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Immunostimulant Fractions of Novel Hexa and Heptasaccharide from Donkey's Milk

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ABSTRACT

The proposed oligosaccharide mixture of Donkey's milk has shown significant stimulation of antibody, delayed type hypersensitivity response to sheep red blood cells in BALB/c mice. The orally treated animals showed a six time increase in haemagglutinating antibody (HA) titre, two times increase in haemolytic plaque-forming cells (PFC) and delayed type hyper-sensitivity (DTH) response. The non-specific immune response of treated animals also showed a two times increase in macrophage migration index (MMI). Two novel oligosaccharides have been isolated from this oligosaccharide mixture obtained from donkey's milk having immunostimulant activity. These compounds were isolated by a combination of gel filtration chromatography, silica gel column chromatography of derivatized oligosaccharides while their homogeneity was confirmed by HPLC. The structures of these isolated oligosaccharides were elucidated on the basis of NMR spectroscopy, mass spectrometry and also with help of Structure reporter groups.

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INTRODUCTION

Milk is a very effective biological fluid responsible for the development of neonates [1] and the oligosaccharides *i.e.* present in milk either in form of free molecules or conjugated with other compounds are also beneficial for new born babies as well as adults. The oligosaccharides isolated from various milk sources are categorized in two classes *i.e.* sialylated oligosaccharide and nonsialylated oligosaccharide. Both classes of oligosaccharides have been tested for their varied biological activities. The sialylated oligosaccharides from pooled human milk recognize cancer associated antigens expressed by most human adenocarcinmas of the breast [2]. The S Le^{X} tetrasaccharide sequence reacts specifically to monoclonal antibody CSLEX 1, obtained after immunization of mouse with human gastric adenocarcinoma memberane proteins [2]. The sialyl-Le^{a} (Lewis^{a}) structure in glycolipids or glycoproteins has been defined as gastrointestinal tumor associated antigen [3] showing that the glycosyltransferases or glycosidases expressed in the lactating mammary gland may

be similar to those found in oncofoetal tissues [4]. Oligosaccharide having the sialyl-Le^x and Le^a or Le^x-i structure have been found in mucin-type glycoproteins and glycolipids in a variety of human cancers [5] similar oligosaccharides with both the sialyl-Le^a and blood group H structure or with both sialyl-Le^a difucosyl (Le^a-Le^x) structure exhibit high affinity to an antibody directed to a human squamous lung carcinomas [6,7]. Bovine milk oligosaccharides have several potentially important biological activities including the prevention of the pathogen binding to the intestinal epithelial and as an nutrients for beneficial bacteria [8]. Oligosaccharide of donkey milk has ability to stimulate non-specific and specific immunological resistance [9] and prevention of athereosclerosis [10].

The earlier workers have elucidated the structure of milk oligosaccharides by chemical degradation and spectroscopic techniques (NMR and FABMS) [11]. Keeping above mentioned basic core units in mind the structures of various milk oligosaccharides were established by comparing the chemical shift (^{1}H NMR and ^{13}C NMR) of anomeric signals and other important signals of isolated milk oligosaccharides with the chemical shifts of known milk oligosaccharides. In this paper we report the immunostimulant activity of donkey milk oligosaccharide mixture and we also report the isolation and structural characterization of two novel oligosaccharides (1) (equinose) and (2) (asinose) from this oligosaccharide mixture.

αGal(1–4)
αGal(1–4)βGlc(1–3)βGal(1–3) ⟩ βGlcNAc(1–3)βGal(1–4)Glc

Equinose (1)

αNeu5Ac(2–6)αGal(1–4)
αFuc(1–3) ⟩ βGlcNAc(1–3)βGal(1–4)Glc

Asinose (2)

EXPERIMENTAL

General procedures: Optical rotations were measured with a Perkin-Elmer 241 automatic polarimeter in 1 cm tube. ^{1}H, ^{13}C and COSY NMR spectra was recorded in $CDCl_3$ and D_2O at 25 °C on a Bruker AM 300 FT NMR spectrometer. FAB mass spectrum was recorded with JEOL mass spectrometer model JMS-SX 102 FAB with DA 6000 data system. The sugars were visualized on thin layer chromatography (TLC) with 50 % aq. H_2SO_4 reagent and on paper chromatography with acetyl acetone and *p*-dimethylaminobenzaldehyde reagents [12]. The absorbent for TLC was silica gel G (SRL) and for column chromatography, silica gel (SRL, 60-120 mesh) was used. Paper chromatography was performed on 3 Whatman No.1 filter paper using ethyl acetate-pyridine (2:1) saturated with H_2O as solvent system. Sephadex G-25 (Pharmacia) was used in gel permeation chromatography. Freeze drying of the compounds was done with the help of a CT 60e (Heto) lyophilizer and centrifuged by a cooling centrifuge Remi Instrument C-23 JJRCI 763. To check the homogeneity of the compounds, reverse phase HPLC system was used equipped with Perkin-Elmer 250 solvent delivering system, 235 diode array detector and G.P. 100 printer plotter. Authentic sample of glucosamine, galactose and glucose were purchased from Sigma- Aldrich Chemicals.

Purification of donkey milk oligosaccharides: Donkey's milk (25 L) was collected from a domestic donkey and was stored at -20 °C. The milk was processed by the method of Kobata *et al.* [13]. It was centrifuged for 15 min at 5000 rpm at -4 °C. The solidified lipid layer was removed by filtration through glass wool column in cold. Ethanol was added to the clear filtrate to a final concentration of 68 % and the resulting solution was left overnight at 0 °C. The white precipitate formed, mainly of lactose and protein was removed by centrifugation and washed twice with 68 % ethanol at 0 °C. The supernatant and washings were combined and filtered through a micro filter (0.24 mm) (to remove remaining lactose) and lyophilized affording crude oligosaccharide mixture (6.62 g). The lyophilized material responded positively to Morgan-Elson test [14] and thiobarbituric-acid [15] assay suggesting the presence of N-acetyl sugars and sialic acid in oligosaccharide mixture. This lyophilized material (mixture of oligosaccharide) was further purified by fractionating it on Sephadex G-25 chromatography using glass triple distilled water as eluant at a flow rate of 3 mi/mm. Each fraction was analyzed by phenol sulphuric acid reagent [16] for the presence of neutral sugar.

Donkey milk oligosaccharide mixture chromatographed over Sephadex G-25 (1.6 × 40 cm) column: The repeated gel filtration was performed by Sephadex G-25 chromatography of crude donkey milk oligosaccharide mixture. The oligosaccharide mixture was packed in a column (1.6 × 40 cm) (void volume = 25 mL) equilibrated with glass triple distilled water (TDW) and left for 10-12 h to settle down. The material was applied onto a Sephadex G-25 column and was eluted for separation of protein and glycoprotein from oligosaccharide mixture (low molecular weight component). Presence of neutral sugars was monitored in all eluted fractions by phenol-sulphuric acid test [16]. The sephadex G-25 chromatography of donkey milk oligosaccharide mixture which was monitored by UV spectrophotometry showed five peaks *i.e.* I, II, III, IV and V. A substantial amount of proteins, glycoproteins and serum albumin were eluted in the void volume which was confirmed by positive colouration with *p*-dimethylaminobenzaldehyde reagent [12] and phenol-sulphuric acid reagent [16]. Fractions under peaks II, III and IV gave a positive phenol-sulphuric acid test which showed the presence of oligosaccharide mixture in donkey milk. They were pooled together and lyophilized.

Acetylation of oligosaccharide mixture: The pooled fraction (II, III and IV) (2.21 g) which gave positive phenol-sulphuric acid test was acetylated with pyridine (45 mL) and acetic anhydride (30 mL) at 60 °C and the solution was stirred overnight. The mixture was evaporated under reduced pressure and the viscous residue was taken in $CHCl_3$ (250 mL) and washed in sequence with 2 N HCl (1 × 25 mL), ice cold 2 N $NaHCO_3$ (2 × 25 mL) and finally with H_2O (2 × 25 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness yielding the acetylated mixture (2.45 g). The acetylation converted the oligosaccharides into their non-polar acetyl derivatives which were resolved very nicely on TLC.

Purification of acetylated milk oligosaccharide on silica gel column: Separation of the acetylated products (2.4 g) was carried over silica gel using varying proportions of C_6H_6:$CHCl_3$, $CHCl_3$ and $CHCl_3$:CH_3OH as eluants. Repeated column chromatography (five times) led to the isolation of two chromatographically pure compounds b (108 mg) and a (132 mg).

Deacetylation of compounds: Compound b (25 mg) obtained from column chromatography of acetylated oligosaccharide mixture was dissolved in acetone (2 mL) and NH_3 (1 mL) was added and left overnight in a stoppered hydrolysis flask. Ammonia was removed under reduced pressure and the product was washed with $CHCl_3$ (3 × 3 mL) (to remove acetamide) and was finally freeze dried giving the deacetylated oligosaccharide (**1**) (18.5 mg) having a comparable retention time as that of R_4. Compound a (28 mg) was deacetylated by the same method as for compound b, resulting in deacetylated compound (**2**) (21 mg) having same retention time as R_7.

Methylglycosidation/acid hydrolysis of compounds 1 and 2: Compound **1** (12 mg) was refluxed with absolute CH_3OH (2 mL) at 70 °C for 18 h in the presence of cation exchange IR-120 (H^+) resin (1 mg). The reaction mixture was filtered while hot and filtrate was concentrated. To a solution of methylglycoside of **1** in 1,4-dioxane (2 mL), 0.1 N H_2SO_4 (2 mL) was added and the solution was warmed for 0.5 h at 50 °C. The hydrolysis was complete after 26 h. The hydrolyzate was neutralized with freshly prepared $BaCO_3$, filtered and concentrated under reduced pressure to afford Glc, Gal, GlcNAc, α and β-methylglucosides identified by on comparison with authentic samples (TLC, PC & $[\alpha]_D$). Methylglycosidation and acid hydrolysis of compound **2** (12 mg) was performed by the same method as for compound **1**, leading to the isolation of α and β-methylglucosides along with Glc, Gal, GlcNAc, Fuc and sialic acid idenfied by comparison with authentic samples (TLC, PC & $[\alpha]_D$).

Methylation/acid hyrolysis of compound 2: Sodium hydride NaH (3 mg) was added to compound **2** (20 mg) in THF (1 mL). The mixture was stirred at room temperature for 1h and then cooled to 0 °C. CH_3I (0.05 mL) as added and the reaction mixture were allowed to reach to room temperature over a period of 3 h. Excess NaH was destroyed by the addition of methanol, the solvents were evaporated and the residue was taken in chloroform. The chloroform solution was washed twice with aqueous NaCl, once with water, dried, filtered and then concentrated. To a solution of methylated compound A in 1,4-dioxane (2 mL), 0.1 N H_2SO_4 (2 mL) was added and the solution was warmed for 0.5 h at 50 °C. The hydrolysis was completed after 22 h exhibiting six spots on TLC. In the hydrolysate, one of the compounds was identified as 6-O-methyl, 2-acetyl amino glucopyranose on comparison with synthetically prepared authentic sample of 6-O-methyl, 2-acetyl amino glucopyranose (TLC, PC).

Evaluation of antigen specific stimulation

Animals: Swiss mice of either sex (20-22 g) from the CDRI colony were used for this study. The animals had access to the standard diet and H_2O.

Treatment: Mice were fed orally at the dose of 2.5 mg/kg for 7 consecutive days. Mice of same group were kept untreated which served as controls.

Non-specific immune response: For non-specific immune response 3 mice each from treated and untreated group were taken and killed by cervical dislocation, peritoneal exudate cells (macrophages) were taken out and used for macrophage migration index (MMI). The migration pattern of peritoneal macrophages *in vitro* was studied according to the method of Saxena *et al.* [17,18]. Briefly, the PEC, packed in a micro-haematocrit capillary tube of uniform diameter, was allowed to migrate in a migration chamber (LAXBRO, India) for 18-24 h. The ratio of the area of migration of macrophages from the control and the experimental animals was expressed as macrophage migration index (MMI). Rests of mice of both the groups were used for antigen specific immune response.

Specific immune response to sheep red blood cells (SRBC): Sheep blood was collected from the jugular vein in Alsever's solution (pH 6.4). The erythrocytes were collected by centrifugation and washed three times with PBS and dispensed in the same medium to give a suspension of 1×10^8 cells/mL. One set each of the treated and control animals (four animals in each group were immunized by injecting i.p. with 1×10^8 SRBC (day Zero). Five days later, haemagglutinating antibody (HA) titre, haemolytic plaque-forming cells (PFC) assay and delayed type hyper-sensitivity (DTH) response to SRBC were determined.

Haemagglutinating antibody (HA) titre: Serum was collected from the blood samples of individual mice by retro-orbital puncture. Antibody levels were determined by the microtitre haemagglutination technique. Briefly, 50 µL aliquots of a two-fold dilution of sera were prepared in 0.15 M PBS (pH 7.2) and dispensed in V shape bottom microtitre plates; 1 % SRBC suspension (25 µL) in PBS was dispensed into each well and mixed thoroughly. After 1.5-2.0 h of incubation at room temperature, the reciprocal of the highest dilution of test sample giving 50 % agglutination essentially according to the method of Hayden [19] was expressed as the HA titre.

Haemolytic plaque-forming cells (PFC) assay: The assay was done according to the technique of Jerne and Nordin [20]. The test was performed in duplicate for each individual mouse and the mean values for the groups were expressed as mean PFC/10^6 spleen cells.

Delayed-type hypersensitivity (DTH): Delayed-type hypersensitivity response to SRBC antigen was induced in mice by the method of Doherty [21]. The groups of SRBC - immunized mice were challenged by injecting 1×10^8 SRBC (50 µL) into the right foot pad. After 24 h, the thickness of the left hind foot pad was measured with the help of Schnelltaster (Kroplin, FRG). The foot pad reaction was expressed in mm as the difference in thickness between the right foot pad injected with SRBC and the other with PBS.

^{1}H NMR of compound 1 (equinose) in D_2O at 300 MHz: 2.25 (s, 3H, $NHCOCH_3$, β-G1cNAc (S_3), 3.25 (t, 1H, *J* = 7.5 Hz, β-Glc (S_1), H-2), 3.30 (t, 1H, *J* = 7.5 Hz, β-Glc (S_6), H-2), 4.00 (d, 1H, *J* = 3.3 Hz, β-Gal (S_4), H-4), 4.09 (d, 1H, *J* = 4.5 Hz, β-Gal (S_2), H-4), 4.46 (d, 2H, *J* = 7.5 Hz, -βGal (S_2 and S_4), H-1), 4.59 (d, 1H, *J* = 7.8 Hz, β- Glc (S_1), H-1), 4.65 (d, 1H, *J* = 8.1 Hz, β-Glc (S_6), H-1), 4.68 (d, 1H, *J* = 7.8 Hz, β-GlcNAc (S_3), H-1), 5.24 (d, 2H, *J* = 3 Hz, α-Glc (S_1), H-1 and αGal (S_5), H-1), 5.26 (d, 1H, *J* = 3.6 Hz, α-Gal (S_7), H-1).

^{13}C NMR values of compound 1 (equinose) in D_2O at 300 MHz:

	C-1	C-2	C-3	C-4	C-5	C-6	-CO	-CH_3
α-Glc (S_1)	92.0	71.8	72.7	78.2	71.1	60.9		
β-Glc (S_1)	95.8	74.3	75.3	78.4	75.6	60.6		
β-Gal (S_2)	102.9	70.9	81.5	68.5	75.8	60.9		
β-GlcNAc (S_3)	102.9	n.d.	75.1	73.7	75.6	60.5	175	24.9
β-Gal (S_4)	101.4	70.9	82.5	69.2	74.07	60.04		
α-Gal (S_5)	91.8	73.7	72.4	70.06	71.3	60.9		
β-Glc (S_6)	95.7	74.3	75.3	78.4	75.5	59.9		
α-Gal (S_7)	92.2	74.7	72.4	69.5	71.3	61.1		

FAB-MS of compound **1**: *m/z* 1232 $[M+K]^+$.

^{1}H NMR of compound 2 (asinose) in D_2O at 300 MHz: δ 1.31 (d, 3H, J = 6.0 Hz, α-Fuc (S_4), -CH_3), 1.73 (αNeu5Ac (S_6), H-3ax), 1.92 (s, 3H, $NHCOCH_3$), 1.99 (s, 3H, $NHCOCH_3$), 2.65 (α-Neu5Ac (S_6). H-3eq), 3.11 (t.1H, J = 8.4 Hz, β-G1c (S_1). H-2), 4.23 (d, 1H, J = 3.6 Hz, β-Gal (S_2), H-4), 4.52 (d, 1H, J = 7.5 Hz, β-Gal (S_2), H-1), 4.72 [d, 2H, J = 7.5 Hz, β-Glc (S_1), H-1 and β-GlcNAc (S_3), H-1], 5.15 (d, 1H, J = 3.6 Hz, α-Fuc (S_4), H-1), 5.24 (d, lH, J = 3 Hz α-Glc (S_1), H-1) 5.27 (d, 1H, J = 3.0 Hz, α-Gal (S_5), H-1).

^{13}C NMR values of compound 2 in D_2O at 300 MHz:

	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9
α-Glc (S_1)	91.9	72.8	72.7	77.2	72.1	61.9			
β-Glc (S_1)	n.d.	75.3	76.3	77.4	75.8	61.8			
β-Gal (S_2)	101.1	72.0	81.5	70.8	76.9	62.0			
β-GlcNAc (S_3)	101.1	n.d.	75.4	81.0	76.8	n.d.			
α-Fuc (S_4)	99.0	71.0	70.8	72.7	68.9	14.0			
α-Gal (S_5)	91.7	73.7	72.4	70.0	71.3	61.4			
α-Neu5Ac (S_7)	175.0	101.1	n.d.	71.0	n.d.	75.0	70.6	73.5	65.0

FAB-MS of compound **2**: *m/z* 1167 $[M+Na]^+$

RESULTS AND DISCUSSION

Compound 1, equinose $[\alpha]_D$ + 45.5° (c, 2, H_2O), $C_{44}H_{75}O_{36}N$ responded positively to phenol-sulphuric acid test [16], Fiegl test [22] and Morgan Elson test [14], indicating the presence of normal and amino sugar(s) in it. ^{13}C and ^{1}H NMR spectra of compound **1** showed eight anomeric carbon and proton signals respectively in them. In the ^{1}H NMR spectrum of compound **1**, the anomeric proton signals appeared as six doublets at δ 4.46 (2H), 4.59 (1H), 4.65 (1H), 4.68 (1H), 5.24 (2H), 5.26 (1H) for eight anomeric protons. These eight anomeric protons could be interpreted for the presence of a heptasaccharide in its reducing form, giving signals for α and β anomeric protons at its reducing end and it was further confirmed by presence of seven anomeric carbon signals for eight anomeric carbons at δ 91.8 (1C), 92.0 (1C), 92.2 (1C), 95.7 (1C), 95.8 (1C), 101.4 (1C), 102.9 (2C) in its ^{13}C NMR spectrum.^{1}H and ^{13}C NMR spectra justifies the eight anomeric signals for heptasaccharides with total integral intensity of seven anomeric proton/carbon. The reducing nature of compound **1** was confirmed by methylglycosylation of compound **1** by CH_3OH/H^+ followed by its acid hydrolysis which led to the isolation of α and β methyl glucosides (3) and Glc (4), Gal (5) and GlcNAc (6) which suggested the presence of glucose at the reducing end. The reducing nature of glucose was further confirmed by the presence of two anomeric proton signals at δ 5.24 (3 Hz) and 4.65 (8.1 Hz) for α- and β-glucose respectively in ^{1}H NMR spectrum of compound **1**. For convenience the seven monosaccharides present in compound **1** have been designed as S_1, S_2, S_3, S_4, S_5, S_6 and S_7 respectively starting from the reducing end. The monosaccharides constituents in compounds **1** were confirmed by its Killiani hydrolysis [23] under strong acidic conditions, followed by paper chromatography and TLC. In this hydrolysis three spots were found on TLC which was found identical with glucose, galactose and GlcNAc by co-chromatography with authentic samples. Confirming the heptasaccharides contained three types of sugar moieties *i.e.* Glc, Gal and GlcNAc. In the 400 MHz ^{1}H NMR of equinose in D_2O Presence of two doublets at δ 5.24 (1H) J = 3 Hz and δ 4.65 (1H) J = 8.1 Hz confirmed the presence of Glc at reducing end. Further presence of another anomeric proton signal at δ 4.46 was due to presence of β Gal moiety in compound **1** which suggested a lactose type of structure at the reducing end of equinose. The presence of lactose moiety as βGal(1→4)Glc was further confirmed by H-2 signal of β-Glc(S_1) as a triplet (SRG) [24,25] at δ 3.30 (J = 7.5 Hz). The third monosaccharide (S_3) present in the heptasaccharide was identified as β-GlcNAc by the presence of an anomeric proton doublet at δ 4.68 (1H) J = 7.8 Hz along with a singlet of amide methyl of N-acetyl glucosamine at δ 2.25. The downfield shifted doublet of H-4 proton of β-Gal(S_2) at δ 4.09 (J = 4.5 Hz) confirmed that C-3 of β-Gal(S_2) is linked to GlcNAc(S_3) moiety (SRG). The presence of two proton anomeric doublet present at δ 4.46 reflects the presence of another Gal moiety (S_4) in heptasaccharide **1**. This doublet contains two anomeric proton signals for two Gal residues (S_2 and S_4) confirmed the LNT structure *i.e.* 13 linkage between Gal(S_4) and GlcNAc(S_3) (SRG). Another anomeric proton signal appearing at β 5.26 as a doublet of 3.6 Hz was due to the presence of α-Gal as a fifth monosaccharide present in the chain of heptasaccharide **1**. By comparison of the spectrum of compound **1** with the LNT and LNnT structures and on the basis of chemical shift analogy, compound **1** appears to have the basic LNT structure with an additional α-Gal residue attached to C-4 of GlcNAc. The presence of one α-Gal and β-Gal residues at C-3 and C-4 position of GlcNAc has caused the crowding and steric hindrances resulting in the downfield shift of α-Gal H-1 resonance (SRG). The presence of two more anomeric proton doublets at β 4.59 (1H) J = 7.8 Hz and δ 5.24 (2H) J = 3.9 Hz showed the presence of one β-Glc and one α-Gal moieties respectively in the heptasaccharide. The pattern of ^{1}H NMR signals of these two sugar units were having the resemblances with the ^{1}H NMR resonance of lactose moiety differing only in the α-glycosidic linkage of Gal moiety (S_7) which was β in S_2. The presence of this lactose moiety was further confirmed by the presence of another triplet of H-2 of β-Glc at δ 3.25 (J = 7.5 Hz). The H-4 doublet of β-Gal (S_4) was observed at δ 4.00 (J = 3.3 Hz) confirmed that this lactosyl type moiety (Galα1→4Glcβ), (S_7→S_6) was attached to β-Gal(S_4) at C-3 position (SRG). The ^{13}C NMR data of compound **1** were also in confirmity with the derived structure. The compound **1** contains seven anomeric signals for eight anomeric carbons at δ 102.9 (β-GlcNAc and β-Gal), 101.4 (β-Gal), 95.8 (β-Glc). 95.7 (β-Glc), 92.2 (α-Gal), 92.0 (α-Glc), 91.8 (α-Gal). All the assignments made in ^{1}H NMR spectrum were confirmed by ^{1}H-^{1}H HOMO COSY experiments [26].

The composition of molecular size of compound **1** was determined by FAB mass spectrum. The FAB mass spectrum of compound **1** showed the highest mass ion peak at *m/z* 1232 which was due to $[M+K]^+$. Further the mass fragments were formed by repeated H transfer in the oligosaccharide and was accompanied by the elimination of terminal sugar less water [27].

Compound **2** asinose $C_{43}H_{72}O_{33}N_2$ was obtained as a viscous syrup $[\alpha]_D$-20° (c, 0.1, H_2O). It responded positively to Fiegl test [22], Morgan-Elson test [14], thiobarbituric acid test [15] and Bromo-Cresol-Green test [12] which indicated the nature of compound as an oligosaccharide containing normal, amino sugar and neuraminic acid in it. The ^{1}H NMR spectrum of compound **2** exhibited five anomeric proton signals for six anomeric protons at δ 4.52 (1H), 4.72 (2H), 5.15 (1H), 5.24 (1H) and δ 5.27 (1H) along with characteristic signal of neuraminic acid at δ 1.73 (H-3ax) and δ 2.65 (H-3eq) which showed the structure of compound **2** as a reducing hexasaccharide containing one sialic acid in it and it was further supported by the ^{13}C NMR spectrum of acetylated compound which contained four anomeric carbon signals at 91.7 (1C), 91.9 (1C), 99.0 (1C) and 101.1 (3C) for six anomeric carbons. The reducing nature of compound **2** was confirmed by methylglycosylation of compound **2** by $MeOH/H^+$ followed by its acid hydrolysis which led to the isolation of α and β methyl glucosides (3) and Glc (4), GlcNAc (5), Gal (6), Fuc (7) and N-acetyl neuraminic acid (8) which suggested the presence of glucose at the reducing end in it. The reducing nature of glucose was confirmed by the presence of two anomeric signals at 5.24 (3 Hz) and 4.72 (7.5 Hz) for α- and β-glucose respectively in ^{1}H NMR spectrum of compound **2**. For convenience the six monosaccharides present in compound **2** have been designed as S_1, S_2, S_3, S_4, S_5 and S_6, respectively starting from the reducing end. The monosaccharides constituents in compounds **2** were confirmed by its Killiani hydrolysis [23] under strong acidic conditions, followed by paper chromatography and TLC. In this hydrolysis five spots were found on TLC which was found identical with glucose, galactose, fucose, GlcNAc and N-acetyl neuraminic acid by co-chromatography with authentic samples. Thus the hexasaccharides contained five types of sugar moieties *i.e.* Glc, Gal, GlcNAc, Fuc and N-acetyl neuraminic acid. In the 400 MHz 1 H NMR of asinose in D_2O presence of two doublets at δ 5.24 (1H) *J* = 3 Hz and 4.72 *J* = 7.5 Hz confirmed the presence of Glc at the reducing end. Further presence of another anomeric proton signal at δ 4.52 was due to presence of β-Gal moiety in compound **2** which suggested a lactose type of structure at the reducing end of asinose. The presence of lactose moiety as βGal(1→4)Glc was further confirmed by H-2 signal of β-Glc(S_1) as a triplet at δ 3.11, *J* = 8.4 Hz (SRG) [24,25]. The downfield shifted H-4 proton of β-Gal (S_2) which appeared as a doublet at δ 4.23, *J* 3.6 Hz, confirmed that β-Gal (S_2) was substituted at C-3 by a G1cNAc (S_3) moiety (SRG), it was supported by the presence of anomeric proton signal as doublet of β-GlcNAc at δ 4.72 (*J* = 7.5 Hz) which was found to be overlapped with the anomeric proton signal of β-Glc (S_1) along with a singlet at δ 1.92 for amide methyl of N-acetyl glucosamine. All the above assignment has resemblance with the chemical shift and splitting pattern of the ^{1}H NMR of LNT and LNnT. The fourth anomeric proton which also appeared as a doublet of 3.0 Hz at δ 5.27 was due to the presence of another Gal (S_5) moiety which was present in the a form. The downfield shift of H-1 of α-Gal (S_5) was derived by comparison with LNT and LNnT, which assigned that Gal (S_5) was present as the fourth monosaccharide unit after GlcNAc (S_3) and is linked to C-4 of GlcNAc (S_3) confirming the 1→4 linkage of S_5 and S_3 (SRG). The fifth anomeric proton appeared as doublet at δ 5.15 of 3.6 Hz which was due to the presence of α-Fuc(S_4) which was further supported by a doublet of three protons of methyl of α-Fuc(S_4) at δ 1.31, *J* = 6.0 Hz. The H-1 resonance of α-Fuc at δ 5.15, *J* = 3.6 Hz was absolutely identical to that observed for α-L-fucosyl group (l→3) linked to GlcNAc (S_3) in oligosaccharide bearing the X determinant, which confirmed the 1→3 linkage between S_4 and S_3 (SRG). The compound **2** also contains a sialic acid residue in it which was confirmed by presence of axial and equatorial H-3 proton signal of sialic acid at δ 1.73 for axial H-3 and δ 2.65 for equatorial H-3 proton along with a three proton singlet of amide methyl of sialic acid appearing at δ 1.99 in its ^{1}H NMR spectrum. The up-field chemical shift of H-3 axial at δ 1.73 and equatorial at δ 2.65 shows that α-Neu5Ac is linked with α-Gal (S_5) by 2→6 linkage (SRG) [27].

The results obtained from the FAB-MS further substantiated the structure of compound **2** which was derived by its ^{1}H and ^{13}C NMR spectra. The molecular weight of compound **2** was confirmed by the highest mass ion recorded at *m/z* 1167 which was due to $[M+Na]^+$. Further the mass fragments were formed by repeated H transfer in the oligosaccharide and was accompanied by the elimination of terminal sugar less water [12].

Immunostimulant activity of donkey's milk: The oligosaccharide fractions from donkey milk processed by the method of Kobata and Ginsburg were analyzed for the biological activity. The pooled oligosaccharide fractions have been examined for immunostimulant activity by the well established method using the mouse/SRBC model. These pooled oligosaccharides, showed promising activity during preliminary screening for antigen specific immune response. These oligosaccharides have also been evaluated for their ability to stimulate non-specific immune response as shown by MMI and LTT test.

Antigen specific immune response: Table-1 presents the data on haemogglutinating antibody (HA) titre, plaque forming cell (PFC) count, delayed type hypersensitivity (DTH) response of mice treated with donkey's milk oligosaccharide mixture. Maximum induction of immune response was observed with respect to HA titre.

Non-specific immune response: It is evident from the data presented in Table-1 that the non-specific immune response of the component by microphage migration index (MMI) and lymphocyte transformation tests. In case of non-specific immune response MMI the macrophages collected from treated and mice showed an index of 2.35 in comparison to untreated animals for which the value of MMI was 1, which suggest that there is an increase in their macrophage mobility. In case of LTT the cell were treated *in vitro* with compounds and showed significant immunostimulant activity by [^{3}H] thymidine incorporation by lymphocytes.

TABLE-1
EFFECT OF DONKEY MILK OLIGOSACCHARIDE MIXTURE ON THE IMMUNE SYSTEM OF SWISS MICE*
Dose: 2.5 mg/Kg; Route: Oral; Schedule: 7 consecutive days

Parameters of immune response		
Antigen-specific	Untreated group	Treated group
HA titre	512.00	3413.00 ± 684.00
PFC/10^6 Spleen cells	103.0 ± 19.00	231.00 ± 126.00
DTH Response	0.45 ± 0.07	0.90 ± 0.14
Non-specific		
MMI	1	2.35 ± 0.98

*Data are based on three separate experiments with four animals each. The values are mean ± S.D. except that of HA titre which is mean ± SEM; Swiss mice for the study were taken from C.D.R.I. Colony.

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