay, S. Bhattacharya, N. C. Mondal and N. Karak, Silver nanoparticles as antibacterial and anticancer materials against human breast, cervical and oral cancer cells, J. Nanosci. Nanotechnol. 17(2017) 968-976.
3. P. P. Banerjee, A. Bandyopadhyay, H. S. Nagesh, R. S. Policegoudra, S. Bhattacharya, N. Karak and A. Chattopadhyay, *Mentha arvensis* (Linn.) mediated green silver nanoparticles trigger caspase 9 dependent cell death in MCF7 and MDA-MB-231 cells, Breast Cancer- Targets and Therapy, 9(2017) 265–278
4. A. Bandyopadhyay, P. P. Banerjee, P. Shaw, M. K. Mondal, V. Das, P. Chowdhury, N. Karak, S. Bhattacharya and A. Chattopadhyay, Cytotoxic and mutagenic effects of *Thuja occidentalis* mediated silver nanoparticles on human peripheral blood lymphocytes, Mater. Focus 6(2017)290-296

Section-C: Details of Grant Utilization

Appendix-A

Details of Assets acquired wholly or substantially out of Govt. grants
Register to be maintained by Grantee Institution

Name of the Sanctioning Authority: *Department of Biotechnology (DBT)*

1.	Sl. No.	211
2.	Name of the Grantee Institution	Tezpur University
3.	No. & Date of sanction order	BT/473/NE/TBP/2012, Dated February 13, 2014
4.	Amount of the sanctioned grant (VU)]	58.81 lakh [33.46 inclu. Equip. 8.16 lakh (TU) + 25.35 lakh
5.	Brief purpose of the grant	To develop polymer supported green non-toxic nanoparticles by using plants of north east India for anticancer application
6.	Whether any condition regarding the right of ownership of Govt. in the Property or other assets acquired out of the grant was incorporated in the grant-in-aid sanction order.	No
*7.	Particulars of assets actually credited or acquired.	*Evolution 300 Double beam UV-VIS spectrophotometer (TU) and Centrifuge (Remi, R-8C, 2 Nos.)*
8.	Value of the assets as on	Rs. (758768 +44174), August 12, 2017
9.	Purpose for which utilised at present	To study the UV-Vis. absorption of nanomaterial and to purify the nanomaterials
10.	Encumbered or not	Not
11.	Reasons, if encumbered	Not applicable
12.	Disposed of or not	Not applicable
13.	Reasons and authority, if any, for Disposal	Not applicable
14.	Amount realised on disposal	Not applicable
15.	Remarks	Working satisfactorily

Niranjan Karak
(PROJECT INVESTIGATOR)
Dr. Niranjan Karak
Professor
Dept. of Chemical Sciences
Tezpur University

19.8.17
(FINANCE OFFICER)
Finance Officer
Tezpur University

(HEAD OF THE INSTITUTE)
Registrar
Tezpur University

* List of equipment purchased indicating the item wise costs may please be provided.

Appendix-B

Utilisation Certificate

(for the financial year ending 31st March 2017)

(Rs. in Lakhs)

1.	Title of the Project/Scheme: *Polymer supported green nano particles: using plants of north east India; studies on toxicity and anticancer property*	
2.	Name of the Organisation:	Tezpur University
3.	Principal Investigator:	Professor Niranjan Karak
4.	Deptt. of Biotechnology sanction order No. & date of sanctioning the project:	BT/473/NE/TBP/2012, Dated February 13, 2014
5.	Amount brought forward from the previous financial year quoting DBT letter No. & date in which the authority to carry forward the said amount was given:	Rs. 4.42222 lakh
6.	Amount received from DBT during the financial year (*please give No. and dates of sanction orders showing the amounts paid*):	Rs. 6.19 lakh BT/473/NE/TBP/2012, Dated February 13, 2014
7.	Other receipts/interest earned, if any, on the DBT grants:	Rs. 329/-
8.	Total amount that was available for expenditure during the financial year (Sl. Nos. 5,6 and 7):	Rs. 10.61551 lakh including interest of Rs.329/- (2016-17)
9.	Actual expenditure (excluding commitments) incurred during the financial year (statement of expenditure is enclosed):	Rs. 10.61551 lakh
10.	Unspent balance refunded, if any *(Please give details of cheque No. etc.):*	Not applicable
11.	Balance amount available at the end of the financial year:	Rs. 0.0 lakh
12.	Amount allowed to be carried forward to the next financial year vide letter No. & date:	Rs. 0.0 lakh

Finance Officer
Tezpur University

1. Certified that the amount of Rs. 10.61551 lakh mentioned against col. 9 has been utilised on the project/scheme for the purpose for which it was sanctioned and that the balance of Rs. 0.0 lakh remaining unutilized at the end of the year has been surrendered to Govt. (vide No. BT/473/NE/TBP/2012, Dated February 13, 2014) will be adjusted towards the grants-in-aid payable during the next year.

2. Certified that I have satisfied myself that the conditions on which the grants-in-aid was sanctioned have been duly fulfilled/are being fulfilled and that I have exercised the following checks to see that the money was actually utilised for the purpose for which it was sanctioned.

Kinds of checks exercised:

1. Payment has been released under the supervision of the Finance Officer.
2. Separate ledger has been maintained for the project equipment and consumable Expenditure.
3. Annual accounts will be audited by C & AG in due course.

Niranjan Karak

(PROJECT INVESTIGATOR)

Dr. Niranjan Karak
Professor
Dept. of Chemical Sciences
Tezpur University

19.8.17

(FINANCE OFFICER)

Finance Officer
Tezpur University

21/8/17

(HEAD OF THE INSTITUTE)

Registrar
Tezpur University

(To be countersigned by the DBT Officer-in-charge)

Appendix-C

Statement of Expenditure referred to in para 9 of the Utilisation Certificate

Showing grants received the Department of Biotechnology and the expenditure incurred during the period from 1st April 2016 to 31st March 2017.

(Rs. in lakhs)

Item	Unspent balance Carried for Ward from Previous year	Grants received from DBT during the year	Other receipts interest earned, if any, on the DBT grants	Total of Col.(2+3+4)	Expenditure (excluding commitments) incurred during in year	Balance (5-6)	Remarks
1	2	3	4	5	6	7	8
A. Non-Recurring							
(i) Equipments	0.13058*	0.00	Nil	0.00	0.00	0.00	
B. Recurring							
(i) Human Resource	3.54648	3.68	Nil	7.22648	7.54103	-0.31455	
(ii) Consumables	-0.04832	2.00	Nil	1.95168	1.92866	0.02302	
(iii)Travel	0.49656	0.25	Nil	0.74656	0.61260	0.13396	
(iv)Contingency	0.1546	0.02	Nil	0.30518	0.13222	0.17296	
(v) Overheads (if applicable)	-0.1135	0.24	Nil	0.38232	0.40100	-0.18680	
(vi)Interest	0.25582**	Nil	0.00329	0.00329	0.0000	0.00329	
Total:	**4.42222**	**6.19**	**0.00329**	**10.61551**	**10.61551**	**0.00**	

Niranjan Karak
(PROJECT INVESTIGATOR)

Dr. Niranjan Karak
Professor
Dept. of Chemical Sciences
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(FINANCE OFFICER) 19.8.17

Finance Officer
Tezpur University

(HEAD OF THE INSTITUTE)

Registrar
Tezpur University

*Reapp. to contingency and **Reapp. to overhead (order no. BT/473/NE/TBP/2013, Dated 07/09/2016)

Annexure A

Manpower Staffing Details (In the financial year wise manner)

NAME OF THE PERSON	NAME OF THE POST	DATE OF JOINING	DATE OF LEAVING	TOTAL MONTHLY SALARY	TOTAL SALARY PAID DURING THE FINANCIAL YEAR	TOTAL SALARY PAID DURING PROJECT PERIOD
Dr. Vijay Das	RA	30.01.2015	March 2017	39600/-	618103/-*	927522/-
Ms. Pallabi Das	JRF	05.06.2015	March 2017	14,000/-**	136000/-	398400/-
Dr. S. N. Harsha	RA	17.06.2014	31.12.2014	22000/-	-	141533/-

*Including arear + HRA

**1st April 2016 to 31st May 2016=12000×2=24000 and 1st June 2016 to 31st January 2016 =14000×8=112000

Niranjan Karak

(Signature of Principal Investigator)

Dr. Niranjan Karak
Professor
Dept. of Chemical Sciences
Tezpur University

19.5.17

(Signature of Accounts Officer)

Finance Officer
Tezpur University

21/5/17

(SIGNATURE OF HEAD OF THE INSTITUTE)

Registrar
Tezpur University

Annexure B

Manpower Expenditure Details (In financial year wise manner):

SANCTIONED POSTS	NUMBER	SCALE OF PAY	ANNUAL OUTLAY	OUTLAY FOR THE ENTIRE PERIOD	REVISED SCALE, IF ANY	REVISED ANNUAL OUTLAY	REVISED PROJECT OUTLAY	ACTUAL RELASES BY DBT	ACTUAL EXPENDITURE	BALANCE
RA	1	39600/-	618103/-*	927522/-	-	-	-	368000/-	754103/-	-386103/-***
JRF	1	14,000/-**	1,36,000/-	1,36,000/	-	-	-			

*Including arear +HRA

**1st April 2016 to 31st May 2016=12000×2=24000 and 1st June 2016 to 31st January 2016 =14000×8=112000

*** Last year arear is adjusted

Niranjan Karak

(Signature of Principal Investigator)

Dr. Niranjan Karak
Professor
Dept. of Chemical Sciences
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19.8.17

(Signature of Accounts Officer)

Finance Officer
Tezpur University

(SIGNATURE OF HEAD OF THE INSTITUTE)

Registrar
Tezpur University

* Details of manpower salary/ fellowship revision alongwith due- drawn statement and arrears requested should be given separately, if applicable.

Due- Drawn Statement

Name of the Project Staff	Month and Year	Due	Drawn	Difference
1. Vijay Kumar Das	1st April 2016 to 31st March, 2017	396000/-	396000/-	Nil
2. Ms. Pallabi Das	1st April 2016 to 31st March, 2017	136000/-	136000/-	Nil

Niranjan Karak

(Signature of Principal Investigator)

Dr. Niranjan Karak
Professor
Dept. of Chemical Sciences
Tezpur Uni

19.8.17

(Signature of Accounts Officer)

Finance Officer
Tezpur University

(SIGNATURE OF HEAD OF THE INSTITUTE)

Registrar
Tezpur University