Eurasian Journal of Analytical Chemistry
ISSN: 1306-3057

ISOLATION OF OLIGOSACCHARIDES AND ANTI-MICROBIAL ACTIVITY OF GADDI SHEEP'S MILK

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Received 09 March 2018 ▪ Revised 19 April 2018 ▪ Accepted 22May 2018

ABSTRACT: *Oligosaccharides are natural constituents of all bacteria, fungi, plants and placental mammals' milk. The milk is a rich source of oligosaccharides with different novel oligosaccharides depends on the nature of their origin in which mammals the milk belongs. Buffalo milk oligosaccharides have resistivity against parasitic infections. Donkey milk oligosaccharides have ability to stimulate non-specific and specific immunological resistance. Goat milk oligosaccharides is useful for intestinal protection and repair after a damage caused by DSS (Dextron sodium sulphate)-induced colitis and their implication in human intestinal inflammation. Goat milk oligosaccharides have anti-inflammatory effects. The cows' milk oligosaccharides reduce the adhesion of enterotoxic Escherichia coli strains of the calf. Remember all bioactive properties of different origin, Gaddi sheep's milk was collected and processed by Modified method of Kobata and Ginsburg and then it was purified by Sephadex G-25 Gel column. The homogeneity is confirmed by reverse phase high performance chromatography and supported by thin layer chromatography.The acetylation of oligosaccharides mixture followed by the silica gel chromatography led to isolation of a novel oligosaccharides. The antimicrobial activity of Gaddi sheep's milk oligosaccharides was investigated against three bacterial namely Staphylococcus aureus, Escherichia coli and Salmonella Typhimurium and two fungal strains namely Candida albicans and Aspergillus niger are selected on the basis of their relevance as human pathogens. All samples of milk oligosaccharide exhibited antimicrobial activity against dermatomycotic fungi and foodborne pathogen bacteria.*

Key words: *Oligosaccharide, bioactivity, bacteria, fungi.*

INTRODUCTION

Oligosaccharides are natural constituents of all bacteria, fungi, plants and placental mammals' milk. 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. In addition to lactose, milk contains numerous glycoproteins and a variety of free oligosaccharides. Oligosaccharides with potential physiological benefit could be found in animal milks. The Elephant milk oligosaccharide fraction contained a high ratio of sialyl oligosaccharide, this may be significant with respect to the formation of brain components, such as gangliosides of the suckling calves. N-acetylneuraminlactose sulphate may play an important role in the nutrition of the rat pups, which is the dominant oligosaccharide in the dog milk[169]. Buffalo milk oligosaccharides have ability to stimulate non-specific immunological resistance of the host against parasitic infections. Donkey milk oligosaccharides have ability to stimulate non-specific and specific immunological resistance. Goat milk oligosaccharides play an important roles in intestinal protection and repair after a damage caused by DSS (Dextron sodium sulphate)-induced colitis and their implication in human intestinal inflammation. Goat milk oligosaccharides have anti-inflammatory effects in rats with trinitrobenzenesulfonic (T) acid induced colitis and may be useful in the management of inflammatory bowel disease. The cows' milk oligosaccharides reduce the adhesion of enterotoxic Escherichia coli strains of the calf. Human milk

oligosaccharides are helpful of postnatal development of the immune system, the protective effect of human milk against viral and bacterial infections, and the enhancement of the bioavailability of minerals. The anti-infective effect of human milk has been partly attributed to the high amount of free oligosaccharides as well as glycoconjugates, and also resistant against digestion, Oligosaccharides with potential physiological benefit could be found in animal milks. The Elephant milk oligosaccharide fraction contained a high ratio of sialyl oligosaccharide, this may be significant with respect to the formation of brain components, such as gangliosides of the suckling calves. N-acetylneuraminlactose sulphate may play an important role in the nutrition of the rat pups, which is the dominant oligosaccharide in the dog milk. Buffalo milk oligosaccharides have ability to stimulate non-specific immunological resistance of the host against parasitic infections. Donkey milk oligosaccharides have ability to stimulate non-specific and specific immunological resistance. Goat milk oligosaccharides play an important roles in intestinal protection and repair after a damage caused by DSS (Dextron sodium sulphate)-induced colitis and their implication in human intestinal inflammation. Goat milk oligosaccharides have anti-inflammatory effects in rats with trinitrobenzenesulfonic (T) acid induced colitis and may be useful in the management of inflammatory bowel disease. The cows' milk oligosaccharides reduce the adhesion of enterotoxic Escherichia coli strains of the calf. Remember all bioactive properties of different origin, Gaddi sheep's milk was collected and processed by Modified method of Kobata and Ginsburg and then it was purified by Sephadex G-25 Gel column. The homogeneity is confirmed by reverse phase high performance chromatography and supported by thin layer chromatography. The acetylation of oligosaccharides mixture followed by the silica gel chromatography led to isolation of a novel oligosaccharides. These crude oligosaccharide mixture are further test for microbial activity.

MATERIAL AND METHODS

2.1. GENERAL PROCEDURE

2.11. Optical rotations were measured with a PERKIN-ELMER 241 automatic polarimeter in 1cm tube. 1H and 13C NMR spectra of oligosaccharides were recorded in D2O and the spectra of acetylated oligosaccharides were recorded in CDCl3 at 250C on a Bruker AM 300 FT NMR spectrometer. The electrospray mass spectra were recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer. The sample (dissolved in suitable solvents such as methanol/acetonitrile/water) was introduced into the ESI source through a syringe pump at the rate 5µl per min.

The ESI capillary was set at 3.5 KV and the cone voltage was 40 V. The spectra were collected in 6s scans and the print outs are averaged spectra of 6-8 scans. The C, H and N analysis were recorded on CARLO-ELBA 1108 an elemental analyzer. The sugars were visualized on TLC with 50% aqueous H_2SO_4 reagent and on Paper Chromatography with acetyl acetone and p-dimethyl amino benzaldehyde reagents. The absorbent for TLC was silica gel G (SRL) and CC silica gel (SRL, 60-120 mesh). PC was performed on Whatman No.1 filter paper using solvent system ethyl acetate-pyridine (2:1) saturated with H2O. Sephadex G –25 (PHARMACIA) was used in gel permeation chromatography. Freeze drying of the compound was done with the help of CT 60e (HETO) lyophylizer and centrifuged by a cooling centrifuged Remi instruments 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 samples of glucosamine, galactosamine, galactose and glucose were purchased from Aldrich Chemicals.

2.12. Isolation of Oligosaccharides by Kobata and Ginsburg Method: 10L Gaddi Sheep milk was collected from a sheep from northern himalayan region and was stored at -20^{0}C. The milk was processed by the method of Kobata and Ginsburg. It was centrifuged for 15 min at 5000 rpm at -4^{0}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^{0}C. The white precipitate formed, mainly of lactose and protein was removed by centrifugation and washed twice with 68% ethanol at 0^{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 (162 g). This lyophilized material (mixture of oligosaccharide) was further purified by fractionating it on Sephadex G-25 chromatography using glass triple distilled water as eluent at a flow rate of 5 min/mm. Each fraction was analysed by phenol sulphuric acid reagent for the presence of neutral sugar. The homogeneity of Gaddi sheep's milk oligosaccharides was further confirmed by reverse phase high performance liquid chromatography [HPLC] which supported by thin layer chromatography [TLC]

2.2. Microbial test

Antimicrobial activity of the milk extracts was assessed against bacteria and fungi

2.21. Disk Diffusion Method: Disc diffusion or the Kirby–Bauer test is classical and commonly used microbiology techniques to determined antimicrobial resistance. A suspension of the milk oligosaccharide (of approximately 1–2 × 10^8 CFU/mL) is prepared to a particular McFarland standard, and then spread evenly onto an appropriate agar.

2.211.Mc Conkey's lactose agar medium and Brilliant green agar medium were used for Staphylococcus aureus, Escherichia coli and Salmonella Typhimurium, respectively and spread over in a petri dish. The agar typically contains (weight/volume). pH adjusted to neutral at 25 °C. The final concentrations of Gaddi sheep's milk oligosaccharides were 25, 20, 15, 10, 5, 2.5, 1.25, 0.75, 0.325 and 0 μg mL^{-1}. Pure culture of the bacteria was taken and 0.1 ml of both culture was spread on the surface of media plate with the help of sterile swab. After the plates were allowed to dry, sterile paper disks containing Gaddi sheep's milk oligosaccharide and double-distilled H_2O were placed on the agar plate surface. Then, the plates were incubated at 37°C for 24hours

2.212. Sabourad's dextrose agar medium was used for Aspergillus niger spread over in a petri dish. pH adjusted to neutral at 25 °C. The final concentrations of Gaddi sheep's milk oligosaccharides were 25, 20, 15, 10, 5, 2.5, 1.25, 0.75, 0.325 and 0 μg mL^{-1}. Pure culture of the fungi was taken and 0.1 ml of both culture was spread on the surface of media plate with the help of sterile swab.After the plates were allowed to dry, sterile paper disks containing Gaddi sheep's milk oligosaccharide and double-distilled H_2O were placed on the agar plate surface. Then, the plates were incubated at 28 °C for 5-7days.

Sabourad's dextrose agar medium was used for Candida albicans spread over in a petri dish. pH adjusted to neutral at 25 °C. The final concentrations of Gaddi sheep's milk oligosaccharides were 25, 20, 15, 10, 5, 2.5, 1.25, 0.75, 0.325 and 0 μg mL^{-1}. Pure culture of the fungi was taken and 0.1 ml of both culture was spread on the surface of media plate with the help of sterile swab. After the plates were allowed to dry, after that sterile filter paper discs were soaked in aqueous solution of the Gaddi sheep's milk oligosaccharide and were placed on the plate inocubated with and double-distilled H_2O at 37 °C for 24hours.

RESULT AND DISCUSSION

1.1. Confirmation of Homogeneity of Gaddi's Milk Oligosaccharide by Reverse Phase HPLC-

Oligosaccharide mixture were quantitatively analysed by reverse phase HPLC. The HPLC system was equipped with Perkin-Elmer 250 solvent delivering system, 235 diode array detector and G.P. 100 printer plotters. The cyano column used for this purpose was a binary gradient system. The eluents were detected at 240 nm. Twenty one peaks were noticed in the sample at the varied retention times from 00.942min. to 19.942min. for convenience the peaks were numbered in their increasing order of retention time i.e. 19.942 min. (R_1),19.592 min. (R_2),19.435 min. (R_3),19.342 min. (R_4),15.575 min. (R_5), 14.700 min. (R_6),09.283 min. (R_7),07.317 min. (R_8),06.742 min. (R_9),05.300 min. (R_{10}),04.742 min. (R_{11}),04.383 min. (R_{12}),04.000 min. (R_{13}),03.758 min. (R_{14}),02.950 min. (R_{15}),02.758 min. (R_{16}),02.375 min. (R_{17}),01.750 min. (R_{18}),01.383 min. (R_{19}),01.167 min. (R_{20}) & 00.942 min. (R_{21}).

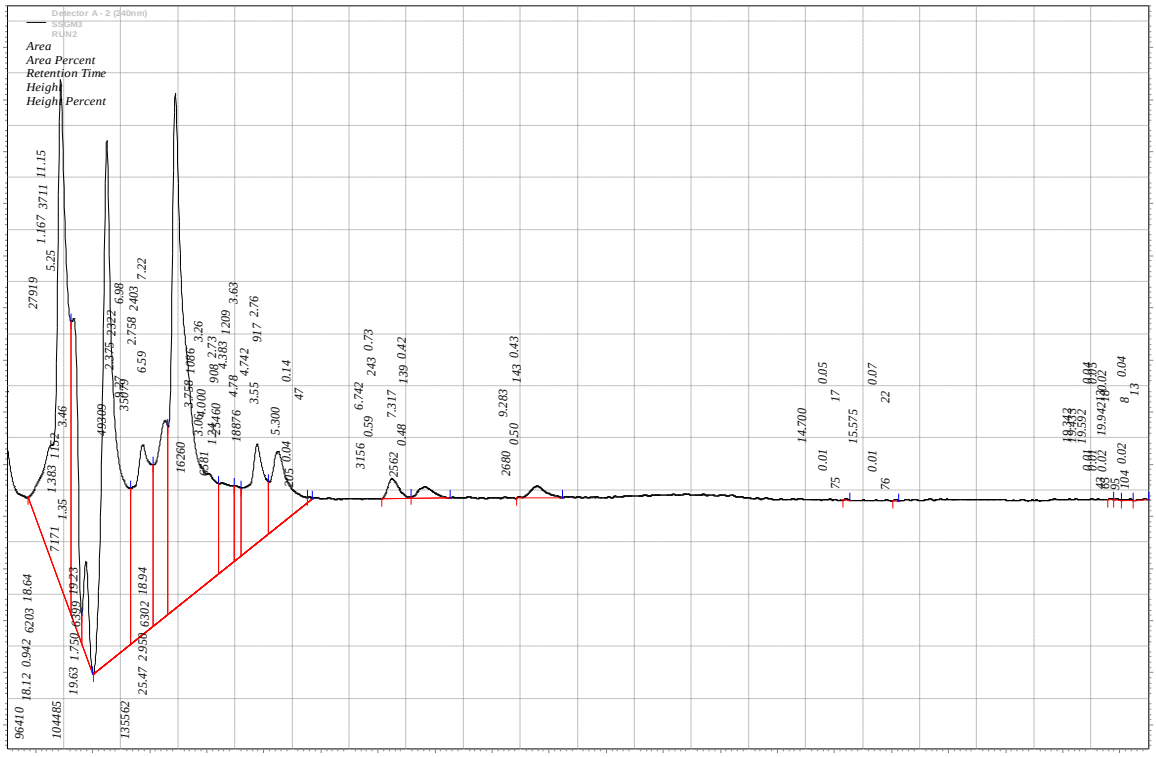


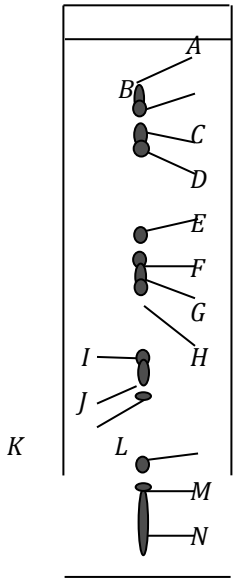
Fig 1: 90% TDW: 10% ACN in water 240 nm--

Table 1: HPLC Table of Crude Gaddi Sheep's Milk Oligosaccharides

PK	Retention time	Area	Area %	Height	Height%
1	19.942	0013	00.04	000104	00.02
2	19.592	0008	00.02	000095	00.02
3	19.435	0018	00.05	000065	00.01
4	19.342	0013	00.01	000043	00.01
5	15.575	0022	00.07	000076	00.01
6	14.700	0017	00.05	000075	00.01
7	09.283	0143	00.43	002680	00.50
8	07.317	0139	00.42	002562	00.48
9	06.742	0243	00.73	003156	00.59
10	05.300	0047	00.14	000205	00.04
11	04.742	0917	02.76	018876	03.55
12	04.383	1209	03.63	025460	04.78
13	04.000	0908	02.73	006581	01.24
14	03.758	1086	03.26	016260	03.06
15	02.950	6302	18.94	135562	25.47
16	02.758	2403	07.22	035079	06.59
17	02.375	2322	06.98	049309	09.27

18	01.750	6399	19.23	104485	19.63
19	01.383	1152	03.46	007171	01.35
20	01.167	3711	11.15	027919	05.25
21	00.942	6204	18.64	096410	18.12

Acetylation of Oligosaccharide Mixture-- 22gm of crude oligosaccharide mixture was acetylated with pyridine (20ml) and acetic anhydride (20ml) at 60^0 C and solution was stirred overnight. The mixture was evaporated under reduced pressure and the viscous residue was taken in $CHCl_3$ (500ml) and washed in with ice cold water. The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness yielding the acetylated mixture (26.50gm). The acetylation converted the free sugars into their nonpolar acetyl derivatives which were resolved nicely on TLC, giving ten spots on TLC ie A,B,C,D,E,F,G,H, I, J, K, L, M & N of which eleven coumpounds were finally separated by column chromatography over silica gel using hexane:chloroform, chloroform and $CHCl_3$:MeOH as eluents. Thin Layer Chromatography (TLC) Of acetylated Gaddi Sheep's milk oligosaccharides.



$CHCl_3$: MeOH (95:5)

Inhibition was observed by any of the milk extract against the above said bacteria and fungi. Pre-known references for comparison in antibacterial and antifungal tests are used. All experimental procedures were performed in triplicates.

3.2. Anti-bacterial Test: The solitary response of Gaddi sheep's milk oligosaccharides on E. coli, S. aureus and S. Typhimurium was testify by scanning electron microscopy and transmission electron microscopy,which showed wrinkled surfaces, bleb-like structures, broken m embranes and wholed cytoplasm compared with normal morphology.

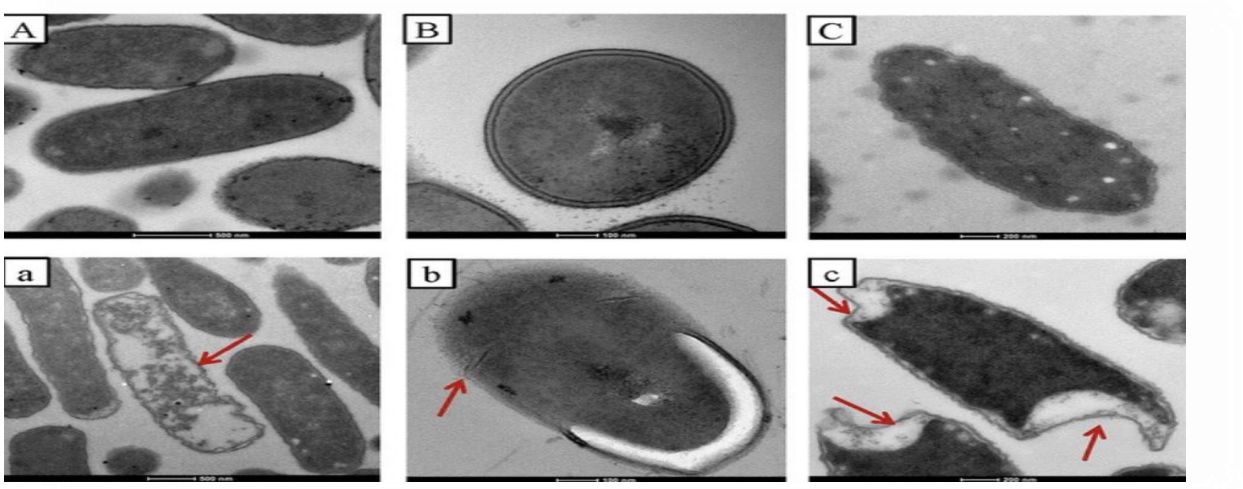


Fig 2: Morphosis of E. coli, S. aureus and S. Typhimurium. cells were observed under high resolution electron microscopy. **A**–**C** Normal E. coli, S. aureus and S. Typhimurium, high resolution electron microscopy. **a**–**c** Gaddi Milk oligosaccharides treated samples.

3.2. Anti-Fungal Test:

Fungi, viz: Aspergillus niger and Candida albicans are used. Antifungal susceptibility testing remains an area of intense interest. Susceptibility testing can be used for drug discovery and epidemiology. Number of reports is available showing efficiency of various milk as antifungal agents. Candida species becoming increasingly important as opportunistic fungal pathogens that frequently cause oral infections in immuno-competent and immune-compromised individuals due to the suppression of local as well as systemic defense mechanism it can also contaminate the failed root canal treated teeth Candida albicans remains the most common infection causing fungus, about 45% of clinical infections are caused by this pathogen. Gaddi sheep's milk oligosaccharides at the concentrations of 0.625, 1.25, 2.5, 5.0, 10, 20mg/ml for each extract have promising antifungal activity against six isolates of Candida albicans.

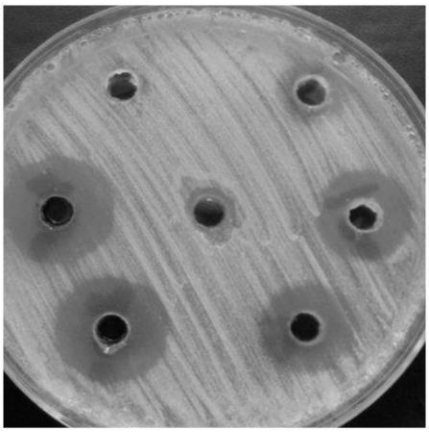


Fig 3: Inhibition zones of Candida albicans growth on Gaddi sheep's milk oligosaccharide the peripheral six wells contained extract concentrations (0.625, 1.25, 2.5, 5.0, 10, 20mg/ml).

We found three bacterial strains viz. E. coli, S. aureus and S. Typhimurium and only one fungal strain viz. C. albicans was significantly sensitive to Gaddi sheep's milk oligosaccharides. The growth of these strains was inhibited nearly 43% and 28%, respectively after 24 h of culture.

The used test-cultures from the genera Aspergillus, which are representatives of carcinogenic, toxigenic, deteriorative and allergenic fungi shows no inhibition. There were no zones of inhibition surrounding the disks that contained only double distilled water (ddH_2O).

CONCLUSION

Finally, on the basis of results obtained from the above study, the antimicrobial activity of Gaddi sheep's milk against Staphylococcus aureus, Escherichia coli and Salmonella Typhimurium, and fungus Candida albicans which frequently cause intense or incurable inflammatory disease of skin. It may be considered as a valuable natural remedies with novel functional properties in cosmetics and pharmaceutical industries.

Acknowledgements

Authors thanks to Dr. Rashmi Singh Associate Professor, Agra, India, for their support.

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