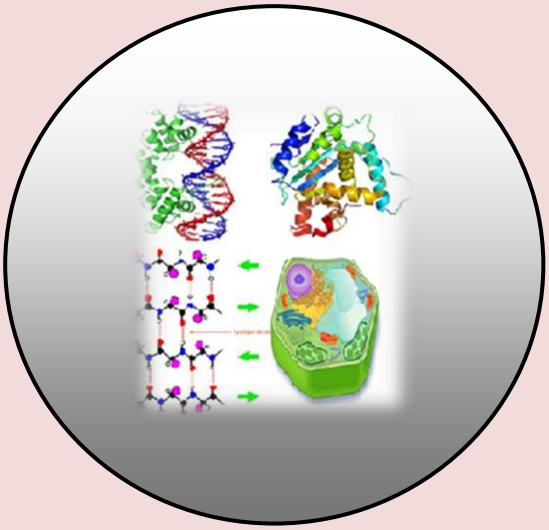
Isolation, Biological Importance and Structure Elucidation of Milk Oligosaccharides by 2-D NMR

By

Manisha Shukla, Shraddha Rathor, Anil Mishra and Desh Deepak

ISSN 2319-3077 Online/Electronic
ISSN 0970-4973 Print

Index Copernicus International Value
IC Value of Journal 82.43 Poland, Europe (2016)
Journal Impact Factor: 4.275
Global Impact factor of Journal: 0.876
Scientific Journals Impact Factor: 3.285
InfoBase Impact Factor: 3.66



J. Biol. Chem. Research
Volume 40 (1), 2023 Pages No. 1-18

Journal of Biological and Chemical Research

An International Peer Reviewed / Referred Journal of Life Sciences and Chemistry

Indexed, Abstracted and Cited: Index Copernicus International (Europe), World Science Index, Polish Ministry of Science and Higher Education (Poland, Europe) Research Bible (Japan), Scientific Journals Impact Factor Master List, Directory of Research Journals Indexing (DRJI), Indian Science. In, Database Electronic Journals Library (Germany), Open J-Gate, J Gate e-Journal Portal, Info Base Index, International Impact Factor Services (IIFS) (Singapore), Scientific Indexing Services (USA), International Institute of Organized Research (I2OR), Cosmos Science Foundation (Germany), Science Library Index (UAE), Eye Source, Swedish Scientific Publication, World Cat, International Innovative Journal Impact Factor, Einstein Institute for Scientific Information {EISI} and Impact Factor.pl - Kompendiumwiedzy o czasopismachnaukowych, Philadelphia citefactor.org journalsindexing Directory Indexing of International Research Journals

Published by Society for Advancement of Sciences®

J. Biol. Chem. Research. Vol. 40, No. 1, 1-18, 2023
(An International Peer Reviewed / Refereed Journal of Life Sciences and Chemistry)
Ms 40/01/016/2023

ISSN 2319-3077 (Online/Electronic)
ISSN 0970-4973 (Print)

Dr. Manisha Shukla
http:// www.sasjournals.com
http:// www.jbcr.co.in
jbiolchemres@gmail.com

RESEARCH PAPER

Received: 30/03/2023 **Revised: 10/06/2023** **Accepted: 11/06/2023**

Isolation, Biological Importance and Structure Elucidation of Milk Oligosaccharides by 2-D NMR

Manisha Shukla, Shraddha Rathor, Anil Mishra and Desh Deepak
Department of Chemistry, University of Lucknow, Lucknow 226007, U.P., India

ABSTRACT

In recent times, the milk oligosaccharides have produced themselves as immuno modulator, brain developer, bone developer, anti-cancer, anti-inflammatory and hypoglycemic agents. Numbers of milks of different animals have been examined for their oligosaccharide contents. Recent researches have shown that the biological activity of any mammalian milk is due to the oligosaccharides residing in them. These oligosaccharides are made up of glucose, galactose, GlcNHAc, GalNHAc, Fucose and sialic acid as there building blocks. They are linked together by O-glycosidic linkages present in α and β configuration. Variation in the structure of any oligosaccharide is also due to the glycosidic linkages present at different positions of monosaccharides. The variation in position of glycosidic linkages, conformation of sugars, configuration of glycosidic linkages results into the formation of novel oligosaccharides. The oligosaccharides formed due to different variations produces straight chain, branched chain, and winged oligosaccharides. The structure elucidation of these oligosaccharides is a challenging task for any natural product chemist. The NMR is the only non-invasive experiment which can confirm the stereoscopic structures of these oligosaccharides. This review article deals in detail with the different 1-D and 2-D NMR experiments for structure elucidation of milk oligosaccharides. The structure elucidation of milk oligosaccharide has been explained by taking the example of Usose, a milk oligosaccharide isolated from milk of Lal Muha Cow. All the NMR spectra's used for interpretation of oligosaccharide have been reproduced for the easier understanding of the readers. This review also contains the methodology and biological activity of milk oligosaccharides.

Keywords: Milk oligosaccharides, NMR and Biological activity.

INTRODUCTION

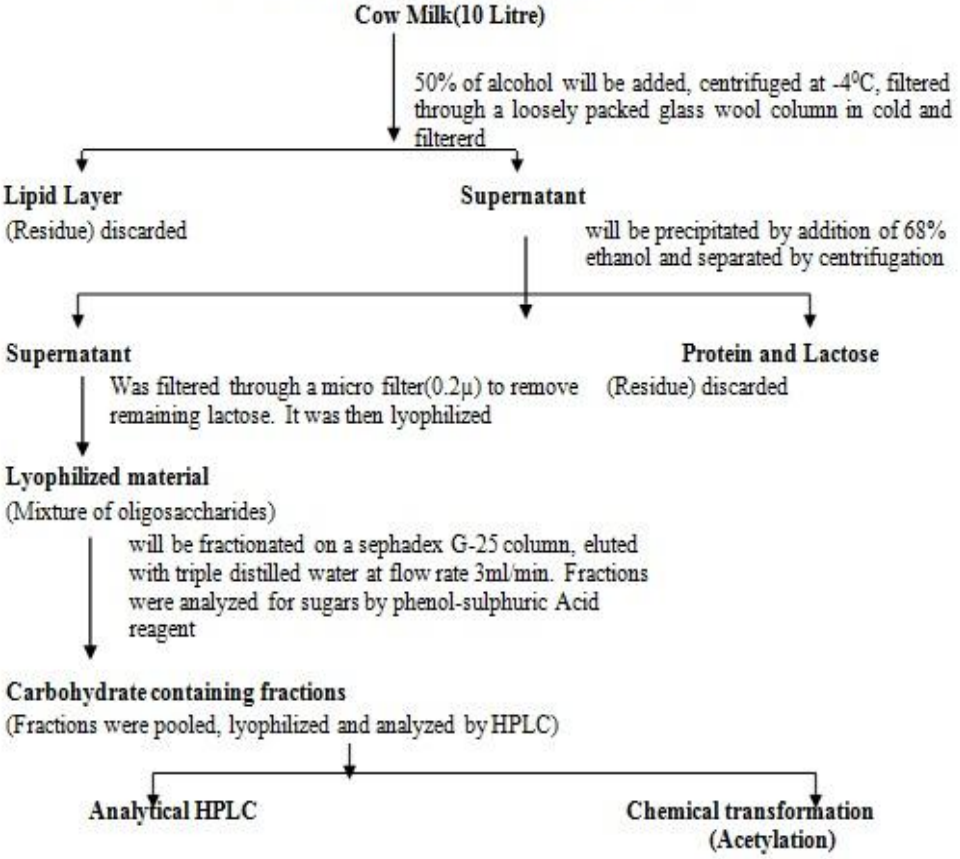
The biological importance of milk has been well defined in Indian / Unani medicinal systems like Charak Sanghita and Ayurveda (Yogini et al., 2017 and Mana et al., 2021). It is well defined that the cow's milk could be a replacement to the mother's milk and is responsible for development of immune system, brain and bones of the neonate (Pietrzak-Fiećko et al., 2020). The cow's milk or any other milk consists of protein, fat, glycoproteins, and carbohydrates (Kumar et al., 2014). The carbohydrate part mainly contains Lactose and oligosaccharides (Chen et al., 2017). The oligosaccharides present in cow milk have been responsible for many of the biological functions for the development of newly born as well as for the younger generations and the elderly persons (Haug et. al., 2007). It is well reported in ancient and recent researches that the contribution of milk has been enormous but it has not been categorically specified that which content of the milk, i.e., fat, protein or oligosaccharide has been mainly responsible for its biological activity (Bleasdale, et al., 2021). Recent researches have shown that different oligosaccharides present in milk, show different biological activities i.e., Immuno modulation, brain development human body growth of infants, antioxidant, anti-inflammatory etc. During the Vedic era the biological activity of the cow milk was glorified by Rishis and Vaidya's at that time, there were limited species of cowsavailable like the Indigenous shyama dhenu (Black Cow) and detailed studies on cow milk could not be performed (Gunjan et al., 2016). In this connection it becomes relevant to inform that oligosaccharide content of the milk varies due to their local habitat including the food they eat and the environment around them. The studies were under taken by various scientists and researchers world over and instead of human milk, milk of various mammals were chosen for their oligosaccharide contents for e.g. elephant, marsupials polar bear, rat etc. were analyzed for their oligosaccharide activity. The biological activities of various milk were found to be as under: The milk oligosaccharides have shown potent biological activities such as antitumor (Schwonzen, et al., 1992), Immunological activities (Srivastava, 1989) Anti-complementary, immuno stimulant (Abe et al., 1983), anticancer (Fang et al., 1985), anti-inflammatory hypoglycemic (Zhang, 2022) and antiviral activities. The oligosaccharides are non-digestive due to β- glycosidic linkages, therefore they play an important role for pre biotic activity, Oligosaccharide Mimics, containing galactose and fucose specifically label tumor cell surfaces and inhibit cell adhesion to fibronectin. They also improve microbiota and fermentation in infants. The galactose and sialic acid present in milk oligosaccharides develop infant's brain (Kuberka, et al., 2019). Donkey milk oligosaccharides possess high degree of immuno stimulant activity hence very helpful in the cure of Athero Sclerosis (Tafaro et al., 2007). The phosphorelated oligosaccharides in bovine and equine colostrum permit the simultaneous delivery of phosphate and calcium. Goat milk oligosaccharides reduce intestinal inflammation (Abdelali et al., 2006), while the elephant milk oligosaccharide containing high ratio of sialic acid is significant for the formation of brain components. The dog milk has predominant, N-acetylneuramin lactose sulphate which plays an important role in nutrition. Mare milk oligosaccharides have shown antioxidant, lipid lowering and post heparin lypolytic activity.

METHODOLOGY

The biological importance of milk has been well defined in Indian / Unani medicinal systems and literature like Ayurveda and Charak Sanghita.

It has been advisable to take up a detailed study of the milk constituents particularly the oligosaccharides present in milk/colostrum's. The presence of oligosaccharides of any milk depends on the fodder and habitat of the animal, therefore it is suggested that the milk of different animals was collected and processed by modified method of Kobata and Ginsberg (Deepak et al., 2020). After collection the equal amount of ethanol was added for its preservation, due to addition of ethyl alcohol the milk thawed separating the proteins and supernatant liquid (water and alcohol). This supernatant liquid then was centrifuged in a cooling centrifuge for 30 min at 5000 rpm at -4°C. The solidified liquid then was filtered through glass wool column under cold atmospheric conditions. Further to this supernatant liquid more ethanol was added to clear the filtrate to a final concentration of 68% ethanol, the resulting solution was left overnight at 0°C which resulted into the formation of a white PPT, formed mainly of lactose and protein which was Centrifuged. This supernatant liquid and it's washing were combined and filtered through micro filter andlyophilized affording crude oligosaccharide mixture. This oligosaccharide mixture was fractionated by gel filtration on a sephadex G- 25 column, and was eluted with triple distilled water. The sephadex chromatography separated the left over glyco proteins from oligosaccharides and lactose. The fractions so obtained were tested by phenol - sulphuric acid test and morgon Elson test which suggested the presence of normal sugars and N-acetyl sugars present in milk oligosaccharide. The fractions containing oligosaccharide mixtures were freeze dried or lyophylised on a lyophiliser for obtaining oligosaccharides in powdered form, then this oligosaccharide mixture was analyzed by analytical HPLC and eluted by triple distilled water containing 0.1 % TFA for checking the homogeneity of oligosaccharide mixture. The eluent was monitored by UV - absorbance at 220 nm or by diode array analyser. This analytical HPLC result was also helpful in defining the oligosaccharides present in the oligosaccharide mixture. Since these oligosaccharides present in the milk were very polar in nature therefore their purification and isolation were a difficult challenge to a chemist. To overcome this problem, it was advised to acetylate this oligosaccharides mixture by acetic anhydride and pyridine which converted the free sugar (oligosaccharide) into their non-polar acetyl derivatives which was resolved nicely on silica gel TLC and was further separated by column chromatography over silica gel. Resulting in the isolation of chromatographically pure oligosaccharides after repeated column chromatography. Further, this chromatographically pure acetylated milk oligosaccharide were deacetylated by mild basic hydrolysis by dissolving them in acetone and ammonia and was left overnight. Further ammonia was removed under reduced pressure and the compound was washed with chloroform and finally it was freeze dried giving the deactylated naturally occurring milk oligosaccharides. In this reaction of basic hydrolysis by acetone in ammonia, it removed all the OAc groups, leaving behind the NHAc group which was present in GlcNAc and GalNAc. Further the purified sample so obtained was extended for chemical degradation / chemical transformation and physicochemical experiments of NMR and Mass spectrometer. In chemical degradation acidic hydrolysis of this oligosaccharide was done for knowing its monosaccharide constituents. For knowing the reducing end monosaccharade of the oligosaccharide it was subjected to its methyl glycosidation followed by its acid hydrolysis which resulted in the isolation of methyl glycoside of the monosaccharide present at reducing end of the oligosaccharide, which is generally glucose, because in any of the oligosaccharides isolated from milk lactose was present at the reducing end of the oligosaccharides, Lactose was glucose (1 – 4) β gal.

Isolation of Cow Milk/colostrum oligosaccharides by Modified Method of Kobata and Ginsburg: (Deepak et. al)



The carbohydrate fractions will beeluted with TDW (containing 0.1%TFA & CH_3CN) at a flow rate 1ml/min., to check homogeneity of the oligosaccharide mixture. The elution was monitored by UV absorbance at 220 nm.

Fractions with (+) test for sugars cannot be separated over silica gel, hence the oligosaccharide mixture will be acetylated with Ac_2O and pyridine converting free sugar into non-polar acetyl derivatives and will be separated by TLC and column chromatography over silica gel. The chromatographically pure oligosaccharides will be analysed by 1H, 13C, COSY, TOCSY, HSQC and HMBC experiments of NMR and Mass spectrometry.

Deacetylation

The chromatographically pure acetylated Milk oligosaccharides will be deacetylatedby ammonia and acetone giving the natural milk oligosaccharides. The chromatographically pure oligosaccharides were analyzed by 1H, 13C, COSY, TOCSY, HSQC and HMBC experiments of NMR and Mass spectrometry

Further for knowing the stereoscopic structure of the oligosaccharide the 1-D and 2-D experiments of NMR i.e., ^{1}H, ^{13}C, COSY, TOCSY, HSQC, HMBC was performed at 400 MHz to 800 MHz spectrometry. Since NMR is the only non-consumable and non-degradable experiment and that too it could be used with the meager quantities up to the extent of microgram. Since the quantities of the oligosaccharides isolated from milk / Colostrum's were very less, even then we can run NMR experiment loaded with Fourier transformation technology [FT – NMR] for getting a good quality NMR spectrum. The first and the foremost experiment of the NMR is ^{1}H NMR which tells the number and nature of the protons present in an oligosaccharide. The most important signal for identification of an oligosaccharide is the anomeric proton signals further with the count of anomeric proton signal present in a oligosaccharide we can fix the number of monosaccharides in a oligosaccharide, which were generally recorded between δ 6.40 to δ 4.2 ppm. So, by counting the anomeric signals in the oligosaccharide spectrum we could justify the number of monosaccharides present in an oligosaccharide. However, the problem arouse when the anomeric signals were overlapped. The next area which also creates confusion in the methine region where the methine or ring protons of the oligosaccharides were observed between δ 3 to δ4 ppm, so this region was also overcrowded due to maximum number of methine protons present in a oligosaccharide and this problem could be resolved by the 2-D NMR experiments. In this series the next experiment was ^{13}C NMR of the oligosaccharide. Due to its long width of ^{13}C NMR i.e., δ 0 to δ 200 ppm the ^{13}C NMR spectrum was more informative than ^{1}H NMR. Further by counting the number of anomeric carbon signals which arise between δ 90 to 110. We could clearly identity and confirm the number of anomeric carbons and hence confirming the number of monosaccharides present in the oligosaccharide. Although the ^{13}C NMR had a long width for the separation of the signals even then sometimes there was an overlap at anomeric signals and other carbons, since the ^{13}C NMR did not have integration facility the overlapping could only be identified by the intensity of the signals. This problem could also be resolved by 2-DNMR experiments. In order to resolve the overlapping of the signals in 'H and ^{13}C NMR signals, the 2-D NMR was performed for unambiguous stereoscopic structures of milk oligosaccharide. The first and the foremost experiment of the 2-D NMR, which was performed by us was HSQC (Heteronuclear single quantum Coherence), this experiment tells the correlation of every carbon to its respective proton counterpart, it also ensures the number of anomeric proton/carbon in a oligosaccharide, therefore, confirming the monosaccharide units in a particular oligosaccharide. This experiment also resolves overlapping problem of anomeric carbons and protons because it gave its signals in the form of cross peaks where , the carbon signal ranges from δ 90 to 110 and the proton ranges for δ 4.2 to 5-2 ppm in ^{1}H NMR respectively. Even if the carbon or proton anomeric signal is overlapped in 1-D NMR it never happens that its counter signal (^{1}H and ^{13}C) also over lapped, therefore results into production of two different Cross peaks in the anomeric region. Hence confirming the results obtained from ^{1}H and ^{13}C NMR therefore again confirming the number of monosaccharide units in a particular oligosaccharide. The next experiment in the series was 2-D TOCSY (Total Correlation Spectroscopy). This experiment was one of the most important experiments for sieving of methine or ring protons of every monosaccharide. Since all the methine protons in acetylated oligosaccharide reside in the region of δ 4.2 to δ5.2 ppm for acetylated methine protons.

Since in the natural oligosaccharide all the ring protons/methine protons give their signal at δ 3.2 - 4.1 ppm which also includes the glycosidically linked ring protons. To differentiate between the ring protons and glycosidically linked ring protons, the NMR experiments are done in acetylated oligosaccharides that cause the difference between the glycosidically linked protons and hydroxylated ring protons. After the acetylation of oligosaccharides, the hydroxylated ring protons are converted to their ester function i.e., presence of O-acetyl groups instead of OH groups, that results into the 1 ppm downfield shift of acetylated methine protons, however the acetylation does not affect the glycosidally linked positions therefore, there is no acetylation at the positions where the glycosidic linkages were present. Hence there is no shift in 1H NMR at the positions where the glycosidic linkages were present. The chemical shift of those methine protons remain intact i.e., between δ 3.2 – 4.1 hence, creating the difference between glycosidically linked centers with the other ring protons which were bearing -OAc group. This region was always overcrowded with the signals which were maximum in case of oligosaccharides, moreover this experiment separate and sieve the methine proton in correlation to its anomeric signal, meaning thereby if it was a hexasaccharide and contained 6 anomeric protons, i.e. A_1, A_2, A_3, A_4, A_5, A_6 all the anomeric signals showed their correlation with all of its ring / methine protons. When we perform this experiment with the acetylated oligosaccharide it also gives information regarding the position of glycosidic linkage in a particular monosaccharide. Because the ring protons which reside in the region δ 4.2 -5.2 does not bear the glycosidic linkage while, the ring protons which are present in the region δ 3.2 – 4.1 gives an indication regarding the presence of glycosidic linkage at that position. The result obtained from TOCSY experiments provide a total correlation between the anomeric signal of a particular monosaccharide with all the ring protons of that particular monosaccharides. However, the TOCSY experiment does not fix the sequence of ring protons in a particular monosaccharide these positions are fixed by COSY (Correlation spectroscopy) experiment incorporating the cross peaks obtained from TOCSY experiment.

The next and the most important experiment was performed in this series was HMBC (Heteronuclear multiple bond Correlation) which gives the information regarding the glycosidic linkage between any two monosaccharides present in the oligosaccharide. This experiment was an example of Hetro COSY experiment which gave the information regarding proton - Carbon correlation up to the multiple bonds that is up to 3 bond distance.

For going for the interpretation of a particular oligosaccharide we start our experiment by HSQC experiment by looking into the cross peaks of anomeric signals obtained by correlations of proton and carbon which reside in the region of δ 4.2 -6.2 for anomeric protons and δ 90 -110 for anomeric carbons. When we observe the particular HSQC NMR experiment, we select the signal which is most downfield shifted in the acetylated oligosaccharide. Since the oligosaccharide present in the natural form as free sugars the reducing end of the oligosaccharide always show α and β anomers in the ratio of 1:3. Moreover, ring in acetylated oligosaccharide the α and β anomers of reducing monosaccharide give their anomeric signals. Here we can see the α anomer is the most downfield shifted anomeric signal while the β anomeric signals thereafter. As we have already explained that there is a ratio of 1:3 between the signal intensity of α and β anomer, we start our interpretation by picking the signal of β anomer.

After picking the anomeric signal of β anomer, we select the chemical shift of these cross-peaks into ^{1}H NMR. This chemical shift is again observed in the TOCSY experiment of the same compound where we found 3 or 4 cross peaks of that particular anomeric proton with correlations to their ring protons. From here we can get idea for the glycosidically linked ring protons by their chemical shift. The protons which give their chemical shift into the region δ 3.2 – 4.1 provides the information regarding the position of glycosidic linkage in that particular monosaccharide with the exception of the monosaccharides having NHAc group in them i.e., GlcNHAc and GalNHAc. Here due to presence of NHAc group the H-2 ring proton of GlcNHAc and GalNHAc appears in the region δ 4.1 - 4.2. Further the results obtained from TOCSY experiments are interpreted by COSY experiments is clearly fix the sequence of methine protons H-2, H-3 , H-4 and H-6 positions of that particular monosaccharide. Now the chemical shift of the signals which reside in the region δ 3.2 – 4.1 clearly indicates the position of glycosidic linkage. There may be 1 to 3 linkages in a particular monosaccharide.

From here we select the chemical shift residing in the region δ 3.2 – 4.1 and further proceed via HMBC experiment from where we get the information regarding the glycosidic linkages. When we observe the HMBC experiment for the signal which reside in the region δ 3.2 -4.1 in the ^{1}H NMR. It gives cross peak with the anomeric carbon of the next monosaccharide in sequence. This cross peak is arised by the correlation of proton signal of the reducing end monosaccharide with the anomeric carbon of next monosaccharide in sequence. Thus sequencing is done.

Upto the extent where all the ring protons are arised in the chemical shift region δ 4.2 – 5.2 in the TOCSY spectrum of that particular anomeric signal, meaning thereby that all the hydroxyl groups in that particular monosaccharide is not involved in any glycosidic linkage. Hence confirm the non-reducing end of the oligosaccharide.

For explaining the whole procedure for the interpretation of NMR experiments we have chosen the compound named as Usose which was isolated from the milk of Lal Muha Cow was taken (Khan et. al). The ^{1}H NMR of Usose showed six doublets for seven anomeric protons signals at δ 5.23 (1H), δ 5.18 (1H), δ 4.58 (1H), δ 4.53 (1H), δ 4.44 (2H) and δ 4.31 (1H). while the HSQC spectrum of acetylated Usose showed six cross peaks of seven anomeric protons and carbons in the region δ 6.24 x 90.31, δ 5.38 x 90.42, δ 5.28 x 92.12, δ 4.68 x 101.87, δ 4.68 x 95.49 and δ 4.46 x 102.02. These crosspeaks suggested that Usose was a hexasaccharide in its reducing form. Moreover, the cross peaks at δ 6.24 x 90.31 and δ 5.38 x 90.42 was due to presence of α and β anomers of the reducing sugar. The presence of six monosaccharides present in hexasaccharide Usose was also confirmed by six doublets at δ 6.24 (1H), δ 5.38 (1H) δ 5.28 (1H) δ 4.68(2H) and δ 4.46 (2H) in the 1H NMR of Usose acetate and it was complemented by anomeric carbon signals at δ 90.31 (1C), δ90.2 (1C), δ 92.12 (1C), δ 95.49(1C), δ 101.87 (1C), δ 102.20 (2C) in the ^{13}C NMR. The nature of reducing sugar S1 was confirmed by anomeric protons / carbon signals at δ 5.38 x δ 90.42 and comparing it with the literature value of β Glc and hence the reducing monosaccharide was confirmed as β-Glc. Further the anomeric proton signals at δ 5.38 J = 8.4 Hz, assigned to β-Glc S1 showed three cross peaks at δ 5.38 (3.50, 4.56 and 5.15) with its TOCSY spectra which were later identified as H-4, H-2 and H-3 of reducing Glc while COSY spectrum of Usose acetate. The peak at δ 3.50 assigned to H-4 of S1 suggested that there was only one position was available for glycosidic linkage in S-1 i.e., H-4 of S1.

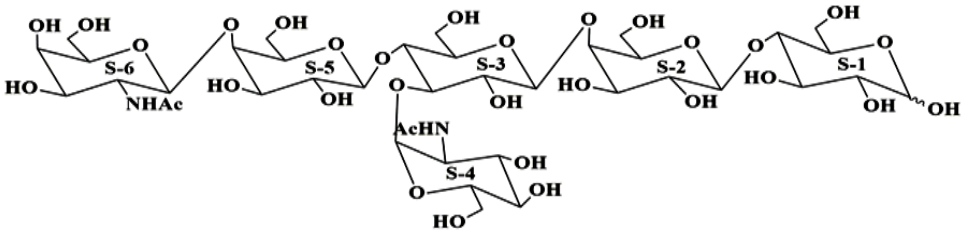
Further to assign the linkage between S-1 and S-2 the chemical shift of δ 3.50 (H-4 of S1) was examined in the HMBC spectrum of Usose acetate which gave cross peak at δ 3.50 x 101.02 which was due to the correlation of H-4 of S1 and C1 of S2 i.e., 101.02 further this anomeric carbon signal at δ 101.02 gave its complementary anomeric proton signal at δ 4.56 (J=9.2Hz), in the HSQC spectrum of Usose acetate. Hence confirming the anomeric proton/ carbon at chemical shift at δ 4.56 and 102.02 for S2 of the oligosaccharide. These anomeric proton/carbon values were having resemblance with the literature value of β-Gal therefore confirming the β-Gal was the next monosaccharide in the sequence the J value of 9.2Hz also confirmed the β configuration of S-2 of the monosaccharide. Hence confirming a β (1→4) glycosidic linkage between S1 and S2. Further the anomeric proton signal at δ 4.56 (S-2) in the TOCSY spectrum of Usose acetate gave three cross peaks at δ 4.56 (3.90, 5.05 and 5.36) which were confirmed as H-4, H-3 and H-2 by COSY spectrum of Usose acetate. The cross peak at δ 4.56 x3.90 suggested that the H-4 of S2 was available for the glycosidic linkage by next monosaccharide unit in the oligosaccharide. Hence this signal at δ 3.90 was again examined at in the HMBC spectrum but there was no cross peak was found in the HMBC spectrum therefore, the signal at δ 3.90 was correlated in the HSQC spectrum which gave a cross peak at δ 4.68 x 73.11. which was due to correlation of H-4 and C-4 of sugar S2 in HSQC spectra. Which was confirmed by Reverse HMBC experiment as a (1→4) linkage between S3 and S2 was confirmed. The anomeric proton signal at δ 4.68 showed its complementary carbon signal at δ 95.49 in HSQC spectrum of Usose acetate. These values of anomeric proton/ carbon were having resemblance with the literature value of β-Glc hence, S3 was confirmed as β-Glc which was also confirmed by the J value of 8.1Hz for the anomeric signal at δ 4.18 assigned to β-Glc S3. Hence confirming its β (1→4) linkage between S3 and S2.

Further the anomeric proton signal at δ 4.68 assigned to β Glc S3 gave three cross peaks at δ 4.68 (δ 3.50, 3.85 and 5.25) in the TOCSY spectrum of Usose acetate. Which was later identified as H-4, H-3 and H-2 respectively by COSY spectrum of Usose acetate. The chemical shift of cross peaks at δ 4.68 x 3.50 and δ 4.68 x 3.85 suggested that in Glc S3 these two positions i.e., H-4 and H-3 was available for glycosidic linkage by next monosaccharide units. The H-3 position of β Glc S3 at δ 3.85 showed a cross peak at δ 3.85 x 71.87 in HSQC spectrum of Usose acetate which showed a long-range coupling with anomeric proton of next monosaccharide S4 i.e., H-1 of S4 and C3 of S3 at δ 5.28 x 71.87 in the reverse HMBC spectrum of acetylated Usose confirming a (1→3) linkage between S4 and S3.

The anomeric proton signal at δ 5.28 showed its complementary carbon signal at δ 92.12 in the HSQC spectrum of Usose acetate. These chemical shift values of anomeric carbon and anomeric proton were having resemblance with literature value of α-GlcNHAc. Therefore, the S4 was confirmed as α GlcNHAc. The coupling constant of GlcNHAc S-4 J=3.9 Hz also confirm a α glycosidic linkage between S4 and S3. Further the anomeric proton signal at δ 5.28 assigned to α-GlcNHAc (S4) gave three cross peaks at δ 5.28 (3.95, 5.04 and 4.60) in its TOCSY spectrum which was later identified as H-2 (containing NHAc group), H-3 and H-4 by COSY spectrum. This anomeric proton did not show any cross peak in the linkage region hence it was confirmed that α-GlcNHAc S4 was present at non-reducing end and none if its -OH groups was involved in glycosidic linkage. As it was already described that β-Glc S3 had two vacant positions at H-3 and H-4.

Further it is already confirmed that H-3 of S3 was linked to α-GlcNHAc but H-4 of S3 was still vacant which was assigned by a peak at δ 3.50 which gave the cross peak at δ 3.50 x 102.03 in the HMBC spectra of Usose acetate confirming a (1→4) linkage between S3 and S5. The anomeric carbon at 102.02 gave its complementary anomeric proton signal at δ 4.56 (J= 9.2) in the HSQC spectrum of Usose acetate. The chemical shift value of S5 in ^{13}C and ^{1}H NMR was δ 102.02 and δ 4.56 were having resemblance with the respective literature values of β-Gal. therefore, it was confirmed that the monosaccharide S5 was β-Gal.

Further the anomeric proton value of β-Gal S5 at δ 4.56 gave three cross peaks at δ 4.56 (δ 3.90, 5.05 and 5.36) in the TOCSY spectrum of the compound. Which were identified as H-4, H-3 and H-2 by the COSY spectrum of Usose acetate. The cross peak at δ 4.56 x δ 3.90 assigned to H-4 of S5 suggested that this position was available for glycosidic linkage by next monosaccharide unit. The ^{1}H NMR signal of H-4 (S5) at δ 3.90 gave a cross peak at δ 3.90 x 101.87 in the HMBC spectrum of Usose acetate. Thus, confirming the 1→4 linkage between S6 and S5. The anomeric carbon of S6 at δ 101.87 gave its complementary anomeric proton signal at δ 4.68 in the HSQC spectrum of Usose acetate. The values obtained for the anomeric carbon and protons of a S6 were having resemblance with the chemical shift value of β-GalNHAc. Hence the S6 monosaccharide was confirmed as β- GalNHAc. The anomeric proton value of δ 4.68 (J=7.9Hz) also confirmed the β glycosidic linkage between S6 and S5. Further the anomeric proton signal at δ 4.68 assigned to β- GalNHAc S6 provided three cross peaks at δ 4.68 (δ 3.85, 4.55 and 5.14) in the TOCSY spectrum of the compound which were later identified as H-2 (containing NHAc group) H-4 and H-3 by COSY spectrum of Usose acetate. This anomeric proton did not show any cross peak in the linkage region and hence it was confirmed that the β-GalNHAc S6 was presrnt at non reducing end and none of its hydroxyl groups were involved in the linkage region. Hence, it was concluded the structure elucidation of an oligosaccharide may be simplified by the NMR experiments using ^{1}H, ^{13}C, HSQC,TOCSY COSY and HMBC experiments which may further be confirmed by mass spectrometric data. This review article justifies the importance of non-invasive experiments of NMR experiments 1D and 2D. In conclusion we could define that by performing the ^{1}H, ^{13}C, HOMO COSY, HETRO COSY, TOCSY, HSQC and HMBC experiments we could define the complete stereoscopic structure of any of the oligosaccharides including the inter glycosidic linkages, conformation of glycosidic linkages and conformation of monosaccharide's present their in. The results so obtained was confirmed by the high-resolution mass spectroscopy which was the M^{+} of the oligosaccharide along with other important mass fragments which were helpful in knowing the conformation and configuration of the structure of the oligosaccharide. Hence on the basis of results interpreted by data of 1H, 13C, HSQC, TOCSY and COSY experiments of NMR and Mass spectrometry, the structure of Usose was explained as given under-



USOSE

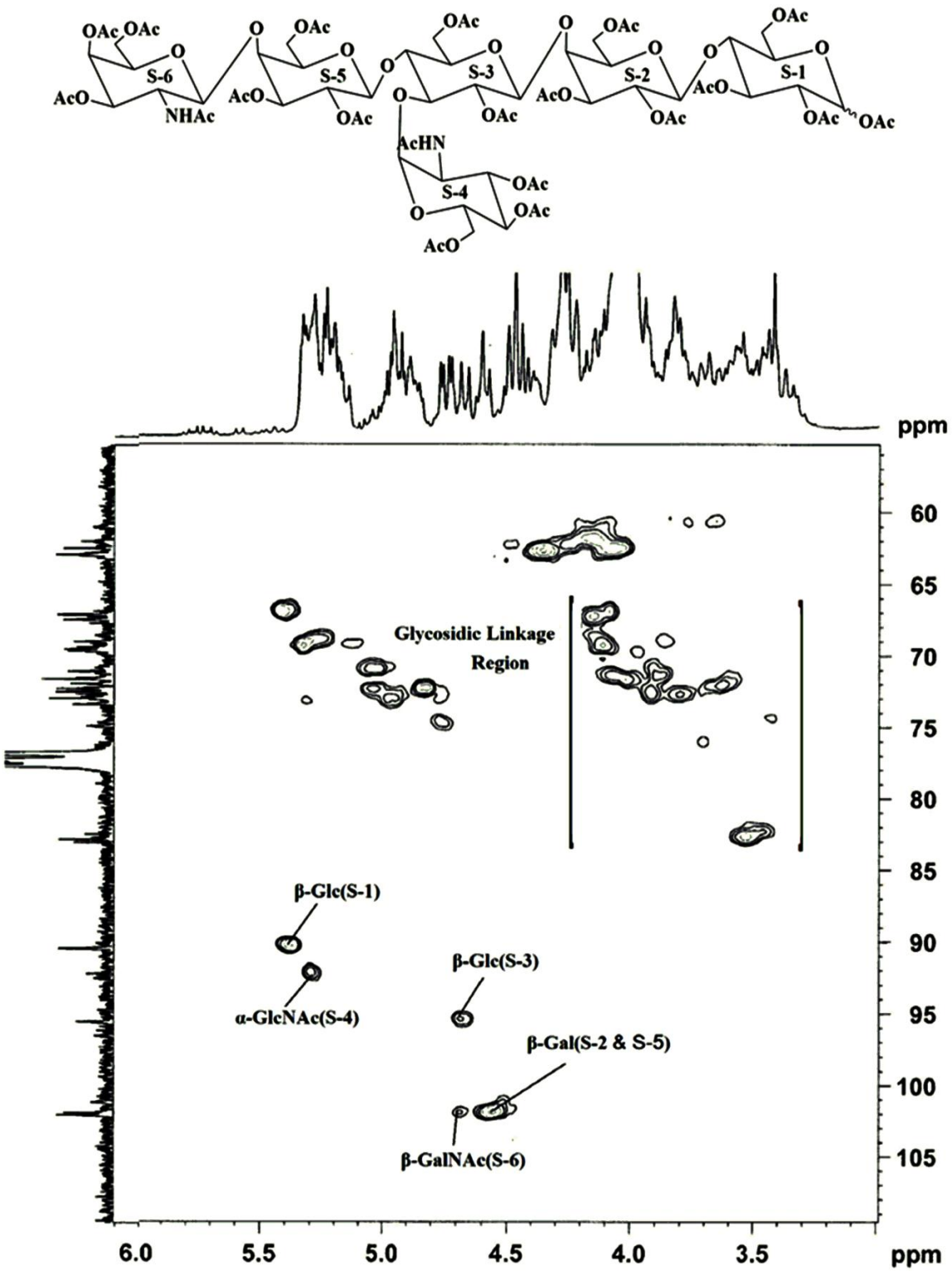


Figure: HSQC Spectrum of Usose Acetate in $CDCl_3$ at 300 MHz.

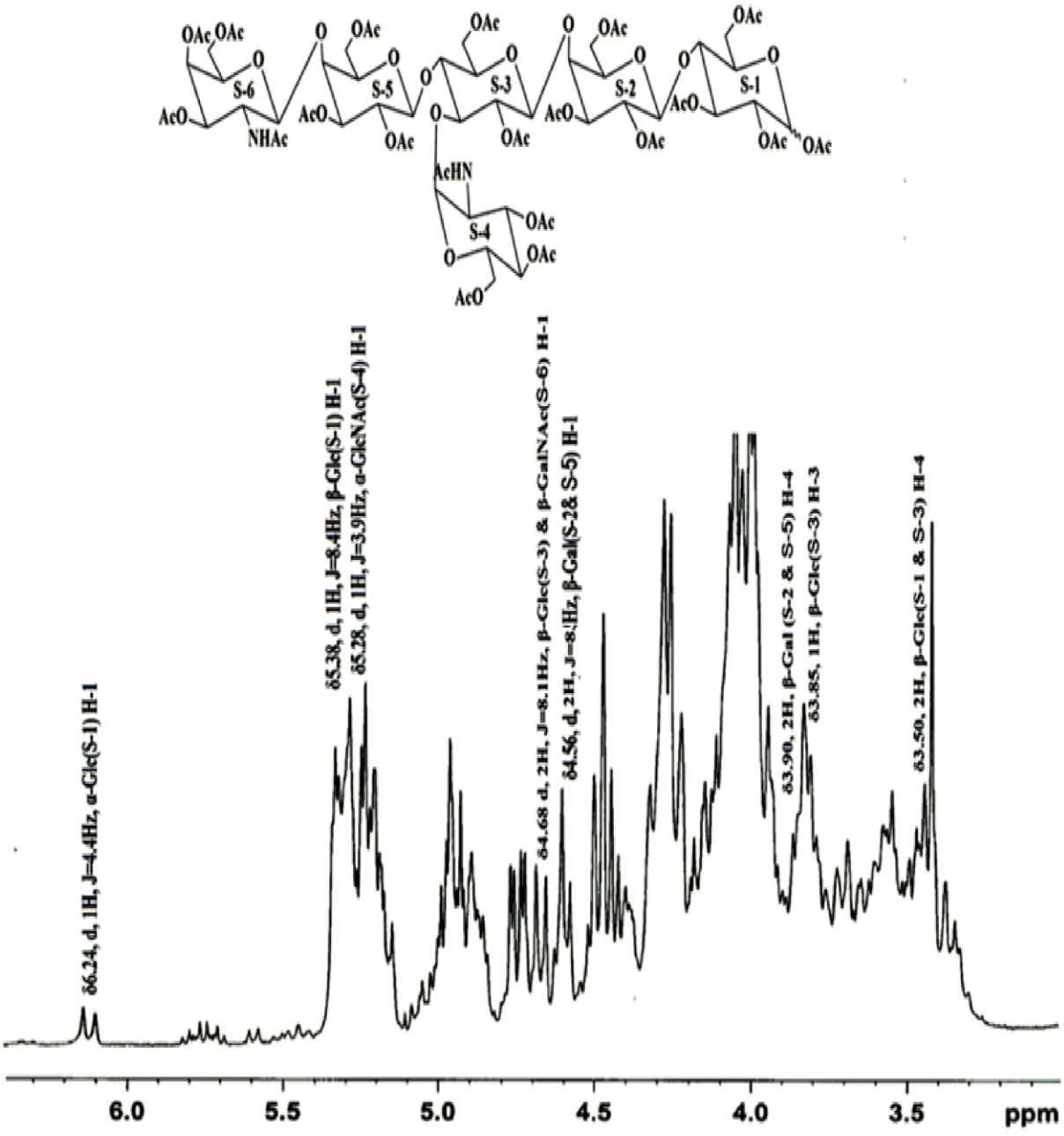


Figure: ^{1}H NMR Spectrum of Usose Acetate in $CDCl_3$ at 300 MHz.

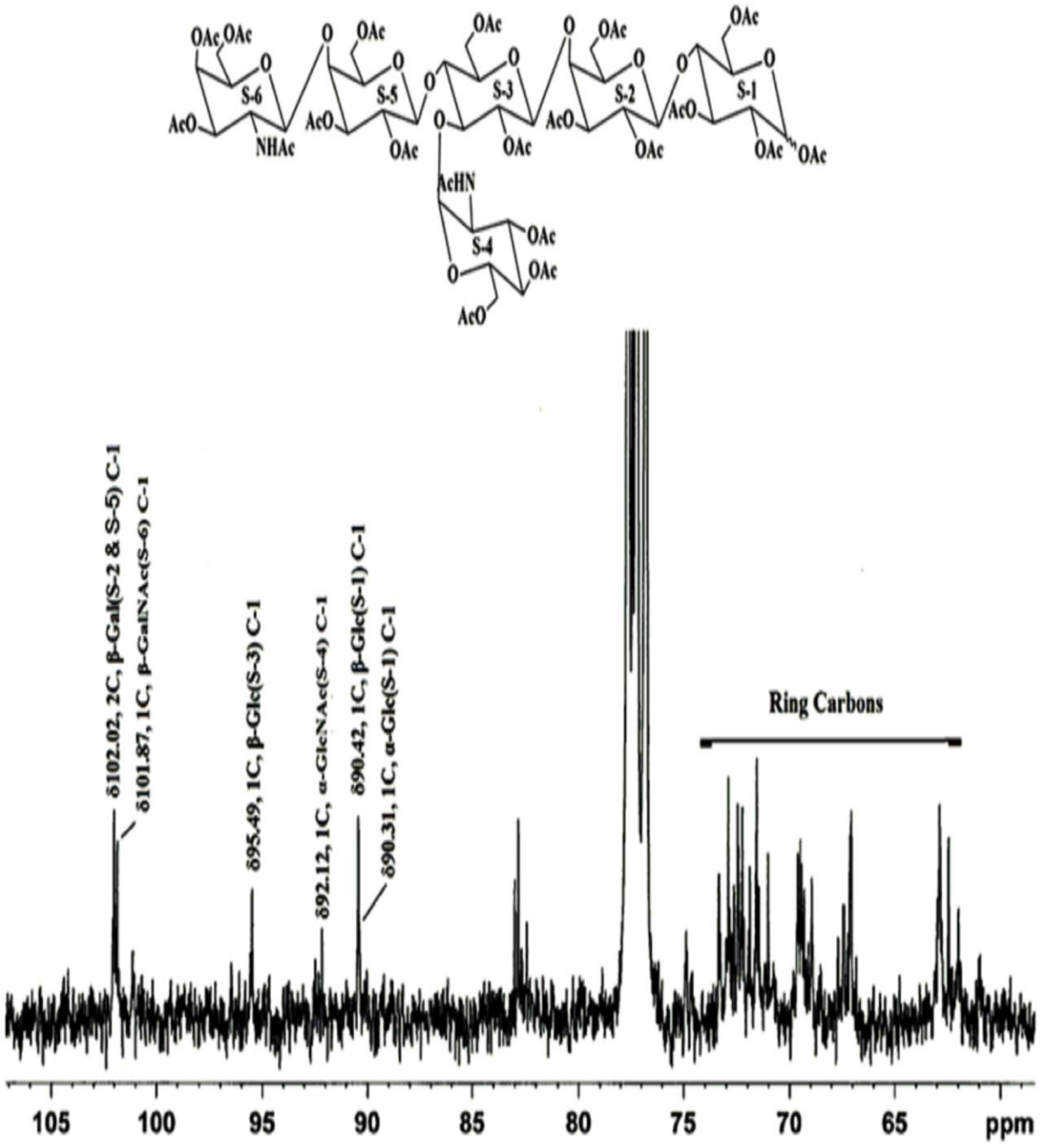


Figure: ^{13}C NMR Spectrum of Usose Acetate in $CDCl_3$ at 75 MHz.

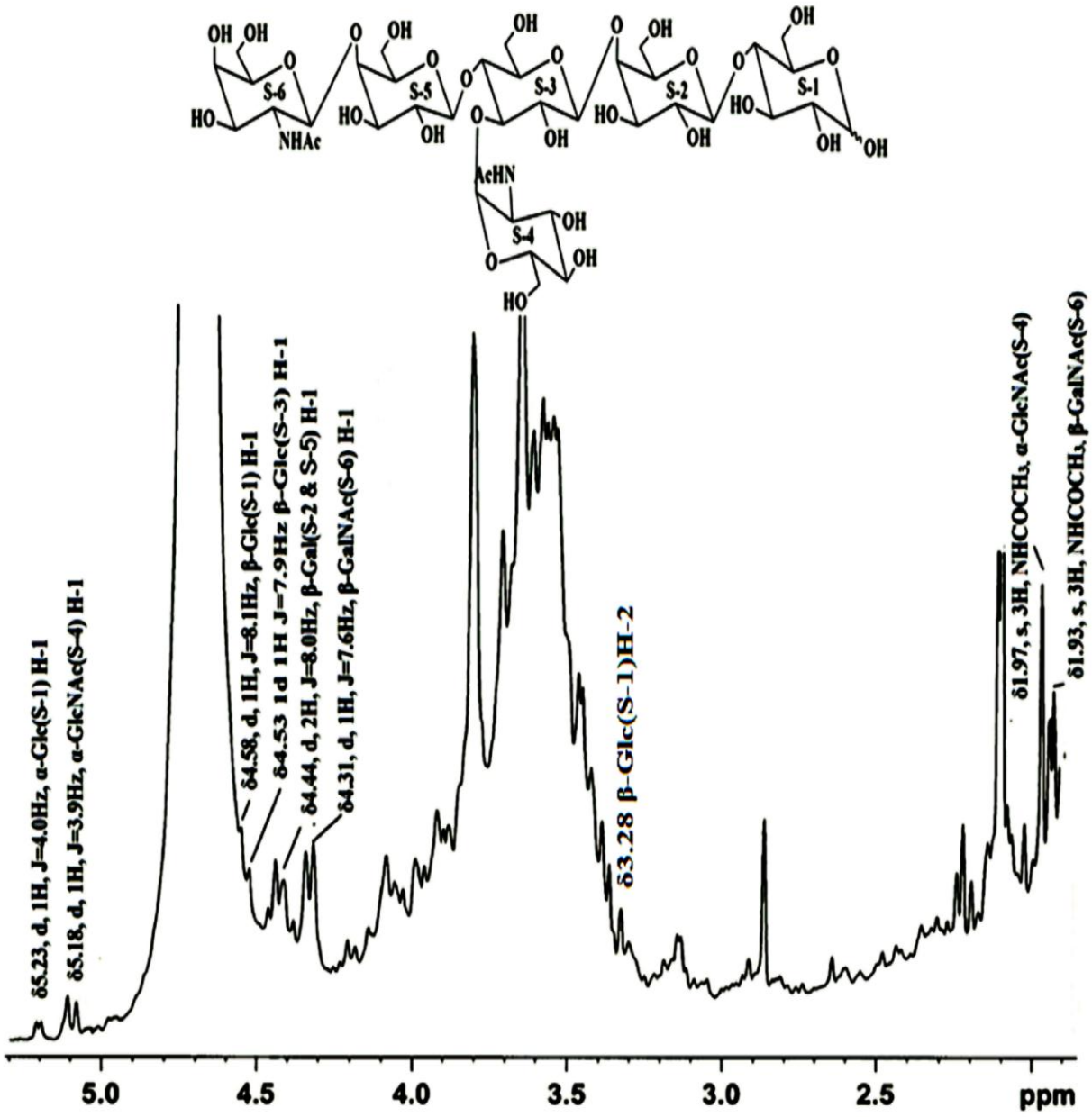


Figure: ^{1}H NMR Spectrum of Usose Acetate in D_2O at 300 MHz.

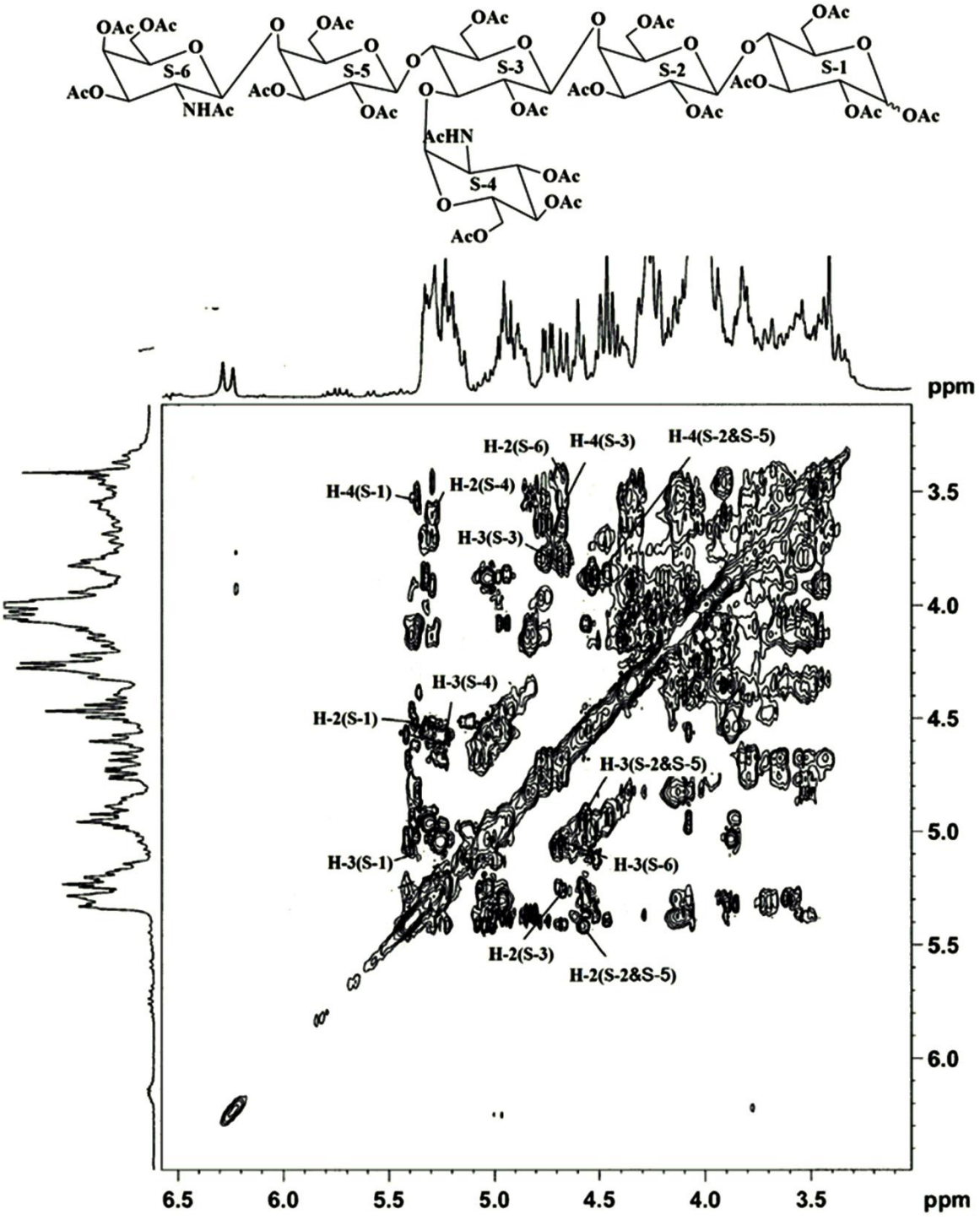


Figure: TOCSY Spectrum of Usose Acetate in $CDCl_3$ at 300 MHz.

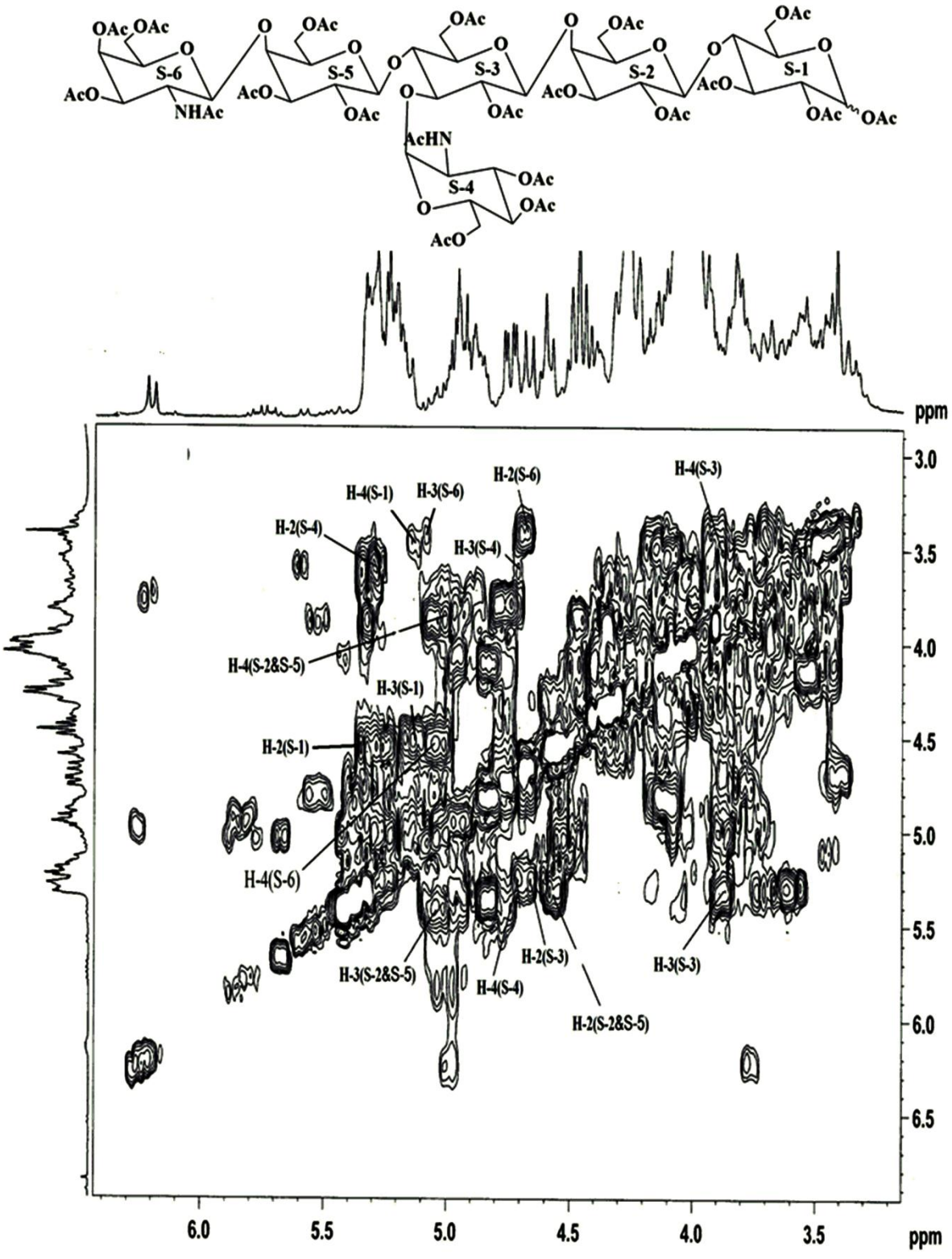


Figure: COSY Spectrum of Usose Acetate in $CDCl_3$ at 300 MHz.

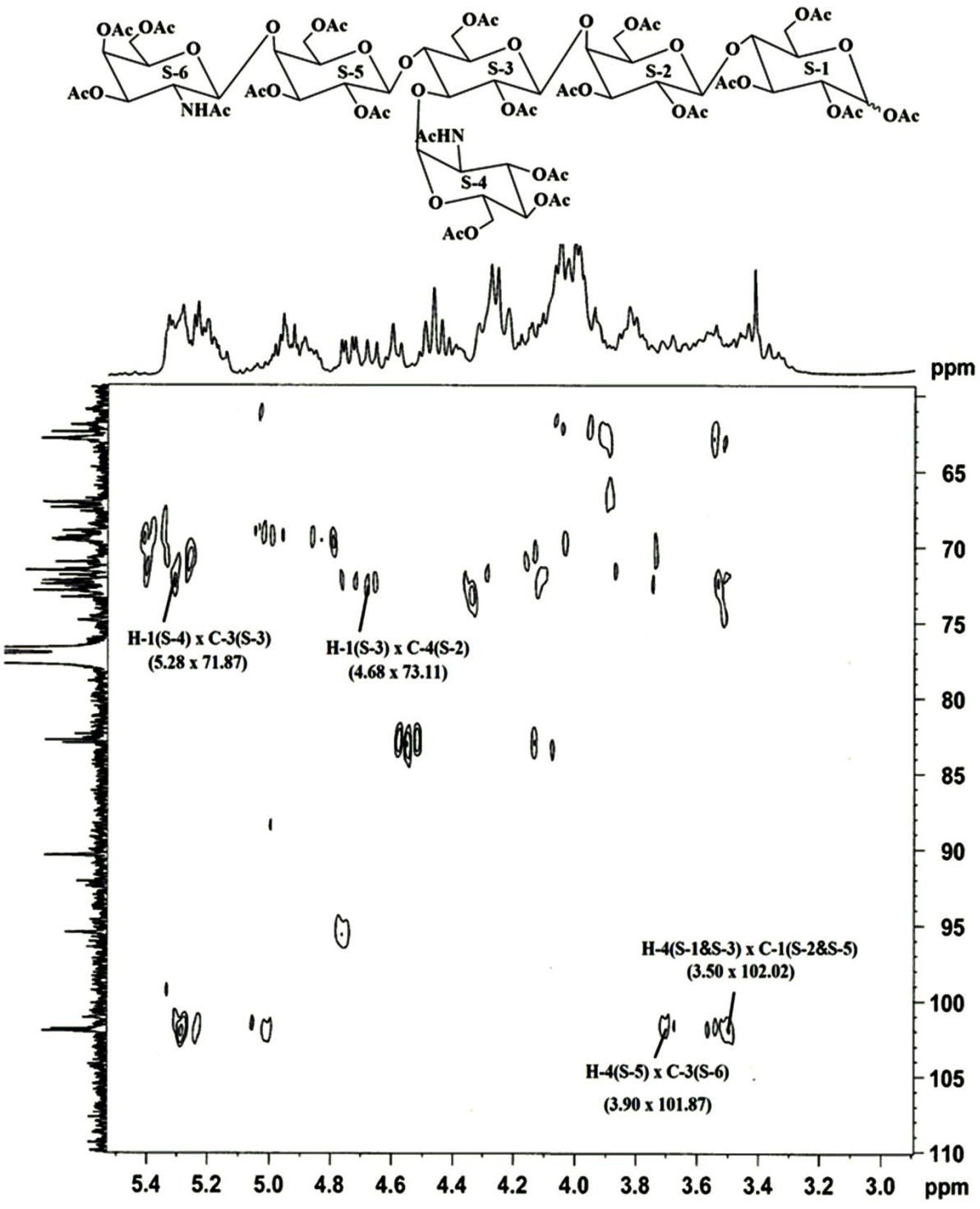


Figure: HMBC Spectrum of Usose Acetate in $CDCl_3$ at 300 MHz.

CONCLUSION

The 1-D (^{1}H and ^{13}C) and 2-D NMR (HSQC, TOCSY, COSY and HMBC) are very useful non-invasive experiment for the structure of complicated oligosaccharide obtained from any natural source.

ACKNOWLEDGMENTS

The authors are thankful to UGC, New Delhi for financial assistance [UGC- Ref. NO.:254/(CSIR-UGC NET DEC.2017].

REFERENCES

Yogini, S. and Williams, L. (2017). A glimpse of Ayurveda – The forgotten history and principles of Indian traditional medicine, *Journal of Traditional and Complementary Medicine*, Jan; 7(1): 50–53.

Mana, D., Mohanan, A. and Venkatesha, R. (2017). Milk and Milk Products in Ayurveda: A Review, *Biol. Life Sci. Forum,* 6(1), 13.

Pietrzak-Fiećko, R. and Anna, M. (2020). The Comparison of Nutritional Value of Human Milk with Other Mammals' Milk, *Nutrients*, May; 12(5): 1404.

Kumar, S., Kumar, K., Suman, S. and Kumar, P. (2014). Cow Milk and Human Health-A Review, *Journal of Dairy Science and Technology*, Volume 3, Issue 3, 2319-3409.

Chen, X. and Michael G. Gänzle (2017). Lactose and lactose- derived oligosaccharides: More than prebiotics, *International Dairy Journal*, Volume 67, Pages 61-72.

Haug, A., Arne, T. Høstmark and Harstad, M. (2007). Bovine milk in human nutrition – A Review, *Lipids Health Dis.;* 6: 25.

Bleasdale, M., Richter, K., Janzen, A., Brown, S., Scott, A., Zech, J. and Wilkin, S. (2021). Ancient proteins provide evidence of dairy consumption in eastern Africa, *Nature Communications* volume 12, Article number: 632.

Gunjan Narain, D., Khare, A. and Deepak, D. (2016). Isolation of Novel Oligosaccharide from Shyama Dhenu (Black Cow) Milk, *J. Biol. Chem. Research*, Volume 33 (2), Page No. 648-654.

Lis-Kuberka, J. and Orczyk-Pawiłowicz, M. (2019). Sialylated Oligosaccharides and Glycoconjugates of Human Milk. The Impact on Infant and Newborn Protection, Development and Well-Being, *Nutrients*.; 11(2): 306.

Tafaro, A., Magrone, T., Jirillo, F., Martemucci, G., D'Alessandro, A., Amati, L. and Jirillo, E. (2007). Immunological properties of donkey's milk: its potential use in the prevention of atherosclerosis, *Curr Pharm Des.*; 13(36):3711-7.

Abdelali, D., Victor, P., Requena Pilar Antonio, M. and Emilia, G. (2006). Goat Milk Oligosaccharides Are Anti-Inflammatory in Rats with Hapten-Induced Colitis, Volume 136, Issue 3, Pages 827-831, 561-712.

Schwonzen, M., Schmits, R., Baldus, S. E., Vierbuchen, M. and Hanish, F. G., Pfreundschuh (1992). Associated epitope. *Br. J. Cancer.,* 65: 559–565.

Abe, K., Mckibbin, J.M. and Hakomori, S. (1983). The monoclonal antibody directed to difucosylated type 2 chain (Fuc alpha 1 leads to 2Gal beta 1 leads to 4 [Fuc alpha 1 leads to 3] GlcNAc; Y Determinant). *Eur J. Biochem Bio Chem* 258: 11793–11797.

Fang, J.N., Proksch, H. and Wagner, H. (1985). Immunologically active polysaccharides of acanthopanax senticosus. Phytochemistry 24: 2619-22.

Srivastava, R. and Kulshretha, K. (1989). Bio reactive polysaccharides from plants, *Phytochem*, 28:2877-2883.

Deepak, D., Gangwar, L. and Sharma, M. (2020). Isolation and Structure Elucidation of a Novel Tetrasaccharide Asose from Shyamadhenu's Milk, *Trends in Carbohydrate Research*, Vol. 12, No. 4, 64-76.

Zhang, M., Chen, Y. and Zhao, C. (2022). Research status of the effect of natural oligosaccharides on glucose metabolism, Vol. 3 issue 6, 54.

Khan, M., Mishra, A. and Deepak, D. (2018). Isolation and structure elucidation of novel hexasaccharide Usose from Lal Muha Cow milk by 2-D NMR, *TCR*, Volume 10(4), 28-40.

Corresponding author: Dr. Desh Deepak, Department of Chemistry, University of Lucknow, Lucknow- 226007, U.P., India
Email: deshdeepakraju@rediffmail.com