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A new arabinoxylan from green leaves of *Litsea glutinosa* (Lauraeae): Structural and biological studies

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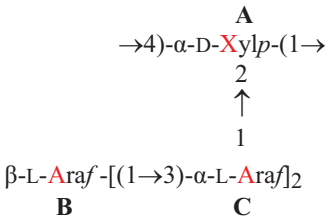
ABSTRACT

A water soluble new arabinoxylan, isolated through hot water extraction from the green leaves of *Litsea glutinosa* (Lauraceae) was found to contain xylose and arabinose in a molar ratio of nearly 1:3. On the basis of NMR (^{1}H, ^{13}C, DQF-COSY, TOCSY, NOESY, ROESY, HMBC and DEPT-135), GLC and GLC–MS analyses, the backbone was established as (1 → 4)-α-D-xylopyranosyl residue, substituted at C-2 with one unit of two adjacently linked (1 → 3)-α-L-arabinofuranosyl residues and the other one was terminated by β-L-arabinofuranosyl residue. The proposed repeating unit of the molecule was established as:



This molecule showed strong splenocyte, thymocyte, and macrophage activations. The optimum doses of the polysaccharide for splenocyte and thymocyte proliferation were observed at 25 μg/mL and 50 μg/mL, respectively. An enhanced production of NO was observed at 100 μg/mL of the polysaccharide.

1. Introduction

In recent years, the importance of polysaccharides has been increasingly recognized in the fields of therapeutics and nutrigenomics. Arabinoxylans, the non-starch polysaccharides in cereals (Izydorczyk & Biliaderis, 1995) exhibit a wide range of functional properties and health benefits. The various sources of arabinoxylans are wheat, rye, corn, barley, oat, rice, and sorghum (Fincher & Stone, 1986). Arabinoxylan isolated from cereals was found to stimulate bowel movement (Lu, Gibson, Muir, Fielding, & O'Dea, 2000a; Lu, Walker, Muir, Mascara, & O'Dea, 2000b), reduce blood sugar (Lu et al., 2000b) and inhibit elevated cholesterol (Lu, Walker, Muir, & O'Dea, 2004). Arabinoxylan (MGN-3) isolated from rice bran was found to sensitize human leukemic cells to death receptor (CD95)-induced apoptosis (Ghoneum & Gollapudi, 2003) and possesses the chemo-sensitizing activity against human breast cancer cells (Ghoneum & Gollapudi, 2008). It also acts as a potent activator of human NK cells (Ghoneum, 1998). A partially hydrolyzed arabinoxylan from corn husk showed the ability to increase immunopotentiating activity in mice (Ogawa, Takeuchi, & Nakamura, 2005). The feruloyl arabinoxylans from rice and ragi showed potent antioxidant activity (Rao & Muralikrishna, 2006). Since, the activity is directly related to the structure, characterization of diverse structure of arabinoxylans is important. The fine structure of arabinoxylans, like the branching at xylan backbone with arabinose residues is thought to bring about the variations in the structure activity relationship (Dervilly-Pinel, Tran, & Saulnier, 2004). In spite of the large amount of knowledge on the structure of arabinoxylans (Izydorczyk & Biliaderis, 1995), the characterization of arabinoxylans obtained from various sources still remains a matter of great interest for chemists and immunobiologists.

Litsea glutinosa (Lauraceae) is a medium-sized tree distributed throughout India. Its bark and leaves are used as a demulcent and mild astringent for diarrhea and dysentery due to balsamic and mucilaginous nature, and the paste of its roots is used as

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poultice in sprain and bruise (Li, 1982). The methanol extract of bark showed antibacterial activity against sixteen tested microorganisms, both gram-negative and gram-positive bacteria (Mandal, Kumar, Majumder, Majumder, & Maity, 2000). The mucilage, isolated from the leaves of *L. glutinosa* exhibit anti-diabetic (type II) and antioxidant property (Palanuvej, Hokputsa, Tunsaringkarn, & Ruangrungsi, 2009). In present study, a new arabinoxylan was isolated from hot water extract of the green leaves of *L. glutinosa* (*Lauraceae*) whose bark is also a source of the same (Herath, Kumar, & Wimalasiri, 1990). The present arabinoxylan reported herein is new since the xylose residues of the backbone here are α-anomers and the arabinose residues, responsible for branching are mixture of α- and β-anomers, which are different from the previously reported arabinoxylans isolated from various sources where the xylose and arabinose residues were found β- and α-anomers respectively with different types of branching. The structural characterization along with splenocyte, thymocyte and macrophage activation of the molecule, arabinoxylan were investigated and reported herein.

2. Materials and methods

2.1. Isolation and purification of polysaccharide

The green leaves (200 g) were collected and boiled with distilled water for 7 h. After filtration through linen cloth followed by centrifugation at 10,000 rpm for 40 min, the supernatant was precipitated in 1:5 (v/v) EtOH. Then the precipitated material was dissolved in water and dialyzed through cellulose membrane (Sigma–Aldrich, retaining $Mw > 12{,}400$) against distilled water followed by freeze-drying to yield 1 g crude polysaccharide, purified by gel permeation chromatography. 30 mg of the polysaccharide was dissolved in 2 mL water and passed through a column (90 cm × 2.1 cm) packed with Sepharose 6B at a flow rate of 12 drops/min. The eluant was collected in test tubes (2 mL each), fitted with a Redifrac fraction collector. 95 test tubes were collected and assayed aliquots of the fraction using the phenol–sulphuric colorimetric assay method (York, Darvill, McNeil, Stevenson, & Albersheim, 1985) and the absorbance was recorded at 490 nm by UV–vis spectrophotometer, model 1601. One fraction was collected and freeze dried yielding 22 mg pure polysaccharide (PS). The purification process was carried out in several times.

2.2. Sugar composition study

3 mg PS was hydrolyzed with 2 M CF_3COOH (2 mL) at 100 °C for 16 h in a boiling water bath and excess acid was completely removed by repeated co-distillation with water. Then, the hydrolyzed product was divided into two parts. One part was subjected to the paper chromatographic study. Another part was reduced with $NaBH_4$ (9 mg), followed by acidification with dilute CH_3COOH, and then co-distilled with pure CH_3OH to remove excess boric acid. The reduced sugars (alditol) were acetylated with 1:1 pyridine–Ac_2O (acetic anhydride) in a boiling water bath for 1 h to obtain the alditol acetates, which were analyzed by GLC and GLC–MS. Quantization was carried out from the peak area by response factors from standard monosaccharides using inositol as standard.

2.3. Methylation experiment

The methylation experiment was carried out by the procedure described by Ciucanu and Kerek (1984). The methylated product was isolated by making partition between $CHCl_3$ and water (5:2, v/v). The organic layer containing product was washed with water for several times and dried. Then, the methylated polysaccharide was hydrolyzed with 90% formic acid (1 mL) at 100 °C for 1 h, and excess formic acid was evaporated by co-distillation with distilled water. The hydrolyzed product was then reduced with sodium borohydride and acetylated with pyridine and Ac_2O to yield alditol acetates, analyzed by GLC and GLC–MS.

2.4. Periodate oxidation

The polysaccharide sample (4.5 mg) was added to 1.5 mL solution of Sodium meta-periodate (0.1 M) and the mixture was kept in dark for 48 h at room temperature (Goldstein, Hay, Lewis, & Smith, 1965). The excess periodate was destroyed by the addition of ethylene glycol, and the solution was dialyzed against distilled water for 4 h. The dialyzed material was reduced with $NaBH_4$ followed by acidification with AcOH (acetic acid). The resulting material was co-distilled with CH_3OH and then divided into two portions. One portion was hydrolyzed by 2 M CF_3COOH for 16 h, followed by alditol acetate preparation. Another portion was methylated using Ciucanu and Kerek (1984) method, followed by alditol acetate preparation and GLC and GLC–MS analyses.

2.5. General methods

Optical rotation was determined with a Jasco Polarimeter model P-1020 at 25 °C. The method of determination of absolute configuration of the monosaccharide constituents was based on Gerwig, Kamarling, and Vliegenthart (1978). Paper partition chromatographic studies (Hoffman, Lindberg, & Svensson, 1972) were performed on Whatmann nos. 1 and 3 mm sheets. Solvent systems used were: (X) BuOH–AcOH–H_2O (v/v/v, 4:1:5, upper phase) and (Y) EtOAc–pyridine–H_2O (v/v/v, 8:2:1). Alkaline silver nitrate solution was used as spray reagent. All GLC experiments were performed on a Hewlett-Packard Model 5730 A having a flame ionization detector and glass columns (1.8 m × 6 mm) packed with 3% ECNSS-M (A) on Gas Chrom Q (100–120 mesh) and 1% OV-225 (B) on Gas Chrom Q (100–120 mesh). The GLC analyses were performed at 170 °C. All GLC–MS experiments were carried out in a Hewlett-Packard 5970 MSD instrument using HP-5 fused silica capillary column. The program was isothermal at 150 °C; hold time 2 min, with a temperature gradient 4 °C/min up to a final temperature of 200 °C. The molecular weight of the polysaccharide was determined by a gel–chromatographic technique (Hara, Kiho, Tanaka, & Ukai, 1982). Standard dextrans T-200, T-70, and T-40 were passed through a Sepharose-6B column and then the elution volumes were plotted against the logarithms of their respective molecular weights. The elution volume of polysaccharide was then plotted on the same graph, and the average molecular weight of the PS was determined.

2.6. NMR studies

The ^1H and ^{13}C NMR spectra were recorded on Bruker Avance DPX 500 spectrometer at 27 °C using inverse probe. For NMR studies, polysaccharide was dried in vacuum over P_2O_5 for several days, and then exchanged with deuterium (Dueñas Chaso et al., 1997) by lyophilizing with D_2O for three times. The deuterium-exchanged polysaccharide (4 mg) was dissolved in 0.7 mL D_2O (99.96% atom 2H, Aldrich). The ^1H and ^{13}C (both ^1H coupled and decoupled) NMR spectra were recorded at 27 °C. Acetone was used as an internal standard (δ 31.05) for ^{13}C spectrum. The ^1H NMR spectrum was recorded suppressing HOD signal (fixed at δ 4.73) at 27 °C using the WEFT pulse sequence (Hård, Zadelhoff, Moonen, Kamerling, & Vliegenthart, 1992). 2D (DQF-COSY) NMR experiment was performed using standard Bruker software. The TOCSY experiment was recorded at mixing time of 150 ms, and complete assignment required several TOCSY experiments having mixing times ranging

from 60 to 300 ms. The NOESY and ROESY mixing delay were 300 ms. The delay time in the HMBC experiment was 80 ms.

2.7. Splenocyte and thymocyte proliferation assay

A single cell suspension of spleen and thymus were prepared from the normal mice under aseptic conditions by frosted slides in phosphate buffer solution (PBS). The suspension was centrifuged to obtain cell pellet. The contaminating RBC was removed by hemolytic Gey's solution. After washing two times in PBS the cells were resuspended in complete RPMI (Rose well Park Memorial Institute) medium. Cell concentration was adjusted to 1×10^5 cells/mL and viability of the suspended cells (as tested by trypan blue dye exclusion) was always over 90%. The cells (180 μL) were plated in 96-well flat-bottom plates and incubated with 20 μL of various concentrations (10–200 μg/mL) of the polysaccharide with lipopolysaccharide (LPS, positive control) of 4 μg/mL. Cultures were set-up for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. Proliferation was checked by MTT assay method (Ohno et al., 1993). Data are reported as the mean standard deviation of six different observations and compared against PBS control (Maiti et al., 2008; Sarangi, Ghosh, Bhutia, Mallick, & Maiti, 2006).

2.8. Assay for macrophage activity by NO (nitric oxide)

Peritoneal macrophages (5×10^5 cells/mL) after harvesting were cultured in complete RPMI (Roswell Park Memorial Institute) media in 96-well plates (Ohno, Hasimato, Adachi, & Yadomae, 1996; Sarangi et al., 2006). The purity of macrophages was tested by adherence to tissue culture plates. The polysaccharide was added to the wells in different concentrations. The cells were cultured for 24 h at 37 °C in a humidified 5% CO_2 incubator. Production of nitric oxide was estimated by measuring nitrite levels in cell supernatant with Greiss reaction (Green et al., 1982). Equal volumes of Greiss reagent (1:1 of 0.1% in 1-napthylethylenediamine in 5% phosphoric acid and 1% sulfanilamide in 5% phosphoric acid) and sample cell supernatant were incubated together at room temperature for 10 min. Absorbance was observed at 550 nm. Lipopolysaccharide (LPS), L6511 of Salmonella enteric serotype Typhimurium (sigma) (4 μg/mL) was used as positive control.

3. Results and discussion

3.1. Fractionation and physical characterization

The crude polysaccharide, isolated from green leaves of *L. glutinosa* through hot water extraction on fractionation through a Sepharose-6B column yielded one homogeneous fraction. The molecular mass of the pure polysaccharide (PS) was estimated as ~1,75,000 Da. The specific rotation was $[\alpha]_D^{25}$ + 36.5 (*c* 0.85, water).

3.2. Chemical analysis

The GLC analysis of alditol acetates of the hydrolyzed PS showed that pure polysaccharide contained xylose (~26%) and arabinose (~74%). The paper chromatographic studies of hydrolyzed PS also supported the presence of same sugar residues. The absolute configurations of sugar units were determined by the method of Gerwig et al. and it was found that xylose and arabinose residues contained D and L configuration respectively. The GLC and GLC–MS analyses of methylated PS revealed the presence of 3-Me-Xyl (~25%); 2,5-Me_2-Ara (~50%); and 2,3,5-Me_3-Ara (~25%). This results indicated that (1 → 2,4)-linked xylopyranosyl or (1 → 2,5)-linked xylofuranosyl, (1 → 3)-linked arabinofuranosyl and terminal arabinofuranosyl moieties were present in a molar ratio of nearly 1:3:1.

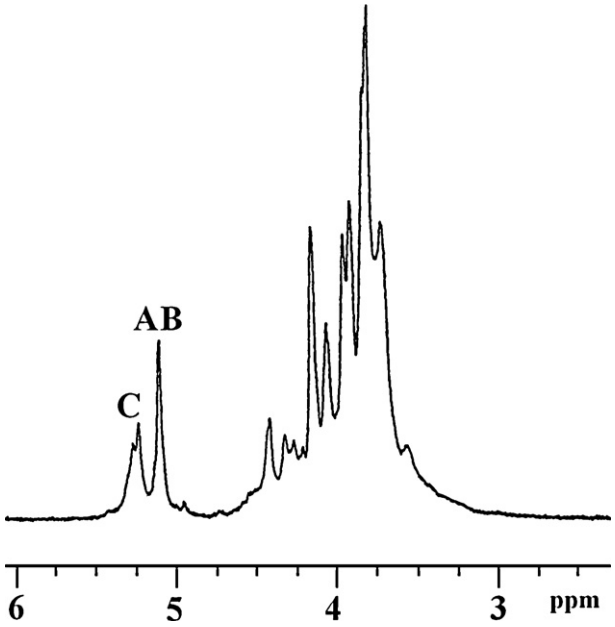


Fig. 1. ^{1}H NMR spectrum (500 MHz, D_2O, 27 °C) of polysaccharide, isolated from the green leaves of *Litsea glutinosa*.

The periodate-oxidized PS upon hydrolysis with 2M CF_3COOH followed by GLC analysis showed the presence of xylose and arabinose in a molar ratio of nearly 1:2. The GLC and GLC–MS analysis of periodate-oxidized, methylated PS revealed the presence of 3-Me-Xyl and 2,5-Me_2-Ara in a molar ratio of nearly 1:2. It was found that one arabinofuranosyl residue of every repeating unit was destroyed during oxidation which supported its presence as a terminal residue. These results also confirmed that one xylose and two arabinose residues were present as (1 → 2,4)-linked xylopyranosyl or (1 → 2,5)-linked xylofuranosyl, and (1 → 3)-linked arabinofuranosyl moiety respectively.

3.3. NMR and structural analysis

The 500 MHz ^{1}H NMR spectrum (Fig. 1) at 27 °C showed the resonances of four anomeric protons at δ 5.09, 5.23, and 5.25. The signal at δ 5.09 composed of two anomeric protons of two different sugar residues, designated as **A** and **B**. Whereas, each of the remaining two signals corresponded to one anomeric proton of same type of two sugar residues and they were collectively designated as **C**. The ^{13}C NMR spectrum (125 MHz, Fig. 2) at 27 °C showed three signals in anomeric region at δ 99.9, 102.2, and 109.6 which

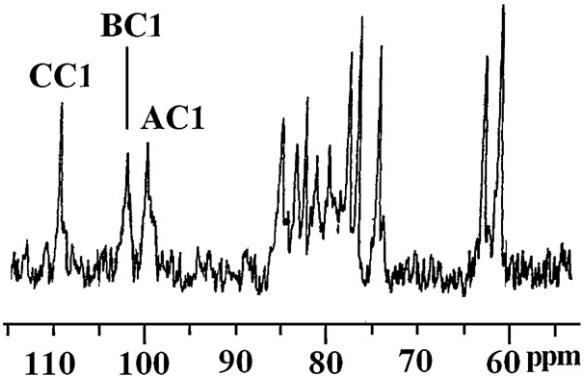


Fig. 2. ^{13}C NMR spectrum (125 MHz, D_2O, 27 °C) of polysaccharide, isolated from the green leaves of *Litsea glutinosa* in D_2O at 27 °C.

Table 1
The ^{1}H NMR[a] and ^{13}C NMR[b] chemical shifts for the polysaccharide isolated from the green leaves of *Litsea glutinosa* in D_2O at 27 °C.

Glycosyl residue	C-1/H-1	C-2/H-2	C-3/H-3	C-4/H-4	C-5/H-5a, H-5b
→2,4)-α-D-xyl*p*-(1→	99.9	81.2	76.7	77.8	61.6
A	5.09	3.72	4.05	3.96	3.75[c], 3.82[c]
β-L-ara*f*-(1→	102.2	77.1	74.5	82.4	63.3
B	5.09	4.14	4.05	3.86	3.67[c], 3.69[c]
→3)-α-L-ara*f*-(1→	109.6	80.6	83.4	85.0	61.6
C	5.23, 5.25	4.40	3.91	4.21	3.77[c], 3.84[c]

[a] Values of the ^{1}H chemical shifts were recorded with respect to the HOD signal fixed at δ 4.73 at 27 °C.
[b] Values of the ^{13}C chemical shifts were recorded with reference to acetone as the internal standard and fixed at δ 31.05 at 27 °C.
[c] Interchangable.

corresponded to anomeric carbons of **A**–**C**, respectively. All ^{1}H and ^{13}C signals (Table 1) were assigned using DQF-COSY, TOCSY, and HMQC experiments.

Residue **A** was assigned as (1 → 2,4)-α-D-xylopyranosyl residue. It was not (1 → 2,5)-linked xylofuranosyl moiety, which was confirmed from the absence downward and downfield signal with respect to δ 61.5 (standard value of C-5 of α-D-xylofuranose) in DEPT-135 spectrum. The coupling constants $^{3}J_{1,2}$ (~3.0 Hz) and $^{1}J_{C,H}$ (~171 Hz) indicated that it was α-anomer. The downfield shift of C-2 (δ 81.2) and C-4 (δ 77.8) of residue **A** with respect to the standard values of methyl glycoside (Agrawal, 1992; Rinaudo & Vincendon, 1982) indicated its (1 → 2,4)-liking.

In case of residue **B**, high chemical shifts of C-2 (δ 77.1), C-3 (δ 74.5) and C-4 (δ 82.4) indicated its furanose conformation. The C-1 signal at δ 102.2 indicated its β-configuration. The values at which all the carbon atoms of residue **B** resonated were more or less same with the standard values of β-L-arabinofuranosyl residue indicating its presence as terminal β-L-arabinofuranosyl moiety. It was supported by the coupling constants $^{3}J_{1,2}$ (~1.6 Hz), $^{3}J_{2,3}$ (~3.5 Hz), and $^{3}J_{3,4}$ (~6.0 Hz) (Hoffmann et al., 1992).

The remaining two residues, designated by **C** were assigned as (1 → 3)-α-L-Ara*f*. The downfield signal at δ 109.6, attributed to C-1 was indicative of α-linked furanosyl conformation. The coupling constant values, $^{3}J_{1,2}$ (~3.5 Hz), $^{3}J_{2,3}$ (~6.0 Hz), and $^{3}J_{3,4}$ (~7.5 Hz) also indicated their presence as reducing residues in furanosyl conformation. The coupling constant $^{1}J_{C,H}$ was found to be 168 Hz. The downfield shift of C-3 at δ 83.4 indicated that those were (1 → 3)-linked moieties.

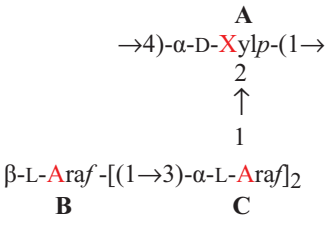
The sequence of sugar residues of the polysaccharide was determined from NOESY (figure not shown) and ROESY (Fig. 3 and Table 2) experiments. The ROE contacts AH-1 to AH-4, CH-1 to AH-2, CH-1 to CH-3, and BH-1 to CH-3 established the following sequences:

Table 2
ROE data for the polysaccharide isolated from the green leaves of *Litsea glutinosa*.

Glycosyl residue	Anomeric proton	ROE contact protons		
	δ	δ	Residue	Atom
→2,4)-α-D-xyl*p*-(1→	5.09	3.96	**A**	H-4
A		3.72	**A**	H-2
β-L-ara*f*-(1→	5.09	3.91	**C**	H-3
B		4.14	**B**	H-2
		3.86	**B**	H-4
→3)-α-L-ara*f*-(1→	5.23/5.25	3.72	**A**	H-2
C		3.91	**C**	H-3

A – (1 → 4) – **A**; **C** – (1 → 2) – **A**; **C** – (1 → 3) – **C**; **B** – (1 → 3) – **C**

The cross couplings, AH-1/AC-4, AC-1/AH-4, CH-1/AC-2, CC-1/AH-2, CH-1/CC-3, CC-1/CH-3, BH-1/CC-3, and BC-1/CH-3 in the HMBC spectrum (Fig. 4, Table 3) confirmed the above sequences. Hence, from NOESY, ROESY, and HMBC experiments the structure of the repeating unit of the polysaccharide was established as:



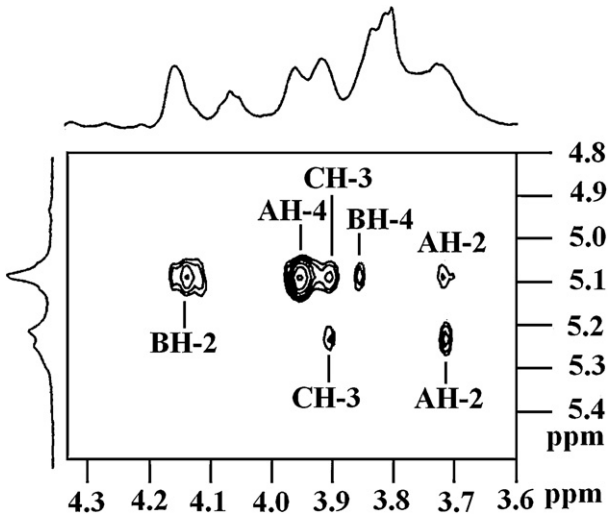


Fig. 3. Part of ROESY spectrum of polysaccharide, isolated from the green leaves of *Litsea glutinosa*. The ROESY mixing time was 300 ms.

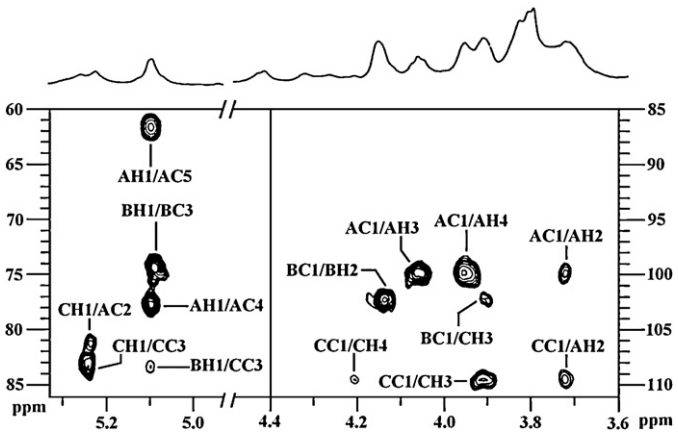


Fig. 4. The HMBC spectrum of polysaccharide, isolated from the green leaves of *Litsea glutinosa*. The delay time in the HMBC experiment was 80 ms.

Table 3
The significant $^3J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide isolated from the green leaves of *Litsea glutinosa*.

Residue	Sugar linkage	H-1/C-1	Observed connectivities		
		δ_H/δ_C	δ_H/δ_C	Residue	Atom
A	→2,4)-α-D-xyl*p*-(1→	5.09	77.8	**A**	C-4
			61.6	**A**	C-5
		99.9	3.96	**A**	H-4
			3.72	**A**	H-2
			4.05	**A**	H-3
B	β-L-ara*f*-(1→	5.09	83.4	**C**	C-3
			74.5	**B**	C-3
		102.2	3.91	**C**	H-3
			4.14	**B**	H-2
C	→3)-α-L-ara*f*-(1→	5.23/5.25	81.2	**A**	C-2
			83.4	**C**	C-3
		109.6	3.72	**A**	H-2
			3.91	**C**	H-3
			4.21	**C**	H-4

3.4. Biological studies

3.4.1. Assay for macrophage activity by NO

Macrophage activation of the polysaccharide was observed in vitro. On treatment with different concentrations of the polysaccharide an enhanced production of NO was observed (Fig. 5a) in a dose dependent manner with optimum production of 18.2 μM NO per 5×10^5 macrophages at 100 μg/mL of the polysaccharide. The various types of polysaccharides like lentinan inhibits tumor growth by stimulating the immune system (Chihara, 1978) through activation of macrophages, T-helper, NK, and other cells.

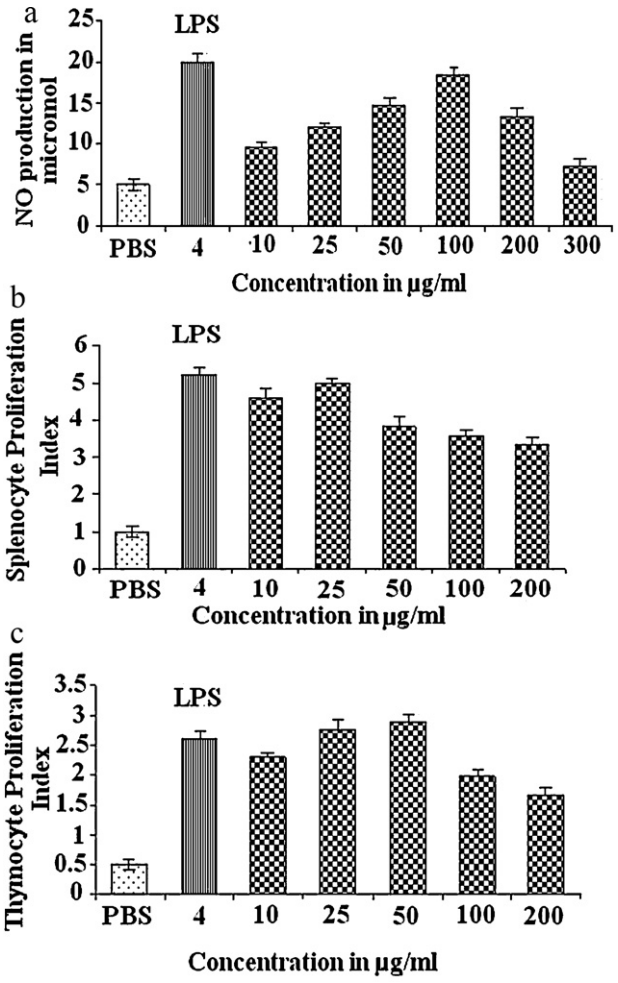


Fig. 5. (a) In vitro activation of peritoneal macrophage stimulated with different concentrations of polysaccharide in terms of NO production. Effect of different concentrations of polysaccharide on (b) splenocyte and (c) thymocyte proliferation.

3.4.2. Splenocyte and thymocyte proliferation assay

Proliferation of splenocyte and thymocyte is an indicator of immunoactivation. The splenocyte and thymocyte activation tests were carried out in mouse cell culture medium with the polysaccharide by the MTT assay method (Ohno et al., 1993). The polysaccharide was tested to stimulate the splenocytes and thymocytes and the results are shown in Fig. 5(b) and (c), respectively. The splenocyte and thymocyte proliferation index as compared to PBS control closer to 1 or below indicates low stimulatory effect on immune system. At 25 μg/mL of the polysaccharide, the splenocyte proliferation index (SPI) was observed maximum as compared to other concentrations. Again 50 μg/mL of that sample showed maximum effect on thymocyte proliferation. Hence, it can be concluded that the concentrations, 25 μg/mL and 50 μg/mL of the polysaccharide are the optimum concentrations for splenocyte and thymocyte proliferation respectively.

4. Conclusion

A water soluble new arabinoxylan was isolated from aqueous extract of the green leaves of *L. glutinosa* (Lauraceae) and purified by gel-filtration chromatography. The reported polysaccharide contained →4)-α-D-Xyl*p*-(1→ as a backbone, substituted at C-2 by β-L-Ara*f*-(1 → 3)-α-L-Ara*f*-(1 → 3)-α-L-Ara*f*-(1→. This molecule showed strong splenocyte, thymocyte, and macrophage activations. This arabinoxylan was new because the xylose residues of the backbone were α-anomers and the arabinose residues, responsible for branching at backbone were mixture of α- and β-anomers, whereas in case of all other arabinoxylans reported earlier, the xylose and arabinose residues were β- and α-anomer respectively with different types of branching.

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