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Structural characterization of an acidic polysaccharide from *Dalbergia sissoo* Roxb. leaves

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ABSTRACT

The composition and structure of an acidic polysaccharide from the leaves of *Dalbergia sissoo* was studied using hydrolytic, methylation, $^{1}H/^{13}C$ heteronuclear multiple quantum coherence (HMQC) and periodate oxidation experiments. The repeating unit of sissoo polysaccharide was found to be composed of α-L-rhamnose, β-D-glucuronic acid, β-D-galactose and β-D-glucose in the molar ratio of 1.00:1.00:2.00:2.33, respectively. The structure of polysaccharide was mainly composed of (1→2), (1→3), (1→4) linkages. Based on extensive laboratory experiments, the structure having the repeating units of the acidic polysaccharide from *sissoo* leaves, with unusual branching, was established.

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1. Introduction

Dalbergia sissoo Roxb. is a large deciduous perennial tree of family Fabaceae. It is naturally distributed in the foothills of the Himalayas from eastern Afghanistan through Pakistan and India to Nepal. It is primarily found below 900 m altitude but ranges naturally up to 1500 m.

Sissoo is one of the most useful multipurpose trees of South Asia. It is mainly grown for the finest quality timber. The heartwood is golden to dark brown, with density of 0.7–0.8 g/cm^3 (at 12% mc), extremely durable and resistant to termites. Young shoots, green pods and leaves contain up to 13% crude protein (dry weight basis) and dry matter digestibility is about 43% (Datt, Datta, & Singh, 2008). The fodder value of the tree is the highest in April and May when other sources of green fodder are scarce.

D. sissoo has been used in folk medicine for the treatment of various digestive disorders, skin diseases along with the conditions like emesis, ulcers, leucoderma, dysentery and stomach troubles (Asif & Kumar, 2009; Brijesh, Daswani, Tetali, Antia, & Birdi, 2006; Hajare et al., 2000). Bark, leaves and roots of *D. sissoo* are used as stimulant and astringent (Hussain, Shahazad, & Zia-ul-Hussnain, 2008; Shah, Mukhtar, & Khan, 2010).

The Ayurvedic system of Medicine in India considers the wood and the bark as aborificant, anthelmintic, antipyretic, aperitif, aphrodisiac, expectorant and refrigerant (Niranjan, Singh, Prajapati, & Jain, 2010; Shah et al., 2010). The wood and the bark are also used in the treatment of anal disorders, blood diseases, burning sensation, dysentery, dyspepsia, leucoderma and skin ailments. In Unani system of medicine, the wood is used for blood disorders, burning sensation, eye and nose disorder, scabies, scalding urine, stomach problems, and syphilis (Ahmad, 2007; Niranjan et al., 2010). The alternative wood is used in India for boils, eruptions, leprosy and nausea (Niranjan et al., 2010). The bark of the tree exhibits potent antioxidant and anti-inflammatory properties (Kumari & Kakkar, 2008).

Dried leaves of *D. sissoo* show antibacterial, anti protozoal, anti inflammatory activity (Hajare, Chandra, Sharma, & Tandon, 2001; Niranjan et al., 2010). Ayurvedic practitioners also prescribe the leaf juice for eye ailments. The alcoholic extract of *D. sissoo* leaves has significant anti-inflammatory effects in different models of inflammation, without any side effect on the gastric mucosa (Hajare et al., 2001). Rural people in India and Nepal use *D. sissoo* leaves for curing animals suffering non-specific diarrhea (Shah et al., 2010). Leaf extract is commonly used to treat sore throat, heart problem, dysentery, syphilis, and gonorrhea (Al-Qura'n, 2008; Duke, 1981; Shah et al., 2010). Leaf extract has also been found to exhibit 200% antioxidant activity in comparison with commonly used antioxidants like Selenium and vitamin E (Shah et al., 2010). The extract has also been found to be effective in the treatment of colorectal cancer along with other conventional treatments with chemotherapeutic agents. The juice of the leaves is used for curing the eye

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and nose diseases and is a strong anthelmintic. It is used in scabies, burning sensation of the body, scalding urine, syphilis, and digestive disorders (Ishtiaq, Khan, & Hanif, 2006). Boiled leaf filtrate is also used to wash hair for removing dandruff and enhance the hair growth (Sultana, Khan, Ahmad, & Zafar, 2006). The mucilaginous polysaccharide of the *sissoo* leaves is responsible for a wide spectrum of medicinal properties leading to its use in treating several ailments like in eye diseases, acute stage of diarrhea, dyspepsia, hemorrhoids and burning sensation, skin excoriations and acute stage of gonorrhea (Kirtikar & Basu, 1981; Rana, Kumar, & Soni, 2009; Sala, 1994).

In view of the strong medicinal activity of the *D. sissoo* leaves mucilage, structural characterization of mucilaginous polysaccharide was under taken. In our previous communication, it has been reported that the polysaccharide, isolated and purified from *D. sissoo* leaves, produced one tri-[I], one hepta-[II] and one nona-[III] saccharides with monosaccharide composition of β-D-glucose and α-L-rhamnose (2:1); α-L-rhamnose, β-D-galactose and β-D-glucuronic acid (1:3:3); and α-L-rhamnose, β-D-galactose and β-D-glucose (1:3:5), respectively (Rana et al., 2009).

In the present communication, detailed structure of *D. sissoo* leaf polysaccharide is being discussed. The structural features of polysaccharide were determined based on extensive chemical and degradative methods and spectroscopic techniques. The results also conclusively established a co-relation among the structures of three oligosaccharides and polysaccharide.

The complete structure of the *D. sissoo* leaf polysaccharide may provide a basis for further studies of the biological functions of the polysaccharide. Structure elucidation of the polysaccharide will also provide a scientific basis for the development of biological and medicinal use of the leaf polysaccharide for futuristic applications.

2. Experimental

The leaves of *D. sissoo* were collected from Forest Research Institute, Dehradun campus in the month of April. The leaves were air dried in the shade for four days and stored under cold conditions (0–5 °C).

2.1. General methods of analysis

Evaporations were performed at or below 40 °C in a rotary evaporator under reduced pressure. All melting points were uncorrected. Optical rotation was measured on Autopol-II, automatic polarimeter (Rudolph Research, Flanders, NJ) at 589 nm, D-lines of sodium. Partition paper chromatography studies were carried on Whatmann 1 and 3 mm sheets using the solvent systems: (S_1) n-butanol:ethanol:water (4:1:5, v/v/v, upper phase); (S_2) n-butanol:pyridine:water (6:4:3, v/v/v), and (S_3) ethyl acetate:acetic acid:n-butanol:water (4:3:2:2, v/v/v/v). Detection was effected with acetonical silver nitrate (R_1) and aniline phthalate spray (R_2). Detection with acetonical silver nitrate was done by treatment with reagents: (a) to silver nitrate solution (12.5 g in 10 ml water), 1 l of acetone was added with continuous shaking. Distilled water was added drop wise with stirring until the white precipitate completely dissolved to form a clear solution. (b) Sodium hydroxide (20 g) was dissolved in 400 ml of ethanol. (c) Aqueous ammonia solution.

The dried chromatograms were dipped and passed through reagent solution (a) for about 5 min, dried at room temperature and passed through reagent (b); when the dark brown spots were visualized, the paper was dipped in reagent (c) for some time with shaking (5–10 min). Finally, the chromatograms were washed with water and dried in air (Trevelyan, Proctor, & Harrison, 1950).

Gas liquid chromatography of the sugar mixtures was carried out on a Shimadzu Gas Chromatograph GC-9A fitted with a flame ionization detector (FID). The samples were analyzed in the form of their alditol acetates on ECNSS-M (3%) on Gas Chrom Q (100–120 mesh) packed into 5 × 1/8 inch. stainless steel column under the following operating conditions; column temperature 170 °C, nitrogen flow rate 35–40 ml/min (C_1). Nuclear magnetic resonance spectroscopy (NMR) of polysaccharide was done on Bruker Avance II 400 NMR spectrometer at 400 MHz at room temperature using D_2O as solvent.

2.2. Isolation and purification of polysaccharide

The polysaccharide from the leaves of *D. sissoo* was isolated and purified by the methods as described in previous communication (Rana et al., 2009). The pure polysaccharide was obtained in the form of a light brown amorphous powder (yield ~ 14%).

2.3. Complete hydrolysis

The pure polysaccharide of *D. sissoo* leaf (0.029 g) was subjected to hydrolysis with sulphuric acid (2 N, 5 ml) for 18 hr on a steam bath. The hydrolysate was cooled, neutralized with saturated solution of barium carbonate by drop wise addition till the pH of the solution reached at 7, filtered and the residue washed with water. The combined filtrate was concentrated.

2.4. Quantitative estimation of glucuronic acid in D. sissoo leaf polysaccharide

The quantitative estimation of glucuronic acid in the polysaccharide was done by the carbazole–sulfuric acid reaction (Dische, 1947, 1950). The intensity of the color developed after reaction in the polysaccharide sample was compared to the standard solutions (10–100 μg/c.c.) of glucuronic acid at 535 nm.

2.5. Reduction of carboxyl groups of polysaccharide

The polysaccharide was reduced by the method of Taylor, Shively, and Conard (1976). A solution of the polysaccharide (0.06 g) in distilled water was adjusted to pH 4.75 and 1 mole of solid 1-ethyl-3-(3-dimethylaminopropyl) carbodimide (EDC) was added. The reaction was allowed to proceed for 45 min at pH 4.75 until hydrogen ion uptake ceases followed by dropwise addition of 3 M solution of sodium borohydride dropwise at 25 °C in 1 h period. The pH was maintained at 7.0 by addition of 4 M hydrochloric acid. The reaction mixture was then made slightly acidic to destroy remaining sodium borohydride if any. The solution was dialysed exhaustively against distilled water and concentrated under reduced pressure to small volume (4 ml).

The reduced polymer was precipitated by addition of three volumes (12 ml) of ethanol, filtered, washed with ethanol (50 ml) and dried (0.043 g). The reduced polysaccharide did not give carbazole–sulphuric acid color test for uronic acid.

2.6. Alditol acetates

Alditol acetates of the hydrolyzed material were prepared by the method of Jansson, Kenne, Liedgren, Lindberg, and Lönngren (1976). Sodium borohydride (0.020 g) was added to hydrolyzates, and the mixture was kept for 18 h at room temperature. The mixture was neutralized by slow addition of dilute acetic acid (6 ml), and concentrated to dryness in the vacuum rotator at 40 °C. Sodium was removed by passing it through cation exchange resin (Dowex-50W-X8). Boric acid was removed by codistillations, in the vacuum rotator with methanol (3 × 5 ml). The residue was treated with

redistilled acetic anhydride and pyridine, 1:1 (4 ml) and refluxed for 6 h. Toluene (6 ml), which gave an azeotrope with acetic anhydride, was added, and the mixture was distilled as above, until the rate of distillation decreases. A new portion of toluene (6 ml) was added and the solution was concentrated to dryness. It was dissolved in water (10 ml) and the acetylated sugars separated by shaking with dichloromethane (4 × 25 ml). Traces of water present in dichloromethane were removed by adding anhydrous sodium sulphate followed by filtration and washing with dichloromethane before concentration.

2.7. Methylation of polysaccharide

Methylation study of polysaccharide was carried out in two parts. The polysaccharide was methylated completely according to the method of Hakomori (1964) followed by Purdie & Irvine (1904) in its native state (0.03 g) and reduced polysaccharide (0.03 g) by Hakomori (1964) method, as the native polysaccharide could be completely methylated by following both the methods sequentially, whereas reduced polysaccharide could be methylated by following only Hakomori method. Each methylated sample was recovered by chloroform extraction. These extracts were evaporated up to dryness (unreduced methylated polysaccharide, 0.015 g; reduced methylated polysaccharide, 0.012 g). After evaporation of chloroform extracts to dryness, the residues were hydrolysed with formic acid (90%, 10 ml) for 1 h on steam bath, the solutions evaporated and treated with aqueous sulphuric acid (0.13 M, 15 ml) for 18 h on a steam bath.

2.8. Periodate oxidation

To a solution of polysaccharide in water (0.2011 g in 50 ml) an aqueous solution of sodium metaperiodate (0.8013 g in 50 ml) was added and the volume of the resultant solution was made up to 250 ml. A blank solution of sodium metaperiodate (0.8000 g in 250 ml water) was also prepared. These were kept in dark at room temperature (25 °C) for 192 h. To determine periodate consumed, an aliquot (5 ml) of the periodate reaction mixture was added to a solution containing distilled water (20 ml), potassium iodide (20%, 2 ml) and sulphuric acid (0.5 N, 3 ml). The liberated iodine was immediately titrated with 0.1 N sodium thiosulphate solution using starch as an indicator (Bobbitt, 1956; Malaprade, 1928; Rankin & Jeanes, 1954).

Liberation of formic acid was determined by the methods reported earlier (Abdel-Akher & Smith, 1951; Bobbitt, 1956; Dayer, 1956; Halsall, Hirst, & Jones, 1947). To an aliquot (5 ml) of the periodate reaction mixture was added acid free ethylene glycol (0.5 ml), followed by an excess of potassium iodide (20%, 5 ml) after 10 min. To the above solution, an excess of 0.01 N sodium thiosulphate (10 ml) was added, and the unused sodium thiosulphate was back titrated with 0.01 N iodine solution using starch as an indicator. A blank solution was titrated concurrently.

3. Results and discussion

The polysaccharide was isolated from air dried leaves in 14% yield as discussed in Section 2. The polysaccharide was found homogenous on size exclusion chromatography on Sephadex G-150 as detailed previously (Rana et al., 2009).

Chemical analysis of pure polysaccharide has shown specific rotation, $[\alpha]_D^{37}$ – 14.6° [c 0.21%, H_2O], ash content 0.36% (on dry basis), nitrogen 0.13%, pH 6.46.

Complete hydrolysis of the polysaccharide followed by paper chromatography revealed the presence of four spots, corresponding to D-galactose, D-glucose, L-rhamnose and D-glucuronic acid, respectively. The presence of uronic acid in the polysaccharide was also confirmed by qualitative and quantitative carbazole reactions (Dische, 1947, 1950). The presence of 15.7% glucuronic acid was determined in the polysaccharide on the basis of carbazole–sulfuric acid color reaction. For the quantitative estimation of sugar mixture, a part of the hydrolysate was converted into its alditol acetate by the method of Jansson et al. (1976) and analysing it by GLC (Bishop, 1964) using ECNSS-M (3%) column employing conditions C_1. It showed that the retention time of the three peaks coincided with the retention time of L-rhamnopyranose, D-galactopyranose and D-glucopyranose. The molar ratio among L-rhamnose, D-galactose, D-glucose was determined as 1.00:2.00:2.33, respectively.

The polysaccharide was then reduced by the method of Taylor et al. (1976) for the conversion of uronic acid moieties into neutral sugars. The reduced polysaccharide was completely hydrolysed and the hydrolysate converted into its alditol acetate derivatives (Jansson et al., 1976) and analyzed by GLC under the same conditions as in the case of unreduced polysaccharide. It showed an increase in the molar ratio of D-glucopyranose, which was due to the presence of D-glucuronic acid. The molar ratio of L-rhamnose, D-galactose and D-glucose was determined as 1.00:2.00:3.33, respectively in the reduced polysaccharide. On the basis of GLC results of reduced as well as unreduced polysaccharide, the molar ratio among L-rhamnose, D-glucuronic acid, D-galactose, and D-glucose was determined as 1.00:1.00:2.00:2.33, respectively.

3.1. Methylation analysis of D. sissoo leaf polysaccharide

Methylation study of the reduced and unreduced polysaccharide was done. The carboxyl groups of glucuronic acid residues in the polysaccharide were reduced by the method of Taylor et al. (1976). The native polysaccharide was methylated by the procedure of Hakomori (1964) followed by Purdie & Irvine (1904) whereas carboxyl reduced polysaccharide was methylated by the procedure of Hakomori (1964). The IR spectrum (KBr) of carboxyl reduced methylated product showed complete absence of —OH band (3590–3225 cm^{-1}) as compared to native polysaccharide but the IR of unreduced methylated polysaccharide showed the considerable presence of —OH group. Therefore, it was again methylated by Purdie's methylation method (Purdie & Irvine, 1904). The IR spectrum (KBr) of resulting unreduced polysaccharide product showed complete absence of —OH band (3590–3225 cm^{-1}) this time indicating clearly complete methylation.

The completely methylated product obtained from native polysaccharide was syrupy, yellowish brown in color, whereas the methylated product obtained from carboxyl reduced polysaccharide was dark brown in color.

The methylated polysaccharide samples were hydrolysed separately and transformed into their alditol acetates according to the method of (Jansson et al., 1976). The peaks corresponding to different methylated sugars were obtained.

On comparison of results obtained from GLC of methylated native polysaccharide and carboxyl reduced polysaccharide (Table 1), there was an increase in 1 mole of 2,3,6-tri-*O*-methyl-D-glucose and 2 moles of 2,3,4,6-tetra-*O*-methyl-D-glucose in case of carboxyl reduced methylated polysaccharide. The 3 moles increase in GLC results of carboxyl reduced polysaccharide suggests the presence of 3 glucuronic acid moieties in the repeating unit of *D. sissoo* polysaccharide. Further, the increased percentage of 2,3,6-tri-*O*-methyl-D-glucose and 2,3,4,6-tetra-*O*-methyl-D-glucose obtained from D-glucuronic acid was 15.7%, which substantiates the results of quantitative determination of D-glucuronic acid by carbazole method.

Based on the methylation study of the *D. sissoo* polysaccharide and from the previously established structures of three constituting oligosaccharide of *D. sissoo* polysaccharide (Rana et al., 2009), it can

Table 1
Molar proportion[a] of the different partially methylated sugars obtained from *D. sissoo* polysaccharide.

Sugars	Molar ratio	
	Native	Carboxyl reduced
2,3-di-*O*-methyl-D-galactose	1	1
2,3,6-tri-*O*-methyl-D-galactose	1	1
2,4,6-tri-*O*-methyl-D-galactose	4	4
3-*O*-methyl-L-rhamnose	1	1
2-*O*-methyl-L-rhamnose	2	2
3,6-di-*O*-methyl-D-glucose	1	1
2,3,6-tri-*O*-methyl-D-glucose[b]	**2**	**3**
3,4,6-tri-*O*-methyl-D-glucose	1	1
2,3,4,6-tetra-*O*-methyl-D-glucose[b]	**3**	**5**

[a] GLC of methylated polysaccharides samples was carried out with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.
[b] Bold values indicate the difference in no. of moles of methylated sugars in native and reduced polysaccharide.

Table 3
^{1}H and ^{13}C NMR chemical shifts (ppm) for *D. sissoo* leaves polysaccharide.

Sugar residues	Assignments (δ)	
	^{1}H	^{13}C
→1)-α-L-Rha*p*	5.21 (H-1)	101.8 (C-1)
→1)-β-D-Glc*p*	4.62 (H-1)	103.0 (C-1)
→1)-β-D-Glc*p*A	4.55 (H-1)	104.2 (C-1)
→1)-β-D-Gal*p*	4.60 (H-1)	105.3 (C-1)
(1→6a, 6b)-β-D-Gal*p*	3.74, 3.92 (H-6a & H-6b)	68.3 (C-6)
(1→4)-β-D-Glc*p*	3.96 (H-4)	78.8 (C-4)
(1→3)-β-D-Gal*p*	4.13 (H-3)	83.6 (C-3)
(1→3)-α-L-Rha*p*	4.44 (H-3)	80.4 (C-3)
(1→2)-β-D-Glc*p*	3.30 (H-2)	84.0 (C-2)
(1→2)-α-L-Rha*p*	4.08 (H-2)	79.2 (C-2)
(1→4)-α-L-Rha*p*	4.38 (H-4)	80.8 (C-4)
(1→4)-β-D-Gal*p*	4.36 (H-4)	77.1 (C-4)
(1→4)-β-D-Glc*p*A	3.71 (H-4)	75.5 (C-4)
—CH3 of – α-L-Rha*p*	1.24, 1.34	19.5, 19.8

be concluded that *D. sissoo* leaf polysaccharide has the structural components shown in Table 2.

3.2. NMR spectroscopy of D. sissoo leaf polysaccharide

The NMR sample used for structural studies was highly viscous and gave relatively broad signals in ^{1}H spectrum. Further, a number of attempts were made for recording of ^{13}C spectrum but the spectrum did not develop properly. Assignment of signals and identification of the sugar moieties was done by heteronuclear multiple quantum coherence (HMQC) followed by comparison of the chemical shifts with published data on similarly substituted sugar residues (Table 3). The signals of two well resolved doublets at 1.24/19.5 and 1.34/19.8 in $^{1}H/^{13}C$ HMQC spectrum of polysaccharide (Fig. 1) were assigned to the CH_3 of rhamnose units. The two pairs of doublets centered at δ 1.24/19.5 and 1.34/19.8 were, respectively assigned to (1→2, 4) linked and (1→3,4) linked α-L-Rha*p* residues (Ali et al., 2011; Kocharova et al., 1989; Yuliya et al., 2004). The anomeric signal at 5.21/101.8 in ^{13}C was assigned for the anomeric position of α-L-Rha*p* (Bystrova et al., 2002; Chattopadhyay et al., 2011; Sengkhamparn, Verhoef, Schols, Sajjaanantakul, & Voragen, 2009). Signals at 4.62/103.0; 4.55/104.2 and 4.60/105.3 were assigned for anomeric configuration of β-D-Glc*p* (Cérantola, Lemassu-jacquier, & Montrozier, 1999; Smiderle et al., 2011; Vincent, Faber, Neeser, Stingele, & Kamerling, 2001), β-D-Glc*p*A (Bartodziejska *et al*., 1999; De Pinto

Table 2
Component sugar residues with linkage in the minimal unit in the structure of *D. sissoo* leaves polysaccharide.

Component Sugar with linkage type	No. of moles in			
	Polysaccharide	Oligosaccharides		
		O-I	O-II	O-III
6 ↓ → 4-β-D-Gal*p*1→	1	–	1	–
→ 4-β-D-Gal*p*1→	1	–	–	1
→ 3-β-D-Gal*p*1→	4	–	1	1
→ 4-α-L-Rha*p*1→ 2 ↑ → 4-α-L-Rha*p*1→	1	–	–	–
→ 4-α-L-Rha*p*1→ 3 ↑ → 4-β-D-Glc*p*1→	2	–	1	1
2 ↑ → 4-β-D-Glc*p*1→	1	–	–	1
→ 4-β-D-Glc*p*1→	2	–	–	–
→ 2-β-D-Glc*p*1→	1	–	–	1
β-D-Glc*p*1→	3	2	–	3
→ 4-β-D-Glc*p*A1→	1	–		–
β-D-Glc*p*A1→	2	–	3	–
→ 4-α-L-Rha*p*2 →	–	1	–	–
→ 3-β-D-Gal*p*	–	–	1	1
Total nos. of units in each repeating unit	19	3	7	9

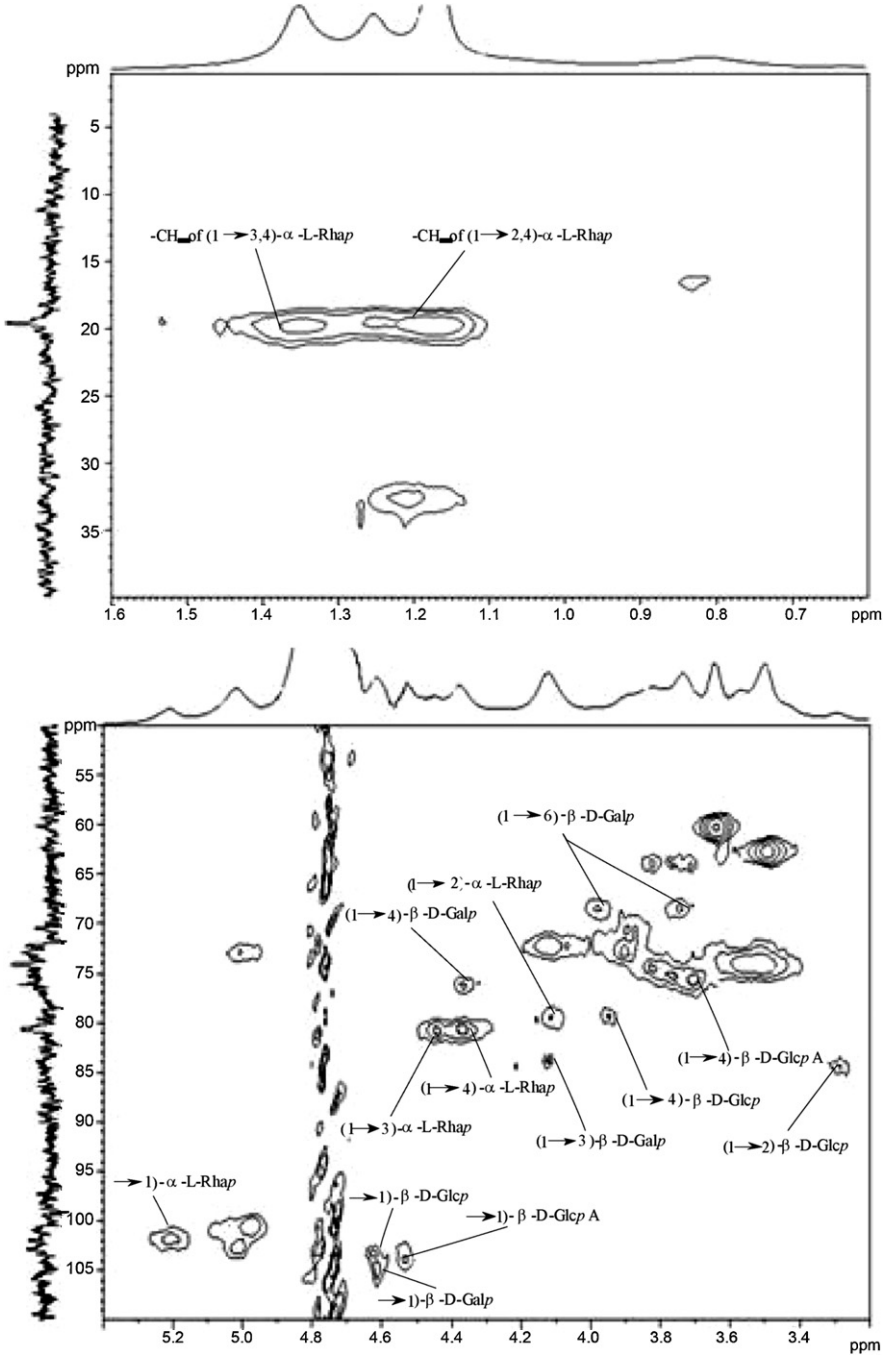

Fig. 1. $^{1}H/^{13}C$-HSQC spectrum of the *D. sissoo* leaves polysaccharide.

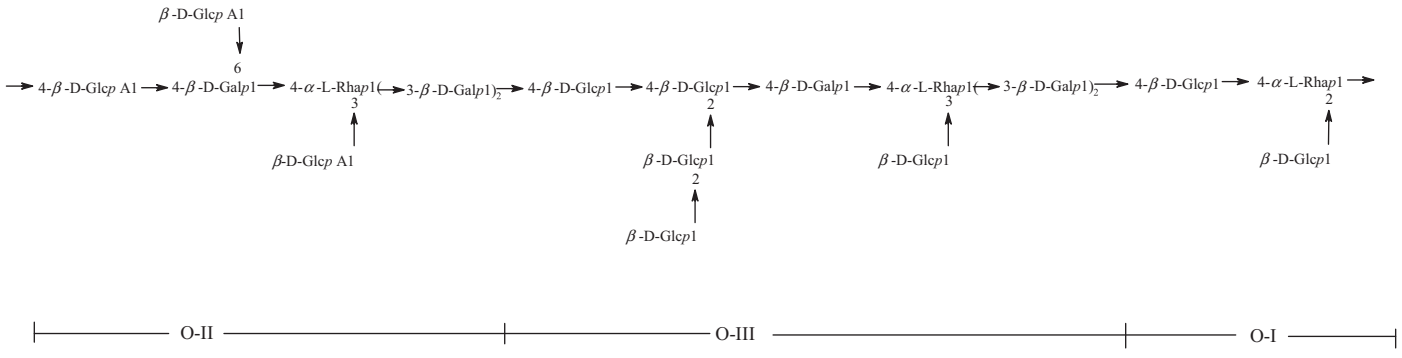

Fig. 2. Repeating unit structure of the *D. sissoo* leaves polysaccharide.

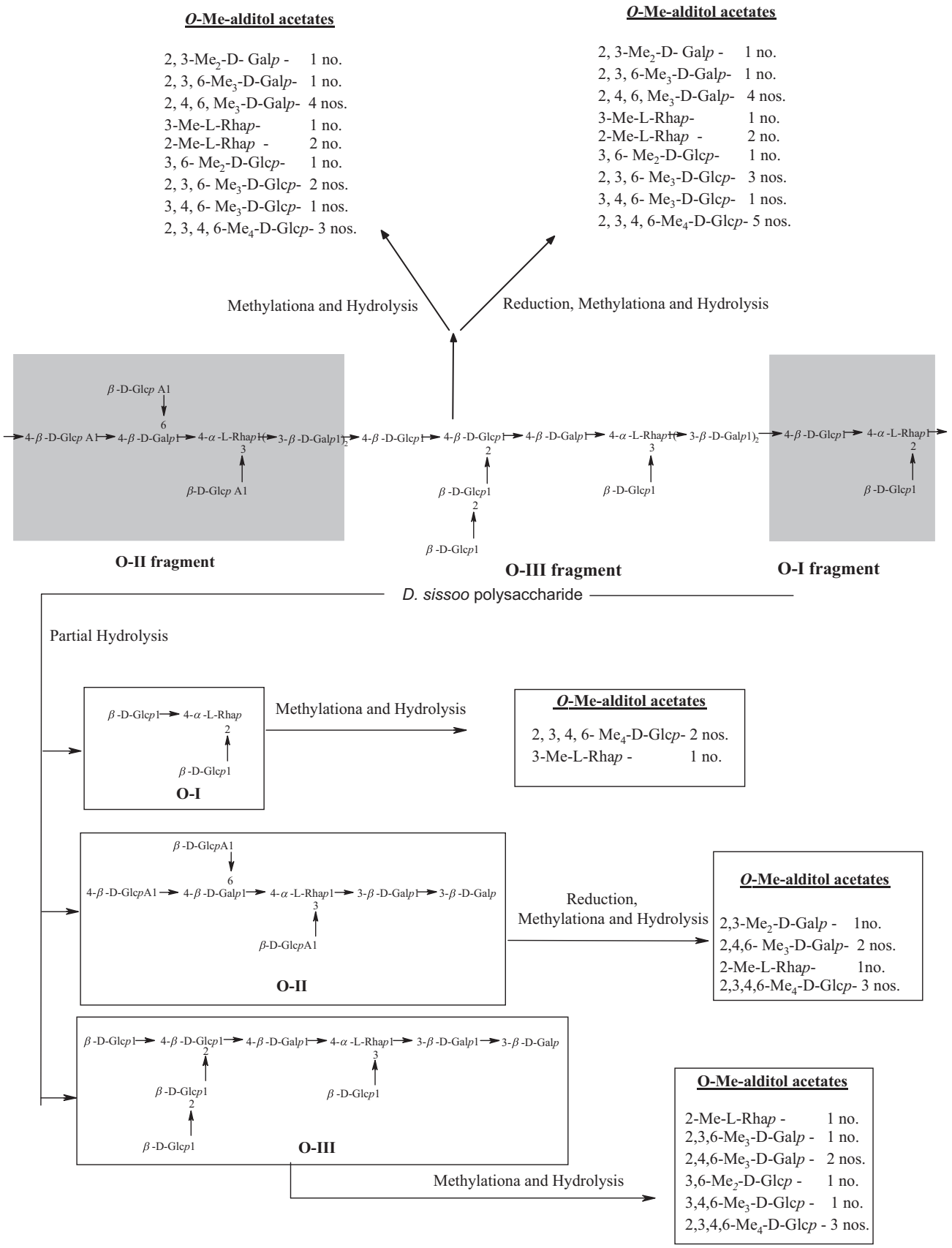

Fig. 3. Schematic representation of oligosaccharide and monosugars generation from *D. sissoo* leaves polysaccharide.

et al., 1995; Redgwell et al., 2011) and β-D-Gal*p* (Kohno et al., 2009; Nimtz et al., 1997; Sun, Cui, Tang, & Gu, 2010) respectively. The signals at 3.74, 3.92/68.3 were assigned to H-6a, H-6b/C-6 of (1→6) linked β-D-Gal*p* (Bergström, Jansson, Kilian, & Sørensen, 2000; Sun et al., 2010; Yu et al., 2009). The signal obtained at 3.96/78.8 was assigned to H-4/C-4 of (1→4) linked β-D-Glc*p* (Karlsson, Jansson, & Sørensen, 1998; Kogan, Matulova, & Michalkova, 2002; Verhoef et al., 2002). Signal at 4.13/83.6 was indicated for H-3/C-3 position of (1→3) linked β-D-Gal*p* (Agrawal, 2002; Golovchenko, Ovodova, Shashkov, & Ovodov, 2002). Three different signals attributed at 4.43/80.4, 4.08/79.2 and 4.38/80.8 were assigned for the third (Ali et al., 2011; Bystrova et al., 2002; Zdorovenko et al., 1999), second (Dinand, Excoffier, Liénart, & Vignon, 1997; Guentas et al., 2001; Sun et al., 2010) and fourth (Capek, Kardosová, & Lath, 1999) positions of (1→3), (1→2) and (1→4) linked α-L-Rha*p*. Signal appeared at 3.30/84.0 was assigned for H-2/C-2 position of (1→2) linked β-D-Glc$_p$ (Kohno et al., 2009; Verhoef et al., 2002; Vincent et al., 2001). Two signals at 4.36/77.1 and 3.71/75.5 indicated the appearance of (1→4) linked β-D-Gal*p* (Golovchenko et al., 2002; Vincent et al., 2001) and β-D-Glc*p*A (Bartodziejska et al., 1999; Ovchinnikova et al., 2004).

To accommodate various types of linkages marked by the methylation analysis of the polysaccharide, and supported by NMR and further taking into consideration the structures of oligosaccharides, the most plausible structure of the repeating unit of the *D. sissoo* leaf polysaccharide is proposed (Fig. 2). The proposed structure accommodates the molar proportion of methylated sugar residues obtained by methylation analysis. It also shows the presence of L-rhamnose, D-glucuronic acid, D-galactose and D-glucose in the molar ratio of 1.00:1.00:2.00:2.33, which corroborates the results obtained by complete hydrolytic studies of the polysaccharide (i.e., 1.00:1.00:2.00:2.33). The scheme given in Fig. 3 is a representation of polysaccharide structure which also indicates the constituting oligosaccharides and O-methyl alditol acetates obtained on GLC analysis of methylated polysaccharide and oligosaccharide.

3.3. Periodate oxidation studies of D. sissoo leaf polysaccharide

Evidence supporting the presence of multiple branching in the framework of polysaccharide has also been obtained from the results of periodate oxidation. The carboxyl reduced polysaccharide when subjected to oxidation with sodium meta periodate, consumed 0.83 mole of periodate per anhydrohexose unit and released 0.26 mole of formic acid per anhydrohexose unit. As the repeating unit of the polysaccharide structure consists of 19 sugar residues, the above results can be interpreted in terms of the consumption of 15.77 mole of periodate with simultaneous liberation of 4.94 moles of formic acid by the repeating unit of the polysaccharide.

Since 2,3-di-*O*-methyl-D-galactose (1 mole), 2,3,6-tri-*O*-methyl-D-galactose (1 mole), 2,3,6-tri-*O*-methyl-D-glucose (3 moles), 3,4,6-tri-*O*-methyl-D-glucose (1 mole) and 2,3,4,6-tetra-*O*-methyl-D-glucose (5 moles) are expected to take part in periodate oxidation, theoretically this would result in the uptake of 16 moles of periodate and simultaneous release of 5 moles of formic acid by repeating unit of polysaccharide. In other words, theoretically the proposed structure (Fig. 2) would consume 0.84 mole of periodate per anhydrohexose unit and liberate 0.263 mole of formic acid per anhydrohexose unit.

Thus, periodate oxidation data of polysaccharide as calculated on the basis of proposed structure and obtained experimentally are found to be in agreement with each other. Therefore, structure (Fig. 2) proposed on the basis of methylation analysis and NMR spectroscopy gets corroborated by the oxidation studies.

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