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New oligosaccharides prepared by acid hydrolysis of the polysaccharides from *Nerium indicum* Mill and their anti-angiogenesis activities

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ABSTRACT

To discover drug candidates with anti-angiogenesis activity for cancer therapeutics, three galactooligosaccharides (OJ1–OJ3) were prepared by acid hydrolysis of the polysaccharides from *Nerium indicum* Mill. Their structures were characterized using sugar analysis, methylation analysis, and both 1D and 2D NMR spectroscopy, complemented by mass spectrometry. They were hexasaccharide (OJ1), a pentasaccharide (OJ2), and an undecasaccharide (OJ3), which was a new linear galactan. Bioactivity testing in vitro showed that OJ2 and OJ3 significantly inhibited the HMEC-1 (human microvascular endothelial cell) cell tube formation.

1. Introduction

Nerium indicum is a white or pink flowering oleander widely grown in China and Southeast Asia. The flowers and leaves of *N. indicum* were used as traditional Chinese medicine to relieve pain and stimulate cardiac muscle.[1] Their poisonous leaves are found to contain various cardiac glycosides, alkaloids, and carbohydrates.[2,3] From *Nerium oleander*, immunologically active acidic pectic polysaccharides have been isolated and characterized.[4] Previously, workers in this laboratory characterized some polysaccharides isolated from *N. indicum*.[5,6] It was reported that pectic polysaccharides may be developed as novel anti-cancer therapeutic targets. Indeed polysaccharides from *N. indicum* have shown anti-tumor, anti-oxidation, immune-stimulating, and neuroprotecting effects.[7–9] However, the problem is that the bioavailability of these macromolecules is very low if they are administrated orally. Oligosaccharides, which have diverse bioactivities,[10–12] are relatively new functional products generally useful as feedstuffs and medicinal agents. For example, the novel anti-angiogenic agent PI-88 is a highly sulfated oligosaccharide that is in Phase III clinic trials.[13] In this study, three oligosaccharides (OJ1, OJ2, and OJ3) were obtained by acid degradation of crude polysaccharides from *N. indicum*. We describe herein the structures of OJ1–OJ3 and report their anti-angiogenesis activities on HMEC-1 cells.

2. Results and discussion

2.1. Chemical features of OJ1–OJ3

OJ1 (35.6 mg), OJ2 (31.8 mg), and OJ3 (27.6 mg) were obtained from 15 g of crude polysaccharides. The average yield of the three isolated oligosaccharides from the crude polysaccharide mixture was 0.237%, 0.212%, and 0.184%, respectively. Purity evaluation showed that OJ1, OJ2, and OJ3 were each showing a single symmetrical peak (data not shown), respectively, on high-performance gel-permeation chromatography (HPGPC). Thus we deduced that they were homogeneous oligosaccharides. The specific rotations $[\alpha]_D$ of OJ1–OJ3 are +61.3, +64.6, and +53.2 (*c* 0.5, H_2O), respectively. GC analysis of the acetylated alditol derivatives of OJ1–OJ3 hydrolysates showed that they were exclusively composed of galactitol. After complete hydrolysis with 2 M trifluoroacetic acid, TLC analysis indicated that OJ1–OJ3 did not contain any uronic acid. This was confirmed by a uronic acid content measurement determined by the 3-phenylphenol (*m*-hydroxybiphenyl) method[14,15] with D-glucuronic acid as the standard (data not shown). The configuration of the three oligosaccharides was assigned as the D configuration by comparing with standard D-galactose, using the GC–MS method of Cases et al.[16] The methylation analysis of OJ1, OJ2, and OJ3 all gave one peak of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-galactitol, representing (1→4)-linked Gal and the other peak of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol, representing 1-linked Gal. The ratio between (1→4)-linked Gal to 1-linked Gal of OJ1, OJ2, and OJ3 is 3.7:10.6, 5.1:9.5, and 6.1:8.7, respectively. The NaOH used in the methylation process may cause degradation of oligosaccharides. This might make the ratio of methylated alditol

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Table 1
Mass spectroscopic analysis of OJ1–OJ3

Compounds	Fragment ions (*m/z*)	Nominal mass (*m/z*)	Type	Molecular weight
OJ1	1008.16	1013.39	$[M+Na]^+$	990.40
OJ2	846.10	851.32	$[M+Na]^+$	828.33
OJ3	1662.53	1824.60	$[M+Na]^+$	1801.61

acetate derivatives inaccurate. The results show that all three compounds (OJ1–OJ3) had a (1→4)-linked galactan linear backbone.[15]

The strong pseudomolecular ion $[M+Na]^+$ peaks of OJ1 and OJ2 were shown at *m/z* 1013.39 and *m/z* 851.32, respectively (Table 1). Hence their molecular weights were supposed to be 990.40 and 828.33, respectively, which were approximate to the calculated molecular weights of a hexasaccharide (990.85) and a pentasaccharide (828.71), respectively (Table 1). The deduction of molecular weights of OJ1 and OJ2 was also supported by comparable recognition using standard maltotetrose, maltopentaose, maltohexose, and maltoheptose on a KS 802 Shodex column (Shoko, Japan). The retention times of four maltose samples on the column were 9.665, 9.357, 9.102, and 8.923 min (data not shown), while the retention time of OJ1 and OJ2 was 9.135 and 9.531 min, respectively (data not shown). On the basis of the above results and the methylation analysis, OJ1 and OJ2 were assigned as a hexasaccharide and a pentasaccharide, respectively. The retention time of OJ3 on the KS802 column was 8.500 min. This suggested that the degree of polymerization of OJ3 was at least equal to or more than eight. The molecular weight of OJ3 was determined using an IonSpec 4.7 T HiRes MALDI Fourier-transform mass spectrometer (Ionspec, Irvine, CA, USA). Fragment ions with fair intensity in the MS spectrum of OJ3 originated from a pseudo molecular ion $[M+Na]^+$ at *m/z* 1824.60, corresponding to the sequential loss of one galactose (*m/z* 1662.53) and two galactose units (*m/z* 1500.47) (Fig. 1 and Table 1). Taken together, we deduced the molecular weight of OJ3 was 1801.61 (Table 1). Since there is a trace impurity mixed with OJ3, which is very difficult (OJ3 was purified on column seven times) to be separated, this causes its mass spectrum to appear as heterogeneous (Fig. 1). Since there is no commercial standard undecasaccharide available, it is difficult to compare the retention time of OJ3 on KS802 with the standard as extra supporting data for the molecular weight determination. The above results suggested that OJ3 was an undecasaccharide.

The ^{13}C NMR spectrum of OJ1 (Fig. 2) shows a signal at 105.63 ppm, indicating the β anomeric configuration for galactose residues.[17] The signal at 97.68 ppm corresponding to C-1 of the residue at the reducing terminus. The anomeric proton signal at 4.60 ppm in the ^1H NMR spectrum of OJ1 was assigned to the H-1 of the β-D-galactose residues, which was in good agreement with the β anomeric configuration supported by the ^{13}C NMR spectrum.[17] The signal at 78.88 ppm was assigned to the C-4 of the (1→4)-linked residues.[17] The signal at 61.99 ppm corresponded to C-6. The ^{13}C NMR spectrum of OJ2 (Fig. 2) contained at least twelve major signals. The anomeric carbon signal of β-Gal*p* was assigned at 105.60 ppm. This also confirmed that the sugar residues of OJ2 were β linked.[16] The signal at 97.64 ppm indicated an anomeric carbon of the β residue at the reducing terminus, while an anomeric signal at ~93.6 ppm corresponded to the reducing terminus of the α anomer due to the mutarotation phenomenon. The signal at 61.97 ppm was assigned to C-6. In the ^1H NMR spectrum, the signal at 4.42 ppm corresponded to the H-1 of the β-D-galactose residues.[17] Similar to the assignment with OJ1, the signal at 78.82 ppm was assigned to C-4 of (1→4)-linked residues.[18] The ^{13}C NMR spectrum of OJ3 (Fig. 2) also showed characteristic signals of the β-Gal*p* residues at 105.62 ppm (anomeric carbon) and 97.68 ppm (reducing-end carbon).[18] The strong signal at 78.88 ppm corresponded to C-4 of the (1→4)-linked residues, which was consistent with the results of the methylation analysis.

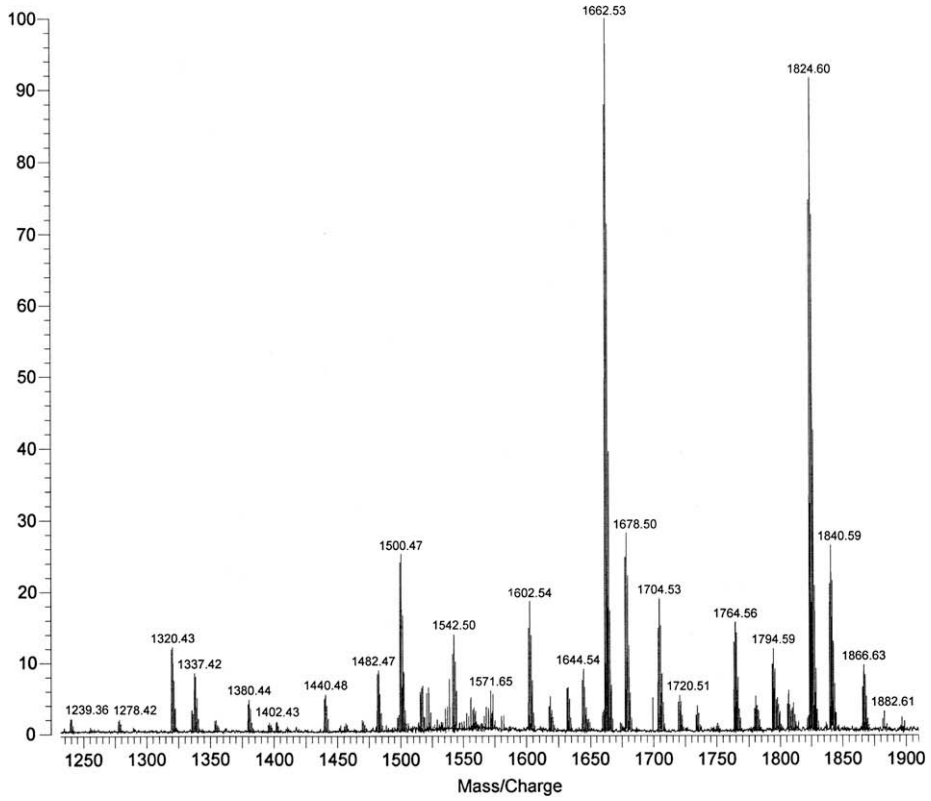

Figure 1. MALDI-MS spectrum of OJ3.

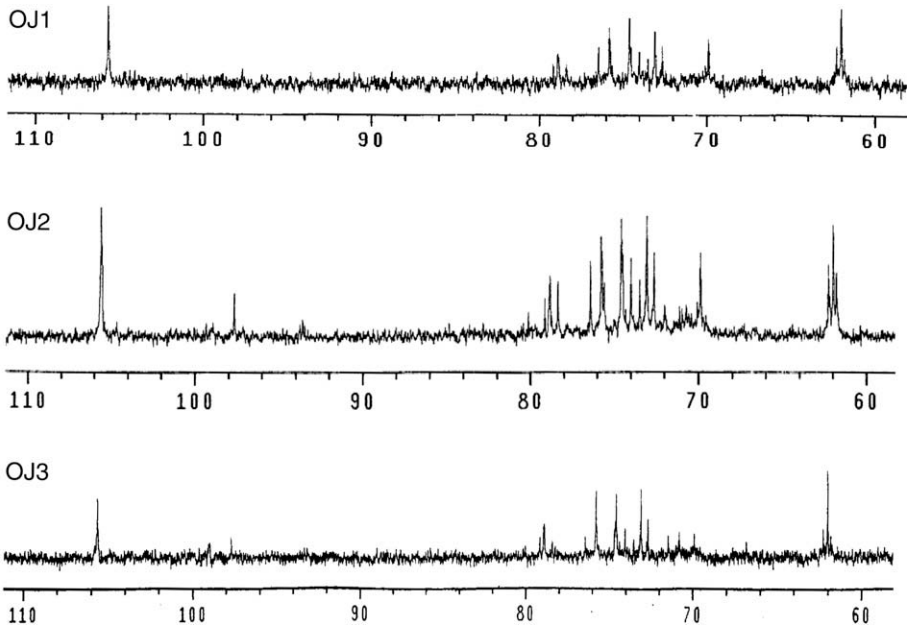

Figure 2. ^{13}C NMR spectra of OJ1–OJ3.

In the ^{1}H NMR spectrum, the signal at 4.52 ppm was assigned to H-1 of the β-D-galactose residues.[17] The two signals at 3.66 and 3.72 ppm could be attributed to the H-6 of the galactose residues.[17] The assignments of the other signals of OJ1–OJ3 were made using CASPER software (http://www.casper.organ.su.se) and corroborated by their HMQC spectrum (data not shown), as summarized in Table 2.

According to all the above data, the structures of OJ1–OJ3 were elucidated unambiguously as follows: OJ1 is β-D-Gal*p*-(1→[4)-β-D-Gal*p*(1→]$_4$4)-D-Gal*p*. OJ2 is β-D-Gal*p*-(1→[4)-β-D-Gal*p*-(1→]$_3$4)-D-Gal*p*. OJ1 and OJ2 are reported from *N. indicum* for the first time. Although the two oligosaccharides have been reported in other plants, no ^{13}C NMR data have been provided so far.[19,20] As an undecasaccharide, β-D-Gal*p*-(1→[4)-β-D-Gal*p*(1→]$_9$4)-D-Gal*p*, OJ3 is reported for the first time as a new oligosaccharide.

Table 2
^{1}H and ^{13}C NMR spectroscopic data of OJ1–OJ3 (ppm)

	Carbon	OJ1		OJ2		OJ3	
		H	C	H	C	H	C
Gal[a]	C-1	4.6	105.63	4.42	105.6	4.52	105.62
	C-2	3.58	73.07	3.41	73.04	3.54	73.08
	C-3	3.67	74.58	3.5	74.56	3.63	74.57
	C-4	4.13	78.88	3.96	78.82	4.06	78.88
	C-5	3.58	75.79	3.45	75.76	3.59	75.77
	C-6	3.69	61.99	3.53	61.97	3.66	62
		3.78		3.62		3.72	
Gal[b]	C-1	4.56	97.69	4.36	97.64	4.48	97.68
Gal[c]	C-2	3.56	72.63	3.32	72.61	3.48	72.65
	C-3	3.65	74.01	3.47	73.98	3.61	74.02
	C-4	3.86	69.89	3.68	69.86	3.78	69.9
	C-5	3.55	76.44	3.39	76.41	3.56	76.43
	C-6	3.67	62.28	3.52	62.25	3.64	62.26
		3.84		3.69		3.75	

The chemical shifts of anomeric C-1 and H-1 of the non-reducing terminus are nearly the same as those of the middle Gal in the hexasaccharide, pentasaccharide, and hendecasaccharide, respectively. The chemical shifts of C-2–C-6 of the reducing terminus are most like those of the middle Gal in the hexasaccharide, pentasaccharide, and hendecasaccharide, respectively (CASPER software, see http://www.casper.organ.su.se).

[a] Gal: middle galactoses.
[b] Gal: reducing terminus.
[c] Gal: non-reducing terminus.

2.2. In vitro anti-angiogenesis activities of OJ1–OJ3

The process of controlling the propagation of blood vessels (anti-angiogenesis) is fundamental to human health,[21] as cancer growth and its spread are angiogenesis dependent. Recently, anti-angiogenesis therapies have been introduced successfully in the clinic, representing a turning point in tumor therapy and the treatment of macular degeneration, heralding a new era for the treatment of several commonly occurring angiogenesis-related diseases.[22] For example, Avastin is an example of an anti-angiogenic agent in clinical use. In recent years, there has been a considerable increase in research on oligosaccharides because of their increasing evidence of function in angiogenesis.[23]

In this study, a tube formation assay[24] in HMEC-1 cells was employed to test the anti-angiogenesis activity of OJ1–OJ3. Clearly, control HMEC-1 formed a network of tubes within 16 h (Figs. 3 and 4). However, tube formation was observed to be disrupted by both OJ2 and OJ3 at a concentration of 100 μM, whereas those treated with OJ1 at the same concentration were not (Fig. 3). Comparatively, OJ3 showed a more robust effect on tube formation than that of OJ2 (Fig. 3). To determine the dose–effect relationship, the influence of OJ3 on tube formation at different concentrations of 2, 20, 200 μM was investigated. The HMEC-1 cells treated with OJ3 at low concentration (2 μM) demonstrated a small impact on tube formation, whereas those treated with a higher concentration (20 μM) differentiated some short tubes or remained dotted on Matrigel; however, these were unable to form a mesh-like network (Fig. 4). When the cells were manipulated with 200 μM of OJ3, there was no significant change on tube formation compared to that induced by the medium concentration (20 μM, Fig. 4). Considering the effects at 100 μM and 200 μM (Figs. 3 and 4), the maximum concentration for tube formation disruption by OJ3 might be 100 μM.

All three glycans possess a similar structure, a (1→4)-linked galactan linear backbone; however, their sizes are different. These characteristics caused OJ1 to show no impact on cell tube formation, while OJ2 and OJ3 exhibited pronounced biological activities.

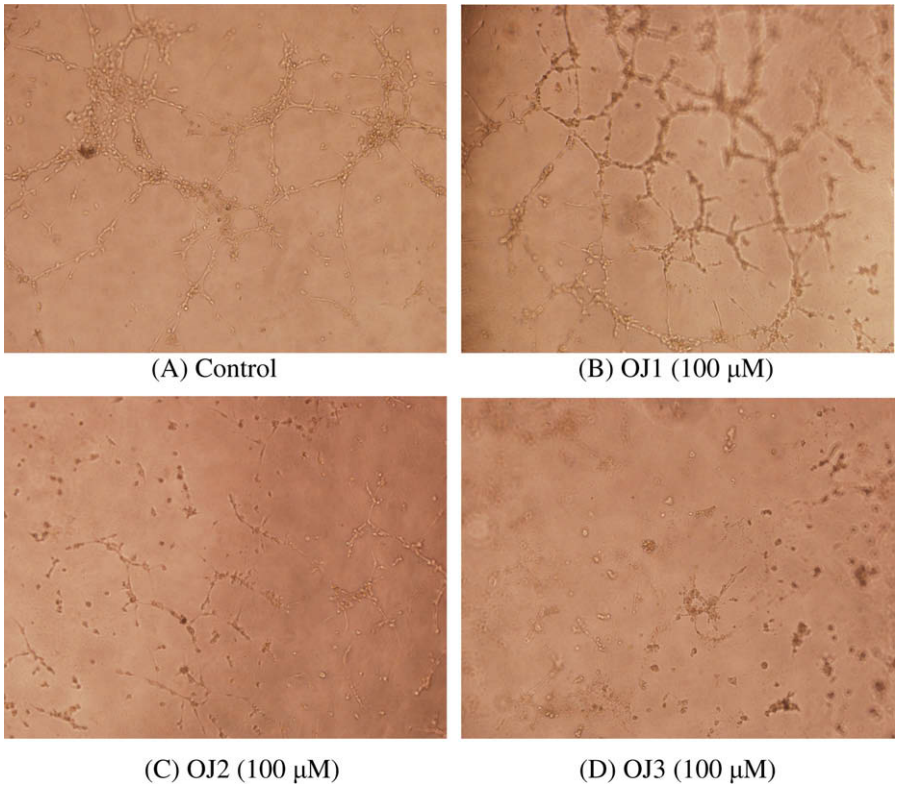

Figure 3. The effects of OJ1–OJ3 on HMEC-1 cell tube formation.

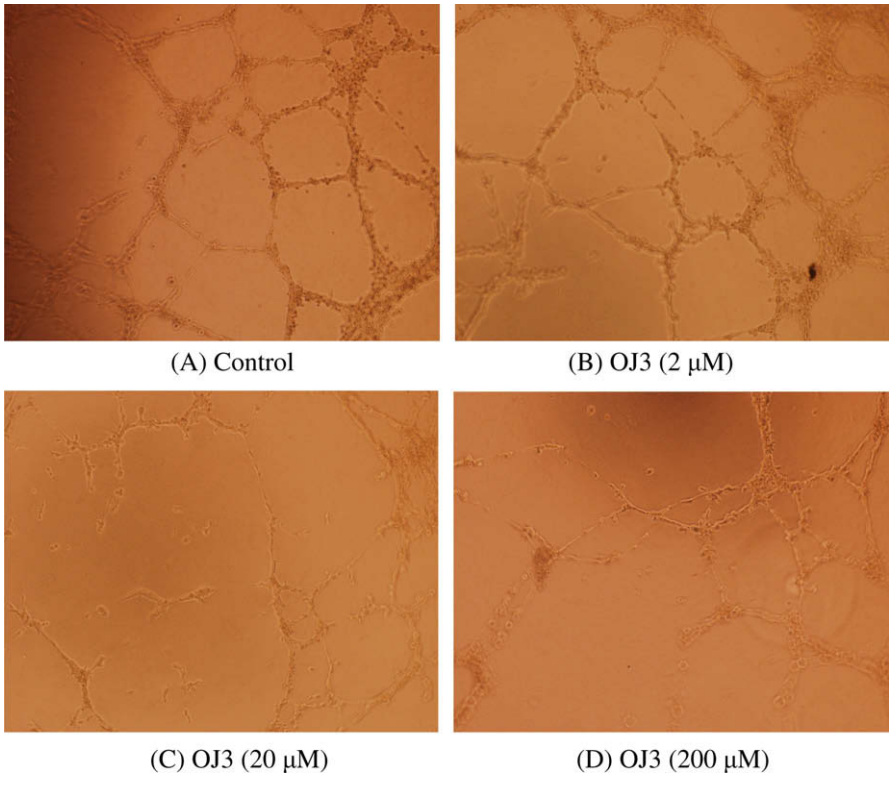

Figure 4. OJ3 may inhibit HMEC-1 cell tube formation. Matrigel was added to wells of a cold 96-well plate (50 μL/well), and then incubated at 37 °C for 30 min to allow gelling. HMEC-1 previously starved overnight were seeded onto the Matrigel. Drugs were added at the same time. Cell culture was incubated at 37 °C for 16 h in a humidified 5% CO2 atmosphere. Photographs were then taken with a digital camera at a magnification of 320×.

We deduced that the conformation of the glycans might play a vital role in the inhibition of tube formation. The five galactoses might comprise a basic functional unit; however, one additional galactose unit might be enough to alter the conformation significantly enough to cause a loss of the bioactivity, while the undecasaccharide might stabilize the conformation of the basic functional unit. Indeed, by using ChemBioDraw software (version 11.0, CambridgeSoft Corporation, USA), three oligosaccharides were modeled (data not shown). In comparison with OJ1 (hexasaccharide), the OJ2 (pentasaccharide) possesses a more flattened pocket structure for cell membrane receptor(s) to possibly bind, which triggers the signaling pathway for tube formation. The OJ3 (undecasaccharide) keeps one part (five galactose residues) as the flat pocket as with OJ2, although it also contains a deeper pocket structure. Definitely, this hypothesis needs to be confirmed once we obtain sufficient material. In fact, sample accumulation and the in vivo animal test of OJ3 is ongoing. Previously, we reported that one sulfated galactan polysaccharide also demonstrated inhibition effect on HMEC-1 tube formation.[24] Combined with this study, the pentasaccharide backbone of the galactan may be the minor structural functional unit for a structure–activity relationship study of the polysaccharide on anti-angiogenesis bioactivity.

There are at least three ways frequently used to obtain oligosaccharides from herbal medicine. The first one is to extract them directly from the herb. The second one is to use enzymes to degrade the polysaccharides. The third one is to hydrolyze the polysaccharides extracted from the herb to obtain the oligosaccharides. The average yield of oligosaccharide directly extracted from the flowers is 30-fold less than that via crude polysaccharide degradation using acid (data not shown), while the enzyme (using a commercial one) degradation costs more money, at least at the bench level. Comparatively, the acidic hydrolysis of polysaccharides is the more economical way to obtain biologically active oligosaccharides from herbal medicine, at least at laboratory level. In recent years, there have been more and more reports that describe functions of oligosaccharides in angiogenesis.[13,23] In this study, three oligosaccharides (OJ1–OJ3) were prepared by the acid degradation of the polysaccharides from *N. indicum*. In vitro bioactivity tests indicated that OJ2 and OJ3 had significant anti-angiogenesis activities on HMEC-1 cells. Thus they could be used as new drug candidates with anti-angiogenesis activities for new cancer therapeutics development.

3. Experimental

3.1. Materials and chemicals

The leaves of *N. indicum* (3.2 kg) were collected in Shanghai on October, 2006 and immediately immersed in 80% aq EtOH. DEAE-cellulose 32 was from Whatman Co. Ltd. Standard monosaccharides, sodium borohydride, and iodomethane were all Fluka products. Dimethyl sulfoxide was from E. Merck. Matrigel was obtained from BD Bioscience (CA, U.S.A). MCDB131 was provided by Gibco. Other reagents were analytical grade unless otherwise claimed.

3.2. Isolation, preparation, and purification

The dried flowers of *N. indicum* were soaked with 95% EtOH for 6 days. The residue was dried in air followed by extraction three times with boiling water. Three vol of 95% EtOH were added to the concentrated supernatant to precipitate the crude polysaccharides.[1] Since crude polysaccharides (258 g) were extracted from 9 kg fresh flowers, the average yield of the extraction of the crude polysaccharide was 2.87%. Ten polysaccharides were purified from the crude polysaccharides by anion-exchange and size-exclusion chromatography, and their homogeneities were confirmed by high-performance gel-permeation chromatography (data not shown). The sizes of the polysaccharide were varied from 5×10^3 to more than 1×10^6 Da (data not shown). Monosaccharide composition analysis showed that the crude polysaccharides were composed of rhamnose, arabinose, galactose, galacturonic acid, xylose, and glucose. Among them, xyloglucan and rhamnogalacturonan were the major polysaccharides in the flowers.[1] Obviously, they were typical plant cell-wall polysaccharides. The crude oligosaccharides were obtained by acid degradation of 15 g of crude polysaccharides (0.2 M trifluoroacetic acid, 100 °C for 4 h). The acid was removed under reduced pressure by repeated evaporations with MeOH. Three vol of 95% EtOH were added to the hydrolysates to precipitate the non-hydrolyzed crude polysaccharides. The supernatant was concentrated. The crude oligosaccharides (5 g) were subjected to DEAE-cellulose column chromatography and eluted stepwise with distilled water, 0.2, 0.4, and 1.0 M NaCl. The distilled water eluate was concentrated, dialyzed, and lyophilized. These oligosaccharides were then subjected to an active carbon column and eluted stepwise with distilled water, 5% EtOH, and 50% EtOH. The 50% EtOH eluate was concentrated, dialyzed, and lyophilized. The resulting oligosaccharides were purified on a Bio-Gel P-2 column and eluted with distilled water to obtain OJ1, OJ2, and OJ3, respectively.

3.3. Analytical methods

3.3.1. Monosaccharide composition analysis[25]

The oligosaccharide samples were hydrolyzed with 2 M trifluoroacetic acid for 2 h at 121 °C. The acid was removed under reduced pressure by repeated evaporations with MeOH, then the hydrolysates were converted to their respective alditol acetates, followed by GC analysis. The GC analyses were carried out on a Finnigan Trace GC 2000 equipped with a flame-ionization detector (FID), using TRB-5MS (30 m × 0.25 mm) as the column. Nitrogen was used as the carrier gas at a flow rate of 1.2 mL/min. GC–MS analysis was performed on a Finnigan Trace GC 2000 equipped with a Finnigan DSQ mass spectrometer, using the same column described above. Helium was used as the carrier gas at 1 mL/min.

3.3.2. Methylation analysis

The vacuum-dried oligosaccharide (2 mg) was methylated twice with iodomethane and powdered sodium hydroxide in dimethyl sulfoxide as described by Needs and Selvendran.[26] The completeness of methylation was confirmed by the disappearance of the hydroxyl absorption in IR (Nujol). The partially methylated alditol acetates were prepared and analyzed by GC–MS.[27]

3.3.3. Spectroscopic methods

ESIMS was employed for oligosaccharides OJ1 and OJ2 MS analysis using a LCQDECAXPplus MS instrument. MS analysis of OJ3 was conducted using an IonSpec 4.7 T HiRes MALDI Fourier-transform mass spectrometer (Ionspec, Irvine, CA, USA), equipped with a ND:YAG laser (355 nm, New Wave, Fremont, USA) for desorption/ionization. Mass spectra were obtained in the positive-ion mode using a laser power of 50% of maximum. The mass spectrometer was calibrated with PEG-400 for each test. All mass spectra were generated by one laser shot to give a single spectrum. Fourier-transform infrared (FTIR) spectra of KBr pellets of the oligosaccharides were recorded on a Perkin–Elmer Series 2000 FTIR spectrophotometer (eight scans, at a resolution of 4 cm^{-1}). For NMR spectroscopy, the lyophilized oligosaccharides were dissolved in D_2O (20–30 mg/mL). The ^{13}C NMR spectra were measured with a Bruker AM-400 spectrometer at room temperature. Samples were

deuterium exchanged by lyophilization twice with D_2O, and chemical shifts are expressed in ppm using acetone as the internal standard at 31.50 ppm.

3.4. Cell culture

HMEC-1 cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were seeded into 96-well plates (Greiner) at a density of 3×10^4 cells/mL in MCDB131 Medium (Gibco category: 10372-019), which contained 10% fetal bovine serum, 20 mM L-glutamine, 5 μg EGF, and antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin).

3.5. Cell tube formation assay

A tube formation assay was employed to test the impact of three oligosaccharides on angiogenesis in vitro.[24] Briefly, Matrigel (BD Biosciences) was thawed at 4 °C on ice. Pipettes and plates used were pre-cooled overnight. Matrigel (50 μL) was coated into each well on a 96-well plate, then incubated at 37 °C for 45 min to allow the gel to coagulate. HMEC-1 cells were incubated in 100 μL serum-free medium overnight. Then the cells were seeded at a density of 5×10^4 cells per well onto the Matrigel in 100 μL of complete medium containing 100 μM of three oligosaccharides or 2, 20, 200 μM of OJ3, or vehicle (as control), respectively. Cell culture was maintained at 37 °C for 16 h in a humidified 5% CO_2 atmosphere. Photographs were then taken with a digital camera attached to inverted phase contrast microscope (Olympus, IX50, Japan) at a magnification of 320×.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2008.10.019.

References

1. Ding, K.; Fang, J. N.; Dong, T.; Tsim, K. W.; Wu, H. *J. Nat. Prod.* **2003**, *66*, 7–10.
2. Institute of Materia Medica, Chinese Academy of Medical Sciences. *Studies on Effective Constituents of Traditional Chinese Medicine*, 1st ed.; The People's Health Press: Beijing, 1972; pp 392–396.
3. Dong, Q.; Fang, J. N. *Carbohydr. Res.* **2001**, *332*, 109–114.
4. Mueller, B. M.; Rosskopf, F.; Paper, D. H.; Kraus, J. *Pharmazie* **1991**, *46*, 657–663.
5. Li, W. Y.; Dong, Q.; Fang, J. N. *Chin. Trad. Herb. Drugs* **1999**, *30*, 891–893.
6. Yu, M. S.; So, K. F.; Fang, J. N. *Brain Res.* **2007**, *1153*, 221–230.
7. Lin, Z. B.; Zhang, H. N. *Acta Pharmacol. Sinica* **2004**, *25*, 1387–1395.
8. Leung, M. Y.; Liu, C.; Koon, J. C. *Immunol. Lett.* **2006**, *105*, 101–114.
9. Yu, M. S.; Lai, S. W.; Lin, K. F.; Fang, J. N. *Int. J. Mol. Med.* **2004**, *14*, 917–924.
10. Carabin, J. G.; Flamm, W. G. *Regul. Toxicol. Pharm.* **1999**, *3*, 268–282.
11. Spiegel, J.; Karabell, P.; Frankos, V. H. *Food Technol.* **1994**, *48*, 85–89.
12. Kim, M. *Nutr. Res.* **2000**, *20*, 1017–1026.
13. Zhao, H.; Liu, H.; Chen, Y.; Xin, X.; Li, J.; Hou, Y.; Zhang, Z.; Zhang, X.; Xie, C.; Geng, M.; Ding, J. *Cancer Res.* **2006**, *66*, 8779–8787.
14. Blumencrantz, N.; Asboe-Hansen, G. *Anal. Biochem.* **1973**, *54*, 484–489.
15. Dong, Q.; Yao, J.; Fang, J. N. *Carbohydr. Res.* **2007**, *342*, 1343–1349.
16. Cases, M. R.; Cerezo, A. S.; Stortz, C. A. *Carbohydr. Res.* **1995**, *269*, 333–341.
17. Liu, L.; Dong, Q.; Fang, J. N. *Carbohydr. Polym.* **2007**, *70*, 304–309.
18. Vogl, H.; Paper, D. H.; Franz, G. *Carbohydr. Polym.* **2000**, *41*, 185–190.
19. Ishii, T.; Ohnishi-Kameyama, M.; Ono, H. *Planta* **2004**, *219*, 310–318.
20. Bonnin, E.; Voragen, A. G. J.; Thibault, J. F. *Enzyme Microb. Technol.* **1996**, *19*, 100–106.
21. Norrby, K. *J. Cell. Mol. Med.* **2006**, *10*, 588–612.
22. Risau, W. *Nature* **1997**, *386*, 671.
23. Slevin, M.; Kumar, S.; Gaffney, J. *J. Biol. Chem.* **2002**, *277*, 41046–41059.
24. Zhang, C.; Yang, F.; Zhang, X. W.; Wang, S. C.; Li, M. H.; Lin, L. P.; Ding, J. *Br. J. Pharmacol.* **2006**, *148*, 741–751.
25. Wang, S.; Annie Bligh, S. W.; Zhu, C.; Shi, S.; Wang, Z.; Hu, Z.; Crowder, J.; Branford-White, C.; Wella, C. *J. Agric. Food Chem.* **2008**, *56*, 2624–2629.
26. Needs, P. W.; Selvendran, R. R. *Carbohydr. Res.* **1993**, *245*, 1–10.
27. Dong, Q.; Jia, L. M.; Fang, J. N. *Carbohydr. Res.* **2006**, *341*, 791–795.