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Characterization and renal protective effect of a polysaccharide from *Astragalus membranaceus*

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ABSTRACT

A water-soluble polysaccharide named as APS was isolated from the roots of *Astragalus membranaceus* by hot water extraction, anion-exchange and gel-permeation chromatography and tested for its renal protective effect. Its structural characteristics were investigated by FTIR, AMLC, NMR spectroscopy, GLC–MS, methylation analysis, periodate oxidation and Smith degradation. Based on the data obtained, APS was found to be an α-(1→4)-D-glucan, with a single α-D-glucose at the C-6 position every nine residue, on average, along the main chain. The glucan has a weight-average molecular weight of about 3.6×10^4 Da. 24 h Urine protein quantification and morphological observation revealed that APS significantly decreased the proteinuria and morphological changes on glomerulonephritis rats induced by cationic Bovine Serum Albumin (C-BSA), indicating APS could have a possible glomerulonephritis therapeutic potential.

1. Introduction

In recent years, as more and more polysaccharides have been reported to exhibit a variety of biological activities, including anti-tumor (Wasser, 2002), immunostimulation (Wasser, 2002; Yamada, 1994), anti-oxidation (Li, Rong, & Wu, 2003a; Li et al. 2003b; Liu, Ooi, & Chang, 1997), etc., the nonstarchy polysaccharides have emerged as an important class of bioactive natural products. In many oriental countries, several immunoceuticals composed of polysaccharides have been accepted such as lentinan, schizophyllan and krestin (Borchers, Stern, Hackman, Keen, & Gershwin, 1999; Liu, Ooi, & Fung, 1999).

Astragalus membranaceus (AM) has a long history of use in the treatment of various renal diseases in Traditional Chinese Medicine (TCM). The previous study in patients and experimental animals showed the effects of Astragalus on the reduction of proteinuria (Deng et al., 2003; Shi et al., 2002; Su, Chen, Hu, Zhou, & Mao, 2000) and hyperlipidaemia (Wang, Li, Song, & Hu, 2002b; Wang et al., 2002a; Yu, Li, Hong, Cai, & Wang, 1999) as well as on immune modulation (Steven, 1998; Wang et al., 2002a, 2002b; Yoshida, Wang, Shan, & Yamashita, 1997; Yu et al., 2001) and renoprotection (Yang, Ma, Wang, Liu, & Zhao, 1997; Zhao, Li, Guo, & Lian, 2000). However, to our knowledge, few studies on the structural features and linkage composition of polysaccharide from AM have been undertaken. Therefore, the present paper was concerned with the isolation, chemical characterization and evaluation of the renal protective effect of a glucan from AM.

2. Materials and methods

2.1. Materials

The roots of *A. membranaceus* (AM) are commercially available in Lanzhou, China and identified by Professor Zhao R.N. in the department of pharmacy, Lanzhou University (Lanzhou, China), by comparison with a voucher specimen collected in the herbarium in the department of pharmacy, Lanzhou University. The coarse powder of the roots was air-dried in the shade and stored in a well-closed vessel for use.

T-series Dextran, DEAE–Sephadex A-25, and Sephadex G-200 were purchased from Amersham biosciences (Uppsala, Sweden). Trifluoroacetic acid (TFA), cationic Bovine Serum Albumin (C-BSA) was purchased from Sigma (St. Louis, MO, USA). All other chemical reagents were analytical reagent grade.

2.2. General methods

The specific rotation was determined at 20 ± 1 °C with an automatic polarimeter (Model WZZ-2B, China). UV–vis absorption spectra were recorded with a Shimadzu MPS-2000 spectrophotometer. The FTIR spectra (KBr pellets) were recorded on a Nicolet 360 FTIR spectrophotometer. Elemental analysis (C, H and N) was conducted on an Elementar Vario EL III instrument. Total carbohydrate content was determined by the phenol–sulfuric acid method as D-glucose equivalents (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Uronic acid content was determined according to an *m*-hydroxydiphenyl colorimetric method in which neutral sugars do

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not interfere (Filisetti-Cozzi & Carpita, 1991). Protein was analyzed by the method of Bradford (1976).

2.3. Extraction and fractionation of polysaccharide

The powdered roots (4.0 kg) of AM were firstly refluxed with methanol to remove lipophilic compounds, and then successively boiled in distilled water for 4 h at 100 °C. After filtration to remove debris fragments, the filtrate was concentrated in a rotary evaporator. Protein was removed with the sevag method (Alam & Gupta, 1986). The crude polysaccharide fraction (26.0 g) was obtained through precipitation with 3 volumes of ethanol and desiccation in vacuo. The precipitate was redissolved in distilled water and applied to a DEAE–Sephadex A-25 column (90 × 5 cm). The column was first eluted with distilled water followed by 0.3 and 0.5 M NaCl, respectively. The yielded fractions were combined according to the total carbohydrate content quantified by the phenol–sulfuric acid method. The main peak was further fractionated on a Sephadex G-200 column (100 × 5 cm) eluted with 0.1 M NaCl to yield three completely separated fractions. The main fraction was collected, dialyzed and lyophilized to get a white purified astragalus polysaccharide (APS, 670 mg, 2.5% of the crude polysaccharide).

2.4. Homogeneity and molecular weight

The homogeneity and molecular weight of APS was determined on a Waters AMLC system (717 plus autosampler and 600 delta AMLC pump) equipped with a TSKgel 4000 PWXL column (7.8 × 300 mm) and a Waters 2414 Refractive Index Detector (RID). A sample solution (20 μl of 0.5%) was injected in each run, with 0.05 mol/L NaCl as the mobile phase at 0.8 ml/min. The AMLC system was precalibrated with T-series Dextran standards (T-10, T-40, T-70 and T-500).

2.5. Monosaccharide analysis

The monosaccharide was analyzed by gas chromatography (GC; GC-9A Shimadzu, Japan) (Li et al., 2003a, 2003b). The APS was hydrolyzed by trifluoroacetic acid to monosaccharide. Monosaccharide was derivatized to acetylated aldononitriles. Xylose, glucose, rhamnose, mannose, and galactose were also derivatized as standard. Acetyl inositol was used as the internal standard. Gas chromatography was used with a capillary column (OV-225, China) and detected with a flame ionization detector (FID). N2 was used as the carrier gas (40 ml/min). The injector temperature was kept at 250 °C (split injection 70:1), and the detector temperature was maintained at 235 °C. The GC station software was Zhida N2000 (Zhida, China).

2.6. Methylation analysis

The methylation analysis of APS (10.0 mg) was performed by the Hakomori method (Hakomori, 1964). The methylated polysaccharide was treated with 90% formic acid (3 ml) for 10 h at 100 °C in a sealed tube. After removal of the formic acid, the residues were heated with 2 M trifluoroacetic acid (2 ml) under the same conditions and the hydrolysate was concentrated to dryness. The methylated sugars were reduced with $NaBH_4$, acetylated with acetic anhydride, and analyzed as the alditol acetates by GLC. The identification of the methylated sugars was analyzed by GLC–MS.

2.7. Periodate oxidation and Smith degradation

The polysaccharide APS (10.0 mg) was dissolved in 0.015 M sodium metaperiodate (30 ml) and kept in the dark at 4 °C, the absorption at 223 nm was monitored every day. The reaction was completed after 120 h and ethylene glycol (0.2 ml) was added to the solution with stirring for 30 min to decompose the excess of the reagent. Consumption of $NaIO_4$ was measured by a spectrophotometric method (Chaplin & Kennedy, 1994; Dixon & Lipkin, 1954) and HCOOH production was determined by titration with 0.01 M NaOH. The reaction mixture was dialyzed against distilled water, and the nondialysate was reduced with $NaBH_4$ (25 mg, 12 h). The pH was adjusted to 5.0, the solution was dialyzed, and the nondialysate was lyophilized, and then hydrolyzed with 2 M TFA at 110 °C for 2 h. The hydrolysate was analyzed by GLC.

2.8. Partial hydrolysis

APS was partially hydrolyzed with a solution adjusted to pH 2.0 (20 ml) with aq. trifluoroacetic acid, at 100 °C, for 18 h. After neutralization with NaOH, a polymeric product (APS-p) was obtained by precipitation with excess EtOH from a small volume of water, and then retained on dialysis with a M_r 2 kDa cut-off membrane. The periodate oxidation and Smith degradation of the precipitate fractions was followed the same procedure as mentioned above.

2.9. Nuclear magnetic resonance spectroscopy

The freeze-dried polysaccharide was kept over P_2O_5 in vacuum for several days and dissolved in 99.96% D_2O. ^1H and ^{13}C NMR spectra were recorded with a Bruker AM 400 MHz spectrometer (operating frequencies 400.17 MHz for ^1H NMR and 100.62 MHz for ^{13}C NMR) at 30 °C. Chemical shifts were reported relative to DSS as internal standard.

2.10. Animal and animal care

Male wistar rats at 110–160 g body weight, obtained from the Gansu Provincial Medical Science Academy, were kept under a 12-h light/dark cycle at 23 °C and a humidity of 60 ± 10%. The animals were allowed 4 weeks to acclimatize. The animal experiments were performed in accordance with local institutional and governmental regulations on the use of experimental animals. The experimental design was based on glomerulonephritis induced by cationic Bovine Serum Albumin (C-BSA) according to the Border method (Border, 1982). A total of 45 rats were randomly divided into three groups, including model (M group), treatment (T group) and control (C group). Rats in the C group were injected with saline through the caudal vein (3 times /week) from week 2 to week 7. Rats in the M and T groups were injected subcutaneously with C-BSA (1.0 mg in 0.5 ml 0.01 M phosphate buffered saline plus 0.5 ml incomplete freund's adjuvant) on day 0, C-BSA (2.5 mg in 1 ml 0.01 M PBS,3 times/week) was injected through the caudal vein from week 2 to week 7 to induce glomerulonephritis. At the same time, rats in the M and C groups received 1.0 ml saline intraperitoneally, and rats in the T group were given APS (10.0 mg/kg body weight) intraperitoneally at the dose volume of 1.0 ml. All rats were treated intraperitoneally once a day.

2.11. Renal protective effect assay

24 h Rat urine was collected by metabolic cage in week 2, week 4, week 6 and week 8. Urine proteins were quantificated by the Coomassie brilliant blue colorimetric method. The frozen sections of the renal cortex were directly dyed with the rabbit-anti-rat IgG labeled FITC and examined under a fluorescence microscope (OLYMPUS IX70). Sections of paraffin wax were taken, stained with hematoxylin and eosin (HE) and observed under a light microscope.

2.12. Statistical analysis

Data were expressed as means ± SD. Data in all the bioassays were statistically evaluated by Student's *t* test and $p < .05$ was considered significant.

3. Results and discussion

3.1. Isolation and structural analysis

The yield of the crude water-soluble polysaccharide from the roots of AM was 0.67% of the fresh material. The crude polysaccharide was separated and sequentially purified through DEAE–Sephadex A-25 and Sephadex G-200, each giving a single elution peak, as detected by the phenol–sulfuric acid assay. The main fraction (APS) was collected for subsequent analyses.

APS appeared as a white powder, $[\alpha]_D^{16}$ +192° (*c* 1.0, H_2O). It had a negative response to the Bradford test and no absorption at 280 or 260 nm in the UV spectrum, indicating the absence of protein and nucleic acid. Elemental analysis found to be free of nitrogen, indicating it was a neutral polysaccharide. In Fig. 1, the GPC profile showed a single and symmetrically sharp peak, indicating that APS was a homogeneous polysaccharide, with a weight-average molecular weight of 3.6×10^4 Da. The total sugar content of APS was determined to be 96.60%. As determined by *m*-hydroxydiphenyl colorimetric method and GC, the polysaccharide did not contain uronic acid. APS was composed of only glucose monomers, as detected by GLC of the alditol acetate derivatives of the components of the APS hydrolyzate. The relatively high positive value of optical rotation (+192°) suggested the dominating presence of α-form glycosidic linkages in APS (Zhao, Kan, Li, & Chen, 2005).

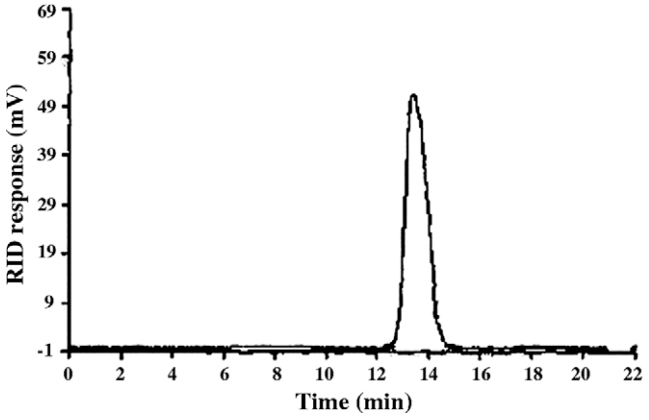

Fig. 1. Profile of APS in HPGPC. The GPC profile showed a single and symmetrically sharp peak, indicating that APS was a homogeneous polysaccharide, with a weight-average molecular weight of 3.6×10^4 Da.

In Fig. 2, the FTIR spectrum of APS showed a strong band at 3420.19 cm^{-1} attributed to the hydroxyl stretching vibration of the polysaccharide. The band at 2930.83 cm^{-1} was due to C–H stretching vibration. The broad band at 1636.52 cm^{-1} was due to the bound water (Park, 1971). The band at 850.81 cm^{-1} was ascribed to α-type glycosidic linkages in the polysaccharide (Barker, Bourne, Stacey, & Whiffen, 1954). The bands at 850.81 and 915.56 cm^{-1} was characteristic of (1→4)-α-glucan. The IR spectrum, together with the high positive value of the specific rotation indicated the presence of α-glycosidic linkages in the APS (Bao, Duan, Fang, & Fang, 2001; Tsumuraya & Misaki, 1979). The absorptions at 1020.85, 1046.68 and 1154.57 cm^{-1} also indicated α-pyranose form of the glucosyl residue.

Periodate oxidation of APS resulted in the values of 1.04 mol periodate consumed and 0.10 mol formic acid produced per sugar residue. After further Smith degradation of the periodate-oxidized APS, the glycerol and erythritol were found with molar ratio 1.1:8.5 by GLC after conversion to the corresponding alditol acetates. It was thus deduced that 1→, (1→6)-, and (1→4, 6) - amounted to 11.5%, with (1→4)-linked glycosyl bonds amounting to 88.5%, respectively. APS was partially hydrolyzed with 0.3 M TFA. After periodate oxidation and Smith degradation, only erythritol was found, indicating APS was a polysaccharide with (1→4)-linked backbone.

The fully methylated product of APS was hydrolyzed with acid, converted into alditol acetates, and analyzed by GLC and GLC–MS (Table 1). APS furnished three types of glucose derivatives in a rel-

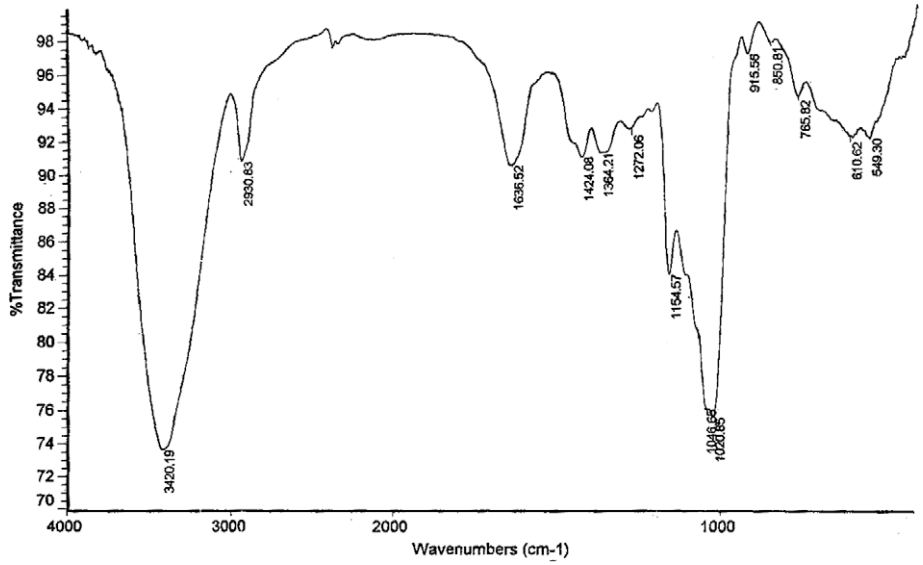

Fig. 2. FTIR spectrum of APS. The FTIR spectrum of APS showed a strong band at 3420.19 cm^{-1} attributed to the hydroxyl stretching vibration of the polysaccharide. The band at 2930.83 cm^{-1} was due to C–H stretching vibration. The broad band at 1636.52 cm^{-1} was due to the bound water. The band at 850.81 cm^{-1} was ascribed to -type glycosidic linkages in the polysaccharide. The bands at 850.81 and 915.56 cm^{-1} was characteristic of (1→4)-α-glucan. The absorptions at 1020.85, 1046.68 and 1154.57 cm^{-1} also indicated -pyranose form of the glucosyl residue.

Table 1
GC and GC–MS data of methylated APS.

Component	T_R*	Molar ratio	MS (*m*/*z*)	Linkage
1,5-di-acetyl-2,3,4,6-tetra-*O*-Me-Glc	1.0	1.0	43, 45, 71, 87, 101, 117, 129, 145, 161, 205	Glc*p*-(1→
1,4,5,-tri-acetyl-2,3,6-tri-*O*-Me-Glc	2.6	8.9	43, 45, 87, 99, 101, 113, 117, 233	→4)-Glc*p*-(1→
1,4,5,6-tetra-acetyl-2,3-di-*O*-Me-Glc	4.3	0.9	43, 101, 117, 127, 261	→4,6)-Glc*p*-(1→

* Retention time of alditol acetate relative to 1,5-di-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

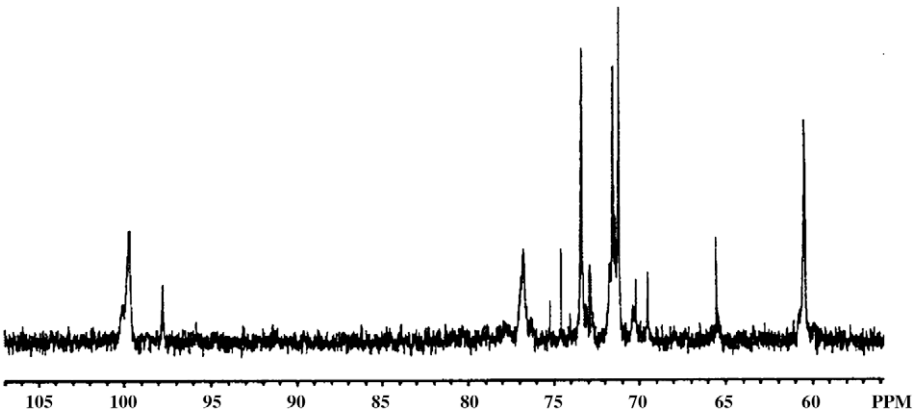

Fig. 3. ^{13}C NMR spectrum of APS. The α-configuration of the D-glucosyl groups was clearly confirmed by the presence of two anomeric peaks in the regions δ99.9 and 97.8 ppm from ^{13}C NMR experiments.

ative molar ratio of 1.0:8.9:0.9 according to the peak areas. The overall results suggested that the polysaccharide APS was a glucan with a (1→4)-linked backbone and (1→6)-linked branches. This was also in accordance with the mode of linkage of glucose present in the polysaccharide by periodate oxidation and Smith degradation.

The ^1H NMR spectrum of APS showed two anomeric protons at δ 5.33 and 4.89, which were assigned as (1→4)-α-D-Glc*p* and (1→6)-α-D-Glc*p*, respectively. This confirmed that the sugar residues were linked α-glycosidically, which is consistent with presence of an IR band 850.81 cm^{-1}. The chemical shifts from 3.4 to 4.0 ppm were assigned to protons of carbons C-2 to C-6 of glycodidic ring (Chauveau, Talaga, Wieruszeski, Strecker, & Chavant, 1996). The α-configuration of the D-glucosyl groups was clearly confirmed by the presence of two anomeric peaks in the regions δ99.9 and 97.8 ppm from ^{13}C NMR experiments (Fig. 3). The ^1H and ^{13}C NMR chemical shifts of APS were showed in Table 2.

On the basis of the above-mentioned results, it can be concluded that APS is composed of a repeating unit having the possible structure as shown in Fig. 4.

3.2. Renal protective effect of APS

In this study, we investigated the renal protective effects of APS on glomerulonephritis rats induced by cationic Bovine Serum Albumin (C-BSA) in vivo. Table 3 showed the proteinuria in M group was increased markedly during week 2, week 4, week 6 and week 8, a significant difference ($p < .05$) compared with C group. After treatment by APS, the proteinuria in T group were decreased significantly in comparison to M group ($p < .05$), whereas there was no significant difference between C group and T group ($p > .05$).The light microscope revealed numerous slightly enlarged renal glomeruli, capillary stenosis and thickened capillary walls with scattered short subepithelial basement membrane projections, as well as the presence of polymorphonuclear leukocytes and monocytes in some capillary lumens in M group (Fig. 5b),whereas morphological changes were much less in T group (Fig. 5c). The fluorescence microscope showed sparse granular deposits of IgG located predominantly in the mesangium and basement membrane in M group (Fig. 6b), whereas IgG deposits were much less in T group (Fig. 6c).

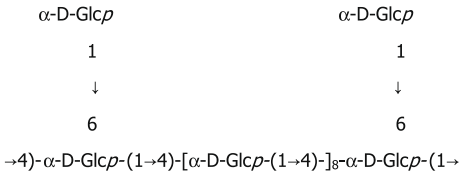

Fig. 4. Predicted structure of polysaccharide APS. The results of the present investigation showed that the polysaccharide of APS was a D-glucan containing α-(1→4)-linked backbone, branched α-(1→6)-linkage.

Table 2
^1H and ^{13}C NMR chemical shifts of polysaccharide APS in D_2O.

Residue	δ^{13}C/^1H (ppm)[a]					
	1	2	3	4	5	6
(1→4)-α-D-Glc*p*	99.9	71.6	73.4	76.9	71.3	60.7
	5.33	3.54	3.87	3.56	3.74	3.70
(1→4)-α-D-Glc*p*	97.8	71.8	73.0	69.6	70.3	65.7
	4.89	3.48	3.63	3.43	3.84	3.64

[a]In ppm downfield relative to the signal for DSS.

Table 3
24 h Urine protein quantification (mg; mean ± SD; $n = 15$).

Group	Week 2	Week 4	Week 6	Week 8
Control	6.60 ± 2.23	6.79 ± 1.99	6.86 ± 1.34	7.79 ± 1.48
Model	8.38 ± 2.30[a]	9.97 ± 2.47[a]	10.94 ± 1.49[a]	12.36 ± 3.14[a]
Treatment	6.20 ± 1.68[b,c]	8.20 ± 1.83[b,c]	8.34 ± 2.28[b,c]	8.73 ± 2.01[b,c]

ANOVA and Turkey's test was used as statistical method.
[a] $p < .05$ when compared to control (model group with control group).
[b] $p < .05$ when compared to model (treatment group with model group).
[c] $p < .05$ when compared to control (treatment group with control group).

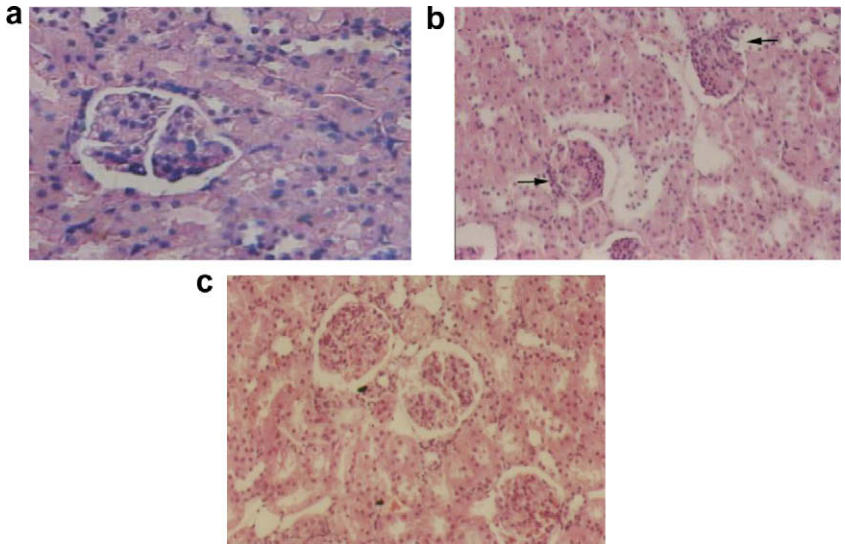

Fig. 5. Morphological observation by Light microscope (40×). The light microscope revealed numerous slightly enlarged renal glomeruli, capillary stenosis and thickened capillary walls with scattered short subepithelial basement membrane projections, as well as the presence of polymorphonuclear leukocytes and monocytes in some capillary lumens in M group (Fig. 5b); whereas morphological changes were much less in T group (Fig. 5c).

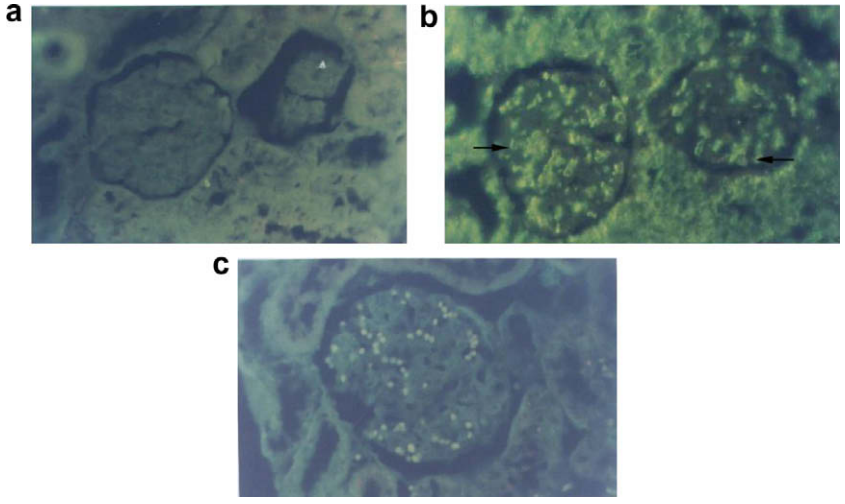

Fig. 6. Morphological observation by Fluorescence microscope (400×). The fluorescence microscope demonstrated sparse granular deposits of IgG located predominantly in the mesangium and basement membrane in M group (Fig. 6b); whereas IgG deposits were much less in T group (Fig. 6c).

Data from this study showed that C-BSA administration developed a renal lesion uniformly consisting of numerous slightly enlarged renal glomeruli, capillary stenosis and thickened capillary walls with scattered short subepithelial basement membrane projections by light microscopy as well as sparse granular deposits of IgG located predominantly in the mesangium and basement membrane in immunofluorescence findings, relative to the 24 h Urine protein elevation. APS had a significant protective effect and is capable of retarding glomerulonephritis development in the C-BSA induced rat model.

4. Conclusion

The results of the present investigation showed that the polysaccharide APS was a D-glucan containing α-(1→4)-linked backbone, branched α-(1→6)-linkage. Preliminary pharmacological tests suggested that APS exhibited significant renal protective effects in vivo.

A common feature of glomerulonephritis is the presence of immuno- inflammatory cells within the kidney. In recent decades, polysaccharides isolated from botanical sources (mushrooms, algae, lichens and higher plants) have shown immunomodulatory role in their broad spectrum of therapeutic properties including renal protection (Schepetkin & Quinn, 2006). The most likely mechanism that APS can protect against C-BSA induced glomerulonephritis in rats can account for the suppression of NF-κB activation and NF-κB-regulated cytokine expressions (Li, Zhang, and Zhao, 2007). The structural and pharmacological results obtained might help enlarge the knowledge of structural correlation to renal protective effect of polysaccharides.

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