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Size and Molecular Flexibility affect the Binding of Ellagitannins to Bovine Serum Albumin

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ABSTRACT: Binding to bovine serum albumin of monomeric (vescalagin and pedunculagin) and dimeric ellagitannins (roburin A, oenothein B and gemin A) was investigated by isothermal titration calorimetry and fluorescence spectroscopy, which indicated two types of binding sites. Stronger and more specific sites exhibited affinity constants, K_1, of 10^4 to 10^6 M^{-1} and stoichiometries, n_1, of 2 to 13 and dominated at low tannin concentrations. Weaker and less-specific binding sites had K_2 -constants of 10^3 to 10^5 M^{-1} and stoichiometries, n_2, of 16 to 30, and dominated at higher tannin concentrations. Binding to stronger sites appeared dependent on tannin flexibility and presence of free galloyl groups. Positive entropies for all but gemin A indicated that hydrophobic interactions dominated during complexation. This was supported by an exponential relationship between the affinity, K_1, and the modeled, hydrophobic accessible surface area and by a linear relationship between K_1 and the Stern-Volmer quenching constant, K_{SV}.

KEYWORDS: *ellagitannins, isothermal titration calorimetry, fluorescence, molecular flexibility, molecular size, hydrophobic accessible surface area*

INTRODUCTION

Many plants, herbal drugs and foods contain not only the widely-studied condensed tannins but also ellagitannins (ETs).[1,2] In fact, ellagitannins are much more common than previously recognized (Salminen – unpublished data). We consume ellagitannins in nuts, berries, fruit juices and wines[3] and interest is growing in the medicinal and health effects of these polyphenols.[3-8]

Dietary polyphenols may prevent diseases through their ability to quench free radicals, which are generated under oxidative stress.[3] Whilst ETs that contain hexahydroxydiphenoyl (HHDP) groups are rapidly metabolized into ellagic acid and urolithins after consumption, C-glucosidic ETs contain a nonahydroxytriphenoyl (NHTP) group and are difficult to hydrolyse.[5] Pedunculagin and gemin A are examples of HHDP-containing ETs and vescalagin and roburin A contain NHTP group(s) (Figure 1). Many ETs have high water solubility and surprisingly high bioavailability.[9] They are also capable of interacting with various molecular targets that affect signaling pathways[10] and can inhibit tumor promotion.[11] For example vescalagin enters cells rapidly,[4] strongly inhibits DNA topoisomerase[12-14] and interacts selectively with actin filaments.[5] Oenothein B is one of the most active ETs in terms of host-mediated antitumor activity and generates an immune response by stimulating interleukin 1 production.[1] Oenothein B and ET metabolites such as ellagic acid and urolithins can also influence histone acetylation/deacetylation and thus inflammatory responses,[15] which play an important role in the development of age-related diseases.

These and many other examples illustrate that tannins are not just non-specific protein-precipitating agents but can also be involved in specific[16] and targeted interactions with certain amino acids.[17] Tannin-protein interactions have been investigated by a wide range of physico-chemical techniques and were summarized previously.[5,18,19] These included competitive binding assays, NMR spectroscopy, circular dichroism, mass spectrometry, infra-red spectroscopy, dynamic light scattering, small angle X-ray scattering, transmission electron microscopy, equilibrium dialysis, nephelometry, calorimetry, fluorescent quenching, ion mobility,[20] and surface plasmon resonance.[21] Isothermal titration calorimetry (ITC) provides information not only on thermodynamic parameters and the strengths of the tannin-protein interaction but also on the stoichiometry of the resulting complex. Other work has shown that both hydrophobic effects[5] and hydrogen bonding are involved.[16,22] Which of these is the dominant factor depends on the precise polyphenol/protein interactions and experimental conditions.[5,23] Both the flexibility and size of the tannins and proteins appear to affect these interactions.[16,24,25] When gallotannin (GT)- and ET-binding to gelatin and BSA were compared, it was found that equilibrium binding constants for flexible GTs with the globular BSA were between 10^4 to 10^5 M^{-1} and for ETs interacting with the highly flexible gelatin these were ca. 10^6 M^{-1}.[19]

ETs are unique amongst tannins as i) they can be isolated as pure compounds (unlike most condensed tannins, which tend to be obtained as closely related mixtures); ii) they possess a wide range of molecular flexibilities (in contrast to gallotannins or condensed tannins); and iii) their water solubilities are inversely correlated to their protein precipitation capacities.[9] ETs, therefore, offer an opportunity for investigating

68 the relative importance of molecular flexibility and size within a series of matched
69 tannin compounds.
70
71 We chose two monomeric and three dimeric ETs that differ in molecular flexibility
72 (i.e. their bond rotational flexibility) and water solubility. Vescalagin and its dimer,
73 roburin A, represent ETs with NHTP groups that are linked to an acyclic glucose.
74 They are consumed in wine that has been stored in oak barrels. Walnuts are a rich
75 source of pedunculagin, which represents ETs with HHDP groups and a cyclic
76 glucose. The dimers, oenothein B and gemin A can be found in several European and
77 Japanese herbal medicines[1,26] and differ in their molecular flexibilities. The water
78 solubilities of these ETs decrease in the following order: vescalagin > pedunculagin ≈
79 roburin A > oenothein B > gemin A.[9]
80
81 The present study investigated the effects of flexibility and size on the
82 thermodynamics of the ET-protein interaction using isothermal titration calorimetry
83 and fluorescence spectroscopy to probe their interaction mechanisms and binding
84 sites. We choose bovine serum albumin (BSA) as the protein-binding partner for two
85 reasons. Firstly, BSA is a widely used model for globular proteins such as Rubisco,
86 which is a major dietary protein.[27,28] Recent studies have shown that the binding
87 affinity in tannin-protein complexes was negatively correlated with digestibility.[29-31]
88 Secondly, albumins are involved in transporting dietary polyphenols[32,33] and drugs to
89 their molecular targets,[34,35] and exhibit a large sequence homology, *e.g.* 76% in the
90 case of BSA and human serum albumin.[36] It is, therefore, important to understand
91 how ellagitannins interact with globular proteins, as this may also impact on protein-
92 drug interactions in the digestive tract and in the blood plasma.[35]

MATERIALS AND METHODS

Ellagitannins. Two monomeric ellagitannins (pedunculagin and vescalagin) and three dimeric ellagitannins (oenothein B, roburin A and gemin A) were extracted from plants and purified as described previously.[37]

Isothermal Titration Calorimetry. Titrations of BSA (purity ≥99%, essentially globulin free, 66 kDa; Sigma, Poole, Dorset, U.K.) with ellagitannins were performed using a NanoITC instrument (TA Instruments Ltd., Crawley, West Sussex, U.K.) as described previously[19,28] with few adaptations. All solutions were prepared in 50 mM citrate buffer at pH 6 and were degassed under vacuum prior to use. In a typical experiment, buffered BSA solution (20 μM) was placed in the 950 μL sample cell of the calorimeter and buffered ellagitannin solution (3 mg mL^{-1}) was loaded into the injection syringe. Ellagitannin was titrated into the sample cell at 298 K as a sequence of 24 injections of 10 μL aliquots for monomeric ellagitannins (pedunculagin and vescalagin) and 48 injections of 10 μL aliquots for dimeric ellagitannins (oenothein B, roburin A and gemin A). The time delay (to allow equilibration) between successive injections was 360 s. The contents of the sample cell were stirred throughout the experiment to ensure thorough mixing.

Raw data were obtained as plots of heat (μJ) against injection number and featured a series of peaks for each injection. These raw data peaks were transformed using the instrument software to obtain a plot of observed enthalpy change per mole of injectant (ΔH_{obs}, kJ mol^{-1}) against molar ratio (see ITC Data Analysis).

Control experiments included the titration of buffered ellagitannin solutions into buffer, buffer into BSA and buffer into buffer; controls were repeated for each BSA concentration. Control experiments of buffer titrated into buffer or protein solutions both resulted in small and equal enthalpy changes for each successive injection of buffer and, therefore, were not further considered in the data analysis.[38] Experimental data were analyzed after subtraction of the tannin into buffer control data from the sample data. Ellagitannin molecules tend to self-associate into aggregates due to hydrophobic groups; therefore, when injected from the syringe into buffer the ellagitannin molecules undergo an endothermic process of deaggregation, analogous to surfactant demicellization.[39] The extent of deaggregation depends inversely on the concentration of ellagitannin already present in the sample cell; therefore, successive injections of ellagitannin into buffer lead to the observation of progressively lower endothermic enthalpy changes as has been illustrated in earlier work.[39] The data are shown after subtraction of the effects of ellagitannin deaggregation, which means that the assumption is made that ellagitannins dissociate prior to binding.

ITC Data Analysis. Estimated binding parameters were obtained from ITC data using the Bindworks™ (Version 3.1.13, Applied Thermodynamics, Hunt Valley, MD, U.S.A.) data analysis program. Data fits were obtained using a model for two independent sets of multiple binding sites. For this model, the analytical solution for the total heat measured (Q) is determined by the formula:

$$Q = V[\mathrm{M}]\left\{\frac{n_1 \Delta H_1 K_1[\mathrm{L}]}{1+K_1[\mathrm{L}]} + \frac{n_2 \Delta H_2 K_2[\mathrm{L}]}{1+K_2[\mathrm{L}]}\right\} \qquad \text{(Equation 1)}$$

where n_1 and n_2 are the molar ratios of interacting species, ΔH_1 and ΔH_2 are the enthalpies, and K_1 and K_2 are the equilibrium binding constants (*syn*. affinity

constants) for each of the two sets of multiple binding sites.[40] The quality of fit was determined by calculation of χ^2; the data fits were acceptable in each case since the χ^2 values were less than the critical values for the appropriate degree of freedom.

Tryptophan Fluorescence Quenching. Fluorescence intensity was recorded using an FP-6200 spectrofluorimeter (JASCO UK Ltd., Great Dunmow, Essex, U.K.) with selective excitation of tryptophan residues in BSA at 295 nm. The excitation and emission slits were 5 nm and the emission spectrum was recorded between 300 and 500 nm. The measured fluorescence intensities are reported without correction for inner filter effects.[41]

Titration of BSA (5 μM, 2 mL) with ellagitannin (3 mg mL^{-1}, 500 μL) was carried out as a sequence of aliquots (10 μL up to volume of 100 μL, 20 μL up to a volume of 460 μL). For the calculation of quenching constants, the data were plotted as a Stern-Volmer plot of F_0/F against [Q] and the quenching constant, K_{SV}, calculated by linear regression.[42] According to the Stern-Volmer equation:

$$\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_{SV}[Q] \qquad \text{(Equation 2)}$$

where, F_0 and F are the fluorescence intensities before and after the addition of the quencher, respectively, k_q is the bimolecular quenching constant, τ_0 is the lifetime of the fluorophore in the absence of quencher, [Q] is the concentration of the quencher (in this case tannin) and K_{SV} is the Stern-Volmer quenching constant.

However, in the case of combined dynamic and static quenching, where the Stern-Volmer plot exhibits an upward curvature (concave towards the *y*-axis) an alternative equation can be used for fitting:[42]

$$\frac{F}{F_o} = 1 + K_{app}[Q] \quad \text{(Equation 3)}$$

where,

$$K_{app} = \left[\frac{F}{F_o} - 1\right]\frac{1}{[Q]} = (K_D + K_S) + K_D K_S[Q] \quad \text{(Equation 4)}$$

The actual plot of K_{app} or $((F/F_o-1)/Q)$ *versus* [Q] yields a straight line with an intercept of K_D+K_S and a slope of K_DK_S.

Molecular Modeling. Molecular modeling was performed with MOE2011.10 (Chemical Computing Ltd., Montreal, Canada). Atomistic models of the respective tannins were built from chemical structures on an atom by atom basis using the Builder Module of MOE. All models were energy minimized to convergence (rms gradient of 0.05 Kcal/mol) using the MMX94x force field by smart minimization (steepest descents, followed by conjugate gradient and truncated Newton-Raphson methods). Physical parameters were calculated from these models using the QuasAR module in MOE. Parameters calculated were accessible surface area (ASA), hydrophobic accessible surface area (ASA_H). Molecular flexibility was calculated by performing a conformational energy search about the torsion angles of each model using Lowmode Molecular Dynamics in the Conformational Analysis module of MOE. Results from conformational analysis were stored in databases of conformations for each tannin indexed by final energy.

RESULTS AND DISCUSSION

This study investigated the interaction between BSA and five ellagitannin compounds using isothermal titration calorimetry and fluorescence spectroscopy. The molecular flexibility and surface areas of five ellagitannins and pentagalloyl glucose were

191 modelled in order to improve our understanding of these interactions.

192

193 **Molecular Flexibility of Ellagitannins.** Molecular simulation was used to assess
194 molecular flexibility in terms of numbers of low energy conformations located and
195 energy of these conformers (Table 1). Modeling experiments[43] demonstrated
196 previously that the nonahydroxytriphenoyl (NHTP) group is much less flexible than
197 the hexahydroxydiphenoyl (HHDP) group (Figure 1) and the low energy conformers
198 of the monomers clearly reflect the extent of these structural constraints: pentagalloyl
199 glucose conformers (n=21) ranged from 110-116 kcal mol^{-1} (no NHTP or HHDP
200 groups), pedunculagin conformers (n=4) from 158-164 kcal mol^{-1} (2 HHDP groups)
201 and vescalagin conformers (n=18) from 229 to 236 kcal mol^{-1} (1 NHTP, 1 HHDP
202 group). The low energy conformers of the dimers followed a similar trend: oenothein
203 B conformers (n=2) had 278-280 kcal mol^{-1} (2 HHDP groups), gemin A (n=1) had
204 323 kcal mol^{-1} (3 HHDP groups) and roburin A conformers (n=4) had 406-412 kcal
205 mol^{-1} (2 NHTP, 2 HHDP groups).

206

207 **Isothermal Titration Calorimetry of Ellagitannin-BSA Interactions and Data**
208 **Fitting**. ITC experiments for ellagitannin titration into BSA were carried out for five
209 ellagitannins (depicted in Figure 1). Figure 2 shows the ITC binding isotherms for the
210 interaction with BSA of vescalagin and its dimer, roburin A, consisting of the
211 experimental data (as points) and the binding model that best fitted to each data set (as
212 lines). For both ellagitannins, the binding interaction led to an exothermic response,
213 which decreased in magnitude with successive titration injections and signifies a
214 saturation of binding sites on BSA.

215

As found in previous studies,[24,39] small but significant exothermic peaks were observed at higher tannin:protein molar ratios at and beyond the point of apparent binding site saturation. This means that titration experiments did not reach zero enthalpy change, even after removing effects from control experiments (*e.g.* tannin titration into buffer). Therefore, we have assumed that the interaction between ellagitannin and BSA has not reached completion at the higher ellagitannin:protein ratios measured. As a consequence, upon fitting the data to a binding model, this long tail of exothermic peaks prevents the use of a model based on a single set of multiple binding sites (one-site model), and, therefore, all data were fitted to a two independent binding site model. A previous study of the interaction between pentagalloyl glucose with BSA or Rubisco described the selection of the two independent binding site model in more detail.[28]

Figure 3 shows the ITC binding isotherms for pedunculagin and the related dimers oenothein B and gemin A. As observed for the vescalagin and roburin A isotherms, the binding isotherm for the dimers shows a clear two-phase shape to the isotherm that signifies two or more binding sites are involved in the interaction. For the oenothein B and gemin A ellagitannins, the titration experiments were extended by increasing the number of titration injections to observe the interaction at higher molar ratios (see Methods for more details). The thermodynamic binding parameters for the ellagitannin-BSA interaction were obtained from a two-site model and are shown in Table 2, which also includes data from our previous study on pentagalloyl glucose binding to BSA for comparison.[28]

Thermodynamic Parameters of Ellagitannin-BSA Interactions in Relation to

Molecular Flexibility. In these studies, the excellent fits of the ITC data have enabled us to probe the effects of different structural characteristics of the ellagitannins on their interaction with BSA. The results will be discussed in terms of monomer-dimer pairs and molecular flexibilities. The weakness of the second binding site allows, however, for some uncertainties in exact values for these fits, but does confirm our previous observations, which included related ellagitannins and suggested that these were able to bind (adsorb) non-selectively.[24,28,39]

The ellagitannin monomers, pendunculagin and vescalagin, show equivalent strengths of interaction (K_1 = 4.2 and 5.2 $\times 10^4$ M^{-1}) and so do the roburin A and oenothein B dimers (K_1 = 2.3 and 6.5 $\times 10^5$ M^{-1}) (Table 2). In contrast, the K_1 affinity constants of pentagalloyl glucose and gemin A were one order of magnitude larger than for the other monomers and dimers (K_1 = 1.8 $\times 10^5$ and 1.8 $\times 10^6$ M^{-1}, respectively). This coincides with the fact, as pointed out above that both have low conformational energies and are flexible molecules. Taken together gemin A has several features, *i.e.* single bonds and 'free' galloyl groups, which may allow selective binding to specific amino acids on the protein surface. These findings are in line with two other studies that reported weaker binding by vescalagin[44] and by its isomer, castalagin,[29] to BSA compared to pentagalloyl glucose. It appears that pentagalloyl glucose and gemin A have optimal structures for BSA binding within the group of tannins studied here.

For both binding sites the interaction in terms of the affinity constants, K_1 and K_2, is approximately one order of magnitude different between monomers and dimers (Table 2). For each of the comparable ellagitannin monomer-dimer pairs, the dimer was shown to bind more strongly than the monomer. It can also be seen that K_2 is one

order of magnitude weaker than K_1. This weaker second binding site is likely to correspond to non-specific adsorption of tannins that may lead to coating of the protein surface[28,45] and eventual precipitation of the tannin-protein complex in some systems. The binding isotherms in Figures 2 and 3 for the dimers show a greater exothermic response than for the corresponding monomers, *e.g.* roburin A *vs* vescalagin (ΔH_1 = -15.3 *vs* -7.5 kJ mol^{-1}), oenothein B *vs* pedunculagin (ΔH_1 = -21.6 *vs* -10.9 kJ mol^{-1}) and gemin A *vs* pentagalloyl glucose (ΔH_1 = -47.4 *vs* -29 kJ mol^{-1}) (Table 2).

A closer look at the monomer series and the dimer series (each having tannins with similar molecular weights) suggests that the strength of binding is inversely related to the minimized energies and increases with a greater potential for rotational flexibility as follows (Table 1). The low energy conformers and binding constants, K_1, of the monomers are: 229-236 kcal/mol and 5.2×10^4 M^{-1} for vescalagin, 158-164 and 4.2×10^4 for pedunculagin, and 110-116 and 1.8×10^5 for pentagalloyl glucose. For the dimer series the corresponding values are: 406-412 kcal/mol and 2.3×10^5 M^{-1} for roburin A, 278-280 and 6.5×10^5 for oenothein B, 323 and 1.8×10^6 for gemin A.

The number of strong (type 1) binding sites increases for ellagitannins with more flexibility and 'free' galloyl groups from n_1 =1.9 for roburin A to 4.1 for oenothein B and 13 for gemin A. The binding parameters for two of the monomer-dimer pairs, *i.e.* the vescalagin-roburin A and pedunculagin-oenothein B pairs, show similarities in the stoichiometry of the interaction. For the vescalagin-roburin A pair the stronger binding site n_1 is 2.5 for the monomer and 1.9 for the dimer; and for the second weaker binding site n_2 is 30 for the monomer and 16 for the dimer. For the

pedunculagin-oenothein B pair n_1 is 2.1 for the monomer and 4.1 for the dimer; and n_2 is 30 for the monomer and 20 for the dimer. However, for the most flexible monomer-dimer pair, which will be able to better support H-bonding, the stoichiometric values were much higher for the stronger binding site: n_1 was 26 for pentagalloyl glucose and 13 for gemin A. The difference reflects the fact that gemin A is twice the molecular size of pentagalloyl glucose. We reported previously that the sum of n_1 and n_2 indicated that 52 pentagalloyl glucose molecules are bound to BSA and this suggested surface coating as the BSA surface can theoretically accommodate 40 to 120 pentagalloyl glucose molecules.[28] Similarly, the sum of n_1 and n_2 for gemin A indicated that 38 gemin A dimers were bound (which equates to 76 'monomers') and matches with the calculated surface area. It also suggests that gemin A due to its flexibility is capable of coating the whole BSA protein just like pentagalloyl glucose.[28] Comparable stoichiometries were found for commercial preparations of the flexible taratannins when assuming a molecular weight of 1500: *i.e.* 48:1 for the taratannin:BSA ratio.[39]

It is, however, of note that this molecular size effect was not observed to the same extent for the other pairs as monomers and dimers had similar n_1-values (2 to 4) and similar sums of $n_1 + n_2$ (*i.e.* 33 for vescalagin/18 for roburin A and 32 for pedunculagin/24 for oenothein B). This indicates that the less flexible structures and especially roburin A were less able to stretch out on the BSA surface.

Many more binding constants are available for flavonoids and condensed tannins than for ellagitannins. Nevertheless, pure flavonoid compounds tend to have comparable affinities as the ellagitannins investigated here. K_a constants with BSA of 10^2 to 10^5

316 M^{-1} were reported for monomeric and polymeric flavanols[19,31] and of 10^4 to 10^5 M^{-1}
317 for flavones and flavonols. [46-49] Examples of polyphenol complexes with human
318 serum albumin include epigallocatechin gallate and diosmetin interactions that
319 exhibited comparable Stern-Volmer affinity constants of ~10^5 M^{-1} from fluorescence
320 data.[50-51]
321
322 The findings also match results from previous studies, where less pure tannins had
323 been used, and confirm that the tannin components of those fractions did indeed
324 dominate the interaction observed.[19,24,39] The present study used a two-site binding
325 model and found K_1 of 10^4 to 10^5 and K_2 of 10^3 to 10^4 M^{-1} for the monomers in BSA
326 complexes. In comparison, data obtained with a single-site model gave K values of
327 ~10^3 M^{-1} for chestnut- and myrabolan-BSA complexes.[24]
328
329 Quideau *et al*[5] reported that most polyphenols, including ellagitannins, when bound at
330 protein surfaces, exhibited affinity constants that were rarely above the micromolar
331 range, *i.e.* K_a of $\leq 10^6$ M^{-1}. However, Xiao *et al*[52] also reported much larger constants
332 of up to 10^8 M^{-1} for some flavanoid and flavonoid - human serum albumin complexes.
333 Not surprisingly, some receptor and enzyme targets had particularly high selectivities
334 with affinity constants at the subnanomolar scale, and varied between ellagitannins
335 even if these were of similar molecular weights.[5,53]
336
337 The data in Table 2 showed positive entropies at both types of binding sites for all but
338 one ellagitannin, i.e. gemin A. Positive ΔS_1 values ranged from 33 to 65 and ΔS_2
339 values from 36 to 54 J mol^{-1} K^{-1} and are indicative of a dominance of hydrophobic
340 interactions.[19,50,54] Gemin A was the only ellagitannin giving a negative entropy, -39 J

mol^{-1} K^{-1}. For comparison purposes, we have included here also the entropies for pentagalloyl glucose, which shows that the highly flexible pentagalloyl glucose and gemin A had noticeably lower entropies at the stronger binding site than the other ellagitannins and were, therefore, apparently better able to form hydrogen bonds.[54] The positive entropies for all ellagitannins at the second binding site are consistent with hydrophobic stacking on the BSA surface, although some uncertainty exists with these values as the affinities were very low for some tannins ($K_2 = 10^3$ M^{-1}). The literature contains examples of both hydrogen bonding and hydrophobic interactions in tannin-protein complexes.[16,19,22,50,55] Taken together, the present study also suggests that both types of bonding interactions occur in these ellagitannin-BSA complexes and slight structural differences appear to dictate, which of these will dominate.

Quenching of the BSA Tryptophan Fluorescence by Ellagitannins. To complement the ITC measurements, ellagitannin binding to BSA was also investigated by fluorescence spectroscopy. These experiments measure the quenching of the intrinsic tryptophan fluorescence of BSA[32] after binding with each of the tannins. Two tryptophan residues are found near the BSA surface, one is within its hydrophobic binding pocket (Trp 212) and the other within the surface region of the protein (Trp 134).[49] Figure 4(a) shows Stern-Volmer plots that describe tryptophan quenching as a function of tannin concentration. In these plots F_0/F increases as fluorescence intensity decreases in the presence of quencher (F) relative to the native fluorescence in the absence of quencher (F_0). The data suggest that the rate of decrease in fluorescence with respect to quencher concentration depends on the molecular size and flexibility of the tannin. The data in Figure 4(a) revealed that four of the tannins fall into either monomer or dimer groups: the monomers, vescalagin

and pedunculagin, exhibit similar quenching behavior, and the dimers, roburin A and oenothein B, also behave similarly to one another. The dimers were more efficient quenchers than the monomers, requiring approximately half the molar tannin concentration to produce equivalent levels of quenching. Indeed, if the quenching data are normalised, as in Figure 4(b) where Stern-Volmer plots are given as a function of 'tannin monomer' concentration, thus removing effects of molecular weight, it can be seen that related monomer-dimer pairs overlay, which demonstrates similar quenching efficiency per monomer unit. In contrast to these four tannins, the most flexible dimer, gemin A, is a much more efficient quencher at much lower concentrations. Pentagalloyl glucose is also a more efficient quencher, K_{sv} ~2 x 10^6 M^{-1},[28] than vescalagin and pedunculagin, but pentagalloyl glucose and gemin A are not a true monomer-dimer pair and thus do not overlay exactly.

The purpose of the Stern-Volmer plot of fluorescence quenching data is to generate a linearised data set that allows assessment of the ability of tannins to quench the BSA tryptophan residues. The slope of the straight line of best fit is termed the Stern-Volmer quenching constant (K_{SV}). From the data shown in Figure 4(a), K_{SV}-values of 10^4 to 10^5 M^{-1} have been calculated from a straight-line plot through the linear region at low tannin concentrations (Table 3) and are comparable with K_{SV}-values of 10^4 M^{-1} for tea, coffee and cocoa extracts[31] and with 6.85 x 10^4 M^{-1} for quenching of human serum albumin by epigallocatechin gallate (EGCg).[50] These fluorescence data complement the ITC data by providing additional support to the findings outlined above. The trend in terms of the quenching constants, K_{SV}, matches that seen from ITC measurements of the affinity constants, K_1, with a linear correlation (R^2 = 0.988) between K_{SV} and the binding affinity constant, K_1 (Tables 2 and 3).

However, it should be noted that each of the Stern-Volmer plots is non-linear and curves towards the *y*-axis with higher tannin concentration. This is not unusual in Stern-Volmer plots and provides information on the type of quenching. An upward slope suggests a combination of both static and dynamic quenching in these ellagitannin-BSA systems.[42] Static quenching is associated with a strong coupling (*e.g.* formation of a ground-state complex) between interacting molecules, whereas dynamic quenching is associated with weak coupling (*e.g.* through-space interactions or collisions).[42] To further confirm that the curvature was indeed due to combined static and dynamic quenching, a modified form of the Stern-Volmer equation was used (see Methods) that is second order with respect to [Q], and this gave as predicted a linear plot (Figure 4(c)).

Polarity of the Tryptophan Environment. Fluorescence spectra can also yield information on another parameter, *i.e.* the position of the emission peak maximum (λ_{em}), which can reflect the polarity of the tryptophan environment.[50] The presence of ellagitannins led to a bathochromic or red-shift in λ_{em} (Table 3), suggesting that the tryptophan environment became more polar upon interaction with ellagitannins. The observed red-shift may suggest a change to the protein's tertiary structure and thus account for the associated increase in the polarity of the tryptophan environment.[28] However, it is difficult to conclude this based solely on the data reported here. A shift to a more polar environment could suggest unfolding of the protein and thus greater solvent exposure or more H-bonding between BSA and the tannins. However, Li *et al*[30] reported a slight blue-shift upon binding of tannic acid (*i.e.* a mixture of gallotannins containing either a central glucose or quinic acid) to ferritin, whereas

Dobreva *et al*[28] measured a red-shift of 5 nm when pure pentagalloyl glucose bound with BSA. The magnitude of the red shift appears to be linked to tannin structure as related monomer-dimer pairs showed some similarities: vescalagin and roburin A generated a 8-9 nm shift, pedunculagin and oenothein B a 4-5 nm shift, but gemin A caused the greatest shift in λ_{em} of 10.3 nm. A similarly large red shift of 12 nm was observed for Trp during EGCg binding to human serum albumin.[50] Other experiments will be needed to establish the precise reasons for the observed differences in red-shifts.

In conclusion, our ITC data suggest that there are two types of binding sites for ellagitannin binding to BSA. A stronger binding site may well be more selective and dependent on tannin flexibility and the presence of free galloyl groups. Indeed, our previous study suggested a link between the number of aromatic residues on the surface of a globular protein, Rubisco, and pentagalloyl glucose binding.[28] This could explain the relationship between K_1 and the hydrophobic accessible surface area, ASA_H (Figure 5), *i.e.* $K_1 = 0.2125 \times e^{0.0114 ASA\_H}$ ($R^2 = 0.965$), and points to the fact that hydrophobic interactions are an important driver for affinity during BSA-ellagitannin complex formation as supported by positive entropies for 5 of the 6 ellagitannins studied here.

`

A second weaker binding site was required to fit our data in order to allow for the binding interactions observed at high tannin:protein molar ratios. This second binding site was associated with high values of n_2 for all tannins and suggests non-selective adsorption of tannin to the protein surface. Such findings suggest tannin binding to BSA is concentration dependent, where specific binding might dominate at low

441 concentrations and non-selective adsorption at higher concentrations of tannin.[16]
442 These studies have shown that ellagitannin binding to BSA increases in strength and
443 affinity for the larger tannins (dimers) compared to their monomer forms. Bond
444 rotational flexibility of the tannin also plays a role by increasing the strength of
445 interaction and number of stronger (possibly hydrogen bonding) binding sites on the
446 protein surface.
447

448 **ABBREVIATIONS**

449 BSA, bovine serum albumin; ET, ellagitannins; GT, gallotannins
450

451 **Funding**

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455

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458

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632

633 **FIGURE LEGENDS**

634

635 **Figure 1.** Structures of ellagitannins and pentagalloyl glucose.

636

637 **Figure 2.** ITC binding isotherms for the interaction of bovine serum albumin with the
638 monomer-dimer pair of (a) vescalagin and (b) roburin A. Data were fitted assuming a
639 model for two-independent binding sites and values obtained from these fits are given
640 in Table 2.

641

642 **Figure 3.** ITC binding isotherms for the interaction of bovine serum albumin with the
643 monomer-dimer pairs of (a) pendunculagin, (b) oenothein B and (c) gemin A. Data
644 were fitted assuming a model for two-independent binding sites and values obtained
645 from these fits are given in Table 2.

646

647 **Figure 4.** Fluorescence quenching of tryptophans in BSA by vescalagin, roburin A,
648 pedunculagin, oenothein B and gemin A plotted as: (a) ratio of initial fluorescence
649 intensity to total fluorescence intensity *versus* ellagitannin concentration; (b) ratio of
650 initial fluorescence intensity to total fluorescence intensity *versus* ellagitannin
651 monomer concentration; and as a (c) modified Stern-Volmer plot to reflect
652 contributions from both static and dynamic quenching (data are shown for vescalagin,
653 roburin A, pedunculagin and oenothein B only).

654

655 **Figure 5**: Relationship between the affinity constant, K_1, and the modeled accessible
656 hydrophobic surface area (ASA_H).

Table 1: Modeling of minimized energies and associated numbers of conformers, accessible hydrophobic surface area (ASA_H) and accessible surface area (ASA).

Compound	**Molecular weight** (Daltons)	**Energies of conformers** (kcal/mol)	**Number of conformers**	**ASA_H nm^2**	**ASA nm^2**
Vescalagin	935	229 to 236	18	253	914
Roburin A	1843	406 to 412	4	407	1587
Pedunculagin	769	158 to 164	4	293	867
Oenothein B	1569	278 to 280	2	524	1485
Pentagalloyl glucose	927	110 to 116	21	373	1041
Gemin A	1841	323	1	574	1748

Table 2: Estimated thermodynamic binding parameters for the interaction between five ellagitannins and pentagalloyl glucose[b] with bovine serum albumin at pH 6. ΔH_1 and ΔH_2 are the enthalpies, and K_1 and K_2 are the equilibrium binding constants for two binding sites.

	Vescalagin	**Roburin A**	**Pedunculagin**	**Oenothein B**	**Pentagalloyl glucose[b]**	**Gemin A**
n_1	2.5±0.5[a]	1.9	2.1	4.1	26	13
K_1 (M^{-1})	5.2 (±2.3) $\times 10^4$	2.3×10^5	4.2×10^4	6.5×10^5	1.8×10^5	1.8×10^6
ΔH_1 (kJ mol^{-1})	-7.5±1.0	-15.3	-10.9	-20.6	-29	-47.4
ΔS_1 (J mol^{-1} K^{-1})	65.1	33.2	51.9	42.2	3.3	-39.3
n_2	30±4	16	30	20	26	25
K_2 (M^{-1})	1.1(±0.07) $\times 10^3$	1.8×10^4	1.1×10^3	3.3×10^4	8×10^2	6.3×10^4
ΔH_2 (kJ mol^{-1})	-6.7±1.3	-8.17	-5.1	-10.3	-29	-11.4
ΔS_2 (J mol^{-1} K^{-1})	35.7	54.0	41.1	51.9	-41.7	53.6
χ^2	0.65±0.16	0.87	1.99	8.07		3.29

[a] ± standard deviation, n = 3. [b] Data are included for comparison (Dobreva *et al.*[28]).

Table 3: Estimated quenching parameters for the interaction of ellagitannins with bovine serum albumin (n=3).

Fluorescence parameters	**Vescalagin**	**Roburin A**	**Pedunculagin**	**Oenothein B**	**Gemin A**
K_{SV} (M^{-1})	0.42 (±0.07) × 10^5	1.03 (±0.07) × 10^5	0.53 (±0.1) × 10^5	1.44 (±0.28) × 10^5	3.13 (±0.06) × 10^5
Bathochromic (red)-shift of λ_{max} (nm)	8.7 ± 0.4	8.7 ± 0.4	4.7 ± 0.9	4.0 ± 1.0	10.3 ± 0.4

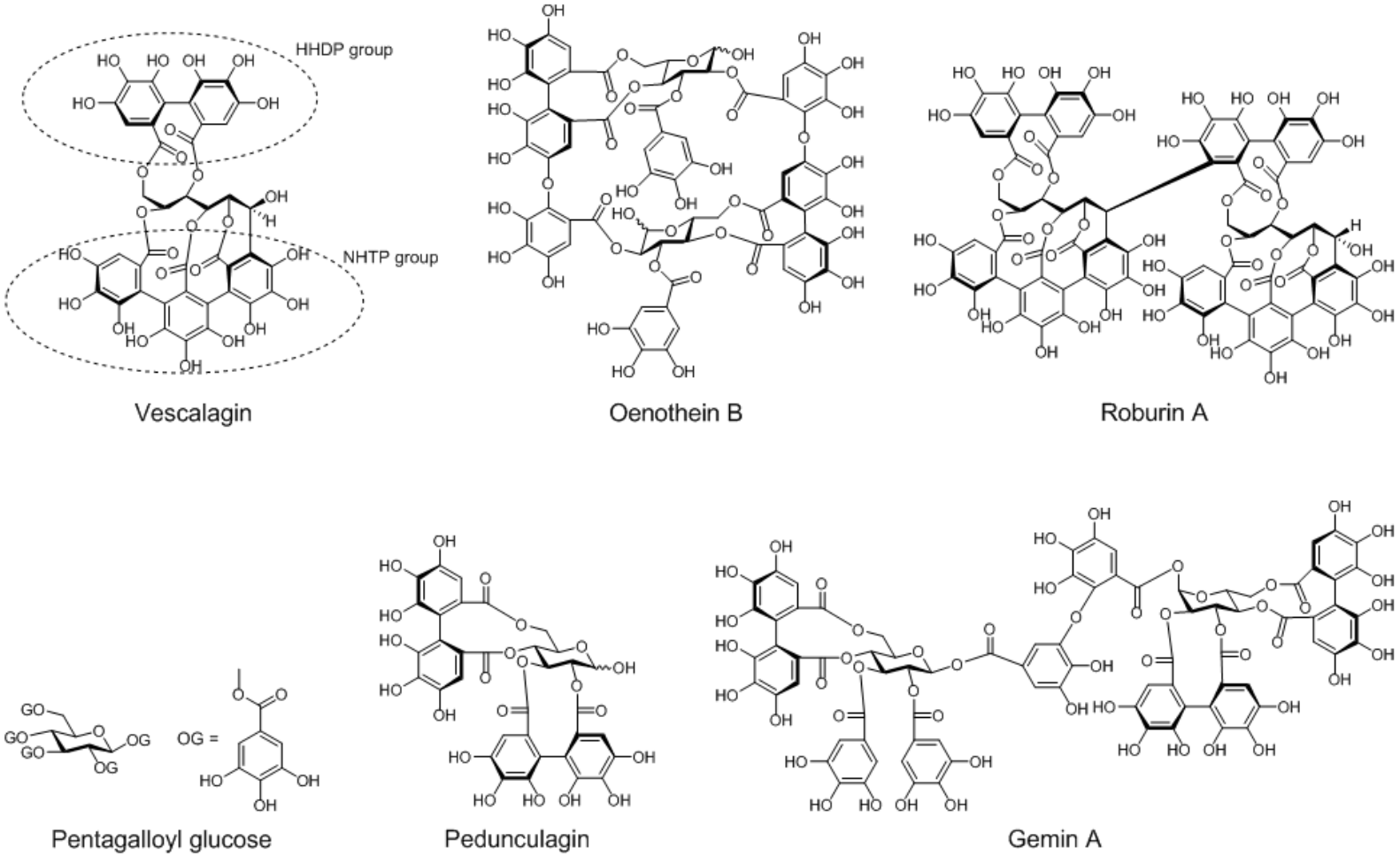

HHDP group
NHTP group
Vescalagin
Oenothein B
Roburin A
GO
OG
OG =
Pentagalloyl glucose
Pedunculagin
Gemin A

Figure 1

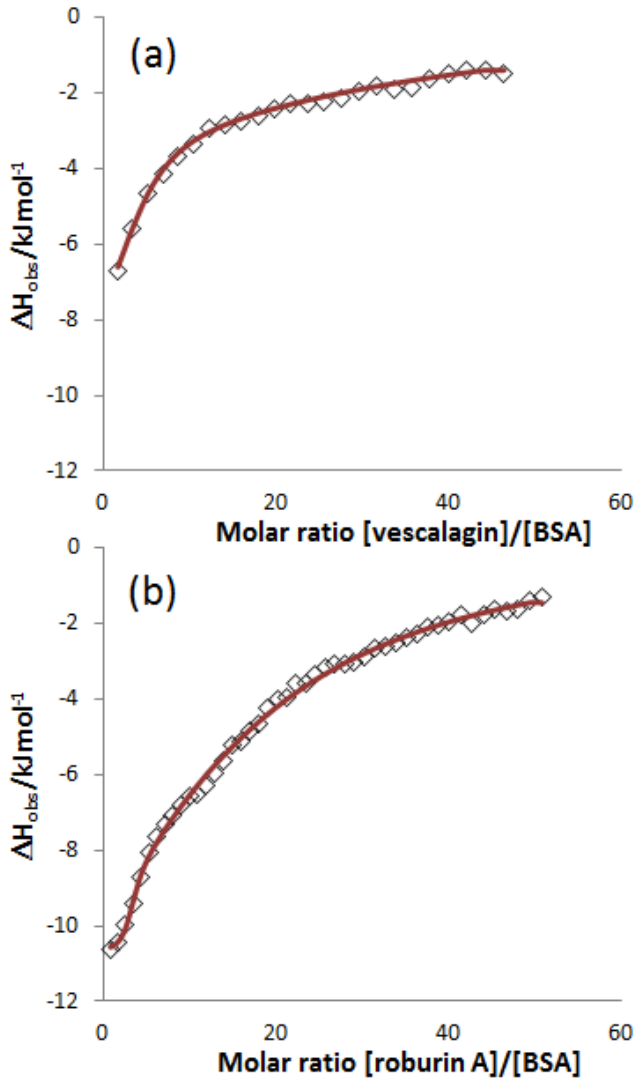

(a)
0
-2
-4
-6
-8
-10
-12
ΔH obs /kJmol-1
0
20
40
60
Molar ratio [vescalagin]/[BSA]
(b)
0
-2
-4
-6
-8
-10
-12
ΔH obs /kJmol-1
0
20
40
60
Molar ratio [roburin A]/[BSA]

FIGURE 2

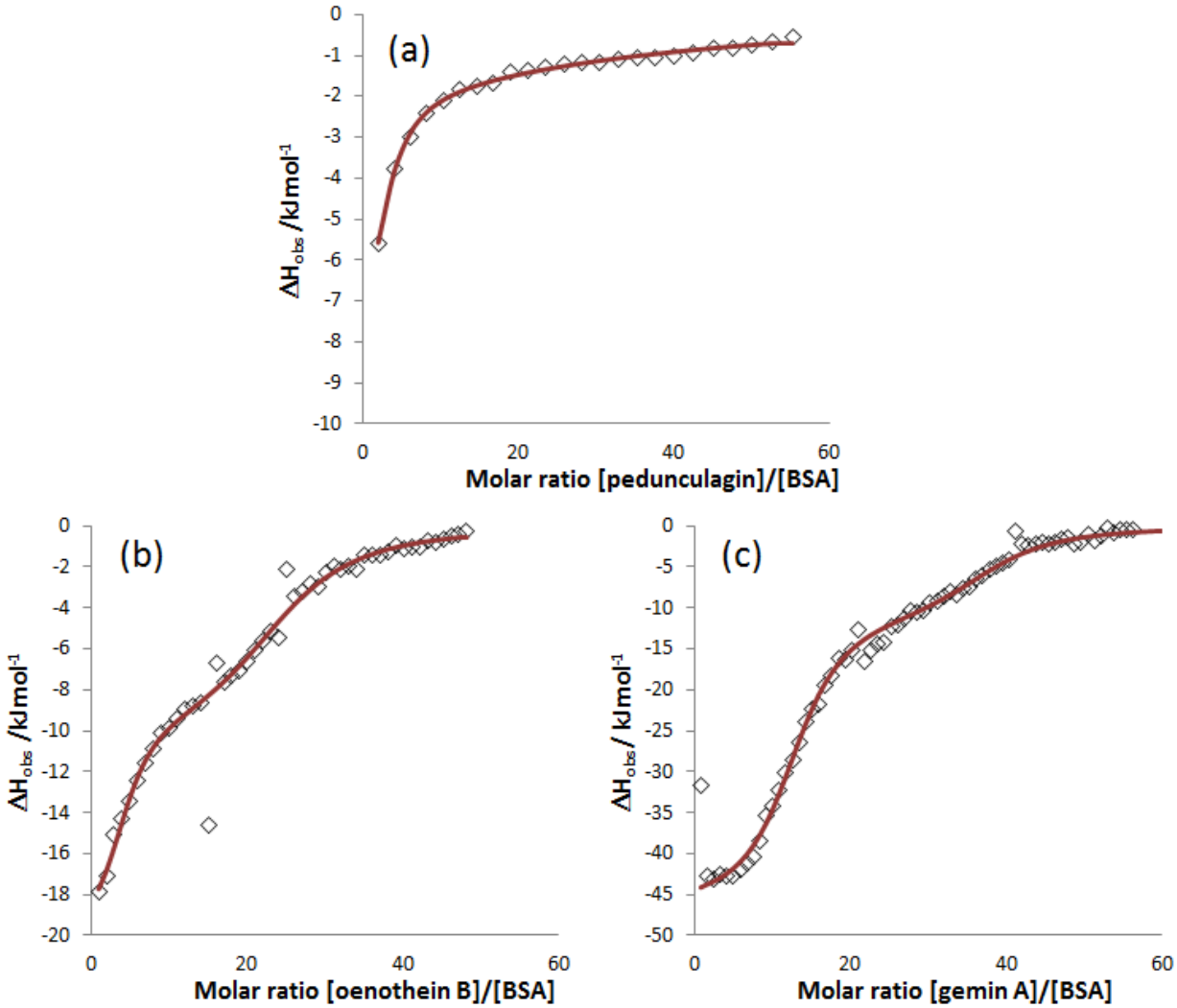

(a)
ΔH_{obs} /kJmol^{-1}
Molar ratio [pedunculagin]/[BSA]
(b)
ΔH_{obs} /kJmol^{-1}
Molar ratio [oenothein B]/[BSA]
(c)
ΔH_{obs}/ kJmol^{-1}
Molar ratio [gemin A]/[BSA]

FIGURE 3

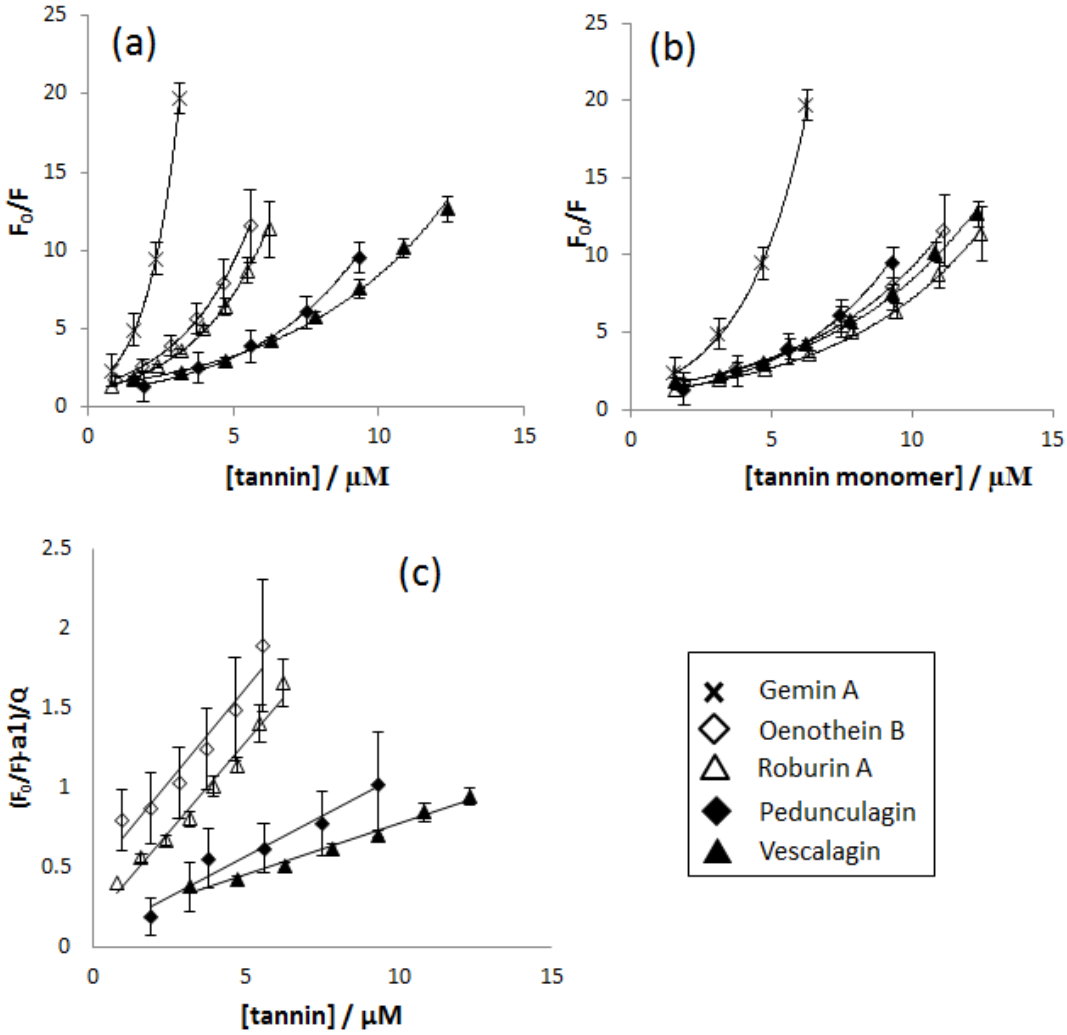

(a)
(b)
(c)
F_0/F
$(F_0/F)-a1)/Q$
[tannin] / µM
[tannin monomer] / µM
Gemin A
Oenothein B
Roburin A
Pedunculagin
Vescalagin

FIGURE 4

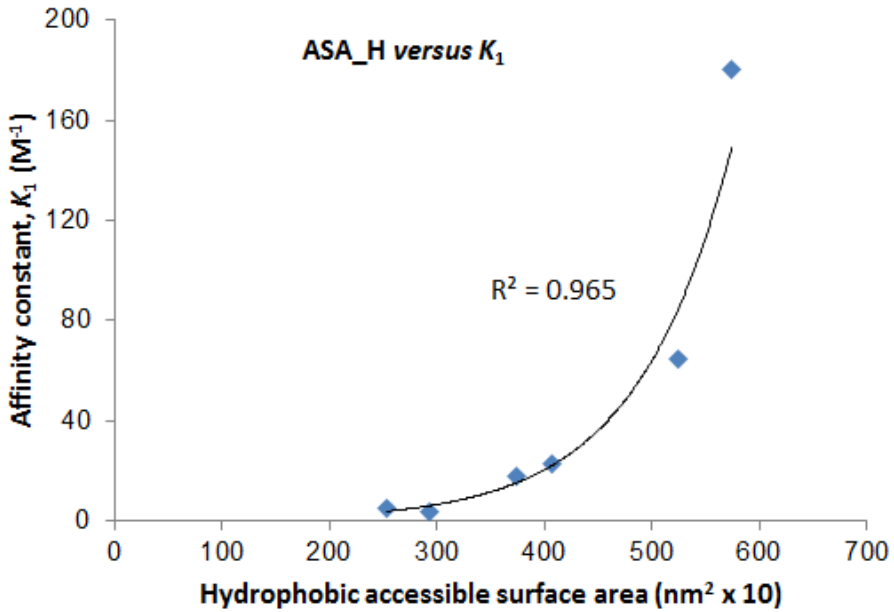
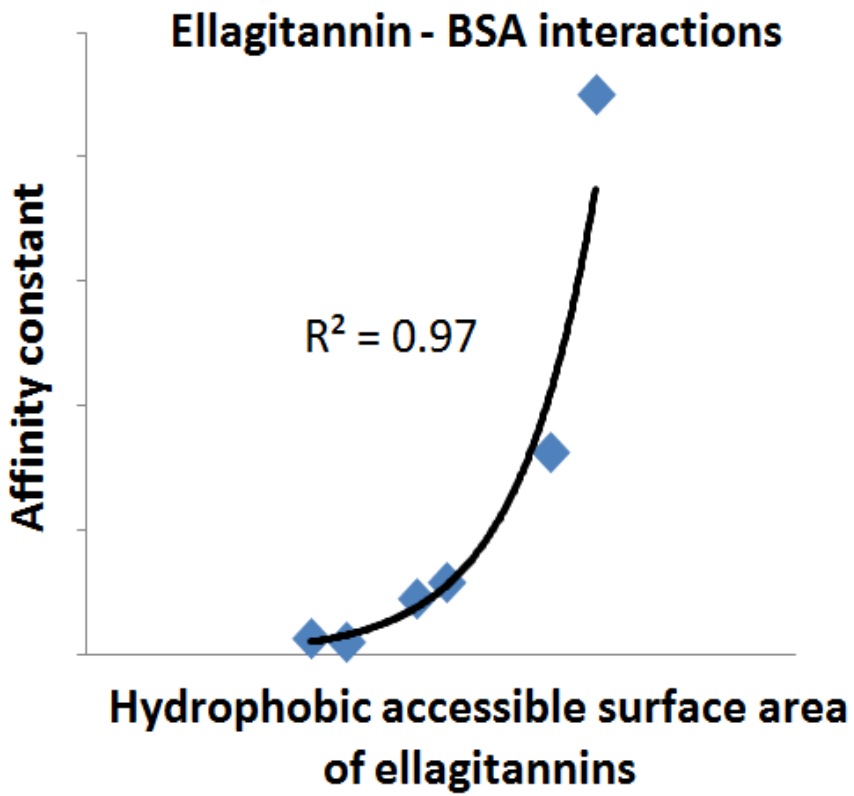

ASA_H versus K_1
Affinity constant, K_1 (M^{-1})
200
160
120
80
40
0
R^2 = 0.965
0
100
200
300
400
500
600
700
Hydrophobic accessible surface area (nm^2 x 10)

FIGURE 5

TOC Graphic


Ellagitannin - BSA interactions
Affinity constant
R² = 0.97
Hydrophobic accessible surface area of ellagitannins