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Biological evaluation of nanosilver incorporated cellulose pulp for hygiene products

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ABSTRACT

Cellulose pulp has a visible market share in personal hygiene products such as sanitary napkins and baby diapers. However it offers good surface for growth of microorganisms. Huge amount of research is going on in developing hygiene products that do not initiate microbial growth. The objective of the present work is to produce antibacterial cellulose pulp by depositing silver nanopowder on the cellulose fiber. The silver nanoparticles used were of less than 100 nm in size and were characterised using transmission electron microscopy and X-ray powder diffraction studies. Antibacterial activity of the functionalized cellulose pulp was proved by JIS L 1902 method. The *in-vitro* cytotoxicity, *in-vivo* vaginal irritation and intracutaneous reactivity studies were done with silver nanopowder incorporated cellulose pulp for introducing a new value added product to the market. Cytotoxicity evaluation suggested that the silver nanoparticle incorporated cellulose pulp is non-cytotoxic. No irritation and skin sensitization were identified in animals tested with specific extracts prepared from the test material in the *in-vivo* experiments. The results indicated that the silver nanopowder incorporated cellulose pulp meets the requirements of the standard practices recommended for evaluating the biological reactivity and has good biocompatibility, hence can be classified as a safe hygiene product.

1. Introduction

Hygiene products made of cellulose pulp and cotton are generally prone to microbial growth resulting in unpleasant odor, color degradation and at times skin irritation. The urging need to address these issues paved way to the development of nanoparticle incorporated textile and hygiene products in the last few years [1]. The antimicrobial potential of silver nano particles prepared by a number of synthetic methods has been found useful in textile products [2–8]. Silver nanoparticles are already used in water purification units, medical products such as scaffolds, catheters, surgical devices and gauze [9–12]. The use of silver nanoparticles in clothes has revolutionized the textile industry [13–17]. This wide use of silver nanoparticle is due to the inhibition of bacterial growth, less inclination for inducing bacterial resistance and broad spectrum antimicrobial properties [10].

Silver is a good antimicrobial agent with less toxicity to mammalian cells [18]. Silver nanoparticles are reported to be more effective in mediating the antibacterial activity as compared to silver compounds [8,11,12]. Antimicrobial properties of silver nanoparticles in particular, are due to the inherent characteristics such as high surface area, small size and high dispersion [2,8]. Though many mechanisms have been put forward to explain the antibacterial activity of silver nanoparticles, it is suggested that the antibacterial activity is due to the generation of silver ions in the aqueous solution which may bind with the proteins on the bacteria cell membrane and inhibit cell respiration and reproduction. Antibacterial activity of silver nanoparticles is enhanced due to its large surface area and better contact with bacterial membrane leading to the breakage of bacterial membrane and disruption of the respiratory chain and the cell division process leading to cell death [11–19].

Cellulose pulp finds application in medical textile and hygiene products (nappies, sanitary napkins, wiping tissues *etc.*). It has a large market potential due to its high liquid adsorption, low toxicity content, softness, light weight and low cost. It is an ideal matrix for making biocompatible and user friendly hygiene products owing to its easy for surface modification [20–23]. At the same time it offers an excellent platform for bacterial growth. Hence methods to impart antibacterial property to cellulose pulp are in demand. Cellulose pulp containing triclosan, quaternary ammonium compounds and silver have been reported in the literature [22]. Silver nanoparticles can be used to impart antibacterial property to cellulose pulp; since it is less toxic to mammalian cells in low concentrations [18]. In the present study we have used cellulose pulp incorporated with silver nanopowder (silver nanopowder size < 100 nm; with proven antibacterial properties) to develop a sanitary napkin. The antibacterial activity was checked by qualitative and quantitative tests. The quantitative antibacterial testing method developed by the Japanese textile industry namely JIS L 1902 method against gram-positive and gram-negative strains of bacteria proved the antibacterial potential of the functionalized cellulose pulp used in sanitary napkin.

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Although several studies on the antibacterial potential of silver nanoparticles are reported; not many studies have been done on the evaluation of toxicity by *in-vitro* tests and *in-vivo* animal models. Previously different studies using silver nanoparticles have shown that lower concentration of silver nanoparticle is found to be non-toxic in nature [24]. In the present work we have focused on the biological evaluation of cellulose pulp with silver nanopowder (<100 nm; with proven antibacterial properties) developed as sanitary napkin. Vagina is a highly sensitive area due to its unique anatomy, microflora and secretions. The vaginal mucosal tissue does not have secretory glands. The drying of the tissue is avoided by a mixture of fluids originating from different sources. It is reported that the composition, volume and rheological properties of vaginal fluids are affected by age, the stage in the menstrual cycle, and sexual arousal [25,26]. So the developed sanitary napkin needs to be assessed for compatibility with the human mucosal surface as it may induce undesirable side-effects on the skin. Hence we have carried out the *in-vivo* tests such as intracutaneous reactivity and vaginal irritation to determine the biological response of animals towards silver nanoparticle incorporated sanitary napkin [27]. The *in-vitro* and *in-vivo* studies enabled us to assess the feasibility and safety of using antibacterial cellulose pulp in sanitary napkin and also to check whether the material meets the biocompatibility standards for such products.

2. Experimental section

2.1. Materials

Cellulose pulp was gifted by the sanitary napkin unit of HLL Lifecare Ltd., Kanagala, India. A commercial silver powder "Silicon di oxide-N9 Pure silver SCBP", herein referred in this paper as "silver nanopowder", was obtained from Resil Chemicals Pvt Ltd., Bangalore, India. According to the product's information, this powder is composed of silver–silicone oil and silicon dioxide composite powder (C.A.S. No. 7440-22-4).

2.2. Characterization of silver nanopowder

A transmission electron microscope (JEOL, JEM-1011; Japan) was used to determine the size, shape and the size distribution of the silver nanoparticles. Samples were prepared by placing a drop of working solution on a carbon-coated standard copper grid (300 mesh) operating at 80 kV. The particle size was further confirmed with X-ray diffraction (Panalytical Empyrean, Radiation:Cu Kα).

2.3. Incorporation of silver nanopowder in sanitary napkin made of cellulose pulp

The sanitary napkins are generally composed of a top perforated nonwoven sheet, the absorption part (fluff pulp or cellulose pulp + tissue paper + silver nano powder) and the back sheet (glue release paper).The sanitary napkin using cellulose pulp incorporated with silver nano powder (0.2–0.4 g per napkin, w/w) as the core was developed in the in-house facility (Fujian Peixin Machine Manufacture Industry Co. Ltd.; Model: PX-HY-700KY-BP) at Sanitary napkin unit, HLL Lifecare Ltd., Kanagala, Belgaum, India.

2.4. Assessing the antibacterial properties

Silver inhibits the growth of microorganisms and hence finds huge application in textile fabrics, hygiene and medical devices [28,29]. The cellulose pulp core of sanitary napkin was evaluated for antibacterial efficiency by a qualitative test such as disc diffusion method. But this test does not comply with real-world use conditions. For this purpose we preferred the quantitative antibacterial test method such as JIS L 1902 method, a method developed in Japan for evaluating the antibacterial properties of textile materials [29]. JIS L 1902 is a more suitable test as it requires only lower level of nutrients in the inoculum for loading levels of bacteria which represent the real-use situation where antibacterial performance counts. JIS L 1902 method was performed with Gram-positive (*Staphylococcus aureus*, ATCC 6538) and Gram-negative (*Klebsiella pneumonia*, ATCC4352) microorganisms. Briefly, the procedure is as follows: the test microorganism is grown in a liquid culture medium. The suspension of test microorganism is standardized by dilution in a nutrient broth. Control and test fabrics were inoculated with microorganisms, in triplicate, and then placed in a sealed container in an incubator for 18 h at 37 °C. Microbial concentrations were determined at "time zero" by elution, dilution and plating. After incubation, microbial concentrations are determined. Reduction of microorganisms relative to initial concentrations and the control fabric is calculated [30,31].

2.5. In-vitro cytotoxicity test

Cytotoxicity test has been carried out based on ISO 10993-5: 2009 (E); Biological evaluation of medical devices — Part 5: Tests for *in vitro* cytotoxicity [32]. An MTT assay was used to evaluate the cytotoxicity of the cellulose pulp core of sanitary napkin. Yellow water soluble MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid) is metabolically reduced in viable cells to a blue-violet insoluble formazan. The percentage of viable cells could be determined by measuring the optical density (OD) value of the formazan.

L929 cells are seeded into 96-well plates and maintained in a culture for 24 h to form a semi-confluent monolayer. They are then exposed to the test compound over a range of concentrations from 10 to 100%. After 24 h exposure, the formazan formation is determined for each concentration and compared to that determined in control cultures. Percentage viability was calculated for each concentration. 1 g of the test sample was sterilized at 121 °C for 15 min to which 10 mL complete MEM medium was added (0.1 g/mL). After the samples were soaked for 24 h at a 37 °C incubator, the solution was collected and stored at 4 °C. L929 fibroblast cells were seeded in a 96-well microplate at a density of 1×10^4 cells per 100 μL of MEM culture medium cells/well at 37 °C under humidified atmosphere containing 5% CO_2 and maintained for 24 h to form a semiconfluent layer. Varying concentrations of the sample extract were added to cells and then incubated for 24 h at 37 °C. 10–100% concentration of sample was used for the studies. After 48 h of sample addition, 25 μL of MTT dye solution (2 mg/mL in PBS) was added to each well and incubated for 4 h. The insoluble formazan crystals formed were determined for each treated concentration of sample and absorbance was read on a microplate reader at 570 nm. The spectrophotometer was calibrated to zero absorbance using culture medium without fibroblast cells. In negative control wells, fresh culture medium was taken and 0.001% SDS (Sodium Dodecyl Sulphate) solution was used as positive control. By measuring the optical density (OD) value of the formazan at 570 nm, the percentage of viable cells was determined. The cell viability was expressed as a percentage of the control as,

$$\text{Viability percentage} = (100 \times \text{OD at 570 nm for extract})/\text{OD at 570 nm for blank.}$$

A paired, t-test was used to test the significance of % viability of fibroblast cells. A p-value of 0.05 was defined to be statistically significant.

2.6. In vivo experiments

All laboratory animals were procured from Division of Laboratory Animal Sciences, BMT Wing, SCTIMST, India. Three male/female albino rabbits weighing not less than 2000 g and previously not used were used for intracutaneous test and six female albino rabbits (<2000 g) were used for vaginal irritation test. The animals were individually housed and maintained under standard conditions (12-h light/dark cycle, 22 ± 3 °C, 30–70% relative humidity) and provided with pelleted food and filtered fresh drinking water *ad libitum* throughout the

experiment. All animal experiments were performed according to OECD guidelines and were proved by the ethics committee of SCTIMST, India.

2.7. Intracutaneous test

Intracutaneous irritation studies were carried out in accordance with the USP34/NF 29: 2011, to evaluate the local responses to the extracts of the cellulose pulp core of sanitary napkin following intracutaneous injection into rabbit [33]. The test procedures, number of animals, test conditions *etc.* are all also fixed based on these guidelines. This study was designed to determine the irritation potential of the physiological saline (PS) and cotton seed oil (CSO) extracts of cellulose pulp core of sanitary napkin. Albino rabbits with body weight not less than 2000 g, were used for the study. Fur of the animal is clipped within 24 h to 4 h prior to the experiment on the dorsal side, close to the skin. In this test the physiological saline (PS) and cotton seed oil extracts (CSO) of the test material were aseptically injected into five sites (0.2 mL/site) on the upper left hand side and right hand side of three rabbits [34]. The PS and CSO alone were injected into five sites on the lower left hand side and lower right hand side of the same rabbits. The grading of erythema and edema of the test and control sites of all animals at 24, 48 and 72 h were recorded as per ISO 10993-10: 2010 (E) [33].

2.8. Vaginal irritation test

This study was designed to evaluate the vaginal tissue response in rabbits following the vaginal application of the physiological saline extract of the cellulose pulp core of sanitary napkin in albino rabbits. Six albino rabbits with body weight not less than 2000 g, were used for the study [33]. This test has been carried out based on ISO 10993-10: 2010 (E); Biological evaluation of medical devices — Part 10: Tests for irritation and skin sensitisation, Clause B.6 Special irritation test: vaginal irritation test. Test sample extract was applied into the vagina of each test rabbit. Here vaginal irritation test has been conducted on six albino female rabbits (three test and three control animals) for five days [34]. The albino rabbits were individually housed and were maintained in a controlled environment with the temperature of 22 ± 3 °C, humidity of 30–70% and light/dark cycle of 12 h with a minimum of 15 fresh air changes per hour. Briefly, the RVI test is performed as follows: 1 mL of test sample extract (extraction temperature: 37 °C; period: 72 h) was applied into the vagina of each test rabbit. A control using the same vehicle was used in parallel with the test. The exposure was repeated every 24 h for five consecutive days. Prior to each treatment the appearance of the vaginal opening and perineum for signs of erythema, edema or discharge as a reaction to the exposure to the test materials was noted and recorded. After the observation period the animals were humanely sacrificed, the vaginal tissue was dissected and examined for signs of irritation, injury to the epithelial layer of tissue and necrosis and was collected in 10% buffered formalin and subjected to histopathological evaluation. An overall individual irritation score is assigned based on a semi-quantitative scoring system which takes into account the endpoints mentioned as: numerical grading 0 = no edema; 1 = very slight edema; 2 = well defined edema; 3 = moderate edema; and 4 = severe edema [34]. The scores for each region are combined, and the total irritation score (irritation index) is then related to human irritation potential as follows: scores of 0 to 0.9 are non-irritant; 1–4.9 are minimal; scores of 5–8.9 are mild; scores of 9–11.9 indicate moderate and scores of 12 and above are indicative of severe irritation potential.

3. Results and discussion

3.1. Characterization of silver nanoparticles

Fig. 1 (A) shows the HRTEM image of the silver nanoparticles. It shows well dispersed particles with a size distribution of less than 100 nm with spherical morphology. The particles are polydisperse in nature with maximum particle size range between 10 and 90 nm. The other images (not shown here) captured also shows similar type of particles. Fig. 1 (B) shows the size distribution of the particles.

The Powder XRD pattern of the silver nanoparticles (data not shown) shows a broad characteristic peak of amorphous silicon dioxide at 22 degrees; mainly due to the amorphous silicon dioxide present in the silver particles. We are not able to see the other characteristic peak of silver and we assume that it is because of the huge amorphous silica peak and very small size of silver particles [35].

3.2. Assessment of cellulose pulp for antibacterial properties

Silver nanoparticles have been demonstrated as an effective biocide against broad-spectrum bacteria including both Gram-negative and Gram-positive bacteria [36–37]. The methods commonly opted to assess the antibacterial efficacy in hygiene products such as AATCC Test method 100 [36], ASTM method and JAFET method are not suited for

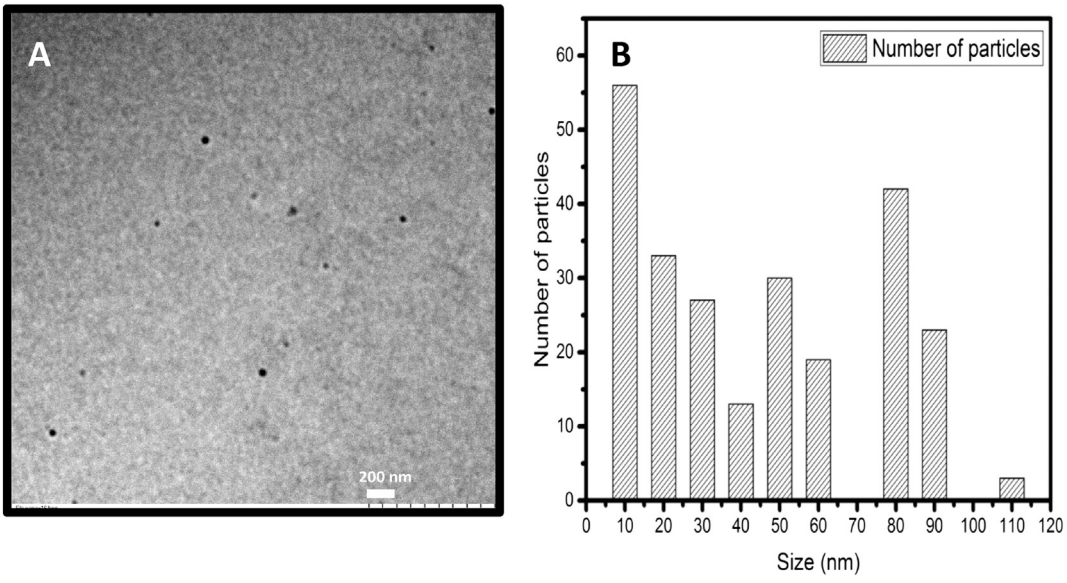


Fig. 1. (A) TEM image of silver nanopowder and (B) shows the histogram showing the size distribution of the particles.

Table 1
Evaluation of antibacterial activity against *Staphylococcus aureus* by JIS L 1902 method.

Sample	Control (UT) (cfu/mL)		Treated (T) (cfu/mL)		Control (UT)			Treated (T)				
	Initial conc used	Final conc observed	Initial conc used	Final conc observed	Log (UT) − 0 h	Log (UT) 24 h	Log (growth on UT) (F)	Log (UT) − 0 h	Log (UT) 24 h	Log (growth on T) (G)	Bacteriostatic activity	% efficacy
	0 h	24 h	0 h	24 h								
B2	1.3×10^5	7.2×10^7	1.3×10^5	7.1×10^4	5.11	7.86	2.74	5.11	4.85	−0.26	3.00	99.94
C3	1.3×10^5	7.2×10^7	1.3×10^5	1.9×10^5	5.11	7.86	2.74	5.11	5.28	0.17	2.58	99.73
D4	1.3×10^5	7.2×10^7	1.3×10^5	2.0×10^4	5.11	7.86	2.74	5.11	4.30	-0.81	3.56	99.97

Bacteriostatic activity = log (growth) on control − log(growth) on treated.
% efficacy = (cfu/mL at 0 h − cfu/mL at 24 h) / (cfu/mL at 0 h) * 100.

functionalized cellulose pulp [29]. JIS L 1902 is a method developed in Japan for evaluating antimicrobial textiles. JIS L 1902 method was performed with *S. aureus* and *K. pneumonia*. At the beginning of the experiment; concentration of *S. aureus* cells was 1.3×10^5 and it was incubated with control and after 24 h of incubation; concentration of *S. aureus* cells was increased to 7.2×10^7. Similarly the number of *K. pneumonia* cells was 1.7×10^5 when incubated with control at 0 h and after 24 h, the number of bacterial cells increased to 5.9×10^7. At the same time all the samples with different concentrations of silver nanopowder (0.2,0.3 & 0.4 g of silver nano powder per napkin, w/w and coded as B2, C3, D4) exhibited a reduction in bacterial count after 24 h. In the case of *S. aureus*; bacterial cells were 1.3×10^5 when incubated with the silver nanopowder treated cellulose pulp (B2). After 24 h, the number of bacterial cells was reduced to 7.1×10^4. Similar behavior was seen in the case of *K. pneumonia* also. All most all the samples showed more or less a similar behavior of bacteriostatic activity and efficiency [19,37–38]. From the data shown in Table. 1 the bacteriostatic value (A) and percentage efficiency are determined as (A) = F − G, where F − growth value on the untreated (control) fabric, F = $lgC_1 - lgC_0$; G − growth value on the treated (test) fabric, G = $lgC_1 - lgC_0$; where lgC_1 is the Log factor of the no. of bacteria from the control fabric after 24 h incubation and lgC_0 is the Log factor of the number of bacteria from the control fabric immediately after incubation (0 hr). Percentage efficacy is calculated as,

$$\%\text{efficacy} = (\text{cfu/mL at 0 h} - \text{cfu/mL at 24 h})/(\text{cfu/mL at 0 h}) * 100.$$

The results are summarized in Tables 1 & 2. The untreated cellulose pulp showed indefinite bacterial growth while there is considerable reduction in the growth of the bacteria on treated, giving a log reduction value >2.00 for both *S. aureus* and *K. pneumonia* under the test conditions. As per JIS L 1902, pass criteria is that bacteriostatic value has to be ≥2. Hence we may conclude that silver nanoparticles of all the three concentrations (0.2 g, 0.3 g and 0.4 g per napkin) are found to have antibacterial property. Hence we have chosen napkins with 0.2 g of silver for our further evaluations since silver may induce cytotoxicity. We may also conclude that silver nanoparticles are active against both Gram positive and Gram negative bacteria.

The mechanism of action of antibacterial property of silver nanoparticles can be explained based on its interaction with bacterial surface. It is reported that interaction between sulphur containing proteins of the bacterial cell membrane and silver nanoparticles leads to cell death in the case of *S. aureus* and *K. pneumonia*. Antibacterial activity of silver nanoparticles depends on the size and shape of the silver nanoparticles as well. It is seen that smaller nanoparticles because of its large surface area can interact with bacteria more effectively and kill them. This effect is more evident in the case of *S. aureus* and *K. pneumonia* [39]. In this study also size of the silver nanoparticles is small (<100 nm) and hence these particles are effective against *S. aureus* and *K. pneumonia* even in small amounts. In this case there is no chemical bonding exists between the silver particles and cellulose pulp. Silver nanoparticles are only entangled in the cellulose pulp matrix. In the moist environment, silver nanoparticles may release silver ions and hence the antibacterial activity found in this case can be attributed to both, the silver nanoparticles and the silver ions released from them [40,41].

3.3. In-vitro and in-vivo biological studies

Though silver nanoparticles are widely exploited as an antibacterial agent; not enough studies on its side effects in human are available. Hence the biocompatibility of silver nanopowder incorporated sanitary napkin needs to be assessed before its use. Biocompatibility can be analysed by performing cell culture and animal experiments for the test material (silver nanopowder incorporated sanitary napkin) by direct and indirect injections of test material extract liquid using standard practices recommended for materials, medical devices and implants for human application.

3.4. In-vitro cytotoxicity studies

MTT reduction assay is based on the mitochondrial metabolic activity of fibroblast cells [42–44]. MTT reduced to colored formazan crystals by the cells can be determined spectrophotometrically. The cell viability after 24 h of incubation with medium released from the sample was determined by measuring the optical density values. The viability of the sample was affected in a concentration dependent manner. Cells retained 90% viability at a concentration of 74.08 mg/mL (74.08%). At 100% (neat) concentration, the sample was found to be toxic to the cells. It is found that the lower the viability percentage values is, the higher the cytotoxic potential is. The % viability for test sample greater

Table 2
Evaluation of antibacterial activity against *Klebsiella pneumonia* by JIS L 1902 method.

Sample	Control(UT) (cfu/ml)		Treated(T) (cfu/ml)		Control (UT)			Treated (T)				
	Initial conc used	Final conc observed	Initial conc used	Final conc observed	Log (UT) 0 h	Log (UT) 24 h	Log (growth on UT) (F)	Log (UT) 0 h	Log (UT) 24 h	Log (growth on T) (G)	Bacteriostatic activity	% efficacy
	0 h	24 h	0 h	24 h								
B2	1.7×10^5	5.90×10^7	1.7×10^5	1.10×10^4	5.23	7.77	2.54	5.23	4.04	−1.19	3.73	99.98
C3	1.7×10^5	5.90×10^7	1.7×10^5	2.50×10^5	5.23	7.77	2.54	5.23	4.40	−0.83	3.37	99.96
D4	1.7×10^5	5.90×10^7	1.7×10^5	2.90×10^4	5.23	7.77	2.54	5.23	4.46	−0.77	3.31	99.95

Bacteriostatic activity = log(growth) on control − log(growth) on treated
% efficacy = (cfu/mL at 0 h − cfu/mL at 24 h) / (cfu/mL at 0 h) × 100.

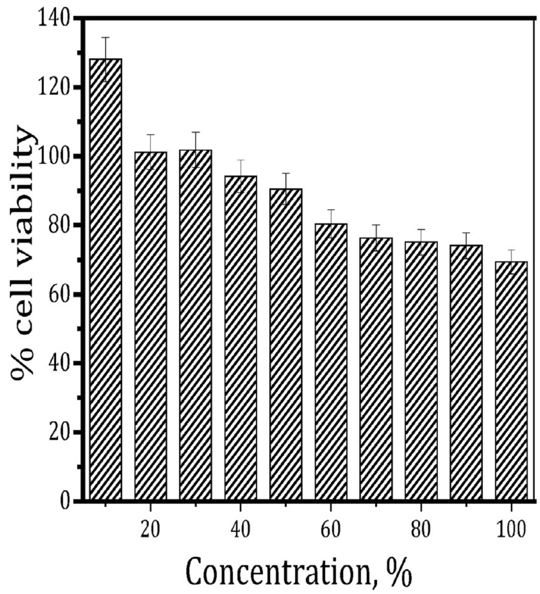


Fig. 2. Cytotoxicity profile of samples incubated with L929 fibroblast cells was expressed as percentage viability of fibroblast cells (n = 10). PC-positive control used was 0.001% SDS (sodium dodecyl sulphate) solution. Note: *values were statistically significant with $p < 0.05$; regression coefficient 0.95.

Table 3
Observation sheet of intracutaneous irritation test (test animals = 3, control = 3).

Animal		Observation at		
		24 h	48 h	72 h
♀	Erythema	Test site		
		CSO = 1,1,1,1,1	CSO = 0,0,0,0,0	CSO = 0,0,0,0,0
		NS = 0,0,0,0,0	NS = 0,0,0,0,0	NS = 0,0,0,0,0
		Control site		
		CSO = 0,0,0,0,0	CSO = 0,0,0,0,0	CSO = 0,0,0,0,0
		NS = 0,0,0,0,0	NS = 0,0,0,0,0	NS = 0,0,0,0,0
	Edema	Test site		
		CSO = 0,0,0,0,0	CSO = 0,0,0,0,0	CSO = 0,0,0,0,0
		NS = 0,0,0,0,0	NS = 0,0,0,0,0	NS = 0,0,0,0,0
		Control site		
		CSO = 0,0,0,0,0	CSO = 0,0,0,0,0	CSO = 0,0,0,0,0
		NS = 0,0,0,0,0	NS = 0,0,0,0,0	NS = 0,0,0,0,0
♂	Erythema	Test site		
		CSO = 1,1,1,1,1	CSO = 0,0,0,0,0	CSO = 0,0,0,0,0
		NS = 0,0,0,0,0	NS = 0,0,0,0,0	NS = 0,0,0,0,0
		Control site		
		CSO = 0,0,0,0,0	CSO = 0,0,0,0,0	CSO = 0,0,0,0,0
		NS = 0,0,0,0,0	NS = 0,0,0,0,0	NS = 0,0,0,0,0
	Edema	Test site		
		CSO = 0,0,0,0,0	CSO = 0,0,0,0,0	CSO = 0,0,0,0,0
		NS = 0,0,0,0,0	NS = 0,0,0,0,0	NS = 0,0,0,0,0
		Control site		
		CSO = 0,0,0,0,0	CSO = 0,0,0,0,0	CSO = 0,0,0,0,0
		NS = 0,0,0,0,0	NS = 0,0,0,0,0	NS = 0,0,0,0,0
♀	Erythema	Test site		
		CSO = 1,1,1,1,1	CSO = 0,0,0,0,0	CSO = 0,0,0,0,0
		NS = 0,0,0,0,0	NS = 0,0,0,0,0	NS = 0,0,0,0,0
		Control site		
		CSO = 0,0,0,0,0	CSO = 0,0,0,0,0	CSO = 0,0,0,0,0
		NS = 0,0,0,0,0	NS = 0,0,0,0,0	NS = 0,0,0,0,0
	Edema	Test site		
		CSO = 0,0,0,0,0	CSO = 0,0,0,0,0	CSO = 0,0,0,0,0
		NS = 0,0,0,0,0	NS = 0,0,0,0,0	NS = 0,0,0,0,0
		Control site		
		CSO = 0,0,0,0,0	CSO = 0,0,0,0,0	CSO = 0,0,0,0,0
		NS = 0,0,0,0,0	NS = 0,0,0,0,0	NS = 0,0,0,0,0

Score for erythema: 0 = no erythema; 1 = very slight erythema; 2 = well defined erythema; 3 = moderate erythema and 4 = severe erythema.

Table 4
Average irritation score.

Animal	Extract	Irritation score	
		NS	CSO
♀	Control	0	0
	Test	0	0.3
♂	Control	0	0
	Test	0	0.3
♀	Control	0	0
	Test	0	0.3
Total mean score		0	0.3

NS, physiological saline; CSO, cotton seed oil.

than or equal to 70% is found to non-toxic (Fig. 2). The % viability of fibroblast cells was statistically significant at a level of p-value < 0.05 when student paired t test was used.

The cytotoxicity study results indicated that the tested sample is non-toxic to the L929 fibroblast cells at the incubated period and concentrations, proving the material noncytotoxic. Zhang et al. studied the cytotoxic effect of six types of silver nanoparticles by MTT assay and it was found that the particles do not exhibit toxic effect at lower concentrations [45]. In this study we have used three different concentrations of silver (0.2 g, 0.3 g & 0.4 g) per sanitary napkin and since all three of them show good antibacterial property we have chosen the lowest amount 0.2 g per napkin for cytotoxicity evaluation and further studies.

3.5. Intracutaneous reactivity test

The skin irritation assay or intracutaneous reactivity test evaluate the irritation potential of a biomaterial when in contact with skin by intradermal injection of rabbits (ISO 10993-10:2010). This test in rabbit was done to investigate the effects of cellulose pulp on dermal exposure with silver nanopowder. The results of the present study indicated that silver nanoparticle incorporated sanitary napkin exhibit a nonirritant behavior and no inflammatory response leading to edema or erythema formation. The grading of erythema and edema of the test and control sites of all animals at 24, 48 and 72 h are tabulated in Table 3. The physiological saline (NS) and cotton seed oil extract of the test material were used in the test. Both the control and test material gave a total mean score of zero in saline while the test material in cotton seed oil extract graded 0.3 (Table 4).

Table 5
Score of discharge, erythema and edema in albino female rabbits after exposure to nano silver powder incorporated cellulose pulp (test animals = 3, control = 3).

Group		Observation at					
		Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Control animal	Discharge	0	0	0	0	0	0
	Erythema	0	0	0	0	0	0
	Edema	0	0	0	0	0	0
	Discharge	0	0	0	0	0	0
	Erythema	0	0	0	0	0	0
	Edema	0	0	0	0	0	0
	Discharge	0	0	0	0	0	0
	Erythema	0	0	0	0	0	0
	Edema	0	0	0	0	0	0
	Discharge	0	0	0	0	0	0
	Erythema	0	0	0	0	0	0
	Edema	0	0	0	0	0	0
Test animal	Discharge	0	0	0	0	0	0
	Erythema	0	0	0	0	0	0
	Edema	0	0	0	0	0	0
	Discharge	0	0	0	0	0	0
	Erythema	0	0	0	0	0	0
	Edema	0	0	0	0	0	0

Score of vaginal tissue reaction: 0 = no edema; 1 = very slight edema; 2 = well defined edema; 3 = moderate edema; and 4 = severe edema.

3.6. Vaginal irritation test

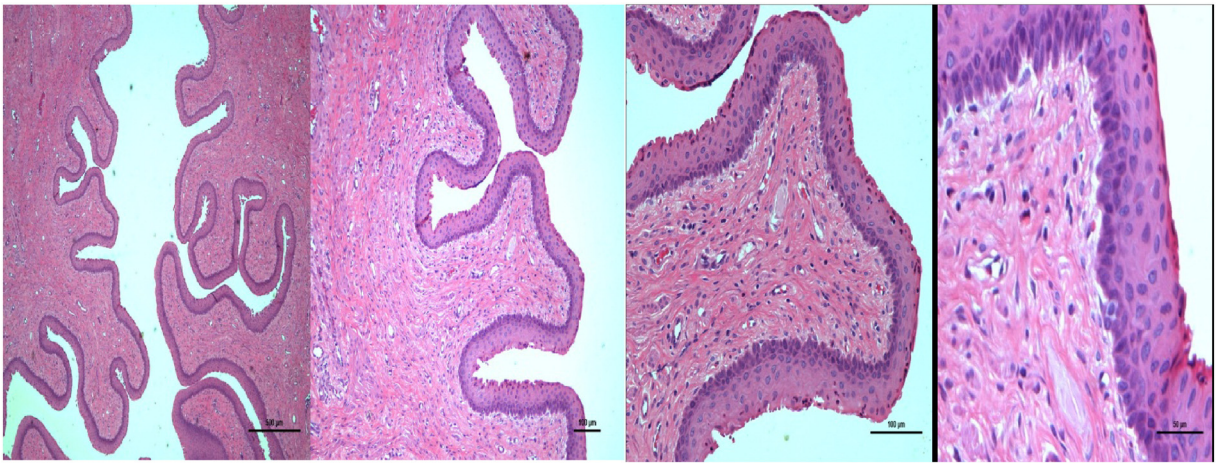
The *in vivo* vaginal irritation test is the preferred choice for vaginal toxicity studies. The assessment of results for vaginal irritation test was based on macroscopic and histological evaluation. Individual results of vaginal irritation performed on albino rabbits are presented in Table 5. Observations were made for 5 consecutive days. The average edema score was 0 and the average erythema score was 0 in both sample and control. The physiological saline extract of the material did not cause any irritation following vaginal application confirming the non-irritant nature of the extract of the test material.

We have selected four identifiable criteria for the 12-point scoring of vaginal irritation namely epithelium, leucocyte infiltration, vascular congestion and odema. The total histopathological score was calculated by adding the quantitative assessment of histopathological findings in the investigated areas of vagina (epithelial ulceration, leukocyte infiltration, vascular congestion or edema) in control and test animal. The irritation index for the test was determined as 0.09 and the test sample is considered as non-irritant. Fig. 3 shows representative vaginal sections of the rabbit exposed to the control and silver nanopowder treated cellulose pulp. There were no visible signs of vaginal inflammation (epithelial ulceration, leukocyte infiltration, vascular congestion or edema) in the control or silver nanocellulose pulp exposed vaginal tissue sections. The epithelial, mucosal and perivascular regions exposed to test sample is unchanged relative to the control section.

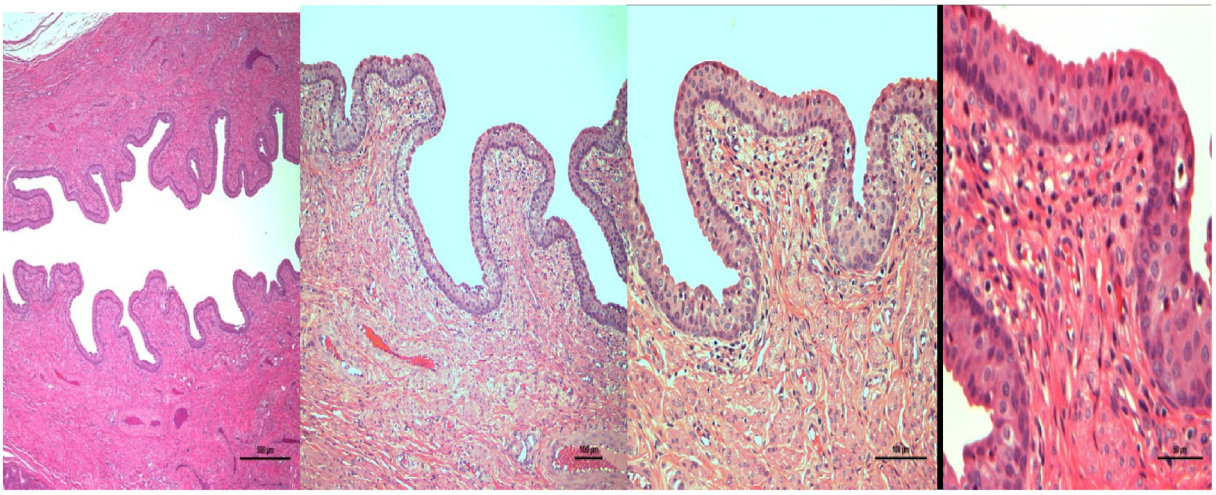
Silver nanoparticles are used in topical vaginal gels to inhibit the transmission of diseases and found to be effective after 48 h post water wash of the applied gel. Lara et al. have studied the effect of silver nanoparticles against HIV-1 IIIB virus showing that those nanoparticles are more effective than silver ions within the non-cytotoxic levels [46]. Further, due to the self-cleansing action of the vaginal tract, the residence times of the foreign particles *i.e.*, drugs or nanoparticles are very much reduced. Hence they have least propensity to cause genital irritation and systemic toxicity [47–50]. Our studies are in agreement with these studies. Hence the sanitary napkin with least amount of silver 0.2 g is found to be effective as an antibacterial agent with no toxicity.

4. Conclusions

Silver nanoparticle incorporated cellulose pulp has been made into sanitary napkins. Different concentrations of silver such as 0.2 g, 0.3 g and 0.4 g of silver have been added into individual cellulose pulp sanitary napkins. Antibacterial property of the different amounts of silver nanoparticle incorporated cellulose pulp has been evaluated and



(a)



(b)

Fig. 3. Hematoxylin and eosin stained sections of rabbit vaginal tissue of control (a) and treated with sample extract (b). No visible signs of vaginal inflammation (epithelial ulceration, leukocyte infiltration, vascular congestion or edema) in the control or silver nanocellulose pulp exposed vaginal tissue sections. Photographs are representative of control and test animal.

found to be antibacterial in nature. Hence cellulose pulp with least amount of silver nanoparticle has been chosen for further studies (0.2 g/napkin). The non-cytotoxic nature of the silver nanoparticle incorporated cellulose pulp was proved by MTT assay. The vaginal irritation test and intracutaneous tests in rabbit were examined to investigate the effects of vaginal exposure and sensitivity on skin by the treated cellulose pulp and were found to be nontoxic. Hence we have optimized the concentration of silver nanoparticle to be used in a cellulose pulp based sanitary napkin to make it antibacterial without toxicity.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.msec.2015.12.072.

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