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Colloidal Aggregation and the *in Vitro* Activity of Traditional Chinese Medicines

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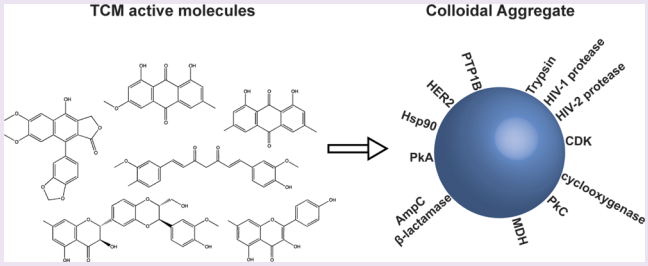
ABSTRACT: Traditional Chinese Medicines (TCMs) have been the sole source of therapeutics in China for two millennia. In recent drug discovery efforts, purified components of TCM formulations have shown activity in many *in vitro* assays, raising concerns of promiscuity. Here, we investigated 14 bioactive small molecules isolated from TCMs for colloidal aggregation. At concentrations commonly used in cell-based or biochemical assay conditions, eight of these compounds formed particles detectable by dynamic light scattering and showed detergent-reversible inhibition against β-lactamase and malate dehydrogenase, two counter-screening enzymes. When three of these compounds were tested against their literature-reported molecular targets, they showed similar reversal of their inhibitory activity in the presence of detergent. For three of the most potent aggregators, contributions to promiscuity via oxidative cycling were investigated; addition of 1 mM DTT had no effect on their activity, which is inconsistent with an oxidative mechanism. TCMs are often active at micromolar concentrations; this study suggests that care must be taken to control for artifactual activity when seeking their primary targets. Implications for the formulation of these molecules are considered.



INTRODUCTION

An intriguing source of bioactive leads for drug discovery is the traditional formularies of preindustrial societies. Whereas some are untrustworthy, others have a long history of observation and optimization. Among these are traditional Chinese medicines (TCMs), which were an integral part of the medical system in China for over 2000 years.[1,2] Their ongoing widespread use has spawned projects to understand the mechanism of action of TCMs and to improve their efficacy, including the purification of their active components, the determination of the molecular targets that they modulate, and the investigation of the role of the whole formulation in their activity.

The large literature in which purified TCM components appear attests to the interest in their structure−activity relationships, as natural products are often involved in drug discovery efforts.[3] Molecules like curcumin, kaempferol, and silibinin have appeared in thousands of scholarly articles, many investigating the molecular targets through which these molecules act (Table 1). Over 10 000 targets have been identified,[4] with the purified TCM components typically acting in the 1 to 200 μM concentration range (Table 1).[5−37] Intriguingly, the targets reported for even a single compound are often unrelated. For instance, curcumin has been reported to inhibit calcium-dependent ATPase,[5] HIV-1 and HIV-2 proteases,[6] human growth factor receptor 2 (HER2),[38] protein kinase A (PKA),[7] protein kinase C (PKC),[8] lipoxygenase,[39] P450 enzymes,[40] tyrosinase,[41] and thioredoxin reductase,[42] among others.

If identifying the molecular targets modulated by TCM molecules is crucial to understanding and optimizing their activity, so is the understanding of whether this activity is well-behaved and obeys classic stoichiometry. Four characteristics of TCM molecules motivated us to investigate their behavior: their resemblance to flavonoids, which can act artifactually via colloidal aggregation;[43−45] their target promiscuity; the relatively high, micromolar concentration ranges in which they act (compared to drugs, whose median dissociation constant for their therapeutic targets is in the 20 nM range[46]); and the apparent importance of formulations involving multiple other components in their *in vivo* efficacy.

Here we investigate the formation of colloidal aggregates by TCM molecules. Colloidal aggregation of small molecules is the dominant mechanism of artifactual inhibition of soluble proteins[45,47] and receptors.[43] These small molecule aggregators form liquid colloid-like particles that act by sequestering[48,49] and partly denaturing[50] their targets on their surfaces.[51] These colloidal particles can be active both in biochemical buffers and in cell culture,[52] affecting the rate and mechanism of penetration of drugs, dyes and reagents into cells.[53] We selected 14 purified TCM molecules for this study on the basis of their structures, availability, and frequency of occurrence in the literature. Most of these compounds formed inhibitory colloidal aggregates, often in a concentration range overlapping that reported for on-target activity. The implications of these results for understanding the

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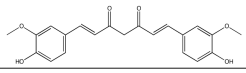
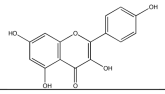
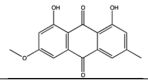
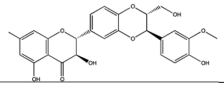
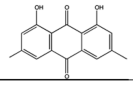
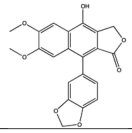
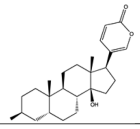
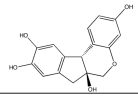
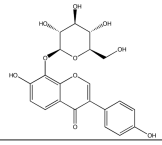
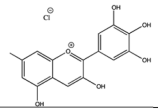
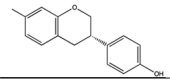
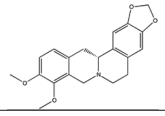
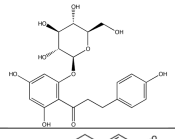
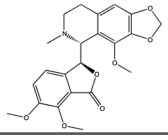
Table 1. TCM Citations, Targets, and Activities

Compound	Structure	Representative molecular targets and EC_{50} values (μM)	Pubmed citations[a]
Curcumin		Ca^{2+}-ATPase (~3), HER2, HIV-1 (100) and HIV-2 protease (250), cyclooxygenase (52), PkA and PkC	6394
Kaempferol		ERR, OATP1B1, CDK (2.5 -51) and trypsin (60, 106)	2960
Physcion		PTP1B (20), MMP (26.8 – 45.3)	244
Silibinin		Hsp90 (250), GLUT4 (60)	957
Emodin		MMP (12.8-20.1), AMPK (0.5), 11 β HSD1(4.2-7.2), trypsin (50), DYRK1A and CLK1 (4.2 – 6.1)	1302
Diphyllin		Protective antigen, DNA topoisomerase II	40
Bufalin		P-glycoprotein (100), CYP3A4 (14.5)	272
Brazilin		PKC	66
Puerarin		I_{K1}, ASICs (38.4)	719
Delphinidin		Histone acetyltransferase, GLO1 (1.9)	550
Equol		ERR (5.8), ERRD	790
Canadine		Coagulation factor III, CDK2(>50)	76
Phlorizin		SGLT1 and SGLT2 (0.02), hCNT (100-200)	2341
Noscapine		OCT2 (2.6), tubulin, MATE1 (34.5)	423

[a]As of 2/26/14.

molecular mechanism of these TCM molecules and for their formulation in complex mixtures will be considered.

RESULTS AND DISCUSSION

Investigating Aggregation of TCM Molecules by DLS and Enzyme Inhibition. Two criteria were used to investigate whether the TCM bioactive compounds formed colloidal aggregates: particle formation and detergent-reversible enzyme inhibition.[48,54] Particle formation was detected and radii measured by dynamic light scattering (DLS). IC_{50} values were measured against model enzymes AmpC β-lactamase and malate dehydrogenase (MDH), which we have widely used as counter-screens

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Table 2. Bioactive Molecules in TCMs Form Particles and Inhibit Model Enzymes

	IC_{50} (μM) vs					
	β-lactamase		MDH			
compound	no detergent[a]	with detergent[b]	no detergent	with detergent	CAC (μM)[c]	radius (nm)
curcumin	12	>30	9	>30	17 ± 0.44	465 ± 30
kaempferol	47	>100	61	>100	81 ± 26	61 ± 20
physcion	7	>20	11	>20	0.32 ± 0.025	320 ± 83
silibinin	ND[d]	ND	12	>100	0.47 ± 0.15	53 ± 16
emodin	27	>100	28	>100	28 ± 2	231 ± 61
diphyllin	55	>100	49	>200	40 ± 4.0	269 ± 49
bufalin	93	>200	101	>200	51 ± 3	1317 ± 394
brazilin	302	>500	1.6	84	57 ± 7	52 ± 10
puerarin	ND	ND	135	156	135 ± 40	78 ± 25
delphinidin	72	148	27	>100	ND	ND
equol	127	>500	ND	ND	ND	ND
canadine	ND	ND	ND	ND	95 ± 11	52 ± 14
phlorizin	ND	ND	ND	ND	ND	ND
noscapine	ND	ND	ND	ND	ND	ND

[a]50 mM potassium phosphate, pH 7.0. [b]50 mM potassium phosphate, pH 7.0 + 0.01% (v/v) Triton X-100. [c]Critical aggregation concentration determined by dynamic light scattering. [d]Not detected to precipitation limit.

for colloidal aggregate formation.[55] Disruption of enzyme inhibition by nonionic detergents, such as Triton X-100, which little affects well-behaved substrates and inhibitors, is characteristic of a colloidal-based inhibition mechanism.

We first investigated the critical aggregation concentration (CAC) of each compound by DLS. This critical point is akin to critical micelle concentrations of detergents, though the resulting colloidal particles are typically much larger (often in the 200 nm radius range) and more polydisperse.[56,57] Of the 14 TCMs, 10 had light scattering intensities and autocorrelation functions that supported particle formation, with radii ranging from 50 to >500 nm (Table 2 and Figure 1). Particles of silibinin and physcion began to be detected at submicromolar concentrations (Table 2), an order of magnitude lower than many of their literature activities (Table 1). The 17 μM CAC value of curcumin overlapped the concentration range of its reported activities, whereas those of kaempferol, emodin, canadine, and bufalin were found to be slightly above their reported activity range in the literature (Table 2). Conversely, the 135 μM CAC value of puerarin, although reproducible, puts it above the range of its bioactivities. For four TCM compounds—equol, phlorizin, noscapine, and delphinidin—no particles were observed by DLS up to their solubility limits, although enzyme inhibition suggests that delphinidin may well form inhibitory aggregates (as discussed below).

To investigate the relevance of colloidal formation on the activity against protein targets, all 14 compounds were tested for inhibition of AmpC β-lactamase and MDH. Of the 14, 11 inhibited one or both of these model enzymes (Table 2, Figures 2 and 3). A total of 10 of these showed detergent-reversible enzyme inhibition against at least one of the two enzymes, and most also formed particles as observed by DLS. The reversal of inhibition by addition of small amounts of nonionic detergent, below the critical micelle concentration (CMC) of that detergent, is a characteristic feature of a colloidal aggregator, one that is hard to reconcile with any other mechanism of inhibition.

We note that although canadine formed particles by DLS, it did not inhibit either counter-screening enzyme. Phlorizin and noscapine showed no inhibition against either enzyme, consistent with their lack of particle formation.

Although there was broad correspondence between colloid-derived IC_{50} values and critical aggregation concentrations (CACs), the two values sometimes differed. Molecules such as physcion and silibinin have CACs a log-order lower than their IC_{50} values, whereas curcumin's IC_{50} was slightly below its DLS-measured CAC (Table 2). IC_{50} values being much higher than CACs is explained by the stoichiometric nature of aggregate-based inhibition and the low concentration of the colloidal particles. These particles are present in the mid-femptomolar to picomolar range at their CACs[58] and sequester enzymes tightly.[59] If the proteins are present at high enough concentrations—which given the low concentration of colloids need not be very high—they will overwhelm the capacity of the colloids to adsorb them, and the effective inhibition will be slight until the colloids rise to such a concentration that they have enough surface to again stoichiometrically match the enzyme. This is why it is possible to diminish the inhibition of colloidal aggregates by increasing enzyme concentration.[56] Conversely, because we rely on DLS to measure CAC and this can miss the scattering of low concentrations of particles, it is also possible to observe particles by enzyme inhibition before they can be observed physically.

If colloid aggregation explains the reported activity of TCMs against some targets, then it should be possible to demonstrate this on these targets. We therefore sought targets that were readily assayed for three of the most highly cited TCMs: emodin, kaempferol, and curcumin. Emodin and kaempferol had been found to inhibit trypsin in the 50 to 100 μM range, values we were able to reproduce in buffer lacking detergent and that were consistent with their CAC values in related buffer[12,13,60] (Tables 2 and 3). Upon addition of 0.01% nonionic detergent Triton X-100, inhibition was essentially eliminated (Table 3, Figure 4A,B). Similarly, the addition of detergent to our HIV-2 protease inhibition assays also resulted in a significant reduction in inhibition by curcumin (Table 3, Figure 4C). The DLS data and enzymology against β-lactamase and MDH suggest that many of the TCMs in this study form colloidal aggregates at relevant concentrations, and these observations against trypsin and HIV-2 protease confirm a colloidal-based mechanism of action on the reported targets themselves.

Investigating the Role of Oxidative Cycling in Enzyme Inhibition. Promiscuous inhibition by (−)EGCG, a polyphenol

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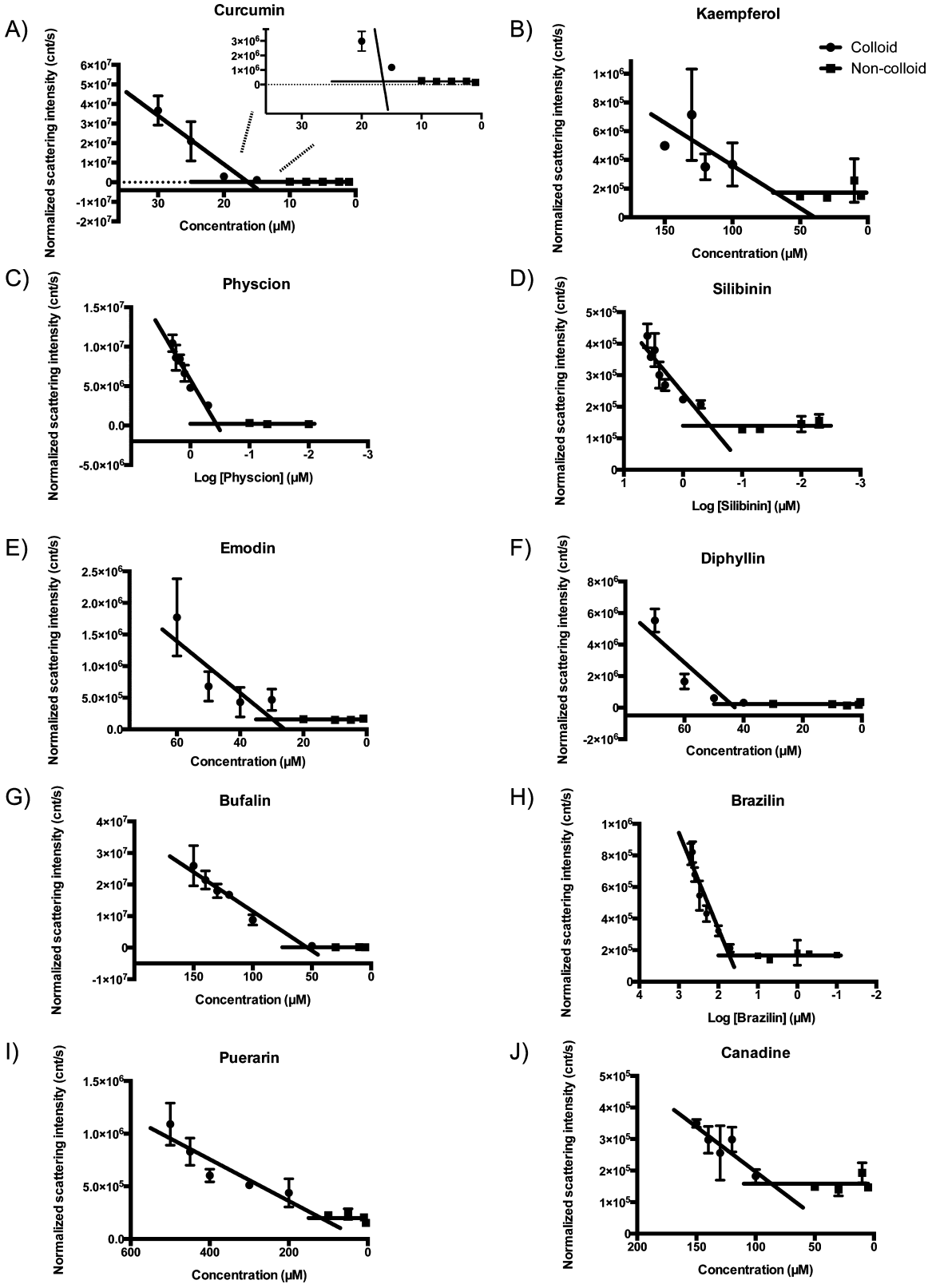

Figure 1. Critical aggregation curves for (A) curcumin, (B) kaempferol, (C) physcion, (D) silibinin, (E) emodin, (F) diphyllin, (G) bufalin, (H) brazilin, (I) puerarin, and (J) canadine. Colloid (●) and noncolloid (■).

found in some TCMs, has been attributed to the reactivity of its auto-oxidation products.[61] Because 10 of our 14 compounds are also polyphenols, we were interested in exploring the contribution of oxidative cycling to their ability to inhibit the two model enzymes. We thus performed enzyme inhibition assays with curcumin, emodin, and physcion in the presence of a reducing agent to discriminate between colloidal and oxidative inhibition. Addition of 1 mM DTT did not significantly affect

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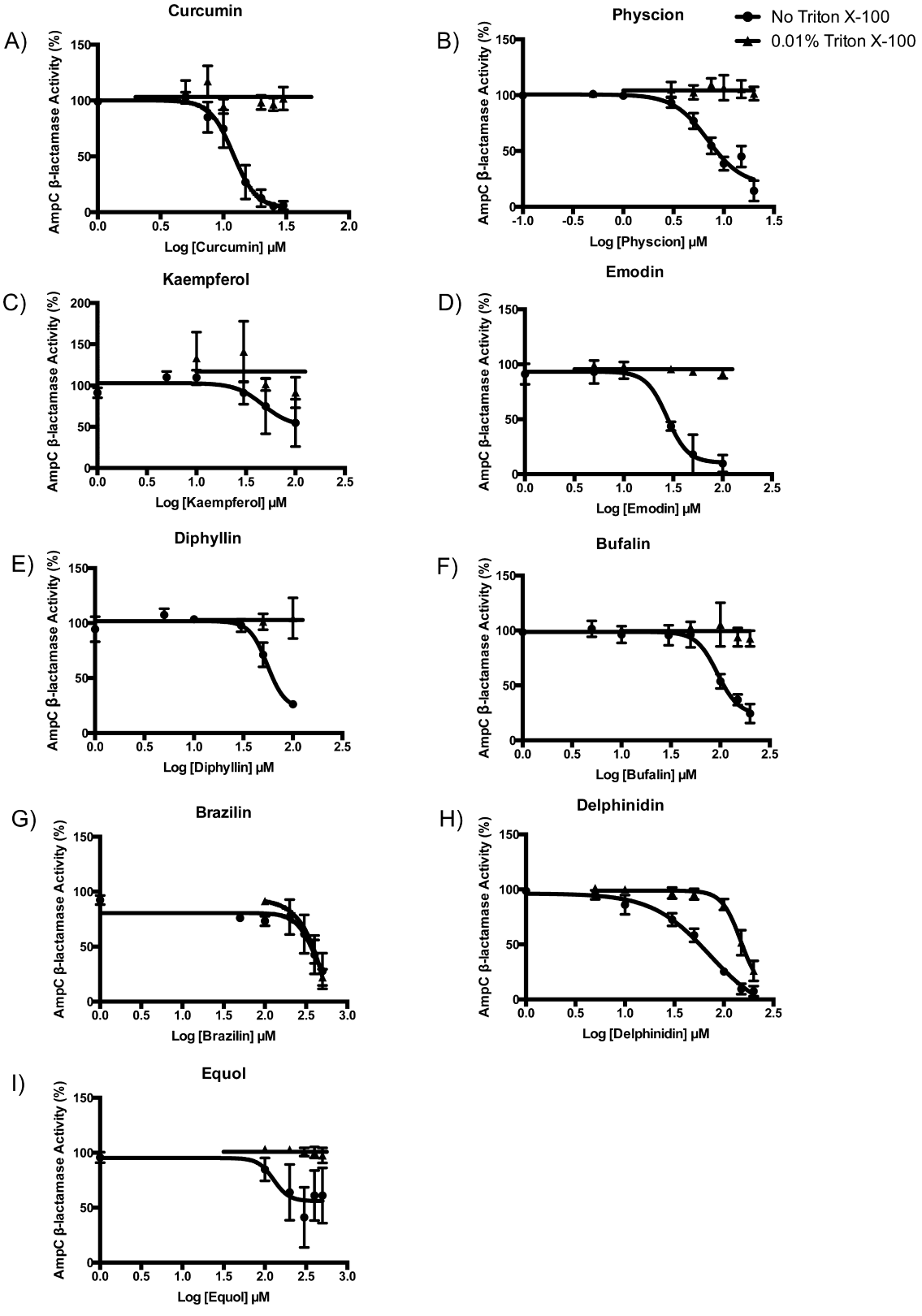

Figure 2. AmpC β-lactamase dose-response curves in the absence (●) and presence (▲) of detergent for (A) curcumin, (B) physcion, (C) kaempferol, (D) emodin, (E) diphyllin, (F) bufalin, (G) brazilin, and (H) delphinidin.

inhibition (Table 4 and Figure 5), suggesting that the observed activity is not influenced by auto-oxidation products. Thus, for these three polyphenols, their promiscuous activity is consistent with a dominant role of colloidal aggregation.

Taken together, these results indicate that curcumin, kaempferol, physcion, silibinin, emodin, diphyllin, bufalin, and brazilin are strong aggregators that exhibit CAC and IC_{50} values in the 1 to 100 μM range. Puerarin, delphinidin, and equol, which

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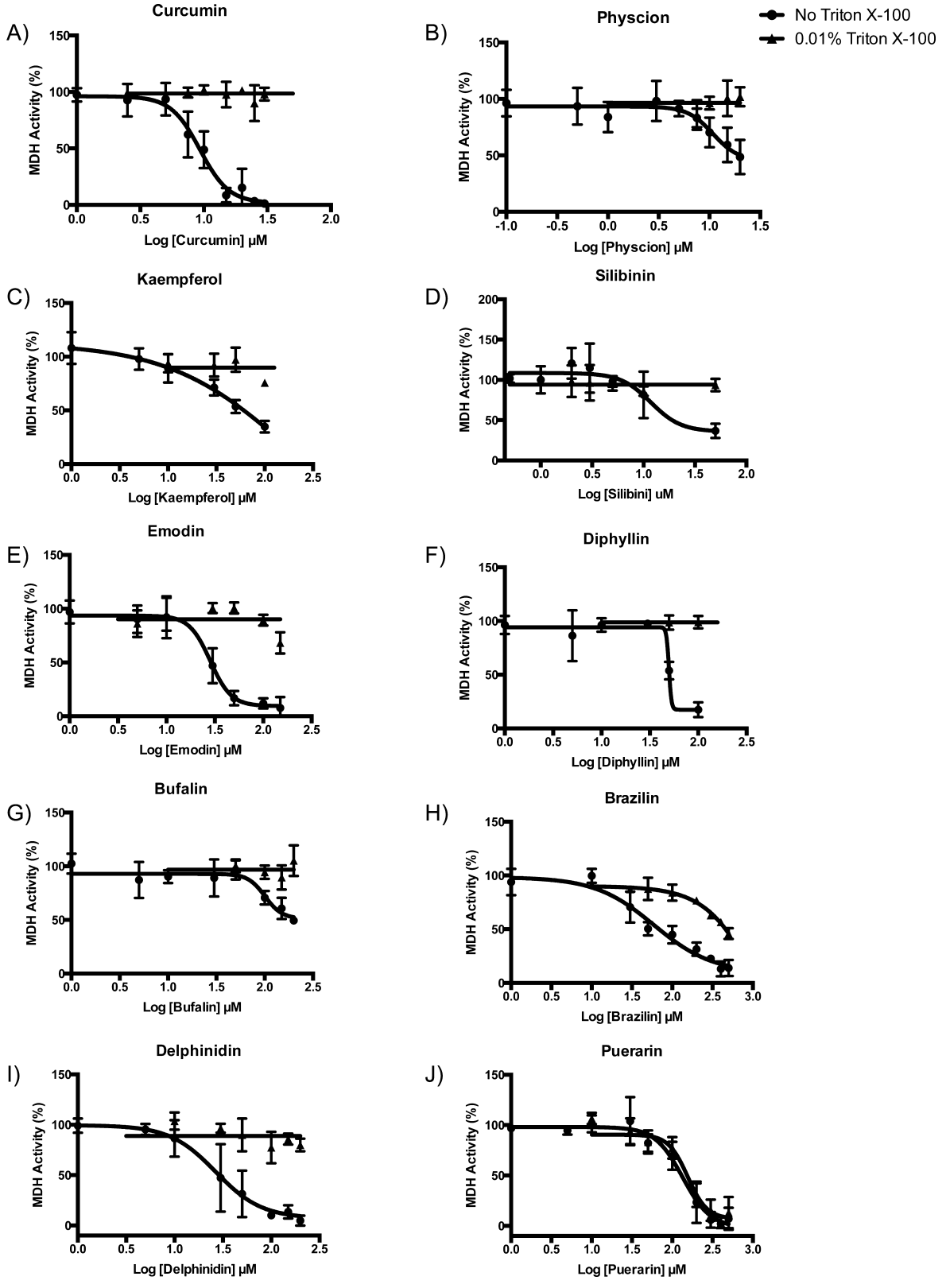

Figure 3. MDH inhibition dose-response curves in the absence (●) and presence (▲) of detergent for (A) curcumin, (B) physcion, (C) kaempferol, (D) silibinin, (E) emodin, (F) diphyllin, (G) bufalin, (H) brazilin, (I) delphinin, and (J) puerarin.

either showed relatively high (poor) CAC values or were only able to satisfy one of the criteria, are likely weaker aggregators. Though we do not discount the role of oxidative cycling in the activity of polyphenols and TCM molecules, such a mechanism does not appear to contribute to the activity of the compounds in this study. For only two of the molecules tested, phlorizin and noscapine, was there no evidence of colloidal aggregation. The lack of enzyme inhibition and the high CAC value for

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Table 3. Inhibition of TCM Targets Can Be Reversed by Nonionic Detergent

	IC_{50} vs trypsin (μM)		IC_{50} vs HIV-2 protease (μM)	
compound	no detergent[a]	with detergent[b]	no detergent[c]	with detergent[d]
emodin	49	>500		
kaempferol	110	>250		
curcumin			19	>100

[a]50 mM potassium phosphate, pH 7.0. [b]50 mM potassium phosphate, pH 7.0 + 0.01% (v/v) Triton X-100. [c]50 mM sodium acetate, pH 5.5, 200 mM sodium chloride, 1 mM EDTA and 0.5 mM TCEP. [d]50 mM sodium acetate, pH 5.5, 200 mM sodium chloride, 1 mM EDTA and 0.5 mM TCEP + 0.01% (v/v) Triton X-100.

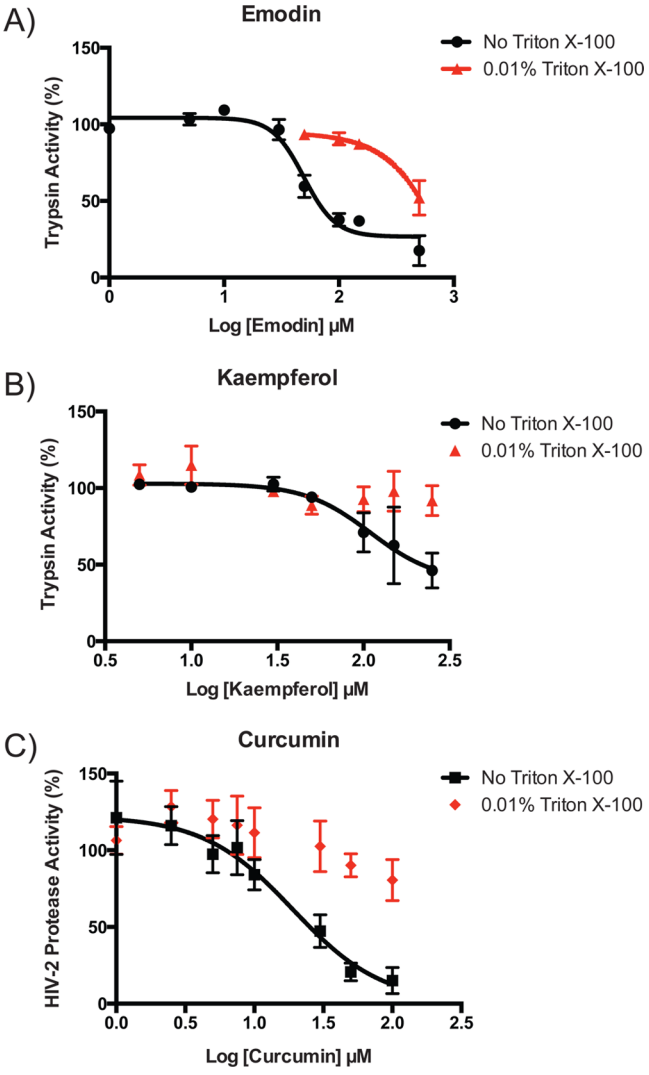

Figure 4. Trypsin inhibition dose-response curves in the absence (black circles) and presence (red triangles) of detergent for (A) emodin and (B) kaempferol. HIV-2 protease inhibition dose response curve in the absence (black squares) and presence (red diamonds) of detergent for (C) curcumin.

canadine suggest that aggregation may also be irrelevant to its activity.

TCMs have been the focus of intense interest, many appearing in hundreds or thousands of scholarly studies as investigators seek the molecular targets underlying the efficacy of these preindustrial-era therapeutics. To understand and to optimize the activity of purified TCM molecules, and to understand if they have reproducible and nonplacebo activities, determining their molecular targets seems crucial. A key observation from this study is that the purified components of TCMs can form colloidal aggregates that act nonspecifically on protein targets, clouding their true mechanism. Of the 14 TCM molecules investigated, 8 are strong aggregators whose IC_{50} values against counter-screen enzymes overlap with literature-reported activities on their putative molecular targets. Consistent with a colloidal mechanism, this inhibition could be reversed by the addition of a nonionic detergent, a treatment that leaves most specific, well-behaved ligand affinities unperturbed. For three of the TCMs, kaempferol, emodin, and curcumin, we were able to show that colloidal aggregation explained their inhibition on their putative literature targets—here trypsin and HIV-2 protease—as nonionic detergent completely disrupted inhibition (Tables 1, 2, and 3).

Table 4. Enzyme Inhibition by Bioactive Molecules in TCMs in the Presence of 1 mM DTT

	IC_{50} vs AmpC β-lactamase (μM)		IC_{50} vs MDH (μM)	
compound	no DTT	with DTT	no DTT	with DTT
curcumin	12	14	9	10
emodin	27	17	28	18
physcion	7	11	11	8

Naturally, not all TCMs tested here are aggregators. Molecules such as puerarin and equol may aggregate at ≥100 μM, but this behavior might only be relevant to those experiments conducted at unusually high concentrations. Correspondingly, for molecules like phlorizin and noscapine, no aggregation could be detected at accessible concentrations. Thus, not all TCM molecules can be tarred with an aggregation brush.[62] Even for those that do aggregate, there are likely targets, conditions, and concentration ranges where this mechanism is not relevant. Finally, it merits re-emphasis that this study reflects only on the *in vitro*, target-based activity of TCM molecules, not their behavior *in vivo*.

What this study does suggest is that colloidal aggregation must be controlled for when seeking molecular targets for TCMs and related molecules. If it is perhaps disturbing that aggregation is so prevalent among TCM molecules, it should also be comforting that controls for aggregation can be readily conducted. They include the addition of nonionic detergents to assays of soluble proteins[44,54,57,63] and some membrane-bound receptors,[43] colloid precipitation by centrifugation,[48,64] increasing target concentration in the assay,[56,65] and more biophysical techniques like DLS, NMR,[66] and surface plasmon resonance.[67] In addition to distinguishing among true and artifactual targets of TCM molecules, this study may help to illuminate the role of formulation in TCM activity, whose practitioners have long argued that it is only in the full mixture, with multiple other components, that TCMs are fully active.[68,69] Although there are many explanations of the importance of formulation, it may be that the strong colloid-forming potential of many TCM molecules can affect their pharmacokinetics and biodistribution, as may be true, too, for some Western formulary drugs.[70] Controlling for colloid formation may not only help with determining their target-based mechanism of action, but also with optimizing their *in vivo* activity, something supported by recent formulation studies of aggregating small molecules.[71,72]

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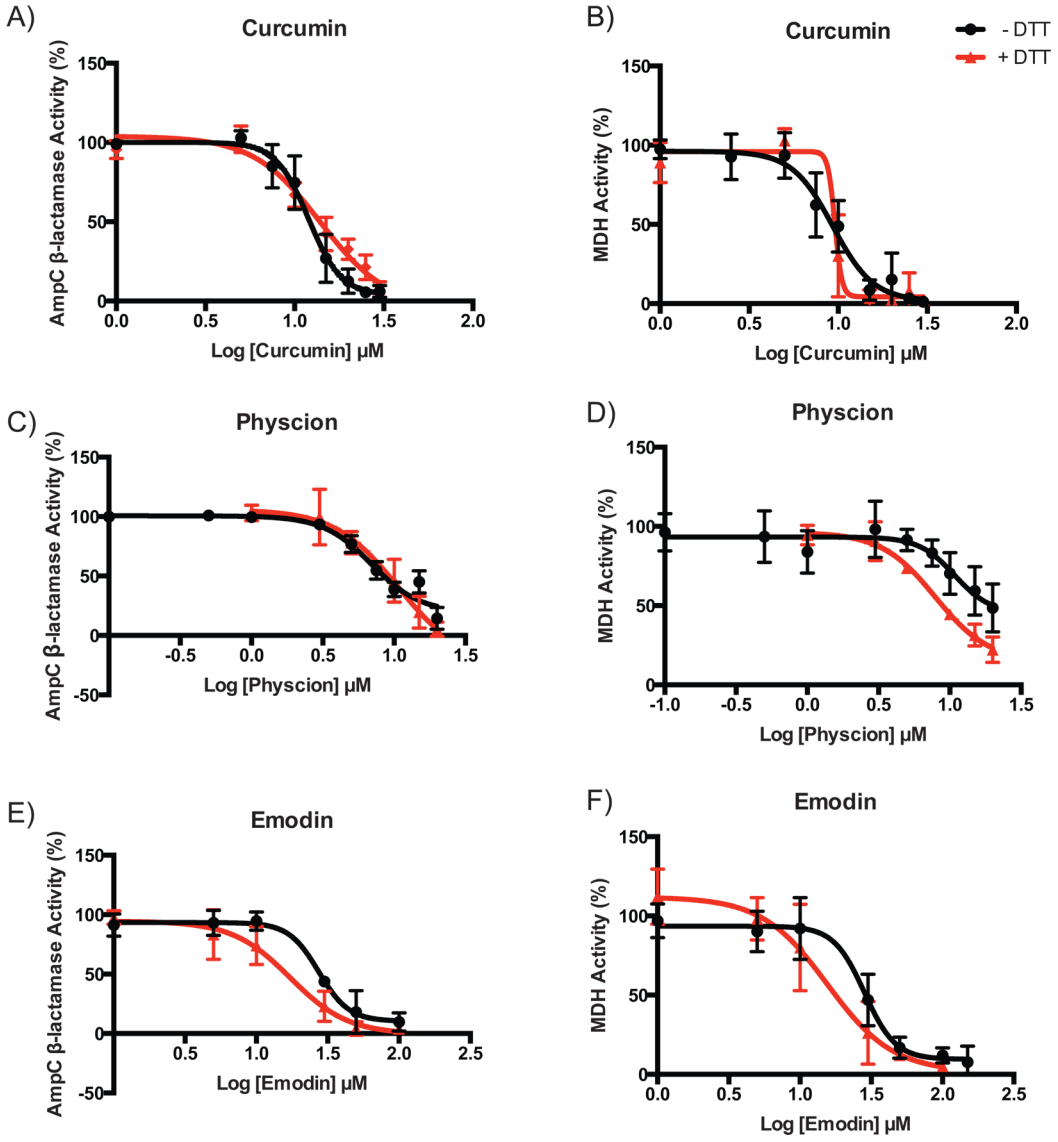

Figure 5. AmpC β-lactamase and MDH inhibition dose-response curves in the absence (black circles) and presence (red triangles) of DTT for (A and B) curcumin, (C and D) physcion, and (E and F) emodin.

MATERIALS AND METHODS

Materials. AmpC β-lactamase was purified from *Escherichia coli* as previously described.[73] Malate dehydrogenase (MDH) was purchased from VWR (M7032) and Sigma-Aldrich (CA97061-502). Trypsin from porcine pancreas (T0303) was purchased from Sigma-Aldrich. HIV-2 protease was kindly provided to us by Dr. Charles Craik (UCSF). CENTA (219574) was purchased from EMD Millipore. Chromogenic trypsin substrate *N*α-benzoyl-DL-arginine 4-nitroanilide hydrochloride (BApNA) (B4875) was purchased from Sigma-Aldrich. Anthranilyl-HIV protease substrate (H-2992) was purchased from BACHEM. Oxaloacetate (O4126), reduced β-nicotinamide adenine dinucleotide (NADH) (N4505), brazilin (PH010674), noscapine (363960, purity: 97%), delphinidin chloride (43725, purity: $\geq$ 95%), kaempferol (60010, purity: $\geq$ 97%), and curcumin (08511, purity: $\geq$ 98%) were purchased from Sigma-Aldrich. Canadine (C175175, purity: 96%) and bufalin (B689510, purity: 98%) were purchased from Toronto Research Chemicals. Equol (S2450, purity: 99%), silibinin (S2357, purity: 99%), puerarin (S2346, purity: 99%), physcion (S2395, purity: 99%), phlorizin (S2343, purity: 99%), and emodin (S2295, purity: 99%) were purchased from Selleck Chemicals. Diphyllin (N038-0217) was purchased from Molport. Triton X-100 (TB0198) was purchased from Bio Basic Canada.

Dynamic Light Scattering. Concentrated DMSO stocks of all 14 compounds were diluted with filtered 50 mM KPi, pH 7.0, with a final concentration of 1% DMSO. Measurements were made using a DynaPro Plate Reader II system (Wyatt Technology) with a 60 mW laser at ~830 nm in either 96- or 384-well plates; this particular instrument had been modified by Wyatt Technology to have a larger laser beam width that is appropriate for detecting large colloidal particles. Data were acquired and processed by the software Dynamics, version 1.7 (Wyatt Technology). The laser power was automatically adjusted by the Dynamics software; the detector angle was 158°. Each radius value reported represents three or more independent measurements at 25 °C at twice the compound's CAC.

CAC Determination. Normalized scattering intensities (counts/sec, cnt/s) were plotted against decreasing concentrations of each compound. Data for colloidal and noncolloidal states were linearly regressed and the intersection point was

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determined to be the critical aggregation concentration. Concentrations are represented as the mean and the standard deviation of at least three independent replicates.

Enzyme Assays. All 14 compounds were tested for inhibition of AmpC β-lactamase and MDH. Emodin and kaempferol were tested for inhibition of trypsin. Stocks of the compounds were typically prepared at 100 times the working concentration in DMSO and subsequently diluted into 50 mM potassium phosphate buffer to yield a final reaction volume of 1 mL. Assays were performed at room temperature in 50 mM potassium phosphate, pH 7.0, with or without 0.01% (v/v) Triton X-100, a concentration below the CMC (0.02%) of this nonionic detergent. Data acquisition was performed by an HP8453a spectrophotometer using kinetic mode in the software UV−Vis ChemStation (Agilent Technologies), which also determined the first-order reaction rates. All reactions were performed in the presence of 1% DMSO to control for its effects on enzyme activity. Additional assays supplemented with 1 mM DTT were performed for curcumin, emodin, and physcion. Curcumin was tested for inhibition of HIV-2 protease. Stocks of curcumin were prepared in DMSO at 100 times the working concentration and subsequently diluted into 50 mM sodium acetate to yield a final reaction volume of 50 μL. These assays were performed at room temperature in 50 mM sodium acetate, 200 mM NaCl, 1 mM EDTA, 0.5 mM TCEP, pH 5.5, with or without 0.01% Triton X-100. Fluorescence data acquisition was performed by a Bio-Tek Synergy H4 microplate reader using the GEN5 software, which also determined the first-order reaction rates.

For AmpC β-lactamase assays, compound and 1 nM enzyme were incubated for 5 min, and the reaction was initiated with 200 μM of the chromogenic β-lactamase substrate CENTA. CENTA was prepared as a 4.6 mM stock in 50 mM potassium phosphate. Hydrolysis of CENTA was monitored at 405 nm. For MDH assays, compound and 2 nM enzyme were incubated for 5 min and the reaction was initiated with 200 μM oxaloacetate and 200 μM NADH, which were freshly prepared on the day of the experiment as 20 mM stocks in 50 mM potassium phosphate buffer. The rate of reaction was monitored spectrophotometrically at 340 nm. For trypsin assays, compound and 1 nM enzyme were incubated for 5 min and the reaction was initiated with 60 μM BApNA. BApNA was prepared as a 200 mM stock in potassium phosphate. Cleavage of BApNA was monitored at 405 nm. For HIV-2 protease assays, curcumin was incubated with 1 μg mL^{-1} enzyme for 5 min, and the reaction was initiated with 0.5 mM anthranilyl HIV protease substrate. Fluorescence of the cleaved substrate was monitored with excitation and emission wavelengths of 280 and 435 nm, respectively. From these assays, an IC_{50} value of 19 μM was obtained for curcumin, which was an order of magnitude stronger than that previously reported.[6] This difference in IC_{50} likely reflects the lower salt concentrations used in our assays (0.2 M vs 1 M) and the use of a FRET-based substrate.[74,75]

Activity was measured in triplicate for at least six different concentrations for each compound. Dose−response curves were plotted, and IC_{50} values were calculated by Graphpad Prism 6 (Graphpad, San Diego, CA), using a sigmoidal dose−response curve analysis with variable slope.

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Notes

The authors declare no competing financial interest.

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