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Full Length Research Paper

Isolation and characterization of sulfated polysaccharide from the *Sargassum pallidum* (Turn.) *C. Ag.* and its sedative/hypnotic activity

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Sulfated polysaccharide from the *Sargassum pallidum* (Turn.) *C. Ag.* was isolated, purified and structurally characterized. The ^{13}C-NMR and IR indicated a characteristic sulfated polysaccharide with 16.4% of sulfate group. The isolated polysaccharide with the molecular range of 10~48 KDa was constituted by xylose (35.92%), mannose (21.86%), arabinose (15.26%), galactose (13.49%), glucose (9.60%) and others (3.88%). The animal experiment results suggested its potential sedative and hypnotic potential activity.

Key words: *Sargassum pallidum* (Turn.) *C. Ag*, polysaccharide, sedative, hypnotic.

INTRODUCTION

There are numerous marine plants in the ocean, which make up the source for important marine pharmaceuticals. *Sargassum pallidum* (Turn.) *C. Ag,* which is one of the marine plants, usually grows in tropical oceans, and is a general food as well as medicine in China. Recorded in traditional Chinese medicine, *Sargassum pallidum* (Turn.) *C. Ag* has been used as a medicinal material for more than one thousand years. The active constituents in *S. pallidum* (Turn.) *C. Ag* have several pharmacological effects, including antitumor and antioxidant activities (Ye et al., 2008) as well as softening- hardness to dissipate stagnation and expectoration to excrete water in traditional Chinese medicine theory (Dong et al., 2002). In this paper, we isolated, purified and characterized polysaccharides from *S. pallidum* (Turn.) *C. Ag.* For the first time, we presented the sedative/hypnotic activity of the polysaccharide from *S. pallidum* (Turn.) *C. Ag.*

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MATERIALS AND METHODS

The fresh marine plant material, *S. pallidum* (Turn.) *C. Ag*, was collected from South China Sea and authenticated by Prof. Zhang Shouyao. A voucher specimen has been deposited in the Center of New Drug Research, Second Clinical Medical College and Zhujiang Hospital, Southern Medical University, Guangzhou, China.

Isolation and purification

The marine plants *S. pallidum* (1.0 kg) were washed quickly with water and decocted twice in water for 1 h. The extract was filtrated and the filtrate was concentrated with heat. After the filtrate was cooled down, 95% ethanol was added slowly until the end concentration of 20% (v/v) and was kept for 1 h. Then 10% $CaCl_2$ was added and kept overnight to precipitate the tannin. The supernatant was obtained by centrifugation at 15000 rpm for 5 min and dialyzed to desalinate with membrane (MWCO: 1 k). The retention was further dialyzed with membrane (10 k). The dialysate was concentrated by heating and lyophilized. The powder of the crude extract dissolved in distilled water was subjected to a Sephadex G-100 gel chromatography (2.6 × 100 cm, Pharmacia) and eluted with 0.1 N NaCl (flow rate: 0.75 ml/min) and monitored by differential refractive index detector (RI, RI200D, Schambeck, SFD GmbH, Germany). The fractions containing polysaccharide of interest were automatically collected, merged, dialyzed with

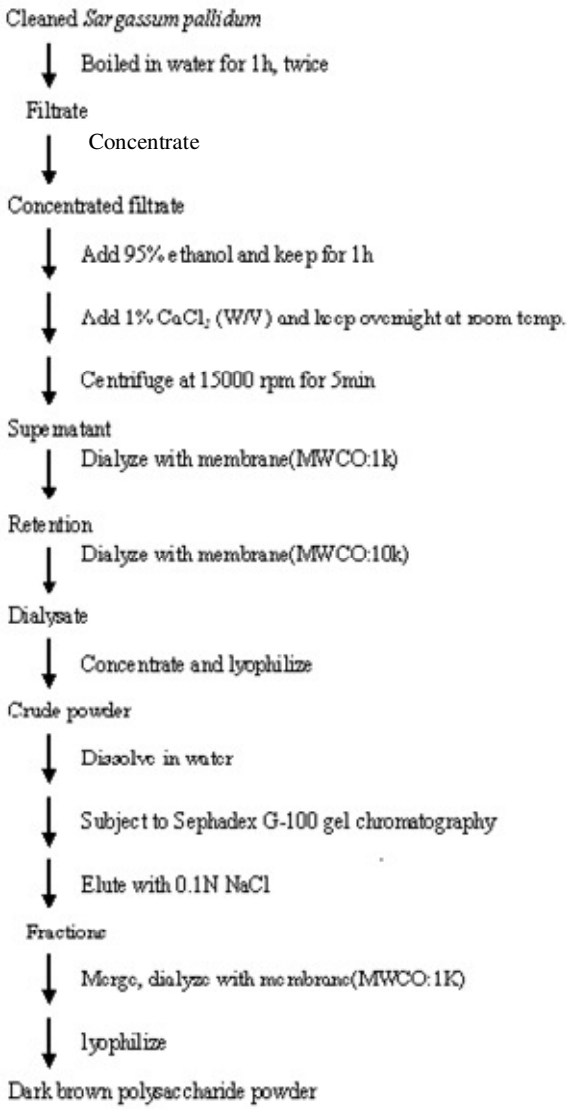

Figure 1. Extraction procedure for the polysaccharide from *Sargassum pallidum* (Turn.) *C.Ag*

membrane (MWCO: 1 k) to desalinate and lyophilized to obtain the dark brown polysaccharide powder. The product was kept in dryer for further use. A classic phenol-sulfuric acid assay was used to quantify the polysaccharide.

Analysis of monosaccharide composition by HPLC

The method of analysis was based on Rioux's (2009) and Bozic et al, (2009) .The monosaccharide components of the polysaccharide were analyzed. Briefly, the polysaccharide powder was dissolved in 2 M trifluoroacetic acid (TFA) and completely hydrolyzed to monosaccharide at 105°C for 4 h. The solution was derived with cyanoacetate and subjected to HPLC (PL Hi2Plex Ca, 9 µm, 300 × 7.7 mm column) analysis. Monopoly saccharide standards were derived in the same way and subjected to analysis. The detector was PL ELS22100. The mobile phase was 0.1 M NaCl buffer with flow rate of 0.6 ml/min and detected with an RI detector. The column temperature was 80°C. The sample volume for injection was 20 µl.

Chemical analysis

The sulfate in the polysaccharide was characterized by a modified $BaCl_2$ turbidimetric method (Zhu et al., 2003). 1.0 mg of purified polysaccharide with the overall polysaccharide more than 95% was hydrolyzed for 2 h at 120°C in 2 N trifluoroacetic acid in a sealed glass tube. The content of sulfate was determined by reference to a standard curve of potassium sulfate solution.

Molecular weight measurement

The method of measurement was based on Yuan et al. (2010). HPLC was used to determine the average molecular weight of the polysaccharide. Series of Dextran (11.5, 41, 71, 267, 580 kDa, Pharmacia) standard sample (2 mg) were dissolved in 0.1 M NaCl and subjected to PL aquagel-OH MIXED (7.5 × 300 mm, 8 µm) column and eluted with 0.1 M NaCl (flow rate: 0.50 ml/min). The polysaccharide was monitored by differential refraction detector. The elution volumes (Ve) were plotted against the logarithm of their respective molecular weights. The elution volumes of the purified polysaccharide in the gel chromatography were plotted in the same graph, and the molecular weights were calculated from the standard curve (Li et al., 2006).

Structure characterization

The monosaccharide types, glucosidic bonds and functional groups could be analyzed by IR to distinguish the vibrations of molecules and polar bonds between the different atoms in the polysaccharide. 2 mg of the polysaccharide powder was mixed with KBr and a sheet was used for IR (170SX FT-IR) spectrum analysis with a scan range of 4000~400 cm^{-1}. Cellulose sulfate was used as a control. Moreover, about 40 mg of the polysaccharide powder was dissolved in D_2O and subjected to ^{13}C-NMR analysis (Bruker AV-500 NMR).

Sedative and hypnotic experiment

The mice were randomized into 4 groups: normal saline group, low dose polysaccharide group, moderate dose polysaccharide group and high dose polysaccharide group. All mice were given normal saline or polysaccharide solution by gastric perfusion. 30 min after administration, pentobarbital of the subliminal dose was given *ip* (25 mg/kg) to each mouse. All mice were observed for 30 min. The number of sleeping mice was counted after 30 min. The abolition of righting reflex was observed as marker for sleep beginning, meanwhile the restoration of righting reflex was marker for sleep end.

RESULTS AND DISCUSSION

Purification of the polysaccharide

The fresh alga *S. pallidum (Turn.) C. Ag* (1.0 kg) was extracted with hot water and precipitated with ethanol. The extraction flowchart is shown in Figure 1. The gel chromatography is shown in Figure 2. The final purified polysaccharide in this protocol was 1.8 g and kept in a

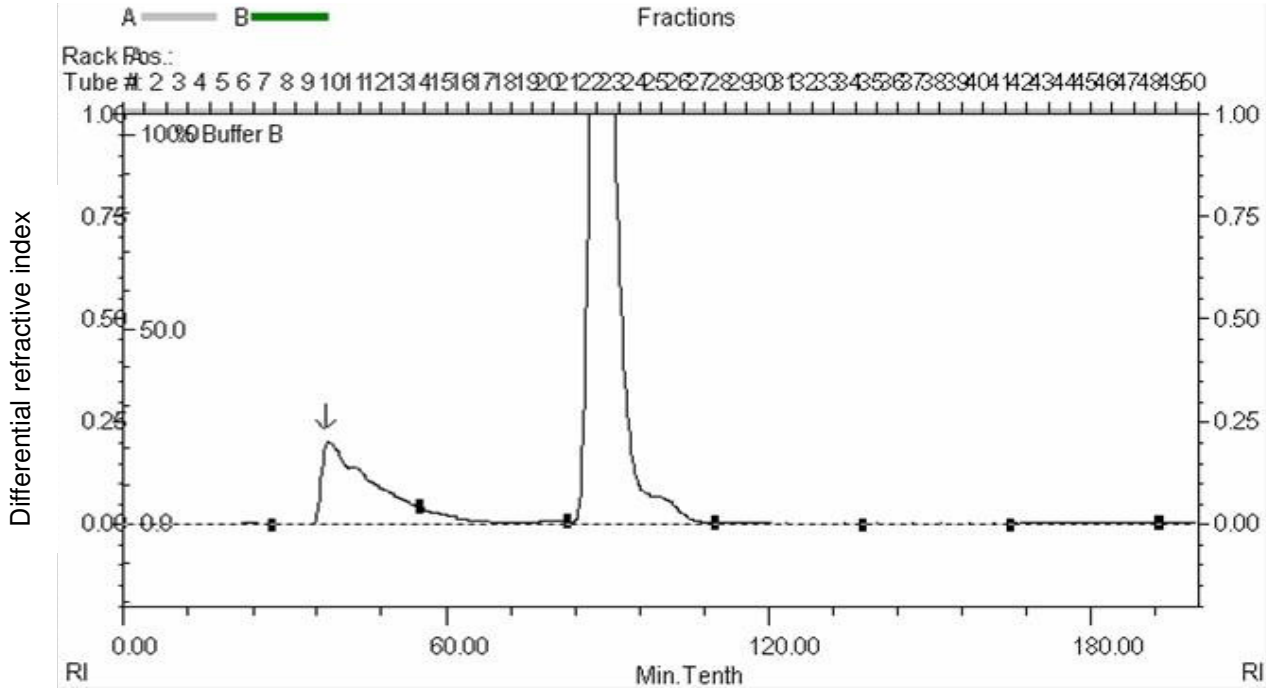

Figure 2. Purification of polysaccharide. The crude powder isolated in Figure 1 was dissolved in water and subjected to Sephadex G-100 gel chromatography and monitored by differential refractive index (RI) detector. After wash with 0.05 N NaCl, the purified polysaccharide was eluted with 0.1 N NaCl. The arrow shows the start time for fraction collection from 36 min to 54 min after Sephadex gel column separation (Y-axis: RI unit; X-axis: time (minute)).

dryer for further use.

Composition analysis of the polysaccharide

The polysaccharide in the earlier purified *S. pallidum* is a dark brown powder with a negative chloride reaction, but positive Molisch reaction. The chemical analysis indicated that the polysaccharide contained 16.4% of sulfate group. The main constituents analyzed by HPLC were xylose (35.92%), mannose (21.86%), arabinose (15.26%), galactose (13.49%), glucose (9.60%) and others (3.88%) (Figure 3 and Table 1).

Determination of molecular weight

The relative molecular weight of polysaccharides from natural plants was always inexact, and was dependent on the processing. Chen et al. (2005) separated a polysaccharide from green tea leaves with the molecular weight of 120 kDa. Another report by Chen et al. (2008) indicated two fractions of water-soluble polysaccharide with the molecular weights of 268 and 42 kDa in green tea leaves. Lee et al. (2006) obtained an acidic polysaccharide from green tea with a molecular weight of 80 kDa. Recently, Monobe et al. (2008) studied the molecular weight distribution of a crude polysaccharide derived from green tea and found that there were three major peaks of around 20×10^4, 1×10^4, and 0.1×10^4 MW. The difference could come from the different extraction methods or raw materials with different source (Harding et al., 2010). The relative molecular weight of the crude polysaccharides in the earlier purified *S. pallidum* was estimated to be 10~48 kDa (Figure 4) and was determined by HPLC way with series of Dextran as references.

Characterization of the structure

The molecular structure of the polysaccharide was characterized by ^{13}C-NMR, and the chemical shift was appeared at 3.5 to 4.0 which is a specific polysaccharide character peak. The infrared (IR) spectrum (Figure 5) indicated that the polysaccharide possessed characteristic peak at 3600~3200, 2900, 1640, 1400, 1200, 1030 and 800 cm^{-1}. Briefly, the wide peak at 3447.6 cm^{-1} indicated the O-H stretching vibration of carbohydrates. The peak at 2940.9 cm^{-1} indicated the C-H stretching vibration of carbohydrates. The peak at 1637 cm^{-1} indicated the characteristic absorbance of carbohydrates. The peak at 1420.3~1384.4 cm^{-1} indicated C-H varied angel vibration of polysaccharide composed of D-glucose, D-mannose, D-xylose and galacturonic acid. The peak at 1255.8 indicated stretching vibration of

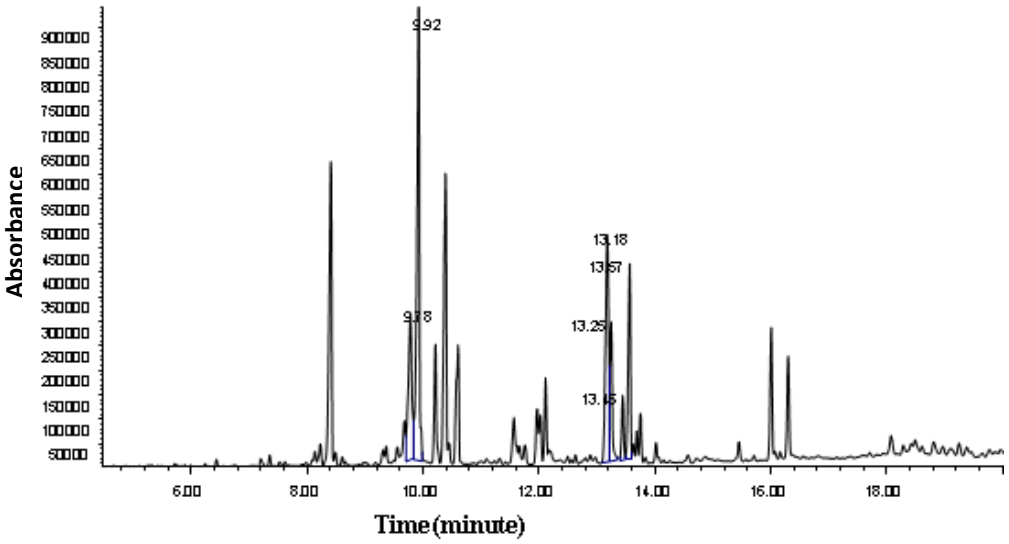

Figure 3. Composition analysis of the polysaccharide from *Sargassum pallidum.* The polysaccharide powder was dissolved in 2 M trifluoroacetic acid (TFA) and hydrolyzed to monosaccharide completely. The solution was derivatized with cyanoacetate and subjected to HPLC analysis. Monosaccharide standard was processed in the same way.

Table 1. Summary of monosaccharide composition.

Monosaccharide	Peak	RT (min)	% of total
Arabinose	1	9.781	15.26
Xylose	2	9.924	39.92
Mannose	3	13.178	21.86
Glucose	4	13.249	9.6
Other	5	13.445	3.88
Galactose	6	13.57	13.49

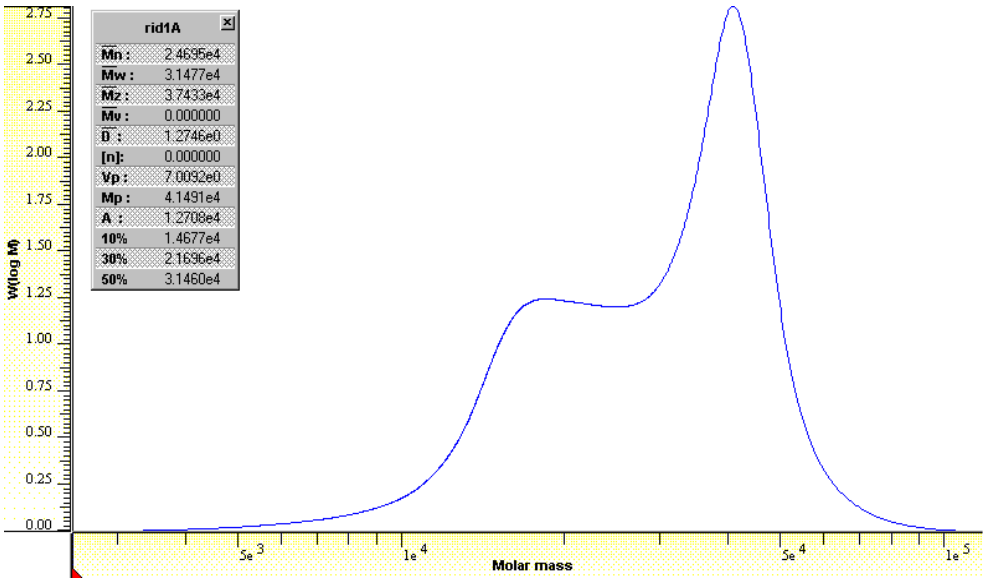

Figure 4. Measurement of molecular weight of the polysaccharide from *Sargassum pallidum.*

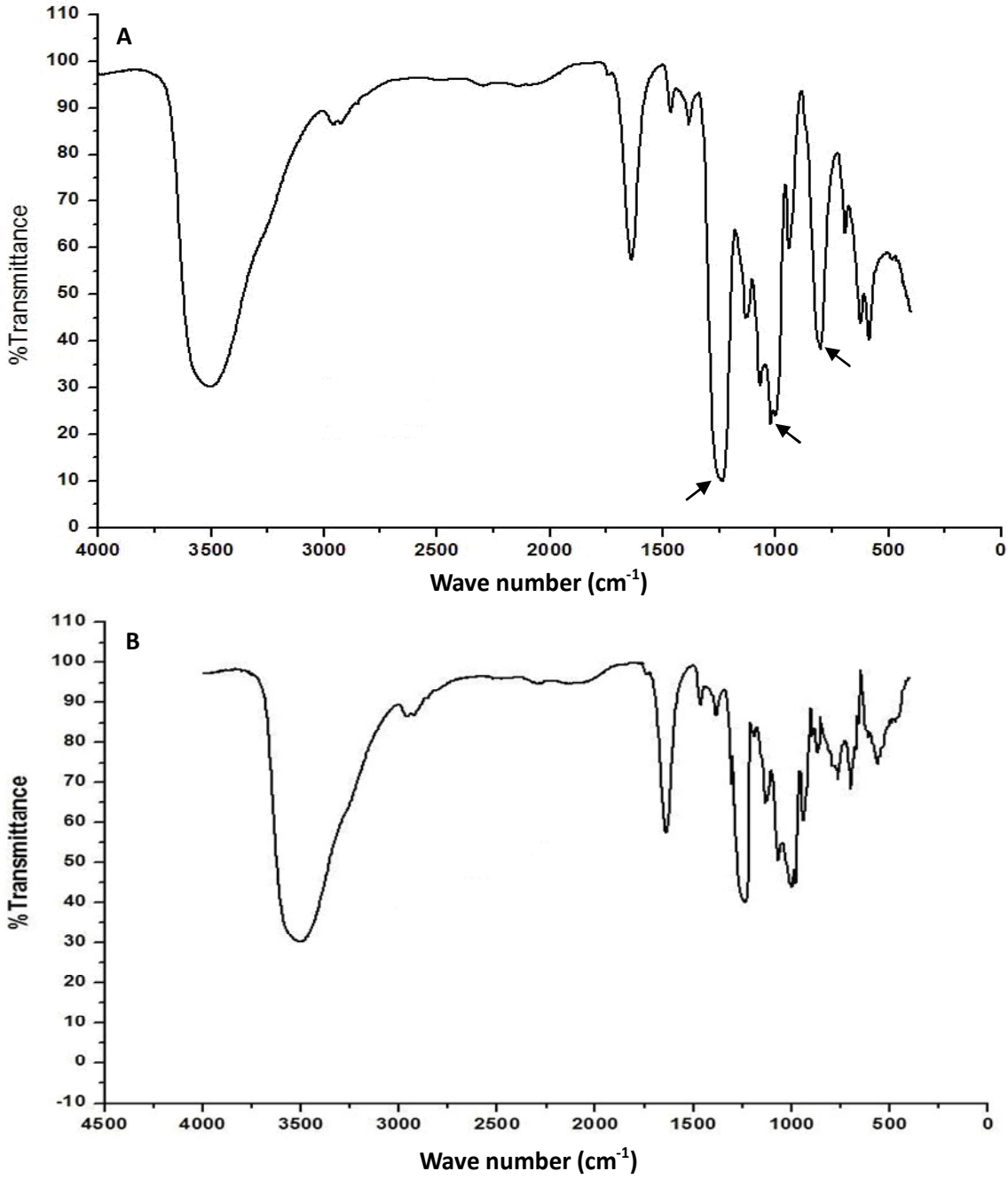

Figure 5. IR spectrum of the isolated polysaccharide from *Sargassum pallidum*. (A) KBr film of the isolated polysaccharide for infrared spectra absorption measurement. About 1 mg of pure dry sample and 300 mg of pure dry KBr were mixed and pressed into a disc. The whole IR spectrum (400 to 4000 cm^{-1}) was recorded on an IR spectrophotometer. The peak at 1255.8 indicated stretching vibration of C=O, peak at 1054.0 indicated stretching vibration of S=O, peak at 838.7 indicated stretching vibration of C-O-S; all these suggested that the polysaccharide possessed sulphuric group, the polysaccharide from *S. pallidum* was a sulfated polysaccharide, (B) IR spectrum for a control sample of sulfated polysaccharide in the data bank.

C=O, peak at 1054.0 indicated stretching vibration of S=O, peak at 838.7 indicated stretching vibration of C-O-S. All these suggested that the polysaccharide possessed sulphuric group, the polysaccharide from *S. pallidum* was sulfated lipopolysaccharide.

Recent reports showed the structural characterizations of polysaccharides in other plants of *Sargassan* (Sinha et al., 2010; Gamal-Eldeen et al., 2009). The carbohydrate

Table 2. The sedative and hypnotic effect of the polysaccharide.

Animal no.	Saline		PS L[1]		PS M[2]		PS H[3]	
	SL[#]	ST*	SL[#]	ST*	SL[#]	ST*	SL[#]	ST*
1	X	X	6.6	14.1	5.8	24.1	4.8	28.3
2	X	X	X	X	5.6	20.6	5.9	25.6
3	X	X	6.2	16.9	6.8	18.8	4.0	30.5
4	7.8	14.6	X	X	5.4	26.5	3.2	29.7
5	X	X	X	X	6.3	15.6	4.3	32.5
6	X	X	5.8	13.7	X	X	3.6	40.3
7	X	X	6.1	15.2	6.2	27.3	X	X
8	X	X	6.9	19.8	X	X	5.7	21.6
9	6.8	16.5	5.0	20.3	5.3	21.6	4.1	28.7
10	X	X	X	X	6.1	23.6	3.8	27.9
11	7.3	12.5	X	X	X	X	4.3	25.8
12	X	X	X	X	5.9	25.6	4.5	32.9
13	X	X	7.3	18.6	4.9	27.6	5.1	30.5
14	X	X	X	X	X	X	4.6	28.9
15	X	X	6.3	15.2	5.8	24.3	4.9	25.2
NSM[4]	3		8		11		14	
Mean ± SD	7.3 ± 0.5	14.53 ± 2.0	6.28 ± 0.7	16.73 ± 2.6	5.83 ± 0.5	23.24 ± 3.8	4. 49 ± 0.8	29.17 ± 4.4

PS L[1]: Polysaccharide low dose-100 mg/kg; PS M[2]: Polysaccharide moderate dose-200 mg/kg; PS H [3]: Polysaccharide high dose-300 mg/kg; NSM[4]: Numbers of sleeping mice; SL: Sleeping latency (minutes); ST *: Sleeping time (minutes). * $p<0.01$ compared to control.

chain which consists of D-galactose, D-mannose, D-xylose, L-fucose and D-glycuronic acid residues in *Sargassum tenerrimum* and *Sargassum latifolium* were similar with our monosaccharide compositions. These suggested that there might be many sulfated polysaccharides existing in *S. Pallidum.* The sulfate group in sulfated polysaccharide may be esterified with the polysaccharide or be binding with metal cation such as Na^+, and $Ca2^+$ (Zhu et al., 2003). In this study, the IR assay and sulfate content determination supported the sulfate ester form.

The sedative action

Sulfated oligosaccharides display an important role in biological processes. They bind proteins through interactions mediated by highly specific sequences (heparin - antithrombin, heparan sulfate - growth factors / herpes simplex virus) or by electrostatic interaction between sulfate groups and cationic sites of proteins. Sulfated oligosaccharides are involved in biological events, such as protein localization at cell surfaces, the control of proteolysis, the modulation of angiogenesis and metastasis of tumors, and the oligomerisation of cell growth factors. The sulfated polysaccharides showed great potential application as new targets for drugs/vaccines/diagnosis developments (Kovensky, 2009). However, insomnia is a common subjective complaint of inadequate sleep. There are many side-effects with established medicine, such as habituation, drowsiness, etc. The herbal medicine and other alternative remedies will provide novel option (Wheatley, 2005). The total flavonoids or total saponins in Semen *Ziziphus jujube* (SZJ) exert hypnotic activity (Peng et al., 2000). The flavonol glycoside from *Goodyera schlechtendaliana* shows sedative activity (Du et al., 2002). Particularly, the sulfated polysaccharide from brown seaweed has sedative and hypnotic effects with a dose dependent manner, and a synergistic central inhibitory effect as diazepam (Sun et al., 2006). The methanol extract of *Abies webbiana* leaves showed synergistic effect on sleeping time induced by standard sedatives in mice (Nayak et al., 2004). However, the limitations of all the previous studies were short of the mechanism (Wheatley, 2005).

The preliminary sedative and hypnotic experiment on mice showed that the polysaccharide from *S. pallidum* reduced sleep latency induced by subliminal (25 mg/kg) sodium pentobarbital, and extended sleep time. The effect was dose-dependent and significant compared to the control ($p<0.01$) (Table 2), which indicated that the polysaccharide in the *S. pallidum* might be a good candidate for the therapy of insomnia in the future.

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