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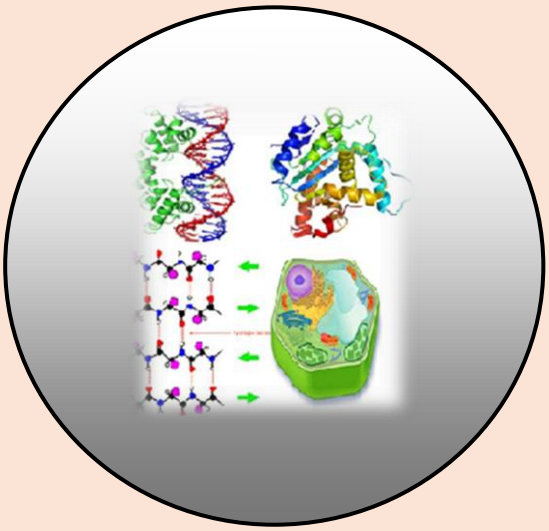

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RESEARCH PAPER

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Isolation and Structure Elucidation of Novel Oligosaccharide Equose from Mare Milk

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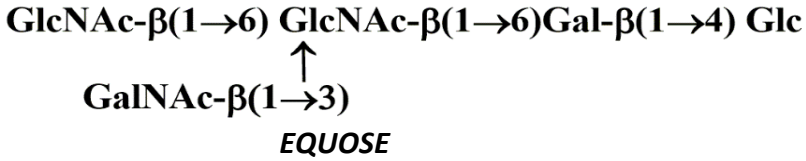
ABSTRACT

Carbohydrate-containing moieties exist in the form of glycosides, glycoprotein, glycoconjugates, oligosaccharides etc. Free oligosaccharides are natural constituents of all placental mammal's milk and also found in bacteria, fungi, plants etc. Several novel oligosaccharides have been identified in animal milks which play an important role in biological activities such as antitumor, immune-stimulant, anti-cancer, anti-complimentary, anticoagulant, anti-inflammatory, hypoglycemic, antiviral and immunological activities. Mare milk is used as antioxidant, lipid lowering agent and mineral absorption regulating agent, it also contribute in transfer of certain nerve impulses and the regulation of blood pressure. With a view to isolate more biologically active novel oligosaccharide, mare milk was collected and processed by the modified method of Kobata and Ginsberg and followed by gel filtration, HPLC and column chromatography. The results obtained from chemical transformation, chemical degradation along with spectroscopic data suggested that it was a pentasaccharides in its reducing form. The glycosidic linkages were confirmed by the splitting pattern of anomeric signals. Further the structure of novel oligosaccharide Equose was confirmed by the 2D NMR studies involving COSY, TOCSY, HSQC techniques along with mass spectrometry, the structure of novel oligosaccharide was confirmed as under:



EQUOSE

Keywords: Mare milk, oligosaccharide and Equose.

INTRODUCTION

Milk is the only naturally source which provides carbohydrates, proteins, fatty acids, lactose and vitamins responsible for growth and immunological support. Milk contains a wide range of prebiotic oligosaccharides impacting various physiological and pathological process like molecular reorganisation, signal transaction and exhibit varied biological activities such as anti-tumour (Schwonzen et al., 1992), anticancer (Fang et al., 1985), immunostimulant (Abe et al., 1983) and immunological activities (Srivastava et al., 2012). Numbers of oligosaccharides have been isolated from milk of many mammalian species including equine, bovine and marine mammals (Kunz et al., 2000, Urashima et al., 2008 and Nakamura et al., 2004). Many important activities are shown by milk of different animals having oligosaccharides with varied structures, like goat milk contain fucosylated as well as sialylated oligosaccharide which shows the supplementary and therapeutic applications (Chaturvedi et al., 1990, Kumar et al., 2016). Elephant milk oligosaccharides have high ratio of sialyl oligosaccharide; this help to the formation of brain components, such as gangliosides of the suckling calves (Osthoff et al., 2007). Buffalo milk oligosaccharide works as an immunostimulant to migration of macrophages (Saksena et al., 1999). Mare milk oligosaccharides promote cellular immune response in vitro both in terms of cellular proliferation and reactive oxidative burst. So it show the activation of one of the innate immune defense mechanism (Srivastava et al., 2014). It has also shown antioxidant, lipid lowering and post heparin lipolytic activity (Singh et al., 2016). Mare milk have an excellent medicinal importance usually in treatment of metabolic gastrointestinal and liver problems and for recovering after surgery and severe illness (Kumar et al., 2017). It also contains more vitamin C, A, B_1, B_2, and B_{12} than cow milk. Due to presence of orot acid in it, it plays an important part in the protection and regeneration of liver cells. Further, mare's milk is a promising alternative to cow's milk for human infants owing to its low fat content and high abundance of whey proteins, including lactoferrin and immunoglobulins (Malacarne et al., 2002) and it also have importance in treatment of inflammatory, in liver, cholesterol and skin disorders (Bremel et al., 1995, Pieszka M.), in search for more biologically active oligosaccharide we have worked on isolation of oligosaccharide from mare milk with the help of modified method of Kobata and Ginsburg (Kumar et al., 2016). In the present study, we have elucidated the structure of a novel mare milk oligosaccharide namely Equose (A) with the help of chemical degradation, chemical transformation, spectroscopic techniques like (^{1}H NMR, ^{13}C NMR and 2D NMR and mass spectrometry).

MATERIAL AND METHODS

GENERAL PROCEDURE

General procedure was same as described in our previous communication (Kumar et al., 2016).

Isolation of Mare milk oligosaccharide by the modified method of Kobata and Ginsberg-

Isolation of Mare milk oligosaccharides was done by the modified method of Kobata and Ginsberg method, which was described in our previous communication (Kumar et al., 2016) except the isolation, was done from 10 litre of mare milk and the yield of oligosaccharide mixture was 315 gm.

Acetylation of Mare milk oligosaccharide mixture

Dry oligosaccharides of pooled fractions obtained from sephadex chromatography (11.5gm) which gave positive phenol-sulphuric acid test (Kumar et al., 2016) were acetylated with pyridine (11.5ml) and acetic anhydride (11.5ml) at 60° C for 24 hr. The mixture was evaporated under reduced pressure and viscous residue was taken in $CHCl_3$ and washed in sequence with 2 N HCl, ice cold 2N $NaHCO_3$ and finally with H_2O.

The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness yielding the acetylated mixture (15.3g). Non-polar acetyl derivative of oligosaccharides were resolved nicely on TLC using $CHCl_3$: MeOH as eluent. Detection of the spots was done by spraying with 50% H_2SO_4 and heat.

Deacetylation of Compound a by NH_3/ Acetone

Compound **a** Equose acetate (50 mg) obtained from column chromatography 3 of acetylated oligosaccharide mixture was dissolved in acetone (5 ml) and 6 ml of NH_3 was added and left overnight in a stoppered hydrolysis flask. After 24 hr ammonia was removed under reduced pressure and the compound was washed with (3 x 5 ml) $CHCl_3$ (to remove acetamide) and the water layer was finally freeze dried giving the deacetylated oligosaccharide Equose (30 mg) having a retention time 1.092 min in HPLC.

Methylation/ acid hydrolysis of compound A

Compound **A** (8 mg) was refluxed with absolute MeOH (2 ml) at 70^0C for 22 h in the presence of cation exchange IR-120 (H^+) resin. The reaction mixture was filtered while hot and filtrate was concentrated. To a solution of methylglycoside of A in 1,4-dioxane (1 ml), 0.1 N H_2SO_4 (1 ml) was added and the solution was warmed for 30 minutes at 50^oC. The hydrolysis was completed after 22 hr. The hydrolyzates were neutralized with freshly prepared $BaCO_3$, filtered and concentrated under reduced pressure to afford α and β-methylglucosides along with the Gal, GlcNAc and GalNAc. Their identification was confirmed by comparison with authentic samples (TLC, PC).

Kiliani Hydrolysis of Compound A

Compound A (5 mg) was dissolved in 2 ml Kiliani mixture (AcOH-H_2O-HCl, 7:11:2) and heated at 100^0C for 1 hr. followed by evaporation under reduced pressure. It was dissolved in 2 ml of H_2O and extracted twice with 3 ml $CHCl_3$. The aqueous residual solution was made neutral by addition of 1-2 drops of 2N NaOH, to it and was evaporated under reduced pressure to afford Glucose, Galactose, GlcNAc and GalNAc on comparison with authentic samples (TLC, PC).

Description of isolated compound Equose

For elemental analysis, this compound was dried over P_2O_5 at 100°C and 0.1 mm pressure for 8 hr.

$C_{36}H_{61}N_3O_{26}$		%C	%H	%N
	Calc.	**45.42**	**6.41**	**4.41**
	Found	**45.45**	**6.42**	**4.42**

It gave positive Phenol-sulphuric acid test, Feigl test, Morgon-Elson test.

δ in D_2O (ppm) : ^{1}H NMR-

δ 2.03 [s, 3H, NHCO$\underline{CH}_3$, β-GlcNAc(S_3)], δ 2.04 [s, 3H, NHCO$\underline{CH}_3$, β-GlcNAc(S_4)], δ 2.13 [s, 3H, NHCO$\underline{CH}_3$, β-GalNAc(S_5)], 3.30 (t, 1H, J = 8.7 Hz, β-Glc(S_1), H-2), 4.04 (t, 1H, J = 5.1 Hz, β-GlcNAc(S_3), H-3), 4.51 (d, 2H, J = 7.5 Hz, β-Gal(S_2) & β-GalNAc (S_5), H-1), 4.57 (d, 2H, J = 7.5 Hz, β-GlcNAc (S_3& S_4), H-1), 4.69 (d, 1H, J = 7.8 Hz, β-Glc(S_1), H-1), 5.22 (d, 1H, J = 3.9 Hz, α-Glc(S_1), H-1).

δ in D_20 (ppm) : ^{13}C NMR -

δ 21.4[NHCO<u>CH</u>$_3$, β-GlcNAc(S_3)], 21.5[NHCO<u>CH</u>$_3$, β-GlcNAc(S_4)] , 24.5 [NHCO<u>CH</u>$_3$, β-GalNAc(S_5)] 92.0, α Glc (S_1) C-1, 95.5 β Glc (S_1) C-1, 100.8 β Gal (S_2) C-1, 102.4 β-GlcNAc(S_3), β-GlcNAc(S_4) &β-GalNAc (S_5) C-1, 173.2 β-GlcNAc (S_3) & (S_4) NH<u>CO</u>CH_3, 175.5 β-GalNAc(S_5) NH<u>CO</u>CH_3.

ES-MS:

951 $[M]^+$ and other fragment ions at 848, 789, 785, 784,720, 702, 667, 650, 618, 608, 560, 558, 499, 482, 466, 465, 440, 423, 406, 384, 357, 342, 325, 316, 278, 276, 260, 242, 187, 118, 117, 99, 83.

RESULT AND DISCUSSION

Compound A, $[\alpha]_D$ +12.5^0 (c, 0.64, H_2O), C_{36} H_{61} O_{26} N_3 gave positive Phenol-sulphuric acid test, Fiegl test and Morgon-Elson test (Singh et al., 2019) indicating the presence of normal and amino sugar(s) in the moiety. The ^{1}H NMR spectrum of A at 300 MHz exhibited six signals in the anomeric proton region as doublets at δ5.22 (1H), 4.69 (1H), 4.57 (2H) and 4.51 (2H) for six protons leading to the presence of six anomeric protons in compound. It was further supported by the appearance of six signals for six anomeric carbons at δ 90.5 (1C), 92.0 (1C) and 100.5 (2C) and 102.4 (2C) in the ^{13}C NMR spectrum of Equose. These data led to the suggestion that A may be a pentasaccharide in its reducing form. The Electrospray mass spectrum of compound A showed the highest mass ion peak at m/z 951 $[M]^+$, which was in support of the composition C_{36} H_{61} O_{26} N_3 with the molecular ion expected at m/z 951 for a pentasaccharide. The five-monosaccharide units present in compound A have been designated as S_1, S_2, S_3, S_4 and S_5 for convenience starting from the reducing end. The Kiliani acid hydrolysis (Kiliani, 1930) of compound A gave four spots on the paper chromatography, which were identified as Glc, Gal, GlcNAc and GalNAc by co-chromatography with authentic samples. Methylglycosidation of A by MeOH/H^+ followed by acid hydrolysis led to the isolation of α and β-methyl glucoside, which suggested the presence of glucose at the reducing end in the oligosaccharide. The reducing and free nature of glucose was further supported by the presence of two anomeric proton signals as doublets and their coupling constants, for α and β Glc at 5.22 (1H, J=3.9 Hz) and δ 4.69 (1H, (J=7.8 Hz) respectively. The presence of a lactosyl moiety i.e. Gal β (1→4) Glc in compound A was confirmed by two doublets of anomeric protons present at δ 4.51 (2H) J=7.5 Hz and δ 4.69 (1H) J=7.8 Hz for Gal (S_2) and β Glc (S_1) residues respectively which is present in lactosyl moiety in compound Equose. This was further confirmed by β Glc (S_1) H-2 signal (a structural reporter group) which appeared as a triplet at δ 3.30, J=8.7 Hz. Further, the ^{1}H NMR spectrum showed another anomeric proton signals appeared as a doublet at δ 4.57 (2H, J=7.5) along with singlet NHAc of three protons at δ 2.03 was due to the presence of GlcNAc (S_3) which is present next in sequence with lactose moiety. The linkage of GlcNAc to the lactose moiety, GlcNAc (S_3) [1→6] β-Gal (S_2) was confirmed by ^{1}H NMR spectrum of Equose, it showed a signal at δ 3.72 for H-6 of Gal (S_2) of lactose which was compared by structure reporter group of this proton. In acetylated ^{1}H-^{1}H COSY and TOCSY spectrum of Equose, showed the H-6 of Galactose appear at upfield region at δ 3.73 ppm, this confirms that the oxygen atom of C-6 are involved in inter residual glycosidation of GlcNAc (S_3) [1→6] β-Gal (S_2). This linkage was also confirmed with the HSQC spectrum of acetylated compound A. In this spectrum the H-6 of Gal (S_2) present at δ 3.73 in ^{1}H axis and its cross peak with C-6 on ^{13}C axis present at δ 72.5 (δ 3.73 xδ 72.5). Hence these data confirms that C-2, C-3 and C-4 positions of Gal (S_2) are not involved in glycosidic linkages with third sugar β-GlcNAc (S_3).

TABLE 1. ^{1}H AND ^{13}C NMR VALUES OF COMPOUND A

	αGlc(S_1)	βGlc(S_1)	βGal(S_2)	βGlcNAc (S_3)	βGlcNAc(S_4)	βGalNAc(S_5)
^{1}H	5.225	4.690	4.510	4.572	4.572	4.510
^{13}C	92.0	95.5	100.8	102.4	102.4	100.8

The fourth anomeric proton present as a doublet at δ 4.572 (2H, J=7.5 Hz) of another β-GlcNAc (S_4) with a singlet of three protons of amide methyl of glucosamine at δ 2.04 ppm, it glycosidically linked to the C-6 of GlcNAc (S_3) because H-6 proton present at δ 3.61 ppm. The linkage GlcNAc (S_4) [1→6] β-GlcNAc (S_3) was further confirmed by ^{1}H-^{1}H COSY and TOCSY spectra of acetylated compound of Equose, In which H-6 of S_3 present at upfield position δ 3.735 ppm, this implies that β-GlcNAc (S_3) was equatorially linked to C-6 to S_4 sugar. It was further supported by HSQC spectrum acetylated Equose, In which H-6 of GlcNAc (S_3) present at δ 3.84 in ^{1}H axis and its cross peak with C-6 on ^{13}C axis present at δ 60.9 (δ 3.84 xδ 60.9). The fifth anomeric proton which appear as a doublet at δ 4.510 (2H, J=7.5 Hz) along with a singlet of three protons at δ 2.13 of another β-GalNAc (S_5), it glycosidically linked to the C-3 of GlcNAc (S_3) because in acetylated spectra of Equose H-3 proton present at δ 3.936 ppm. It was supported by COSY spectra of GlcNAc (S_4), the HSQC spectrum of acetylated Equose also confirms that the H-3 of GlcNAc (S_3) give cross peak at (δ 3.936 xδ 70.8) which strongly indicated that GalNAc (S_5) moiety β glycosidically linked to S_3 at C-3 position.

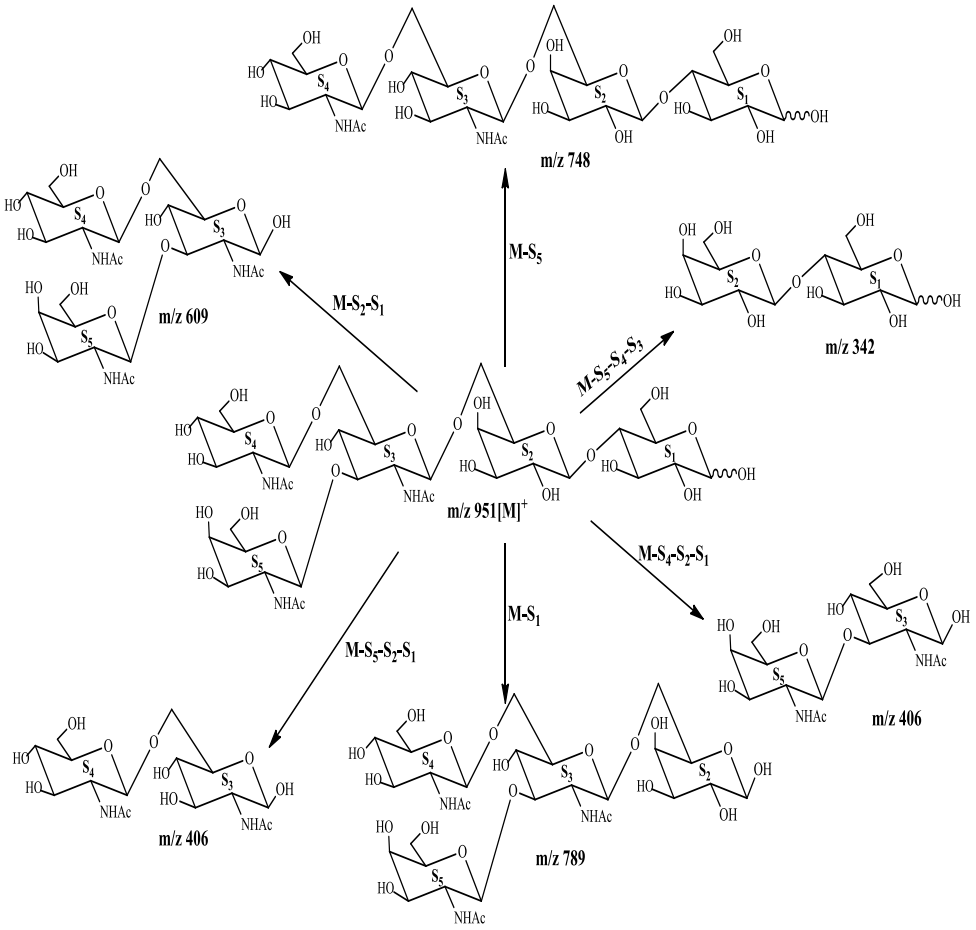
The heteronuclear single quantum-coherence (HSQC) spectrum of acetylated product of compound A confirmed the anomeric assignments by showing the ^{1}H and ^{13}C axis cross peaks of α-Glc at δ6.25 x δ 89.1 and β-Glc at δ 5.6 x 91.5. It also contains other cross peaks one of β- Gal at δ 4.45 x δ 100.8, one β-GalNAc and two β-GlcNAc moieties at δ 4.421 x δ100.8,δ4.48 x δ101.1, δ4.432 x δ101.1 respectively. Based on the pattern of chemical shift of ^{1}H, ^{13}C, HOMOCOSY, TOCSY and HSQC NMR experiments of acetylated compound A and ^{1}H, ^{13}C, HOMOCOSY spectroscopic data of deacetylated A was interpreted that the compound A was a branched pentasaccharide molecule having one Glc, one Gal, one GalNAc and two GlcNAc moieties. So compound A was interpreted branched pentasaccharide having following structure.

GlcNAc-β(1→6) GlcNAc-β(1→6)Gal-β(1→4) Glc
↑
GalNAc-β(1→3)

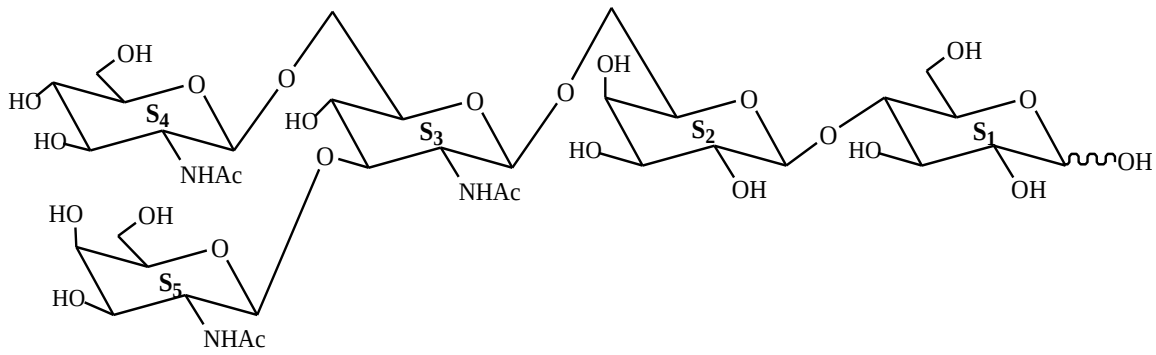
Equose

The sequence of the monosaccharides in **Equose** was confirmed by the results obtained from chemical transformation, chemical degradation / acid hydrolysis and comparative NMR study of Equose with the literature value of known compounds. All the assignment made in ^{1}H NMR was confirmed by ^{1}H-^{1}H HOMOCOSY experiments. Further the Electrospray Mass Spectrometric data of compound **Equose** helped in substantiating the sequence of monosaccharide units in it. The highest mass ion peak was recorded at m/z 951, confirming the molecular weight of Equose as $[M]^{+}$ 951. Further the mass fragments were formed by repeated H transferin the oligosaccharide and was accompanied by the elimination of terminal sugar less water. The Pentasaccharide was fragmented to give mass ion peak at m/z 748 (951-S_5) was due to the loss of terminal GalNAc (S_5) sugar, further m/z 342[748- (S_4) & (S_3)], with sugar residual loss of GlcNAc (S_4) & (S_3).

The structure of novel compound **Equose** was interpreted as –



ES-MS FRAGMENTS OF COMPOUND 'A' EQUOSE



EQUOSE

The m/z at 951 showed $[M]^+$ molecular ion peak and it further fragmented to give m/z at 893[951- $NHCOCH_3$], 824[893-H_2O-3OH], 789[824-H_2O-OH] and from other way it fragmented to give ion peak at m/z at 848[951-5OH-H_2O], 789[848-CH_2C=O-OH], 785[848-CH_2OH-CH_3OH], 720[785-CH_2OH-2OH], 702[720-H_2O], 667[702-H_2O-OH],650[667-OH], 608 [650-CH_2C=O], 558[608-CH_3OH-H_2O], 499[558-OH-CH_2C=O], 482[499-OH], 465[482-OH], 440[482-CH_2C=O], 423[440-OH], 406[440-2OH], 316[406-OH-CH_2OH-CH_2C=O], 357[406-CH_2OH-H_2O]. The molecular ion peak m/z 748 obtained from M^+ 951 with loss of non reducing S_4 or S_5 sugar molecule respectively and this tetrasaccharide molecule further fragmented to give m/z at 667[748-CH_3OH-CH_2OH-H_2O], 650 [667-OH], 618[650-CH_3OH], 560[618-$NHCOCH_3$], 466[560-CH_2OH-CH_2OHCHO], 384[466-3OH-CH_2OH], 316[384-CH_3OH-$2H_2O$], 242[316-CH_3OH-CH_2C=O] obtained. With loss of three sugars (M^+951-S_5-S_4-S_3) m/z at 342 obtained, which is molecular mass of disaccharide and it further fragmented to give m/z at 278[342-2CH_3OH],261[278-OH], 260[278-H_2O], 242[260-H_2O], 175[242-CH_3OH-H_2O-OH], 116[175-CH_2C=O-OH], 143[175-CH_3OH], 83[143-CH_2OHCHO], 118[242-CH_2OHCHO-CH_3OH], 83[118 -OH- H_2O]. In other way of fragmentation of disaccharide m/z 342 fragmented to m/z 222[342-CH_2OHCHO-CH_2C=O-H_2O], 143[222-2CH_2OH-H_2O], 187[222 -OH-H_2O], 118[187-3OH-H_2O]. The m/z 325[342-OH], 276[325-OH-CH_3OH] also obtained from m/z 342 in other fragmentation pathway.

The different types of fragmentation pathway of compound **Equose** M^+ 951 shows its respective molecular mass ion fragments at m/z 789[M-S_1], 748[M-S_5 or S_4], 609[M-S_2-S_1], 406[M-S_5-S_2-S_1 or M-S_4-S_2-S_1 or M-S_3-S_2-S_1] and at m/z 342[M-S_3-S_2-S_1], which helps in confirmation of monosaccharide residues in native milk oligosaccharide **Equose**.

Based on the pattern of chemical shift of ^1H, ^{13}C, HOMOCOSY, TOCSY and HSQC NMR experiments, chemical transformation, chemical degradation / acid hydrolysis and Electro-Spray Mass spectrometric studies of Equose and acetylated Equose.

CONCLUSION

In summary, the novel milk oligosaccharide Equose was a pentasaccharide has been isolated from mare milk and its structure was elucidated with the help of ^1H, ^{13}C, 2D NMR spectroscopy and mass spectrometry as above.

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