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Hemostatic, antibacterial biopolymers from *Acacia arabica* (Lam.) Willd. and *Moringa oleifera* (Lam.) as potential wound dressing materials

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Acacia arabica and *Moringa oleifera* are credited with a number of medicinal properties. Traditionally gum of *Acacia* plant is used in the treatment of skin disorders to soothe skin rashes, soreness, inflammation and burns while *Moringa* seed extracts are known to have antibacterial activity. In the present study the potential of the polymeric component of aqueous extracts of gum acacia (GA) and the seeds of *M. oleifera* (MSP) in wound management was evaluated. The results revealed that both biopolymers were hemostatic and hasten blood coagulation. They showed shortening of activated partial thromboplastin time and prothrombin time and were non-cytotoxic in nature. Both showed antibacterial activity against organisms known to be involved in wound infections with MIC ranging from 500-600 µg mL^{-1} for GA and 300-700 µg mL^{-1} for MSP. They were biodegradable and exhibited water absorption capacity in the range of 415 to 935%. The hemostatic character coupled to these properties envisions their potential in preparation of dressings for bleeding and profusely exuding wounds. The biopolymers have been further analysed for their composition by Gas chromatography.

Keywords: Antimicrobial, Biodegradable, Cytotoxicity, Gum acacia, Hemostatic, *Moringa* seed polymer, Polysaccharides, Wound healing

Hemostatic dressings that can check bleeding from serious wounds are a primary need in both civilian as well as military trauma centres, where nearly 85% combat patients succumb to injuries due to uncontrolled hemorrhage[1-4]. Though human fibrinogen, thrombin and some synthetic hemostatic materials have been developed, their practical applicability is limited due to their cost and scarce availability[5]. A need thus exists to develop inexpensive hemostatic agents based on readily available materials. In the present study the biopolymers obtained from *Moringa oleifera* and *Acacia* sp. plants have been explored as wound healing agent. These plants are abundantly available in India, Pakistan, Bangladesh, Afghanistan, Australia, America, Africa and Iran[6-9].

Both plants are pharmacologically important and parts of these plants are used in rural areas for curing a number of ailments[10]. *Acacia arabica* (Lam.) Willd. a member of Fabaceae produces gum, which is used traditionally as emollients, astringent and demulcent on irritated mucous membranes[11,12]. *Moringa oleifera* Lam. (Moringaceae) on the other hand is nature's medicine cabinet. Coagulant properties of its seed extract have been used in water treatment to reduce turbidity[13]. These extracts have also been found to be effective as hepatic carcinogen metabolizing enzymes, antioxidants and as a cure of skin papillomagenesis in mice[14]. A seed based ointment had been reported to have an effect similar to neomycin against pyoderma in mice caused by *Staphylococcus aureus*[15]. However, there is no study on the systematic use of the seed associated biopolymer of *M. oleifera* as hemostatic dressings. Wound dressings need to be non hemolytic, non-cytotoxic and absorbent especially in case of bleeding wounds[16,17]. An antimicrobial property is an added advantage. Biodegradability of any modern wound dressing is essential. The present communication discusses properties of biopolymers from seed extract of *Moringa oleifera* and gum acacia for the preparation of prospective hemostatic wound dressings for effective management of bleeding wounds.

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Materials and Methods

Chemicals—Chitosan purchased from Hi-Media, India having 110,000-150,000 viscosimetric molecular weight and ≤ 40% degree of acetylation (calculated by the method of Baxter *et al.*[18]) was characterised along with the plant biopolymers as a reference material since it has been widely studied as a wound management material. All the experiments were performed in triplicates and reported as mean values.

Polymer extraction—Moringa pods were collected from local plantations and dried. *Moringa* was identified by Prof. Surendra Bhatnagar (retd.) (Department of Environmental Biology, Rewa University, Rewa) and the voucher specimen is preserved in department of Microbiology, MDS University, Ajmer. After drying the seeds with their hulls around it were ground to a fine powder. Aqueous extracts were prepared using 10 g powder in 100 mL distilled water and stirred for 30 min[8]. After filtration, the filtrate was treated with three volumes of chilled isopropyl alcohol and refrigerated overnight for the precipitation of biopolymer (BP). In the same way water soluble polymeric part of gum acacia (Hi-Media, India) was separated from a 20% solution. The gum was dissolved in distilled water at 80 °C with stirring and subsequently precipitated with isopropyl alcohol after removal of insoluble components by vacuum filtration.

Characterization of polymers—Carbohydrate content of the plant BP was measured by anthrone method using glucose as a standard[19], and protein was estimated after Lowry *et al.*[20] using bovine serum albumin as a standard. Its sulphate content was measured by the modified $BaCl_2$-gelatin method[21] using 5 mg BP which was further dissolved in 1 mL deionized water for estimations. HCl was used as a blank and potassium sulphate (20-200 µg mL^{-1}) dissolved in 1 *N* HCl was used as a standard for calibration. Uronic acid (UA) content of BPs was measured after the modified meta-hydroxy biphenyl method[22] using 4 *M* sulfamic acid in 1% (w/v) BP solution. Glucuronic acid (10-100 µg mL^{-1}) was used as a standard for the calibration of the method.

GC analysis—Monomer sugar composition of the BP was determined by Gas chromatography of alditol acetate derivatives of sugars with inositol as an internal standard[23]. The samples (200 µg) were hydrolyzed with 2 *N* 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.

Thrombogenicity—Gravimetric method was followed to evaluate thrombus formation by the BPs[24]. For this the BPs were suspended in the phosphate buffered saline (PBS, *p*H 7.4) to give a concentration of 1% (w/v) in a Petri plate at a constant temperature of 30 °C. Further, blood coagulation time was determined by the modified method of Hu *et al.*[25] using 1% plant BP solution (w/v in PBS) for estimation of activated partial thromboplastin time (APTT) and prothrombin time (PT).

Hydrophobicity—Hydrophobicity of BP was determined using modified method of Tielen *et al*[26]. An aliquot (5 mL) of each polymeric suspension prepared in PBS (1%, w/v) was dispensed into a clean test tube. After obtaining OD_{580} (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_{580} was measured (A_1). The degree of hydrophobicity was calculated as $[(A_0-A_1)/A_0] \times 100$.

Hemolytic activity—Blood from healthy donors was obtained from blood bank at Jawaharlal Nehru Hospital, Ajmer. Hemolytic activity of the BPs was tested by the direct contact method of ASTM F 756-00[27]. Acid citrate dextrose (ACD) blood (1 mL) was mixed with 7 mL of BP solution (2%, w/v in PBS). Positive and negative controls were prepared by adding the ACD blood to water and PBS respectively in the same ratio. The tubes were incubated at 37 °C and gently inverted twice every 30 min to maintain contact of the blood with the material. After incubation for 3 h, the tubes

were centrifuged at 8000 *g* for 15 min. The haemoglobin released by hemolysis was measured spectrophotometrically at 540 nm. Percentage hemolysis was calculated as under:

$$\text{Hemolysis } (\%) = \frac{OD_s - OD_{nc}}{OD_{pc} - OD_{nc}} \times 100$$

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

Cytotoxicity test—Vero cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and incubated at 37 °C in a humidified incubator, with 5% CO_2. After confluent monolayer propagation to detach cells from the culture flask, trypsinization was done with 0.25% trypsin solution. The cells were transferred to a screw-capped plastic tube, centrifuged and washed twice with PBS. The cells were reseeded in new culture medium and the concentration was adjusted to ~60,000 cells mL^{-1} with the help of haemocytometer. Tissue culture microtiter plates (96 wells) were inoculated with 50 µL BP suspension (1 %, w/v in 0.9% NaCl), followed by the addition of 50 µL of the cell suspension (~3000 cells) in quadruplicate. The same amount of test substance was added to the blank wells containing no cells. Negative control wells were prepared by adding culture media without test substance. After incubation for 24 h, 20 µL of 0.001% of 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 it was analyzed with a fluorometer at 590 nm against blank[28].

Antibacterial activity—Antibacterial activity was determined using the disc diffusion method[29]. 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. Bacterial cell suspension (100 µL) was spread on the surface of Mueller-Hinton agar plates. Discs (5 mm) impregnated with BP solution (1 mg mL^{-1}) were placed on the inoculated surface. Inhibition zones were measured after 24 h incubation at 37 °C. Chloramphenicol was used as a positive control.

Minimum inhibitory concentration (MIC)—The MIC was determined according to Zgoda and Porter[30] with some modifications. Dilutions of the BP were prepared in the range of 100-1000 µg mL^{-1}. Each polymeric dilution (100 µL) was added to the wells in a microtiter plate in triplicate followed by addition of 100 µL of bacterial cell suspension (10^8 cells mL^{-1}). All the bacterial strains referred above were tested at all dilutions. Wells having sterile distilled water and the bacterial medium served as positive control whereas wells with biopolymeric suspension and without microorganism 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 in each well. The plates were further incubated for 24 h. Reduction of the colorless TTC to red color formazan was an indicator of the cell viability. MIC was read as the concentration of biopolymer where no color could develop.

Water absorption capacity—Water absorption capacity of the BPs was determined by the tea bag method[31]. Dried and labelled tea bags were filled with 200 mg polymer and immersed in 200 mL distilled water in a beaker. After 45 min the bags were removed, drained, dabbed on paper towel to remove excess water and weighed. Empty tea bags were used as control. % water absorption was calculated as: $[(W_1-W_2)/W_p] \times 100$, where W_1, W_2 and W_p are the weights (g) of the wet bag with BP, wet bag without BP and the dried BP respectively.

Biodegradability—To determine the biodegradability of BPs, amount of reducing sugar formed after enzymatic degradation was estimated[32]. For this purpose 2 mL of aqueous BP solution (0.5%, w/v) was taken in a test tube followed by addition of 1 mL of enzyme solution (cellulase: 4U and β-glucosidase: 144U mL^{-1}) in separate sets. Incubation was done at 37 °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 dinitro salisylic acid (DNS) reagent. Glucose was used as a standard. For color development all the tubes were placed in a boiling water bath for 15 min, cooled down to room temperature and OD_{575} was recorded.

Statistical analysis—All experiments were performed using completely randomized design and values are presented as arithmetic mean. Standard deviation (SD) was calculated using Microsoft excel statistical tool.

Results and Discussion

Characterization of plant biopolymers—GA and MSP contained 762 and 564 µg of anthrone reactive carbohydrate, 47 and 325 µg protein, 0.3 and 1.3 µg sulphate along with 103 and 62 µg uronic acid mg^{-1} dry weight, respectively. GA and MSP were dominated by pentose sugars. Table 1 showed their monosaccharidic composition analysed by Gas chromatography.

Thrombogenic behaviour of biopolymers—Accelerating and strengthening the clotting of blood is pertinent to combat wound treatment where nearly 85% deaths are caused by severe bleeding[33]. Both the BPs from *Acacia* and *Moringa* plants were thrombogenic as weight of the clots obtained was significantly higher than the control (Fig. 1). For a majority of biomaterials especially ones designed to act as implants this is an undesirable feature, however if the BP is intended to be used on bleeding wounds, thrombogenicity leads to hemostasis. This can be of great importance in improving coagulation and cicatrisation process of the wound[34,17].

Thrombus that forms by all known biomaterials is partly due to platelet mediated reaction and partly due to coagulation of blood plasma itself, in proportions that depend upon the surface chemistry of the biomaterials. Hydrophobic surfaces are known to promote the coagulation cascade by enhancing protein adsorption[35] however BPs used in the present study are primarily hydrophilic (Table 2) and yet thrombogenic. Chitosan and MSP are known to be cationic in nature[36,37]. Thus presumably MSP enhances blood clotting like chitosan by interacting with negative charges of cell membranes of erythrocytes[38,39]. GA on the other hand was also hydrophilic but is known to be anionic[40] and yet showing maximum thrombogenic ability. This perhaps induces blood coagulation *via* contact activation of the inactive zymogen factor XII to factor XIIa which occur when blood comes in contact with hydrophilic surfaces having anionic groups[41-43].

The blood coagulation cascade includes the intrinsic, extinsic and common pathway. APTT and PT tests are used to examine the intrinsic and extrinsic pathways, respectively. Fig. 2 shows the blood clotting time of the studied BPs. APTT was reduced 27, 13 and 12% while PT was reduced at the rate of 63, 17 and 10% by GA, MSP and chitosan, respectively. Thus the BPs enhance the coagulation

Table 1—Monosccharide composition of plant biopolymers analysed by Gas chromatography

Monosaccharides	Sugar monomers (%) Gum acacia	*Moringa* seed polymer
Ribose	-	-
Arabinose	80	22
Xylose	-	36
Glucose	-	14
Mannose	-	14
Galactose	25	14

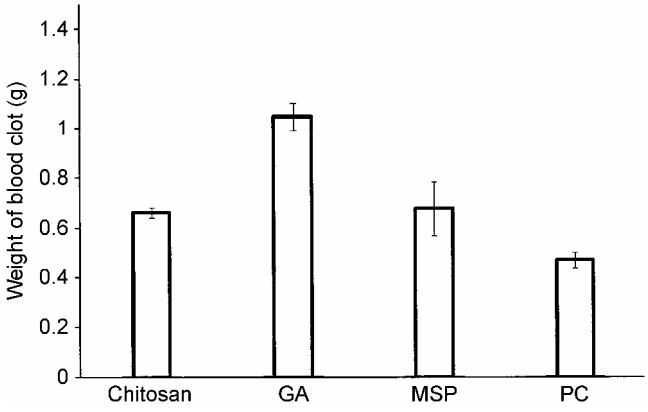

Fig. 1—Thrombogenic index of GA, MSP, chitosan *vis a vis* glass used as positive control (PC). [The polymers are thrombogenic as the weight of blood clot obtained was higher than the positive control. Values are mean ± SD)].

Table 2—Hydrophobicity, viability of Vero cell lines when exposed to 1% biopolymer along with its water absorption capacity

[Values expresed in % are mean ± SE]

Sample	Hydrophobicity	Viability	Water absorption
Chitosan	27.83 ± 5.0	85.30 ± 2.1	378.74 ± 2.7
GA	31.35 ± 0.6	84.60 ± 1.5	953.36 ± 2.2
MSP	16.44 ± 0.5	83.30 ± 2.9	415.41 ± 3.3
NC*	-	97.60 ± 3.7	-

*NC, Negative control for cell lines without polymer

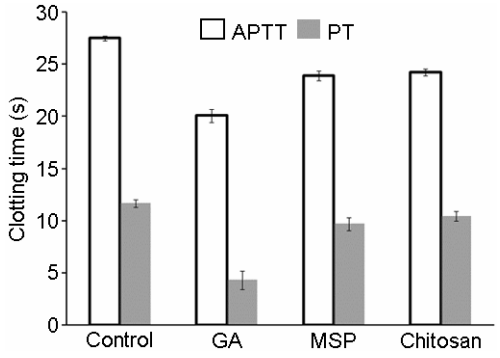

Fig. 2—PT and APT time of GA, MSP and chitosan against control.

cascade in presence as well as absence of tissue factor. Since, these plant BPs are hydrophilic they may also reduce coagulation time by increasing the viscosity of blood[44].

Hemolytic and cytotoxic effect—ASTM F 756-00 (2000)[27] classifies materials into three categories based on their hemolytic index. Those showing >5% hemolysis are considered hemolytic, between 2-5% are slightly hemolytic while the ones having <2% hemolysis are non hemolytic materials. All the three polymers could hence be categorized as slightly hemolytic with 3.44, 3.83 and 4.21% hemolysis observed for GA, MSP and chitosan, respectively. The risk of hemolytic character is evaluated against the clinical benefits and thus like chitosan both the plant BPs have hemolytic potential within acceptable limits and may be used as good wound healing agents. Besides this, derivatization can be used to alter the hemolytic ability as is known for chitosan which shows non hemolytic character when grafted with 2-hydroxyethyl methacrylate and vinyl monomers[17]. Resazurin assay is often used to study *in vitro* cytotoxicity of polymeric components. Nearly 83-85% cells remained viable (Table 2) in presence of BPs, suggesting that they are non-cytotoxic.

Antibacterial activity—An important character of a dressing material is to provide protection against infection. The wound dressing may either have an innate antibacterial activity or can be impregnated with antimicrobial agents like silver or iodine. The BPs used in the present study were evaluated against *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Bacillus licheniformis* (ATCC 14580) and *Staphylococcus aureus* (ATCC 25923). Both the BPs showed good activity against all the four tested bacterial strains. The Minimal Inhibitory Concentration (MIC) of the BPs is given in Table 3. The cationic polymers chitosan and MSP interact with the anionic membrane of the bacterial cell thereby disrupting it[45]. MSP is also known to have a cationic Flo polypeptide that acts directly and non-specifically upon bacterial membranes causing leakage of cytoplasmic content[46] whereas, GA contains cyanogenic glycosides that exhibit antibacterial properties[47].

Table 3—Antibacterial activity of biopolymers expressed as minimal inhibitory concentration (MIC μg mL^{-1}) against bacteria associated with wound infections

	P. aeruginosa	*E. coli*	*B. licheniformis*	*S. aureus*
Chitosan	300	600	400	700
GA	600	600	500	600
MSP	400	700	300	400

Water absorbing capacity—For bleeding and moderate to highly exuding wounds the selected dressing should be able to absorb high volume of fluid[48]. MSP, GA and chitosan showed water absorbing capacity 3.17 and 8.53 and 2.78 g H_2O g^{-1} dry weight respectively (Table 2). Thus they could be used as dry dressings to control bleeding wounds and for wounds releasing high amount of exudate[49,50].

Biodegradability—Enzymatic biodegradability of the polymer is an important characteristic to determine the use of polymer and its recalcitrance. Although all the studied BPs showed biodegradability measured in terms of reducing sugars released by enzymatic degradation with β-glucosidase and cellulase (Fig. 3), the extent of degradation varied. β-glucosidase that acts upon β,1→4 bonds linking two glucose or glucose substituted molecules is reported to be inactive on polysaccharides. However the present results showed that chitosan was most susceptible to degradation by β-glucosidase. Zhang and Neau[51] also reported degradation of chitosan by β-glucosidase prepared from almond emulsion however they suspected the presence of a contaminant in the commercial preparation, which may also be the case in the present study.

GA is an arabiongalactan with β-(1→3) galactose back bone with linked branches of arabinose and rhamnose terminating in glucouronic acids. Besides this, a smaller fraction of high molecular weight arabinogalactan protein complex is also present[52].

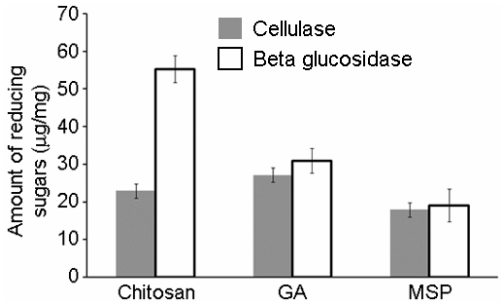

Fig. 3—Reducing sugar (μg. mg^{-1}, mean ± SD) released after enzymatic degradation of GA, MSP and chitosan by cellulase and β-glucosidase as an indicator of biopolymer biodegradability.

Both cellulase and β-glucosidase showed degradation of GA to the same extent. MSP was less degradable being a more complex molecule with xylose, glucose, mannose and galactose.

It can be concluded that the polymeric component of aqueous extracts from gum acacia and *Moringa* seeds have potential to be used as dry hemostatic dressings for bleeding wounds. Owing to their good water absorption capacity they can also be applied on exuding wounds. Since, these BPs are readily available and may be cheap than the conventional wound dressings they shall be a boon to developing countries where advanced wound dressings are still not available.

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