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# Structural identification and cytotoxic activity of a polysaccharide from the fruits of *Lagenaria siceraria* (Lau)

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ABSTRACT

A water-soluble polysaccharide, isolated from fruiting bodies of *Lagenaria siceraria,* is composed of methyl-α-D-galacturonate, 3-*O*-acetyl methyl-α-D-galacturonate, and β-D-galactose in a ratio of nearly 1:1:1. Compositional analysis, methylation analysis, periodate oxidation, and NMR studies ($^{1}$H, $^{13}$C, 2D-COSY, TOCSY, NOESY, HMQC, and HMBC) revealed the presence of the following repeating unit in the polysaccharide:

OAc
↓
3
→4)-α-D-Gal*p*A6Me-(1→2)-α-D-Gal*p*A6Me-(1→4)-β-D-Gal*p*-(1→

This polysaccharide showed cytotoxic activity in vitro against human breast adenocarcinoma cell line (MCF-7).

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Bottle gourd is an excellent fruit in nature having all the essential constituents required for normal and sound health. It cures pain, ulcers, fever, asthma, and other bronchial disorders.[1] The fruit extract of *Lagenaria siceraria* possesses cardioprotective[2] as well as anti-inflammatory[3] effects. It is also used as a purgative and a diuretic.[4,5] The antihepatotoxic activity[6] of different fractions of the ethanolic extract of fruit was evaluated and showed significant activity in a dose of 250 mg/kg. Modern phytochemical screening methods showed the presence of triterpenoid, cucurbitacins B, D, G, H[7–9] and flavone C-glycosides[10] in fruits. The seeds of *L. siceraria* are used in the treatment of headache and pain[1,11] and a novel ribosome inactivating protein, lagenin isolated from it reported to show antitumor and anti-HIV activities.[12]

Detailed structural works on polysaccharide isolated from the stem of *L. siceraria* were carried out and reported[13] by our group in *Carbohydrate Research*. Here, in this case, a different polysaccharide from the fruits (pepos) of *L. siceraria* was isolated from hot water extract followed by acetic acid treatment and gel filtration. We are reporting herein the detailed structural studies and cytotoxic activity of this polysaccharide on breast adenocarcinoma cell line (MCF-7).

The molecular weight of the polysaccharide was estimated as ~78,000 Da from a calibration curve prepared with standard dextrans.[14] The pure polysaccharide has $[\alpha]_D^{25}$ +11.6 (*c* 0.68, water). Paper chromatographic analysis[15] of the hydrolyzed product showed the presence of galacturonic acid and galactose only. The GLC analysis of the alditol acetates of the sugars showed the presence of galactose and carboxyl-reduced polysaccharide on hydrolysis followed by GLC examination of the corresponding alditol acetates which also showed the presence of galactose. The absolute configurations of the sugars were determined by the method of Gerwig et al.[16] taking intact and carboxyl-reduced polysaccharide. The polysaccharide was methylated by the Ciucanu and Kerek method[17] and then hydrolyzed. The alditol acetates of methylated product were analyzed by GLC–MS analysis and showed the presence of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-galactitol only. This result indicates that either (1→4)-galactopyranosyl or (1→5)-galactofuranosyl moiety may be present in the polysaccharide. The carboxyl-reduced polysaccharide[18] was methylated, and alditol acetates of methylated sugars were identified by GLC–MS analysis, which showed the presence of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-galactitol and 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-galactitol in a molar ratio of nearly 2:1. This result indicates that (1→4)-linked galactopyranose or (1→5)-galactofuranose, (1→4) and (1→2)-linked galacturonic acid may be present in the polysaccharide. Then, a periodate oxidation experiment was carried out with this polysaccharide. The periodate-oxidized, reduced material was

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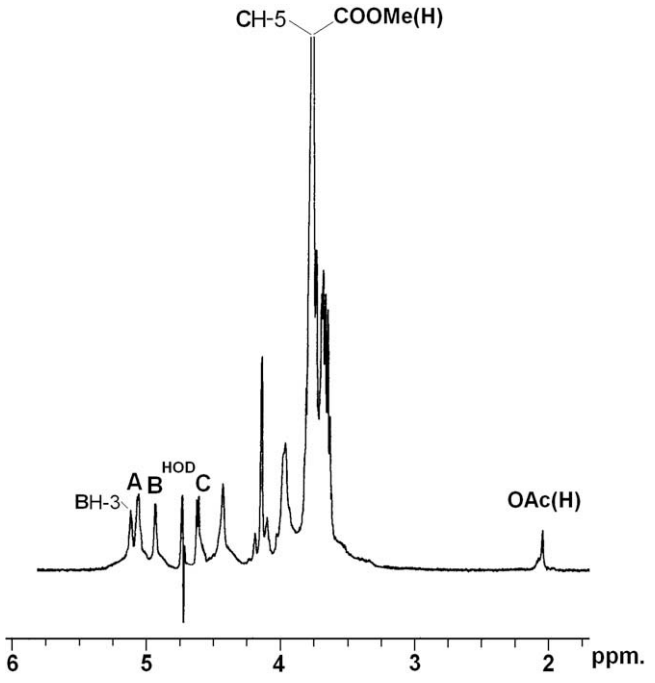


**Figure 1.** $^{1}$H NMR spectrum (500 MHz, $D_2O$, 27 °C) of polysaccharide, isolated from the fruits of *Lagenaria siceraria.*

hydrolyzed and the paper chromatographic studies showed the presence of galacturonic acid only. Thus, the periodate oxidation study only confirms the presence of a (1→4)-linked galactopyranosyl or a (1→5)-galactofuranosyl residue, but the linkage analysis of galacturonic acid remains unconfirmed due to its survival under the same conditions. Hence, its linkage information was confirmed through NMR experiments.

The $^{1}$H NMR (500 Hz) spectrum of the polysaccharide (Fig. 1) showed three anomeric proton signals at $\delta$ 5.06, $\delta$ 4.94, and $\delta$ 4.62 ppm in a ratio of nearly 1:1:1. The sugar residues were assigned as **A, B**, and **C** according to their decreasing anomeric chemical shifts (Table 1). In the $^{13}$C NMR (125 MHz) spectrum (Fig. 2, Table 1), three anomeric carbon signals appeared at $\delta$ 104.7, $\delta$ 100.7, and $\delta$ 99.8 ppm in a ratio of nearly 1:1:1. Further $\delta$ 53.3 ppm and 20.5 ppm were assigned for carbomethoxy carbon and acetyl carbon, respectively. All the $^{1}$H and $^{13}$C signals were assigned using DQF-COSY, TOCSY, HMQC, and HMBC (Fig. 3) NMR experiments.

Residue **A** has an anomeric chemical shift at $\delta$ 5.06 and $J_{H\text{-}1,\ H\text{-}2}$ ~2.8 Hz and $J_{H\text{-}1,C\text{-}1}$ ~170 Hz, indicating an α-linked residue. The spin system of this residue, which consisted of only five protons with a relatively high chemical shift of the H-5 signal ($\delta$ 4.43) and weak coupling between H-3, H-4, and H-5, indicated that residue **A** is a D-galacturonosyl moiety. The C-1 signal of residue **A** at $\delta$ 99.8 was confirmed by the appearance of cross-peak **A**C-1, **B**H-2 in the HMBC experiment (Fig. 3, Table 3). The C-4 peak of residue **A** at $\delta$ 79.4 showed a down field shift compared to that of standard methyl glycosides[19] due to the effect of glycosylation. The presence of a carboxy methyl group in residue **A** is confirmed by the appearance of intra-residual coupling between ester carbonyl carbon ($\delta$ 171.0) and carboxy methyl proton ($\delta$ 3.78) in HMBC experiment (Fig. 3, Table 3). These results indicate that residue **A** is (1→4)-α-D-Gal*p*A6Me.

Residue **B** has an anomeric proton chemical shift at 4.94 (unresolved) and $J_{H\text{-}1,C\text{-}1}$ ~171 Hz indicating that it is an α-linked residue. This residue showed only five proton signals with two relatively high chemical shifts of H-5 ($\delta$ 4.43) and H-3 ($\delta$ 5.12) observed. The high chemical shift of H-3 ($\delta$ 5.12) is due to the presence of an acetyl group at the C-3 of this residue. The appearance of intra-residual coupling between acetyl carbonyl carbon ($\delta$ 171.0) and H-3 ($\delta$ 5.12) in the HMBC experiment (Fig. 3, Table 3) indicates that the acetyl group is attached at the C-3 of residue **B**. The anomeric carbon chemical shift of moiety **B** at $\delta$ 100.7 was confirmed by the presence of cross-peak **B**C-1, **C**H-4 in the HMBC experiment (Fig. 3, Table 3). The down field shift of C-2 ($\delta$ 74.9) compared to that of standard methyl glycosides[19] was due to the effect of glycosylation. The appearance of intra-residual coupling between carbonyl carbon ($\delta$ 171.0) and carboxymethyl proton ($\delta$ 3.78) in the HMBC experiment clearly indicates that the carboxyl group of galacturonic acid is present as a methyl ester. These indicate that residue **B** is (1→2)-3-*O*-Ac-α-D-Gal*p*A6Me.

Residue **C** has an anomeric proton chemical shift at 4.62 ppm. A large coupling constant $J_{H\text{-}1,H\text{-}2}$ value (~7.8 Hz) and $J_{H\text{-}1,C\text{-}1}$ value (~160 Hz) indicates that it is a β-linked residue. The $J_{H\text{-}2,H\text{-}3}$ value (~9.0 Hz) and the $J_{H\text{-}3,H\text{-}4}$ value (~3.5 Hz) for residue **C** indicate that it is a β-D-galactosyl residue. The C-1 signal of residue C at 104.7 ppm was confirmed by the presence of cross-peak **C**C-1, **A**H-4 in the HMBC experiment (Fig. 3, Table 3). The down field shift of C-4 ($\delta$ 78.7) with respect to standard methyl glycosides[19] indicates that residue **C** is present as a (1→4)-β-D-galactopyranosyl residue and not as a (1→5)-β-D-galactofuranosyl moiety whose anomeric carbon signal appears nearly at $\delta$ 109.6 ppm. Hence, the presence of a galactose in the polysaccharide in the furanose form does not arise.

The sequence of glycosyl residues of the polysaccharide was determined from NOESY (Fig. 4, Table 2) as well as from ROESY experiments (Fig. not shown) followed by confirmation with an

**Table 1**
$^{1}$H NMR[a] and $^{13}$C NMR[b] chemical shifts of the polysaccharide isolated from the fruits of *Lagenaria siceraria* recorded in $D_2O$ at 27 °C

| Sugar residue | H-1/C-1 | H-2/C-2 | H-3/C-3 | H-4/C-4 | H-5/C-5 | H-6a/6b/C-6 | COOMe | 3-*O*-COMe |
|---|---|---|---|---|---|---|---|---|
| →4)-α-D-Gal*p*A6Me-(1→ | 5.06 | 3.82 | 3.97 | 4.11 | 4.43 | | 3.78 | |
| **A** | 99.8 | 68.9 | 68.1 | 79.4 | 72.2 | 171.0 | 53.3 | |
| →2)-3-*O*-Ac-α-D-Gal*p*A6Me-(1→ | 4.94 | 3.70 | 5.12 | 3.97 | 4.43 | | 3.78 | 2.04[c] |
| **B** | 100.7 | 74.9 | 72.2 | 68.9 | 73.7 | 171.0 | 53.3 | 171.0[d], 20.5[e] |
| →4)-β-D-Gal*p*-(1→ | 4.62 | 3.66 | 3.68 | 4.14 | 3.78 | 3.74, 3.64 | | |
| C | 104.7 | 72.2 | 68.9 | 78.7 | 70.9 | 61.1 | | |

[a] The values of chemical shifts were recorded keeping HOD signal fixed at $\delta$ 4.73 ppm.
[b] The values of chemical shifts were recorded with reference to acetone as internal standard and fixed at $\delta$ 31.05 ppm at 27 °C.
[c] The values of the acetyl methyl proton.
[d] The values of the acetyl carbonyl carbon.
[e] The values of the acetyl methyl carbon.

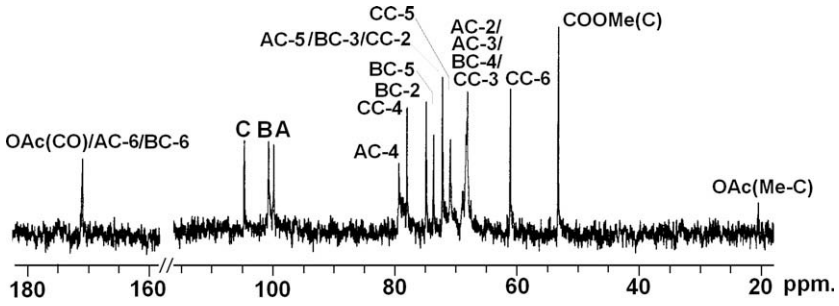


**Figure 2.** $^{13}$C NMR (125 MHz, $D_2O$, 27 °C) spectrum of polysaccharide, isolated from the fruits of *Lagenaria siceraria*.

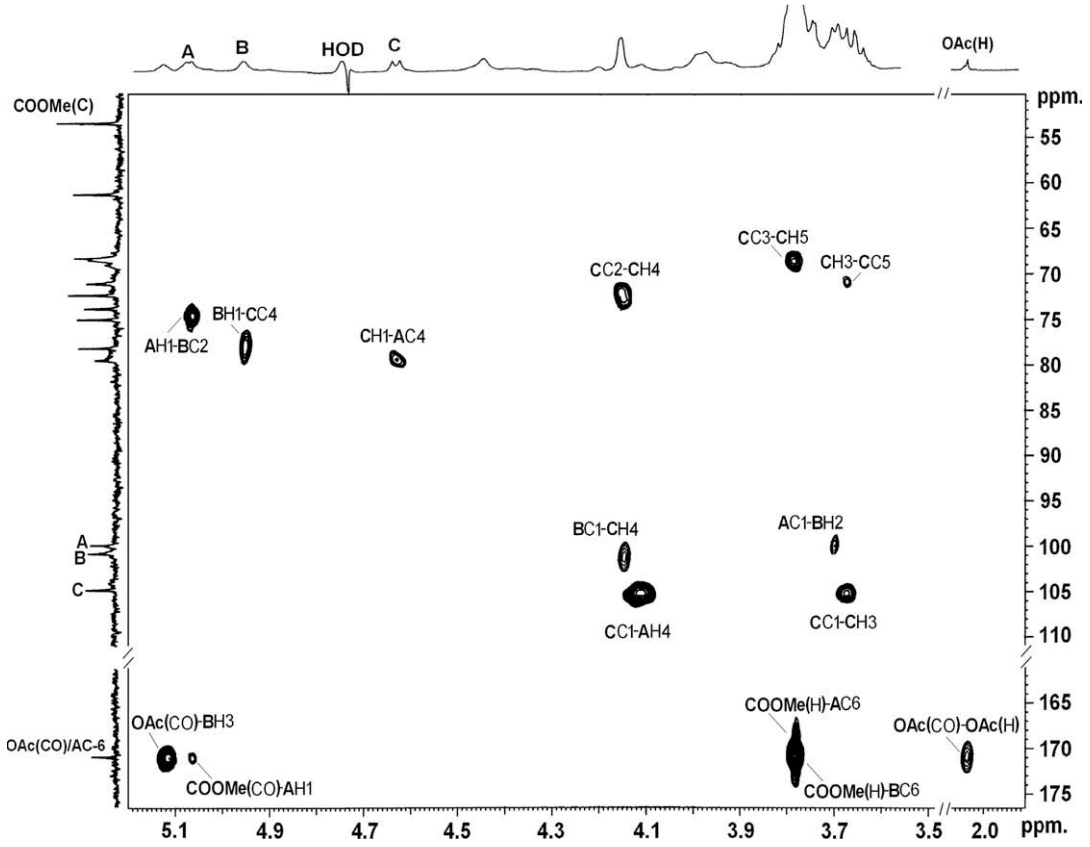


**Figure 3.** HMBC spectrum of polysaccharide, isolated from the fruits of *Lagenaria siceraria*. The delay time in the HMBC experiment was 80 ms.

**Table 2**
NOE data for the polysaccharide isolated from the fruits of *Lagenaria siceraria*

| Glycosyl residue | Anomeric proton | NOE contact to proton | |
|---|---|---|---|
| | $\delta_H$ | $\delta_H$ | Residue, atom |
| →4)-α-D-Gal*p*A6Me-(1→ | 5.06 | 4.94 | **B**H-1 |
| **A** | | 3.70 | **B**H-2 |
| | | 4.43 | **A**H-5 |
| | | 3.97 | **A**H-3 |
| | | 4.11 | **A**H-4 |
| →2)-3-*O*-Ac-α-D-Gal*p*A6Me-(1→ | 4.94 | 5.06 | **A**H-1 |
| **B** | | 3.70 | **B**H-2 |
| | | 3.97 | **B**H-4 |
| | | 4.14 | **C**H-4 |
| | | 4.43 | **B**H-5 |
| →4)-β-D-Gal*p*-(1→ | 4.62 | 3.66 | **C**H-2 |
| **C** | | 3.68 | **C**H-3 |
| | | 4.14 | **C**H-4 |
| | | 4.11 | **A**H-4 |

**Table 3**
The significant $^{3}J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide isolated from the fruits of *Lagenaria siceraria*

| Residue | Sugar linkage | H-1/C-1 | Observed connectivities | | |
|---|---|---|---|---|---|
| | | | $\delta_H/\delta_C$ | Residue | Atom |
| **A** | →4)-α-D-Gal*p*A6Me-(1→ | 5.06 | 74.9 | **B** | C-2 |
| | | 99.8 | 3.70 | **B** | H-2 |
| **B** | →2)-3-*O*-Ac-α-D-Gal*p*A6Me-(1→ | 4.94 | 78.7 | **C** | C-4 |
| | | 100.7 | 4.14 | **C** | H-4 |
| **C** | →4)-β-D-Gal*p*-(1→ | 4.62 | 79.4 | **A** | **C-4** |
| | | 104.7 | 4.11 | **A** | H-4 |
| | | | 3.68 | **C** | H-3 |
| | | COOMe ($\delta_H$) | Observed connectivities | | |
| | | | $\delta_C$ | Residue | Atom |
| **A** | →4)-α-D-Gal*p*A6Me-(1→ | 3.78 | 171.0 | **A** | C-6 |
| **B** | →4)-3-*O*-Ac-α-D-Gal*p*A6Me-(1→ | 3.78 | 171.0 | **B** | C-6 |
| | | 3-*O*-COMe ($\delta_C$) | Observed connectivities | | |
| | | | $\delta_H$ | Residue | Atom |
| **B** | →4)-3-*O*-Ac-α-D-Gal*p*A6Me-(1 | 171.0 | 5.12 | **B** | H-3 |

HMBC experiment. From inter-residue NOE contacts (Fig. 4, Table 2), the following sequences were established:

OAc
↓
3
α-D-Gal*p*A6Me-(1→2)-α-D-Gal*p*A6Me-(1→
**A** **B**

OAc
↓
3
→2)-α-D-Gal*p*A6Me-(1→4)-β-D-Gal*p*-(1→
**B** **C**

→4)-β-D-Gal*p*-(1→4)-α-D-Gal*p*A6Me-(1→
**C** **A**

The sequence was further confirmed by the HMBC experiment (Fig. 3, Table 3). Cross-peaks were found between H-1 of residue **A** ($\delta$ 5.06) and C-2 of residue **B** (**A**H-1, **B**C-2); C-1 of residue **A** ($\delta$ 99.8) and H-2 of residue **B** (**A**C-1, **B**H-2). Similarly, cross-peaks were found between H-1 of residue **B** ($\delta$ 4.94) and C-4 of residue **C** (**B**H-1, **C**C-4); C-1 of residue **B** ($\delta$ 100.7) and H-4 of residue **C** (**B**C-1, **C**H-4). Cross-peaks were also observed between H-1 of residue **C** ($\delta$ 4.62) and C-4 of residue **A** (**C**H-1, **A**C-4); C-1 of residue **C** ($\delta$ 104.7) and H-4 of residue **A** (CC-1, AH-4) along with intra-residual coupling between C-1 of residue **C** with its own H-3 (**C**C-1, **C**H-3). Other intra-residual interactions (**C**H-3, **C**C-5) and (**C**C-3, **C**H-5) were observed. Another two intra-residual couplings between the carboxymethyl proton ($\delta$ 3.78) and the ester carbonyl carbon ($\delta$ 171.0) of residue **A** and residue **B** were observed. Intra-residual coupling between acetyl carbonyl carbon ($\delta$ 171.0) and H-3 ($\delta$ 5.12) atom of residue **B** [OAc (carbonyl carbon), **B**H-3], and also cross-peak between acetyl carbonyl carbon ($\delta$ 171.0) and acetyl methyl proton ($\delta$ 2.04) of residue **B** [OAc (carbonyl carbon), OAc (methyl proton)] were observed.

Therefore, on the basis of the above results, the following trisaccharide-repeating unit in the polysaccharide is assigned

OAc
↓
3
→4)-α-D-Gal*p*A6Me-(1→2)-α-D- Gal*p*A6Me-(1→4)-β-D-Gal*p*-(1→
**A** **B** **C**

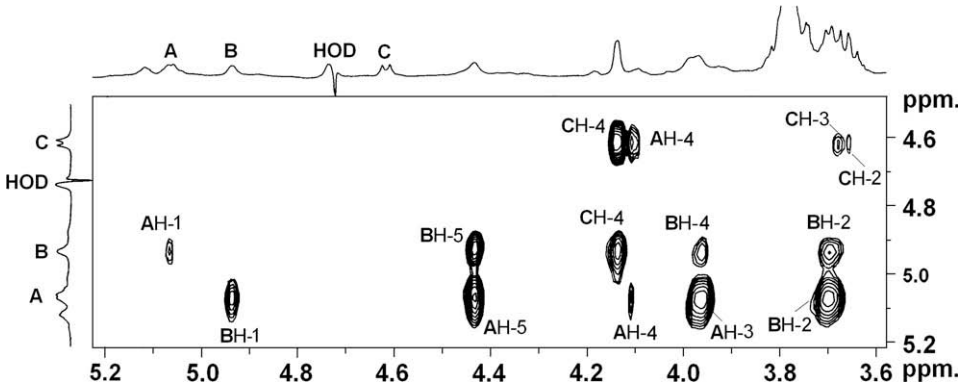


**Figure 4.** NOESY spectrum of polysaccharide, isolated from the fruits of *Lagenaria siceraria.* The NOESY mixing time was 300 ms.

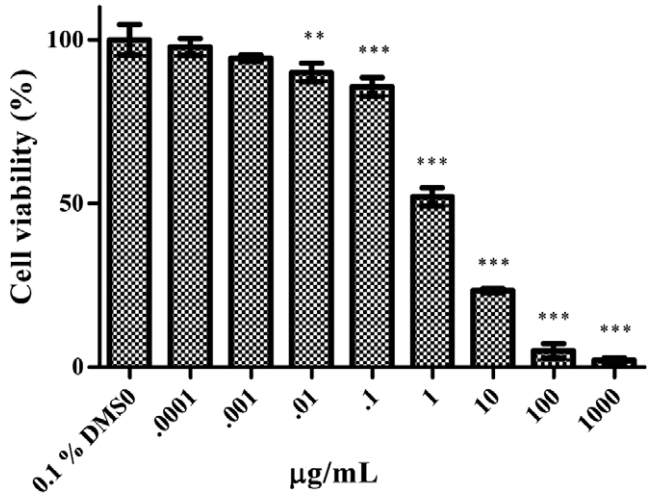


**Figure 5.** Dose-dependent growth inhibitory effects of polysaccharide on MCF-7. Cells were treated with polysaccharide and incubated for 72 h. Data represent average ± SD of three independent experiments each performed in triplicate. * represent level of significance with $P$ (< 0.05) with respect to control (0.1% DMSO). Statistical analysis was done by one-way ANOVA using Graph PadPrism 5.

Cytotoxicity of the polysaccharide was determined by the MTT dye-reduction assay.[20] The polysaccharide showed the $IC_{50}$ value at 1.0 μg/mL concentration against breast adenocarcinoma MCF-7 cell line (Fig. 5). The cell cycle analysis[21] was carried out with this concentration of polysaccharide. There is an increase in sub ($G_0/G_1$) or hypo diploid stage from 3.88% to 20.18% indicating programmed cell death/apoptosis (Table 4). These results indicate that the polysaccharide has a cytotoxic activity in vitro against breast adenocarcinoma cell line (MCF-7).

## 1. Experimental

### 1.1. Isolation and purification of the polysaccharide

The fresh fruit of *L. siceraria* (2.0 kg) was collected from the local market and washed with water. It was boiled with distilled water for 4 h and filtered through a linen cloth. The filtrate was centrifuged at 9000 rpm (using a Heraeus Biofuge stratos centrifuge) for 45 min at 4 °C, and the crude polysaccharide (3.5 g) was isolated as described in our previous papers.[22–24] The crude polysaccharide (32 mg) was purified by gel permeation chromatography on Sepharose 6B column. One homogeneous fraction (test tubes 28–43) was collected and freeze dried, yield — 25 mg. The purification process was carried out in several lots.

### 1.2. General methods

Paper chromatographic studies were performed as described previously.[15,22,23] Optical rotation was measured on a Jasco polarimeter, model P-1020 at 25 °C. For monosaccharide analysis, the polysaccharide sample (3.0 mg) was hydrolyzed with 2 M $CF_3COOH$ (2 mL), and the analysis was carried out as described earlier.[13] The molecular weight of the polysaccharide was determined as reported earlier.[13,14,22,23] The absolute configuration of the monosaccharide constituent was assigned according to Gerwig et al.[16] The same procedure was applied with carboxyl-reduced polysaccharide where the methyl ester of galacturonic acid was converted to galactose and the determination of configuration of galactose indicated that galacturonic acid was present as D-configuration. Both the polysaccharide and the carboxyl-reduced polysaccharide[18] were methylated according to the Ciucanu and Kerek method.[17] All gas–liquid chromatography–mass spectrometric (GLC–MS) analyses were performed on Hewlett–Packard 5970A automatic GLC–MS system, using an HP-5 capillary column (25 m × 25 mm). The program was isothermal at 150 °C; hold time was 2 min, with a temperature gradient of 4 °C $min^{-1}$ up to a final temperature of 200 °C. Periodate oxidation experiment was performed with this polysaccharide as described in the earlier report.[13] NMR experiments were carried out as reported in our previous papers.[13,25,26]

### 1.3. Cell line

Human breast adenocarcinoma cell line (MCF-7) was cultured in Dulbecco's Modified Eagle's Medium: Nutrient Mix F-12 (D-MEM/F-12) w/15 mM Hepes buffer and L-glutamine, supplemented with sodium bicarbonate (1.2 g/L), penicillin (100 U/mL), streptomycin (100 μg/mL). Cells were incubated at 37 °C in 5% carbon dioxide, 95% humidity, and supplemented with 10% fetal bovine serum (complete medium). Cells were subcultured in the same medium under the same conditions after reaching 70–80% confluency and were maintained in the log phase for further experiments.

### 1.4. Cell viability assay by MTT

Cytotoxicity was determined by the MTT dye-reduction assay. The methodology described is a modification of the original MTT colorimetric assay developed by Mosmann.[20] Cells were harvested from maintenance cultures in the exponential phase and were counted by a hemocytometer using trypan blue solution. The cell suspensions were dispensed (200 μL) in triplicate into 96-well culture plates at optimized concentrations of 5000 cells/well in a complete medium. After a 24 h recovery period, the medium was taken out and redispensed in 200 μL of incomplete medium supplemented with 2% FBS. For medium inhibition concentration ($IC_{50}$) determination, dose-response curves were conducted with a series of different concentrations ranging from 0.1 ng/mL to 1 mg/mL of the polysaccharide. In control wells, only culture medium (100 μL) with vehicle (0.1% DMSO in 2% medium) was added. After an additional 72 h incubation period, the medium in each well was aspirated and replaced with 100 μL MTT working solution (1 mg/mL). The cells were incubated at 37 °C for 4 h, and then the medium was aspirated and replaced with 100 μL DMSO to dissolve the formazan crystals that are formed. The culture plates were shaken for 5 min, and the absorbance of each well was read at 540 nm.

### 1.5. Cell cycle analysis

Cells were cultured in 60 mm culture dish in a complete medium (DMEM/F12). After 24 h, the medium was aspirated and an

**Table 4**
Cell cycle changes and apoptosis in polysaccharide-induced breast adenocarcinoma cell line (MCF-7)

| Time (h) | Sub $G_0$ | $G_0/G_1$ | S | $G_2/M$ |
|---|---|---|---|---|
| Control | 3.88 ± 0.88 | 31.40 ± 3.63 | 20.82 ± 4.18 | 44.56 ± 5.44 |
| 12 h-Treated polysaccharide | 7.74 ± 1.26 | 25.60 ± 3.60 | 16.24 ± 3.76 | 51.30 ± 1.82 |
| 24 h-Treated polysaccharide | 20.18 ± 4.82 | 22.76 ± 4.24 | 14.58 ± 2.58 | 33.22 ± 3.22 |

Data represent mean ± SD.

incomplete medium with 5% FBS was added. Time response curve for polysaccharide (1.0 μg/mL) was studied for 12 and 24 h along with control. The cells were harvested and fixed in 70% ethanol, maintained at −20 °C overnight for resolving sub $G_0$ stage. Then, the cells were washed with ice cold PBS (pH 7.4) and resuspended in 1 mL of PI Master mixture (40 μg/mL PI, 100 μg/mL RNaseA in PBS), followed by incubation at 37 °C for 1 h. Apoptotic cells were determined by their hypochromic sub-diploid staining profiles. The distribution of cells in the different cell cycle phases was analyzed from the DNA histogram using Becton–Dickinson FACS Caliber flow cytometer and CELL QUEST software.[21]

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