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Bioactive polysaccharides from the stems of the Thai medicinal plant *Acanthus ebracteatus*: their chemical and physical features

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Abstract—Crude water-soluble polysaccharides were isolated from *Acanthus ebracteatus* by hot water extraction followed by ethanol precipitation after pre-treatment with 80% ethanol. The crude polysaccharides were separated into neutral and acidic polysaccharides by anion-exchange chromatography. The neutral polysaccharide (A1001) was rich in galactose, 3-*O*-methylgalactose and arabinose, whereas the acidic polysaccharide (A1002) consisted mainly of galacturonic acid along with rhamnose, arabinose and galactose as minor components indicating a pectin-type polysaccharide with rhamnogalacturonan type I (RG-1) backbone. 3-*O*-Methylgalactose is also present in the acidic fraction. Both neutral and acidic fractions showed potent effects on the complement system using pectic polysaccharide PM II from *Plantago major* as a positive control. A small amount of 3-*O*-methylgalactose present in the pectin seemed to be of importance for activity enhancement in addition to the amount of neutral sugar side chains attached to RG-1. The relationship between chemical structure and effect on the complement system of the isolated polysaccharides is considered in the light of these data. The presence of the rare monosaccharide 3-*O*-methylgalactose may indicate that this can be used as a chemotaxonomic marker. The traditional way of using this plant as a medical remedy appears to have a scientific basis.

Keywords: *Acanthus ebracteatus*; 3-*O*-Methylgalactose; Pectin; Complement fixing activity

1. Introduction

Acanthus ebracteatus Vahl (Acanthaceae, English name: Sea Holly, Thai name: Ngueak Plaa Mo) is a mangrove plant distributed in Southeast Asia. This plant is used for the treatment of a wide range of diseases. For example, the boiled seeds are commonly used in Malaysia as a cough remedy, the seeds are also used for poulticing boils, or a decoction is drunk against boils. In China, the plant is prescribed against hepatosplenomegaly, hepatitis, lymphoma and asthma. In Thailand, the root and stem are used for treating skin diseases and for longevity.[1] Crushed leaves are used as a poultice on inflamed joints.[2] A decoction of the whole plant is used for treating conditions associated with inflammation.[3]

Some biological activities of this plant have previously been reported: the organic extracts were reported to have anti-mutagenic[4] and anti-tumour promoting properties,[5] and the aqueous extract was reported to inhibit eicosanoid synthesis.[3] The chemical constituents from this plant have also been investigated, but only the low-molecular weight constituents have been isolated and identified.[6] Due to the important physiological role of the complement immune system, complement modulation, either inhibition or stimulation, is related to various diseases and considered as an interesting target

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for drug development. Several plant polysaccharides are known to possess complement-modulating activity.[7] Such polysaccharides might be present in this plant.

To our knowledge, however, the isolation, purification and characterisation of polysaccharides from *A. ebracteatus* and subsequent evaluation of biological activity (complement fixing activity) have not yet been performed. In the present study, we report the fractionation and characterisation of polysaccharides isolated from *A. ebracteatus* and their effect on the complement system. The relationship of the chemical features of the polysaccharides with their activity is also considered.

2. Results and discussion

2.1. Isolation of polysaccharide fractions

A60 was separated into two main fractions (A601 and A602) and A100 also gave two main fractions (A1001 and A1002) after elution on the DEAE-Sepharose fast-flow column as shown in Figures 1 and 2 for A60 and A100, respectively.

Fractions A601 and A1001 were regarded as neutral polysaccharides as they were eluted with water and no acidic sugar was found in these fractions. Polysaccharides eluted with NaCl solution from anion-exchange chromatography are regarded as acidic polysaccharides. This is in keeping with the results from the sugar composition analysis for A602 and A1002, which were eluted in the range of 0.5–0.9 M NaCl as galacturonic acid was found in considerable amounts.

A602 and A1002 were considered to be major polysaccharide constituents for the corresponding water-soluble crude extracts. Appearance, yield, protein content and sugar composition of the polysaccharide fractions are shown in Table 1. All fractions had low

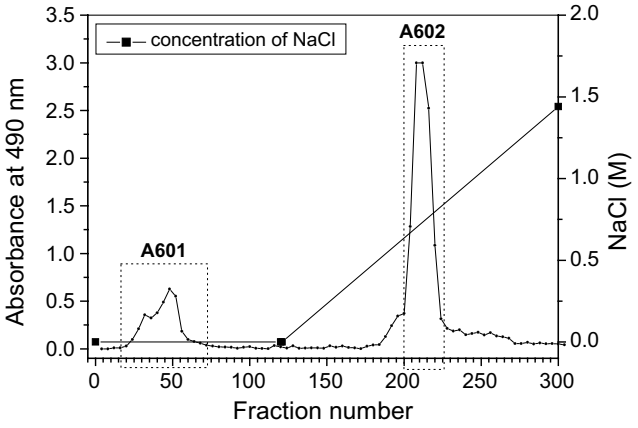

Figure 1. Anion-exchange chromatography of A60 on DEAE-Sepharose fast-flow.

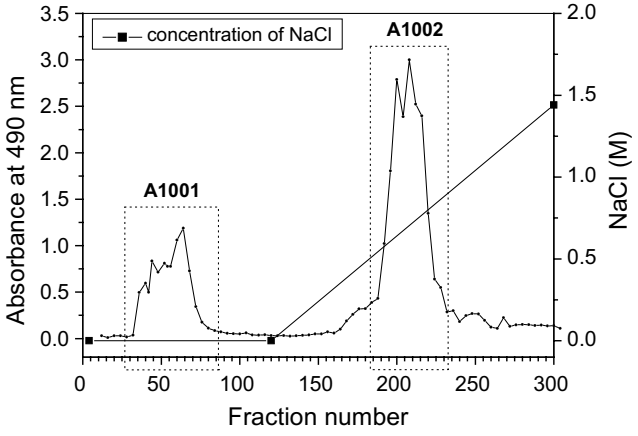

Figure 2. Anion-exchange chromatography of A100 on DEAE-Sepharose fast-flow.

Table 1. Appearance, yield, protein content and sugar composition (mol%) of polysaccharide fractions obtained after separation of the crude extracts on the DEAE-Sepharose fast-flow column

	60 °C water extract		100 °C water extract	
	A601	A602	A1001	A1002
Appearance	Cream, fluffy	White, fluffy	White, fluffy	White, fluffy
Yield[a]	20.0	50.2	10.1	48.9
Protein content (w/w)	4.8	6.4	5.9	6.2
Sugar composition[b]				
Ara	9.1	11.0	13.3	6.1
Rha	0.9	12.3	0.8	7.5
Fuc	Trace	0.8	Trace	0.3
Xyl	0.6	1.4	1.1	0.8
Man	3.6	1.4	2.3	0.4
Glc	4.3	3.1	9.9	2.2
Gal	55.0	18.7	39.6	11.0
3-*O*-Me-Gal	26.5	1.3	33.0	1.9
GalA	—	50.0	—	69.8

[a] Calculated as weight % of applied material.
[b] Mol% of total carbohydrate content.

Table 2. Glycosidic linkage composition (mol%) of the polysaccharide fractions obtained after separation of the crude extracts on the DEAE-Sepharose fast-flow column

Sugar	Type of linkage	Fraction			
		A601	A602	A1001	A1002
Ara	T*f*	1.8	2.5	2.7	2.3
	1,3	1.0	—	0.7	—
	1,5	4.9	7.2	6.0	3.8
	1,3,5	1.4	1.3	2.1	—
	1,2,5	—	—	1.8	—
Rha	T	—	3.1	—	—
	1,2	—	7.3	0.8	7.5
	1,2,4	—	1.9	—	Trace
Fuc	T	—	—	Trace	0.3
Xyl	T	—	—	1.1	0.8
Man	T	3.6	1.4	2.3	—
Glc	T	Trace	Trace	0.9	Trace
	1,4	4.3	3.1	7.9	—
	1,6	—		0.4	—
	1,4,6	—	—	0.6	—
Gal[a]	T*p*	7.8	3.5	8.3	1.7
	T*f*	4.1	—	1.5	—
	1,4	55.8	9.8	56.3	5.3
	1,6	4.6	1.4	2.2	—
	1,4,6	9.5	3.9	4.3	5.3
	1,2,4		1.4		0.5
GalA	T	—	1.8	—	Trace
	1,4	—	45.4	—	69.8
	1,3,4	—	2.0	—	—
	1,2,4	—	0.8	—	Trace

[a]As combined amount of galactose and 3-*O*-Me-galactose.

protein contents (<6.5%). As can be seen in Tables 1 and 2, A601 and A1001 shared some similarities in terms of types of main sugar components and their glycosidic linkages present. Both fractions were rich in galactose, 3-*O*-methylgalactose, arabinose and glucose in decreasing order. However, a significant difference in galactose content between these two was observed, and the relative amount of 3-*O*-methylgalactose was higher in A1001 than A601. The glucose content in A1001 was higher than A601, probably arising from starch solubility (a positive test with I_2/KI was observed for A601 and A1001) due to the higher extraction temperature and this will not be considered further. A602 and A1002 were rich in galacturonic acid, galactose, rhamnose and arabinose in decreasing order, but there was a significant difference in the amount of galacturonic acid between the latter two, 3-*O*-methylgalactose was also present in small amounts. This indicates that the fractions A601 and A1001 most likely contained the same type of polysaccharide(s) and this was also evidently the case for fractions A602 and A1002. It also indicates that there is no significant difference in the type of polysaccharides obtained due to different temperature extraction. However, the polysaccharide content obtained from the 100 °C extract was much higher than those obtained from 60 °C extract: this finding is relevant since in Thai traditional medicine, this plant is prepared as a decoction (boiled in water). We will now focus on A1001 and A1002 since these are likely to be the constituents present in the highest concentration in the traditional preparation.

2.2. Structural features

The types of linkages present in A1001 and A1002 determined by methylation and GC–MS analysis are shown in Table 2. Galacturonic acid was the main sugar component in A1002, most of which is $(1\rightarrow4)$-linked. Due to the dominant feature of A1002 consisting of a linear chain of $(1\rightarrow4)$-linked galacturonic acid units (smooth region), A1002 is considered to be a pectin-type polysaccharide. Along with the presence of a significant amount of $(1\rightarrow2)$-linked rhamnose residues, we presumed that the $(1\rightarrow4)$-linked galacturonic acid units were most probably interrupted by the insertion of $(1\rightarrow2)$-linked rhamnose residues giving a typical Type I rhamnogalacturonan backbone (RG-1). The neutral sugars, more specifically, the galactose and arabinose units are possibly attached to RG-1 as complex neutral side chains of arabinogalactans on position 4 of rhamnose, and may give rise to the so-called 'hairy region', the second feature of pectin-type polysaccharides. Some of the galactose units represent also the 3-*O*-methylgalactose found in the polymer. The other neutral sugars

like xylose and fucose are present in trace amounts and these types of neutral sugars can also be found in pectins.[8] However, the fine structure of the neutral sugar side chains has not yet been determined.

A1001 contained mainly galactose and 3-*O*-methylgalactose along with arabinose as a minor component, while xylose, mannose and rhamnose were present in less amounts. The amount of (1→4)-linked galactose calculated from methylation based on the amount of 2,3,6-tri-*O*-methylgalactose was found to be higher than the total amount of galactose calculated from methanolysis. This indicates that some of the 2,3,6-tri-*O*-methylgalactose had been derived from 3-*O*-methylgalactose. Since iodomethane (CH_3I) was used as a methylating agent, the precise amount of linkage composition of galactose and 3-*O*-methylgalactose as determined from the amount of methylated sugar products cannot be established. To investigate the linkage type of 3-*O*-methylgalactose, the methylation analysis will be performed in a future study using CD_3I instead of CH_3I. However, our current study suggests that galactose and 3-*O*-methylgalactose were likely to be mainly (1→4)-linked. Arabinose residues were the third most abundant sugar units in this fraction, most of these were (1→5)-linked and shown to be considerably branched at C-2 or C-3. Arabinose residues can fulfil a variety of roles—as building blocks for isolated arabinans, linked to galactan chains, in isolated arabinogalactans commonly found in the primary cell wall of plants[9] and they are also frequently associated with the isolation of pectins.[10]

The chemical structure of A1001 and A1002 were also studied by ^{13}C NMR and ^{1}H NMR spectroscopy. The ^{13}C NMR spectrum of A1001 (Fig. 3) provided relevant information on the anomeric configuration in the polymer. A sharp signal at 108.5 and 68.0 ppm were tentatively assigned to C-1 and C-5 of (1→5)-linked α-arabinofuranosyl residues. The signals at 104.5 and 105.3 ppm were assigned to C-1 of (1→4)-linked β-galactopyranosyl and 3-*O*-Me-galactopyranosyl residues, respectively. The signals at 71.6, 74.0, 78.6 and 75.5 ppm were assigned to C-2, C-3, C-4 and C-5 of the β-galactopyranosyl residues, respectively. The region 61.8–62.2 ppm relates to the signal from the C-6 of the galactose and the 3-*O*-methylgalactose units, and C-5 of arabinofuranosyl residues that are not linked through C-5. The intense sharp signal at 58.5 ppm represented the carbons of OCH_3 groups from 3-*O*-methylgalactose, typical chemical shifts for OCH_3 carbons fall in the range of 55–61 ppm.[11] This is in good agreement with the result from sugar composition analysis, which found significant amounts of 3-*O*-methylgalactose. A1001 also contained acetyl groups in the polymer as indicated by the presence of signals at 21.2 and 174.8 ppm, which belonged to methyl carbons and carbonyl groups of acetyl groups, respectively. This suggests that some of

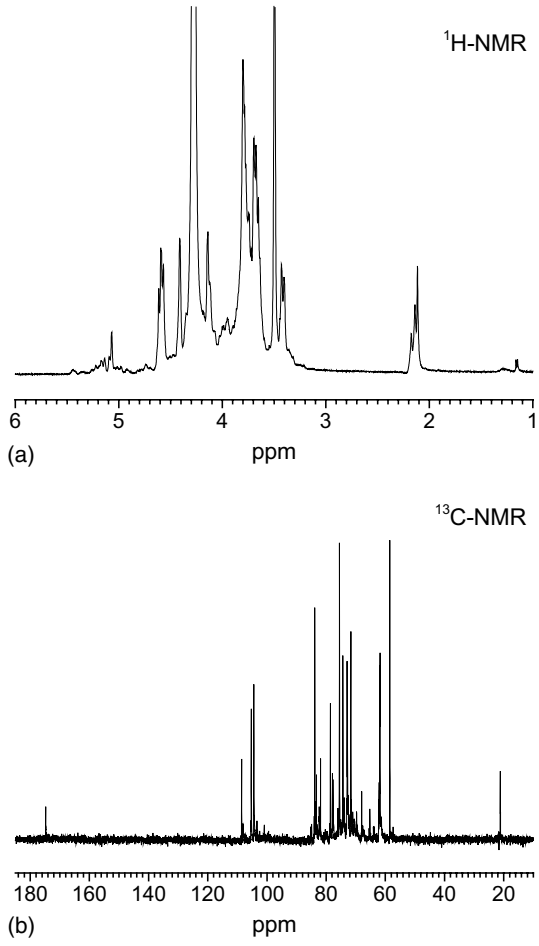

Figure 3. ^{1}H and ^{13}C NMR spectra for A1001.

hydroxyl groups on the neutral sugars in the polymer were acetylated and further structural details will be the subject of a future study. The proton NMR for A1001 the group of signals between 4.5 and 4.6 shows the presence of the anomeric protons for β-linked galactose and 3-*O*-methylgalactose. Signals at 3.7, 3.8, 4.1, 3.7 and 3.9 ppm were assigned to H-2, H-3, H-4, H-5 and H-6 of (1→4)-linked β-galactopyranosyl residues, respectively. The proton NMR for A1001 also shows that a significant amount of acetyl groups are present in the polymer with the group of signals in the region 2.1–2.2 ppm. The signal at 3.5 represents the protons of the methyl group in 3-*O*-methylgalactose. In the ^{13}C NMR spectrum for A1002 (Fig. 4), the signals from the main sugar components were found. Signals were assigned in accordance with previous work[10,12–14] as shown in Table 3. The spectrum had an intense resonance at 53.8 ppm representing the methyl carbon of the methyl ester (COO*C*H3). This indicates that A1002 is a highly methyl-esterified pectin. However, the degree of methylation was not determined. The spectrum also had a small signal at 21.2 ppm, which probably belongs to methyl carbons of acetyl groups ($OCOCH_3$) as neutral sugars and/or galacturonic acid could be acetylated[14]

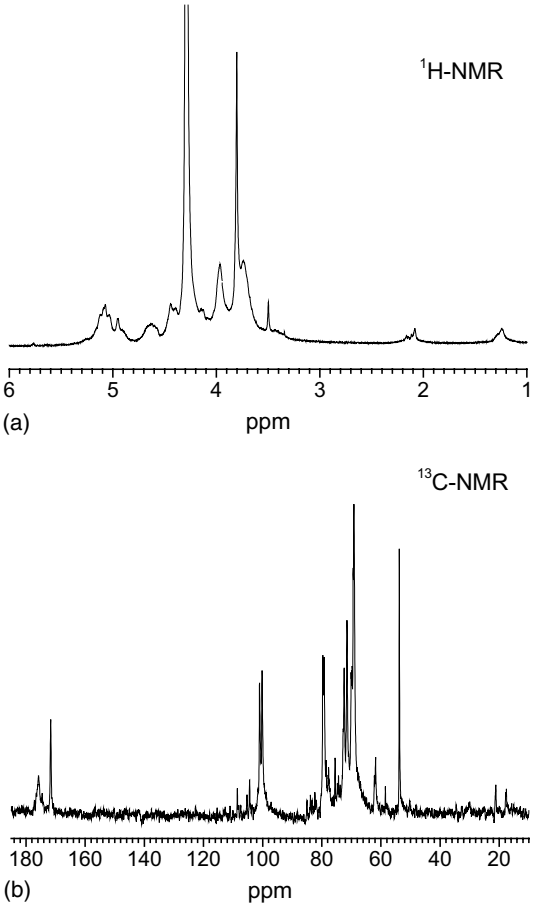

Figure 4. ^{1}H and ^{13}C NMR spectra for A1002.

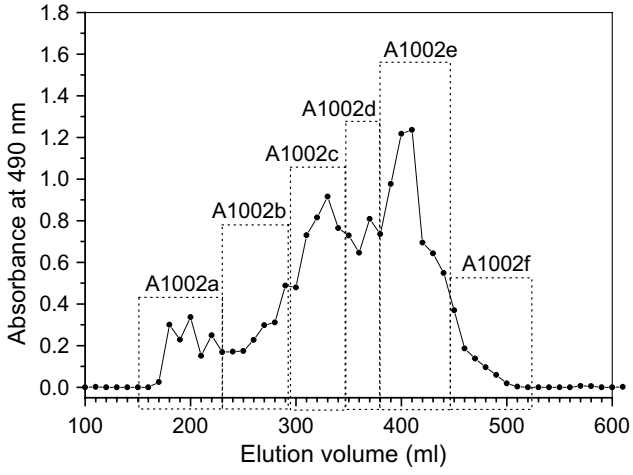

Figure 5. Size-exclusion chromatography of A1002 on Sephacryl 400.

and 58.5 ppm the OCH_3 of the 3-*O*-methylgalactose. Proton NMR of A1002 had a signal at ~1.3 ppm representing the methyl group at position 6 of rhamnose. Signals at 2.1 ppm from acetyl protons and 3.5 ppm from the methyl protons in 3-*O*-methylgalactose are seen.

A1002 was further fractionated by SEC into six fractions: A1002a, A1002b, A1002c, A1002d, A1002e and A1002f (Fig. 5). Yield and sugar composition for all A1002 sub-fractions are shown in Table 4. Interestingly, all major sugar components especially galacturonic acid, galactose, arabinose and rhamnose were found in all A1002 sub-fractions, but it was surprising that the rare sugar 3-*O*-methylgalactose was confined mainly to A1002a in an amount of approximately 14%. This indicated that all fractions contained pectin-type polysaccharides.

The presence of a considerable amount of 3-*O*-methylgalactose in A1001 and also a fair amount in A1002a is interesting and this may be of chemotaxonomical importance. The fact that this sugar could be of such an importance is also a conclusion drawn by Popper et al.[15] They have stated that the presence of 3-*O*-methylgalactose in substantial amounts may be an autapomorphy, meaning a character that uniquely defines a taxon.[16] Popper et al. found 3-*O*-methylgalactose in the lycophyte primary cell walls, and gave an overview of the presence of this sugar in various taxa of green plants was given.[15] It was concluded that amongst the lower plants this substance is mainly, for the time being, found in lycophytes. Extracellular polysaccharide produced by the Chlorococcaean *Ankistrodesmus densus* also contains 3-*O*-methylgalactose in substantial amounts.[17] It is interesting to note that this sugar also appears in significant amounts in the carbohydrate moiety of gastropod haemocyanins, which are glycoproteins.[18,19] 3-*O*-methylgalactose was isolated from the bacteria *Actinomadura madurae*, and was first called madurose. This sugar is an important component of the cell wall of *Actinomadura* species.[20] It also appears to be a feature of

Table 3. Summary of ^{13}C chemical shifts for A1002

Residues	Chemical shift (ppm)				
	C-1	C-2+3	C-4	C-5	C-6
α-(1→4)-Gal*p*A	100.2	69.2–69.7	79.2–79.7	71.5	175.8
6-Me-Gal*p*A	101.0	69.2–69.7	79.2–79.7	71.5	171.7
β-(1→4)-Gal*p*	104.4	72.5	n.d.	n.d.	61.8
α-(1→5)-Ara*f*	108.6	n.d.	n.d.	n.d.	—
α-(1→2)-Rha*p*	n.d.	n.d.	n.d.	n.d.	17.6

n.d. = not determined.

Table 4. Yield, weight average molecular weight (M_w) and sugar composition (mol%) of A1002 sub-fractions obtained after size-exclusion chromatography of A1002

	Fraction						
	A1002a	A1002b	A1002c	A1002d	A1002e	A1002f	PM II[a]
Yield[b]	8.6	8.6	24.0	16.0	31.4	3.4	—
Molecular weight (kDa)	1500 ± 30	276 ± 20	58 ± 6	42 ± 3	32 ± 4	29 ± 4	46–48
Sugar composition[c]							
Ara	7.3	7.1	5.6	4.3	3.9	4.3	8.8
Rha	5.9	5.7	5.6	5.5	5.5	5.7	4.2
Fuc	0.6	0.3	0.3	0.3	Trace	0.3	—
Xyl	0.7	0.6	0.6	0.6	0.5	0.6	—
Man	1.4	—	—	—	—	—	—
Glc	5.1	2.1	1.5	1.6	1.5	2.3	7.3
Gal	16.2	13.1	10.0	8.2	6.9	8.1	8.0
3-*O*-Me-Gal	13.9	1.8	0.5	Trace	Trace	Trace	—
Total neutral sugars	51.1	30.7	24.1	20.5	18.3	21.3	28.3
GalA	49.1	69.4	75.9	79.5	81.8	78.7	71.1

[a]Data obtained from Ref. 25.
[b]Calculated as weight % of applied material.
[c]Mol% of total carbohydrate content.

the Actinomycetes and is proposed to be a taxonomic marker for these.[21]

2.3. Homogeneity and molecular weight

SEC coupled to refractive index (RI) and multi-angle laser-light scattering (MALLS) detectors was used to assess the homogeneity and molecular weight of polysaccharide fractions. This technique can provide absolute molecular weight and its distribution. The RI gives a signal proportional to concentration whereas the light scattering signal from MALLS depends on both concentration and molecular weight. Figure 6 shows the SEC/MALLS chromatogram of A1001, a broad heterogeneous RI profile probably arising from a mixture of polysaccharides in keeping with the speculation from the sugar composition analysis (Table 1). The overlapping of LS and RI peaks indicates that about 40% of the mixture is high-molecular-weight polysaccharide as estimated from the area under the RI peak eluted between 11.6 and 17.7 mL. The relatively large RI peak eluting near total permeation volume indicated that A1001 contained a significant amount of low-molecular-weight polysaccharide, which coincides with very low LS signal. However, no molecular weights were estimated from Figure 6 because the RI peaks were not well separated. Figure 7 shows the SEC/MALLS chromatogram for A1002: the shoulder in the RI peak at ~14.5 mL coincides with the main light-scattering peak suggesting the presence of high-molecular-weight polysaccharide component, whereas the main component based on RI profile (concentration profile) had a peak elution volume ~17.5 mL. The weight average molecular weight (M_w) over the whole peak was (180 ± 10) kDa. An additional small RI peak eluted between 20.5 and 22.1 mL indicating the total permeation volume. Recalling the results from sugar composition analysis of A1002 and its

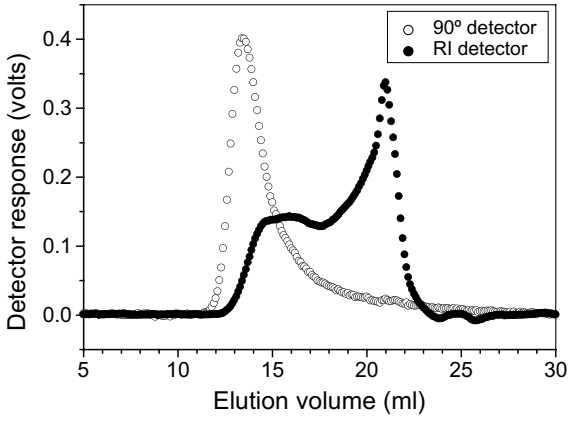

Figure 6. Light-scattering and refractive-index profiles from size-exclusion chromatography coupled to multi-angle laser-light scattering of A1001.

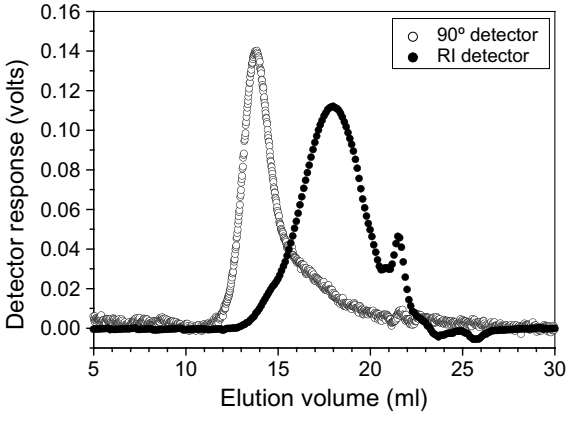

Figure 7. Light-scattering and refractive-index profiles from size-exclusion chromatography coupled to multi-angle laser-light scattering of A1002.

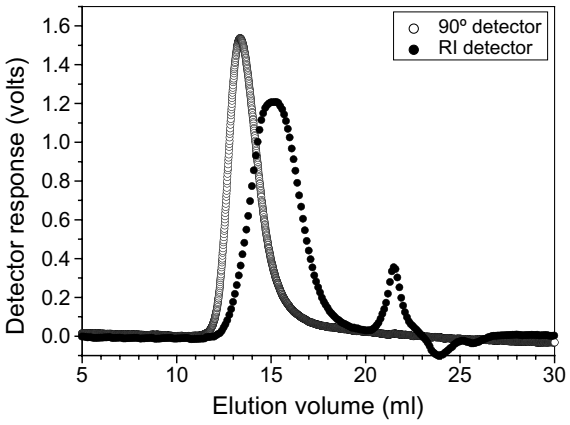

Figure 8. Light-scattering and refractive-index profiles from size-exclusion chromatography coupled to multi-angle laser-light scattering of A1002a.

sub-fractions, we presumed that neutral sugars as neutral sugar side chains are associated with (RG-1), a characteristic of pectin-type polysaccharide.

Now consider the further fractionated samples (A1002a to A1002f). Figure 8 shows a relatively single narrow distribution of RI and LS profiles for sample A1002a, a fraction with by far the highest percentage of total neutral sugars, which may indicate either neutral sugars are an integral part of the pectin (not being separate chains of neutral sugars) or the presence of separate chains of neutral sugars, noncovalently bound to pectin. The peak of A1002a appears to correspond to the shoulder of the main RI peak of A1002 (Fig. 7). Figure 9 shows RI chromatograms of all the A1002 sub-fractions; they all appeared as single symmetrical peaks and increasing peak elution volume from A1002a to A1002f indicated the decreasing molecular weight, which agrees with the M_w values obtained from SEC/MALLS as shown in Table 4. The increasing order from the lowest M_w fraction (A1002f) to highest M_w fractions probably originates from the increasing in a number or size of the neutral side chains, mainly arabinogalactans.

According to the sugar analysis results, the RI profiles and M_w values for all A1002 sub-fractions, it can be concluded that all A1002 sub-fractions contained pectin-type polysaccharide, which has molecules similar in fundamental structure, but differ in number and/or length of neutral sugar side chains giving rise to the differences in molecular weight. But the A1002a and A1002b differed greatly in the molecular weight from the others, especially A1002a, which also was the only fraction with a significant amount of 3-*O*-methylgalactose.

2.4. Effect on the complement system

The effect of A1001 and A1002 on the complement system were compared to that of PM II, which has been known to possess this activity.[22] Both A1001 and A1002 exhibited higher activity than PM II as can be seen in Figure 10. The activity for both fractions was concentration dependent at the range of low concentrations studied and reached maximum at the concentration $\geqslant 67\,\mu g/mL$, with an ICH_{50} (concentration inhibiting the haemolysis by 50%) of approximately 10 μg/mL compared to approximately 50 μg/mL for PM II. Both A1001 and A1002 are therefore considered as polysaccharides with very high effect on the complement system. A1002 sub-fractions were also subjected to the assay and their concentration–activity profiles are shown in Figure 11. It was interesting to note that the two fractions A1002a and A1002b with much higher molecular weight than the other four fractions had the highest effect on the complement system, much higher than that for the original A1002. The four samples with the lower molecular weights had activities comparable to that of PM II. This indicated that the fraction with larger

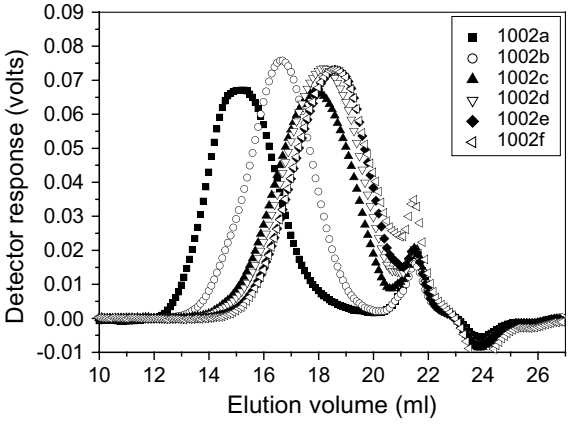

Figure 9. Refractive-index profiles from size-exclusion chromatography coupled to multi-angle laser-light scattering of A1002 sub-fractions.

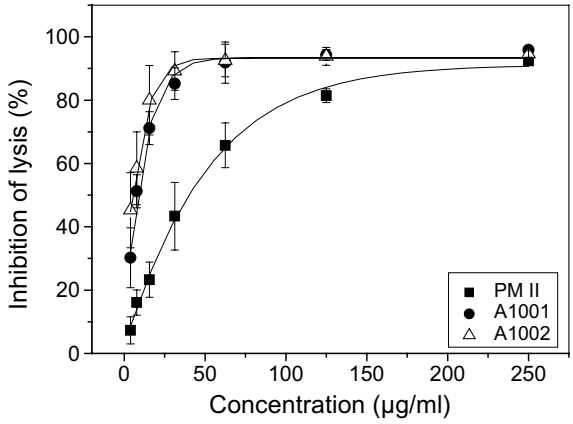

Figure 10. Concentration-dependent effect of A1001 and A1002 on the inhibition of haemolysis. Results are presented as mean ± SD of triplicate determinations.

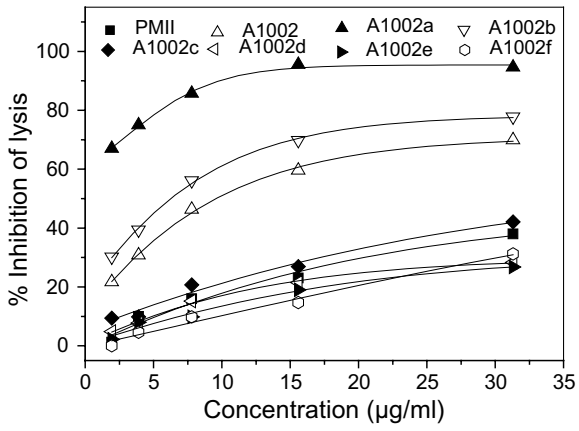

Figure 11. Concentration-dependent effect of A1002 sub-fractions on the inhibition of haemolysis. Results are presented as mean of duplicate determinations.

amounts of neutral sugar side chains had higher activity, as mentioned earlier, the difference in M_w among A1002 sub-fractions was partly due to the amount of neutral sugar side chains. But the difference in activity can also be related to the much higher molecular weights of A1002a and A1002b compared to the other four. This agrees very well with the conclusion from other workers: the effect on the complement system of pectin-type polysaccharides is related to the number of neutral sugar side chains in the ramified region of pectin.[23–25] This means that the ramified region from each pectin had a more potent complement activating activity than the corresponding original pectin, whereas the polygalacturonic acid backbone has less or negligible activity.[26,27] For the polymers investigated in this paper—the presence of 3-*O*-methylgalactose may also play role for the activity. This type of sugar has only been found in a few polysaccharide present in land plants, one of them is from the bark of *Ulmus glabra* Huds., but the effect on the complement system has not been investigated.[28]

It is well known that not all pectins have effects on the complement system.[23] It has been summarised that the ramified regions may be responsible for effects on the complement system and a β-(1→6)-linked galactose side chain attached to the rhamnogalacturonan backbone is the minimum requirement for this activity.[23] This can possibly explain why A1002 had the activity and also PM II, despite the differences between galactan side chains in A1002 and PM II where the former are β-(1→4,6)-linked, whereas the latter are β-(1→3,6)-linked. Notably both contained branched 3,5-arabinan side chains, whose presence has also been reported to effect the complement system in a way, which correlates with the presence of this type of side chains.[27]

Comparing molecular weight and sugar composition between A1002c, A1002d, A1002e and A1002f with those of PM II (see Table 4) shows striking similarities and they all appeared to have comparable activity as shown in Figure 11. This may indicate that these four fractions from *A. ebracteatus* share common structures with PM II.

A1001 appears to contain a mixture of polysaccharides as indicated by the SEC/MALLS chromatogram (Fig. 6). It is not clear at present, which polysaccharide is responsible for the activity unless further purification is performed. The presence of (1→2,5) and (1→3,5)-linked arabinose units, in this fraction may possibly be responsible for the activity. This is because certain arabinans, for example, α-(1→2,5)-arabinan isolated from *Zizyphus jujuva*,[29] α-(1→3,5)-arabinan with α-(1→4)-glucan complex from *Bupleurum falcatum*[30] and α-(1→3,5)-arabinan with β-6-linked galactose from *Artemisia princeps*[31] have been reported to have an effect on the complement system. Arabinose units would be part of arabinogalactans as mentioned earlier. However, most of the arabinogalactans with effects on the complement system, have been reported to be arabinogalactans type II, that is, the arabinogalactans possessing a backbone of β-3,6-galactan[26] whereas (arabino)galactan in A1001 comprised of a β-4-galactan backbone (arabinogalactans type I). Apart from galactan in arabinogalactans either isolated or as attached to galacturonan in pectins, only 4-linked galactan with glucan isolated from *Dipsacus asperoides* roots has been reported to exhibit effects on the complement system.[32]

Fraction A1002a had a high 3-*O*-methylgalactose content and also had a very high activity and it may well be the high activity of the neutral fraction (A1001) is caused by the 3-*O*-methylgalactose containing polymer. Further studies on this neutral fraction will be performed in order to discover what polysaccharides are the most potent and the structure–activity relation will also be investigated.

3. Concluding remarks

Polysaccharides with effect on the complement system that have been isolated from *A. ebracteatus* are heteropolysaccharides. The neutral polysaccharide is rich in galactose, 3-*O*-methylgalactose and arabinose, whereas the acidic polysaccharide consists mainly of galacturonic acid, galactose, arabinose and rhamnose featuring a pectin-type polysaccharide with a small amount of 3-*O*-methylgalactose. Both exhibit higher effects on the complement system than PM II. The chemical feature of A1002 consolidates previous findings that pectin-type polysaccharides rich in neutral sugar side chains with certain type of linkages have effects on the complement system. But the high amount of 3-*O*-methylgalactose also present in the pectin seemed to be of importance for enhancing the activity.

Further fractionation of A1001 will be performed in the near future to see which polysaccharide is responsible for the activity as well as more detailed chemical structural analysis of A1002.

The presence of 3-*O*-methylgalactose is unique, as only a few examples of this monomer have been reported in the literature from higher plants. The presence of this monosaccharide may be of chemotaxonomic importance.

The complement-fixation assay used cannot differentiate between activation and inhibition of the complement system. Due to this the polysaccharide fractions will be subjected to more specific assay(s) to find the mode of action and to support the traditional use of this plant. Our present studies would appear to indicate that polysaccharides may be the important active ingredient in traditionally prepared remedies.

4. Experimental

4.1. Plant materials

The stems of *A. ebracteatus*, which were collected in Thailand, were supplied from Medicinal Plant Analytical Development Section, Research and Development Institute, The Government Pharmaceutical Organization (GPO), Thailand.

4.2. Extraction and fractionation

4.2.1. Extraction. Ground dried stems with residual moisture content of 16.8% w/w (500 g) were first pre-extracted with 80% ethanol to remove coloured materials and the extraction was repeated twice. Subsequently, the dried ethanol extracted residue was extracted with about 10 L of water at 60 °C for 3 h, filtered through gauze and centrifuged to remove any water-insoluble materials. The aqueous extract was concentrated under reduced pressure at 40 °C and lyophilised. The lyophilised sample was re-dissolved in 1.5 L of water, then the polysaccharides in the solution were precipitated by the addition of 2 volumes of ethanol. The resulting gel-like precipitate was dissolved in water and dialysed against distilled water in a Spectrapor dialysis tubing (molecular weight cut off = 3500 Da). The nondialysable portion was again centrifuged to remove insoluble material, the supernatant was lyophilised and a brown crumbly mass was obtained (A60, yield: 0.34% w/w calculated from dry plant material).

After extracting the ground stem in water at 60 °C, the residue was further extracted in water at 100 °C, treated similarly as described above and a light brown crumbly mass was obtained (A100, yield: 0.71% w/w calculated from dry plant material).

4.2.2. Fractionation. Anion-exchange chromatography: 1.2 g of A60 was fractionated by anion-exchange chromatography on a column (25 × 5 cm, i.d.) of DEAE-Sepharose fast-flow (Pharmacia). The sample was dissolved in distilled water and left overnight. The solution was filtered through a 0.8 μm membrane filter and applied onto the column. The column was first eluted with distilled water followed by a NaCl gradient (0–2 M). Fractions of 12 mL were collected and monitored for the presence of carbohydrate using phenol–sulfuric acid assay.[33] Fractions containing carbohydrate were pooled, dialysed and lyophilised. Similarly, 2.5 g of A100 was dissolved, filtered and applied onto the column as described above. Fractionation profiles for A60 and A100 are illustrated in Figures 1 and 2, respectively.

Size-exclusion chromatography: the acidic fraction from A100 designated as A1002 (500 mg) was further fractionated on a column (80 × 3 cm, i.d.) of Sephacryl 400, eluted with 10 mM NaCl. Fractions containing carbohydrate were pooled, dialysed and lyophilised. The SEC elution profile of A1002 is shown in Figure 5.

4.3. Quantitative determination of protein content

The protein content of the samples was determined by Micro BCA™ Protein Assay Reagent Kit (U.S. Patent 4,839,295; Pierce, Rockford, IL) using albumin as protein standard.

4.4. Determination of the sugar composition of polysaccharides

The sugar composition analysis was determined by methanolysis and gas chromatography (GC). Briefly, the polysaccharide samples were subjected to methanolysis with 4 M HCl in anhydrous methanol for 24 h at 80 °C. Mannitol was added before methanolysis was performed as an internal standard. This was followed by trimethylsilylation: the trimethylsilylated samples were subjected to gas chromatographic analysis.[34]

4.5. Determination of the glycosidic linkage composition of polysaccharides

The glycosidic linkage analysis was determined by methylation and gas chromatography-mass spectroscopy (GC–MS). Prior to methylation, the samples containing uronic acid (A602 and A1002) were essentially reduced to the corresponding neutral sugars at the polymer level.[35] After this reduction, methylation of the polymers was carried out according to the procedure previously described[36] followed by GC–MS analysis of the derived partially methylated alditol acetates.[34,37]

4.6. ^{1}H and ^{13}C NMR

Samples A1001 and A1002 were dissolved in D_2O at concentrations of approximately 30 mg/mL. ^{1}H and ^{13}C NMR spectroscopy were performed with a Bruker DRX 400 at 400.2 and 100.6 MHz, respectively, at 70 °C. The

chemical shifts were expressed in ppm relative to the resonance of the internal standard, 3-trimethylsilyl-1-propanesulfonic acid (sodium salt).

4.7. Homogeneity and molecular weight determination

Homogeneity and molecular weights of the polysaccharide fractions were determined by size-exclusion chromatography coupled to multi-angle laser-light scattering (SEC-MALLS). Each sample was dissolved at a concentration of 2 mg/mL in phosphate buffered saline, pH 7.0, $I = 0.1$.[38] Solutions (100 μL) were injected on to the column systems consisting of a Phenomenex guard column, TSK G6000PW and TSK G4000PW connected in series. An eluent identical to the solvent used to dissolve the samples was pumped at the flow rate of 0.8 mL/min. Details of instrumentation and software analysis of molecular weight were as previously described.[39]

4.8. Complement fixing activity of the polysaccharides

Samples were subjected to a complement-fixation assay (Method A)[22] using polysaccharide fraction PM II from *Plantago major* as a positive control.[34]

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