ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 12, Issue 2, 2019

Online - 2455-3891
Print - 0974-2441

Research Article

ANTI-*HELICOBACTER PYLORI* ACTIVITY AND IMMUNOSTIMULATORY EFFECT OF OLIGOSACCHARIDE ISOLATED FROM *AVERRHOA CARAMBOLA* L.

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Received: 02 July 2018, Revised and Accepted: 05 October 2018

ABSTRACT

Objective: The main goals of the study were to isolate oligosaccharide as a secondary metabolite from indigenous fruit, *Averrhoa carambola* L. (star fruit), and analyze its anti-*Helicobacter pylori* and immunostimulatory potential.

Methods: The oligosaccharide (S3W) isolated using adsorption chromatography was partially characterized using tandem mass spectrometry in atmospheric pressure chemical ionization/electron spray ionization (APCI/ESI) positive and negative mode and nuclear magnetic resonance. The use of microtiter well method determined anti-*H. pylori* activity in the form of percentage inhibition of microbial growth, while the determination of the immunostimulatory potential of S3W implemented a protection of DNA, buccal cells, and macrophages against damage by oxidant and N-nitroso-N- methylurea (MNU) and percentage splenocyte proliferative potential.

Results: S3W was a (1→2) β-heptaglucosaccharide with (4→6) α-branching at every second residue. APCI/ESI-positive mode showed B and A fragmentation patterns, while the negative mode showed abundance of A and C fragments. Anomeric hydrogen displayed integration values at 4.8 and 4.2 ppm indicating the presence of α-glucopyranose and β-glucopyranose. The compound protected DNA from oxidant-induced fragmentation. It expressed anti-*H. pyloric* activity with an IC_{50} value of 10.71 µg/ml. At 20 µg/ml, S3W protected buccal cells and macrophages significantly from damage due to oxidants and MNU carcinogens, while >50% splenocyte proliferation was induced as compared to that of an untreated control group.

Conclusion: Our studies constitute the first report on the significant immunostimulatory activity of the oligosaccharide isolated from star fruit. The study, thus, supports its application as a therapeutic potential in curbing gastric diseases caused by *H. pylori*.

Keywords: *Helicobacter pylori*, Macrophages, Splenocytes, Immunostimulation, Oligosaccharides.

 DOI: http://dx.doi.org/10.22159/ajpcr.2019.v12i2.28244

INTRODUCTION

The Gram-negative bacterium, *Helicobacter pylori*, can colonize in human gastric mucosa of an infected individual without causing clinical illness [1,2]. The development of gastric adenocarcinoma, gastric lymphoma, and mucosa-associated lymphoid tissue lymphoma in association with long-term *H. pylori* infection has classified *H. pylori* as a carcinogen [3-6]. It induces inflammation, reactive oxygen species (ROS) accumulation, infiltration and activation of immune cells, and oxidative damage to DNA in the gastric mucosa by protecting itself from death through instigating apoptosis to macrophages [2,7-9]. Its clinical manifestation is associated with factors such as host genetic factors, virulence factor of the bacteria, and its environment [10]. Hence, *H. pylori* on prolonged existence survives by manipulating the host immune response and creates a prosurvival and tolerant environment for itself which is harmful for the host. Treatment of *H. pylori* infection uses a combination of minimum two antibiotics and gastric acid inhibitors which often causes side effects such as nausea, antibiotic resistance, and recurrence [5]. The search for a safe and effective non- antibiotic agent has rekindled the interest in alternative medicine in the form of natural drugs. Herbal formulation or isolation of bioactive compounds is widely investigated, particularly in indigenous fruits known for their folkloric medicinal value [11]. Development of carbohydrates and carbohydrate-based molecules in drug discovery in the form of glycomics has attained increased recognition [12]. Recent advances in oligosaccharide research have established its application in therapy for areas such as inflammation, immunity, oncology, neurodegenerative diseases, and infection [13,14]. Synthetic oligosaccharides tested for its antibiotic potential against *H. pylori* were studied [15], while carbohydrates present in porcine milk were found to mediate prevention of *H. pylori* colonization [16]. However, substantial reports on the effect of oligosaccharides from plants against the bacteria are limited despite its antimicrobial potential. The challenge could be due to the natural oligosaccharides possessing diverse structural and size heterogeneity, challenging the ability to determine its detailed structure. Using biochemical tools such as chromatographic separations for isolation and characterization using spectral analysis such as mass spectra (MS) and nuclear magnetic resonance (NMR), drug discoveries have obtained success rates. Our study, thus, identifies the oligosaccharides isolated from star fruit and explores its potential as an anti-*H. pyloric* agent through immunostimulatory effects for the first time since the fruit is known to alleviate problems of the gut.

Starfruit (*Averrhoa carambola* L.) is a tree fruit with a distinctive star shape in cross-section. The edible fruit of the Oxalidaceae family is available locally in various products such as pickles, jam, or jelly. The powdered seed concoction of the fruit is traditionally used for its medicinal properties to treat hemorrhoids, fever, eczema, diarrhea, and asthma [17,18]. Extensive reports on the strong free radical scavenging potential of juice and residue of star fruit cultivated in Singapore and Indonesia have attributed its capacity to the rich procyanidin polymers and β-carotene [19]. Antioxidant and immunomodulatory effects of star fruit leaves were evidenced through increased antibody titer values and hematological profile in cyclophosphamide-induced immunosuppressed mice [20]. Our earlier reports established the antioxidant potential and anticancer properties of star fruit [21,22]. The present investigation is, therefore, the first to report the isolation of an oligosaccharide from star fruit (S3W) and its anti-*H. pylori* potential through its immunostimulatory ability.

METHODS

Plant material

A. carambola fruit was procured from the local market of Mysore, Karnataka, during July–September. A herbarium specimen was deposited at the Herbarium Center, DOS in Botany, Manasagangothri, Mysuru (VS. No. DOSBWK05).

Preparation of extract

About 500 g of fresh fruit was blended and extracted with 1000 ml of 60% acetone/water at 30°C for 3 h under continuous agitation and filtered using a muslin cloth. The residue was reextracted using 500 ml of solvent mixture under the same conditions and the filtrates pooled. After concentration in a rotary evaporator (Buchi, Germany), the aqueous portion was dried in a hot-air oven at 50°C.

Isolation of star fruit oligosaccharide

A sequence of biphase extraction on the aqueous extract of star fruit included using 10% ethyl acetate in methanol, 20% ethyl acetate in methanol, and 30% ethyl acetate in methanol, wherein the aqueous phase in each period of extraction was collected and subjected to further biphase separation. At 40% ethyl acetate in methanol, the obtained monophase was dried at 40°C. 5 g of the dried extract was reconstituted in methanol and subjected to ethyl acetate equilibrated silica gel chromatography of 300 mm × 10 mm column size using silica gel 60–120 mesh. A gradient of ethyl acetate: methanol (10:0, 8:2, 6:4, 4:6, 2:8, and 0:10) as mobile phase resulted in six fractions (S1-S6), of which fraction S3 was subjected to preparative thin-layer chromatography using the mobile phase ethyl acetate: methanol:water (1.65:9:1.35) and observed under UV. The blue fluorescing spot at Rf 0.63 (Supplementary material 1) was extracted using methanol and dried at 40°C to give S3W. S3W underwent analysis by phenol- sulfuric acid method to confirm the presence of carbohydrates [23] and was further subjected to liquid chromatography -electron spray ionization (ESI)/atmospheric pressure chemical ionization (APCI)- MS/MS, and ^{1}H and ^{13}C NMR.

High-performance liquid chromatography (HPLC)-ESI-mass spectrometry (ESI-MS) analyses of S3W

A BDS HYPERSIL C-18 column (150 mm × 4.6 mm, 5 µm particle size) equipped with PDA/UV detector with 280 nm as the detecting wavelength was used for chromatographic separations in room temperature (27°C) under the following conditions: 1 ml/min; solvent A, 10% acetic acid in water; solvent B, and 15% methanol in water starting from 0 to 20 min (40–52% A), 20 to 40 min (52–80% A), and 40 to 60 min (80% A).

The Synapt G2 HDMS ESI/APCI-Hybrid Quadrupole, Time-of-flight tandem mass spectrometer (Waters, USA) was used to identify S3W. The heated capillary and spray voltage were maintained at a temperature of 275°C and 4.5kV. Nitrogen is operated at 40 psi for sheath gas flow rate and 26 psi for auxiliary/sheath gas flow rate. The full scan MS from m/z 50–2000 were acquired in positive and negative ion mode with a scan speed of 1 s per scan. The MS was performed using argon as collision gas, operated at 0.1 mtorr.

NMR spectroscopy

Proton and carbon NMR spectra were obtained using Agilent 400MR DD2 NMR spectrometer at 400 MHz. Sample dissolved in 700 µl of dimethyl sulfoxide (DMSO)-d6 with concentration of 30 and 15 mg/ml for ^{13}C and ^{1}H, respectively, was prepared and transferred to a 5 mm NMR tube, wherein the analysis was performed at 45°C. The compound was identified by comparing spectra with that reported in literature [24,25].

Bacterial strains and cultivation

H. pylori were isolated and confirmed as per the protocol of Belagihalli and Dharmesh [5].

Inhibition of *H. pylori* viable colony count method

Bactericidal activity of the star fruit compound was determined using 96-well microtiter plate method [2]. 100 µl suspension of 10^8 colony-forming units/ml was treated with 100 µl of distilled water (control) and S3W in concentration range of 2–10 µg/ml and absorbance read at 620 nm using an automatic ELISA microplate reader (Thermo Fisher, USA). The control consisted of *H. pylori* treated with sterile distilled water. The suspensions were incubated for 3 days at 37°C under microaerophilic atmosphere and the absorbance read again in the same wavelength after agitation. All experiments were performed 3 times. The absorbance obtained before and after incubation was compared, and percentage inhibition using the following formula was used.

$$\text{Percentage inhibition} = 1 - \frac{\text{OD of test}}{\text{OD of control}}$$

The effectiveness of S3W at killing *H. pylori* was expressed as percentage inhibition of bacterial growth (i.e., percentage of bacteria killed) compared to that of control.

DNA protection assay

DNA protection assay was conducted by the inhibition of Fenton's reagent induced strand breaks in lambda phage DNA by S3W [26]. The control group contained 5 µl of phage DNA and 5 µl Fenton's reagent (30 mM H_2O_2, 500 µM ascorbic acid, and 800 µM ferric chloride) made up to 25 µl with distilled water, while the test group contained 5 µl phage DNA and 5 µl Fenton's reagent followed by addition of 10 µl of 1 mg/ml of S3W. The final volume was made up to 25 µl with distilled water, and the reaction mixtures were incubated for 45 min at 37°C. The strand break inhibition observed of test group was compared to that of control group using 0.9 % agarose gel electrophoresis by staining with ethidium bromide.

Buccal cell collection technique

A clean toothpick full of cheek buccal cells from healthy consenting donors was agitated in 2 ml cold phosphate buffer saline (PBS) (100 mM, pH 7) and centrifuged at 2500 rpm at 4°C for 10 min. The supernatant was aspirated and the cell pellet resuspended in 100 µl PBS.

Effect of S3W on buccal cells exposed to oxidant (ascorbic acid and $FeSO_4$) and N- methyl N-nitrosourea (MNU)

To determine the effect of exposure of buccal cells to S3W *in vitro*, buccal cells were tested immediately after collection [27]. Buccal cells (1×10^4 cells/well) were exposed to 500 µM ascorbic acid and 500 µM $FeSO_4$, and treated with and without 10 µg/ml S3W for 1 h at 37°C. Similarly, another set of buccal cells was treated with a carcinogen – MNU (10 µg/ml for 1×10^4 cells/well) and exposed to the presence and absence of 10 and 20 µg/ml S3W for 1 h at 37°C. 1 µl of dye mix containing acridine orange and ethidium bromide of 100 µg/ml each was mixed to 25 µl cell suspension of treated and untreated cells. They were observed under the fluorescence microscope at ×40 (Olympus). Staining pattern between the cells was compared and cytoprotective ability of S3W was determined.

Animals

Experiments were performed using 6–8 weeks old Swiss albino mice, weighing 20–25 g. Animals were maintained in accordance with the OECD guidelines, and experiments were performed with the regulations of Farooqia College of Pharmacy, Mysuru, India (FCP/EC- 5/273/2014- 2015).

Isolation of peritoneal macrophages and cell culture

Macrophages were isolated by peritoneal lavage from male Swiss albino mice [2]. The peritoneal cavity was washed with ice cold PBS supplemented with 20 U/ml heparin and 1 nM EDTA. Care was taken not to cause internal bleeding while collecting macrophages in the exudates. The cells were cultured in 60 mm Petri dishes in RPM1640 media supplemented with 10% FBS, 50 µg/ml penstrap for 24 h at 37°C in a humidified atmosphere of 5% CO_2 in CO_2 incubator. Non-adherent cells were removed by vigorously washing 3 times with ice-cold PBS. Cell viability was evaluated by trypan blue exclusion and viable cells not

<95% was used for further studies. The Petri dishes containing the cells were divided into the following groups:

- Group I: Untreated cells (control)
- Group II: Cells treated with oxidant (ascorbic acid with $FeSO_4$)
- Group III: Cells treated with MNU
- Group IV: Cells treated with oxidant + 20 µg/ml S3W
- Group V: Cells treated with MNU + 10 µg/ml S3W
- Group VI: Cells treated with MNU + 20 µg/ml S3W

The cells were stained with a mixture of acridine orange and ethidium bromide dye and morphologically analyzed under fluorescence microscope. The protocol described was in accordance to that reported by Mahapatra *et al*. with slight modifications [27].

Splenocyte proliferation assay

Effect of S3W on splenocyte proliferation was tested by MTT assay [28] wherein splenocyte suspension (1×10^6 cells/ml) in complete RPMI 1640 medium was incubated in different concentrations of S3W (0–10 µg/ml) dissolved in 0.1% DMSO in PBS. Control splenocytes include those treated with 0.1% DMSO in PBS only. After incubation for 48 h at 37°C in 5% CO_2 humidified atmosphere, the medium was removed and the adherent splenocytes were washed twice with PBS. 15 µL of MTT stock solution (5 mg/ml) was added to the culture medium for 4 h at 37°C. Absorbance was measured at 450 nm using microplate reader. Percentage splenocyte proliferation was calculated using the following formula:

$$\text{Percentage proliferation} = 1 - \frac{\text{OD before incubation}}{\text{OD after incubation}}$$

Statistical analysis

The assays were conducted in triplicates and data are represented as mean ± standard deviation. All statistical analysis was conducted using Origin 5.0.

RESULTS

Anti-*H. pylori* activity of S3W

Inhibitory potential of S3W against *H. pylori* growth analyzed by bacterial growth inhibition method is shown in Fig. 1. S3W expressed an IC_{50} value of 10.71 µg/ml.

DNA damage protection assay

DNA damage occurs through Fenton reaction generated by oxidants and carcinogens causing increase of its mobility in electrophoresis. Retardation of the S3W-treated DNA indicated that S3W recovered DNA from damage by the hydroxyl radicals (Fig. 2).

Protection of buccal cells from ascorbic acid/$FeSO_4$ oxidant and MNU carcinogen damage

The cytoprotectivity on buccal cells treated with oxidant and MNU carcinogen is indicated in Fig. 3a-d. Intact viable cells stained green as they were bound more effectively by acridine orange than by ethidium bromide from the acridine orange/ ethidium bromide dye mixture whereas nuclear components of damaged cells stained more orange due to better interaction with ethidium bromide than to acridine orange. The oxidant and carcinogen induced clustering, cell disruption, and echnocytic type morphological alteration, whereas cells treated with S3W were normal indicating protection. Our results showed S3W-alleviated oxidative stress and protected the cells from undergoing cell damage.

Protection of peritoneal macrophages against oxidant ($FeSO_4$+ascorbic acid) and MNU carcinogen damage

Macrophage cellular damage was caused by oxidation induced by ROS generated by $FeSO_4$ and ascorbic acid and reactive nitrogen species (RNS) generated by MNU (Fig. 4b and d). Cellular damage was first evident by fragmented nucleus followed by degraded cytoplasm and membrane deregulation. These distinctive characters implicated that the oxidants induced apoptosis. Results show that treatment of macrophages with S3W protected the macrophages exposed to

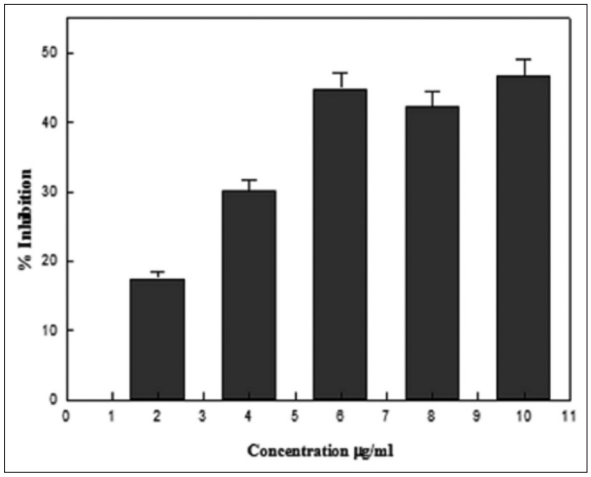

Fig. 1: Anti-*Helicobacter pylori* activity of S3W is expressed as percentage inhibition wherein its IC_{50} value is 10.7±0.192 µg/ml (n=4)

Fig. 2: Protection against DNA damage by S3W

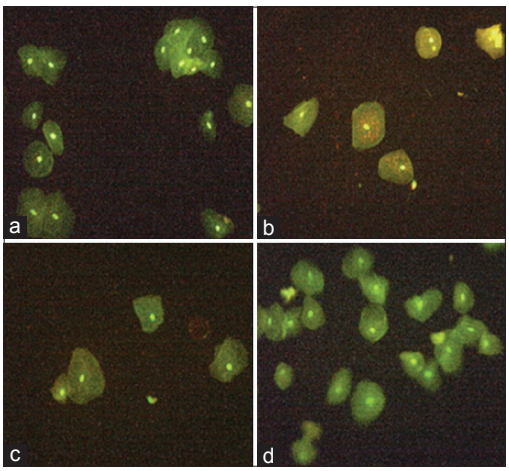

Fig. 3: Fluorescent microscopic view of buccal cells protected N-nitroso-N-methylurea (MNU). (a) Control (b) buccal cells treated with MNU (c) buccal cells treated with 10 µg/ml S3W (d) buccal cells treated with 20 µg/ml S3W

the oxidants and MNU from entering apoptosis (Fig. 4c, e, and f) by retaining the morphology of normal cell (Fig. 4a). Once again, S3W at 20 µg/ml showed better macrophage protective effect against MNU carcinogen.

Immunostimulatory activity as evaluated by proliferation of splenocytes by MTT assay

The star fruit oligosaccharide induced significant immunostimulatory activity indicated by a distinct splenocyte proliferation increase. The proliferation was dose dependent, wherein splenocyte proliferated by 37 and 55.7% at 10 and 20 µg/ml concentration of S3W, respectively (Fig. 5). The resultant expression showed that S3W could be a potential mitogen.

Liquid chromatography–MS of star fruit oligosaccharide

The carbohydrate eluted at 2.574 min (Fig. 6a). The MS/MS spectra of isolated oligosaccharide generated by collision-induced dissociation (CID) were evaluated by assigning all product ions using the Domon-Costello nomenclature [29]. Fig. 6b and c shows MS^2 spectra of protonated and deprotonated oligosaccharides. The pattern of fragmentation indicated that the oligosaccharide was a low molecular weight sugar having O-2 linkages at the first and second residues with possible branching at C-4. Protonated oligosaccharides are prone to cleaving exclusively at glycosidic bonds [30]. However, due to prolonged exposure to higher voltage, the oligomer underwent cross-ring cleavage along with the expected glycosidic linkages. The protonated oligosaccharide displayed mass of m/z 1465. S3W (Fig. 6b) showed non-reducing B_1 and B_2 glycosidic cleavages at m/z 1303 and 978

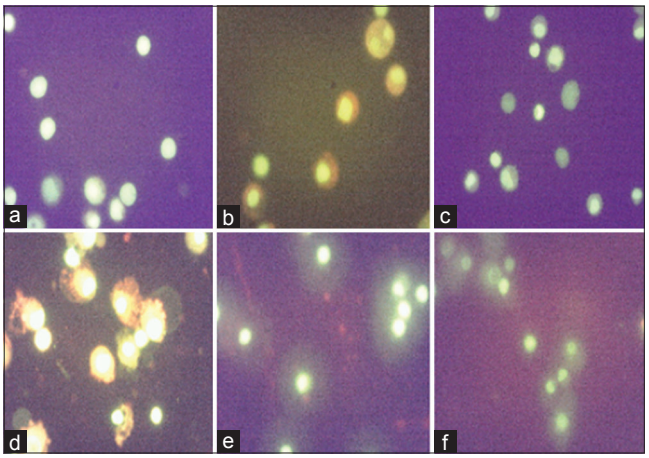

Fig. 4: Fluorescent microscopic view of macrophages protected against oxidant and N-nitroso-N-methylurea (MNU) (a) Control (b) macrophages treated with oxidant ($FeSO_4$+ascorbic acid) (c) macrophages treated with 20 µg/ml S3W (d) macrophages treated with MNU carcinogen (e) macrophages treated with 10 µg/ml S3W (f) macrophages treated with 20 µg/ml S3W

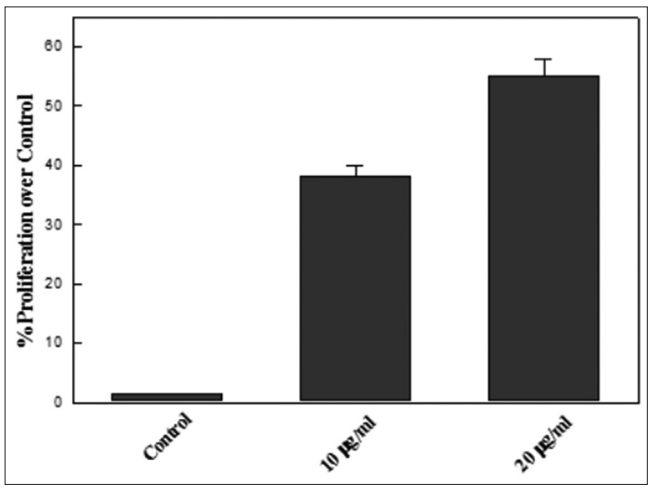

Fig. 5: Percentage proliferation of splenocytes treated with S3W as an indicator of immunostimulatory effect of S3W (n=4)

involving homolytic cleavage at (1→2) β- linkage bonds to release single glucose residue without the glycosidic oxygen at non-reducing terminal of backbone chain of m/z 162 and one (1→2) β-linked backbone glucose residue connected by a (4→6) α linkage. This accounted for mass loss of m/z 325. High-energy CID produced reducing fragmentation due to $^{0,2}A_3$ and $^{0,2}A_4$ cleavage which generated fragment ions at m/z 853 and 669, respectively. A unique feature of the MS was observation of a series of A-type cross-ring cleavages and C-type cleavage (Fig. 6b) bearing fragment ions of m/z 44 of varying relative intensities from m/z 405 to m/z 669. The formation of the fragment ions at m/z 44 was possibly due to the OH or $–CH_2OH$ group of the precursor ion being on the same ring carbons adjacent to the ring oxygen. Cross-link cleavages are frequently seen at reducing side of sugars [30]. Further, fragmentation led to the release of ions at m/z 388 and 361 due to the loss of $–CHCH_2OH$ releasing non- reducing terminal glucose of side chain and reducing terminal of glucose backbone of m/z 180 each.

Fig. 6c shows the deprotonated MS of the oligosaccharide. According to Domon and Costello nomenclature, a series of A and C type fragments ions are expected in MS at negative mode [31]. Hence, fragmentation could have started from the non-reducing end of the oligosaccharide. The spectrum contained $^{1,5}A_1$ and C- type fragmentation to release m/z 1331 and 1295 although the molecular ion peak of m/z 1475 was not detected. $^{1,5}A_{1α}$, C_2, and $^{1,5}A_4$ fragmentation released fragment ions at m/z 1007, 971, and 683, respectively. The glucose backbone continued through a series of A- and C-type cleavage releasing ions of m/z 359, 323, and 215 (Fig 6c). The fragmentation pattern in negative mode was a characteristic of a (1→2) β-linked saccharide with alternating (4→6) α-branching.

NMR spectroscopy of S3W

From information of coupling constant (Supplementary material 2) at 8 Hz and 3.6 Hz, 4.231 ppm was assigned for β-D-glucose and 4.883 to α-D-glucose. The resonances of one of the methylene protons of the branch point glycosyl repeat unit at 3.225 ppm were similar to that of integrated area of the anomeric proton suggesting a singly branched side chain of (4→6) α-linkages. The ratio of the areas from resonances assigned to H1 of side chain residue at and H1 of the (1→2) β-linked repeat units in the polymer indicated that the side chain attached to the backbone on average every two repeat units. Coupling constant of 11.2 Hz at 4.161 ppm was assigned to $3J_{H2\text{-}H3}$. 92.630 ppm was assigned to α-C-1 of (4→6)- α side chain (SC) and 97.281 to β- C-1 of reducing terminal (RT) of (1→2)-β-glycosyl backbone. 104.591 ppm was assigned to β-C-1 of non- reducing terminal, while 102. 389 ppm was assigned to β- C-1 of internal residues. The presence of (1→2)-β-bonding was evident by the presence of resonance at 81.444 to 83.751 ppm for C-2 at the reducing terminals, while resonance at 75.253 ppm was assigned to C-2 of non-reducing terminal. Single branching at (4→6)-α produced C-4 resonance at 77.096 to 77.156 ppm and C-6 resonance at 69.279–69.302 ppm. Unsubstituted C-4 resonated at 70.334–71.010 ppm, while that by C-6 at 61.653–63.712 ppm. The rest of the proton and carbon NMR assignments are summarized in Table 1.

The resonance and MS analysis agreed with each other indicating the oligosaccharide likely to possess a (1→2) β-linked heptasaccharide backbone with alternating (4→6) α-linked glycosyl branches (Fig. 7).

DISCUSSION

Glycobiologists have stated the wide scope for the applications of novel carbohydrate-based drugs particularly that of oligosaccharides to modulate the immune system since native polysaccharides and glycoconjugates have their large size (MW 10–1000 kDa) as one of the limitations for their value in drug discovery. Oligosaccharide as a secondary metabolite from star fruit was isolated using adsorption methods and partially characterized for the first time. Separation of carbohydrates is extensively carried out using high-pressure liquid chromatography while MS is a widely used tool to study its structure [31]. ESI is one of the common methods for carbohydrate analysis. However, the hydrophilicity of carbohydrates decreases its ionization efficiency,

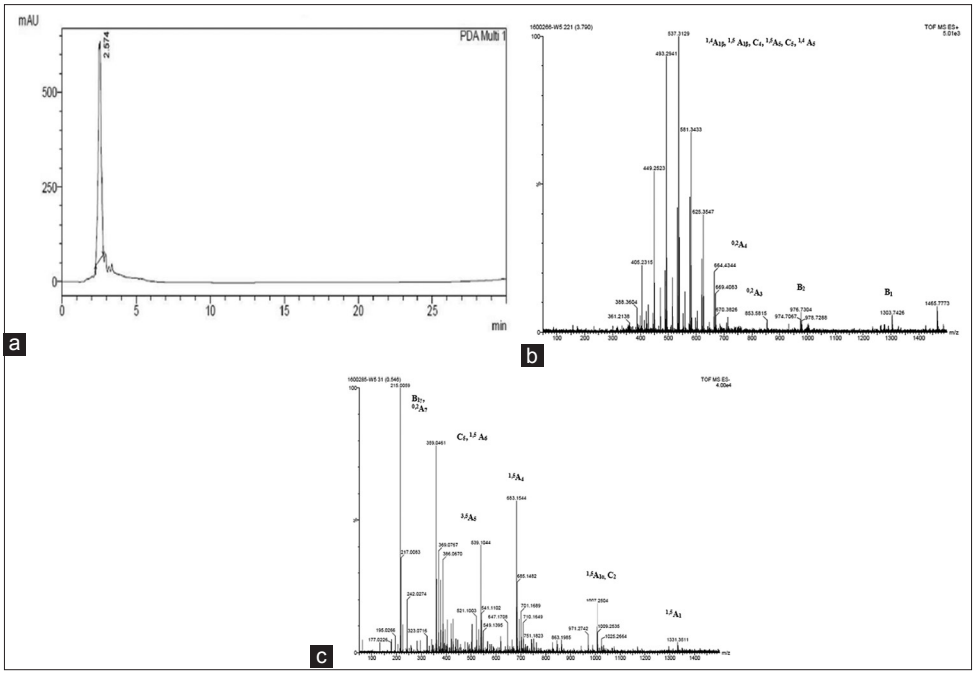

Fig. 6: High-performance liquid chromatography (HPLC)-electrospray ionization-mass spectrometry of S3W (a) HPLC chromatogram of S3W (b) MS/MS of protonated S3W (b) deprotonated S3W

Table 1: Chemical shifts ^{1}H and ^{13}C NMR spectroscopy assigned for S3W

Chemical shift ppm					
Proton assignment	**(1→2)-β-linked backbone chain**	**(1→2)-α- β-linked NRT**	**(1→2) β- linked RT**	**(4→6)- α- Br**	**SC1**
H1	4.251	4.251	4.231	4.251	4.883
H2	3.509	3.249	3.257	3.089	3.662
H3	3.613	3.418	3.407	3.706	3.113
H4	3.348	3.348	3.367	3.798	3.089
H5	3.638	3. 377	3.367	3.798	3.349
H6	3.787	3.798	3.787	4.131	4.133
H6’	3.662	3.613	3.638	3.613	3.688
C1	102.389	104. 597	97.281	101.569	92.630
C2	83. 751	75.253	82. 294	71.010	72.762
C3	77.096	77.096	77.096	83.751	73.521
C4	70.334	71.010	71.010	77.156	71.010
C5	77.156	78.416	77.156	75.775	75.258
C6	63.299	61.653	64.817	69.848	63.482

particularly in increasing molecular weight [32]. Hence, an APCI/ESI hybrid was used to improve the efficiency of the ionization of the oligosaccharide. Substantial use of MS for determining carbohydrate structure using the nomenclature of Domon and Costello has been recorded [33-37], wherein CID on carbohydrates produces distinct CID fragmentation explaining the linkage type and anomeric configuration. This, along with ESI to ionize oligosaccharides connected to tandem MS (MS/MS), is useful tool to elucidate carbohydrate structure. Protonated oligosaccharide cleavage generally occurs at the non-reducing side of the glycosidic oxygen (B- and Z-type). In negative mode, fragmentation patterns with C-and A-type ions are more common [25] compared to B-type fragments. The cross-link cleavages exposed the type of linkage present in the oligo/polysaccharides. The isolated oligosaccharide in negative mode fragmented into distinctive ions of mass m/z 108, 215, and 195 which characterized the underivatized oligosaccharide as to possess a β (1→2) glycosidic linkage despite formation of abundant internal fragments. NMR is the most efficient technique to structurally analyze glycans [32]. It provides most of the experimental data to determine glycosidic linkages of carbohydrates from natural sources. In ^{1}H NMR spectroscopy, the anomeric protons are generally located at 4.2–5.8 ppm, while ring protons are found in the 3.2–4.5 ppm region. The large ^{13}C chemical shift differences due to conformational and configurational changes made ^{13}C NMR spectroscopy a useful instrument to elucidate structures of star fruit oligosaccharides. Carbons involved in glycosylation exhibit a large downfield shift of approximately 4–10 ppm [38]. Slight upfield shifts at C3, C4, and C5 of 0.5∼2 ppm due to the β-effect of 1→2 linkage were evident. Downfield shifts of 7–8 ppm indicated the β (4→6) linkage of the side chain. Based on comparison with resonances reported for β (1→2) glucans, distinctive shifts for ^{13}C and ^{1}H were assigned [24]. The results were in agreement with that reported. Characteristic resonance for anomeric carbon and hydrogen revealed the oligosaccharide to be a β (1→2)-linked glycan with α (4→6) branching. It is, however, essential to employ 2D NMR analysis to completely elucidate the oligosaccharide.

Macrophages are critical components in immunity as they interact with T cells, B cells, natural killer cells, dendritic cells, neutrophils, and fibroblasts to phagocytize microorganisms in an attempt to kill them using ROS/RNS. Buccal cells, on the other hand, are the first barriers during inhalation or ingestion route, capable of metabolizing proximate carcinogens to reactive products, thus representing a preferred

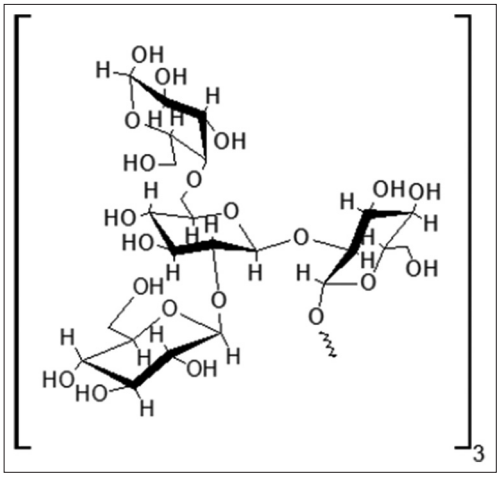

Fig. 7: Proposed structure of S3W

target site for early genotoxic events induced by carcinogens [39,40]. Eradication of *H. pylori* is strongly recommended to overcome gastric diseases such as gastric cancers as it plays the predominant role of being the main etiological agent for these gastric disorders. *H. pylori* hampers immunostimulation for its survival and causes the overproduction of ROS and RNS which itself harms host cells such as marcophages, neutrophils, and splenocytes [2]. Studies have suggested that such self-harm could be mainly due to ROS being able to eliminate any bacteria within the vicinity of the phagocyte, but separation between the phagocytes and the bacteria in the lumen curbs efficient elimination of *H. pylori* [3]. As a result, excess production of ROS and RNS damages DNA beyond its repair capabilities. This prolonged combined damage can also initiate the process of carcinogenesis. The therapeutic compounds are thus required to bear properties that enhance immunostimulatory effects and prevent progressive tumorogenesis. Kojima *et al.* observed that carbohydrate carriers reduced the affinity of *H. pylori* adhesion to antigens on gastric mucosa [41] which might explain the limitations of using carbohydrates as anti-*H. pylori* agents. The *in vitro* studies of the effect of star fruit glycan, however, showed promising potential. S3W was highly potent against *H. pylori* proliferation and its mechanism can be attributed to its anti-radical and immunostimulatory properties. Macrophages exposed to oxidants and MNU, a carcinogen most notably to cause adenocarcinoma of the stomach [42], were protected by S3W from undergoing damage significantly. Enhancement of phagocyte function is an established therapeutic application against microbial infection and cancer. Carbohydrates isolated from various medicinal plants possessed ability to regulate macrophage function resulting from its immune-stimulating activity [43,44]. β-glucans are postulated to act as pathogen-associated molecular pattern (PAMP) which binds to typical macrophages and dendritic cell surface receptors called pattern recognition receptors (PRRs) that detect innately non-self-molecules such as PAMPs. On binding, the PRRs can induce cascade of signals activating immune cells [45]. Thus, the ability of S3W to behave as a PAMP can be analyzed in future studies. Spleen as a blood filter responds to systemic infection by trapping blood born antigens wherein splenocytes in coordination with B and T lymphocytes help in pathogen clearance in the body primarily through antibody formation and secretion. An increase in the concentration of splenocytes enhances acquired immunity and is an important criterion of immunostimulation [26]. Elaborate records of the effect of oligosaccharides such as nigerose oligosaccharides, asparagine-like oligosaccharides, synthetic β (1→6) branched β (1→3) gluco-hexose, fructooligosaccharides, chitosan oligosaccharides, and many more in augmentation of splenocyte proliferation resulting in its production of many cytokines such as interleukin-2 (IL-2), interferon-γ, IL-6, and tumor necrosis factor α are available [35,46-49]. There, however, are no substantial reports of immunomodulatory effect of (1→2) α/β linked oligosaccharides. The present study is the first to reveal a star fruit oligosaccharide which is most probably (1→2)- β- linked, expressing for the first time the significant mitogenic property evident by the enhanced splenocytes proliferation by more than 50% at 20μg/ml compared to untreated splenocytes. To our knowledge, such significant reports of star fruit oligosaccharides have not been published elsewhere. It is evident that the use of oligosaccharides from natural sources for its anti-*H. pylori* potential, particularly, is in its infancy. There is a wide scope to extensively study and further establish oligosaccharides in drug discovery. Our investigation is the first to highlight the distinct potential of (1→2)- β, (4→6) – α linked oligosaccharide isolated from fruit of star fruit as an agent against gastric cancer caused by *H. pylori* through its immunomodulatory functions. Future studies are encouraged to analyze immune factors released as a result of immunomodulation by the star fruit oligosaccharide and establish the molecular mechanism.

CONCLUSION

Star fruit oligosaccharide showed anti-*H. pylori* activity through immunomodulatory effects by protecting DNA from damage caused by free radicals, cytoprotection from carcinogen like MNU, and stimulating proliferation of splenocytes. The oligosaccharide has a therapeutic potential for application in immunotherapy. Further research to understand the molecular mechanism by which the oligosaccharide can protect and induce proliferation of immune cells to promote anti *H. pylori* activity is encouraged.

ACKNOWLEDGMENT

The authors thank the University Grants Commission, New Delhi, India, for their financial support (Project F.No. 37-519 (2009) SR), Director of Pooja Bhagavat Memorial Mahajana Education Centre, PG Wing of SBRR Mahajan First Grade College and Mahajana Education Society, Mysore, India, for providing the infrastructure for conducting the experiments, Dr. Shylaja M Dharmesh, CFTRI for her valuable guidance in conducting the bioassays, and Institute of Excellence, University of Mysore, Mysuru, for rendering their facilities to perform the spectral analysis. Wethroe Kapfo thanks the Council for Scientific and Industrial Research, Government of India for financial support as Senior Research Fellowship (08/6/6 (0001)/2014- EMR- I).

AUTHORS' CONTRIBUTIONS

Jyoti Bala Chauhan: Conceptualized and designed the experiments. She has guided in data analysis and edited the manuscript.

Wethroe Kapfo: performed the experiments, analyzed the data and prepared the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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