ISSN: 2394-1391

Indian Journal of Biology
Volume 3 Number 2, July - December 2016
DOI: http://dx.doi.org/10.21088/ijb.2394.1391.3216.3

Original Article

Antibacterial and Toxicity of *Dillenia indica* L. Based Green Colloidal Silver Nanoparticle (CSNP)

Jyoti Prasad Saikia*, Rajesh Saikia, Yutika Nath*, Alak Kumar Buragohain*,***, Bhaskarjyoti Gogoi***

Received on 04.06.2016, **Accepted on** 16.06.2016

Abstract

D. indica fruit juice based SNP synthesis has been carried out using previously established method. The SNP were characterized using UV-Vis, furrier transform infrared spectroscopy (FTIR) and transmission electron microscopy (TEM). The CSNP were found to be antimicrobial and chemotactic positive. The cytotoxicity against RAW 246.7 (murine macrophage) cell line is found to be moderate at 10% (v/v) of CSNP in culture medium. Moderate toxicity has been observed against *Vigna radiata* Linn. seed and *Oryza sativa* (*variety Ranjit*) seeds. $NaBH_4$ alone and $NaBH_4$ based CSNP is found to be promoting germination of plant seeds.

Keywords: Siver Nanoparticle; Dillenia Indica; Antibacterial; Cytotoxicity; Phytotoxicity.

Introduction

Colloidal SNP is a requirement of the present day. Green methods are only hope for CSNP synthesis and supply with respect to its increasing demand. Out of the recent CSNP synthesis research a major amount of publications are focused on green CSNP synthesis. Comparison of these green CSNP with respect to the chemically synthesized CSNP is essential. Some of the green CSNP are found to be better in some aspects compared to their chemical counterpart. The variation in green CSNP is due to mixture of chemicals present in plant, animal and microbial extract. These chemicals sometimes act as reducer or stabilizer or both.

Researchers also suggested that green CSNP will be less toxic and hazardous for the environment as part of their precursor is from a living organism. The toxicity should be tested for scientific validity of the claims.

Dillenia indica L. fruits are sour in taste and are used in the preparation of jam and jellies and as flavouring agent for curries. Traditionally the juice of leaves, bark and fruits are mixed and given orally for the treatment of cancer and diarrhoea [1]. The plant is reported to contain betulinic acid, betulin, cycloartenone, n-hentriacontanol and α-sitosterol [1]. Fruit of *D. indica* also contain about 34% of total phenolics in methanolic extract and polysaccharide like an arabinogalactan [2]. These literatures suggest that *D. indica* fruit juice contain all the needed chemicals required for synthesis of CSNP. In our previous publication we have already synthesized the CSNP using *D. indica* fruit juice [3].

Cytotoxicity of nanomaterial is always evaluated using animal cell line only [4, 5]. Research on effect of nanomaterials on plant system is scanty. In many cases the plants also get exposed to these nanomaterials. Any effect on them can adversely affect the ecological balance and productivity in crop field. Therefore in the present study the cytotoxicity is evaluated on animal cell line as well as plant seeds.

In the light of above information, a project has been design to synthesized, characterize, test antimicrobial, chemotaxis and toxicity properties of the colloidal silver nanoparticle.

Author's Affiliation: *Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur-784028, Assam, India. **Department of Physics, Tezpur University, Tezpur-784028, Assam, India.***Dibrugarh University, National Highway 37, Dibrugarh - 786004, Assam, India

Reprint's Request: Jyoti Prasad Saikia, 106, Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur-784028, Assam, India.
E-mail: jyotizone@gmail.com

Materials and Methods

Materials and Chemicals

D. indica is collected from Sonitpur, Assam, India. $AgNO_3$ (A.R.) is obtained from Merck, India. Sodium borohydride is obtained from Fluka Chemicals, USA.

D. Indica Extract Preparation

The process for extract preparation is followed from Singh *et al.* [3] with modifications. Ripped *D. Indica* fruits were collected from the tree, Sonitpur, Assam. The fruit is washed properly to remove all debris and 25g calyx washed gently with sterile distilled water. The calyx was cut into 0.5 cm^3 pieces and homogenized using mixer grinder (Philips HL1629 Mixer grinder, Bajaj Electronics India) with 100 ml sterile distilled water. The grinding was done until liquefied mixture get formed. Liquefied mixture was filtered using double-layered cheese cloth to remove solid debris. Filtrate was centrifuged at 5000 rpm for 30 min (Spinwin MC-02, Tarsons) and supernatant was collected. The supernatant was further subjected to 0.45 mm filtration followed by 0.22 mm filtration. The filtrate is used for synthesis of colloidal silver nanoparticle (CSNP) and will be referred further as *D. indica* extract.

Synthesis and Characterization of the Silver Nanoparticle

Synthesis was performed following the method described by Singh *et al.* [3] with minor modifications. 20 ml of $AgNO_3$ (0.5 M) was mixed with 50 ml of the extract in 4 ml distilled water. The solution was made slightly basic by adding 100 ml 0.01 M KOH and the final volume was adjusted to 5 ml with distilled water. Control silver nanoparticle was synthesized using $NaBH_4$ following the method described by Phukon et al. [6] and labelled as 'PC'. Different types of silver nanoparticle and negative controls synthesized are given in Table 1.

The characterization of the CSNP was performed using UV-Vis wavelength scanning from 200-800 nm, transmission electron microscopy (TEM) JEOL JEM 2100, Japan. Fourier transform infrared spectroscopy (FTIR) of Nicolet Impact 410 spectrometer.

Antibacterial Activity

The antibacterial assay was done by using the Agar Well Diffusion method described by Bharali et al. [7]. Briefly the bacterial culture was adjusted to the McFarland standard No. 0.5 before the tests. The media used for the assay was Mueller Hinton Agar (Himedia). The test was performed against *Bacillus subtilis* (MTCC 121) and *Escherichia coli* (MTCC 40). Streptomycin (STR) (50 mg / ml, Sigma) was taken as the positive control. The test was performed in triplicates.

Chemotaxis Analysis

Chemotaxis analysis was performed following the method described by Bharali et al. [7]. The bacteria used for analysis were *Staphylococcus aeurius* (MTCC 3160) and *Klepsiala pneunomonia* (MTCC 618).

Cytotoxicity Assay

Cytotoxicity assay was performed on RAW 246.7 (murine macrophage) cell line and *Vigna radiata* Linn. and *Oryza sativa* (*variety Ranjit*) seeds.

Mouse macrophage cells were plated in 96 well plates ($1x10^4$ cells / well) and kept for 24 h using DMEM media supplemented with 10% FBS (fetal bovine serum) 200 U / mL penicillin, 100 μg / mL streptomycin, 0.3 g / mL L-glutamine and 2 mM $NaHCO_3$ in 5% CO_2 atmosphere at 37°C so that cells can reach confluency. The cells were then treated with $AgNO_3$ (0.001M), distilled water (control), DISNP and $AgNO_3$(0.001M) solutions of volume 1, 5 and 10 mL and then incubated for 24h. After incubation 20 mL of 3(4, 5dimethyl-2-thiazolyl)2,5-diphenyl-2H-tetrazoliumbromide (MTT) is poured to each well and incubated. After incubation for 4 h the MTT solution was discarded without disturbing the formed formazone crystal and 100 mL of MTT solvent was added to each well to dissolve the complex. The optical density of the solution was measured at 580 nm using Multiskan Go equipment (Thermo Scientific, India).

The toxicity on plant cells were evaluated using germination process. In short, 2.50 g (dry weight) of *Vigna radiata* Linn. and *Oryza sativa* (*variety Ranjit*) seeds were surface sterilized and then soaked in 50 mL volume of distilled water, $AgNO_3$, PC, DISNP and DIEX for 24h. After imbibitions the seeds were washed thoroughly under sterile condition and allowed to germinate in germination chamber under ambient temperature and light / dark phase using plant tissue culture facility. Number of seeds germinated was recorded after 7 days.

Results

Silver Nanoparticle Synthesis and Characterization

Synthesis of colloidal silver nanoparticle using *D.*

indica extract is successful. Visually the colour of the DISNP colloid is golden yellow. Formation of CSNP in DISNP sample is confirmed using UV-Vis wavelength scanning 200-800 nm (Figure 1, A). The peak of absorption for DISNP and PC are at 420 and 410 nm, respectively. Less intensity of DISNP (after 1 day) compared to PC might be due to less amount of CSNP formation in the previous. The phenomenon might be due to weak organic reducers present in *D. indica* compared to sodium borohydride [1]. The intensity of the DISNP peak after 1 day is comparable to results obtained by Grishchenko *et al.* [8] using arabinogalactan. The DISNP intensity increases with time (Figure 1, B and C; after 27 days) and become comparable with PC. Intensity of the negative controls (Figure 1, A) are low and no signature of CSNP has been observed during the study. The symmetry of the peak is not disturbed during 27 days of storing at room temperature and slight increase in width is observed, suggest that DISNP nanoparticles are uniform [9].

DISNP peak shifted from 420 to 440 nm during 27 days period. This might be because of slight increase in the particle size due to slight agglomeration or increase in the particle size (Figure 1, C).

Table 1: Composition of different samples

Name	$AgNO_3$ 0.5M (ml)	*D. indicia* extract (ml)	KOH 0.01M (ml)	Distilled water (ml)
DISNP	20	50	100	4830
AGDI	20	50	0	4930
DIKOH	0	50	100	4850
AGKOH	20	0	100	4980

NB: PC was prepared using 10 ml 0.001M $AgNO_3$ and 30 ml 0.002M $NaBH_4$.

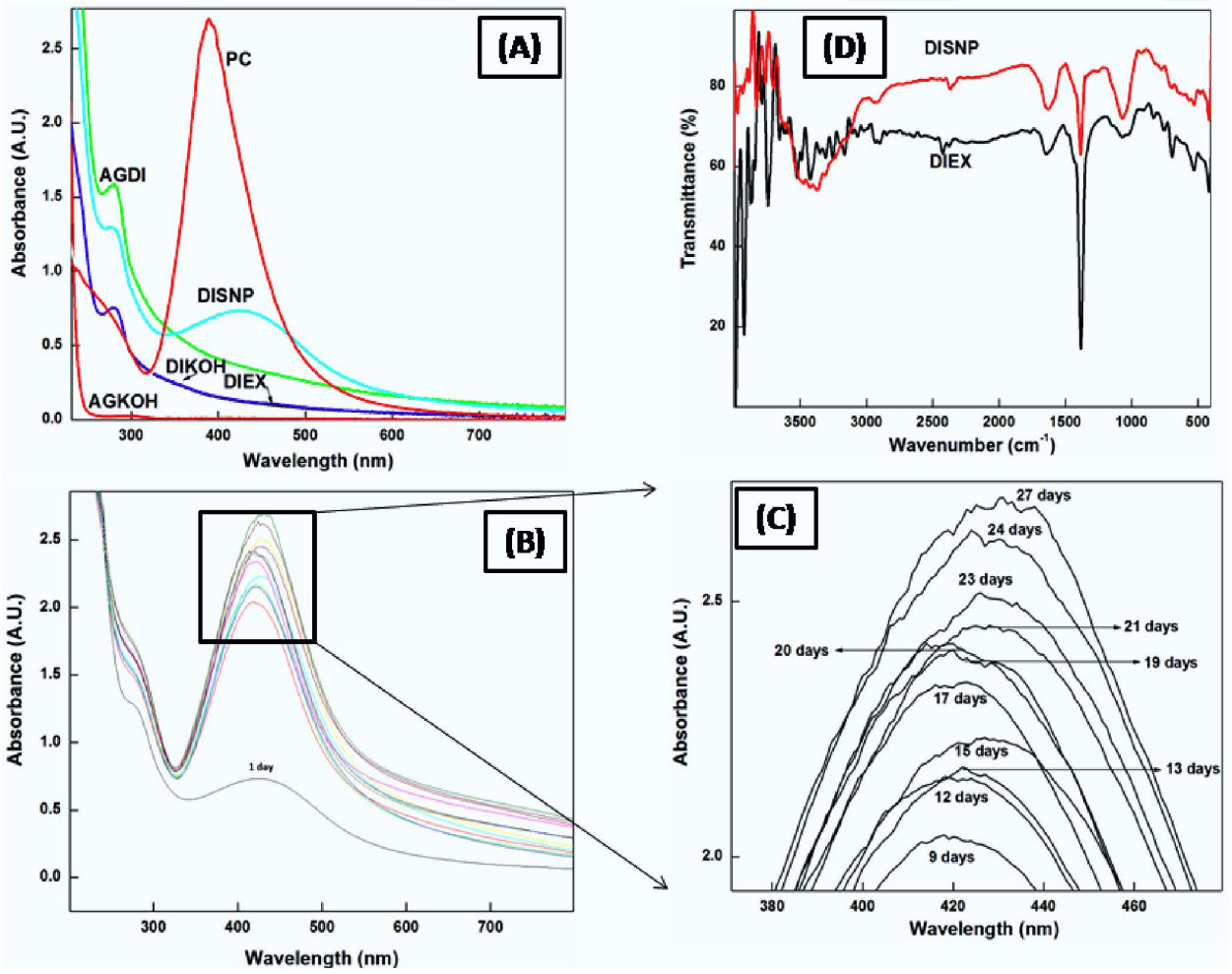

Fig. 1: Spectroscopic characterization of colloidal silver nanoparticle, **(A)** UV-Vis absorption spectra of different samples after 24 h (1 day) of incubation, **(B)** UV-Vis absorption spectra of DISNP with respect to different days (up to 27th day), **(C)** Enlarged and day wise labelled UV-Vis absorption spectra of DISNP, **(D)** FTIR analysis of DISNP and DIEX

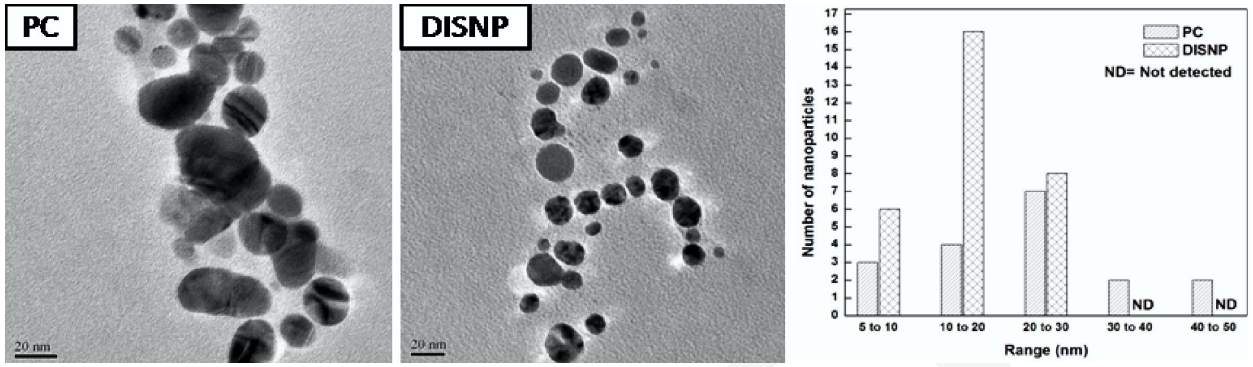

Fig. 2: TEM images of PC and DISNP. Size range wise distribution of nanoparticles as calculated from the figure is presented as bar diagram (right)

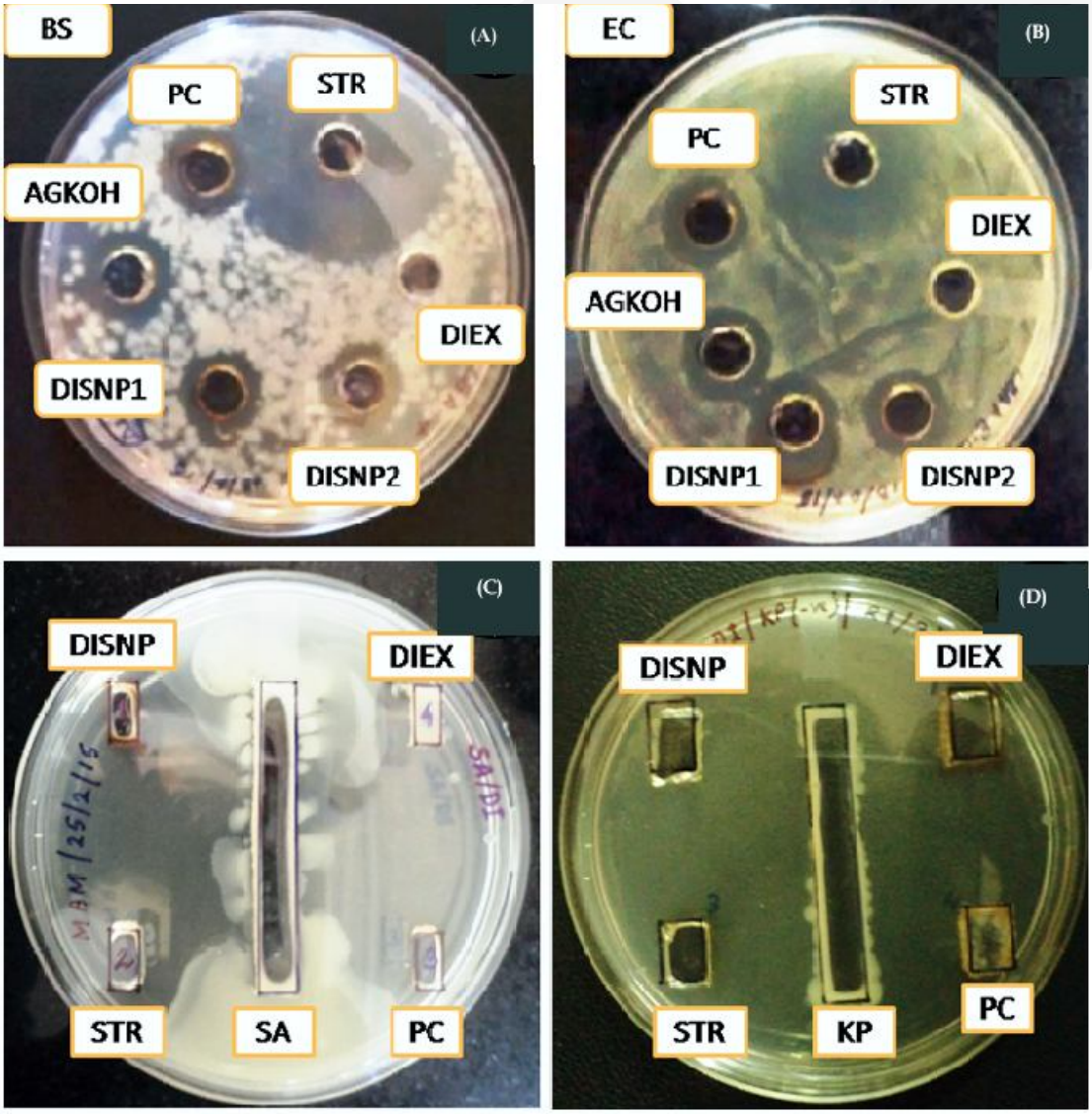

Fig. 3: Antibacterial **(a and b)** and chemotaxis **(c and d)** activity of nanoparticles, **(a)** Antimicrobial activity of silver nanoparticles against *B. Subtillis* (BS), **(b)** Antimicrobial activity of silver nanoparticles against *E. coli* (EC), **(c)** Chemotaxis activity of *S. aeurius* (SA) towards silver nanoparticles, **(d)** Chemotaxis activity of *K. pneunomonia* (KP) towards silver nanoparticles, DISNP1=27 days old DISNP, DISNP2=30 days old DISNP, STR= Streptomycin. Graphical representation of antibacterial assay is presented as bar diagram.

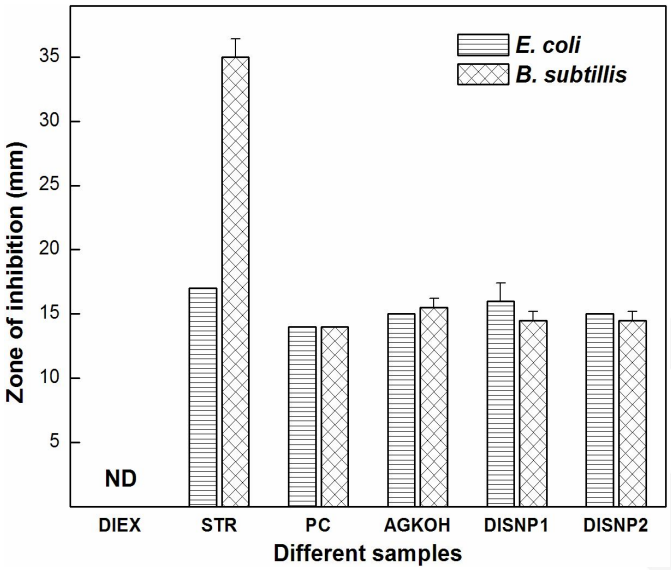

Fig. 3 (graph)

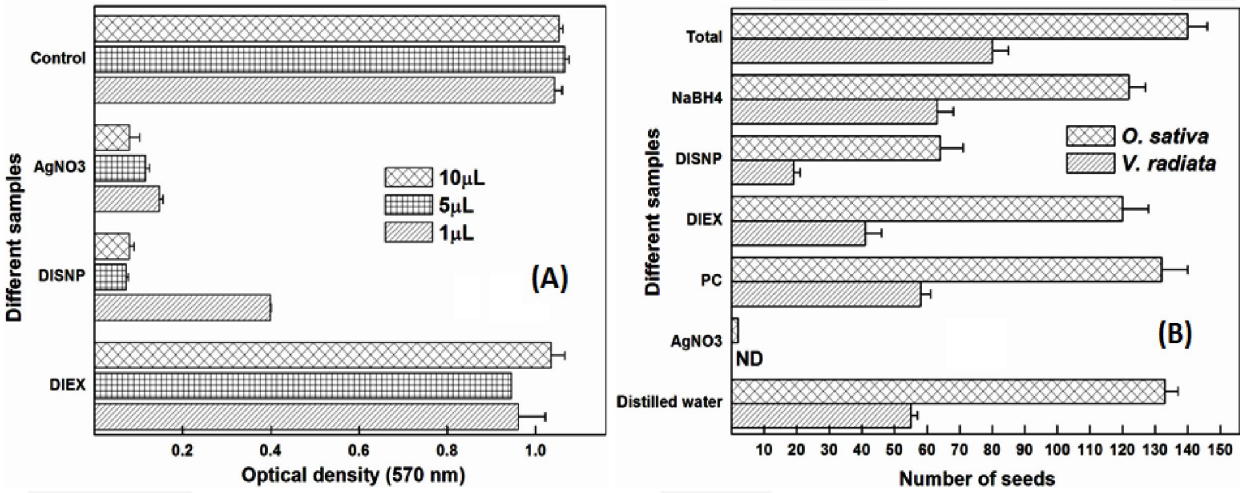

Fig. 4: Toxicity assay of silver nanoparticles in **(A)** RAW 246.7 (murine macrophage) cell line, **(B)** seeds of *V. radiata* and *O. sativa.*

Figure 1, D shows the FTIR analysis of 27 day old DISNP and DIEX. DISNP follows the pattern of DIEX except in the region of 4000 to 3000 cm^{-1}. This might be related to bound water to the compounds and suggest DISNP have more bound water compared to the *D. indica* extract (DIEX). TEM analysis presented in Figure 2 shows 10-50 nm size range for PC and 5-30 nm for DISNP. This image further confirms the formation of CSNP using *D. indica* extract. The bar diagram presented in Figure 2 shows that maximum number of particle in TEM image of PC are in the range of 20-30 nm whereas the same for DISNP is in the range of 10-20 nm.

Antibacterial and Chemotaxis Analysis

Figure 3 (a & b) shows antibacterial activity of all samples against *B. subtillis* and *E. coli*, except DIEX. PC is well known for its antibacterial activity, AGKOH might be showing antibacterial activity due to toxicity of $AgNO_3$ and alkaline KOH. DISNP1 (27 days old DISNP) and DISNP2 (30 days old DISNP) do not have any significance difference in their antimicrobial activity.

Figure 3 (c and d) shows the chemotaxis activity of the *S. aeurius* and *K. pneunomonia* towards DISNP, DIEX, STR and PC. *K. pneunomonia* does not show any growth outside the well. *S. aeurius* show positive growth towards *D. indica* extract (DIEX). Growth of *S. aeurius* towards PC and STR are comparable. DISNP is found to be chemo-attractant for *S. aeurius*. The result for PC coincides with our previously reported finding Bharali *et al.* [7]. Figure 4 (B), shows the number of seeds germinated with respect to different treatment and figure 4 (A) viability of the mouse macrophage cells with respect different treatment, represented in the form of optical density. The seed germination

number was found to be varying significantly with respect to different pre-germination soaking medium for a time period of 24 h. From figure 4 (B) it was clear that soaking seeds in 0.001M $AgNO_3$ solution is detrimental and prevent the germination. Distilled water and PC are found to be non toxic to the germination process. DIEX was found to be slightly preventing the germination. DISNP was found to an intermediate toxic in the process of germination.

In case of mouse macrophage cell line, the toxicity corresponds directly to lower optical density as observed for $AgNO_3$ and DISNP. 1mL DISNP was found to be significantly less toxic compared to other two higher concentrations whereas DIEX does not show any toxic effect for the applied concentration as the absorbance of the solution was found to be equal to the control cells.

Discussions

In our previous research the data has been observed for about 6 days with absorption of 1.2 units [3]. Presently the analysis was done further up to 27 days and the observed absorbance is equivalent to PC, suggest that all $AgNO_3$ got reduced to Ag^0, then clump and formed nanoparticle. Shifting of the DISNP peak (Figure 1, B and C) towards longer wavelength and slight broadening, suggest increase in the size of the nanoparticle [10].

The TEM images of the nanoparticle presented in Figure 2 propose that DISNP particle are smaller compared to PC (chemically synthesized particles). The bar diagram presented in Figure 2 clearly suggests that most of the PC particles fall under the size range of 20-30 nm. The data is slightly higher compared to previous result presented by Solomon *et al.* [10] as 12 nm. The size range observed in the present study 5-50 nm for PC is also found to bigger compare to Song *et al.*'s [11] report of 30-40 nm. In case of DISNP the maximum particle fall under the range of 10-20 nm and show a narrow size distribution (5-30 nm) compared to PC (5-50 nm). These suggest that DISNP based synthesis is better compared to chemical synthesis method (using $NaBH_4$) for obtaining smaller sized nanoparticle with a narrow size distribution. The credit of narrow size distribution of DISNP might be attributed to natural surfactant molecules present in DIEX [12]. The polysaccharide like an arabinogalactan present in the DIEX might be acting surfactants and preventing clumping and thereby preventing formation of larger particles [13, 2]. During filter sterilization of the DIEX a huge amount of mucilaginous substances might get separated from the *D. indica* extract. Presence of those might further stabilize the nanoparticle. A future study can be conducted in unfiltered DIEX and additional stability provided therefore.

Antimicrobial activity presented in Figure 3 (a & b) suggest that DIEX do not have any antimicrobial property. Abdille *et al.* [14] suggested that lowest amount of total phenolics (1.4%) from *D. indica* fruit is recovered using water as solvent. The low amount of polyphenolic constituents might be responsible for not having antibacterial property. In case of *B. subtillis* treated with streptomycin (STR) the antimicrobial property was found to be highest (35.0 ± 1.4 mm). The same in case of PC (14.01.2), AGKOH (15.50.7), DISNP1 and DISNP 2 (14.50.8) are not significantly different from each other. PC is well known for its antibacterial activity against most of the bacteria with exceptions *B. subtillis* (MTCC 441) [7]. Similarly, in case of *E. coli* with streptomycin (STR) treatment show highest zone of inhibition (17.0ND, ND= not detected). Same in case of PC (14.0ND), AGKOH (15.0ND), DISNP1 (15.0ND) and DISNP 2 (15.01.4). In sample AGKOH the zone of inhibition might be due to the combine action of AgNO3 and un-reacted alkaline KOH. DIEX do not show any antibacterial activity against *E. coli*. These results suggest that antibacterial value addition to the DIEX can be done by synthesizing silver nanoparticle in fruit extract and it might be useful in increasing the shelf life of the fruit sap.

Literature on chemotaxis assay using silver nanoparticle and bacteria are scanty. In our previous research we found the same *S. aeurius* (MTCC 3160) was found to be highly chemotactic towards PC. In the present research a moderate chemotaxis was observed towards the PC [7]. Movement of the *S. aeurius* towars the well containing streptomycin (STR) is further less compared to PC (Figure 3, c). Movement of bacteria towards DISNP and DIEX is prominent. This suggests that DISNP might have the capacity to attract and then kill bacteria. This might be an added advantage over PC. In case of *K. pneunomonia* no movement was observed towards any of the well (Figure 3, d). Similar result was observed when *E. coli* (MTCC 40) is used for the assay (figure not presented). Since DIEX contain some amount of polysaccharide and other sugars a positive chemotaxis is expected at least towards it. The negative result might be due to the lack of mobility by the bacterial strains. During our literature search we have not found any literature suggesting lack of mobility by the above said strains.

To perform the toxicity of the DISNP towards mammalian cells, MTT assay was performed. From the Figure 4 (A) it has been found that the cells are

more sensitive to $AgNO_3$ and DISNP and hence only few cells can survive upon the treatment of $AgNO_3$ and DISNP. Even though at lower volume of given concentration i.e. 1 µl, toxicity reduced to half of the control cells. In case of DIEX, it did not show any toxic effect to the cultured macrophage cells. This happens only because of the presence of more numbers of live cells forming more numbers of formazone complexes by reacting with MTT solution. Henceforth the absorbance obtained in this case was more which was found equivalent to the absorbance of the control cells.

Germination of seeds follows a normal course of secretion of giberellic acid from embryo which give the signal to aleuron layer of cells to secret alpha-amylase. The alpha-amylase enzyme degrades the endosperm food reserve (major component starch) to produce reducing sugar. Therefore, the success of germination is depends a lot on degradation of starch. The data regarding toxicity of DISNP against plant seeds (*O. Sativa* and *V. mungo*) are presented in Figure 4B with respect to number of seeds germinated when DISNP, distilled water, DIEX, $AgNO_3$ and PC were used as imbibitions medium for 24h. The toxicity of DISNP was found to be moderate (Figure 4, B) against *O. Sativa* compared to *V. mungo* seeds. $AgNO_3$ in equal concentration with DISNP and PC is found to highly toxic to both seed types. It is a surprise to observe that PC is completely nontoxic (seen equal number of germination with distilled water) and even more number of seeds germinate in PC compared to distilled water in case of *V. mungo*. Chemically the PC contains Silver nanoparticle and excess of $NaBH_4$ (10 ml 0.001M $AgNO_3$ + 30 ml 0.002M $NaBH_4$). $NaBH_4$, being a strong reducer might be responsible for degradation of starch and doing so it promote germination. The hypothesis is yet to establish by performing an amylase assay for starch degradation.

Comparing Figure 4 (A) and (B) it can be summarised that DISNP is less toxic than $AgNO_3$, have comparable antimicrobial activity and size distribution of the nanoparticle is better than the $NaBH_4$ based nanoparticle (PC). Further research to evaluate the mechanism of stimulating germination by PC is necessary. *D. indica* based nanoparticle might be toxic to mammalian cells and plant seed. Therefore, one should be merely suggest a green nanoparticle will be always less toxic until the toxicity assay is performed and confirmed.

Acknowledgement

Authors would like to acknowledge the financial and instrumentation assistance received from UGC and Department of Biotechnology, New Delhi with sanction no. BT/22/NB/2011 and BT/HRD/01/002/2007.

References

1. D. Kumar, S. Mallick, J.R. Vedasiromoni, B.C. Pal, Anti-leukemic activity of *Dillenia indica* L. fruit extract and quantification of betulinic acid by HPLC, Phytomedicine 2010; 17: 431-435.
2. D. Gandhi, P. Mehta, *Dillenia indica* Linn. and *Dillenia pentagyna* Roxb.: Pharmacognostic, Phytochemical and Therapeutic aspects, J. Appl. Pharm. Sci. 2013; 3: 134-142.
3. S. Singh, J.P. Saikia, A.K. Buragohain, A novel 'green' synthesis of colloidal silver nanoparticles (SNP) using *Dillenia indica* fruit extract, Coll. Surf. B 2013; 102: 83-85.
4. S. Thakore, P.S. Rathore, R.N. Jadeja, M. Thounaojam, R.V. Devkar, Sunflower oil mediated biomimetic synthesis and cytotoxicity of monodisperse hexagonal silver nanoparticles, Mater. Sci. Eng. C 2014; 44: 209-215.
5. X. Zhang, Z. Li, X. Yuan, Z. Cui, H. Bao, X. Li, Y. Liu, X. Yang, Cytotoxicity and antibacterial property of titanium alloy coated with silver nanoparticle-containing polyelectrolyte multilayer, Mater. Sci. Eng. C 2013; 33: 2816-2820.
6. P. Phukon, J.P. Saikia, B.K. Konwar, Enhancing the stability of colloidal silver nanoparticles using polyhydroxyalkanoates (PHA) from Bacillus circulans (MTCC 8167) isolated from crude oil contaminated soil, Coll. Surf. B 2011; 86: 314-318.
7. P. Bharali, J.P. Saikia, S. Paul, B.K. Konwar, Colloidal silver nanoparticles/rhamnolipid (SNPRL) composite as novel chemotactic antibacterial agent, Int. J. Biol. Macromol. 2013; 61: 238-242.
8. L.A. Grishchenko, S.A. Medvedeva, G.P. Aleksandrova, L.P. Feoktistova, A.N. Sapozhnikov, B.G. Sukhov, B.A. Trofimov, Redox reactions of arabinogalactan with silver ions and formation of nanocomposites, Russ. J. Gen. Chem. 2006; 76: 1111-1116.
9. S. He , J. Yao , P. Jiang , D. Shi , H. Zhang , S. Xie , S. Pang , H. Gao, Formation of Silver Nanoparticles and Self-Assembled Two-Dimensional Ordered Superlattice, Langmuir. 2001; 17: 1571–1575.
10. S.D. Solomon, M. Bahadory, A.V. Jeyarajasingam, S.A. Rutkowsky, C. Boritz, Synthesis and Study of Silver Nanoparticles, J. Chem. Educ. 2007; 84: 322-325.
11. K.C. Song Affiliated with Department of Chemical and Biochemical Engineering, Konyang University, S.M. Lee, T.S. Park, B.S. Lee, Preparation of colloidal silver nanoparticles by chemical reduction method, Korean J. Chem. Eng. 2009; 26: 153-155.

12. J.P. Saikia, Hair waving natural product: *Dillenia indica* seed sap, Coll. Surf. B 2013; 102: 905-907.

13. A.J. Kora, S.R. Beedu, A. Jayaraman, Size-controlled green synthesis of silver nanoparticles mediated by gum ghatti (*Anogeissus latifolia*) and its biological activity, Org. Med. Chem. Lett. 2 (2012) 17. URL: http://www.orgmedchemlett.com/content/2/1/17

14. M.H. Abdille, R.P. Singh, G.K. Jayaprakasha, B.S. Jena, Antioxidant activity of the extracts from *Dillenia indica* fruits, Food Chem. 2005; 90: 891-896.