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Exopolymers from *Tolypothrix tenuis* and three *Anabaena* sp. (Cyanobacteriaceae) as novel blood clotting agents for wound management

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ABSTRACT

Rapid initiation of clotting is critical to trauma patients. In the present study exopolymers (EPs) from four desert cyanobacteria including *Tolypothrix tenuis* and three species of *Anabaena* have been discovered as potential hemostatic biomaterials. The EPs showed reduction in activated partial thromboplastin time (APTT) and prothrombin time (PT) by 16–41% and 12–65%, respectively. Besides hastening blood clotting, the EPs could absorb 7.1–25.9 g H_2O g^{-1} EP and displayed 7.1–18.1% hydrophobicity. They were noncytotoxic and biodegradable. The EP from *Anabaena* sp. showed strong antibacterial activity against *E. coli*, *S. aureus* and *B. licheniformis*. These results suggest that cyanobacteria, the microscopic phototrophs growing rapidly over simple mineral medium could prove to be a novel source of affordable hemostatic dressings for the traumatic wounds in underdeveloped and developing countries. Compositional analysis of the EPs showed them to be consisting of mainly carbohydrate (17–50%), protein (4.4–7.2%), uronic acid (4.7–9.5%) and sulphate (0.6–6.6%). Their viscometric molecular weight ranged from 539 to 3679 kDa. They were further characterized using GC–MS and FTIR.

1. Introduction

In the first four decades of life, trauma is the leading cause of death and remains a significant cause in later life. Exsanguination accounts for 40–45% of total fatalities in the trauma setting (Sauaia et al., 1995). The incidence of hemorrhage related death amongst potentially survivable casualties in the wars in Iraq and Afghanistan was found to be between 83 and 87% (Kelly et al., 2008). Thus an early and effective hemorrhage control is important to save lives. Conventionally hemorrhage is controlled either by manual pressure, with a tourniquet or using hemostatic agents. A variety of hemostatic biomaterials are currently available that include gelatin foams, fibrin glues, variously derived collagen, cellulose, thrombin and albumin based agents; however none is without its drawbacks (Barnard and Miller, 2009) and thus discovery of novel material or modification of existing material is required. Glucosamine containing polysaccharides such as chitin and its deacetylated product chitosan are being developed as advanced wound management aids (Muzzarelli, 2009). The hemostatic mechanism of chitosan involves agglutination of red blood cells, possibly due to its intrinsic polycationic properties and non-specific binding to cell membranes. It enhances primary hemostasis mechanisms by promoting platelet adhesion, aggregation and activation (Chou et al., 2003; Okamoto et al., 2003). Further wound healing is accelerated by enhancing the function of inflammatory cells and subsequently promoting granulation and organization (Dai et al., 2011). Fully *N*-acetylated chitin is also found in some diatoms like *Thalassiosira fluviatilis* (Falk et al., 1966) that are known to reduce the coagulation time (Pusateri et al., 2003) and have been developed as hemostats (Seyednejad et al., 2008).

Marine algae are also a rich source of anionic sulfated polysaccharides such as sulfated fucans which affect blood clotting and fibrinolysis. They exhibit strong anticoagulant and other bioactive properties also (reviewed by Jiao et al., 2011). Alginate, composed of guluronic acid and mannuronic acid, a polysaccharide from marine brown algae has also been shown to be useful as a hemostatic agent however it is not effective for high pressure bleeding wounds and needs secondary dressing (Glick et al., 2013; Solanki & Solanki, 2012). Microporous polysaccharide hemospheres derived

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from purified plant starch have also been evaluated (Murat et al., 2006).

Polysaccharides are also abundantly produced by desert cyanobacteria of which some are sulphated. They have promising rheological properties for practical applications as emulsifiers, stabilizers and thickening agents. They can also be exploited as soil conditioners and biosorbents for heavy metal removal (Li et al., 2001). However their application has been poorly investigated in biomedical field except as antiviral agents (Senni et al., 2011). Since cyanobacteria grow considerably faster and can be easily cultivated with minimal nutritional requirements, the exopolymeric component of these forms may prove to be an inexpensive, readily available dressing material for hemorrhage control. In this study exopolymers produced by three species of *Anabaena* and *Tolypothrix tenuis* have been explored for their hemostatic activity along with other properties like cytotoxicity, water absorption capacity, antibacterial activity, bacteriostasis and biodegradability that are pertinent to a wound dressing agent.

2. Material and methods

2.1. Organisms and culture conditions

Four cyanobacterial strains *viz Anabaena* sp., *Anabaena anomala*, *Anabaena oryzae* and *T. tenuis*, isolated from the arid soils and water samples at or near Ajmer, Rajasthan, India were grown in BG-11 medium at 29 ± 1 °C in a 12:12 h::L:D cycle under 17–20 μmol photons s^{-1} m^{-2} illumination. Double distilled water was used for all preparations and all experiments were conducted in triplicate. The strains were deposited in the Arid Zone Algae Collection of Department of Microbiology, MDSU, Ajmer and assigned numbers AZAR 2224, AZAR 2214, AZAR 2221 and AZAR 2102, respectively.

2.2. Yield and solution properties of the EPs

EP was extracted from 30d old cultures by the modified method of Vincente-Garcia et al. (2004) using two volumes of chilled acetone for precipitation and leaving overnight at 4 °C. The precipitated EP was separated by centrifugation at 6000 g for 10 min, oven dried at 60 °C, weighed and yield expressed as mg EP produced per mL of the culture. Chitosan purchased from Hi-Media, India was included in the study for comparison. It had 16 ± 1.61% degree of acetylation, calculated from the values of absorbance in FTIR at 1320 and 1420 cm^{-1} using the equation $A_{1320}/A_{1420} = 0.382 + 0.031$ DA (Czechwska-Biskup et al., 2012).

Dilute solution viscometry of 0.1% EP solutions in double distilled water at 37 °C was determined using Ostwald viscometer (Barnes et al., 1989) while pH and Eh of these solutions was measured at 37 °C using Eutech CyberScan pH 2100 pH/Ion Meter.

2.3. Compositional analysis of the EPs

Carbohydrate and protein content of the EPs were measured after Roe (1955) and Lowry et al. (1951), respectively. Estimations were done by taking aliquots, from 1 mg mL^{-1} EP stock, prepared in double distilled water. Glucose (0–100 μg mL^{-1}) for carbohydrate and bovine serum albumin (0–200 μg mL^{-1}) for protein were used as standards.

Sulphate content of the EP was estimated by the modified $BaCl_2$-gelatin method (Dodgson, 1961). Gelatin (0.5%, w/v) was dissolved in warm water (60–70 °C), cooled and stored overnight at 4 °C. The following day, $BaCl_2$ (0.5%, w/v) was added and the resultant cloudy solution ($BaCl_2$-gelatin reagent) was allowed to stabilize for 2–3 h at room temperature before use. EP (5 mg) was dissolved in 1 mL deionized water in a glass ampoule. Then 1 mL of HCl (1N) was added to hydrolyse it at 105 °C for 17 h. Cooled, hydrolysed sample was transferred into a test tube containing 3.8 mL of 3% trichloroacetic acid (TCA) solution. To this mixture, 1 mL of $BaCl_2$-gelatin reagent was added, mixed and left at room temperature for 15–20 min. The turbidity formed was measured at 360 nm. 1 mL HCl treated similarly was used as a blank. Potassium sulphate (20–200 μg mL^{-1}) dissolved in 1N HCl was used as a standard for calibration.

Uronic acid (UA) content in the EP was measured following the modified *m*-hydroxydiphenyl method (Filisetti-Cozzi & Carpita, 1991). Briefly, 40 μL of 4 M sulfamic acid was added to 0.4 mL of EP solution (1%, w/v) in a test tube and vortexed. To this, 2.4 mL of sodium tetraborate (75 mM) solution prepared in concentrated H_2SO_4 was added and vortexed. The test-tube was then placed in boiling water bath (100 °C) for 20 min followed by immediate shifting to vessel containing crushed ice. After cooling to room temperature, 0.08 mL of 0.15% (w/v) *m*-hydroxydiphenyl prepared in 0.5% NaOH was added to it and mixed well. The pink color developed at room temperature was measured within 5 min at 525 nm against blank (distilled water treated similarly). Glucuronic acid (10–100 μg mL^{-1}) was used as a standard for the calibration of the method.

Monomer sugar composition of the EPs was determined by GC–MS using alditol acetate derivatives of sugars with inositol as an internal standard (Swardeker et al., 1965). The samples (200 μg) were hydrolyzed with 2N trifuoroacetic acid (200 μL) in a Teflon lined screw capped tube at 120 °C for 2 h. The acid was removed by rotavac, followed by three washings with water. Then $NaBH_4$ was used for reduction followed by mixture of acetic anhydride and pyridine (1:1). Finally alditol acetates of the monosaccharides were extracted with dichloromethane and analysed by GC (Agilent 6820 with FID and HP5 fused silica capillary column (30 m × 0.25 mm I.D.). Nitrogen was the carrier gas with flow rate of 1.5 mL min^{-1}. Initial oven temperature was set at 180 °C for 5 min then ramped to 220 °C with a rise of 5 °C/min. FID was set to 300 °C and the inlet to 250 °C.

2.4. FTIR characterization and viscometric molecular weight determination

The major structural groups in the EP were detected using Fourier Transform Infrared (FTIR) spectroscopy. Pellets were prepared by grinding and mixing pre-dried (60 °C, 24 h) EP with KBr in the ratio of 1:100 in a pestle and mortar. The mixture was then pressed at 10 tonnes pressure to convert to a thin film for the scan (Czechwska-Biskup et al., 2012). Transmittance over the range 4000 to 800 cm^{-1} was recorded, as an average of 100 scans with a resolution of 4 cm^{-1} on FTLA2000 laboratory FT-IR system (ABB Bomem Inc.).

Dilute solution viscometry method was used for the determination of relative molecular weight of the EPs (Kumar et al., 2013). Polymer solutions of 0.025–0.4% were prepared in double distilled water. Two solutions of dextran of known molecular weight (Hi-Media, India), D-40 ($M_w = 40{,}000$) and D-70 ($M_w = 70{,}000$) were also prepared. The flow time of the polymer solutions and solvent (double distilled water for exopolymers; 0.2N HCl for chitosan) at 25 °C was determined using Ostwald viscometer. Relative viscosity (η_{rel}) was calculated as t_s/t_o where t_s is the flow time of solution and t_o is the flow time of the solvent. Specific viscosity η_{sp} at different concentrations was calculated as $\eta_{sp} = (\eta_{rel} - 1)$ and the intrinsic viscosity was obtained by plotting η_{sp}/C against C (abscissa), where C is the concentration of the polymer solution (%). Extrapolating the curve to $C = 0$ gives the value of the intercept (i) according to the straight line equation $y = bx + i$ which is a measure of intrinsic viscosity.

Molecular weight of the EP was determined using the Mark–Houwink equation $[(\eta)_{Poly} = KM^a]$. The constants K and a

were determined for EP and chitosan assuming solution properties relative to dextran. Experimentally determined intrinsic viscosities of dextran standards, D-40 and D-70 of molecular weights 40 and 70 kDa respectively were used to solve for a and K in equations 1 and 2.

$$\log_{10}(\eta)_{\text{D-40}} = \log_{10}K + a\log_{10}M_{\text{D-40}} \quad (1)$$

$$\log_{10}(\eta)_{\text{D-70}} = \log_{10}K + a\log_{10}MD_{\text{D-70}} \quad (2)$$

2.5. Hemostatic potential

Thrombus formation by the EP was measured gravimetrically after Imai and Nose (1972). Chitosan (0.02 g) was suspended in 1 mL PBS and to this 300 μL of 0.1N HCl was added to dissolve the chitosan. The volume was then made upto 2 mL using PBS. The cyanobacterial EP was dissolved in PBS to give a final concentration of 1% and left for complete hydration for 12 h. Two mL of polymer suspension was taken in a Petriplate and 1 mL ACD blood (9 mL blood in 1 mL acid citrate dextrose (ACD)) was added to the suspension and also to an empty Petriplate that served as a positive control. Blood clotting was initiated by adding 0.02 mL of a 0.1 M calcium chloride solution and was stopped by the addition of 5 mL distilled water after 45 min. The resultant clots were fixed by adding 5 mL formaldehyde (36%, v/v). Excess water was removed using a blotting paper and the clot was weighed.

Blood coagulation time was determined by the modified method of Hu et al. (2012) using 1% solution of the exopolymers (w/v in PBS) for determination of activated partial thromboplastin time (APTT) and prothrombin time (PT).

2.6. Hydrophobicity

Hydrophobicity of EP was determined using modified method of Tielen et al. (2010). Five mL of each exopolymeric suspension (1%, w/v in PBS) was dispensed into a clean test tube. After obtaining OD_{550} (A_0), 300 μL of hexadecane was added. The tubes were vigorously agitated for 1 min and then allowed to stand for at least 15 min to separate the phases. The lower aqueous phase was gently drawn out into a cuvette using a sterile Pasteur pipette and OD_{550} (A_1) was measured again. The degree of hydrophobicity was calculated as

$$DH = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100 \quad (3)$$

where DH is the degree of hydrophobicity, A_0 initial absorbance and A_1 absorbance after phase separation.

2.7. Hemolysis, cytotoxicity and antibacterial activity

Hemolytic activity was tested by the method described in American Society for Testing and Materials (ASTM F 756-00, 2000). Haemoglobin released on hemolysis was measured spectrophotometrically at 540 nm. Percentage hemolysis was calculated as

$$\%\text{Hemolysis} = \left[\frac{(\text{OD}_s - \text{OD}_{nc})}{(\text{OD}_{pc} - \text{OD}_{nc})}\right] \times 100 \quad (4)$$

where OD_s, OD_{nc} and OD_{pc} are optical densities of sample, negative control and positive control, respectively.

Cytotoxicity test was carried out on Vero cell lines (Zeni et al., 2008), for which 1% (w/v) of EP suspension was prepared in 0.9% NaCl solution. Cells were grown in RPMI 1640 containing 10% foetal bovine serum and incubated at 37 °C in a humidified incubator with 5% CO_2 in the atmosphere. After confluent monolayer propagation trypsinization was done with 0.25% trypsin solution to detach cells from the culture flask. The cells were transferred to a screw-capped plastic tube, centrifuged, washed twice with PBS and then reseeded in fresh culture medium. The concentration was adjusted to ~60,000 cells mL^{-1} with the help of hemocytometer. EP suspension (50 μL) was added in quadruplicate in a 96 well tissue culture microtiter plate and each was supplemented with 50 μL of the cell suspension (~3000 cells).

The same amount of test substance was added to the blank wells (without any cells). Negative control wells were prepared by adding culture medium without test substance. After incubation for 24 h, 20 μL of 0.001% resazurin was added to the test wells and further incubated for 4 h. The reduction of resazurin to resorufin was an indicator of metabolic activity and was analyzed with a fluorometer at 590 nm against blank.

Antibacterial activity was determined by the disc diffusion method (Yamac & Bilgili, 2006). Overnight grown bacterial cultures of *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Bacillus licheniformis* (ATCC 12759) and *Staphylococcus aureus* (ATCC 25923) were adjusted to 10^8 CFU mL^{-1} with 0.5 Mc Farland standard. The bacterial cell suspension (100 μL) was spread on the surface of Mueller-Hinton agar plates. Discs containing 1 mg mL^{-1} EP solution were placed over the inoculated surface. Inhibition zones were measured after 24 h incubation at 37 °C. Commercially available chloramphenicol discs were used as positive control.

Minimum inhibitory concentration (MIC) values were determined according to Zgoda and Porter (2001) with some modifications. EP dilutions in the range of 100–1000 μg mL^{-1} were prepared and 100 μL of each polymeric dilution was added to the wells in a microtiter plate in triplicate followed by addition of 100 μL of bacterial cell suspension (10^8 cells). All the bacterial strains referred above were tested at all dilutions. Wells having sterile distilled water and the growth medium served as positive control whereas uninoculated wells with EP suspension served as the negative control. The plates were covered and incubated for 24 h at 37 °C. After incubation 50 μL of 2,3,5-triphenyl tetrazolium chloride (TTC) from a stock of 100 μg mL^{-1} was added to each well. The plates were further incubated for 24 h. Reduction of the colorless TTC to red color formazan was an indicator of cell viability. MIC was read as the concentration of biopolymer where no color could develop.

2.8. Water absorption capacity

Water absorption capacity was determined by the tea bag method (Kurane & Nohta, 1994). Percentage water absorption was calculated using following equation:

$$\%\,\text{Water absorption} = \left[\frac{((W_1 - W_2) - W_p)}{W_p}\right] \times 100 \quad (5)$$

where W_1, W_2 and W_p are the weight (g) of the swollen EP including the bag, weight of the wet bag without EP and initial weight of the dried EP, respectively.

2.9. Biodegradability

Biodegradability of the EP was assessed by determining the reducing sugar content after enzymatic degradation (Miller, 1959). Two mL of aqueous EP solution (0.5%, w/v) was mixed with 1 mL of enzyme solution (cellulase: 4U and β-glucosidase: 144 U mL^{-1}) in separate test tubes. Incubation was done at 40 °C for 1 h. Controls were prepared in the same way and placed on ice. After incubation, the enzyme activity was stopped by adding 3 mL dinitrosalisylic acid (DNS) reagent. Glucose was used as standard. For color development all the tubes were placed in boiling water bath for 15 min and then cooled down to room temperature. OD was recorded at 575 nm.

Table 1
Yield and solution properties of exopolymers.

	Anabaena sp.	*A. anomala*	*A. oryzae*	*T. tenuis*
Yield ($mg\,mL^{-1}$)	3.15 ± 0.41	0.82 ± 0.57	1.60 ± 0.26	0.34 ± 0.29
Eh (mV)	−191.5 ± 4.8	−151.1 ± 1.6	−64.1 ± 1.8	−115.3 ± 1.2
pH	10.02 ± 0.05	8.80 ± 0.08	7.98 ± 0.03	8.85 ± 0.11

3. Results and discussion

3.1. Yield and solution properties of the EPs

The four cyanobacterial strains produced 0.34–3.15 mg EP mL^{-1}, respectively in 30d. The EP from *Anabaena* sp. showed maximum viscosity of 2.11 ± 0.05 cP followed by *A. anomala* (1.76 ± 0.02 cP), *T. tenuis* (1.68 ± 0.01 cP) and *A. oryzae* (1.57 ± 0.02 cP). EP from *Anabaena* sp. had an Eh of −191.5 ± 4 followed by *A. anomala*, *T. tenuis* and *A. oryzae* (Table 1) indicating a strong electron donating ability of these EPs. All the aqueous solutions of the EPs were alkaline in nature with pH ranging from 8 to 10.

3.2. Compositional analysis of the EPs

The EPs from *Anabaena* sp., *A. anomala, A. oryzae* and *T. tenuis* were primarily polysaccharidic, composed of 509 ± 3.4, 170 ± 2.3, 251 ± 3.5, 356 ± 4.5 $\mu g\,mg^{-1}$ anthrone reactive carbohydrate and 72 ± 2.1, 47 ± 2.2, 56 ± 2.2, 44 ± 1.5 $\mu g\,mg^{-1}$ protein, respectively. Cyanobacterial exopolymers rich in sulphate and uronic acids are primarily anionic in nature (Sutherland, 1994). The EPs from *Anabaena* sp., *A. anomala, A. oryzae* and *T. tenuis* contained 13 ± 0.6, 13 ± 0.7, 66.5 ± 1.2 and 6.9 ± 0.8 $\mu g\,mg^{-1}$ sulphate and 58 ± 3.0, 95 ± 4.6, 47 ± 4.0 and 67 ± 4.2 $\mu g\,mg^{-1}$ UA, respectively thus could be considered as anioinic EPs.

Monosaccharide composition of cyanobacterial EP is depicted in Table 2. EPs of all the three *Anabaena* strains exhibited arabinose sugar monomer in highest concentration (28–60%) followed by glucose and galactose in *Anabaena* sp. and *A. oryzae*. In *A. anomala* galactose contributed more than glucose. In contrast EP from *T. tenuis* was dominated by glucose (41%).

3.3. FTIR characterisation and viscometric molecular weight determination

FTIR spectra of chitosan and the cyanobacterial EPs showed a single peak in the 2956–2850 cm^{-1} region due to CH stretching of $-CH_3$ and $>CH_2$ groups (Fig. 1a and b). The broad band formed between 3600 and 3200 cm^{-1} was due to OH stretching while 1570–1540 cm^{-1} (amide II) band formed by N–H bending vibrations of proteins of the EPs. Amide I band at ~1650 cm^{-1} was overlapped by the antisymmetric and symmetric vibrations of C=O bonds from carbohydrate groups forming a sharp peak at ~1600 cm^{-1} (Rochas et al., 1986) except for *A. anomala* that showed a discrete amide I band at 1683 cm^{-1}. The absence of peak at 1655 cm^{-1} in chitosan is indicative of the product being deacetylated and the peak at 1604 cm^{-1} indicates the prevalence of NH_2 group (Czechwska-Biskup et al., 2012). The peak at 1463 cm^{-1} may be attributed to scissoring (bending) vibration of the CH_2 group next to the carbonyl group while the peak at 1430 cm^{-1} depicted C—O—H in plane bending in carbohydrates (Kweon et al., 2001). Presence of S=O (sulfo) groups formed bands around 1255 cm^{-1} region due to asymmetric vibrations while that between 800 and 820 cm^{-1} in the polysaccharidic region was due to C—O—S of sulphate esters of hexoses (Nie et al., 2006). *A. oryzae* showing maximum amount of sulphate also showed a broad band at 1253 cm^{-1}. Peaks in the region of 1130–1160 cm^{-1} were dominated by glycosidic linkages (Nikonenko et al., 2002). All cyanobacterial EPs showed band between 850 and 840 cm^{-1} that represent presence of D-galactose-4-sulphate in their structure (Rochas et al., 1986). The region between 1200 and 800 cm^{-1} was dominated by C—O—C and C—O ring vibrations of polysaccharides (Nikonenko et al., 2002). Functional group vibrational assignments in the FTIR spectra of the EPs are listed in Table 3.

Relative molecular weight of EPs as measured by viscometry was 3679 ± 83.03 kDa for *Anabaena* sp. EP followed by *T. tenuis* (1953 ± 46.81 kDa), *A. anomala* (864 ± 13.65 kDa) and *A. oryzae* (539 ± 39.17 kDa), while chitosan had a molecular weight of 68 ± 1.43 kDa. The values of a and k were 0.755 and 2.9×10^{-4}

Table 2
Monosaccharide composition (%, w/w) of cyanobacterial exopolymers analysed by GC-MS.

Monosaccharides	*Anabaena* sp. (%)	*A. anomala* (%)	*A. oryzae* (%)	*T. tenuis* (%)
Ribose	9 ± 3.2	–	–	4 ± 2.2
Arabinose	28 ± 2.5	51 ± 1.6	60 ± 4.9	18 ± 3.1
Xylose	11 ± 4.5	5 ± 2.5	-	8 ± 3.0
Glucose	22 ± 3.8	15 ± 3.9	22 ± 4.2	41 ± 4.6
Mannose	9 ± 2.9	4 ± 2.2	2 ± 1.4	12 ± 3.7
Galactose	21 ± 3.3	25 ± 5.6	16 ± 2.8	17 ± 4.5

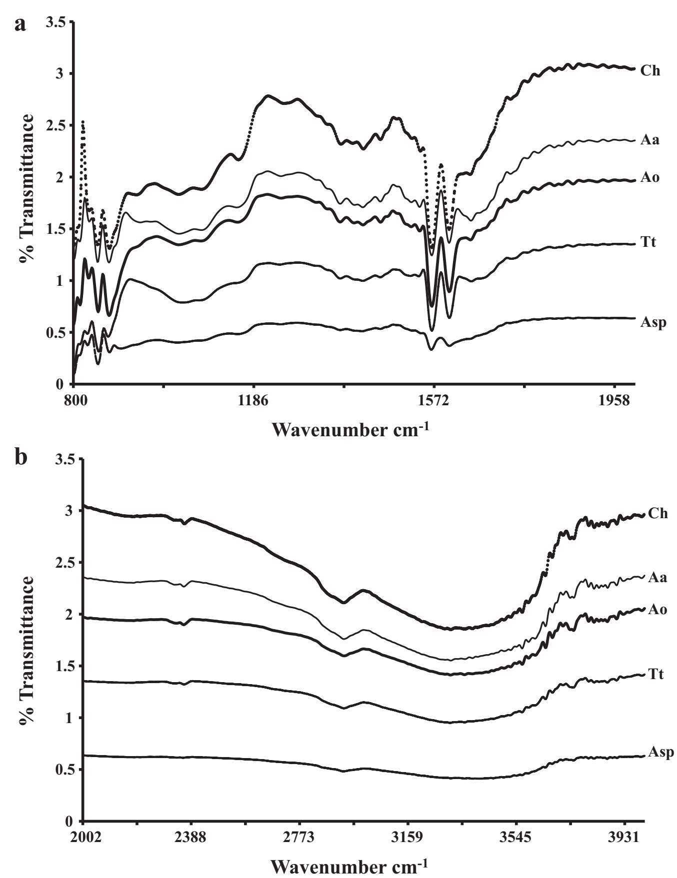

Fig. 1. FTIR spectra of cyanobacterial exopolymers. Asp (*Anabaena* sp.), Aa (*Anabaena anomala*), Ao (*Anabaena oryzae*), Tt (*Tolypothrix tenuis*), ch (chitosan).

Table 3
Proposed functional group vibrational assignments for FTIR spectra of cyanobacterial exopolymers and chitosan.

Functional group vibrations	*Anabaena* sp.	*A. oryzae*	*A. anomala*	*T. tenuis*	Chitosan
Wavenumber (cm^{-1})					
O–H stretching and hydrogen bonding	3388	3334	3232	3313	3488
Aliphatic CH stretching (symmetric and asymmetric of $-CH_3$ and $>CH_2$ of carbohydrates)	2950	2948	2937	2950	2890
C=O stretching of carboxylic acid	–	–	1745	1751	–
C=O stretch of amide group, uronic acid	1652	1650	1674	1683	–
Asymmetric vibration of COO^- group	1608	1606	1595	1604	1604
NH bending of NH (Amide II) and/or C=O stretching arising from proteins	1568	1566	1544	1579	1570
CH_2 bending	1463	1460	1461	1465	1465
Symmetric vibration of COO^- group	1419	1421,1404	1429	1434, 1423, 1400	1430
C=O symmetric stretch of carboxylate and/or Amide III	1375	1375	1379	1379	1390
N-acetyl glucosamine	–	–	–	–	1340
Asymmetric vibrations of S=O	1255	1243	1237	1234	1255
C-O-C stretching, glycosidic linakages	1161	1157	1153	1163	1153
C–O, C–C stretch vibrations and C–OH bending of polysaccharides	1085	1085	1085	1075	–
Free primary amino group (NH_2) at C_2 position of glucosamine	–	–	–	–	1097
C–O–C group vibrations in the cyclic structure of carbohydrates	1031	–	1027	1043	1040
Polysaccharidic region	916, 879, 852, 802,779	906, 881, 852, 813, 800	908, 867, 848, 808	865, 827, 802,777,715	1161, 1000, 908, 885, 875

respectively with water as a solvent and 0.814 and 2×10^{-4} respectively, with 0.2 N HCl at 25 °C.

3.4. Hemostatic potential of EPs

Hemostasis is a desirable property in a topical wound healing agent intended to be used for bleeding wounds. Hemostasis also enhances coagulation and cicatrisation process of the wounds (Dos Santos et al., 2006). The blood clot formed by the cyanobacterial EPs was heavier vis a vis glass (positive control) (Fig. 2) and thus are thrombogenic. Maximum thrombogenicity was shown by the EP from *Anabaena* sp. followed by that of *A. anomala, A. oryzae*, chitosan and *T. tenuis*. The blood coagulation system includes extrinsic, intrinsic and the common pathway. APTT and PT are used to determine the effect of a material on the intrinsic and the extrinsic pathways, respectively. APTT and PT were significantly shorter when blood plasma was in contact with the EPs than with the control (Fig. 3). APTT was reduced by 41, 29, 20, 28 and 16% while PT by 65, 52, 40, 53 and 12% due to EPs of *Anabaena* sp., *A. anomala*, *A. oryzae*, *T. tenuis* and chitosan, respectively. The EP from *Anabaena* sp. displayed maximum Thrombogenicity and shortest coagulation time and thus may prove to be an excellent hemostatic agent.

3.5. Hydrophobicity

Thrombogenicity is related to the surface characters of a material. Different factors such as morphology, hydrophilicity and surface chemical composition have been proposed to relate to blood compatibility (Chen et al., 2000). All EPs showed hydrophilic character (Table 5). Hong et al. (2011) showed that hydrophilicity may be desirable for the formation of fibrin network and for the stabilization of the blood clot. It has been reported earlier that biomaterials induce blood coagulation *via* contact activation of the inactive zymogen factor XII to factor XIIa when in contact with hydrophilic surfaces having anionic groups (Zhuo et al., 2005). Besides hydrophilicity EPs with a superabsorbent character are suggested to increase the coagulation potential by increasing blood

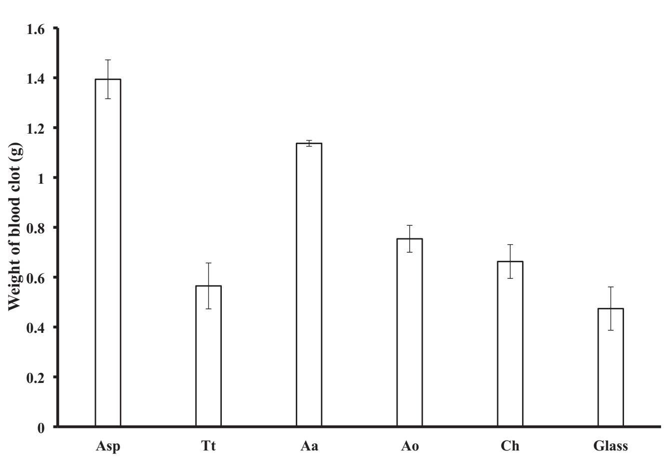

Fig. 2. Thrombogenic index of cyanobacterial EPs and chitosan *vis a vis* glass used as positive control. Asp (*Anabaena* sp.), Aa (*Anabaena anomala*), Ao (*Anabaena oryzae*), Tt (*Tolypothrix tenuis*), ch (chitosan). Bars depict mean values ± SD.

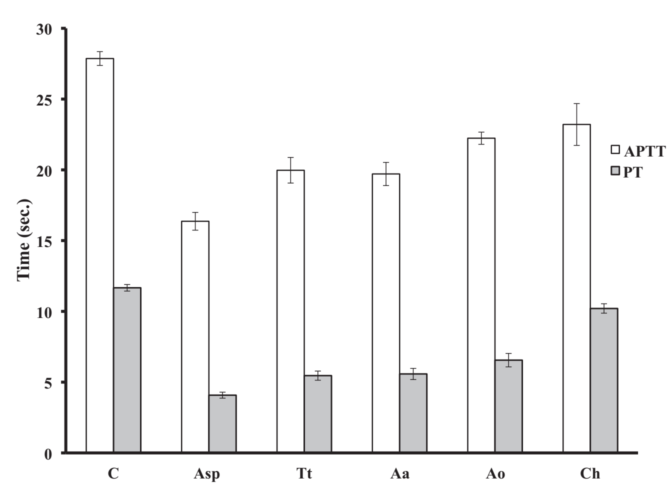

Fig. 3. APTT and PT for cyanobacterial exopolymers and chitosan, C: control, Asp: *Anabaena* sp., Aa: *Anabaena anomala*, Ao: *Anabaena oryzae*, Tt: *Tolypothrix tenuis*, ch: chitosan. Bars depict mean values ± SD.

Table 4
Hemolysis (%) and non-cytotoxic (% cell viability) activity of cyanobacterial exopolymers.

	% Hemolysis	% Cell viability
Anabaena sp.	4.02 ± 0.6	86.6 ± 2.5
A. anomala	2.20 ± 0.1	83.2 ± 1.7
A. oryzae	3.02 ± 1.2	84.8 ± 1.5
T. tenuis	1.53 ± 0.7	83.4 ± 4.2
Chitosan	4.21 ± 0.4	85.3 ± 2.7
NC*	–	97.6 ± 1.8

NC*, negative control for cell lines without exopolymer

viscosity and allowing better interactions amongst the blood cells (Gersh et al., 2009).

3.6. Hemolytic, cytotoxic and antibacterial activity of exopolymers

Ideally biomaterials to be used in contact with blood should be non hemolytic however as reported in literature (ISO-10993-4), it is not possible to define a universal level of acceptable or unacceptable amounts of hemolysis. Although by definition a blood-compatible material should be non-hemolytic, the truth is that in practice several medical devices cause hemolysis. This means when such hemolytic effect takes place, it is important to make sure that clinical benefits overcome the risks and that the values of hemolysis are within acceptable limits. As per ASTM F 756-00 (2000) classification the EP of *Anabaena* sp., *A. anomala, A. oryzae* could be characterized as slightly hemolytic showing 2.2 to 4.02% hemolysis, while that of *T. tenuis* was non hemolytic as compared to chitosan. All were non-cytotoxic showing 83–85% cell viability after 24 h exposure (Table 4). Some *Anabaena* strains are known to produce neurotoxins. Although neurotoxicity was not tested, the noncytotoxicity of the EPs eliminates possible presence of toxins, which are known to be cytotoxic against Vero cell lines (Lakshmana Rao et al., 2002). Besides, in most cases these toxins are either intracellular or cell bound (Pietsch et al., 2002).

Majority of open wounds become contaminated by bacteria which might delay healing by competing with host cells for nutrients and oxygen and cause damage *via* cytotoxic enzymes and waste products that interfere with the host immune response (Marks et al., 1983). Protection of wounds from pathogenic bacteria is a challenge met through application of antibiotics. Antibiosis inherent in a wound healing agent is clinically relevant and an attractive alternative to strong antiseptics. Cyanobacterial polysaccharides that contain sulphate and uronic acids are known to exhibit antibacterial and antiviral activities (Pereira et al., 2011).

Antibacterial activity of cyanobaterial EPs was evaluated against *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and *Bacillus licheniformis* (ATCC 14580) that are important infectious agents in wounds. All EPs showed good activity against all the four tested bacterial strains (Fig. 4). Best activity was observed for the EP from *Anabaena* sp. with an MIC of 200 μg mL^{-1} against *E. coli, S. aureus* and *B. licheniformis* and 700 μg mL^{-1} for *P. aeruginosa*. Chitosan also showed an inherent antibacterial property with minimum MIC for *B. licheniformis* followed by E. coli. It has been proposed that interaction between positively charged chitosan molecules and negatively charged microbial cell membranes leads to the disruption of the microbial membrane (Dai et al., 2011).

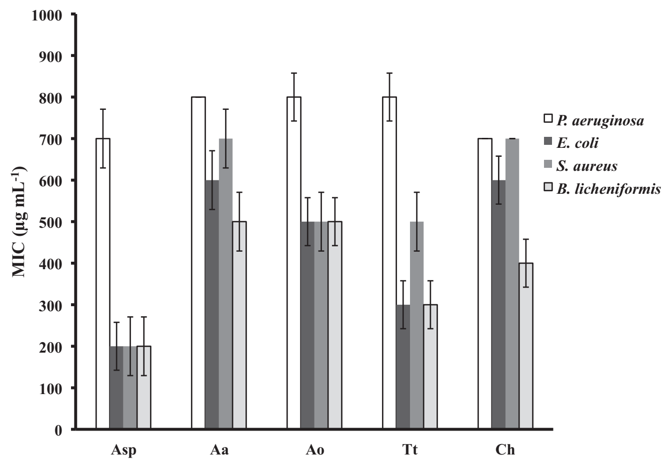

Fig. 4. Antibacterial activity of the exopolymers expressed as minimal inhibitory concentration (MIC μg mL^{-1}) against wound infection causing bacteria.

3.7. Water absorption capacity of EPs

Cyanobacterial EPs showed water absorption in the range of 716 to 2588% (Table 5). This property had an inverse relation with the hydrophobicity of the material. Water or fluid absorption capacity is of utmost importance for bleeding wounds. The EP from *Anabaena* sp. could absorb 25.9 g H_2O g^{-1} EP and can be labelled a superabsorbent polymer after Zohuriaan-Mehr & Kabiri (2008). Such polymers expand after absorbing fluid to occlude the wound and create back pressure to stop bleeding (Peng, 2010). Water absorption helps in concentrating erythrocytes, platelets and clotting factors in the wound and thereby promotes coagulation (Gersh et al., 2009). Besides being of importance in combat and traumatic wounds, this property is also helpful in wounds releasing high amount of exudates (Gorham, 1991).

3.8. Biodegradability

Biodegradability of a dressing material is an important characteristic for waste disposal. The study shows that all the EPs along with chitosan were biodegradable, as measured in terms of reducing sugars released by enzymatic degradation with β-glucosidase and cellulase (Fig. 5), however the extent of degradation varied with the two enzymes. EPs from *A. oryzae* and *T. tenuis* were easily degraded by cellulase which is a group of enzymes that hydrolyse cellulose while β-glucosidase hydrolyzes the terminal non-reducing residues in β-D glucosides. Both cellulase and β-glucosidase act upon β-1→4 glycosidic bonds to release glucose (Bisaria & Mishra, 1989). β-glucosidase is reported to be inactive on polysaccharides, however our studies showed that amongst the four, EP of *Anabaena* sp. was most susceptible to degradation by β-glucosidase followed by chitosan and *A. anomala.* In another study by Zhang & Neau (2001) degradation of chitosan was observed by β-glucosidase prepared from almond emulsion. However the authors suspected the presence of a chitinase as a contaminate in the commercial preparation.

Table 5
Hydrophobicity (%) and water absorption capacity (%) of the cyanobacterial exopolymers.

	% Hydrophobicity	% Water absorption
Anabaena sp.	7.2 ± 0.8	2590 ± 260
A. anomala	18.1 ± 1.5	716 ± 174
A. oryzae	16.9 ± 2.3	1230 ± 160
T. tenuis	17.7 ± 1.9	935 ± 420
Chitosan	27.8 ± 2.9	278 ± 12

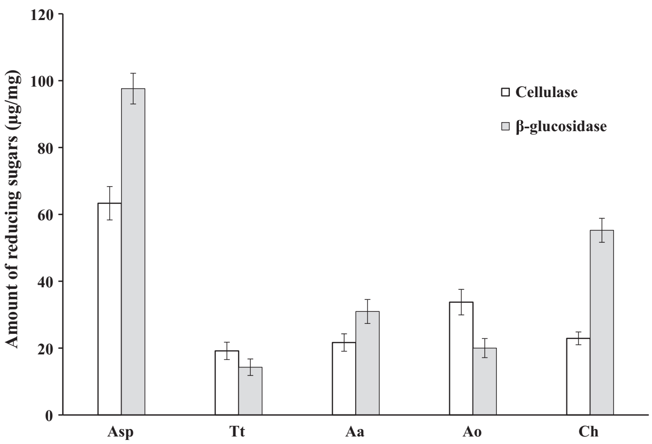

Fig. 5. Reducing sugar released from the cyanobacterial exopolymers and chitosan after enzymatic degradation with cellulase and β- glucosidase. Bars depict mean values ± SD. Asp: *Anabaena* sp., Aa: *Anabaena anomala*, Ao: *Anabaena oryzae*, Tt: *Tolypothrix tenuis*, ch: chitosan.

4. Conclusion

The hydrophilic EP extracted from the four arid zone cyanobacteria i.e. *Anabaena* sp., exhibited strong thrombogenicity and shorter blood coagulation time. This was found to be noncytotoxic and slightly hemolytic. EP exhibited antibacterial activity against common wound infecting bacteria. Besides these, its super absorbing character makes it an effective agent for hemorrhagic wounds. The study thus opens up a hidden treasure of biomaterials for wound management that are yet to be explored.

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