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Characterization of *Acacia mangium* Tannin Fractions Extracted with Different Organic Solvents

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The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), nuclear magnetic resonance and gel permeation chromatography were introduced to characterize the structures of tannins in diethyl ether fraction, ethyl acetate fraction and water fraction extracted from *Acacia mangium* bark. The spectra obtained through MALDI-TOF MS analysis revealed that the tannins in each fraction were predominantly consisted of (epi)catechin repeating units combined with (epi) gallocatechin and (epi) afzelechin. Also, procyanidin, prodelphinidin and propelargonidin features were all presented in both MALDI-TOF MS and ^{13}C NMR spectra. For these oligomer units, both *cis*- and *trans*- form were observed through ^{13}C NMR analyses. The results from GPC test revealed that tannins in diethyl ether fraction, ethyl acetate fraction and water fraction were primary consisted of dimer and trimer, pentamer and hexamer, nonamer and decamer, respectively. Based on the information obtained from MALTI-TOF MS, ^{13}C NMR and GPC, the structures of tannins in each fraction were finally made clear. The results could provide a valuable reference for the fine applications of *Acacia mangium* tannin.

Keywords: *Acacia mangium* tannin, Structure, MALDI-TOF MS, ^{13}C NMR, GPC.

INTRODUCTION

Acacia mangium is a fast-growing tree species and widely distributed in tropical and sub tropical area[1]. It is a member of the mimosa family and native to northern Australia, New Guinea and Indonesia[2]. In the late 1980s, large scale plantings of *A. mangium* had been in progress in southeast China for making pulp wood in plantation forests. Nowadays, it has already become one of the most important commercial plants in China and south Asia[3]. *A. mangium* tannin (AMT) is composed of flavan-3-ol repeating units and belongs to the condensed type[4]. "Angular" structure, "twice-angular" structure and relative higher molecular weight were considered as the prominent structural features of the AMT molecule[5]. These structural features were also proposed to be closely associated with its performance in tanning and adhesive industries.

Like other vegetable tannins, AMT is a kind of natural product with polydispersity properties. The molecular AMT are composed by similar repeating units but different in polymerization degrees and molecular weights[6]. Meanwhile, this structural heterogeneity is considered as the most important property of tannins[7]. It had been proved that reactions between collagen and AMT were differed with respect to the molecular weight of the AMT[8]. However, structures of vegetable tannins are also essential to their properties and chemical reactivity and ultimately determine their commercial values[9-11]. In this study, *A. mangium* bark was extracted with aqueous acetone, the extraction was then extracted with petroleum ether, diethyl ether and ethyl acetate successively and then tannins with different molecular weight were obtained. The characterizations of AMTs in each fraction were investigated with MALDI TOF-MS, ^{13}C NMR and GPC, meanwhile, possible structures were also clarified. The results could provide a valuable reference for fine applications of the AMT.

EXPERIMENTAL

Preparation of *A. mangium* tannins[8]: According to the previous research, air dried *A. mangium* bark (5 years of its tree age, collected from Baise Tree Farm in Guangxi, China) was smashed and extracted with aqueous acetone (70 %), afterwards, the extraction was degreased with petroleum ether and then extracted with ethyl acetate and diethyl ether in order to obtain the AMTs. After reduced pressure distillation and freeze-drying, tannins in water fraction, ethyl acetate fraction and diethyl ether fraction were properly prepared.

Purification of *A. mangium* tannins: The crude AMTs (1 g) was purified using a chromatography on Sephadex LH-20 column (48.5 cm × 2.5 cm i.d.). Each fraction was eluted with 1500 mL 50 % aqueous methanol on the Sephadex LH-

20 to remove impurities, tannins in the column was then eluted with 70 % aqueous acetone and the eluant were properly collected. After reduced pressure distillation and freezing dried, the purified AMTs were finally obtained.

MALDI-TOF MS analysis: Purified *A. mangium* tannin (4 mg) was dissolved in 1 mL 30 % aqueous acetone, then subjected to a Bruker Reflex III MALDI-TOF mass spectrometer (Germany). Cesium chloride and dihydroxy-benzoic acid were used as matrix to enhance ion formation process. The pulsed nitrogen (337 nm) with 3 ns of laser pulse duration was used as an irradiation source. The ions were detected under a positive-ion mode at an accelerating voltage of 20.0 kV. Polyethylene (3000 Mw) was used as a standard sample for system calibration[12].

^{13}C NMR analysis: 100 mg of each AMT was dissolved in 1 mL deuterated aceton solution and transferred into a NMR tube. The NMR analysis was performed on a Varian Unity Inova 400 NMR spectrometer (American) at 125.78MHz.

GPC analysis: 1 mg of AMT was dissolved in 1 mL water and then injected to a 150-C ALC/GPC instrument (American), a combination of μ-Styrage GPC column (10, 50, 100 and 1000 nm) was used to measure the molecular weight of tannins. Mobile phase was pumped into the column at the flow-rate of 1.0 mL/min, 150 bars at 20 ºC. A molar mass standard curve was obtained using monodispersed polystyrene as a standard sample.

RESULTS AND DISCUSSION

MALDI-TOF MS analysis: MALDI TOF mass spectra of the AMTs in water fraction (Fig. 1), ethyl acetate fraction (Fig. 2) and diethyl ether fraction (Fig. 3) showed a clear repetitive pattern of peaks. These series of peaks allow for the identification of specific oligomer series present in the tannin sample. Fig. 1 showed a series of major peaks at 1194, 1482, 1786, 2075, 2363, 2651, 2941, 3226, 3512 (serious A). In this series, peaks were separated by 288 and 304 Da. Similarly, a serious of major peaks at 1194, 1482, 1770, 2059, 2347, 2635, 2924 and 3210 Da (serious B) were shown in Fig. 2. Peaks in serious B were all separated by 288 Da. In Fig. 3, major peaks were 617 and 905 Da (serious C), which was also separated by 288 Da. These regular 288 Da mass increasing indicated that (epi) catechin (288 Da) is the prime structure unit of all the AMTs[13]. In other words, the AMTs in water fraction, ethyl acetate fraction and diethyl ether fraction were mainly procyanidin.

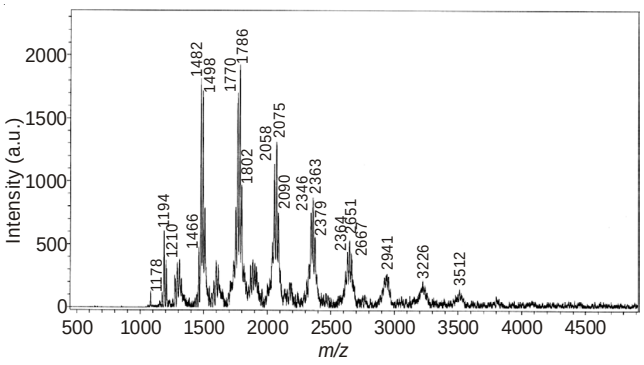


Fig. 1. MALDI TOF mass spectra of AMT in water fraction

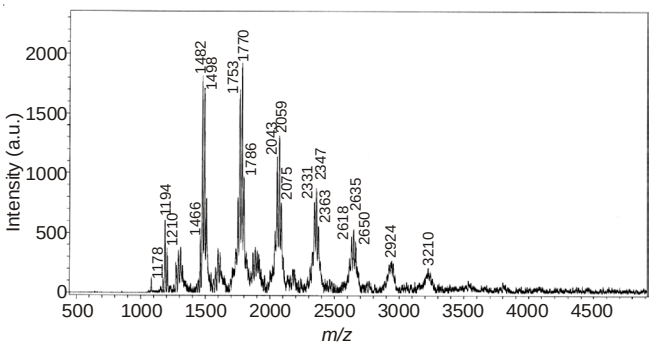


Fig. 2. MALDI TOF mass spectra of AMT in ethyl acetate fraction

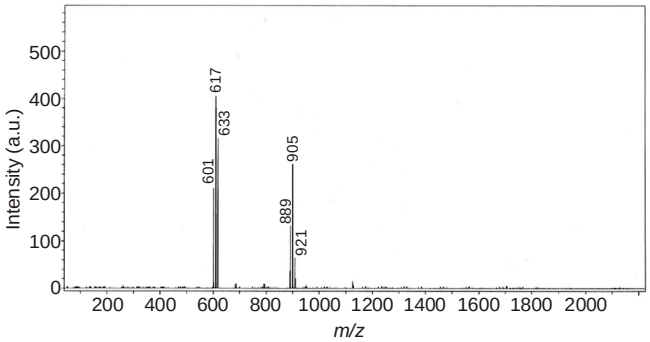


Fig. 3. MALDI TOF mass spectra of AMT in diethyl ether fraction

Similar patterns were discovered in other peaks with lower intensity, such as: 1178, 1466, 1770, 2058, 2346, 2634, 1210, 1498, 1802, 2090, 2379 and 2667 Da in Fig. 1, 1178, 1466, 1753, 2043, 2331, 2618, 1210, 1498, 1786, 2075, 2363 and 2650 Da presented in Fig 2, 601, 889, 633 and 921 Da in Fig. 3. In these series, peaks were also separated by 288 and 304 Da. Meanwhile, a regular 16 Da mass increase or decrease was found compared these peaks to the adjacent ones in series A, B and C (Fig. 4a), indicating the existence of (epi) afzelechin (272Da) and (epi) gallocatechin (304 Da) which was 16 Da more or less compared to (epi) catechin (288 Da) (Fig. 4b). These evidences indicated the AMTs in each fraction were mainly composed of procyanidin coexisted with prodelphinidin and propelargonidin. The chemical structure of flavan-3-ol monomer units and condensed tannins is presented in Fig. 5.

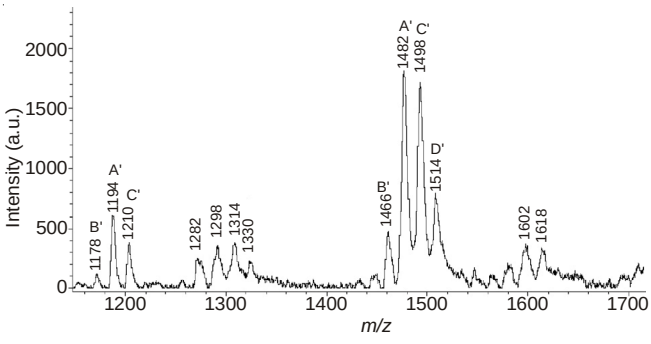
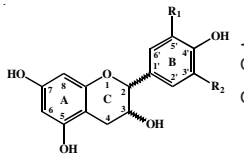


Fig. 4. MALDI TOF mass spectra of AMT in water fraction (1200-1700Da)



R_1	R_2	R_3	Flavan-3-ol	Condensed tannins
OH	H	H	(epi) catechin	procyanidin
OH	OH	H	(epi) gallocatechin	prodelphinidin
H	H	H	(epi) afzelechin	propelargonidin

Fig. 5. Chemical structure of flavan-3-ol monomer units and condensed tannins [Ref. 7]

The number of repeating units in each oligomer was calculated by using the following expression: $[M + Na]^+ = 23.0 + 2.0 + 272.0a + 288.0b + 304.0c$ (Table-1). The calculated data were basically same as the data observed in MALDI-TOF MS spectra, which also proved that (epi) afzelechin (epi), catechin and (epi) gallocatechin were the basic structural units of AMTs in water fraction, ethyl acetate fraction and diethyl ether fraction.

TABLE-1
CALCULATED AND OBSERVED MASS OF AMTs FROM WATER FRACTION ACETATE FRACTION AND DIETHYL ETHER FRACTION BY MALDI-TOF MS SPECTRA

Polymers	a	b	c	Calculated* $[M+Na]^+$	Observed $[M+Na]^+$
Dimer	1	0	1	601	601
	0	1	1	617	617
	0	0	2	633	633
Trimer	0	3	0	889	889
	1	0	2	905	905
	0	1	2	921	921
Tetramer	0	4	0	1178	1178
	1	1	2	1194	1194
	0	2	2	1210	1210
Pentamer	2	1	2	1466	1466
	1	2	2	1482	1482
	1	0	4	1514.5	1498
	0	1	4	1514	1514
Hexamer	1	4	1	1754	1753, 1754
	1	3	2	1770	1770
	0	4	2	1786	1786
	1	1	4	1802	1802
Heptamer	2	3	2	2043	2043
	0	6	1	2059	2058, 2059
	1	3	3	2075	2075
	2	0	5	2091	2090, 2091
Octamer	1	7	0	2315	2315
	2	4	2	2331	2331
	1	5	2	2346	2346
	0	7	1	2347	2347
	3	0	5	2363	2363
	2	1	5	2379	2379
Nonamer	1	7	1	2619	2618, 2619
	1	6	2	2635	2634, 2635
	3	1	5	2651	2650, 2651
	2	2	5	2667	2667
Decamer	1	8	1	2908	2907
	2	5	3	2924	2924
	3	2	5	2940	2941
	0	7	3	2956	2954
	2	2	6	2972	2971
Undecamer	1	6	4	3210	3210
	2	5	4	3228	3226
	1	6	4	3244	3240
	3	1	7	3260	3256
	1	4	6	3276	3272

*Calculated masses were based on the equation: $[M + Na]^+ = 23.0 + 2.0 + 272.0a + 288.0b + 304.0c$, where 23.0 is the mass of Na^+, 2.0 is the terminal H^+, a represents the number of (epi) af zelechin unit, b represents the number of (epi) catechin units, c represents the number of (epi) gallocatechin unit.

^{13}C NMR analysis: The ^{13}CNMR spectra of AMTs in water fraction, ethyl acetate fraction and diethyl ether fraction were shown in Figs. 6-8. The spectrum shows a distinct signal at 144-145, 145-146 and 157 ppm. Signals at these regions were assigned to C3' and C4' of B ring in (epi) catechin units (procyanidin), C3', C4' and C5' of B ring in (epi) gallocatechin units (prodelphinidin), C4' of B ring in (epi) afzelechin units (propelargonidin), respectively[14]. The ^{13}C NMR spectra indicated AMTs in water fraction, ethyl acetate fraction and diethyl ether fraction were mainly composed of procyanidin. Meanwhile, there are also fewer amounts of prodelphinidin and propelargonidin. This result was also supported by the MALDI-TOF MS spectrum.

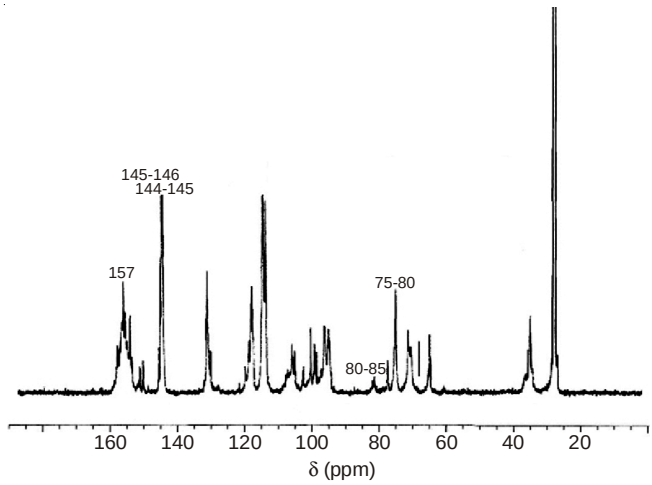


Fig. 6. ^{13}C NMR spectra of AMT in water fraction

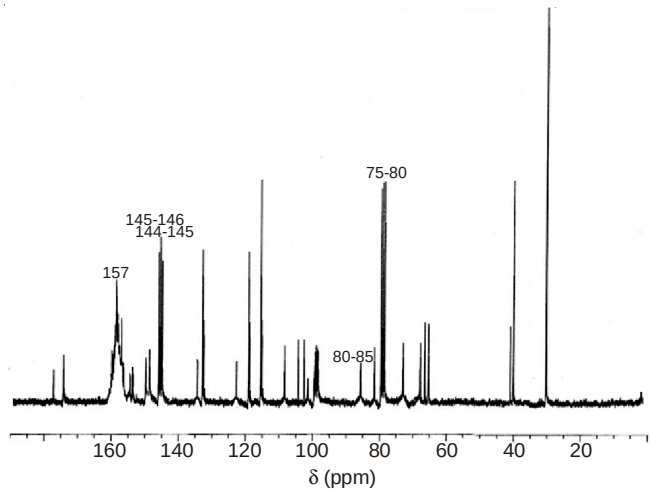


Fig. 7. ^{13}C NMR spectra of AMT in ethyl acetate fraction

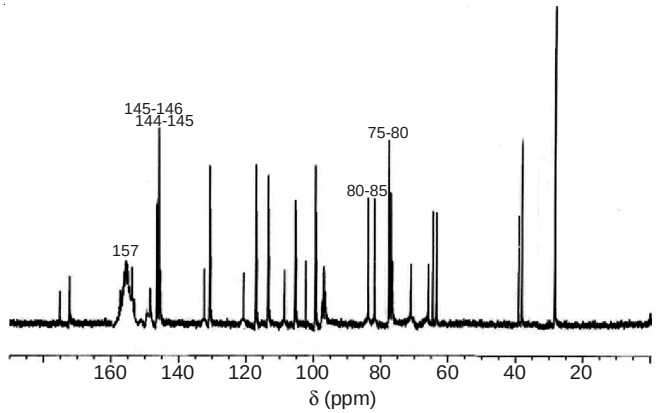


Fig. 8. ^{13}C NMR spectra of AMT in diethyl ether fraction

The ^{13}CNMR spectra between 70-90 ppm are assigned to the stereochemistry of the C ring[14]. C2 gives a resonance line at 76 ppm for the *cis*- and at 84 ppm for the *trans*-form. Both signals were clearly visible in the spectra of AMTs in water fraction, ethyl acetate fraction and diethyl ether fraction. It indicated both stereoisomers were existed in C2 and C3 site of structure units of AMT in each fraction.

The C4-C8 interflavonoid links were presented at 115-110 ppm, meanwhile C4-C6 links were presented at 105 ppm. Figs. 6-8 indicated the existence of both C4-C6 and C4-C8 links in the AMTs in water fraction, ethyl acetate fraction and diethyl ether fraction. In other words, linear and branched chain structures were all presented in AMTs.

Average polymerization degrees of *A. mangium* tannins: Table-2 showed that the M_n and average polymerization degree of AMTs had quite a large difference. For the AMT in diethyl ether fraction, the M_n had a relatively small molecular mass (415 Da), which was equal to 2-3 flavan-3-ol units. On the contrary, the molecular weight of AMT in water fraction was the highest at 2808 Da, which was equal to 9-10 flavan-3-ol units. The molecular weight of AMT in ethyl acetate fraction showed a medium value (1788 Da), which was equal to 5-6 flavan-3-ol units. Based on the result above, structures of AMTs in water fraction, ethyl acetate fraction and diethyl ether fraction were deduced as following (Fig. 9).

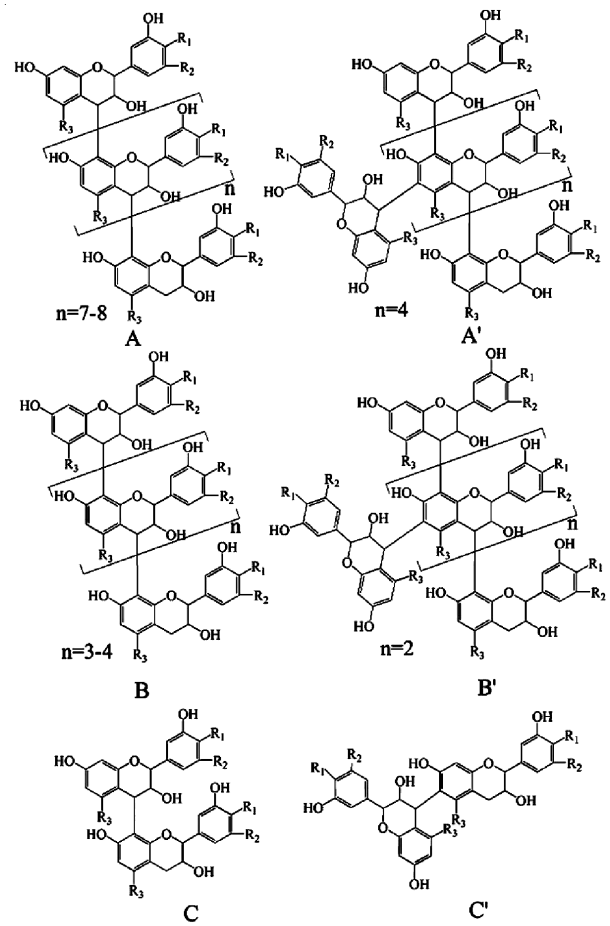


Fig. 9. Structure of AMTs in each fraction. (A and A': Water fraction, B and B': ethyl acetate fraction, C and C': diethyl ether fraction; A, B, C: linear structure, A', B', C' : branched structure; $R_1 = R_2 = H$, R_3 = OH propelargonidin; $R_1 = R_3$ = OH, R_2 = H procyanidin, $R_1 = R_2 = R_3$ = OH prodelphinidin

TABLE-2
M_n AND AVERAGE POLYMERIZATION DEGREES OF *A. mangium* TANNINS

	M_n (Da)	Average polymerization degree
Water fraction	2808	9-10
Ethyl acetate fraction	1788	5-6
Diethyl ether fraction	415	2-3

Conclusion

MALDI-TOF MS and ^{13}CNMR analysis indicated *A. mangium* tannins (AMTs) in water fraction, ethyl acetate fraction and diethyl ether fraction were all composed by (epi) afzelechin, (epi) gallocatechin and (epi) catechin. In other words, AMTs in each fraction were mainly procyanidin and coexisted with prodelphinidin and propelargonidin. Both *cis*- and *trans*-form, linear and branched structure features of these oligomers were observed through ^{13}C NMR spectra. The results from GPC test revealed that tannins in diethyl fraction were consisted of dimer and trimer. Pentamer and hexamer were primary component in ethyl acetate fraction, for water fraction, it was mainly consisted of nonamer and decamer. Based on these results, the structures of tannins in each fraction were eventually made clear. The results could provide a valuable reference for the fine applications of *A. mangium* tannin.

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