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Sulfation of *Aegle marmelos* gum: Synthesis, physico-chemical and functional characterization

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ABSTRACT

The present investigation was aimed at optimizing the conditions for preparing sulfated derivative of gum obtained from partially ripe fruits of *Aegle marmelos*. Elemental analysis, FTIR-ATR and NMR studies confirmed successful sulfation. The ratio of chlorosulfonic acid to pyridine exerted maximum influence on the degree of substitution followed by reaction temperature and reaction time. The sulfated derivative showed higher swelling in both acidic and alkaline pH as compared to unmodified gum. It also possessed higher negative zeta potential, higher viscosity, work of shear, firmness, consistency, cohesiveness and index of viscosity as compared to both unmodified gum as well as sodium alginate. Sulfated derivative was superior to unmodified gum and sodium alginate in terms of antimicrobial and anticoagulant activity. The sulfated sample appears to be a potential substitute over the unmodified gum sample and sodium alginate for modulating physicochemical properties of food and drug release dosage forms.

1. Introduction

Polysaccharides have drawn the attention of biochemical and nutritional researchers in recent years due to their various biological activities (Cui, Tao, Xu, Guo, & Xu, 2007; Zhao et al., 2006). Many studies have demonstrated that biological activities of polysaccharides can be modified by modification of their functional groups. Molecular modification and structural improvement of polysaccharide appear to elicit excellent physiological properties in maintaining health and preventing diseases (Alban & Franz, 2000; Coothan, Anthony, Sreenivasan, & Palaninathan, 2007).

Sulfated polysaccharides contain sulfur groups in place of hydroxyl groups. Sulfation of polysaccharides has been reported for a variety of polysaccharides such as fucoidan (Soeda, Kozako, Iwata, & Shimeno, 2000), curdlan (Alban & Franz, 2000), fucan (Nishino & Nagumo, 1992), chitosan (Baumann & Faust, 2001), dextran and pullulan (Mahner, Lechmer, & Nordmeier, 2001). The reagents used were mainly chlorosulfonic acid in pyridine (Py) (Geresh, Mamontov, & Weinstein, 2002), piperidine N-sulfonic acid (Yoshida, Nakashima, Yamamoto, & Uryu, 1993) or sulfur trioxide complexes with pyridine (Wu et al., 1998), triethylamine (Soeda et al., 2000) or DMF (Alban, Schauerte, & Franz, 2002). The solvents used were usually formamide (FA), DMF, DMSO and pyridine. However, due to the structural complexity of polysaccharides, one sulfation method resulting in predictable derivatives of a certain polysaccharide was not easily applicable to another polymer. In addition, the different biological activities of sulfated polysaccharides, such as anticoagulation (Huynh, Chaubet, & Jozefonvicz, 2001), antithrombotic activity (Mourao et al., 2001), antiviral activity (Amornrut et al., 1999), anti-inflammatory property (Winkelhake, 1991), antiangiogenetic property (Paper, Vogl, Franz, & Hoffman, 1995), antiproliferative property (Hoffman & Paper, 1993) have been known to be strongly dependent on their structure, i.e. degree of sulfation (DS) (Alban et al., 2002), molecular weight (MW) (Barbucci, Lamponi, Magnani, & Renier, 1998) and glycosidic branching (Mulloy, Mourao, & Gray, 2000; Yoshida et al., 1993). Derivatives of BFG have not been reported earlier. However, sulfated derivatives of polysaccharides are known to exhibit higher water solubility (Wang, Zhang, Li, Hou, & Zeng, 2004) and used as texturing agents in food, as food supplements and in drug delivery (Pomin, 2010). Therefore, it is important to establish an appropriate sulfated method for a certain polysaccharide.

Chlorosulfonic acid–pyridine (CSA–Pyr) method is the most popular method employed for sulfating polysaccharides. Many studies have indicated that CSA–Pyr method possesses advantages

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of high yield, high DS and convenient manipulation (Wang, Li, & Chen, 2009). The ratio of CSA to Pyr, reaction temperature and reaction time are important factors in this method. Polysaccharide modification performed under different conditions is known to result in sulfated polysaccharides with various DS and bioactivities.

The unripe fruits of *Aegle marmelos*, commonly known as 'Bael' in Hindi language are reported to contain bioactive compounds such as carotenoids, phenolics, alkaloids, pectins, tannins, coumarins, flavonoids and terpenoids (Maity, Hansda, Bandyopadhyay, & Mishra, 2009; Suvimol & Pranee, 2008). The fruit is edible and has been recommended for use as antiamoebic and antihistaminic (Baliga, Bhat, Pereira, Mathias, & Venkatesh, 2010). Seeds of unripe bael fruit contain gum enveloped around each seed. The bael fruit gum (BFG) is reported to contain D-galactose (54.26%, w/w), L-arabinose (6.1%, w/w), L-rhamnose (18.83%, w/w) and glucouronic acid (20.8%, w/w) (Mandal & Mukherjee, 1980). The branching heteropolysaccharide feature allows the preparation of sulfated compounds that would be difficult to obtain by a synthetic approach.

The literature, however, does not report any studies with respect to sulfation of BFG. In this paper, nine sulfated BFG (sBFG), with various degrees of sulfation (DS), were prepared by applying orthogonal design by using CSA–Pyr method. The prepared sBFGs were evaluated for their chemical and functional characteristics and these values were compared with those obtained for sodium alginate.

2. Materials and methods

2.1. Materials

Bael fruits, partially ripe were collected from local market. BFG (clean with no extraneous material) was used in the experimental analysis. Gum samples collected were stored in airtight polypropylene jars in desiccated condition. De-ionized (Milli-Q) water was used for all experiments. Sodium alginate (SA), dimethyl formamide (DMF), CSA and Pyr were purchased from Himedia Laboratories, Mumbai, India. All other chemicals used were of analytical reagent grade.

2.2. Antinutritional factors

Dried pulp from unripe *Aegle marmelos* fruits was tested for the presence of antinutritional factors. The extracted gum was tested only for the factors that presented positive results for the dried pulp.

2.2.1. Trypsin and α-amylase inhibitors

The trypsin inhibitory activity was determined according to Kunitz modified by Arnon (1970), using casein as the enzyme substrate. Trypsin inhibitory unit (TIU) was defined as the difference between the units observed in the maximum activity and the activity of the samples containing the inhibitors. The activity of α-amylase inhibitor was determined according to Deshpande, Sathe, Salunkhe, and Comforth (1982), using starch as the substrate for the enzyme. One unit of α-amylase inhibitor was defined as the amount of inhibitor that inhibits one unit of α-amylase.

2.2.2. Hemagglutinating activity

Hemagglutination assays, using rabbit erythrocytes, were carried out following the method described by Moreira and Perrone (1977) with modifications. The extract (1%, w/v, dried pulp) prepared in 0.05 mol L^{-1} acetate buffer pH 5.0 was serially diluted with 0.15 mol L^{-1} NaCl solution. One milliliter of a 2% erythrocyte suspension was added to an equal volume of the sample and the mixture incubated at 37 °C for 30 min followed by 30 min of resting at room temperature (25 °C). The tubes were centrifuged at 2000 × *g* for 1 min and the last tube showing visible agglutination was considered equivalent to minimal hemagglutinating concentration.

2.2.3. Phytic acid determination

The phytic acid content was determined by the method described by Latta and Eskin (1980) with modifications for resin DOWEX-AGX-4 according to Ellis and Morris (1986). A standard curve of phytic acid (Sigma, P8810) was done and the results were expressed as mg g^{-1} of the sample.

2.2.4. Total tannins

The analysis of total tannins in the extract (1%, w/v, dried pulp in 0.05 acetate buffer pH 5.0) was made according to Hagerman and Butler (1989). Tannin concentration in the sample was measured using a standard curve of tannic acid.

2.2.5. Saponins determination

Presence of saponins was detected by employing the methodology described by Duarte, Yassumoto, and Cecy (1990). 100 mg of dried pulp sample was suspended in 20 mL of distilled water and incubated in boiling water for 5 min. After incubation, the mixture was cooled to room temperature, filtered through a nylon membrane and the volume adjusted to 100 mL with distilled water. Serial dilutions (10^{-1} to 10^{-5}) were made using distilled water and the tubes were vortexed for 15 s followed by 15 min of incubation at room temperature (25 °C). The presence of persistent foam after incubation indicated existence of saponin.

2.2.6. Alkaloids

The phytochemical analysis to evaluate the presence of alkaloids was carried out following methodology described by Costa (2001). One gram of *A. marmelos* dried pulp or extracted gum was dissolved in 10 mL of 1% (v/v) H_2SO_4 solution, and the mixture was incubated for 2 min in boiling water. The solution was filtered and aliquots of 1 mL were added to tubes containing 40 mL of Dragendorff reagent. The formation of orange-red precipitate indicates the presence of alkaloids. To confirm result, 1 mL of the filtrate was added to tubes containing 40 mL of Mayer reagent. The formation of precipitate confirms the presence of alkaloids.

2.3. Extraction of BFG

Bael fruit gum was extracted by modifying the method reported by Rai, Tiwary, and Rana (2012). In brief, partially ripe bael fruits were collected from the *A. marmelos* tree. The hard woody and spherical fruits were carefully broken down into two equal parts. The amber colored viscous, very sticky, translucent gummy substance along with the seeds and pulp separating the fruit outer wall was marked as the desired portion. This gum along with seeds was collected in a beaker containing 2% (v/v) glacial acetic acid solution. The slurry was boiled on water bath for 45 min with continuous stirring and stored overnight. The slurry was filtered through muslin cloth to remove debris. The gum was precipitated from the filtered slurry by adding acetone. The precipitates were dried in a vacuum oven at 50 °C and grounded to obtain light brown fine powder. The gum was further purified by dialysis (Himedia-60 LA390-5MT) and purified BFG was obtained by freeze drying.

2.4. BFG sulfation

2.4.1. Experimental design

Nine reaction conditions were adopted for studying the influence of molar ratio of CSA to Pyr, reaction time and reaction temperature on sulfation of BFG (Table 1). These experiments were

Table 1
Sulfation of BFG.

Reaction conditions				Results	
Products	A CSA:Pyr	B Temperature (°C)	C Time (h)	Yield (mg)	DS
sBFG1	1:4	45	1	121	1.83
sBFG2	1:4	70	2	138	3.68
sBFG3	1:4	95	3	34	1.36
sBFG4	1:6	45	3	75	0.88
sBFG5	1:6	70	1	62	0.97
sBFG6	1:6	95	2	53	0.58
sBFG7	1:8	45	2	62	0.27
sBFG8	1:8	70	3	52	0.19
sBFG9	1:8	95	1	29	0.05

in accordance to the orthogonal test design designated as L_9 (3^3) by Wang et al. (2009).

2.4.2. Preparation of sulfating reagent

The sulfation reagent, complex of CSA and Pyr was prepared by slowly adding CSA to Pyr (25 mL) filled in three-necked flask, with continuous stirring and cooling the mixture in an ice bath so as to maintain the temperature between 4 and 10 °C. The ratio of CSA to Pyr is given in Table 1.

2.4.3. Sulfation reaction

Sulfation of BFG was carried out by using the method described by Yoshida, Yasuda, Mimura, and Kaneko (1995). 200 mg BFG was suspended in 20 mL anhydrous dimethyl formamide (DMF), and the sulfation reagent was added. The mixture was processed under different conditions (Table 1). After the reaction, the compound was cooled to room temperature, neutralized with 2.5 M NaOH and precipitated with 95% ethanol. The sediment was re-dissolved with water and dialysed (Himedia-60 LA390-5MT) against tap water for 48 h and distilled water for 24 h to remove pyridine, salt and potential degradation products. At last, nine sulfated polysaccharides named sBFG1, sBFG2, sBFG3, sBFG4, sBFG5, sBFG6, sBFG7, sBFG8, sBFG9 were collected after lyophilizing.

2.5. Chemical characterization

2.5.1. Elemental analysis of BFG

Lyophilized sBFG samples were analyzed for sulfur content (%*S*) by using element analyzer (Elementar, Analysen systeme, Germany). Accurately weighed 0.5 mg of sample was heated to 1150 °C and the corresponding element was determined by using a thermal conductivity detector. Degree of sulfation (DS) was calculated according to the following equation:

$$\mathrm{DS} = \frac{0.162 \times (\%S/32)}{100 - [(80/32) \times \%S]}$$

2.5.2. FTIR analysis

FTIR spectra of BFG and sBFG2 samples were recorded on a FTIR-ATR spectrophotometer (Alfa, Bruker, Berlin, Germany). The lyophilized dry powder was mixed with KBr and pressed into pellets. The FTIR spectra were obtained between wavelengths of 4000–500 cm^{-1}.

2.5.3. NMR studies

^1H NMR spectra were recorded using D_2O as solvent for BFG and sBFG2 (2.5%, w/v) using Bruker Avance II 400 NMR spectrometer at 25 °C and frequency of 400 MHz (Cui, Phillips, Blackwell, & Nikiforuk, 2007).

2.6. Functional properties

2.6.1. Swelling Index

The BFG and sBFG2 samples were soaked in distilled water, HCl (0.1 N) or phosphate buffer pH 1.2, 6.8 or 7.4 respectively (100 mL) for 24 h. The swollen material was then removed and weighed after superficial drying using a blotting paper. The swelling index (SI) was calculated as:

$$\mathrm{SI} = \frac{w_f - w_i}{w_i}$$

where w_f is the weight of swollen material and w_i is the initial weight of the dry material.

2.6.2. Zeta potential studies

The zeta potential of BFG and sBFG2 were measured by using Zetasizer 4 (Malvern Instrument Ltd., UK). The temperature of the samples was controlled at 25 °C. The zeta potential measurements were performed by using an aqueous dip cell in an automatic mode. Samples were diluted with HPLC water (MilliQ Synergy Systems, Millipore) and placed in capillary measurement cell.

2.6.3. Rheological behavior

2.6.3.1. Solution preparation. BFG, sBFG2 or SA sample was dissolved in distilled water at varying concentrations (0.5–5.0%, w/v) using a magnetic stirrer (2MLH, REMI Elektrotechnik Limited, Vasai, India) for 3 h and then centrifuged (using the C-24 BL, REMI Elektrotechnik Limited, Vasai, India) for 25 min at 25 °C at a speed of 2500 rpm to remove insoluble matter.

2.6.3.2. Viscosity analysis. BFG as well as sBFG2 samples of various concentrations (0.5–5%, w/v) were analyzed for viscosity using Brookfield viscometer (Brookfield DV-1 Prime, Bruker, Berlin, Germany) at temperature of 25 °C maintained using refrigerated circulating water bath.

2.6.4. Instrumental texture measurements

Back extrusion (BE) and cone penetration (CP) mechanical tests were performed for investigating the rheological behavior. Both experiments were performed using a TA XT Plus Texture Analyzer (Stable Micro Systems Ltd., Godalming, U.K.) equipped with a 300 N load cell. For performance of BE tests, a rig (model A/BE, Stable Micro Systems) was used consisting of a flat 35 mm diameter perspex disc plunger that was driven into a larger perspex cylinder sample holder (50 mm diameter) to force down into the BFG, sBFG2 or SA samples and flow it upward through the concentric annular space between plunger and the container. The measuring cup was filled with 30 ± 1 g of BFG, sBFG2 or SA sample. The test was replicated eight times at a pretest speed of 1.0 mm s^{-1}, test speed of 2.0 m s^{-1} at a distance of 50 mm above the top of the sample, penetrated to a depth of 10 mm, and returned to starting position. The plunger was cleaned after each measurement. At this point (most likely to be the maximum force), the probe returned to its original position. The maximum positive force of extrusion (firmness [N]), area of the curve (consistency [N.s]), maximum negative force due to back extrusion (cohesiveness [N]) and the negative area of extrusion (index of viscosity [*N·s*]) were documented as descriptors of rheological behavior of these samples. For performing the CP tests, spreadability rig (HDP/SR, Stable Micro Systems) was used. It consisted of a 45° conical perspex probe (P/45C) that penetrated a conical sample holder containing 5 ± 0.1 g of BFG, sBFG2 or SA sample. Product was penetrated to a distance of 50 mm at 3 mm/s compression rate. The work required to accomplish penetration was calculated from the area under the curve (work of shear [N s]).

2.7. Antimicrobial effect

The antimicrobial activities of BFG, sBFG2 and SA samples against *Bacillus cereus* and *Escherichia coli* were examined. *B. cereus* and *E. coli* were inoculated in nutrient broth and incubated at 37 °C for 24 h. The gum solutions (90 μL) with three different concentrations (0.5, 1.0, 2.0 mg/mL) were added to the culture broth (10 μL) which was incubated at 37 °C for 18 h, then the absorbance of the culture broth was measured at 540 nm. The microbial inhibition effect was calculated as follows;

$$\text{Inhibition effect}\,(\%) = \left[\frac{\text{Abs of control} - \text{Abs of sample}}{\text{Abs of control}}\right] \times 100$$

2.8. Anticoagulant activity

The anticoagulant activity of BFG, sBFG2 and SA sample was determined by using the method of Matsubara et al. (2001). For the activated partial thromboplastin time (APTT) assay, the derivative, sBFG2 (50 μL in distilled water at different concentrations of 25, 50, 100 μg/mL) was mixed with the plasma (50 μL) and incubated at 37 °C for 2 min. Then, APTT assay reagent (100 μL) was added to the resulting solution and further incubated at 37 °C for 6 min. After the addition of 20 mM $CaCl_2$ (100 μL), the clotting time was recorded and compared with that of heparin (Himedia Laboratories Ltd., Mumbai, India). In the prothrombin time (PT) assay, PT assay reagent (100 μL) preincubated at 37 °C for 10 min was added to the solution of the sulfated derivative (50 μL) and plasma (50 μL) and the clotting time was then measured. The prothrombin time (international normalized ratio) was obtained from the clotting time ratio between the sample and control.

3. Results and discussion

3.1. Antinutritional factors

The results obtained, showed absence of hemagglutinating activity, saponins and trypsin and α-amylase inhibitors in the *A. marmelos* dried pulp. The absence of these antinutritionals improves the nutritional value of the *A. marmelos* and consequently of the gum extracted. Alkaloids are secondary metabolites of plants that serve as defense mechanisms against predation by many microorganisms, insects and other herbivores. Results obtained in Dragendorff and Mayer tests evidenced presence of alkaloids in dried pulp of *A. marmelos* fruit but these compounds were absent in extracted gum.

The *A. marmelos* dried fruit pulp presented 1.07 mg g^{-1} of phytic acid. Phytate is considered an antinutritional factor mainly due to its ability to bind essential dietary minerals, proteins and starch, which consequently reduces their bioavailability. Although several authors consider phytic acid an antinutritional factor, studies point out that this compound is an important additive, with antioxidant properties that can be exploited in manufacturing bread, pasta and meat products (Oatway, Vasanthan, & Helm, 2001). In addition, several authors have reported beneficial effects of phytic acid against cancer (Midorikawa, Murata, Oikawa, Hiraku, & Kawanishi, 2001; Vucenik & Shamsuddin, 2006). Tannins are a special group of phenolic compounds that can react with proteins or minerals decreasing their bioavailability (Ferreira, Nogueira, Souza, & Batista, 2004). Although we detected as much as 1.21 mg tannic acid per 100 mg^{-1}, there was no phytic acid or tannins in the extracted gum, in all tested conditions. These results, associated with the absence of amylase and trypsin inhibitors, hemagglutinating activity, improve the safety and the nutritional quality of the BFG.

3.2. Chemical characterization

3.2.1. The yield and DS of BFG

The yield and DS of sBFG samples obtained by employing orthogonal experimental design are listed in Table 1. The results indicated highest yield of 138 mg of sBFG2 followed by 121 mg of sBFG1. The lowest yield was 29 mg of sBFG9. The DS of sBFG followed the order: sBFG2 > sBFG1 > sBFG3 > sBFG5 > sBFG4 > sBFG6 > sBFG8 > sBFG7 > sBFG9 and were 3.68, 1.83, 1.36, 0.97, 0.88, 0.58, 0.19, 0.07 and 0.05, respectively. Analysis on the orthogonal array design indicated that the most predominant influence on DS was exerted by variable *A* (molar ratio of CSA to Pyr). The extent of the impact of variables on DS followed the order: variable *C* (reaction time) < *B* (reaction temperature) < *A* (molar ratio of CSA to Pyr). Many researchers have observed that an increase in reaction temperature, prolongation of reaction time and enhancement of the molar ratio of CSA to Pyr contributed to high DS (Wang et al., 2009). The results of the experiments revealed that the DS of products increased rapidly with increasing molar ratio of CSA to Pyr. However, at very high proportion of CSA, hardening and difficulty in stirring was encountered. The DS content increased when the reaction temperature was increased to 70 °C from 45 °C, but it decreased dramatically at higher temperature of 95 °C. This indicated that the reaction required relatively mild condition. Wang et al. (2009) had shown the rate of reaction to be higher in the primary stage where about 85% of the possible substitution occurs within the first hour. This observation was confirmed by our experiment. *A. marmelos* gum is highly branched in nature with terminal units of galactose, galacturonic acid, rhamnose and arabinose (Roiy, Mukherjee, & Rao, 1977). The ratio of the sugar terminal units to sugars in the chains is 1:2. The gum contains 1 → 3 linkages in the galactose backbone wherein galactose sugar hydroxyls at positions 2, 4 and 6 are free. Further, in the terminal hexose sugar units, four hydroxyls at position 2, 3, 4 and 6 are free since at terminal positions the sugars are non-reducing. The linkage at 1 position in non-reducing sugars make four hydroxyls free unlike in case of reducing sugars where terminal sugar units require branching point i.e. one hydroxyl at position 2, 3, 4 or 6 for linkage. Hence, four hydroxyls present in galactose can be envisaged to be easily amenable to sulfation. The higher DS of 3.68, thus, could be possibly due to the easy amenability of terminal units with four hydroxyls or three hydroxyls and one carboxylic group in each unit of BFG. The data presented in Table 1 shows low yield of sBFG samples obtained by employing reaction temperatures >70 °C. Further, the DS was observed to decrease significantly on increasing the reaction temperature to 95 °C. This could be attributed to partial degradation of BFG at higher temperature. Degradation of BFG was manifested in the sample becoming hard and non-workable when the reaction was carried out at 95 °C. However, the influence of inter- and/or intramolecular dehydrations in making the sample hard at high temperature could not be ruled out. Therefore, the degradation and sulfation could be thought to be a group of competing reactions. The data summarized in Table 1 suggested that moderate temperature of 70 °C and prolonged reaction time of 2 h were optimum time for obtaining highest DS in sBFG2 sample.

3.2.2. FTIR analysis

Fig. 1 depicts the ATR spectra of BFG and sBFG2 samples. In the spectrum of BFG (Fig. 1A), the absorption bands at 1613 cm^{-1} and 1421 cm^{-1} could be ascribed to asymmetrical and symmetrical COO^- stretching vibration, respectively. The disappearance of these two absorption bands in the spectra (Fig. 1B) of the sulfated sample (sBFG2) and appearance of a new band at 1734 cm^{-1} suggested C=O stretching vibration. Furthermore, the disappearance of this

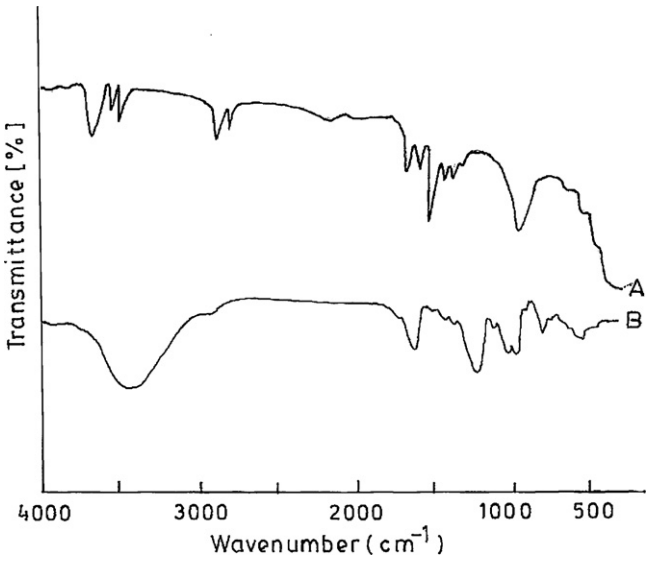

Fig. 1. FTIR-ATR spectra of (A) BFG and (B) sBFG2.

band on treatment of sBFG2 with dilute NaOH solution indicated it to be due to C=O stretching vibration of carboxylic acid moieties present in sBFG2 sample. Fig. 1B revealed additional two bands each at 1258 cm^{-1} and 854 cm^{-1}. These bands could be ascribed to asymmetrical S=O stretching vibration and symmetrical C—O—S vibration possibly associated to a C—O—SO_3 group, respectively. Besides these, a new band appeared at 1636 cm^{-1}. This could be related to the unsaturated bond formed in the sulfation process. However, it could also be due to O—H deformation vibration of H_2O that usually occurs at 1640 cm^{-1} (Chatjigakis et al., 1998).

3.2.3. NMR studies

The ^{1}H NMR spectrum of BFG (Fig. 2A) revealed two singlets at high field (δ 1.10 ppm (s), δ 1.11 ppm (s). This could be related to the environments of methyl groups of rhamnose and the protons linked to C-6 (δ 3.65, δ 3.70 ppm) and C-4 of galactose (δ 3.98, 4.28 ppm), respectively and suggests the existence of two different galactose derivatives (Agrawal, 1992). The anomeric protons could be assigned to β-sugar residues (δ 4.92–4.96 ppm) and the α-sugar residue (δ 5.1–5.3 ppm), as reported earlier (Agrawal, 1992). The H-1 resonance for methyl β-L-Ara p (δ 5.00 ppm) and methyl α-L-Ara p (δ 4.58 ppm) were also observed as reported previously for other gums (Mitzutani, Kasai & Tanaka, 1980). The anomeric region contains the proton signals (δ 5.078, 5.068 ppm) attributed to α-L-rhamnose (Agrawal, 1992). The three closely neighbored signals observed in the ^{1}H NMR spectrum (δ 4.15, δ 4.02 and δ 3.84 ppm) can be assigned to H-1 of α-glucose (Samuelson, Paulsen, Wold, Knutsen, & Yamada, 1998). The ^{1}H NMR spectrum of BFG showed signals corresponding to β-D-galactopyranose, α-L-arabinofuranose, α-L-rhamnose, β-D-glucuronic acid and 4-O-methyl-α-D-glucuronic acid as represented in Fig. 2A. A complete assignment of the 1H signals of the 4-O-methyl β-D-glucuronic acid was established. The H-1 (δ 4.48 ppm) signals proved β-configuration of 4-Omethyl-β-D-glucuronic acid. The O-methyl group could be assigned to 1H at δ 3.5 ppm.

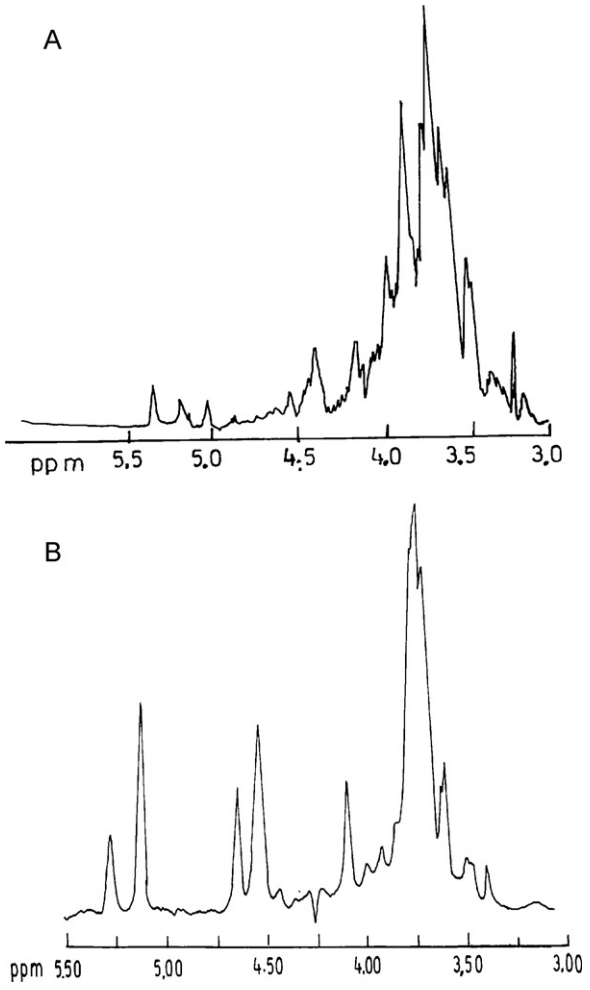

Fig. 2. NMR spectra of (A) BFG and (B) sBFG2.

The ^{1}H NMR spectra of sBFG2 is somewhat complex (Fig. 2B). The signals from the α anomeric proton at δ 5.13 and 5.28 were assigned to 3,6-α-L-anhydrogalactose and α-L-galactose-6-sulfate, respectively. The H-1 of β-D-galactose was linked to α-L-galactose 6-sulfate and that of β-D-galactose was linked to 3,6-α-L-anhydrogalactose, at δ 4.43 and 4.54, respectively. The comparison of signals before and after sulfation of BFG is shown in Table 2. Further, a number of attempts were made to record the ^{13}C spectrum, however the same could not be obtained due to higher viscosity of the sample. This is similar to our earlier problem of recording ^{13}C spectra of the polysaccharide samples due to very low concentration ^{13}C atoms in the solution (Rana, Kumar, & Soni, 2012).

Table 2
^{1}H NMR chemical shifts for BFG.

Residue	^{1}H chemical shift					
	H-1	H-2	H-3	H-4	H-5	H-6
β-D-Galactose	4.54	3.62	3.75	4.12	3.72	3.76
3,6-α-L-Anhydrogalactose	5.13	4.09	4.53	4.64	4.55	4.10
α-L-Galactose-6-sulfate	5.28	3.85	3.94	nd	nd	4.30
β-D-Galactose linked to α-L-galactose-6-sulfate	4.43	3.72	nd	nd	nd	3.51

nd, not detected.

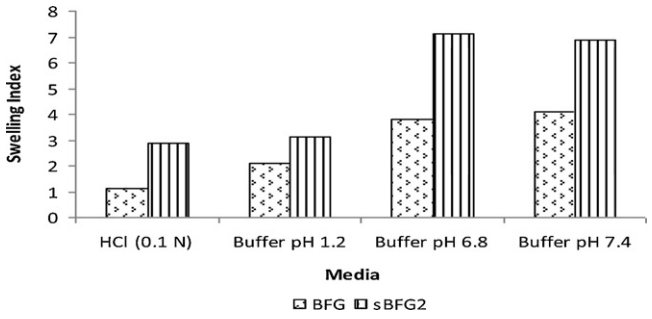

Fig. 3. Swelling index of BFG and sBFG2 in various pH enviornments.

3.3. Functional properties

3.3.1. Swelling index

The swelling characteristics of BFG and sBFG2 were investigated in 0.1 N HCl and buffers of pH 1.2, 6.8 or 7.4. The swelling of BFG in different media was observed to follow the order pH 7.4 = pH 6.8 > pH 1.2 = HCl (0.1 N) (Fig. 3). The sBFG2 sample also exhibited the same pattern (Fig. 1). However, the SI of sBFG2 was higher than that of BFG in any buffer. It is well established that low swelling in acidic pH restricts the release of drugs from dosage forms. At the same time, high swelling in alkaline pH would be useful for sustaining drug release for prolonged period as the dosage form travels down the g.i.t (Rai et al., 2012). Therefore, higher magnitude of swelling of sBFG2 compared to BFG can be expected to be useful for modulating the drug release from dosage forms.

3.3.2. Zeta potential

Aqueous dispersions of BFG and sBFG2 yielded an acidic pH. This behavior of BFG and sBFG2 is similar to that observed for other polysaccharides and was attributed to presence of uronic acid and sulfate units in their structure, respectively (Cui, 2005). The zeta potential of BFG and sBFG2 were, respectively, −16.7 mV and −41.27 mV, indicating their polyelectric effect in pure water. The high negative zeta potential of both the samples suggests their utility in enforcing gum–polymer or gum–ion interactions for modulating drug release characteristics. The higher zeta potential of sBFG2 sample could be attributed to greater electronegative character of SO_3^{2-} groups, which would exhibit higher magnitude of crosslinking thus yielding greater cross-linked density.

3.3.3. Rheological behavior

The viscosity of both BFG and sBFG2 samples was observed to increase with an increase in their concentrations. However, the increase in viscosity of sBFG2 samples was much greater than for BFG samples. Further, sBFG2 exhibited significantly greater ($p < 0.05$) viscosity as compared to BFG dispersions of comparable

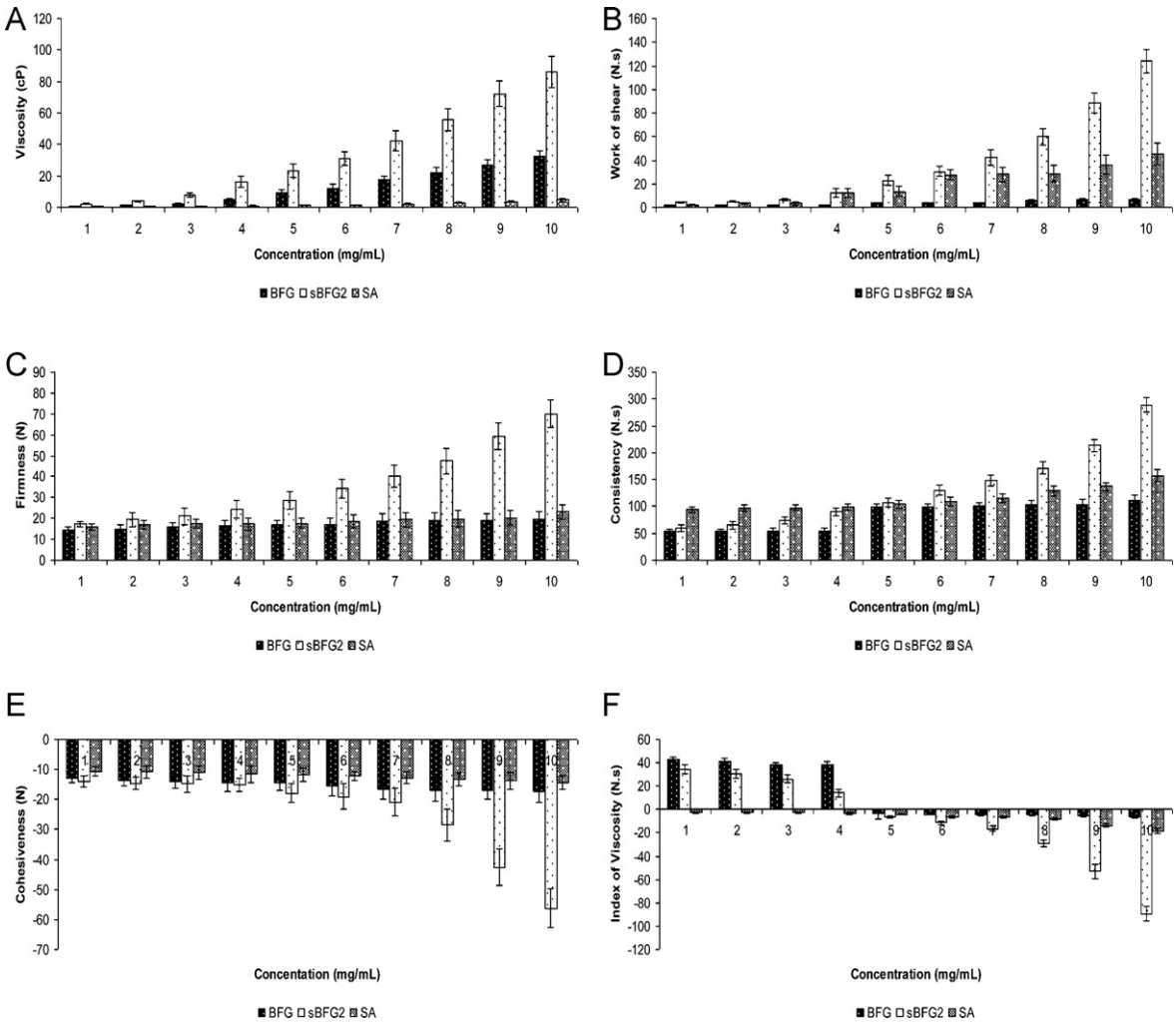

Fig. 4. Comparison of viscosity and textural behavior of BFG, sBFG2 and SA: (A) viscosity, (B) work of shear, (C) firmness, (D) consistency, and (E) cohesiveness (*F*) index of viscosity.

concentration (Fig. 4A). Accordingly the force required to accomplish penetration of probe to a fixed depth (work of shear) was observed to be greater for sBFG2 samples (Fig. 4B). The SI of sBFG2 sample was greater than that of BFG solution in both the acidic as well as alkaline pH (Fig. 3). The higher SI of sBFG2 sample is supported by its higher zeta potential, due to which, greater quantity of water molecules would have got associated with it.

3.3.4. Texture analysis

It is worthy to note that neither BFG sample nor SA sample exhibited any significant ($p < 0.05$) change in the firmness (Fig. 4C) and cohesiveness (Fig. 4D) values with their increasing concentration. Also, the consistency (Fig. 4E) and index of viscosity (Fig. 4F) were not markedly affected by change in their concentration. However, the firmness (Fig. 4C), cohesiveness (Fig. 4D), consistency (Fig. 4E) and index of viscosity (Fig. 4F) of sBFG2 sample increased with increase in concentration.

Firmness is a measure of the maximum force required to extrude a sample from concentric annular space between plunger and container. Hence, it is logical to expect greater firmness for samples of sBFG2 that had exhibited greater viscosities at different concentrations (Fig. 4A) as compared to both SA and BFG samples. The fact that sBFG2 samples showed greater firmness the area under the curve obtained was also greater, it reflected higher consistency values with increasing concentrations. Similarly, the less viscous SA and BFG samples did not exhibit any marked change in the maximum force due to back extrusion (cohesiveness). Whereas, the samples of sBFG2, that exhibited greater viscosities with increasing concentrations, revealed greater cohesiveness and thereby, greater index of viscosity.

SA has been widely investigated for its use in modifying drug release dosage forms and food preparations (Jensen, Knudsen, Viereck, Kristensen, & Astrup, 2012) due to its viscous behavior. A critical appraisal of the results revealed that the viscosity of SA samples was less than those of BFG and sBFG2 samples. Parameters obtained from texture analysis evidently indicated that firmness, cohesiveness, consistency and index of viscosity of SA was comparable to BFG. sBFG2 was significantly ($p < 0.05$) superior to both SA and BFG with respect to these parameters. The fact that these parameters are important indicators of the performance of viscosifying agents in food and pharmaceuticals, sBFG2 could be suggested to offer a great potential over widely used SA for use in pharmaceutical dosage forms and food products.

3.4. Antimicrobial effect

Antimicrobial activities of BFG, sBFG2 and SA samples were evaluated using *B. cereus* and *E. coli*. All the samples exhibited a dose dependent increase in inhibition of *B. cereus* as well as *E. coli* (Fig. 5). The activities of both BFG and sBFG2 samples at all dose levels were observed to be significantly more ($p < 0.05$) against *E. coli* as compared to *B. cereus*. However, SA was observed to be significantly more ($p < 0.05$) effective at all dose levels against *B. cereus* as compared to *E. coli*. These results suggested that sulfation had enhanced the antimicrobial activity of gum. It was previously reported that the bacteriostatic activity of carrageenans was critically dependent on the presence of sulfate groups (Yamashita, Sugita-Konishi, & Shimizu, 2001). Cellulose sulfate also exhibited antimicrobial activity against *Neisseria gonorrhoeae* and *Chlamydia trachomatis* which was explained by the interaction between proteoglycan receptors and their target cell ligands (Anderson et al., 2002). It is difficult to understand the reason for more microbicidal activity of sBFG2 against *E. coli*. However, the observations are supported by earlier reports of Park, Choi, and Chang (1995) who observed greater microbicidal activity of pectin hydrolysate against gram negative bacteria (*E. coli* and *Acetobacter aceti*) than gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*).

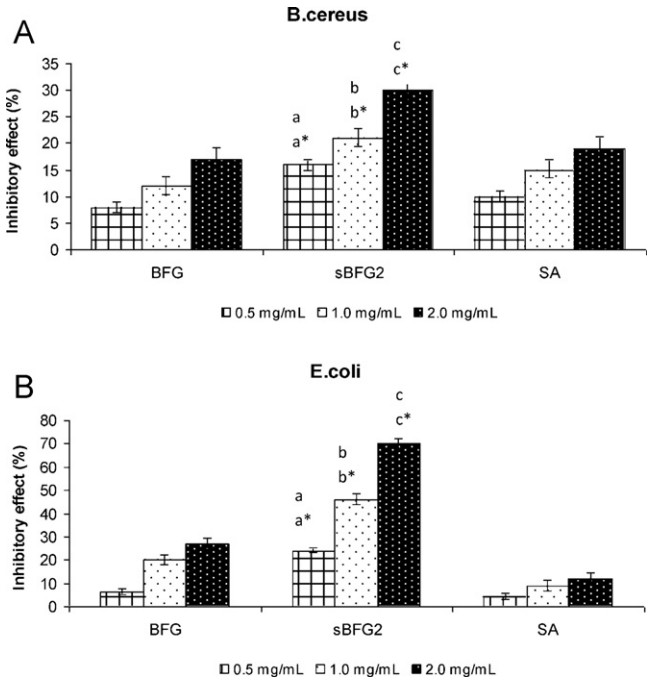

Fig. 5. Antimicrobial activities of BFG, sBFG2 and SA against: (A) *B. cereus* and (B) *E. coli*. $n = 3$ for each group; Values are mean ± S.D. Data of the results was analyzed using ANOVA followed by Tukey's multiple range test. a = $P < 0.05$ vs. BFG 0.5 mg/ml; a* = $P < 0.05$ vs. SA 0.5 mg/ml. b = $P < 0.05$ vs. BFG 1.0 mg/ml; b* = $P < 0.05$ vs. SA 1.0 mg/ml. c = $P < 0.05$ vs. BFG 2.0 mg/ml; c* = $P < 0.05$ vs. SA 2.0 mg/ml.

3.5. Anticoagulant activity

Blood coagulation is a complex process involved in the sequential activation of clotting factors, ultimately leading to formation of insoluble fibrin (Renne et al., 2005). The disorders of coagulation can give rise to an increased risk of bleeding and/or clotting. Since, heparin is a well established effective anticoagulant, the anticoagulant activity of sulfated gum (sBFG2) was compared with that of heparin. Fig. 6 presents the effect of sulfation on the anticoagulant activity of gum samples. The sulfated gum derivative significantly ($p < 0.05$) prolonged prothrombin time (PT) in a concentration-dependent manner. Thus, the incorporation of sulfate groups into the gum structure appeared to be responsible for the anticoagulant activity since native gum hardly exhibited anticoagulant effect. sBFG2 exhibited more than 4 min for APTT (activated partial thromboplastin time), when used in the concentration range of 50–100 μg/mL. This could be explained by the anionic characteristic of the sulfated gum that interacted with positively charged coagulant proteins, thus, improving the anticoagulant activity. Similar anticoagulant effects have been reported for other sulfated polymers such as β-glucan (Bae, Chang, Kim, & Lee, 2008), chitosan (Huang, Du, Yang, & Fan, 2003), galactan (Matsubara et al., 2001), and galactomannan (Mestechkina et al., 2008). Therefore, the results confirmed that enhanced anticoagulation activity of sBFG2 sample to be due to the introduction of sulfate groups into the gum chains. The prolonged PT and APTT indicate the inhibition of extrinsic and intrinsic coagulation pathways, respectively (Bayele, Murdock, Perry, & Pasi, 2002). Therefore, the increased PT and APTT in Fig. 6 indicated that the anticoagulant activity of the sulfated gum was possibly involved in the regulation of both intrinsic and extrinsic coagulation pathways.

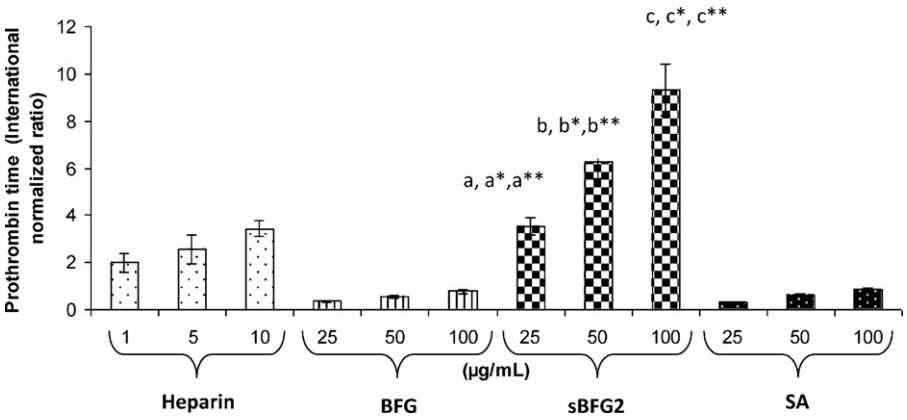

Fig. 6. Comparison of anticoagulant activity of heparin with BFG, sBFG2 and SA. $n = 3$ for each group; values are mean ± S.D. Data of the results was analyzed using ANOVA followed by Tukey's multiple range test. a = $P < 0.05$ vs. heparin 1.0 μg/ml; a* = $P < 0.05$ vs. BFG 25 μg/ml; a** = $P < 0.05$ vs. SA 25 μg/ml. a = $P < 0.05$ vs. heparin 5.0 μg/ml; a* = $P < 0.05$ vs. BFG 50 μg/ml; a** = $P < 0.05$ vs. SA 50 μg/ml. a = $P < 0.05$ vs. heparin 10.0 μg/ml; a* = $P < 0.05$ vs. BFG 100 μg/ml; a** = $P < 0.05$ vs. SA 100 μg/ml.

4. Conclusion

The sulfation of BFG was successfully accomplished and optimized to 3.68° of sulfation. The ratio of CSA to Pyr and temperature of reaction was observed to play a vital role in influencing the degree of substitution. The sulfated derivative (sBFG2) was found to be superior to the widely used polysaccharide SA in terms of work of shear, firmness, cohesiveness, consistency and index of viscosity parameters. Furthermore, sBFG2 exhibited absence of antinutritional factors, better antimicrobial activity and ability to prolong prothrombin time as compared to BFG and SA. Overall, the results suggested that sBFG2 could be exploited for diverse applications instead of SA.

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