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Protein-Binding Affinity of *Leucaena* Condensed Tannins of Differing Molecular Weights

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ABSTRACT: Depending on their source, concentration, chemical structure, and molecular weight, condensed tannins (CTs) form insoluble complexes with protein, which could lead to ruminal bypass protein, benefiting animal production. In this study, CTs from *Leuceana leucocephala* hybrid were fractionated into five fractions by a size exclusion chromatography procedure. The molecular weights of the CT fractions were determined using Q-TOF LC-MS, and the protein-binding affinities of the respective CT fractions were determined using a protein precipitation assay with bovine serum albumin (BSA) as the standard protein. The calculated number-average molecular weights (M_n) were 1348.6, 857.1, 730.1, 726.0, and 497.1, and b values (the b value represents the CT quantity that is needed to bind half of the maximum precipitable BSA) of the different molecular weight fractions were 0.381, 0.510, 0.580, 0.636, and 0.780 for fractions 1, 2, 3, 4, and 5, respectively. The results indicated that, in general, CTs of higher molecular weight fractions have stronger protein-binding affinity than those of lower molecular weights. However, the number of hydroxyl units within the structure of CT polymers also affects the protein-binding affinity.

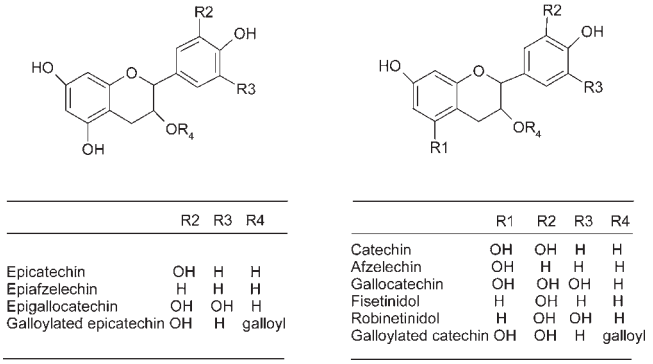
KEYWORDS: condensed tannins, molecular weight, structure, protein binding, leucaena

INTRODUCTION

Condensed tannins (CTs) are widely distributed in the plant kingdom with polymers of flavanol units linked by carbon−carbon bonds that are not susceptible to degradation and with an average molecular mass ranging from 288 to >5000 Da (Figure 1).[1] Condensed tannins have both beneficial and adverse effects on animal nutrition. At a moderate concentration, tannins could enhance milk production, wool growth, ovulation rate, and lambing percentage and reduce internal parasite burdens;[2−4] they could also reduce bloat when a very low level was applied.[5] Recently, it was indicated that CT-containing forages have the potential to reduce methane gas production in ruminants.[6−8]

As an important secondary compound in forages, CTs were suggested to have potential in promoting protein utilization in ruminant animals.[9] It has been reported that CTs could form stable and insoluble complexes with proteins at pH 3.5−7.0. Therefore, dietary proteins that form complexes with CTs escape rumen degradation. The complexes dissociate, and proteins are released in the abomasum. This enables the absorption of amino acids from the small intestine and generally results in better utilization of protein by the host animals.[10,11] Incorporation of CTs from several species of *Leucaena* to bind protein have shown a N digestibility of >78% postruminally (in the small intestine).[12] Bermingham et al.[13] found that amino acid absorption markedly increased ($P < 0.05$) in the small intestine of sheep fed sulla (*Hedysarum cornarium*, 64 g CT/kg DM) but not sainfoin (*Onobrychis viciifolia*, 38 g/kg DM). Therefore, differences in the effects of CTs on amino acid absorption may be associated with differences in the chemical structures of CTs.[14]

Apart from chemical structure and source of CTs, the ability of CTs to interact with protein to form insoluble CT−protein complexes is affected by multiple factors, including plant variety and their concentration, biological activity, and molecular weight.[15−17] Recent studies have suggested that CT polymer structure as affected by monomeric composition and molecular weight could be the primary determining factor for protein-binding affinities.[18,19] Huang et al.[8] determined protein-binding affinity of CTs of the Malaysian local *Leucaena leucocephala* and its hybrid [62-2-8 *Leucaena* hybrid Bahru (LLB)] and reported that although the molecular weights of the CTs from the two genera were nearly identical, the protein-binding affinity of the



	R2	R3	R4
Epicatechin	OH	H	H
Epiafzelechin	H	H	H
Epigallocatechin	OH	OH	H
Galloylated epicatechin	OH	H	galloyl

	R1	R2	R3	R4
Catechin	OH	OH	H	H
Afzelechin	OH	H	H	H
Gallocatechin	OH	OH	OH	H
Fisetinidol	H	OH	H	H
Robinetinidol	H	OH	OH	H
Galloylated catechin	OH	OH	H	galloyl

Figure 1. Basic units of condensed tannins.[1]

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Table 1. Composition of the Five CT Fractions Estimated from Q-TOF LC-MS Spectra

CT		obsd mass (m/z)	I^a ($\times 10^4$)	ion detected	calcd mass (Da)	possible assignments[b]	DP[c]	CT type
fraction 1	peak 1	1225.2950	2.8	$[M + Na]^+$	1202	304*3 + 288 +2	4	B
	peak 2	1241.2670	3.5	$[M + Na]^+$	1218	304*4 + 2	4	B
	peak 3	1313.2750	3.5	$[M + Na]^+$	1290	288*3 + 272 + 152 + 2	4	B
	peak 4	1397.2440	2.0	$[M + H]^+$	1396	288*5 +2 (A type*3)	5	A
	peak 5	1567.2100	4.5	$[M + H]^+$	1566	288*2 + 272*4 + 2	6	B
	CT av M_n[d]	1348.6						
fraction 2	peak 1	871.5707	1.4	$[M + Na]^+$	848	288*2 + 272 + 2	3	A
	peak 2	871.5697	2.4	$[M + Na]^+$	848	288*2 + 272 + 2	3	A
	peak 3	1225.2930	0.1	$[M + Na]^+$	1203	304*3 + 288 + 2	4	B
	CT av M_n	857.1						
fraction 3	peak 1	727.4818	1.1	$[M + H]^+$	726	(288*2 + 152 + 2) − 2H	2	A
	peak 2	741.4972	0.45	$[M + H]^+$	740	(288 + 304 + 152 + 2) − 4H	2	A
	CT av M_n	730.1						
fraction 4	peak 1	727.4797	1.2	$[M + H]^+$	726	(288*2 + 152 + 2) − 2H	2	A
	CT av M_n	726.0						
fraction 5	peak 1	437.1942	2.6	$[M + H]^+$	436	(288 + 152 + 2) − 6H	1	
	peak 2	427.2459	2.0	$[M + H]^+$	426	272 + 152 + 2	1	-
	peak 3	443.3204	1.7	$[M + H]^+$	442	288 + 152 + 2	1	-
	peak 4	461.3245	2.4	$[M + Na]^+$	438	(288 + 152 + 2) − 4H	1	-
	peak 5	427.2461	2.2	$[M + H]^+$	426	272 + 152 + 2	1	-
	peak 6	427.2461	1.4	$[M + H]^+$	426	272 + 152 + 2	1	-
	peak 7	531.3872	1.6	$[M + H]^+$	530	(2*72 + 2) − O	2	B
	peak 8	436.3403	1.1	$[M + H]^+$	435	(288 + 152 + 2) − 7H	1	-
	peak 9	471.3664	1.4	unknown	471	unknown	1	-
	peak 10	633.1517	0.65	$[M + Na]^+$	610	304*2 + 2	2	B
	peak 11	515.3719	1.2	$[M + H]^+$	514	(2*72 + 2) − 2O	2	B
	peak 12	492.4034	2.2	unknown	492	unknown	1	-
	peak 13	781.1885	1.0	$[M + Na]^+$	758	(304*2 + 152 + 2) − 4H	2	B
	peak 14	855.2002	0.5	$[M + Na]^+$	832	272*2 + 288 + 2	3	A
	peak 15	855.2002	1.0	$[M + Na]^+$	832	272*2 + 288 + 2	3	A
	CT av M_n	497.1						

[a] I = absolute intensity ($\times 10^4$). [b] *, 304, 288, 272, and 152 represent the calculated molecular weights of (epi)gallocatechin, (epi)catechin/(epi)robinetinidol, (epi)fisetinidol/(epi)afzelechin, and galloyl derivatives. [c] DP, degree of polymerization. [d] CT average M_n was calculated with the equation $M_n = (\Sigma (m/z)_i I_i)/(\Sigma I_i)$.

hybrid was significantly higher than that of the local. It was hypothesized that CT structure might play a role in that.

This study is a followup to that of Huang et al.[8] with the primary objective of examining the relationship between molecular weight and structure of CTs with protein-binding affinity. The effects of differing molecular weight CT from different factions extracted from LLB were used as materials for this study.

MATERIALS AND METHODS

Plant Material. LLB was harvested from the research farm of the Department of Animal Science, Universiti Putra Malaysia (3° 00′ 18.88″ N, 101° 42′ 15.05″ E) between 9:00 a.m. and 10:30 a.m. by cutting tips of about 30 cm from the youngest fully expanded leaves from several trees. The harvested sample was immediately brought back to the laboratory, freeze-dried prior to grinding through a 1.0 mm sieve, and stored at 4 °C in an airtight dark container pending further analysis.

Extraction and Purification of Condensed Tannins. CTs were extracted from freeze-dried LLB using aqueous acetone and diethyl ether as described by Terrill et al.[20] The CTs were extracted from 200 mg of freeze-dried samples in 200 mL of extraction solvent [70% (v/v) aqueous acetone containing 0.1% (w/v) ascorbic acid] in a shaker at room temperature for 20 min prior to centrifugation at 3500*g* for 10 min. The pellet was then used for another two rounds of extraction with 150 and 100 mL of extraction solvent, respectively. The supernatant was filtered under vacuum to remove any particulate plant residues. The filtrate was then washed three times, each time with an equal volume of diethyl ether in a separation funnel to remove chlorophyll, pigments, and low molecular weight phenolic acids. Traces of acetone and diethyl ether in the extracts were further evaporated under vacuum in a rotary evaporator at <40 °C.

The extracts were kept in a 500 mL bottle, and an equal volume of 40% methanol (v/v) was then added. The mixed solution was purified by using Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden)

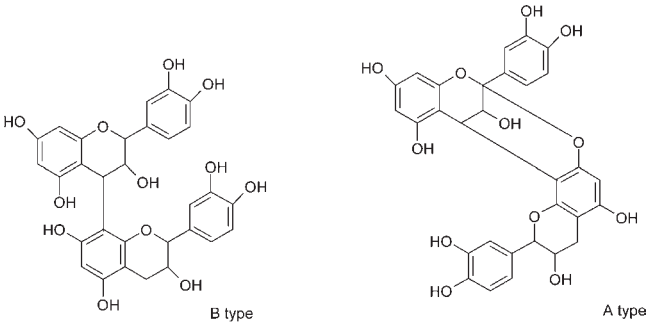

Figure 2. Model type A (right) and type B (left) of condensed tannins.[21].

packed in a 40 cm × 16 mm i.d. XK16 column (GE Healthcare Bio-Sciences AB), with 40% (v/v) methanol and 80% (v/v) acetone as the two respective purification solvents.[21] In the purification process, low molecular weight phenolics were eluted with 40% (v/v) methanol, and the CTs were eluted with 80% (v/v) aqueous acetone. The purified CTs eluted with traces of aqueous acetone were evaporated using a Büchi rotary evaporator (Büchi Labortechnic, Flawil, Switzerland). The purified CTs were then lyophilized and stored at 4 °C in the dark.

Fractionation of the Purified CTs. Different fractions of the CTs were separated by using a 40 cm × 16 mm i.d. XK16 column (GE Healthcare Bio-Sciences AB) packed with about 20 g of Sephadex G-25 (GE Healthcare Bio-Sciences AB). The purified CT powder was dissolved in 50% (v/v) acetone to a mixture concentration of 1 mg/mL. The flow rate (1.2 mL/min) was strictly controlled using a peristaltic pump (Gilson, Villiers Le-bel, France). The mixture was eluted through the column for 5 min. Therefore, about 6 mg of purified CT powder was applied to the column for purification. After that, 50% (v/v) acetone was used for elution of 16 min prior to collection of the analytes (CT fractions). Each fraction was then collected every 0.5 min, and the spectral reading of absorbance at 350 nm was recorded. All of the fractions collected were later grouped into five fractions on the basis of their spectral readings.

Molecular Weight Determination by Q-TOF LC/MS. The number-average molecular weights (M_n) of different molecular weight fractions of the purified CTs were determined by Q-TOF LC-MS (Agilent Technologies, Inc., Santa Clara, CA). One microliter of the CT solutions (1 mg/mL) was applied to the Q-TOF LC-MS. Prior to this analysis, the CT solutions were applied to HPLC analysis whereby a 5 μm Symmetry C18, 3.9 × 150 μm (Waters, Wexford, Ireland), was used as HPLC column. The separation by HPLC was carried out at a flow rate of 500 μL/min. Solvent A was water containing 0.1% formic acid, and solvent B was acetonitrile. The gradient elution was 0−20% B, 0−20 min; 20−40% B, 21−30 min; 40−100% B, 31−40 min; and return to the initial condition for 20 min. For the Q-TOF LC-MS, the mass spectrometer was operated under positive ion mode and scanned from m/z 100 to 3000 with a scanning rate of 1.4. The drying gas (nitrogen) and sheath gas (nitrogen) were set at 8 and 11 L/min, respectively. The electron spray ionize voltage was 3500 V, and the orifice potential was 40 V. Mass spectral data were processed using Agilent Mass Hunter Workstation Software-Offline Qualitative and Quantitative Analysis. The M_n of the different CT fractions by Q-TOF LC-MS was calculated using the following equation:[22]

$$M_n = \frac{\sum (m/z)_i I_i}{\sum I_i}$$

I = absolute intensity.

Protein-Binding Affinity Determination of CTs. The protein-binding affinity of the purified CTs was determined using a protein precipitation assay as described by Huang et al.,[8] which was modified according to the procedure of Makkar et al.[23]

The equation for protein-binding data was analyzed using a nonlinear regression procedure. The curve for purified CTs was fitted to a sigmoid curve: $Y = a/(1 + b \times \exp^{(-c \times x)})$, where Y = mg of BSA precipitated and x = mg of extracted CTs incubated. The protein-binding affinity of CTs was expressed as the b value, which represents the quantity of CT that is needed to bind half of the maximum precipitable BSA.

Statistical Analysis. Data were subjected to one-way analysis of variance (ANOVA). Means separations, when there were differences ($P < 0.05$) between treatments, were carried out using Duncan's procedure. Data were analyzed using SPSS 15.0 version.

RESULTS AND DISCUSSION

Molecular Weights of Different CT Fractions from LLB. In general, the molecular weights of polyphenols in plants range from 100 to 10000 Da. CTs are oligomeric and polymeric proanthocyanidins consisting of various flavan-3-ol units. It has been demonstrated that the protein-binding affinity could increase with the increase in the degree of polymerization (DP)[24] and, thus, molecular weight.[18] However, due to structural diversity and complexity, the characterization of CTs is a difficult task.[25] Only recently have studies on the molecular weights and structures of CTs from different sources been carried out.[26−28]

Mass spectrometry has enabled significant advances in the qualitative and quantitative analysis of CTs. In mass spectrometry, the positive mode has been demonstrated to be more suited for the study of CTs, and their mass data were reported as naturally occurring $[M + Na]^+$, $[M + H]^+$, and $[M + K]^+$ adducts.[29]

The direct Q-TOF LC-MS analysis of the different CT fractions from LLB showed ion series within the mass range of 290−1567 Da (Table 1). The ion series of CTs mainly correspond to hydrogen, ammonia, and sodium adduct series of (epi)catechin, (epi)afzelechin, and (epi)gallocatechin oligomers and galloylated derivatives with mass differences of 288, 272, 304, and 152 Da, respectively. The spectrum of the CTs showed DP ranging from 1 to 6, indicating that CTs of LLB are made up of a complex set of different flavanol units. The structures of the CTs were obtained by determination of the theoretical or calculated mass. The theoretical mass corresponds to the different classes of CTs, which can be calculated as the accumulated number of (epi)catechin, (epi)afzelechin, and (epi)gallocatechin oligomers and galloylated derivatives.[29] B and A types represent different linkages between units (Figure 2). However, this method does not allow us to distinguish the different stereoisomers.

As shown in Table 1, the number-average molecular weight (M_n) of the first fraction was 1348.6 Da with DP from 4 to 6; the highest molecular weight was peak 3, which might be CT pentamer or hexamer, depending on the different combinations of the flavanol units. The second fraction consisted of three peaks, namely, CT trimer, trimer, and tetramer. The different types of CTs in this fraction were detected with sodium ion. The M_n of the second fraction was 857.1 Da. Two peaks were detected in the third fraction, and both were identified as galloylated CT dimer with a difference of 16 Da in their molecular weights. On the other hand, only one peak was detected in the fourth fraction, with a M_n of 726.0 Da and DP of 2, and it was possibly an A-type CT. There were 15 peaks detected with molecular weight (M_n) ranging from 316 to 855 Da for the fifth fraction, and the DP of the fifth fraction could be up to 3. Most of the different types of

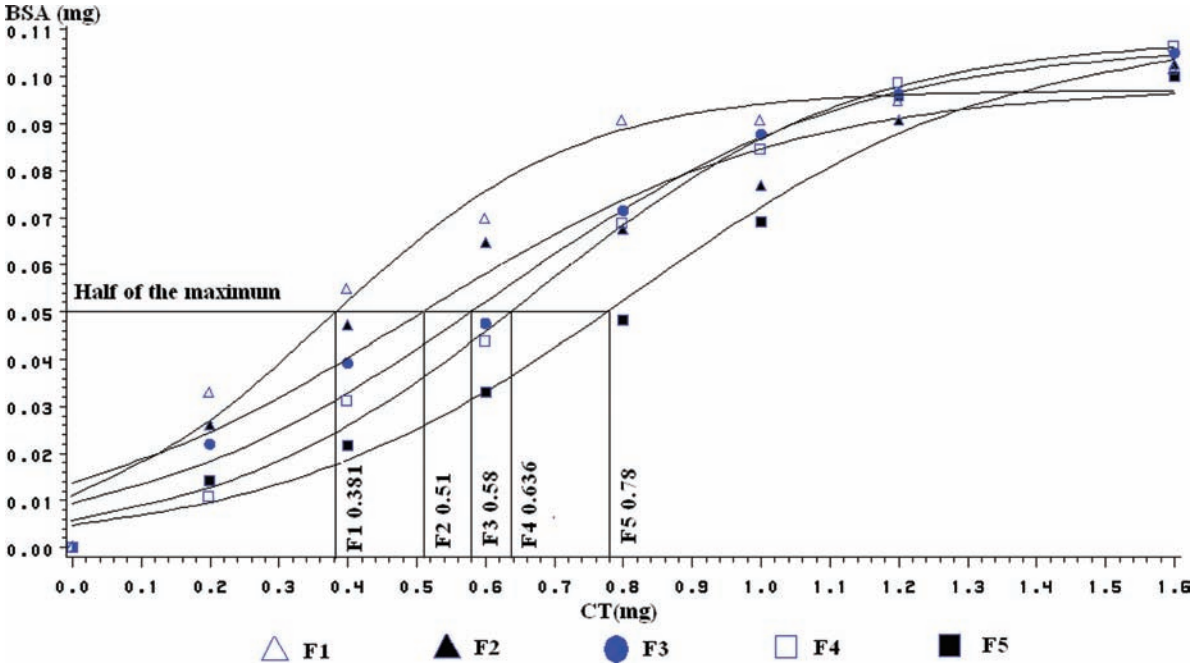

Figure 3. Protein-binding affinities of different molecular weight CT fractions from LLB. The *Y*-axis represents the bonded BSA value, whereas the *X*-axis represents different CT levels.

CTs from the fifth fraction are possibly B type CTs with galloyl derivatives.

As mentioned previously, Q-TOF LC-MS was used in this study to examine the molecular weights of CTs from LLB of the different fractions as it could estimate both their molecular weights and structures. The calculated M_n values were 1348.6, 857.1, 730.1, 726.0, and 497.1 Da, respectively, for the first to the fifth fractions. The above result shows that the molecular weights of the CTs decreased from the first to the fifth fraction, thus indicating the successful fractionation of the CTs into different molecular weights as expected from the use of the Sephadex G-25 column. Their mass data were mainly of $[M + Na]^+$ and $[M + H]^+$ adducts. Results of this study indicated that various DPs, up to hexamer, were found in the different CT fractions from LLB with molecular weights ranging from 426 (Table 1, fraction 5, peak 2) to 1566 Da (Table 1, fraction 1, peak 5). The structures, calculated by the accumulation of constituent units such as (epi)catechin, (epi)fisetinidol, (epi)afzelechin, (epi)gallocatechin, and (epi)-robinetinidol and galloyl derivatives, indicated that A and B type CTs were found in the LLB (as shown in Figure 2, B type CTs contain two more H atoms than A type CTs), with the A type mainly found in the CTs of higher DP (≥ 3).

Protein-Binding Affinity of CTs of Different Molecular Weight Fractions. The *b* value, the amount of CT used to bind half of the maximum precipitable BSA, is used to denote the protein-binding affinity of CTs in this study. That is, when the *b* value is smaller, the protein-binding affinity of the CTs is stronger.

The *b* values of the different molecular weight fractions of CTs are presented in Figure 3. Results of the study show that the *b* value of the first fraction (0.381) was significantly ($P < 0.05$) lower than those of the other fractions, indicating that the protein-binding affinity of the first fraction was the highest among the five fractions of CTs extracted from LLB. On the other hand, the *b* value of the fifth fraction (0.780) was significantly ($P < 0.05$) higher than those of the other fractions, indicating its protein-binding affinity was the lowest. Generally, *b* values increased gradually from fraction 1 to fraction 5, with values of 0.381, 0.510, 0.580, 0.636, and 0.780 for fractions 1, 2, 3, 4, and 5, respectively. These results suggest that the protein-binding affinities of higher molecular weight CTs are stronger than those of the lower molecular weight CT fractions (Table 2).

Table 2. Protein-Binding Affinities of Different Molecular Weight CT Fractions Using Bovine Serum Albumin (BSA) as the Reference Protein

fraction	molecular weight	*b* value[a]
F1	1348.80	0.381 ± 0.023 a
F2	857.01	0.510 ± 0.046 b
F3	730.06	0.580 ± 0.106 bc
F4	726.00	0.636 ± 0.043 c
F5	494.56	0.780 ± 0.049 d

[a] Means with different letters are significantly different ($P < 0.05$). The *b* value, the CT quantity that is needed to bind half of the maximum precipitable BSA, is used to denote the protein-binding affinity of CTs in this study. That is, when the *b* value is smaller, the protein-binding affinity of the CTs is stronger.

The primary objective of several recent studies[18,30,31] on plant CTs focused on the capability of the CTs to bind protein to enhance bypass protein for better utilization of dietary protein in ruminant animals, although not all CTs could be functional as useful bypass protein. Whereas CTs from *Lotus corniculatus* significantly ($P < 0.05$) increased the absorption of essential amino acids from the small intestine, there was no significant ($P < 0.05$) effect for *Lotus pedunculatus*.[14] The effect and action of CTs were associated with the structural differences of CTs, as mentioned in the Introduction.

From the literature, it was evident that the protein-binding affinity of CTs differed among plant varieties[32−34] and within the

Leucaena genus.[18] The latter[18] reported that the larger-sized CTs (fractionated by a size exclusion chromatography procedure) of *Leucaena pallida* and *Leucaena trichandra* had stronger protein-binding capacity than those of the smaller-sized CTs; however, the above relationship was not observed for *Leucaena leucocephala*. Huang et al.,[8] on the other hand, found that protein-binding affinity differed between two *Leucaena* genera with nearly identical molecular weights. The results of the above studies thus suggest that, in general, the protein-binding affinity of CTs is positively, but not necessarily, related to their molecular size. This is because the monomeric composition of the CTs may also influence their respective protein-binding affinity.

Our results indicated a clear difference ($P < 0.05$) in protein-binding affinity among all five fractions, with the larger molecular weight fractions exhibiting stronger protein-binding affinity than the smaller molecular weight fractions, except for fraction 3, which was not different from those of fractions 2 and 4 (Table 1). The nonsignificant difference in protein-binding affinity between fractions 3 and 4 could be explained by the similarity of their molecular weights (730.1 vs 726.0 Da). However, molecular weight may not be the sole factor influencing protein-binding affinity. This is reflected by the fact that although their molecular weights differed by about 100 units (857.1 vs 730.1 Da), the protein-binding affinities of fraction 2 ($b = 0.517$) and fraction 3 ($b = 0.580$) were not different. We propose that the above observation is due to similarity in their monomeric composition, especially the number of hydroxyl units. Although fraction 2 mainly consisted of A type CT with (epi)catchin, (epi)fisetinidol, and (epi)afzelechin as its constituent units and fraction 3 was mainly made up of (epi)catchin, (epi)gallocatechin, and (epi)robinetinidol and galloyl derivatives, the numbers of total hydroxyl units of the two fractions were nearly identical, that is, 13 and 12 or 11, depending on the linkages of the constituent units, for fractions 2 and 3, respectively.

The results of this study thus reaffirmed previous reports that the protein-binding affinity of CTs is positively related to their molecular weights, with higher molecular weight CTs exhibiting stronger protein-binding affinity. However, the above may not be true for CTs with intermediate molecular weights fractionated using the size exclusion chromatography procedure. It has been reported that CT−protein interactions are most frequently based on hydrophobic and hydrogen bonding;[35] thus, the number of hydroxyl units (important functional units influencing the hydrogen and hydrophobic bonding) should also be taken into account. To the best of our knowledge, this experiment is the first to quantify the molecular weights of the differing molecular weight CT fractions extracted from *Leucaena* species and their relationship with protein-binding affinity; therefore, no published data are available for direct comparisons.

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ABBREVIATIONS USED

CTs, condensed tannins; Da, dalton; DP, degree of polymerization; LLB, *Leucaena* hybrid Bahru; M_n, number-average molecular weight.

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