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By

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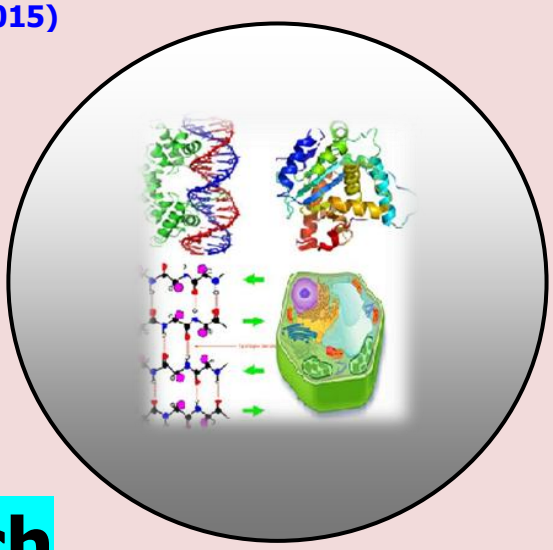

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Isolation and Structure Elucidation of Novel Nonasaccharide from Gaddi Sheep Milk

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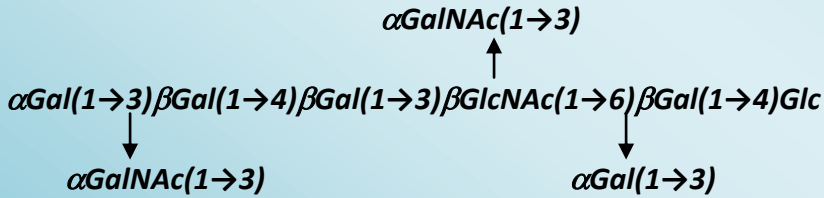
ABSTRACT

Carbohydrates play a critical role in many biological processes and diseases including cancer inflammation and infection. Milk oligosaccharides are a unit of nondigestible carbohydrate consisting of at least three monosaccharides linked by diverse glycosidic bond. Oligosaccharides are an important class of bioactive natural products and are emerging as potent drugs against fatal diseases like cancer and AIDS. Many oligosaccharides has been isolated from various milk which have exhibited potent biological activities such as anti-tumor, anti-viral, anti cancer, anti-inflammatory, anti-coagulant, immunostimulant and hypoglycemic activities and they are also used as certain biomarkers. Keeping in mind the biological activities of sheep milk as bioactive inhibitory, hypertensive defense, control of microbial infection, affect in cardiovascular, nervous and immune system, it was collected, and processed by the modified method of Kobata and Ginsburg involving deproteination, centrifugation and lyophilzation followed by gel filtration, column chromatography and HPLC, affording crude oligosaccharide mixture which on acetylation and silica gel column chromatography afforded a purified novel compound Arieose. The isolated structure of oligosaccharide was elucidated by chemical transformation, chemical degradation, NMR (^{1}H, ^{13}C and 2D COSY, TOCSY HSQC) techniques and mass spectrometry.



Arieose

Keyword: Carbohydrate, milk oligosaccharide and Arieose.

INTRODUCTION

Carbohydrates are ubiquitous firewood of life and play important roles in cellular recognition, structural material in cell walls, insect and shell fish exoskeletons (Lindhorst 2007). Carbohydrates are present in free monosaccharides, oligosaccharides, polysaccharides and with vital component of glycoconjugates such as glycolipids, glycoproteins, glycopeptides and glycosylated natural products. Glycosylated natural products are commonly use as antimicrobial drugs and now as emerging anti-cancer drug candidates (Hung et al., 2006)**.** Oligosaccharides play an essential role in many molecular processes impacting eukaryotic biology, diseases and exhibit varied biological activities such as immunostimulant, hypoglycemic, anti-tumor, antiviral, anticancer, anticoagulant, anti-complementary, immunological and anti-inflammatory activities (D.W. Ehresmann et al., 1979, Piere Jacques et al., 1982). The milk is a rich source of oligosaccharides which can provide number of novel oligosaccharides depends on the nature of their origin in to which mammals the milk belongs. Milk oligosaccharides are also fucosylated and sialylated which decrease the attachment of bacteria and viruses in intestinal cells. The ability of pathogens to bind to specific oligosaccharide seems to be intrinsically correlated with their structure. Neutral oligosacchride containing HexNAc block adhesion of pathogens that cause diarrhea (*Vibrio cholerae*) and pneumonia (*Streptococcus pneumoniae*) [G. V. Coppa et al. 2006, H. H. Tong et al.,1999] while neutral fucosylated oligosaccharide have been shown to inhibit adhesion of other pathogens (i.e., *Campylobacter jejuni* and diarrheagenic *E. coli*) that cause gastrointestinal disorders [A. L. Morrow et al., 2004]. Acidic oligosaccharides containing sialic acid are able to block adhesion of *Helicobacter pylori* which causes peptic ulcers and other gastric diseases (Chia-Chien Hsieh et al., 2014). Preclinical data suggest that Human milk oligosaccharide confer numerous physiological benefits, including immunomodulation of the host improved cognition modulation of intestinal motility, perfusion microbial production of short-chain fatty acids, prevention of pathogen attachment and other metabolites (Tasawan Thongaram et al., 2017). Sheep milk is a well balanced nutrient and shows various course of action such as absorption of nutrients, digestion, growth, development of various organs and plays a specific character to the resist outer infection (Egito et al., 2002). It contains proteins, peptides, low fat content and amino acids (Ashok kumar et al., 2015). The protein of Sheep milk is an essential source of bioactive inhibitory, control of microbial infection and hypertensive defense. The peptides present in sheep milk affect the cardiovascular, nervous and immune system. Sheep milk is a rich source of fucosylated oligosaccharides which constitute a powerful innate immune system of human (Sharon and Ofek, 2000). Keeping in mind the biological activity of Gaddi milk and oligosaccharide present therein, it was collected in bulk and was processed by modified method of Kobata and Ginsburg (Kobata A. et al 1970) for obtaining its oligosaccharides constituents. We have isolated a novel oligosaccharide from the Gaddi milk and then its structure was elucidated with the help of chemical degradation, chemical transformation and spectroscopic method like ^{1}H NMR ^{13}C NMR and 2D NMR (COSY TOCSY, HMBC, HSQC) technique as well as mass spectrometry. The structure of these basic core oligosaccharides were assigned by chemical degradation and spectroscopic techniques by previous workers (Dorland et al.,1977) and further, the structures of various milk oligosaccharides were determined by comparing the (^{1}H NMR) chemical shifts of anomeric signals and other

important signals of unknown milk oligosaccharide with the chemical shifts of LNT and LNnT (Dua, et al., 1983) In the present study, analogies between chemical shifts of certain 'structural reporter group resonances' were used to make protons resonance assignments as well as structural assignments of the oligosaccharides (Fournet, et al., 1978), ancillary techniques such as Deacetylation, chemical degradations, NMR technique and FAB mass spectroscopy were used for unambiguous determination of the structure.

MATERIAL AND METHODS

General procedure

General procedures were same as described in our previous articles (Muzeeb Khan et al., 2016).

Isolation of Gaddi milk oligosaccharide by Kobata and Ginsburg method-

10 litres milk was collected from a Gaddi Sheep then processesed by method of Kobata and Ginsberg. For this method, milk was collected and stored at -20°C for 12 hours and 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 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 was washed twice with 68% ethanol at 0°C. The supernatant and washings were combined and filtered through a microfilter and lyophilized affording crude oligosaccharide mixture (267 gm).

Acetylation of Gaddi milk oligosaccharide mixture

60.00 g of pooled fractions which gave positive phenol-sulphuric acid test were acetylated with pyridine (60ml) and acetic anhydride (60ml) at 60°C and the solution was stirred overnight. The mixture was evaporated under reduced pressure and the viscous residue was taken in $CHCl_3$ (3 x 300ml) and washed in sequence with 2N-HCl (1x 100ml), ice cold 2N-$NaHCO_3$ (2 x 100ml) and finally with H_2O (2 x 100ml). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness yielding the acetylated mixture (65.50g).

Purification of Acetylated milk oligosaccharide on Silica Gel Column

The acetylated oligosaccharide mixture (22 g) was purified by column chromatography. the silica was used in the ratio of 1:100 using various proportions of Hexane $CHCl_3$, $CHCl_3$, $CHCl_3$:MeOH mixture which was resolved into twelve fractions namely I(1.045g), II(0.154g), III(0.220g), IV(280), V(1.280m), VI(0.350m), VII(0.520g), VIII(4.510g), IX(4.850g), X(2.250g), XI(0.100g) and XII(2.800g) respectively. These fractions were containing mixture of two to four compounds. Repeated column chromatography of fraction V led to the isolation of one chromatographically pure compound c (25mg).

Deacetylation of Compound c (Arieose)

Compound c (25.0mg) obtained from column chromatography 2 of acetylated oligosaccharide mixture was dissolved in acetone (2ml) and 3 ml of NH_3 was added and left overnight in a Stoppard hydrolysis flask. After 24h ammonia was removed under reduced pressure and the compound was washed with (3 x 5ml) $CHCl_3$ (to remove acetamide) and the water layer was finally freeze dried giving the deacetylated oligosaccharide C (21.0mg).

Description of Isolated Compound C (Arieose)

Substance c (141.90 mg) obtained from fraction 26-57 of chromatography–5. On deacetylation of 25mg of substance C with NH_3 / acetone it afford substance C (21.0mg) as a viscous mass, $[\alpha]_D$ +138.72^0(c, 4, H_2O) .

For experimental analysis, this compound was dried over P_2O_5 at 100^0 C and 0.1 mm pressure for 8 hr.

$C_{60}H_{101}N_3O_{46}$	%C	%H	%N
Calcd.	45.03	6.32	2.63
Found	45.02	6.31	2.61

δ in D_2O: ^{1}H NMR

δ5.194 [d, 1H, J=3.8Hz, αGlc (S_1), H-1], δ5.085 [d, 1H, J=3.6Hz, αGal (S_7), H-1], δ5.045 [d, 1H, J=3.6Hz, αGal (S_6), H-1], δ5.031 [d, 1H, J=4.8 Hz, αGal (S_8), H-1], δ5.011 [d, 1H, J=4.8Hz, αGal (S_9), H-1], δ4.655 [d, 1H, J=7.8Hz, βGlc (S_1'), H-1], δ4.594 [d, 1H, J=7.8Hz, βGlcNAc (S_3), H-1], δ4.519 [d, 2H, J=7.8Hz, βGal (S_2) & βGal (S_4), H-1], and δ4.502 [d, 1H, J=6.9Hz, βGal (S_5), H-1], δ4.059 [t, 1H, J=6.1, βGlcNAc (S_3), H-2], δ3.544 [t, 1H, J=5.9, βGlc (S_1'), H-2], δ2.042 [s, 6H, αGalNAc (S_8) & αGalNAc (S_9), $NHCOCH_3$] and δ1.956 [s, 3H, βGlcNAc (S_3), $NHCOCH_3$].

δ in D_2O: ^{13}C NMR

δ172.06[βGlcNAc (S_3), $NHCOCH_3$], δ171.34 [αGalNAc (S_8) & αGalNAc (S_9), $NHCOCH_3$], δ102.10 [βGlcNAc (S_3), C-1], δ101.60 [βGal (S_2) & βGal (S_4), C-1], δ97.40[β Gal (S_5), C-1], δ95.80 [αGalNAc(S_9)], δ94.20 [αGalNAc(S_8), C-1], δ91.50[αGal (S_6), C-1], δ90.80[αGal (S_7), C-1], δ89.90[βGlc(S_1'), C-1] and δ88.20 [αGlc (S_1), C-1], δ20.76 [βGlcNAc (S_3), $NHCOCH_3$] and δ20.56 [αGalNAc (S_8) & αGalNAc (S_9), $NHCOCH_3$].

δ in $CDCl_3$: ^{1}H NMR (Acetylated)-

δ6.255 [d, 1H, J=3.8Hz, αGlc (S_1), H-1], δ5.678[d, 1H, J=7.8Hz, βGlc (S_1'), H-1], δ5.087 [d, 1H, J=3.6Hz, αGal (S_7), H-1], δ5.058 [d, 1H, J=3.6Hz, αGal (S_6), H-1], δ5.037 [d, 1H, J=4.8 Hz, αGal (S_8), H-1], δ5.017 [d, 1H, J=4.8Hz, αGal (S_9), H-1], δ4.604 [d, 1H, J=7.8Hz, βGlcNAc (S_3), H-1], δ4.577 [d, 2H, J=7.8Hz, βGal (S_2) & βGal (S_4), H-1], and δ4.536 [d, 1H, J=6.9Hz, βGal (S_5), H-1], δ2.069[s, 3H, αGalNAc (S_8), $NHCOCH_3$], δ2.054 [s, 3H, αGalNAc (S_9), $NHCOCH_3$] and δ1.998 [s, 3H, βGlcNAc (S_3), $NHCOCH_3$].

δ in $CDCl_3$: ^{13}C NMR (Acetylated)-

δ173.08 [αGalNAc (S_8), $NHCOCH_3$], δ172.86[βGlcNAc (S_3), $NHCOCH_3$], δ173.00 [αGalNAc (S_9), $NHCOCH_3$], δ102.15 [βGlcNAc (S_3), C-1], δ101.89 [βGal (S_2), βGal (S_4), αGalNAc(S_8)& αGalNAc(S_9), C-1], δ98.97 [β Gal (S_5), C-1], δ91.52[βGlc(S_1'), αGal (S_6)& αGal (S_7), C-1], and δ88.94 [αGlc (S_1), C-1], δ20.78 [βGlcNAc (S_3), $NHCOCH_3$] and δ20.56 [αGalNAc (S_8) & αGalNAc (S_9), $NHCOCH_3$].

ES Mass

m/z 1623$[M+Na+H]^+$, m/z 1599$[M]^+$, m/z 1437 , m/z 1401, m/z 1368 , m/z 1312, m/z 1275, m/z1210, m/z 1180, m/z1120, m/z1109, m/z 1102, m/z 1072, m/z 1063, m/z 1027, m/z 1010, m/z 945, m/z 910, m/z 945, m/z 884, m/z 880 , m/z 852 , m/z 824, m/z 805, m/z 802, m/z 745, m/z 742, m/z 707 , m/z 704, m/z 687, m/z 643, m/z 582, m/z 559, m/z 548 , m/z 504, m/z 499, m/z 455, m/z 422, m/z 393 , m/z 384, m/z 375 , m/z 364, m/z 357, m/z 345 , m/z 342, m/z 303, m/z 281, m/z 264, m/z 261 , m/z 222, m/z 203, m/z 183, m/z 180, m/z 156, m/z and 150 m/z 144.

RESULT AND DISCUSSION

Structure elucidation of 'Arieose' isolated from Gaddi milk oligosaccharide

Compound Arieose, $C_{60}H_{101}N_3O_{46}$, $[\alpha]_D$ +138.72, gave positive Phenol-sulphuric acid test (Dubois et al.,1956), Feigl test (Feigl 1975), Morgon-Elson test (Partridge et al., 1948) showing the presence of normal and amino sugar(s) in the compound. The HSQC spectrum of acetylated compound at 300 MHz exhibited ten cross peaks for ten anomeric proton and carbon signals at δ 6.255 x 88.94, δ5.678 x 91.52, δ5.087 x 91.52, δ5.058 x 91.52, δ5.037 x 101.89, δ5.017 x 101.89, δ4.604 x 102.15, δ4.577 x101.89, δ4.577 x101.89 and δ4.536 x 98.92 indicating that the Arieose may be a nonasaccharide in its reducing form giving signals for α and β anomers of glucose in its reducing end. The nonasaccharide nature of acetylated compound Areiose was further confirmed by the presence of ten anomeric carbon and proton at δ88.94(1C), δ91.52(3C), δ98.92(1C), δ101.89(4C) and δ102.15(1C) in ^{13}C NMR and δ6.255(1H), δ5.678(1H), δ5.087(1H), δ5.058(1H), δ5.037(1H), δ5.017(1H), δ4.604(1H), δ4.577(2H) and δ4.536(1H) in ^{1}H NMR, respectively. Methylglycosidation of Arieose by MeOH/H^+ followed by its acid hydrolysis led to isolation of α and β-methyl glucoside, which confirmed the presence of glucose at the reducing end of the oligosaccharide. It was further confirmed by the presence of two anomeric proton signals at δ 5.194 and δ 4.655 for α- and β-Glc in ^{1}H NMR of Arieose in D_2O. The nine monosaccharides present in compound have been designated as S_1, S_2, S_3, S_4, S_5, S_6, S_7, S_8 and S_9 for convenience starting from reducing end. To confirm the monosaccharide constituents in compound, it was hydrolysed under strong acidic conditions. In Killiani hydrolysis under strong acid condition, it gave four monosaccharides i.e. glucose, galactose, N-acetylgalactosamine and N-acetylglucosamine, confirming that the nonasaccharide is consist of four types of monosaccharide units i.e. glucose, galactose, N-acetylgalactosamine and N-acetyl-glucosamine. Since the glucose was present in its reducing form which was supported by ^{1}H NMR of Arieose in D_2O which contains two anomeric proton signals for α- and β-Glc at δ 5.194 (J=δ4.8Hz) and at δ 4.655 (J=δ8.4Hz) (Urashima et al., 2002, Urashima et al., 2004, Urashima et al., 2006). Further the presence of another anomeric proton doublet signal at 4.519 (J=δ7.8Hz) was due to presence of β-Gal (Urashima et al., 2004, Urashima et al., 2006) moiety in the Arieose. β-Glc (S_1') H-2 signal as a triplet at δ3.544 in the downfield region (Urashima et al., 2006, Gronberg et al., 1990), indicated that both the equatorially oriented hydroxyl groups at C-3 and C-4 of the reducing β-Glc (S_1') were substituted and are involved in glycosidation, suggested the presence of a lactosyl moiety i.e β-Gal(1→4)Glc with a substitution on 3-position of Glc(S_1)(SRG) (Gronberg et al., 1990). The coupling constant of anomeric signal with J value of δ7.8Hz shows the β-configuration of anomeric linkage between S_2→S_1. It was also supported by the presence of β-Glc H-4 proton resonance at δ4.002 in acetylated derivative of Arieose. Another anomeric signal, which appeared at δ5.085, was due to presence of α-Gal (S_7) moiety. The α-Gal(1→3)Glc linkage was confirmed by reporter group value (Urashima et al., 1997a, Urashima et al., 2006) of α-Gal at H-1 at δ5.085 with H-2 triplet of S_1 and also supported the presence of α-Gal (S_6) moiety at non-reducing end. The splitting pattern of anomeric signal with J value of δ3.6Hz shows the α-configuration of anomeric linkage at S_7→S_1.It was further confirmed by the upfield shifted values of H-3 of S_1 at δ3.904 and H-5 of S_7 at δ4.138 in Arieose acetate. Further the presence of two anomeric proton doublet at δ4.594 (J=δ8.4Hz) and δ 5.031 (J=δ3.6 Hz) along with two signals of amide methyl group at δ1.956 and δ2.042, respectively was due to the presence of β-GlcNAc (S_3) and α-GalNAc (S_8) moieties.

The position of anomeric proton resonances at δ4.594 and δ 5.031 suggested that β-GlcNAc (S_3) and α-GalNAc (S_8) may be (1→6) (Urashima et al., 2006, Haeuw-Fievre et al., 1993) and (1→3) (Urashima et al., 2003b, Haeuw-Fievre et al., 2006) linked to β-Gal (S_2), respectively (SRG). The coupling constant of anomeric signals with J value of δ7.8Hz and δ4.8Hz shows the β and α-configuration of anomeric linkages, among S_3→S_2 and S_8→S_2, respectively. It were further confirmed by the presence of H-3 & C-3 resonance of β-Gal (S_2), of Arieose acetate at δ3.882 & δ73.48 and H-6 & C-6 resonance of β-Gal (S_2), of Arieose acetate at δ3.785 & δ77.20, respectively with the H-4 proton resonance of β Gal (S_2) at δ 4.116 (Strecker et al., 1992). Further another anomeric signals appeared at δ4.519 (J=δ7.8 Hz) was due to presence of a β-Gal moiety (S_4). The linkage between β-Gal (S_4) and β-GlcNAc (S_3) was confirmed by the chemical shift value of this β-Gal moiety (S_4), that is identical with the chemical shift value of β-Gal (S_2) of lactosyl moiety (SRG) (Chaturvedi et al., 1988, Chaturvedi et al., 1990, Haeuw-Fievre et al., 2006). The splitting pattern of anomeric signal with J value of δ7.8Hz shows the β-configuration of anomeric linkage at S_4→S_3.This was further confirmed by COSY, HSQC and TOCSY NMR spectra of Arieose acetate by assignments of ascertained the positions of H-3 proton of β-GlcNAc (S_3) at δ 3.852 which implies that H-3 of β-GlcNAc(S_3) were involved in the glycosidation. Another anomeric proton signals appeared as doublet at δ5.011 (J=δ3.3Hz) along with signals of amide methyl group at δ2.042 was due to the presence of another α-GalNAc (S_9) moiety in Arieose. The position of anomeric proton value of α-GalNAc (S_9) confirmed that α-GalNAc (S_9) was linked to H-3 of β-Gal (S_4)(SRG) (Urashima et al., 2003b, Haeuw-Fievre et al., 2006). The coupling constant of anomeric signal with J value of δ4.8Hz shows the α-configuration of anomeric linkages between S_9→S_4. It was further confimed by the presence of H-3 & C-3 resonance of β-Gal (S_4) of Arieose acetate at δ3.986 & δ77.20, respectively.

Table 1- ^{1}H and ^{13}C NMR values in D_2O -

Moieties	^{1}H NMR	^{13}C NMR	Coupling Constt.(J)
α-Glc (S_1)	5.194	88.20	3.8
β-Glc (S'_1)	4.655	89.90	7.8
β-Gal (S_2)	4.519	101.60	7.8
β-GlcNAc (S_3)	4.594	102.10	7.8
β-Gal (S_4)	4.519	101.60	7.8
β-Gal (S_5)	4.502	97.40	6.9
α-Gal (S_6)	5.045	91.50	3.6
α-Gal (S_7)	5.085	90.80	3.6
α-GalNAc (S_8)	5.031	94.20	4.8
α-GalNAc (S_9)	5.011	95.80	4.8

Further another anomeric proton signals appeared as doublet at δ4.502 (J=δ6.9Hz) was due to the presence of another β-Gal (S_5) moiety in Arieose. The position of anomeric proton value of β-Gal (S_5) confirmed that β-Gal (S_5) was linked to H-4 of β-Gal (S_4) (Urashima et al., 2006). The coupling constant of anomeric signal with J value of δ6.9Hz shows the β-configuration of anomeric linkage between S_5→S_4. It was further confimed by the presence of H-4 & C-4 resonance of β-Gal (S_4) of Arieose acetate at δ4.024 & δ76.57, respectively.

The next anomeric signal appeared at δ 5.045 were recognized for the presence of another α-Gal (S_6) moiety. The presence of anomeric proton resonance of α-Gal (S_6) at δ5.045 confirmed the α-Gal (S_6) was (1→3) linked to β-Gal (S_5) and also supported the presence of α-Gal (S_6) moiety at non-reducing end (SRG) (Urashima et al., 2003b, Haeuw-Fievre et al., 2006). The splitting pattern of anomeric signal with J value of δ3.6Hz shows the β-configuration of anomeric linkage between S_6→S_5. It was further confimed by the presence of H-3 and C-3 resonance of β-Gal (S_5) of Arieose acetate at δ3.785 and δ 77.00 with H-5 of α-Gal (S_6) in upfield region at δ4.153, respectively.

Table 2. ^{1}H NMR values of acetylated Areiose in $CDCl_3$-

Moieties	H-1	H-2	H-3	H-4	H-5	H-6	-CH_3
α - Glc (S_1)	6.255	5.140	3.852	3.821			
β - Glc (S'_1)	5.6785	5.180	3.904	4.002			
β - Gal (S_2)	4.577	4.993	3.785	4.116	4.355	3.882	
β - GlcNAc(S_3)	4.604	4.100	3.852	5.278			1.998
β - Gal (S_4)	4.577	4.998	3.986	4.024			
β - Gal (S_5)	4.536	5.033	3.785	5.114			
α - Gal (S_6)	5.058	4.534	4.299	5.247	4.138		
α - Gal (S_7)	5.087	4.539	4.285	5.218	4.153		
α - GalNAc(S_8)	5.037	3.775	5.218	4.599			2.069
α - GalNAc(S_9)	5.017	3.785	5.247	4.582			2.054

The ^{13}C NMR values of anomeric carbons and ring carbons of Arieose are given in table. The various values of ring carbons are in accordance with ^{13}C value of their respective monosaccharide, which also supports the derived structure.

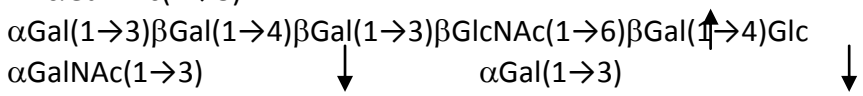
Table 3.- ^{13}C NMR values of acetylated Areiose in $CDCl_3$-

Moieties	C-1	C-2	C-3	C-4	C-5	C-6	-$CONH_2$	-CH_3
α - Glc (S_1)	88.94	69.14	75.75	77.00				
β - Glc (S'_1)	91.52	69.01	76.57	75.62				
β - Gal (S_2)	101.89	69.40	73.48	60.83	68.49	77.20		
β - GlcNAc(S_3)	102.15	61.73	77.42	69.01			172.86	20.78
β - Gal (S_4)	101.89	69.61	77.20	76.57				
β - Gal (S_5)	98.92	68.39	77.00	69.01				
α - Gal (S_6)	91.52	69.01	61.73	69.40				
α - Gal (S_7)	91.52	68.49	61.45	69.14				
α - GalNAc(S_8)	101.89	61.45	69.40	68.40			173.08	20.56
α - GalNAc(S_9)	101.89	60.83	69.61	68.49			173.00	20.56

The nonasaccharide nature of compound was further confirmed by the spectral studies of acetylated derivative of compound. The heteronuclear single quantum coherence (HSQC) spectrum of acetylated compound confirmed linkages in ^{1}H and ^{13}C NMR spectra by showing

cross peaks of α-Glc (S_1) H-3 and C-3 at δ 3.852 x 75.75 showed (1→3) linkage of S_7 and S_1 and also its H-4 and C-4 at (δ 3.821 x 77.00) shows (1→4) linkage of S_2 and S_1, β-Glc (S_1) H-3 and C-3 at (δ3.904 x 76.57) showed (1→3) linkage of S_7 and S_1 and also H-4 and C-4 at (δ4.002 x 75.62) showed (1→4) linkage of S_2 and S_1 i.e. its 3-position and 4-position of Glc (S_1) were involved in linkage, β-Gal (S_2) H-3 and C-3, H-6 and C-6 at (δ3.785 x 73.48) and (δ3.882 x 77.20) showed (1→3) & (1→6) linkages of $S_3 \rightarrow S_2$ and $S_8 \rightarrow S_2$ respectively. β-GlcNAc(S_3) H-3 and C-3 at (δ3.852 x 77.42) showed (1→3) linkage of S_4 and S_3, β-Gal (S_4) H-3 and C-3, H-4 and C-4 at (δ3.986 x 77.20) and (δ4.024 x 76.57) showed (1→3) & (1→4) linkages of $S_5 \rightarrow S_4$ and $S_9 \rightarrow S_4$, β-Gal (S_5) H-3 and C-3 at (δ3.785 x 77.00) showed (1→3) linkage of S_6 and S_5 showing in the same chemical region in acetylated and deacetylated spectra. It was further confirmed by the presence of same peaks in COSY and TOCSY spectrum. The result obtained from the ES mass spectrum further substantiated the structure of Arieose which was derived by its ^{1}H and ^{13}C NMR spectra. The highest mass ion peak were recorded m/z 1623 which was due to $[M+H+Na]^{+}$. It confirmed that the molecular weight of compound was 1599. Further the mass fragments were formed by repeated H transfer in the oligosaccharide and was accompanied by the elimination of terminal sugar less water. The fragmentation pathway confirmed the sequence of monosaccharide units in the nonasaccharide. The nonasaccharide fragment mass ion peak at m/z 1599(M) on further fragmentation gave the octasaccharide mass ion peak at m/z 1437(I) which was obtained by the loss of S_6 sugar unit i.e. Gal(S_6). The octasaccharide mass ion peak fragmented to give mass ion peak at m/z 1275(II), which was due to the loss of S_5 sugar unit i.e. Gal(S_5) sugar unit linked to S_6 of the oligosaccharide (M-S_6) which was supported by its respective fragment at m/z 180, this confirmed the presence of Gal (S_6) at the non-reducing end. The heptasaccharide mass ion fragment at m/z 1275(II) further fragmented by the loss of other terminal sugar i.e. GalNAc (S_9), gave the corresponding hexasaccharide mass ion fragment at m/z 1072(III). The mass ion peak at m/z 1072 further fragmented to give mass ion fragment for pentasaccharide moiety which was arose by loss of sugar (S_4) [Gal]. It was accounted for the mass ion fragment at m/z 910(IV). This pentasaccharide mass ion fragment on further fragmentation gave tetrasaccharide segment (V) at m/z 707, by loss of sugar (S_3) [GlcNAc].This on further fragmentation gave a trisaccharide segment (VI) at m/z 504 by loss of sugar (S_8) [GalNAc]. This trisaccharide mass ion fragment on further fragmentation gave an important disaccharide segment (VII) at m/z 342, by loss of sugar (S_7) [Gal], which on further fragmentation give a monosaccharide fragment (VIII) at 180 by loss of sugar (S_2) [Gal].(scheme-2 & 3). The nonasaccharide mass ion peak at m/z 1599 in the spectrum of compound C also showed other supporting mass ion peaks which are shown in scheme-5.The other important signals obtained at m/z 1193[M-S_8,S_9], m/z 869[M-S_6,S_7,S_8,S_9], m/z 748[M-S_1,S_2,S_6,S_7,S_8 or M-S_4,S_5,S_6,S_7,S_8], and m/z 691[M-S_1,S_2,S_3,S_7,S_8]. The anchoring nature of Sugar-3 (GlcNAc) in the compound was confirmed by the mass ion fragment at m/z=748 [M-S_1,S_2,S_6,S_7,S_8]. The other supporting mass fragments obtained at m/z 1510 (1599-$NHCOCH_3$,CH_2OH), m/z 1437 (1510-CH_2CO,CH_2OH)confirmed the nonasaccharide nature of compound. The nonasaccharide m/z 1599 on fragmentation gave octasaccharide m/z 1437(M-S_6), which was further confirmed by its other fragments ions at m/z 1312 (1437-CH_2OHCHO, H_2O,HCHO,OH),m/z1210(1312-CH_2CO,CH_2OHCHO),m/z1109(1210- CH_2OHCHO,H_2O,CH_2OH) m/z 1063 (1109-$2H_2O$), m/z 1027 (1063-$2H_2O$), m/z 945 (1027-CH_2CO,HCHO), m/z 910 (945-H_2O,OH), m/z 1401 (1437-$2H_2O$), m/z 1368 (1401-$2H_2O$,CH_2OH), and m/z 1275 (1368-$NHCOCH_3$,H_2O).

The octasaccharide m/z 1437 on fragmentation gave heptasaccharide m/z 1275 (1437-S_5), which was further confirmed by its other fragments ions at m/z 1238 (1275-H_2O,OH), m/z 1180 (1238-$NHCOCH_3$), m/z1120 (1180-CH_2OHCHO),m/z 1102 (1120-H_2O), and m/z 1072 (1102-HCHO). The heptasaccharide m/z 1275 on fragmentation gave hexasaccharide m/z 1072 (1275-S_9), which was further confirmed by its other fragments ions at m/z 1010 (1072-2CH_2OH), m/z 945 (1010- H_2O,OH,HCHO), m/z 884 (945-CH_2OH,HCHO), m/z 824 (884- CH_2OHCHO), m/z 704 (824-CH_2CO,H_2O,CH_2OHCHO), m/z 687 (704-OH), m/z 559 (687-CH_2CO,HCHO), and m/z 499 (559-CH_2OHCHO). The hexasaccharide m/z 1072 on fragmentation gave pentasaccharide m/z 910 (1072-S_4), which was further confirmed by its other fragments ions at m/z 852 (910-$NHCOCH_3$), m/z 805 (852-HCHO,OH), m/z 745 (805-CH_2OHCHO), m/z 687 (745-$NHCOCH_3$), m/z 880 (910-HCHO), m/z 802 (880-CH_2CO), m/z 742 (802-CH_2OHCHO), m/z 687 (742-H_2O,OH,HCHO), and m/z 707 (742-H_2O,OH). The pentasaccharide m/z 910 on fragmentation gave tetrasaccharide m/z 707 (910-S_3), which was further confirmed by its other fragments ions at m/z 643 (707-$NHCOCH_3$), m/z 582 (643-CH_2OH,HCHO), m/z 548 (582-H_2O,OH,CH_2OH), m/z 499 (548-CH_2OH,H_2O), m/z 422 (499-CH_2CO,HCHO), m/z 364 (422-$NHCOCH_3$), m/z 303 (364-CH_2OH,HCHO), and m/z 504 (582-2H_2O,CH_2CO). The tetrasaccharide m/z 707 on fragmentation gave trisaccharide m/z 504 (707-S_8), which was further confirmed by its other fragments ions at m/z 455 (504-H_2O,2CH_2OH), m/z 393 (455-CH_2OH), m/z 375 (393-H_2O), m/z 357 (375-H_2O), m/z 303 (357-H_2O,OH,HCHO), m/z 261 (303-OH), m/z 183 (261-CH_2OHCHO), m/z 144 (183-CH_2OH,H_2O), m/z 345 (393-$NHCOCH_3$), m/z 303 (345- CH_2CO), m/z 384 (455-CH_2OH,HCHO), and m/z 342 (384-CH_2CO). The trisaccharide m/z 504 on fragmentation gave disaccharide m/z 342 (504-S_7), which was further confirmed by its other fragments ions at m/z 281 (342-HCHO,CH_2OH), m/z 264 (281-OH), m/z 222 (264-CH_2CO), m/z 203 (222-OH), m/z 156 (203-HCHO,OH), and m/z 180 (222-CH_2CO). The disaccharide m/z 342 on fragmentation gave monosaccharide m/z 180(342-S_2), which was further confirmed by its other fragments ions at m/z 150(180-HCHO), and m/z 144(180-2H_2O). Based on the results obtained from chemical degradation chemical transformation, mass spectrometry and ^1H , ^{13}C, HOMOCOSY, TOCSY , HSQC NMR, the structure of the isolated novel nonasaccharide, Areiose was deduced as-



CONCLUSION

In summary, the novel milk oligosaccharides namely as **C (**Arieose) has been isolated from Gaddi milk and elucidated with the help of ^1H, ^{13}C, 2D NMR spectroscopy and mass spectrometry.

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