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Original Article

Vigna mungo Seed Non-Destructive Based Colloidal Silver Nanoparticle Synthesis and Toxicity

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Abstract

Silver nanoparticle synthesis needs reduction. Seeds of plant are known to exude reducing agent during imbibition. Combining these two ideas we synthesize novel red coloured colloidal silver nanoparticle without destroying the *Vigna mungo* seeds. Rather than going with the general idea that green method will produce less toxic nanoparticle, we evaluate the toxicity in germinating seeds and RAW cell line along with antimicrobial and chemotaxis test. The result suggests that silver nanoparticles are antibacterial positive without chemotactic property. The cytotoxicity against RAW 246.7 murine macrophage cell linewas found high. Phytotoxicity was also found potent in case of *Vigna mungo* (dicot) and *Oryza sativa* (monocot) seed with respect to inhibition of germination.

Keywords: Silver Nanoparticle; *Vigna Mungo*; Toxicity; Antibacterial; Chemotaxis.

Introduction

Why SNP?

Silver nanoparticle (SNP) synthesis, application and other related studies provide 43,300 different scientific papers in 'Google Scholar' search during last five years (2010-14). About half of these (21,600) are related to key word 'green silver nanoparticle'. The immense thrust on 'green' might be due the immense probability of novelty. Researcher try to categorise the CSNP synthesis with respect to the green precursor used [1]. Every plant species extract is unique to its composition though the major chemical compounds have similarity with other species. CSNP synthesis is a highly sensitive process and show great variation with slight change in reduction medium (including presence of minor chemicals).

Exploring the same opportunity during our literature survey we found many different plant enzymes / proteins, acids, polysaccharides, pectin and vitamins were exploited for the synthesis of colloidal silver nanoparticle [1-3]. Many of the methods used food items during synthesis. The logic behind might be edible will be less toxic compared to non edible. Industrialization of these CSNP synthesis methods might have impact on respective food prices. There are several reports of green silver nanoparticle synthesis from *Lantana camara*, *Plumeria alba*, *Nyctanthes arbor-tristis*, *Piper longum*, Sunflower like plant sources [4-8]. The literatures on application of food industry by-product like'seed exudates' for CSNP synthesis are scanty.

Black gram (*Vigna mungo*) seeds highly priced and common in Indian meals. The seed has been reported for hypolipidemic, immunostimolatoryetc [9,10]. Very few reports are available on *V. mungo* seeds exudates. Unlike other legume seeds the seed exudates of *V. mungo* do not contain detectable sugar [11]. Like all other legume seeds *V. mungo* seeds during imbibitions secrets flavonoids and nitrogenous metabolites such as alkaloids, terpenoids, peptides and amino acids [12].

Bioactivity of colloidal silver nanoparticle has been

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evaluated by number of researcher with respect to antibacterial,chemotaxisand cytotoxicity [13-16]. Antibacterial property of the green silver nanoparticle has been evaluated by many different researchers[7, 6, 4, 17]. Chemotaxis property of colloidal silver nanoparticle has been reported by Bharali et al., Kirschling,Sosenkova and Egorova, and Tran et al. [15, 18-20]. Silver nanoparticle application for UV-protection has been reported for textile fabrics [21-23]. Colloidal silver nanoparticle effect on Cys-Cysdisulfide bond is reported by López-Tobar et al. [24].

Cytotoxicity of colloidal silver nanoparticle is reported by Yu, Samberg et al. and Hatipoglu et al. [25-27].

Materials and Methods

Chemicals and consumables: *Vigna mungo* seeds were collected from Sonitpur, Assam, India. $AgNO_3$ (A.R.) is obtained from Merck, India. Sodium borohydride is obtained from Fluka Chemicals, USA.

Green Synthesis Method

V. mungo seed Exudates Preparation (VMEX)

Five gram (5.00 g) *V. mungo* seeds were surface sterilized with 70% ethanol in sterile distilled water (v / v) for 5 min and then washed with sterile distilled water to remove traces of ethanol. The seeds were soaked in 100 ml sterile distilled water for 24 h. The seed exudates obtained were filtered using Whattman no. 1 filter paper. The filtrate is further filter sterilized using 0.22 mm nitrocellulose filter paper assembly. The filtrate is stored at $4^{O}C$ for further use and labelled as VMEX.

Protein, Reducing Sugar and Polyphenol Estimation of VMEX

For the experiment 50 g seeds were surface sterilized as mentioned above and soaked in 200 ml sterile distilled water for 24h. Exudates of 5 ml for protein, 5 ml for reducing sugar and 5 ml for polyphenol estimation were collected at 3^{rd}, 6^{th}, 12^{th} and 24^{th} h of imbibitions. The total volume of the exudates is measured during every collection using a sterile measuring cylinder under sterile condition inside laminar air flow cabinet. These actual volumes were used later for calculating the total amount of protein, reducing sugar and polyphenol exude by seed at a particular time. Protein and reducing sugar estimation was performed using Bradford's andanthrone method respectively. Total polyphenol estimation was done using Cordenunsi*et al.*'s method [28]. In short 500 mL extract was mixed with 2.5 ml 0.2 N Folin's reagent and incubated at room temperature for 5 min followed by addition of 2.0 ml saturated Na_2CO_3 (75 g / L) and incubation for 90 min at 30°C. After incubation, absorption was measured at 765 nm using gallic acid as standard.

Silver Nanoparticle Synthesis Using VMEX

50 mL 0.5M $AgNO_3$ solution was mixed with 4850 mL VMEX. The solution is mixed properly and then 100 mL of 0.01M KOH solution was added. Immediately, red colloidal silver nanoparticle solution is observed and labelled as VMSNP. Other negative controls were prepared accordingly and composition is presented 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 Analysis

Antimicrobial analysis was performed following the method described by Bharali et al. (2013) [15]. The test was performed against *Staphylococcus aureus* (MTCC 3160) and *Escherichia coli* (MTCC 40). A total of 6 wells were prepared in Muller Hilton Agar (Himedia, India) and CSNP, negative controls and streptomycin (STR) (50 mg / ml) solution of 100 ml is poured into respective wells. After antimicrobial activity evaluation VMSNP2 is excluded from further study as it has been confirmed that it is only a bigger sized slowly synthesized silver nanoparticle. Therefore, VMSNP1 is referred hereafter as VMSNP.

Antibacterial Activity

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

Chemotaxis Assay

The assay for chemotaxiswas performed following

the method described by Bharali et al. (2013) [15]. The bacteria used for analysis were *Staphylococcus aeurius* (MTCC 3160) and *Klepsiala pneunomonia* (MTCC 618).

- 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 distilled water (control), VMSNP 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, VMSNP1 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

Biochemical Characterization of VMEX

The biochemical characterization of VMEX with respect to protein, reducing sugar and polyphenol is presented in Figure 1. Reducing sugar content is found to be the highest followed by polyphenol and protein. With respect to soaking time period leaching of all content is found to be increasing.

Synthesis and Characterization of the Nanoparticle

VMSNP1 start turning into a red colour solution from a colourless one, after addition of 100 mL KOH. The red colour becomes deeper with respect to time. Figure 2 shows the UV-Vis absorption spectroscopic analysis of the VMSNP1 nanoparticle along with other negative and positive controls. In case of negative control VMSNP2 (without KOH) it has been seen that nanoparticle formation takes more time compared to VMSNP1 (with KOH). Nanoparticle formation is confirm from the absorption between 400-500 nm in PC, VMSNP1 and VMSNP2, which is not visualised in other negative controls including *V. mungo* exudates.

For further confirmation and to evaluate the nanoparticle shape and size TEM and FTIR analysis were performed and presented in Figure 3. As seen in the Figure 3 (a, b and c) the nanoparticle size of VMSNP2 is bigger compared to PC and VMSNP1. The fact is correspond with broad absorption spectra of VMSNP2 compared to VMSNP1 as presented in Figure 2(c).

Antibacterial and Chemotaxis Activity

The result obtained for antibacterial and chemotaxis property of the VMSNP1 is presented in Figure 4. Except VMEX all other samples are found to be antibacterial including VMSNP1 (Figure 4, a and b). *Staphylococcus aeurius* is found to be chemotactic and VMSNP is not a good chemo-attractant compared to glucose and PC. *Klepsiala pneunomonia* is found to have poor chemotactic property and slight movement towards glucose and PC has been detected.

Cytotoxicity Assay

Figure 4 (a) viability of the mouse macrophage cells with respect to treatment with different samples, represented in the form of optical density and figure 4 (b) shows the number of seeds germinated with respect to different treatment.

In case of mouse macrophage cell line, the toxicity corresponds directly to lower optical density as observed for $AgNO_3$ and VMSNP. VMSNP toxicity is found to be equivalent to $AgNO_3$, whereas VMEX does not show any toxic effect for the applied concentration as the absorbance of the solution was found to be slightly more than the controls treated with sterile distilled water (Figure 5, a).

Figure 5 (b) shows number of seeds germinated under different sample treatment. Total in the figure suggest the total number of seeds from each species. Numbers represented against all other sample represent the number of seeds germinated after soaking in the sample solution for 24h. Silver nitrate solution was found to be highly toxic as it is already known to inhibit germination by increasing

sensitivity to abscisic acid during germination through its inhibitory effects on ethylene activity [29]. VMEX have distilled water like activity. VMSNP was found to be highly toxic and reduce the number of germinating seed to more than five and three folds in case of *V. mungo* and *O. sativa* seeds respectively. The effect on *O. sativa* might be little less because of the hard seed coat compared to *V. mungo*. Germinating seeds in PC treatment was found to slightly more which is reported by many researchers as toxic for germination. As there are excess of $NaBH_4$ is present in PC (10 ml 0.001M $AgNO_3$+30 ml 0.002M $NaBH_4$) therefore, same concentration $NaBH_4$ is also evaluated for the germination assay. $NaBH_4$ is found to be non toxic rather it is a germinations stimulator at least in case of *V. mungo*.

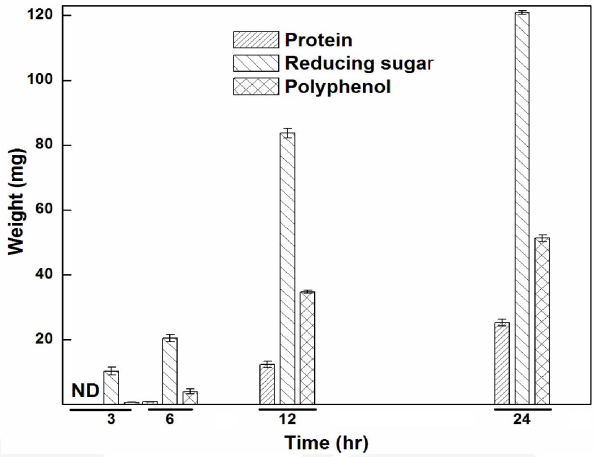

Fig. 1: Total protein, reducing sugar and polyphenol leached out of 50 gm*Vigna mungo*(dw) seed at different time intervals. ND= not detected.

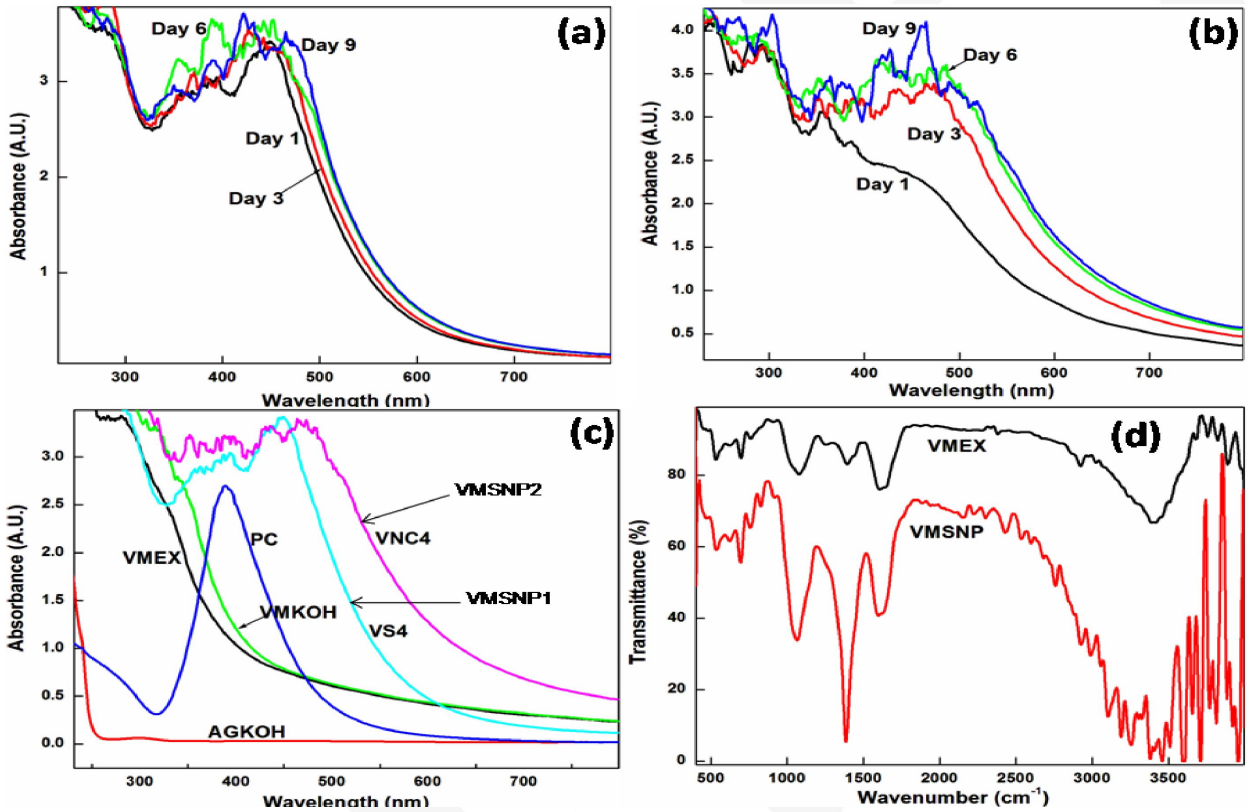

Fig. 2: UV-Vis absorption spectroscopy of *Vigna mungo* based silver nanoparticle. Day wise absorption spectra of VMSNP1 (a), VMSNP2 (b) and comparative absorption spectra of different samples on day 3 (c).FTIR spectra of VMEX and VMSNP1 (d).

Table: 1: Composition of VMSNP and negative controls

Name of the sample	$AgNO_3$ (0.5M) ml	VMEX ml	KOH (0.01) ml	Distilled water ml
VMSNP1	50	4850	100	0
VMSNP2	50	4850	0	100
VMEX	0	4850	0	150
AGKOH	50	0	100	4850
VMKOH	0	4850	100	50

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

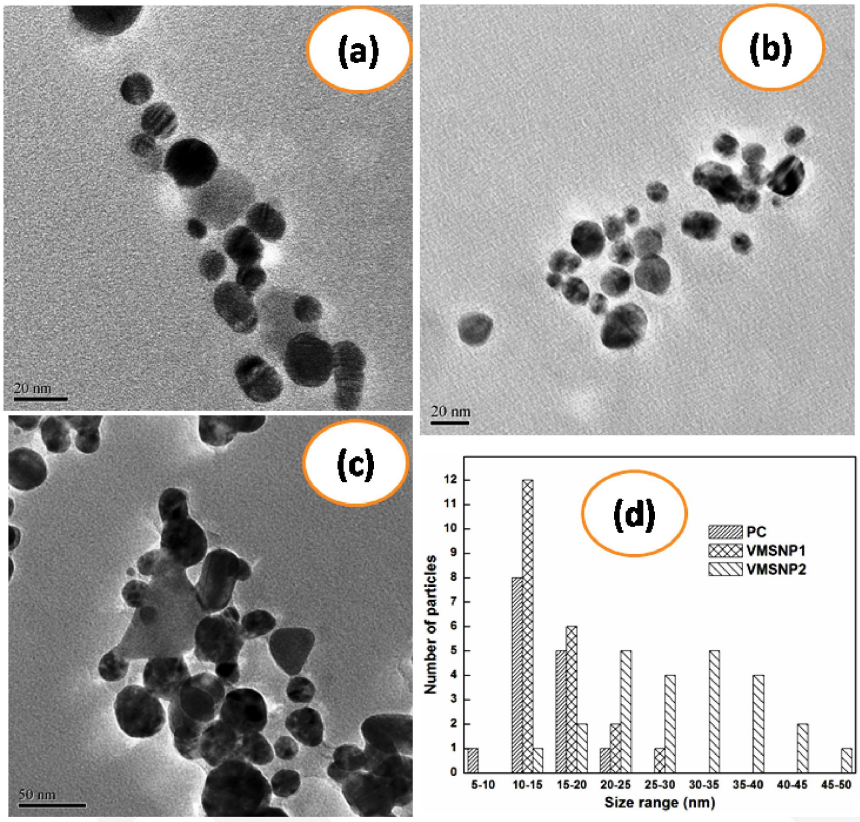

Fig. 3: TEM image of PC (a), VMSNP1 (b) and VMSNP2 (c). Number of particle in different range in presented from the TEM figures (d)

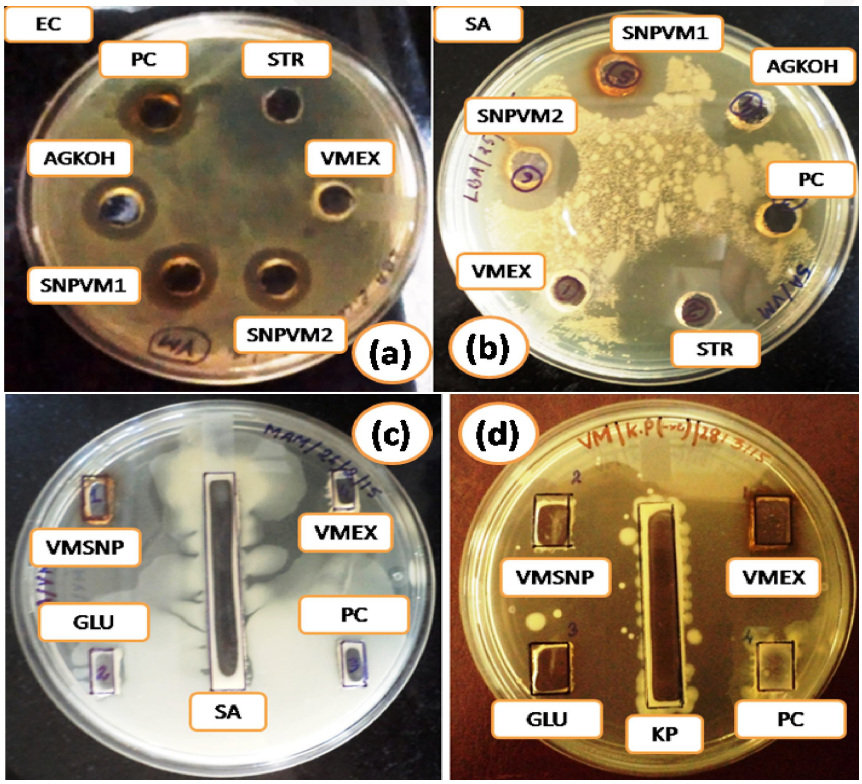

Fig. 4: Antibacterial (a and b) and chemotaxis (c and d) activity of SNPVM1. EC=*Escherichia coli* (MTCC 40), SA=*Staphylococcus aureus*(MTCC 3160), SA=*Staphylococcus aeurius* (MTCC 3160), KP=*Klepsiala pneunomonia* (MTCC 618), GLU=glucose solution, VMSNP=VMSNP1 and STR=streptomycin.

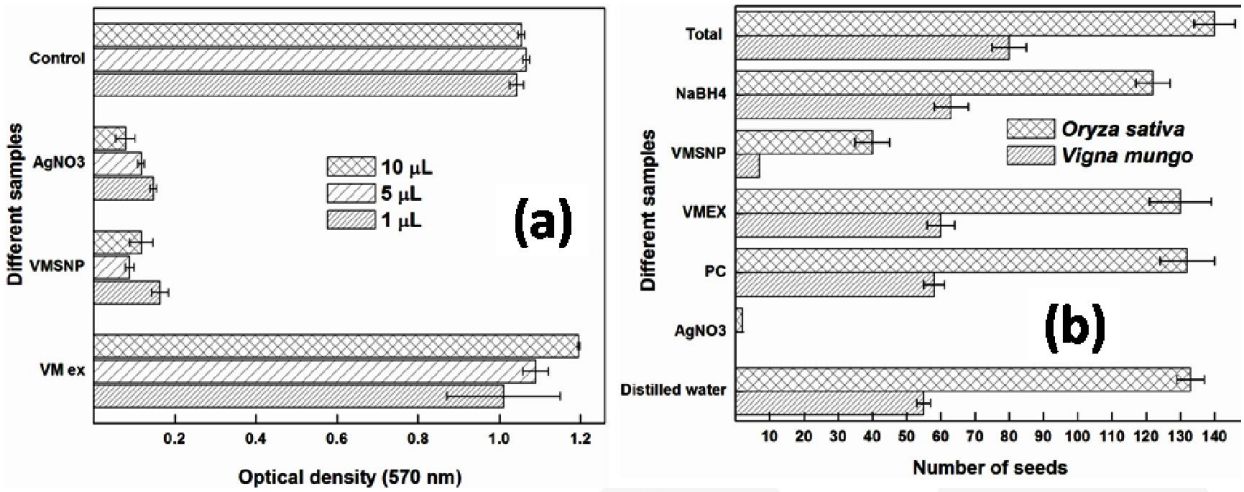

Fig. 5: Toxicity assay of silver nanoparticles in (A) RAW 246.7 (murine macrophage) cell line, (B) seeds of *V. radiata* and *O. sativa*.

Discussion

Ahmed et al. reviewed the green nanoparticle size reported for different plant extract showing 0.5 to 150 nm ranges using different plant extract [30]. In their review they did not referred any article using seed exudates for silver nanoparticle synthesis. As presented in Figure 3 (d) the maximum number of silver nanoparticles of sample PC and VMSNP1 are present the range of 10-15 nm and pattern of distribution of particle size range of PC and VMSNP1 are similar. Maximum numbers of VMSNP2 particles are found in the range of 20-35 nm range. The size distribution of PC and VMSNP1 is also narrow (PC 5-25 nm, VMSNP1 10-30 nm) compared to wide size distribution of VMSNP2 (10-50 nm). This suggest that for narrow size distribution and small sized nanoparticle the reduction method should be fast and in the present case KOH perform the same. Further, it can be observed in Figure 3 (a, b and c) the PC and VMSNP1 particle morphology is spherical and the same for VMSNP2 is irregular and triangular.

Antimicrobial activity of all the samples was found to be equal and no significant difference is found except streptomycin and VMEX [30]. Another noticeable event is that the exudates (VMEX) alone do not have the antimicrobial activity but on addition of $AgNO_3$, antimicrobial activity is incorporated tomicrobesusceptible exudates rich in reducing sugar, protein and polyphenol. From the chemotaxis assay it is clear that KP strain might not have taxis or movement. SA shows good chemotaxis and VMEX was found to be a chemo-attractant for SA but VMSNP is not. This suggests that VMSNP might be a good repellent for SA.

The toxicity assay suggests that he VMSNP is highly toxic to animal cell line like $AgNO_3$. Similar toxicity is found in germination test with two different plant seeds. These results suggest that green nanoparticles should not declare merely nontoxic rather their toxicity should be evaluated from case to case basis.

Conclusion

Silver nanoparticle synthesized using *V. mungo* seed exudates without destroying the seeds is a novel method. Green nanoparticle is found to be highly toxic to animal cell line and plant seeds. Nanoparticles are found to be repelling activity towards *Staphylococcus aeurius* suggesting possible application in bacteria repelling surface fabrication. $NaBH_4$ germination stimulating activity might be submerging the toxicity of PC type silver nanoparticle to some extent. Further investigation is needed to evaluate the mechanism of PC mediated germination stimulation of *V. mungo* seeds. The present approach of using food industry waste like seed exudates might provide more novel nanoparticle and will not harm the medicinal plants and biodiversity on extensive use.

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