A unique polysaccharide containing 3-O-methylarabinose and 3-O-methylgalactose from *Tinospora sinensis*

Shipra Nagar\(^a, b, c, *\), Andreas Hensel\(^a\), Petra Mischnick\(^b\), Vineet Kumar\(^c\)

\(^{a}\) Institute of Pharmaceutical Biology and Phytochemistry, University of Münster, D-48149, Münster, Germany
\(^{b}\) Institute for Food Chemistry, Technische Universität Braunschweig, Schneidemüllerstr.20, D-38106, Braunschweig, Germany
\(^{c}\) Chemistry Division, Forest Research Institute, Dehradun, 248006, India

**Abstract**

*Tinospora sinensis* (Lour.) Merrill is of great therapeutic significance in Indian traditional medicine. Crude polysaccharides were isolated from methanol pre-extracted stems of dried material by successive extractions with cold water, hot water and NaOH (0.25 mol/L) in 0.98, 0.55 and 0.70 % yields respectively. Cold water soluble polysaccharides (CWSP) were purified and fractionated by ion exchange chromatography on DEAE-Sephadex. Neutral polysaccharides were further fractionated on Sepharose CL6B to yield three fractions TW1, TW2, TW3. The study further focuses on structural elucidation of TW1. TW1 was obtained in 0.8 % yield relative to CWSP, with MW of 1.6 x 10\(^5\) Da. It was composed of 3-O-methyl-arabinose, 3-O-methyl-galactose and galactose in molar ratio of 1:6.3:0.9 respectively. Based on per-deuteromethylation, NMR and ESI-MS analyses, TW1 was composed of 1,4-linked 3-O-methyl-β-D-galactopyranose and β-D-galactopyranose backbone with branching at O-6 of 3-O-methyl-β-D-galactosyl residues by 1,5-linked 3-O-methyl-α-L-arabinofuranoside chains. 3-O-methyl-arabinose and 3-O-methyl-galactose have first ever been reported in any polysaccharide and *Tinospora* genus, respectively.

1. Introduction

*Tinospora sinensis* (Lour.) Merrill syn. *T. malabarica* Miers, ex Hook. f., *Coccus chondrodendron* DC., *T. tomentosa* (Cleb.) Hook f & Thompson, *C. malabaricus* DC., *Campylos sinensis* Lour., *Menispermum malabaricum* Lam., *C. tomentosa* Cleb., *Menispermum cordifolium*, *Menispermum tomentosum* (Cleb.) Roxb. (Haque, Jantan, & Abbas, 2017) commonly known as Giloy, is a deciduous climber with rambling stems, bearing aerial roots from branches. The twiner is found all over India, China, Nepal and South East Asia (Chi et al., 2016; Haque et al., 2017). It is now considered as a vulnerable species due to its extensive utilization in traditional medicine and consequent exploitation of the natural habitat (Bhatnagar, Karhi, & Tandon, 2002). *T. sinensis* is utilized in Indian and Chinese folk medicinal systems for fumigation in piles, ulcerated wounds and preparation of medicated baths for liver-complaints (Haque et al., 2017). The twiner has been reported to be used in stimulating blood circulation, easing pain and decreasing swelling (Xu, Yu-Kui, & Wei, 2010); in treating debility, dyspepsia, fever, inflammation, syphilis, ulcers, bronchitis, jaundice, urinary and skin diseases and as a muscle and joints relaxant. Boiled roots are given in fever while fresh leaves and stems are used in chronic rheumatism (Fuhrman, Volkova, & Rosenblat, 2000; Haque et al., 2017). In Chinese traditional medicine, the stems have been used in the treatment of bruises with pain and lumbar muscle strain (Li et al., 2004). Aqueous and ethanolic extracts of *T. sinensis* have been reported to exhibit strong pharmacological potential. They have been investigated for anti-inflammatory (Li, Lin, Myers, & Leach, 2003), hepatoprotective (Maurya et al., 2009), anti-diabetic (Yonemitsu, Fukuda, & Kimura, 1993), immunomodulatory and adaptogenic (Manjrekar, Jolly, & Narayanan, 2000) activities. The ethanolic extract and its n-butanol fraction have been known to possess significant in vitro anti-leishmanial activity (Singh et al., 2008). Dried extracts obtained from ethanol (80%), aqueous and ethyl acetate fractions were found to exhibit hypoglycemic effects (Do, Le, Nguyen, & Nguyen, 2007).

A number of standard formulations and patents on *T. sinensis* ascribing to its huge spectrum of activities have been reported (Bao, Zhao, & Bu, 2001; Fan & Li, 2007; He, 2009; Ma, 2010; Nima, 2010; Ye & Liao, 2005). Concerning phytochemical composition, the occurrence of gly-
cosides, sterols, tannins, flavonoids, polyphenols, alkaloids, sesquiterpenes and esters have been described. The stems have been reported to contain a phenolic glycoside (Yonemitsu et al., 1993), two dinoditerpene glucosides (Yonemitsu, Fukuda, Kimura, Isobe, & Komori, 1995); two lignin glucosides (Li et al., 2004), a dinocerol diterpenoid glycoside (Dong et al., 2010); three clerodane diterpene glycosides, a cadinane sesquiterpene glycoside tinosinenside (Li, Wei, Fu, & Koike, 2007); trans-syringin, 3'-demethylphillyrin, sesquiterpene glycoside, vanillin, daunderoxol and β-sitosterol (Ren et al., 2008); alkaloids such as magnoflorine, berberine and also the occurrence of tinosporicine, menispermacide, palmatine, and (+)-malabaridole has been mentioned in literature (Srinivasan, Unnikrishnan, Shree, & Balachandran, 2008).

In view of primary metabolites from the genus, various polysaccharides of diverse structural characteristics have been isolated from T. cordifolia and reported to possess a range of different bioactivities with immunomodulating properties being dominant (Kumar & Nagar, 2014). As for instance, immuno-protective novel RR1 polysaccharide (Nair et al., 2004; Nair, Melnick, & Ramachandran, 2006), immunomodulatory G1 – 4A polysaccharide (Desai, Ramkrishnan, Chintalwar, & Sainis, 2007; Raghu et al., 2009) and an immunologically active arabinogalactan polysaccharide (Chintalwar et al., 1999) have been reported. Also antioxidant and radioprotective arabinogalactan polymer (Goel, Prem, & Rana, 2002; Subramanian, Chintalwar, & Chattopadhyay, 2002; Subramanian, Chintalwar, & Chattopadhyay, 2003) has been isolated from T. cordifolia. In contrast to the manifold studies on polysaccharides from T. cordifolia, no studies have been performed concerning the isolation and structural elucidation of polysaccharides from T. sinensis. Such work has been considered as a prerequisite for understanding the bioactivity and the use of such polysaccharides from T. sinensis. Therefore the following study aimed to characterize the neutral polysaccharide from the stem of T. sinensis.

2. Materials and methods

The stems of T. sinensis were collected from Forest Research Institute, Dehradun. A voucher specimen was identified by Dr. H. B. Naithani, Systematic Botany Discipline, Botany Division, Forest Research Institute, Dehradun-248006 and accession number (164274A, 164274B, 164274C) was obtained from Herbarium, Botany Division, Forest Research Institute, Dehradun. The stems were dried in shade.

2.1. General methods of analyses

If not stated otherwise, all chemicals were of analytical grade, purchased from Sigma and VWR. The polysaccharide standards, arabinogalactan (from larch wood) and galactomannan (from locust bean gum) were purchased from Sigma-Aldrich. All standard and sample solutions were freshly prepared before use. Distilled water was used in isolation and purification protocols while Millipore water was used in all other procedures throughout the experimentation. The molecular weight (MW) was determined by High Performance Size Exclusion Chromatography (HP-SEC) of Agilent Technologies 1200 Series with Suprema® (PSS, Mainz) 100 Å and 3000 Å column as stationary phase and ammonium acetate buffer 0.15mol/L at pH 4.5 as mobile phase. The HP-SEC was coupled to a refractive index detector, Agilent series 1200 RID. The column was calibrated with pullans standards (10, 22.8, 47.3, 11.2, 212, 404 and 788KDa). Colorimetric tests were evaluated by measurements of UV absorbance by Tercan-Reader Sunrise. Gas chromatography (GC) of the reference standards and sample mixtures was carried out on Shimadzu GC-2010 SPL-2010 using HP-SMS and ZB-SMS stationary phases. Gas Chromatography-Mass Spectrometry (GC–MS) analysis was carried on Agilent Technologies 6890GC System employing the same capillary columns as above. The operating conditions for alditol acetate analysis were as follows: double ramp program i.e. first ramp with initial temperature 160°C for 1 min, program rate of 0.5°C per min up to 190°C, followed by second ramp of program rate of 20°C per min up to final temperature 250°C. Partially methylated alditol acetates were analyzed by GC and GC-MS under a single ramp program with initial temperature 170°C for 1 min, program rate of 1°C per min up to final temperature 210°C for 10 min. Helium was used as mobile phase in both the programs. Electrospray ionization ion trap mass spectrometry (ESI-IT-MS) recordings of oligomeric mixtures were performed in the positive ion mode on Esquire LC 00681 (Bruker Daltonics). 1H and 13C NMR measurements were obtained at 600 MHz and 150 MHz, respectively (Agilent VNMR5000). 2D-NMR data (HSQC) were obtained under the same conditions.

2.2. Isolation of polysaccharides

The shade dried stems of T. sinensis were chopped into small pieces followed by pulverization. The ground plant material (3kg) was subjected to Soxhlet extraction with methanol for 19h. The methanol extracted plant material (81% yield) thus obtained was air dried and further used for isolation of Cold Water Soluble Polysaccharide (CWSP). The material (2430g; 550g, taken in each batch) was stirred vigorously in distilled water (3L) at 12°C for 3h. The stirred mixture was filtered with a four layered muslin cloth and centrifuged in order to remove the water insoluble part. The clear supernatant was concentrated under reduced pressure to 300mL. This concentrated aqueous extract (300mL) was poured dropwise into 900mL of ethanol (96% v/v) under continuous stirring. The suspension was kept overnight at 4°C, and precipitated polysaccharides were collected after centrifugation. The resulting polysaccharide pellet was dissolved in 100mL of water, re-precipitated by pouring this solution dropwise into ethanol 96% and centrifuged to give the polysaccharide pellet. The so obtained pellet was dried in an oven at 40°C to yield brown crude CWSP (5.4g, 0.98%, related to the herbal material on dry mass basis).

The cold water insoluble residue obtained after the 1st extraction was subjected to subsequent 2nd, 3rd and 4th extractions with distilled water at 12°C for 3h each followed by isolation of crude CWSP as mentioned above. The so obtained polysaccharides were combined. The insoluble residue obtained after the four cold water extraction steps was subjected to extraction of Hot Water Soluble Polysaccharide (HWSP), wherein the residue was vigorously stirred twice with 3L of hot water at 90–95°C for 3h successively. The hot water extract was processed subsequently in the same manner as described for CWSP. The insoluble residue obtained after cold and hot water extractions was subjected to the isolation of Alkali Soluble Polysaccharide (ASP). The residue was stirred twice successively with 2L of NaOH 0.25 mol/L at 15°C for 3h. The supernatant obtained after centrifugation of the mixture was neutralized with 2M HCl and concentrated to 300mL volume, which was processed further in the same manner as described for CWSP. The residue left after eight successive extractions with cold water, hot water and alkali treatment was finally discarded. The yields and extraction conditions of CWSP, HWSP and ASP have been provided in Table 1.

2.3. Purification and fractionation of raw polysaccharides

Crude CWSP was purified by ion exchange chromatography (IEC) using DEAE Sephadex® (GE, Germany), equilibrated in potassium phosphate buffer (KPB), pH 6.0. 220mg of CWSP was loaded in each batch and eluted by use of a step gradient of water, 0.1M KPB, 0.25M KPB, 0.5M KPB and 1M KPB at pH 6.0. 85 fractions were collected per gradient step with a fraction size of 10mL. Carbohydrates within the eluted fractions were detected by resorcinol sulfuric acid assay
Table 1  
Yields related to dried herbal material and extraction conditions of CWSP, HWSP & ASP.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Extraction Conditions</th>
<th>Yields (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CWSP</td>
<td>polysaccharide isolated by cold water extraction at 12°C</td>
<td>0.98</td>
</tr>
<tr>
<td>HWSP</td>
<td>polysaccharide isolated by hot water extraction at 90.95°C</td>
<td>0.55</td>
</tr>
<tr>
<td>ASP</td>
<td>polysaccharide isolated by alkali extraction (0.25M NaOH) at 15°C</td>
<td>0.70</td>
</tr>
</tbody>
</table>

(Monsigny, Petit, & Roche, 1988). Carbohydratecontaining fractions were pooled and dialyzed (SpectraPor® cellulose membranes MWCO 3500Da, Roth, Germany) for 72h against distilled water, followed by lyophilization of the remaining solution. Neutral polysaccharides, obtained within the water eluate were further fractionated by gel permeation chromatography (GPC) on Sepharose CL6B (GE, Germany) by elution with water. GPC column was calibrated using standard dextrans (5, 50, 73.5 and 270kDa, Sigma, Germany) prior to analysis. The neutral polysaccharide sample was fractionated into three fractions i.e. TW1, TW2 and TW3. The mean MW of samples was determined by HPSEC-RID against pullulans as standard (10, 22.8, 47.3, 11.2, 212, 404 and 788kDa).

2.4. Determination of protein content

Protein content was determined by Bradford assay, 1976. The assay was performed as instructed by the manufacturer Bio-Rad (Hercules, USA). 80 μL of the test solutions (c = 0.1–0.5mg/mL) were taken in 96-well plate followed by addition of 20μL of the Bio-Rad Protein Assay solution. The mixture was mixed vigorously and incubated for 10 min at room temperature (15°C). The absorbance of the subsequent blue color thus obtained was measured at λ = 595 nm, whereby the blind value (absorbance of reaction mixture without sample) was subtracted from the absorption of test mixtures. The protein content was determined by a calibration curve of bovine serum albumin (cBSA = 5, 10, 25, 50, 75, 100 μg/mL).

2.5. Monosaccharide composition

Monosaccharide composition of TW1 was determined qualitatively and quantitatively by GC analysis of alditol acetates (Herrmann et al., 2012) after hydrolysis of the polymer by trifluoroacetic acid (2M TFA) at 120°C for 2h, followed by reduction with 0.25M NaBD₄ at 60°C for 2h, and acetylation in the presence of acetic anhydride and N-methylimidazole at room temperature (15°C) for 15 min. The alditol acetates of samples and standards were subjected to GC–MS and GC-FID analyses. The GC–MS chromatograms were studied only for comparing retention time (tₑ) and fragmentation pattern, while GC-FID chromatograms were analyzed for quantification of the respective monomers.

2.6. Methylation analysis

Per-deuteromethylation of polysaccharides was performed according to Harris et al. modified method (Harris, Henry, Blakeney, & Stone, 1984), and standard arabinogalactan and galactomannan polysaccharides were taken as control. About 2–3mg polysaccharide was dissolved in 100μL anhydrous DMSO. After complete solubilization, 20μL of freshly prepared potassium-dimethyl reagent 3mol/L was added under argon atmosphere and continuous stirring followed by addition of 5μL of ice cooled methyl iodide-d₅. These steps were repeated four times. Finally, 60μL of 3M potassium-dimethyl reagent was added and reaction mixture was vortexted; then 15μL CD₃J was further added and the reaction mixture was vigorously stirred for 30min at 25°C. Subsequently, 3mL of a mixture of dichloromethane and methanol (2:1) was vortexed with DMSO solution of per-deuteromethylated samples and the reaction was stopped by adding 2mL water. The reaction mixture was centrifuged (1500×g, 2min) to facilitate the phase separation. The upper layer was discarded and the lower layer was washed five times with 2mL water each time. Finally the upper water phase was removed completely. The remaining moisture in DCM phase was eliminated by adding 2mL of 2,2-dimethoxypropane and 20μL of glacial acetic acid, which were then evaporated completely at 60°C under a stream of nitrogen.

The per-deuteromethylated polysaccharide was then derivatized to the respective partially deuteromethylated alditol acetates (PMAA; Voiges, Adden, Rinking, & Mischnick, 2012). The etherified polysaccharide sample was hydrolyzed with TFA (2mol/L), followed by reduction with NaBD₄ and acetylation with pyridine and acetic anhydride. The PMAA thus obtained were subjected to GC–MS and GC-FID analysis.

2.7. Partial degradation and ESI–MS analysis

1–2mg of polysaccharide was subjected to partial hydrolysis with 600μL of 0.5M HCL at 100°C for 20min. The hydrolyzates were cooled and mineral acid was removed by adding 1 mL acetone (×4) and evaporating the reaction mixture under a stream of nitrogen to dryness. Finally, 1 mL methanol was added and the oligomeric mixture was subjected to ESI-IT-MS analysis.

2.8. NMR analysis

22–25mg isolated polysaccharide was dissolved in 0.75mL D₂O (99.95%) and filtered through prewashed cotton. ¹H, ¹³C and HSQC NMR data were recorded at 26°C, and peak of HDO at 4.76 ppm was taken as reference in ¹H NMR as well as HSQC spectra. The parameters for ¹H NMR acquisition were as follows: Irradiating frequency at field strength of 600 MHz was 599.74 MHz; 32 scans were recorded with acquisition time 3.4 s, relaxation delay of 1s and spectral width of 9615.38 Hz. For ¹³C NMR acquisition, spectrometer frequency at field strength 150MHz was 150.82 MHz; 5000 scans were taken with acquisition time 0.86 s, relaxation delay of 1s and spectral width of 37878.8 Hz. Same parameter settings of ¹H and ¹³C were employed for 2D HSQC NMR recordings. Data analysis was achieved with MestReNova software, version 11.0.2–18153.

3. Result and discussion

Crude polysaccharides, extracted from the stems of T. sinensis with cold water, hot water and aqueous sodium hydroxide (CWSP, HWSP and ASP) have been isolated in 0.98, 0.55 and 0.70 % (w/w) yields respectively, related to the dried herbal material. For further investigation, CWSP was fractionated by IEC into neutral fraction TW (6.26 % (w/w), related to CWSP) and three acidic fractions i.e. TB, TC, TD (7.09, 3.41 and 2.08 % (w/w) respectively w.r.t CWSP). The neutral fraction was further fractionated by GPC to yield three fractions TW1, TW2, TW3 (yields: 0.8 %, 1.59 %, and 3.61 % (w/w) respectively related to CWSP). Subsequent structure elucidation was performed on TW1.

Protein was found to be absent in TW1. The polysaccharide was found to be homogeneous by HPSEC-RID technique with a polydispersity index of 1.5 and a mean molecular weight was of 1.64×10⁵Da as...
shown in Fig. 1. This is not an absolute mass of polymer but related to calibration with pullulan standards.

### 3.1. Monosaccharide composition

Monosaccharides obtained from TW1 after TFA hydrolysis were converted to alditol acetates and subjected to qualitative GC-MS and quantitative GC-FID analyses. The respective MS spectra revealed the presence of 3-O-Me-pentose (11.9%), 3-O-Me-hexose (75.5%) and galactose (10.9%) as major constituents and rhamnose (1.6%) in lower amounts. Alditol acetate of the only commercially available 3-O-methyl derivative of hexoses i.e. 3-O-Me-glucose was prepared for comparison of chromatographic retention times, but this methylated compound was found to be absent in the TW1 hydrolysate. The four constituent monomers i.e. 3-O-Me-hexose, 3-O-Me-pentose, galactose, and rhamnose, were present in molar ratio 6.3:1:0.9:0.1.

### 3.2. Linkage analysis

In order to identify both unknown methylated monosaccharides (3-O-Me-pentose and 3-O-Me-hexose), TW1 was subjected to per-deuteromethylation to conserve the native —OCH₃ groups and distinguish them from —OCD₃ groups by the characteristic mass shift of 3amu in mass spectrometry. The deuto-etherified polysaccharides were then converted to partially deuteromethylated alditol acetates (PMAA) and subjected to GC-MS analysis. Guar and galactomannan were used as control polysaccharides to verify the correctness of the used methylation procedure and to gain detailed linkage information from the respective PMAAs. PMAA peaks in the GC–MS chromatogram of TW1 corresponded to 1,5-linked 3-O-Me-pentose, terminal-galactose, terminal-3-O-Me-hexose, 1,4-linked galactose, 1,4-linked 3-O-Me-hexose and 1,4,6-linked 3-O-Me-hexose (Table 2). Rhamnose was not detected in methylation analysis, owing to its presence in meager amount. The co-eluted peaks were identified on the basis of pair of signals (e.g. m/z 165 and 168; Table 2) occurring for the same fragmentation with mass shift of +3amu in mass spectrum. In Fig. 2, the combination of m/z 121, 165/105 and 211/151 and acetyl substituents at 1,5-position indicated that —OCH₃ group was present natively at C3 in hexas, i.e. the residue is terminal 3-O-Me-hexose whereas m/z 121, 168/108 and 214/154 with same acetyl substitution and same linkage indicated the presence of —OCH₂-CH₂-OH at position 3 of hexas (galactose) due to per-deuteromethylation. Intriguingly, it has been observed that terminal 3-O-Me-hexose co-eluted with terminal galactose and 1,4-linked 3-O-Me-hexose co-eluted with 1,4-linked galactose, it revealed that the methyl monomer was 3-O-Megalactose. Furthermore, 1,5-linked-3-O-Me-pentose was co-eluted with 1,5-linked-arabinose in GC–MS chromatogram of PMAA of one of the acidic polysaccharides TB2 fractionated from CWSP. Since the retention time of this co-eluted peak in TB2 sample is same as in case of TW1, it indicates that the other unknown monomer was 3-O-Me-arabinose. The fourth peak with fragment ions at m/z 121, 261 – 261 showed the presence of acetyl groups at 1,4,5 and 6 positions and —OCH₃ group at C3, i.e. the residue is 1,4,6-linked 3-O-Me-hexose. It was also referred as derivative of galactose since alditol acetate method revealed the presence of only one type of 3-O-Me hexose monosaccharide in TW1.

### 3.3. Partial degradation of TW1

TW1 was partially hydrolyzed by 0.5M HCl and the mixture of oligomers obtained was subjected to ESI-IT-MS in positive ion mode, wherein the oligosaccharides were detected as [M + Na]⁺ and [M + K]⁺ as illustrated in Fig. 3. (The initials used in Fig. 3 for 3-O-Me-Galp, Galp and 3-O-Me-Araf are MG, G and MA respectively.) The color code signifies oligomers of different compositions. For instance, the oligosaccharide with degree of polymerization 5 (DP 5) in blue color is made up of one G, one MA and three units of MG and other oligomers of DP 4 and DP 3 (in blue color) have the similar composition that may belong to same larger oligomer obtained in partial hydrolysis. The complete assignment of all the signals has been given in Table 3a. Amongst the signals, the oligosaccharides observed at m/z 377, 407, 423, 523,

---

**Table 2**

<table>
<thead>
<tr>
<th>tₚ (min)</th>
<th>PMAA</th>
<th>Constituents (Deduced linkage)</th>
<th>Fragmentation (primary fragment ions, m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17.63</td>
<td>3-O-Me-2-O-Me-d₂-arabinitol-1,4,5-triacetate</td>
<td>1,5-linked 3-O-Me-Ara</td>
<td>121; 165 → 105; 189 → 129</td>
</tr>
<tr>
<td>20.09</td>
<td>2,3,4,6-tetra-O-Me-d₂-galactitol-1,5-diacetate</td>
<td>1,6-Gal</td>
<td>121; 168 → 108; 167 → 107,132; 214 → 154</td>
</tr>
<tr>
<td>23.51</td>
<td>3-O-Me-2,4,6-tri-O-Me-d₂-galactitol-1,5-diacetate</td>
<td>3-O-Me-Gal</td>
<td>121; 165 → 105; 167 → 107,132; 211 → 151</td>
</tr>
<tr>
<td>28.68</td>
<td>2,3,6-tri-O-Me-d₂-galactitol-1,4,5-triacetate</td>
<td>1,4-linked Gal</td>
<td>121; 168 → 198; 239 → 179; 48</td>
</tr>
<tr>
<td></td>
<td>3-O-Me-2,6-di-O-Me-d₂-galactitol-1,4,5-triacetate</td>
<td>1,4-linked 3-O-Me-Gal</td>
<td>121; 165 → 105; 236 → 176; 48</td>
</tr>
<tr>
<td></td>
<td>3-O-Me-2,3-2,4,6-tetraacetate</td>
<td>1,4,6-linked 3-O-Me-Gal</td>
<td>121, 261 → 201</td>
</tr>
</tbody>
</table>

---

**Fig. 1.** HPSEC-RI chromatogram of TW1; Molar mass range is calibrated with pullulan standards.
553, 583, 599, 729, 759, 775, 861, 891, 951 denote methyl glycosides of corresponding oligomers, formed as a product of a side reaction (methanolysis) during work up of partial degradation. This can be explained by considering the m/z signals 363 and 377. The regular m/z for a disaccharide of MA + MG would be 363 (as M + Na), while m/z 377 corresponds to the respective methyl glycoside (as M – OMe + Na), which could be formed in the methanol solution prepared for ESI-MS analysis after evaporation of aqueous HCl, leaving acid traces.

Since the respective linkages between the different monomers of TW1 have already been determined by GC-MS analysis of PMAA derivatives, ESI-MS followed by MS/MS measurements of partially hydrolyzed polysaccharide was performed to support the linkage data obtained from PMAA analysis and to determine the sequence of residues in oligomers, leading to the fine structure of the polymer. Since no disaccharide of G-MA was observed in the spectra, it was inferred that MA cannot be directly linked to G. On the contrary, MA and G are linked to MG individually. The ions at m/z signals representing oligomers (Fig. 3) were subjected to tandem MS to study the respective fragmentation pattern of the monomers (Mischnick, 2011). The MS/MS spectra obtained for ions with m/z 363 (Fig. 4a) and 379 (Fig. 4b) has been discussed here and the monomers are written with specific colors to explain the fragmentation and sequence more vividly. Now, the m/z 363 corresponds to a disaccharide containing MA and MG and the possible sequence of this disaccharide could be MA-MG or MG-MA. When MG is present at reducing end (Table 3b), 0.3A2 ring cleavage would occur to give a fragment at m/z 259 [M – 104 + Na]+ along with Y1 and B1 fragments at m/z 217 [M – 146 + Na]+ and 169 [M-194 + Na]+, respectively. The presence of ion [M – 104 + Na]+ and the lack of M-60 or -74 confirms existence of -OCH3 group at C3 and free -OH group at C4 in MG. On the other hand, when MA is present at reducing end (Table 3b), similar 0.3A2 ring cleavage would occur to give a fragment ion at m/z 259 [M – 104 + Na]+. Y1 and B1 fragments at m/z 187 [M – 176 + Na]+ and 199 [M – 164 + Na]+ have also been observed, respectively. Though, both the combinations are possible but MA-MG moiety revealed greater probability of occurrence due to higher intensity of signals appearing in the spectrum.

For the MS/MS spectrum of the ion at m/z 379 (Fig. 4b), two combinations, G-MG and MG-G, would be possible. When MG is present at the reducing end (Table 3b), 0.3A2 ring cleavage would occur to give a fragment ion at m/z 275 [M – 104 + Na]+, while 0.2X1 ring cleavage may also occur to give a fragment ion of m/z 259 [M – 120 + Na]+. Ideally, the former always dominates the latter, however such cleavage (0.2X1) has been reported to take place in some fragmentation studies (Wende et al., 2016) despite blocked reducing end. Y1 and B1 fragments are also observed at m/z 217 and 185, respectively. Conversely, in case of MG-G (Table 3b), loss of 60 (0.2A2), 90 (0.2A2) and 120 (0.2A2) reveals the linkage G-(6 → 1)-MG. However, no such linkage was detected in methylation studies and G was found to be present only in 1,4-linkage in 8% in TW1. Hence in this case, [G-MG-Na]+ dimer is supported by PMAA analysis.

The MS/MS spectra of other ions were also recorded and interpreted with respect to linkage position. The fragmentation data supported the findings of the methylation analysis and also revealed that unlike bacterial polysaccharides, the constituting monosaccharides in TW1 were arranged in a random fashion. The possible sequences in the oligosaccharides obtained were found to be linear chains of 1,4-linked MGs; 1,5-linked MAs linked to MG at C6 and 1,4-linked MG and G.

3.4. NMR analysis of TW1

TW1 was subjected to 1H, 13C and HSQC NMR spectroscopy. The respective NMR spectra are displayed in Fig. 5a-c. Extensive literature survey was carried out for signal assignment of the constituents present.
in TW1 and it was found that very limited data is available in the present literature for 3-O-Me-Galp and no data is available for 3-O-Me-Araf. In order to deal with this problem, trimers of 1,5-linked 3-O-Me-α-Araf and 1,4-linked 3-O-Me-β-Galp were simulated in silico on website http://www.nmrdb.org/, and the corresponding NMR data (1H NMR and 13C NMR) were generated to study the effect of native 3-O-methylation at C3 on the chemical shifts δc and δH of C and H to which OCH3 is linked and on the adjacent C and H. Based on this information, the anomeric and other ring carbons and protons of methylated monosaccharides were assigned. 1H NMR spectrum (Fig. 5a) showed six signals in anomic region, out of which the dominant signals were at δ 4.58, 4.98 and 5.23 ppm. The major signal at δ 4.58 was assigned for anomeric protons of →4)-3-O-Me-β-Galp(1→3)-O-Me-α-Araf(1→ and →4)-β-Galp(1→ (Habibi, Mahrouz, & Vignon, 2005). The signals at δ 4.98, 5.06, 5.09 and 5.23 were correlated to the anomeric protons of →4)-3-O-Me-α-Araf(1→ (He et al., 2016), →5)-3-O-Me-α-Araf(1→ (Habibi et al., 2005), α-Rhap(1→ and 3-O-Me-α-Araf(1→ (Kang et al., 2011) respectively. The signal of methyl group at 1.2 ppm was referred to the C6 of α-Rhap. The protons at δ 3.42 and 3.45 ppm indicated the presence of methoxy group at C3 in 3-O-Me-α-Araf and 3-O-Me-β-Galp (Mandal et al., 2015) respectively. The 13C NMR spectrum showed three major signals at the anomeric region. The signals at δ 110.20 and 107.04 ppm referred to C1 of →5)-3-O-Me-α-Araf(1→ and 3-O-Me-α-Araf(1→ (Ding et al., 2015; Dourado, Cardoso, Silva, Gama, & Coimbra, 2006), respectively, while the peak observed at 105.7 ppm was assigned to anomeric carbons of →4)-3-O-Me-β-Galp(1→ and →4)-β-Galp(1→ (Habibi et al., 2005). There were two small signals observed at 101.94 and 98.71 ppm. The former was identified as anomeric carbon of α-Rhap(1→ (Kang et al., 2011) and the latter interpreted as C1 of →4)-3-O-Me-α-Araf(1→ (He et al., 2016). The two peaks had a very low intensity owing to the low content of Rhap and 3-O-Me-α-Araf as well as low sensitivity of 13C NMR technique. All the data obtained by 1H NMR and 13C NMR was in agreement with the respective HSQC data and literature (Dourado et al., 2006; Habibi et al., 2005; Mandal et al., 2015; Shakhmatov, Atukmaev, & Makarova, 2016). The C/H values obtained for three residues are given in Table 4a and Table 4b.

The δc and δH values assigned for →4)-β-Galp(1→ were in agreement with literature (Habibi et al., 2005; Mandal et al., 2015; Shakhmatov et al., 2016) as given in Table 4a. In case of →5)-3-O-Me-α-Araf(1→, it has been observed from the respective in silico simulations that →OCH3 group at C3 affect the chemical shift values δc and δH up to three bonds, which implies that δc and δH values for C1/H1 and C5/H5 would remain same as for →5)-α-Araf(1→; the same has been supported by the literature as shown in Table 4b (Dourado et al., 2006; Habibi et al., 2005). The →OCH3 group was identified at δ 59.03/3.42 in 1H NMR, 13C NMR and HSQC. C2/H2 could not be determined, while δc/δH for C3/H3 was assigned to be at 83.53/ 4.08. The assignment of C3/H3 can be explained as follows: When →OH linked at C3 is substituted by →OCH3, the respective δc value shift downfield with a δc of 3.77 ppm while δH shifts upfield with a slight decrease of 0.255 ppm. Hence the values of C3/H3 at δ 83.53/ 4.08 for →5)-3-O-Me-α-Araf(1→ were justified with respect to the C3/ H3 δ values 78.53/4.11 for the non-methyalted counterpart (Habibi et al., 2005). In Table 4a, the signals for →4)-3-O-Me-β-Galp(1→ were assigned by using δc/δH values obtained for →4)-β-Galp(1→ as reference. Since →4)-3-O-Me-β-Galp(1→ residue constitutes about 75% of the TW1 polysaccharide, the signals with highest intensities denoted the same. The signals for C1/H1, C5/H5 and C6/H6 peaks were found to be identical with those of →4)-β-Galp(1→, since they are not affected by the substitution at C3. The C2/H2 δc/δH values were close in the two residues (→4)-β-Galp(1→ and →4)-3-O-Me-β-Galp(1→ with an upfield shift of 0.05 ppm in →4)-3-O-Me-β-Galp(1→ in 1H NMR. The values δc/δH observed at 85.30/3.40 have been assigned to C3/H3 of →4)-3-O-Me-β-Galp(1→ owing to the downfield effect due to →OCH3 group. When →H linked to C3 is replaced by →CH3, δc value is shifted downfield with a Δδc of 9.39/4.55 ppm while δH goes upfield with a slight decrease of 0.32–0.34 ppm (Carbonero et al., 2008; He et al., 2016), thereby rationalizing the above assignment. On the other hand, for C4/ H4 the presence of →OCH3 group at neighboring C-atom cause a decrease in δc value and a slight increase in δH value. Conversely, both δc and δH increase when C4 is in linkage. So the shift values for C4/H4 would experience a net effect from both these factors.

Summarizing, a tentative structure (Fig. 6) for the TW1 polysaccharide, isolated from the stems of T. sinensis is proposed. This polymer structure is based on the combination of chromatographic and spectroscopic methods. TW1 was found to encompass a backbone of 1,4-linked 3-O-Me-β-D-Galp with a random distribution of 1,4-linked β-D-Galp in the backbone, and some of them (1,4-linked 3-O-Me-β-D-Galp moieties) branched at O-6 with short (1),5-linked 3-O-Me-α-L-Araf side chains.
The structure was found similar to that of type I arabinogalactans found in pectins with a backbone of 1,4-linked β-D-galactopyranosyl and 1,5- and 1,3-linked α-L-arabinofuranosyl residues in branching at C6 position of galactose. It is assumed that 3-O-Me-β-Galp and β-Galp are present in D-configuration and 3-O-Me-α-Araf is present in L-configuration in TW1 polysaccharide, since similar kind of configuration has been reported in pectins (Aspinall, 1983; Leivas, Iacomini, & Cordeiro, 2015).

### Table 3b
Fragmentation for disaccharides of m/z 363 and 379.

<table>
<thead>
<tr>
<th>m/z signal Fragmented [M+Na]</th>
<th>Constituent monomers</th>
<th>Sequence</th>
<th>Ring Cleavage</th>
<th>Sequence</th>
<th>Y1/B1</th>
<th>Ring Cleavage</th>
</tr>
</thead>
<tbody>
<tr>
<td>363</td>
<td>MG, MA</td>
<td>MA-MG</td>
<td>Y1/B1 217/169</td>
<td>MG-MA</td>
<td>187/199</td>
<td>259 (3(\alpha)β)</td>
</tr>
<tr>
<td>379</td>
<td>MG, G</td>
<td>G-MG</td>
<td>Y1/B1 217/185</td>
<td>MG-G</td>
<td>203/199</td>
<td>319 (3(\alpha)β)</td>
</tr>
</tbody>
</table>

### 4. Conclusion

A novel neutral polysaccharide TW1 has been isolated from stems of *Tinospora sinensis* and characterized. The polysaccharide was found to possess a unique composition of 3-O-Me-α-Ara residues and larger content of 3-O-Me-β-Gal residues. TW1 with mean molecular weight of 1.64 \times 10^{5} Da was found to be composed of 3-O-Me-α-Araf, 3-O-Me-β-Galp and β-Galp in a molar ratio of 1.0:6.3:0.9, respectively, and
Fig. 5. (a) $^1$H NMR spectrum of TW1 (recorded in D$_2$O (99.95%) at 304 K; HDO peak at 4.76 ppm was taken as reference). (b) $^{13}$C NMR spectrum of TW1 (recorded in D$_2$O (99.95%) at 304 K). (c) HSQC NMR spectrum of TW1 (recorded in D$_2$O (99.95%) at 304 K; HDO peak at 4.76 ppm was taken as reference).
Carbohydrate Polymers 1,4 residue in NMR 2018

Table 4a

| Site  | Chemical shifts found by HSQC | Chemical shifts found by HSQC | Habibi et al. (2005) | Shakhmatov et al. (2016) | Mandal et al. (2015) | Simulated in silico chemical shifts
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(1→) 3-O-Me-β-Galp-</td>
<td>(1→) 3-O-Me-β-Galp-</td>
<td>(1→) 3-O-Me-β-Galp-</td>
<td>(1→) 3-O-Me-β-Galp-</td>
<td>(1→) 3-O-Me-β-Galp-</td>
<td>(1→) 3-O-Me-β-Galp-</td>
</tr>
<tr>
<td>C1/H1</td>
<td>105.92/4.57</td>
<td>105.92/4.57</td>
<td>105.21/4.57</td>
<td>107.3/4.65</td>
<td>103.3/4.42</td>
<td>102.29/4.95</td>
</tr>
<tr>
<td>C2/H2</td>
<td>72.94/3.66</td>
<td>72.72/3.71</td>
<td>72.70/3.48</td>
<td>75.3/3.68</td>
<td>70.8/3.59</td>
<td>69.25/3.29</td>
</tr>
<tr>
<td>C3/H3</td>
<td>85.30/3.60</td>
<td>74.36/3.70</td>
<td>76.2/3.76</td>
<td>81.4/4.09</td>
<td>82.6/3.28</td>
<td></td>
</tr>
<tr>
<td>C4/H4</td>
<td>73.76/4.44</td>
<td>78.60/4.07</td>
<td>80.5/4.17</td>
<td>68.0/3.89</td>
<td>72.1/3.42</td>
<td></td>
</tr>
<tr>
<td>C5/H5</td>
<td>77.12/3.64</td>
<td>75.30/4.65</td>
<td>77.6/3.72</td>
<td>76.6/3.98</td>
<td>72.1/3.52</td>
<td></td>
</tr>
<tr>
<td>C6/H6a</td>
<td>63.67/3.67, 3.67</td>
<td>63.67/3.76, 3.76</td>
<td>61.94/3.80, 3.80</td>
<td>63.6/3.82, 3.79</td>
<td>61.24/3.89</td>
<td></td>
</tr>
<tr>
<td>H6b</td>
<td>3.80</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OCH₃</td>
<td>59.88/3.45</td>
<td></td>
<td></td>
<td></td>
<td>59.9/3.45</td>
<td>59.48/3.22</td>
</tr>
</tbody>
</table>

* A hypothetical trisaccharide of 3-O-Me-β-Galp-(1→4)-β-Galp-(1→4)-3-O-Me-β-Galp was used for in silico simulation and its corresponding ¹H and ¹³C NMR spectra were generated to indicate the respective C/H shift values.

Table 4b

| Site  | Chemical shifts found by HSQC | Dourado et al. (2006) | Habibi et al. (2005) | Simulated in silico chemical shifts
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(1→) 3-O-Me-α-Araf</td>
<td>(1→) 3-O-Me-α-Araf</td>
<td>(1→) 3-O-Me-α-Araf</td>
<td>(1→) 3-O-Me-α-Araf</td>
</tr>
<tr>
<td>C1/H1</td>
<td>110.0/5.04</td>
<td>110.4/5.10</td>
<td>108.4/5.03</td>
<td>109.02/4.64</td>
</tr>
<tr>
<td>C2/H2</td>
<td>84.1/4.15</td>
<td>79.4/4.05</td>
<td>78.53/4.11</td>
<td>80.77/3.53</td>
</tr>
<tr>
<td>C3/H3</td>
<td>83.53/4.08</td>
<td>85.1/4.22</td>
<td>83.20/4.16</td>
<td>82.59/3.78</td>
</tr>
<tr>
<td>C4/H4</td>
<td>68.14/3.76, 3.76</td>
<td>69.1/3.83, 3.81</td>
<td>68.0/3.83</td>
<td>66.69/4.14</td>
</tr>
<tr>
<td>OCH₃</td>
<td>59.03/3.42</td>
<td>57.45/3.25</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* A hypothetical trisaccharide of 3-O-Me-α-Araf with 1,5 linkage was used for in silico simulation and its corresponding ¹H and ¹³C NMR spectra were generated to indicate the respective C/H shift values.

Fig. 6. Tentative structure of TW1 containing backbone of 1,4-linked 3-O-Me-β-D-Galp and β-D-Galp with branching at O-6 of 3-O-methyl-β-D-galactosyl residues by 1,5-linked 3-O-methyl-α-L-arabinofuranoside.

Compassing a linear chain of 1,4-linked 3-O-Me-β-Galp and 1,4-linked β-Galp with branching of 1,5-linked 3-O-Me-α-Araf side chains at C6 of 3-O-Me-β-Galp. The polymer has a similar linkage pattern as it is known for type I arabinogalactans in peptin.

Interestingly, 3-O-Me-Gal has never been reported in Tinospora genus. The residue, ‘3-O-Me-Gal’ has been reported in polysaccharides isolated from fungi (Pleurotus citrinopileatus (Brito et al., 2018; He et al., 2016); Phellinus igniarius (Yang, Zhang, & Zhang, 2007); Macropliota dolichula (Samanta et al., 2015)); bacteria (Klebsiella pneumoniae (Mandal et al., 2015)), algae (Grifola frondosa (Cui et al., 2013; Oliveira et al., 2018); Gracilaria padumadensis (Kondaveeti, Kumar, Ganesan, & Siddhanta, 2014); Aposphalea ayalii (Watt, O’Neill, Percy, & Brasch, 2002)), and plants (Selaginella apoda (Popper, Sadler, & Fry, 2001); Castanea sativa Mill and Citrus aurantifolia (Bacon & Cheshire, 1971); Sassafras albidum (Springer, Takahashi, Desai, & Kolecki, 1965)), wherein 3-O-Me-Gal is present in polysaccharides as terminal residue or in 1,6-linked backbone or branching. 1,4-Linked 3-O-Me-Gal has been reported in the polysaccharides isolated from the bark of Ulmus fulva and U. glabra (Barsett & Paulsen, 1992). However, this carbohydrate residue is not present as a major constituent in 1,4-linkage in the backbone but as part of the side chains in polysaccharides isolated from the above mentioned species. Hence these facts established that 1,4-linked 3-O-Me-β-Galp has been reported as a major constituent in the backbone of a polysaccharide in Tinospora genus for the first time. Further, extensive literature survey revealed that 3-O-Me-Araf has until now not been reported as building block of plant polysaccharides to the best of authors’ knowledge. Since T. sinensis has been utilized ethnopharmacologically for centuries, the novel polysaccharide iso-
lated may be further investigated for its physiochemical properties and potential biological activities.

Acknowledgements

One of the authors (SN) is grateful to DAAD [Deutscher Akademischer Austauschdienst (English: German Academic Exchange Service)] for providing funding as well as opportunity to perform a part of this research work in Germany. Thanks are also due to Dr. H. B. Naitiani, Systematic Botany Discipline, Botany Division, Forest Research Institute, Dehradun-248006 for authentication of the plant material.

References


Barrett, H., Paulsen, B., 1988. Rhamnose, isolation, purification and identification of acidic poly-
 saccharides from the inner bark of Ulmus glabra Huds. Carbohydrate Polymers 17 (2), 137–144.


Brito, P.J., Carbonero, E., Schilling, S.R., Silva, E.V., Ruthes, A.C., Liou, L.M., & Iaco-


Chi, S., Shi, G., Han, D., Wang, W., Liu, Z., Liu, B., 2016. Genus Tinospora: ethnophar-
 macology, phytochemistry, and pharmacology. Evidence-Based Complementary and Alternative Medicine 2016, 1–32.


 latory polysaccharide from Tinospora cordifolia, modulates macrophage responses and protects mice against lipopolysaccharide induced endotoxic shock. International Immunopharmacology 7 (10), 1375–1386.


ocin-induced diabetes. Tap Chi Duoc Hoac 1, 8–11.


Dourado, F., Cardoso, S.M., Silva, A.M., Gama, F.M., Coimbra, M.A., 2006. NMR structural elucidation of the arabinan from Prunus dulcis immunological active pectic polysac-
charides. Carbohydrate Polymers 66 (1), 27–33.

Fan, J., Li, P., 2007. Lotion containing traditional chinese medicine for treating rheuma-
 tosis and rheumatoid arthritis. Faming Zhiuan Shengqi Gouming Shoumingshi. Chi-

tioxidants & Redox Signalling 2 (4), 491–496.

Goel, H.C., Kumar, L.P., Rana, S.V.S., 2002. Free radical scavenging and metal chelation by Tinospora cordifolia, a possible role in radioprotection. Indian Journal of Experi-
 mental Biology 40, 727–734.

Habibi, Y., Mahrouz, M., Vigoun, M.K., 2005. Arabinian-rich polysaccharides isolated and characterized from the endocarp of the seed of Ompintia flavus-indica prickly pe-
e fruit. Carbohydrate Polymers 60 (3), 319–329.

Haque, A., Jantan, I., Bukhari, S.N.A., 2017. Tinospora species: An overview of their mod-


