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

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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-Sephacel. 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 1.6×10^5 Da. It was composed of 3-O-methyl-arabinose, 3-O-methyl-galactose and galactose in molar ratio of 1.0:6.3:0.9 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., *Coccilus chondodendrum* DC., *T. tomentosa* (Colebr.) Hook f & Thomson., *C. malabaricus* DC., *Campylus sinensis* Lour., *Menispermum malabaricum* Lam., *C. tomentosus* Colebr., *Menispermum cordifolium*, *Menispermum tomentosum* (Colebr.) 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 (Bhattara, Karki, & 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-

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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 dinorditerpene glucosides (Yonemitsu, Fukuda, Kimura, Isobe, & Komori, 1995); two lignin glucosides (Li et al., 2004), a dinorclerone 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, daucosterol and β -sitosterol (Ren et al., 2008); alkaloids such as magnoflorine, berberine and also the occurrence of tinosporicide, menispermicide, palmatine, and (+)-malabarolide 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 $\rightarrow$ 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 0.15 mol/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 pullulans standards (10, 22.8, 47.3, 11.2, 212, 404 and 788 kDa). Colorimetric tests were evaluated by measurements of UV absorbance by Tecan-Reader Sunrise. Gas chromatography (GC) of the reference standards and sample mixtures was carried out on Shimadzu GC-2010 SPL-2010 using HP-5MS and ZB-5MS 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 00081 (Bruker Daltonics). ^1H and ^{13}C NMR measurements were obtained at 600 MHz and 150 MHz, respectively (Agilent VNMRs 600). 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 (3 kg) was subjected to Soxhlet extraction with methanol for 19 h. 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 (2430 g; 550 g, taken in each batch) was stirred vigorously in distilled water (3 L) at 12 °C for 3 h. 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 300 mL. This concentrated aqueous extract (300 mL) was poured dropwise into 900 mL 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 100 mL 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 black brown crude CWSP (5.4 g, 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 3 h 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 3 L of hot water at 90–95 °C for 3 h 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 2 L of NaOH 0.25 mol/L at 15 °C for 3 h. The supernatant obtained after centrifugation of the mixture was neutralized with 2 M HCl and concentrated to 300 mL 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 Sephacel™ (GE, Germany), equilibrated in potassium phosphate buffer (KPB), pH 6.0. 220 mg of CWSP was loaded in each batch and eluted by use of a step gradient of water, 0.1 M KPB, 0.25 M KPB, 0.5 M KPB and 1 M KPB at pH 6.0. 85 fractions were collected per gradient step with a fraction size of 10 mL. 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.

Samples	Extraction Conditions	Yields (%)
CWSP	polysaccharide isolated by cold water extraction at 12 °C	0.98
HWSP	polysaccharide isolated by hot water extraction at 90–95 °C	0.55
ASP	polysaccharide isolated by alkali extraction (0.25 M NaOH) at 15 °C	0.70

(Monsigny, Petit, & Roche, 1988). Carbohydrate-containing fractions were pooled and dialyzed (SpectraPor® cellulose membranes MWCO 3500 Da, Roth, Germany) for 72 h 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 270 kDa, 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 788 kDa).

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 ($c = 0.1$ – 0.5 mg/mL) were taken in 96-well plate followed by addition of 20 µL 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 $\lambda = 595$ 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 (2 M TFA) at 120 °C for 2 h, followed by reduction with 0.25 M NaBD₄ at 60 °C for 2 h, 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 (t_R) 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 2–3 mg polysaccharide was dissolved in 100 µL anhydrous DMSO. After complete solubilization, 20 µL of freshly prepared potassium-dimsyl reagent 3 mol/L was added under argon atmosphere and continuous stirring followed by addition of 5 µL of ice cooled methyl iodide-*d*₃. These steps were repeated four times.

Finally, 60 µL of 3 M potassium-dimsyl reagent was added and reaction mixture was vortexed; then 15 µL CD₃I was further added and the reaction mixture was vigorously stirred for 30 min at 25 °C. Subsequently, 3 mL 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 2 mL water. The reaction mixture was centrifuged (1500 × g, 2 min) to facilitate the phase separation. The upper layer was discarded and the lower layer was washed five times with 2 mL water each time. Finally the upper water phase was removed completely. The remaining moisture in DCM phase was eliminated by adding 2 mL of 2,2-dimethoxypropane and 20 µL 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, Rinken, & Mischnick, 2012). The etherified polysaccharide sample was hydrolyzed with TFA (2 mol/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

1–2 mg of polysaccharide was subjected to partial hydrolysis with 600 µL of 0.5 M HCl at 100 °C for 20 min. 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

22–25 mg isolated polysaccharide was dissolved in 0.75 mL 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 1 s and spectral width of 9615.38 Hz. For ¹³C NMR acquisition, spectrometer frequency at field strength 150 MHz was 150.82 MHz; 5000 scans were taken with acquisition time 0.86 s, relaxation delay of 1 s 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–RI technique with a polydispersity index of 1.5 and a mean molecular weight was of 1.64×10^5 Da 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 6.3:1.0:0.9:0.1.

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 $-\text{OCH}_3$ groups and distinguish them from $-\text{OCD}_3$ groups by the characteristic mass shift of 3 amu in mass spectrometry. The deuterio-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) occurred for the same fragmenta-

tion with mass shift of +3 amu 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 $-\text{OCH}_3$ group was present natively at C3 in hexose, *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 $-\text{OCH}_3\text{-d}_3$ at position 3 of hexose (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-Me-galactose. 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 $\rightarrow$ 201 showed the presence of acetyl groups at 1,4,5 and 6 positions and $-\text{OCH}_3$ 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 $[\text{M} + \text{Na}]^+$ and $[\text{M} + \text{K}]^+$ 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,

Fig. 1. HPSEC-RI chromatogram of TW1; Molar mass range is calibrated with pullulan standards.

Table 2
Deduced Carbohydrate linkages in PMAA of TW1.

t_R (min)	PMAA	Constituents (Deduced linkage)	Fragmentation (primary fragment ions, m/z)
17.63	3-O-Me-2-O-Me- d_3 -arabinitol-1,4,5-triacetate	1,5-linked 3-O-Me-Ara	121; 165 $\rightarrow$ 105; 189 $\rightarrow$ 129
20.09	2,3,4,6-tetra-O-Me- d_3 -galactitol-1,5-diacetate	<i>t</i> -Gal	121; 168 $\rightarrow$ 108; 167 $\rightarrow$ 107, 132; 214 $\rightarrow$ 154
	3-O-Me-2,4,6-tri-O-Me- d_3 -galactitol-1,5-diacetate	<i>t</i> -3-O-Me-Gal	121; 165 $\rightarrow$ 105; 167 $\rightarrow$ 107, 132; 211 $\rightarrow$ 151
23.51	2,3,6-tri-O-Me- d_3 -galactitol-1,4,5-triacetate	1,4-linked Gal	121; 168 $\rightarrow$ 108; 239 $\rightarrow$ 179; 48
	3-O-Me-2,6-di-O-Me- d_3 -galactitol-1,4,5-triacetate	1,4-linked 3-O-Me-Gal	121; 165 $\rightarrow$ 105; 236 $\rightarrow$ 176; 48
28.68	3-O-Me-2-O-Me- d_3 -galactitol-1,4,5,6-tetraacetate	1,4,6-linked 3-O-Me-Gal	121; 261 $\rightarrow$ 201

Fig. 2. EI-MS fragmentation of 2,3,4,6-O-Me-*d*₃-galactitol-1,5-di-O-acetate and 3-O-Me-2,4,6-O-Me-*d*₃-galactitol-1,5-di-O-acetate.

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), ^{0,3}A₂ 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 ^{0,3}A₂ 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), ^{0,3}A₂ ring cleavage would occur to give a fragment ion at *m/z* 275 [M – 104 + Na]⁺, while ^{0,2}X₁ 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 (^{0,2}X₁) 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 (^{0,2}A₂), 90 (^{0,3}A₂) and 120 (^{2,4}A₂) 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

Fig. 3. ESI-MS spectrum (positive mode) of oligosaccharide mixture obtained from partial hydrolysis of TW1; initials used for 3-O-Me-Galp, Galp and 3-O-Me-Araf are MG, G and MA, respectively; colors signify the oligosaccharides of different compositions.

Table 3a

Complete assignment of m/z signals in ESI-MS spectrum of TW1; (MG = 3-O-Me-Galp, G = Galp, MA = 3-O-Me-Araf).

m/z Signals	Degree of Polymerization (DP)	Constituent Monomers	Complexed Ion
363	2	MA, MG	Na
377	2	[MA, MG]-OMe	Na
379	2	G, MG	Na
393	2	MG ₂	Na
407	2	[MG ₂]-OMe	Na
409	2	MG ₂	K
423	2	[MG ₂]-OMe	K
509	3	MA ₂ , MG	Na
523	3	[MA ₂ , MG]-OMe	Na
525	3	MA, G, MG	Na
539	3	MA, MG ₂	Na
541	3	MA, G, MG	K
553	3	[MA, MG ₂]-OMe	Na
555	3	G, MG ₂	Na
569	3	MG ₃	Na
571	3	G, MG ₂	K
583	3	[MG ₃]-OMe	Na
585	3	MG ₃	K
599	3	[MG ₃]-OMe	K
671	4	MA ₂ , G, MG	Na
685	4	MA ₂ , MG ₂	Na
687	4	MA ₂ , G, MG	K
701	4	MA, G, MG ₂	Na
715	4	MA, MG ₃	Na
717	4	MA, G, MG ₂	K
729	4	[MA, MG ₃]-OMe	Na
731	4	G, MG ₃	Na
745	4	MG ₄	Na
747	4	G, MG ₃	K
759	4	[MG ₄]-OMe	Na
761	4	MG ₄	K
775	4	[MG ₄]-OMe	K
831	5	MA ₃ , MG ₂	Na
847	5	MA ₂ , G, MG ₂	Na
861	5	[MA ₂ , G, MG ₂]-OMe	Na
863	5	MA ₂ , G, MG ₂	K
877	5	MA, G, MG ₃	Na
891	5	[MA, G, MG ₃]-OMe	Na
893	5	MA, G, MG ₃	K
907	5	G, MG ₄	Na
921	5	MG ₅	Na
923	5	G, MG ₄	K
937	5	MG ₅	K
951	5	[MG ₅]-OMe	K

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 δ_C and δ_H 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 anomeric 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 $\rightarrow$ 4)-3-O-Me- β -Galp-(1 $\rightarrow$ 3-O-Me- β -Galp-(1 $\rightarrow$ and $\rightarrow$ 4)- β -Galp-(1 $\rightarrow$ (Habibi, Mahrouz, & Vignon, 2005). The signals at δ 4.98, 5.06, 5.09 and 5.23 were correlated to the anomeric protons of $\rightarrow$ 4)-3-O-Me- α -Galp-(1 $\rightarrow$ (He et al., 2016), $\rightarrow$ 5)-3-O-Me- α -Araf-(1 $\rightarrow$ (Habibi et al., 2005), α -Rhap-(1 $\rightarrow$ and 3-O-Me- α -Araf-(1 $\rightarrow$ (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 indi-

cated 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 $\rightarrow$ 5)-3-O-Me- α -Araf-(1 $\rightarrow$ and 3-O-Me- α -Araf-(1 $\rightarrow$ (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 $\rightarrow$ 4)-3-O-Me- β -Galp-(1 $\rightarrow$ and $\rightarrow$ 4)- β -Galp-(1 $\rightarrow$ (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 $\rightarrow$ (Kang et al., 2011) and the latter interpreted as C1 of $\rightarrow$ 4)-3-O-Me- α -Galp-(1 $\rightarrow$ (He et al., 2016). The two peaks had a very low intensity owing to the low content of Rhap and 3-O-Me- α -Galp 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 δ_C and δ_H values assigned for $\rightarrow$ 4)- β -Galp-(1 $\rightarrow$ 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 $\rightarrow$ 5)-3-O-Me- α -Araf-(1 $\rightarrow$, it has been observed from the respective *in silico* simulations that —OCH₃ group at C3 affect the chemical shift values δ_C and δ_H up to three bonds, which implies that δ_H and δ_C values for C1/H1 and C5/H5 would remain same as for $\rightarrow$ 5)- α -Araf-(1 $\rightarrow$; 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 δ_C/δ_H for C3/H3 was assigned to be at δ 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 δ_C value shift downfield with a $\Delta\delta_C$ of 3.77 ppm while δ_H shifts upfield with a slight decrease of 0.255 ppm. Hence the values of C3/H3 at δ 83.53/4.08 for $\rightarrow$ 5)-3-O-Me- α -Araf-(1 $\rightarrow$ were justified with respect to the C3/H3 δ values 78.53/4.11 for the non-methylated counterpart (Habibi et al., 2005). In Table 4a, the signals for $\rightarrow$ 4)-3-O-Me- β -Galp-(1 $\rightarrow$ were assigned by using δ_C/δ_H values obtained for $\rightarrow$ 4)- β -Galp-(1 $\rightarrow$ as reference. Since $\rightarrow$ 4)-3-O-Me- β -Galp-(1 $\rightarrow$ 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 $\rightarrow$ 4)- β -Galp-(1 $\rightarrow$, since they are not affected by the substitution at C3. The C2/H2 δ_C/δ_H values were close in the two residues [$\rightarrow$ 4)- β -Galp-(1 $\rightarrow$ and $\rightarrow$ 4)-3-O-Me- β -Galp-(1 $\rightarrow$] with an upfield shift of 0.05 ppm in $\rightarrow$ 4)-3-O-Me- β -Galp-(1 $\rightarrow$ in ¹H NMR. The values δ_C/δ_H observed at 85.30/3.40 have been assigned to C3/H3 of $\rightarrow$ 4)-3-O-Me- β -Galp-(1 $\rightarrow$ owing to the downfield effect due to —OCH₃ group. When —H linked to C3 is replaced by —CH₃, δ_C value is shifted downfield with a $\Delta\delta_C$ of 9.3–9.45 ppm while δ_H 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 δ_C value and a slight increase in δ_H value. Conversely, both δ_C and δ_H 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.

Fig. 4. (a) MS² Fragmentation of m/z signal 363; Stereochemistry is not considered in this fragmentation scheme. (b) MS² Fragmentation of m/z signal 379; Stereochemistry is not considered in this fragmentation scheme.

Table 3b

Fragmentation for disaccharides of m/z 363 and 379.

m/z signal Fragmented [M + Na]	Constituent monomers	Sequence 1	Y1/B1	Ring Cleavage	Sequence 2	Y1/B1	Ring Cleavage
363	MG, MA	MA-MG	217/169	259 (^{0,3} A ₂)	MG-MA	187/199	259 (^{0,3} A ₂)
379	MG, G	G-MG	217/185	275 (^{0,3} A ₂) 259 (^{0,2} X ₁)	MG-G	203/199	319 (^{0,2} A ₂) 289 (^{0,3} A ₂) 259 (^{2,4} A ₂)

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).

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 1.64×10^5 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) ^1H NMR spectrum of TW1 (recorded in D_2O (99.95%) at 304K; HDO peak at 4.76 ppm was taken as reference). (b) ^{13}C NMR spectrum of TW1 (recorded in D_2O (99.95%) at 304K). (c) HSQC NMR spectrum of TW1 (recorded in D_2O (99.95%) at 304K; HDO peak at 4.76 ppm was taken as reference).

Table 4a¹H NMR and ¹³C NMR chemical shifts (δ, ppm) for →4)-3-O-Me-β-Galp-(1→ residue in TW1.

	Chemical shifts found by HSQC	Chemical shifts found by HSQC	Habibi et al. (2005)	Shakhmatov et al. (2016)	Mandal et al. (2015)	Simulated <i>in silico</i> chemical shifts ^a
Site	→4)-3-O-Me-β-Galp-(1→	→4)-β-Galp-(1→	→4)-β-Galp-(1→	→4)-β-Galp-(1→	3-O-Me-β-Galp-(1→	→4)-3-O-Me-β-Galp-(1→
C1/H1	105.92/4.57	105.92/4.57	105.21/4.57	107.3/4.65	103.3/4.42	102.29/4.95
C2/H2	72.94/3.66	72.72/3.71	72.70/3.48	75.3/3.68	70.8/3.50	69.25/3.29
C3/H3	85.30/3.40	–	74.36/3.70	76.2/3.76	81.4/4.09	82.6/3.28
C4/H4	73.76/4.44	78.60/4.07	78.53/4.11	80.5/4.17	68.0/3.89	72.1/3.42
C5/H5	77.12/3.64	77.12/3.64	75.30/3.65	77.6/3.72	76.6/3.98	72.1/3.52
C6/H6a, H6b	63.67/3.67, 3.67	63.67/3.76, 3.76	61.94/3.80, 3.80	63.6/3.82, 3.79	–	61.24/3.89
OCH ₃	59.88/3.45	–	–	–	59.9/3.45	59.48/3.22

^a 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 4b¹H NMR and ¹³C NMR chemical shifts (δ, ppm) for →5)-3-O-Me-α-Araf-(1→ residue in TW1.

	Chemical shifts found by HSQC	Dourado et al. (2006)	Habibi et al. (2005)	Simulated <i>in silico</i> chemical shifts ^a
Site	→5)-3-O-Me-α-Araf-(1→	→5)-α-Araf-(1→	→5)-α-Araf-(1→	→5)-3-O-Me-α-Araf-(1→
C1/H1	110.00/5.04	110.4/5.10	108.40/5.03	109.02/4.64
C2/H2	–	84.1/4.15	82.40/4.08	81.00/4.08
C3/H3	83.53/4.08	79.4/4.05	78.53/4.11	80.77/3.53
C4/H4	83.82/4.16	85.1/4.22	83.20/4.16	82.59/3.78
C5/H5a, H5b	68.14/3.76, 3.76	69.1/3.91–3.89, 3.83–3.81	68.22/3.81	66.69/4.14
OCH ₃	59.03/3.42	–	–	57.40/3.25

^a 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.

comprising 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 pectin.

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); *Macrolepiota dolichaula* (Samanta et al., 2015)]; bacteria [*Klebsiella pneumoniae* (Mandal et al., 2015)], algae [*Grifola frondosa* (Cui et al., 2013; Oliveira et al., 2018); *Gracilaria pudumadamensis* (Kondaveeti, Kumar, Ganesan, & Siddhanta, 2014); *Apophlaea alyallii* (Watt, O'Neill, Percy, & Brasch, 2002)], and plants [*Selaginella apoda* (Popper, Sadler, & Fry, 2001); *Castanea sativa* Mill and *Citrus aurantiifolia* (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 physicochemical properties and potential biological activities.

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References

- Aspinall, G.O., 1983. The polysaccharides Vol. II, Academic Press, New York, pp 126.
- Bacon, J.S.D., Cheshire, M.V., 1971. Apiose and mono-O-methyl sugars as minor constituents of the leaves of deciduous trees and various other species. *Biochemical Journal* 124 (3), 555–562.
- Bao, Y., Zhao, B., Bu, R., 2001. A herbalogical study on traditional Mongolian medicine lideri. *Journal of Chinese Medicinal Materials* 24 (11), 823–825.
- Barsett, H., Paulsen, B.S., 1992. Separation, isolation and characterization of acidic polysaccharides from the inner bark of *Ulmus glabra* Huds. *Carbohydrate Polymers* 17 (2), 137–144.
- Bhattara, N.K., Karki, M., Tandon, V., 2002. Report of the CAMP workshop in Nepal. *Medicinal Plant Conservation* 8, 28–30.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72 (1–2), 248–254.
- Brito, D.R., Carbonero, E.R., Viana, S.R., Silva, E.V., Ruthes, A.C., Lião, L.M., & Iacomini, M. (2018). Partially methylated galactans containing different proportions of 3-O-methyl-galactose from *Pleurotus citrinopileatus*. *Carbohydrate Research*, accepted manuscript, doi: 10.1016/j.carres.2018.02.002.
- Carbonero, E.R., Gracher, A.H.P., Rosa, M.C.C., Torri, G., Sasaki, G.L., Gorin, P.A.J., et al., 2008. Unusual partially 3-O-methylated α -galactan from mushrooms of the genus *Pleurotus*. *Phytochemistry* 69 (1), 252–257.
- Chi, S., She, G., Han, D., Wang, W., Liu, Z., Liu, B., 2016. Genus *Tinospora*: ethnopharmacology, phytochemistry, and pharmacology. *Evidence-Based Complementary and Alternative Medicine* 2016, 1–32.
- Chintalwar, G., Jain, A., Sipahimalani, A., Banerji, A., Sumariwalla, P., Ramakrishnan, R., et al., 1999. An immunologically active arabinogalactan from *Tinospora cordifolia*. *Phytochemistry* 52 (6), 1089–1093.
- Cui, F., Zan, X., Li, Y., Yang, Y., Sun, W., Zhou, Q., et al., 2013. Purification and partial characterization of a novel anti-tumor glycoprotein from cultured mycelia of *Grifola frondosa*. *International Journal of Biological Macromolecules* 62, 684–690.
- Desai, V.R., Ramkrishnan, R., Chintalwar, G.J., Sainis, K.B., 2007. G1-4A, an immunomodulatory polysaccharide from *Tinospora cordifolia*, modulates macrophage responses and protects mice against lipopolysaccharide induced endotoxic shock. *International Immunopharmacology* 7 (10), 1375–1386.
- Ding, H.H., Cui, S.W., Goff, H.D., Chen, J., Wang, Q., Han, N.F., 2015. Arabinan-rich rhamnogalacturonan-I from flaxseed kernel cell wall. *Food Hydrocolloids* 47, 158–167.
- Do, N.L., Le, T.X., Nguyen, T.T.N., Nguyen, T.T.Q., 2007. Phytochemical composition *Tinospora sinensis* (Lour) Merr. and its hypoglycemic effects on mice with streptozotocin-induced diabetes. *Tap Chi Duoc Hoc* 47, 8–11.
- Dong, L.P., Chen, C.X., Ni, W., Xie, B.B., Li, J.Z., Liu, H.Y., 2010. A new dinorclerone diterpenoid glycoside from *Tinospora sinensis*. *Natural Product Research* 24 (1), 13–17.
- Dourado, F., Cardoso, S.M., Silva, A.M., Gama, F.M., Coimbra, M.A., 2006. NMR structural elucidation of the arabinan from *Prunus dulcis* immunobiological active pectic polysaccharides. *Carbohydrate Polymers* 66 (1), 27–33.
- Fan, J., Li, P. (2007). Lotion containing traditional chinese medicine for treating rheumatism and rheumatoid arthritis. *Faming Zhuanli Shenqing Gongkai Shuomingshu*. Chinese Patent Application: CN 2005-10040926 20050705.
- Fuhrman, B., Volkova, N., Rosenblat, M., 2000. Mechanism of action of antioxidants. *Antioxidants & Redox Signalling* 2, 491–496.
- Goel, H.C., Kumar, I.P., Rana, S.V.S., 2002. Free radical scavenging and metal chelation by *Tinospora cordifolia*, a possible role in radioprotection. *Indian Journal of Experimental Biology* 40, 727–734.
- Habibi, Y., Mahrouz, M., Vignon, M.R., 2005. Arabinan-rich polysaccharides isolated and characterized from the endosperm of the seed of *Opuntia ficus-indica* prickly pear fruits. *Carbohydrate Polymers* 60 (3), 319–329.
- Haque, A., Jantan, I., Bukhari, S.N.A., 2017. *Tinospora* species: An overview of their modulating effects on the immune system. *Journal of Ethnopharmacology* 207, 67–85.
- Harris, P.J., Henry, R.J., Blakeney, A.B., Stone, B.A., 1984. An improved procedure for the methylation analysis of oligosaccharides and polysaccharides. *Carbohydrate Research* 127 (1), 59–73.
- He, P., Zhang, A., Zhou, S., Zhang, F., Linhardt, R.J., Sun, P., 2016. Structural elucidation of polysaccharide containing 3-O-methyl galactose from fruiting bodies of *Pleurotus citrinopileatus*. *Carbohydrate Research* 434, 72–76.
- He, L. (2009). Orthopedic chinese medicine plaster and preparation method thereof. *Faming Zhuanli Shenqing*. Chinese Patent Application: CN 2009-10015741 20090601.
- Herrmann, A., König, S., Lechtenberg, M., Sehlbach, M., Vakhrushev, S.Y., Peter-Katalin, J., et al., 2012. Proteoglycans from *Boswellia serrata* Roxb. and *B. carteri* Birdw. and identification of a proteolytic plant basic secretory protein. *Glycobiology* 22 (11), 1424–1439.
- Kang, J., Cui, S.W., Phillips, G.O., Chen, J., Guo, Q., Wang, Q., 2011. New studies on gum ghatti (*Anogeissus latifolia*) part II. Structure characterization of an arabinogalactan from the gum by 1D, 2D NMR spectroscopy and methylation analysis. *Food Hydrocolloids* 25 (8), 1991–1998.
- Kondaveeti, S., Kumar, S., Ganesan, M.S., Siddhanta, A.K., 2014. Galactans of *Gracilaria pudumadamensis* (Gracilariiales, rhodophyta) of indian waters. *Natural Product Communications* 9 (9), 1341–1344.
- Kumar, V., Nagar, S., 2014. Studies on *Tinospora cordifolia* monosugars and correlation analysis of uronic acids by spectrophotometric methods and GLC. *Carbohydrate Polymers* 99, 291–296.
- Leivas, C.L., Iacomini, M., Cordeiro, L.M., 2015. Structural characterization of a rhamnogalacturonan 1-arabinan-type 1 arabinogalactan macromolecule from starfruit (*Averrhoa carambola* L.). *Carbohydrate Polymers* 121, 224–230.
- Li, R.W., Lin, G.D., Myers, S.P., Leach, D.N., 2003. Anti-inflammatory activity of Chinese medicinal vine plants. *Journal of Ethnopharmacology* 85 (1), 61–67.
- Li, W., Koike, K., Liu, L., Lin, L., Fu, X., Chen, Y., et al., 2004. New lignan glucosides from the stems of *Tinospora sinensis*. *Chemical and Pharmaceutical Bulletin* 52 (5), 638–640.
- Li, W., Wei, K., Fu, H., Koike, K., 2007. Structure and absolute configuration of clerodane diterpene glycosides and a rearranged cadinane sesquiterpene glycoside from the stems of *Tinospora sinensis*. *Journal of Natural Products* 70 (12), 1971–1976.
- Ma, J. (2010). Cosmetic composition containing oxytropis falcate bunge, Yadahuang and vitamin e for repairing skin injury. *Faming Zhuanli Shenqing Application: Chinese Patent* CN 2010-10251240 20100810.
- Mandal, A.K., Sen, I.K., Maity, P., Chattopadhyay, S., Chakraborty, R., Roy, S., et al., 2015. Structural elucidation and biological studies of a novel exopolysaccharide from *Klebsiella pneumoniae* PB12. *International Journal of Biological Macromolecules* 79, 413–422.
- Manjrekar, P.N., Jolly, C.I., Narayanan, S., 2000. Comparative studies of the immunomodulatory activity of *Tinospora cordifolia* and *Tinospora sinensis*. *Fitoterapia* 71 (3), 254–257.
- Maurya, R., Gupta, P., Chand, K., Kumar, M., Dixit, P., Singh, N., et al., 2009. Constituents of *Tinospora sinensis* and their antileishmanial activity against *Leishmania donovani*. *Natural Product Research* 23 (12), 1134–1143.
- Mischnick, P., 2011. Mass spectrometric characterization of oligo- and polysaccharides and their derivatives. *Mass spectrometry of polymers—new techniques*. Springer, Berlin Heidelberg, 105–174.
- Monsigny, M., Petit, C., Roche, A.C., 1988. Colorimetric determination of neutral sugars by a resorcinol sulfuric acid micromethod. *Analytical Biochemistry* 175 (2), 525–530.
- Nair, P.R., Rodriguez, S., Ramachandran, R., Alamo, A., Melnick, S.J., Escalon, E., et al., 2004. Immune stimulating properties of a novel polysaccharide from the medicinal plant *Tinospora cordifolia*. *International Immunopharmacology* 4 (13), 1645–1659.
- Nair, P.K.R., Melnick, S.J., Ramachandran, C. (2006). *Tinospora* polysaccharide as immunostimulant for treatment of proliferative diseases or infection. *US Patent Application: 2006/0009501 A1*, 2006.
- Nima, C. (2010). Tibetan medicinal composition for treating diabetes and its preparation method. *Faming Zhuanli Shenqing*. Chinese Patent Application: CN 2009-10216182 20091110, 2010.
- Oliveira, G.K.F., Da Silva, E.V., Ruthes, A.C., Lião, L.M., Iacomini, M., Carbonero, E.R., 2018. Chemical structure of a partially 3-O-methylated mannogalacturonan from edible mushroom *Grifola frondosa*. *Carbohydrate Polymers* 187, 110–117.
- Popper, Z.A., Sadler, I.H., Fry, S.C., 2001. 3-O-Methyl-D-galactose residues in lycophyte primary cell walls. *Phytochemistry* 57 (5), 711–719.
- Raghu, R., Sharma, D., Ramakrishnan, R., Khanam, S., Chintalwar, G.J., Sainis, K.B., 2009. Molecular events in the activation of B cells and macrophages by a non-microbial TLR4 agonist, G1-4A from *Tinospora cordifolia*. *Immunology Letters* 123 (1), 60–71.
- Ren, Y., Tang, Q., Zhang, Z., Chen, L., He, H., Hao, X., 2008. Chemical constituents from *Tinospora sinensis*. *Tianran Chanwu Yanjiu Yu Kaifa* 20 (278–279), 282.
- Samanta, S., Nandi, A.K., Sen, I.K., Maity, P., Pattanayak, M., Devi, K.S.P., et al., 2015. Studies on antioxidative and immunostimulating fucogalactan of the edible mushroom *Macropleota dolichaula*. *Carbohydrate Research* 413, 22–29.
- Shakhmatov, E.G., Atukmaev, K.V., Makarova, E.N., 2016. Structural characteristics of pectic polysaccharides and arabinogalactan proteins from *Heracleum sosnowskyi* Manden. *Carbohydrate Polymers* 136, 1358–1369.
- Singh, N., Kumar, A., Gupta, P., Chand, K., Samant, M., Maurya, R., et al., 2008. Evaluation of antileishmanial potential of *Tinospora sinensis* against experimental visceral leishmaniasis. *Parasitology Research* 102 (3), 561–565.
- Springer, G.F., Takahashi, T., Desai, P.R., Kolecki, B.J., 1965. Cross reactive human blood-group H (O) specific polysaccharide from *Sassafras albidum* and characterization of its hapten. *Biochemistry* 4 (10), 2099–2113.
- Srinivasan, G.V., Unnikrishnan, K.P., Shree, A.R., Balachandran, I., 2008. HPLC estimation of berberine in *Tinospora cordifolia* and *Tinospora sinensis*. *Indian Journal of Pharmaceutical Sciences* 70 (1), 96–99.
- Subramanian, M., Chintalwar, G.J., Chattopadhyay, S., 2002. Antioxidant properties of a *Tinospora cordifolia* polysaccharide against iron-mediated lipid damage and γ -ray induced protein damage. *Redox Report* 7 (3), 137–143.
- Subramanian, M., Chintalwar, G.J., Chattopadhyay, S., 2003. Radioprotective property of polysaccharide in *Tinospora cordifolia*. *Indian Journal of Biochemistry and Biophysics* 40, 22–26.
- Voiges, K., Adden, R., Rinken, M., Mischnick, P., 2012. Critical re-investigation of the alditol acetate method for analysis of substituent distribution in methyl cellulose. *Cellulose* 19 (3), 993–1004.

- Watt, D.K., O'Neill, S.A., Percy, A.E., Brasch, D.J., 2002. Isolation and characterisation of a partially methylated galacto-glucurono-xylo-glycan, a unique polysaccharide from the red seaweed *Apophloeoea lyallii*. *Carbohydrate Polymers* 50 (3), 283–294.
- Wende, F.J., Gohil, S., Mojarradi, H., Gerfaud, T., Nord, L.I., Karlsson, A., et al., 2016. Determination of substitution positions in hyaluronic acid hydrogels using NMR and MS based methods. *Carbohydrate Polymers* 136, 1348–1357.
- Xu, F., Yu-Kui, R., Wei, T., 2010. Monitoring of nine trace elements in Chinese herbal medicine *Tinospora sinensis* (Lour.) Merr by ICP-MS. *Asian Journal of Chemistry* 22 (9), 7429–7431.
- Yang, Y., Zhang, A.Q., Zhang, J.S., 2007. Identification of 3-O-methyl galactose in polysaccharide isolated from *Phellinus igniarius*. *Chinese Journal of Pharmaceutical Analysis* 27 (7), 1007–1010.
- Ye, Y., & Liao, Z.A. (2005). Chinese medicinal composition for treating rheumatic arthralgia, rheumatism and joint pain, and preparation method thereof. Faming Zhuanli Shenqing. Chinese Patent Application: CN 2005-10034580 20050509.
- Yonemitsu, M., Fukuda, N., Kimura, T., 1993. Studies on the constituents of *Tinospora sinensis*; I. Separation and structure of the new phenolic glycoside tinosinen. *Planta Medica* 59 (06), 552–553.
- Yonemitsu, M., Fukuda, N., Kimura, T., Isobe, R., Komori, T., 1995. Studies on the constituents of the stems of *Tinospora sinensis*, II. Isolation and structure elucidation of two new dinorditerpene glucosides, tinosinides A and B. *European Journal of Organic Chemistry* 1995 (2), 437–439.