

Optimized Turmeric Extracts have Potent Anti-Amyloidogenic Effects

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Abstract: Inhibition of β -amyloid ($A\beta$) accumulation and $A\beta$ fibril ($fA\beta$) formation from $A\beta$ are attractive therapeutic targets for the treatment of Alzheimer's disease (AD). While previous studies have shown anti-amyloidogenic effects of curcumin *in vitro* and *in vivo*, no studies have examined optimized turmeric extracts enriched in curcuminoids or turmerones. Three standardized turmeric extracts, HSS-838, HSS-848, and HSS-888, were prepared with different chemical profiles to investigate their potential therapeutic benefits for AD. These extracts were fingerprinted by DART TOF-MS to reveal the significant chemical complexity. In addition four curcuminoids (curcumin, tetrahydrocurcumin, demethoxycurcumin and bisdemethoxycurcumin) were also examined. We measured the effects of the extracts and curcuminoids, on the aggregation of $A\beta$ by using a thioflavin T cell-free assay and the secretion of $A\beta$ from human neuronal cells (SweAPP N2A cells) *in vitro*. All three extracts and the curcuminoids showed dose-dependent inhibition of $fA\beta$ aggregation from $A\beta_{1-42}$ in the cell-free assay, with IC_{50} values of $\leq 5 \mu\text{g/mL}$. However, only HSS-888, curcumin and demethoxycurcumin significantly decreased $A\beta$ secretion ($\sim 20\%$) in SweAPP N2A cells. Interaction matrices were used to examine possible synergistic interactions between HS-888 and the other extracts and the individual curcuminoids on $A\beta$ aggregation. Only simple additive effects were observed for the $A\beta$ aggregation inhibition, supporting the notion that the known curcuminoids are not strong inhibitors of this activity. However, HSS-888 showed strong inhibition of $A\beta$ aggregation and secretion, thus indicating that there are novel bioactive molecules in this extract that might be important leads for future AD drug discovery efforts.

Keywords: Alzheimer's disease, curcuminoids, turmeric extracts, amyloid aggregation, amyloid secretion, DART TOF-MS.

INTRODUCTION

Alzheimer's disease (AD), the most common cause of dementia among the elderly, is characterized by cognitive deterioration, progressive memory loss, and behavioral problems. Pathologically, AD is characterized post mortem by the presence of senile plaques, neurofibrillary tangles, and neuronal cell loss. The accumulation of amyloid- β ($A\beta$), produced as a cleavage product of the amyloid precursor protein (APP), both as soluble aggregate oligomers and senile plaques is a neuropathological hallmark of AD [1]. A fundamental aspect of the current $A\beta$ cascade hypothesis is that $A\beta$ accumulation in the brain initiates a series of pathological reactions that result in tau aggregation and neuronal dysfunction that are the primary causes of dementia [2].

Roles for neuroinflammation and oxidative damage have also been implicated in neurodegeneration, and may play an important role in the neuropathogenesis of AD [3, 4]. For example, $A\beta$ can produce H_2O_2 [5] and reactive oxygen species (ROS) that may mediate plaque-induced neurotoxicity [6-8]. Recently it has been shown that inhibition of mitochondrial respiratory capacity and oxidative stress elevates β -secretase protein levels and activity as well as $A\beta$ levels

[9]. In addition, mechanical disruption of mitochondrial electron transport activities by amyloid accumulation in this organelle leads to loss of ROS scavenging function as well as loss in energetic capabilities required for neuronal cell maintenance and activities [10, 11].

At present, the number of therapeutic options for AD is severely limited. Currently marketed drugs for AD do not prevent or reverse this disease and are approved only for the management of symptoms [12]. Driven by the clear unmet medical need and a better understanding of the biology and pathophysiology of AD, the number of drugs in development for this indication has increased dramatically in recent years [13]. Because drug discovery using synthetic drugs is expensive, complex, and vastly inefficient, many groups have turned their attention to screen natural products and botanical extracts, especially where therapeutic uses and benefits have been documented by traditional medicine systems [14, 15]. For example, our group recently found that EGCG, the major polyphenolic found in green tea, works both *in vitro* and *in vivo* to reduce amyloid production by promoting α -secretase activity [16]. In addition, curcumin represents a hopeful approach for treating, delaying, and/or preventing the progression of AD [17]. Curcumin (diferuloylmethane) is an orange-yellow component of turmeric (*Curcuma longa*), a spice often found in curry powder. Traditionally known for its anti-inflammatory effects, curcumin has been shown, in the

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last two decades, to be a potent therapeutic agent with reported beneficial effects in arthritis, allergy, asthma, atherosclerosis, heart disease, diabetes, and cancer [18-22]. *In vitro* studies have shown that curcumin attenuates inflammatory activation of brain microglial cells [22, 23]. Curcumin also inhibits the formation of A β oligomers and fibrils *in vitro* [24, 25]. Other studies have shown that curcumin prevents neuronal damage [26], reduces both oxidative damage [27-29] and amyloid accumulation [25] in a transgenic mouse model of AD. Lastly, human clinical trials have shown that curcumin is safe and has broad anti-inflammatory properties [30].

Recently, Garcia *et al.* [31] used multiphoton microscopy (MPM) and longitudinal imaging to evaluate *in vivo* and in real-time the effects of systemic curcumin administration on existing A β deposits using aged APP^{sw}/PS1^{de9} transgenic mice. They found that curcumin clears and reduces plaques and partially restores the altered neuronal pathology near and away from plaques [31]. This study further supports evidence that curcumin has beneficial effects in reducing the pathology and neurotoxicity of AD in transgenic mice.

While previous studies have shown anti-amyloidogenic effects of curcumin, no studies have examined "optimized" turmeric extracts enriched in the curcuminoids. Commercially available curcumin extracts used for research and for clinical trials vary considerably, but often contain about 75% curcumin (Cur), 15% demethoxycurcumin (DMC), and 5% bisdemethoxycurcumin (BDMC). In addition, some extracts also contain very low levels of tetrahydrocurcumin (THC), one of the naturally occurring metabolites of curcumin. Various studies have shown that curcumin and DMC were less stable than BDMC, whereas the reduced curcumin metabolite, THC, is the most stable curcuminoid. Turmeric and most commercial turmeric extracts are also rich in the lipid-soluble turmerones. The turmerones include several species with α -turmerone, β -turmerone and γ -turmerone typically being the most abundant in turmeric. The precise role of turmerones in AD is unclear, though they have established anti-inflammatory and anti-oxidative activities which could reduce neurotoxicity [32].

Optimized botanical extracts must be developed to produce standardized, dose-reliable, and concentrated botanical extracts necessary to not only meet FDA regulations for botanical drug development, but to provide efficacious and safe herbal medicines. Moreover, optimized botanical extracts under IND for human therapeutic indications must be produced in facilities following GMP and cGMP standards. The development of Direct Analysis in Real Time (DART) Time-of Flight Mass spectrometry [33] has allowed for the rapid characterization of the chemical complexity of botanical extracts, and has allowed for the chemical compositions of standardized extracts to be defined [34, 35]. In the present study, three optimized and standardized proprietary extracts were prepared from Turmeric (*Curcuma longa* L.) root with different chemical profiles, and Cur, DMC, BDMC, and THC, curcuminoid standards, were used to investigate the potential therapeutic effects for AD. Using fluorescence spectroscopic analysis with thioflavin T in a cell-free assay and A β secretion from human neuronal cells (SweAPP N2A cells), we examined the three extracts, HSS-888, HSS-838,

and HSS-848 and the curcuminoid standards on the aggregation and secretion of A β *in vitro* with the goal to further understand the functional roles of the complex chemistries of the extracts.

MATERIALS & METHODS

Turmeric Extracts and Curcuminoids

Two standardized turmeric (*Curcuma longa* L.) extracts, HSS-888 and HSS-838, were prepared using supercritical CO₂ and affinity chromatography and were provided by HerbalScience Singapore Pte Ltd. One additional extract was prepared from a 100% ethanol (USP) extract of HSS-838, termed HSS-848, which was enriched in turmerones (>98%) and depleted in curcuminoids (<2%). Curcuminoid standards, curcumin (Cur), tetrahydrocurcumin (THC) and demethoxycurcumin (DMC) were purchased from Chromadex Inc. (Irvine, CA), and bisdemethoxycurcumin (BDMC) was purified from a Cur source from Chromadex Inc. (Irvine, CA). The purity of the curcuminoid standards was evaluated using DART Time-of-Flight (TOF) mass spectrometry [38]. All of the standards were >97% pure while the Curcumin standard was 81% Cur, 12% DMC and 2% BDMC.

Chemical Analyses of Extracts and Curcuminoids

All extracts and curcuminoids were characterized by DART Time-of-Flight Mass spectrometry (TOF-MS). The JEOL DARTTM AccuTOF-mass spectrometer (JMS-T100LC; Jeol USA, Peabody, MA) was used for chemical analysis of the turmeric extracts and the curcuminoid standards. DART TOF-MS was executed in positive ion mode [M+H]⁺. The needle voltage was set to 3500V, heating element to 250°C, electrode 1 to 150V, electrode 2 to 250V, and helium gas flow between 1.98 and 2.76 liters per minute. For the mass spectrometer, the following settings were loaded: orifice 1 set to 10V, ring lens voltage set to 5V, and orifice 2 set to 5V. The peak voltage was set to 1000V in order to give peak resolution beginning at 100 *m/z*. The microchannel plate detector (MCP) voltage was set at 2500V. Calibrations were performed internally with each sample using a 10% (w/v) solution of PEG 600 (Ultra Chemical, North Kingston, RI) that provided mass markers throughout the required mass range of 100 to 1000 *m/z*. Calibration tolerances were held to 10 mmu. Samples (as dry powders) were introduced into the DART helium plasma using the closed end of a borosilicate glass melting point capillary tube until a signal was achieved in the total-ion chromatogram (TIC). The next sample was introduced when the TIC returned to baseline levels.

A searchable database of botanical masses and molecular formulas developed by HerbalScience Group LLC was used for the identification of both known and unknown chemicals present in the turmeric extracts and the curcuminoid standards. Molecular identifications were searched and verified against the NIST/NIH/EPA Mass Spectra Database [36] and/or the Dictionary of Natural Products [37] database when applicable.

Aggregation Assay (Thioflavin T Assay)

The presence of A β ₁₋₄₂ fibers was monitored in solution by thioflavin T fluorescence as previously described [38,

39]. Briefly, triplicate 15 μL samples of $\text{A}\beta_{1-42}$ (25 μM , 95 $\mu\text{g}/\text{mL}$) in 50 mM Tris-HCl buffer (pH 7.4) were removed after incubation of the peptide solution in the presence or absence of optimized turmeric extracts (HSS-888, HSS-838, HSS-848) or the curcuminoid standards (Cur, DMC, BDMC, and THC) at concentrations from 0 to 30 $\mu\text{g}/\text{mL}$ for up to 120 hours at 37°C. These peptide solutions were each added to 100 μL of 10 μM Thioflavin T in 50 mM glycine/NaOH buffer (pH 9.0) in a black-walled 96-well plate, incubated for 30 minutes at 25°C before the characteristic change in fluorescence was monitored ($\text{EX}_{450\text{nm}}$ and $\text{Em}_{482\text{nm}}$) following binding of thioflavin T to the amyloid fibers by using a Molecular Devices SPECTRAMax GEMINI plate reader. Triplicate samples were scanned three times before and immediately after the addition of the peptide solutions. Results show the mean value of the triplicate samples \pm the difference between those mean values.

$\text{A}\beta_{1-42}$ ELISA Assay

Conditioned media were collected and analyzed at a 1:1 dilution using the method as previously described [40] and values were reported as percentage of $\text{A}\beta_{1-42}$ secreted relative to control (conditioned medium from untreated N2a SweAPP cells). Quantification of total $\text{A}\beta$ species was performed according to published methods [41, 42]. Briefly, 6E10 (capture antibody) was coated at 2 $\mu\text{g}/\text{mL}$ in phosphate buffered saline (PBS; pH 7.4) into 96-well immunoassay plates overnight at 4°C. The plates were washed with 0.05% (v/v) Tween-20 in PBS five times and blocked with blocking buffer (PBS with 1% BSA, 5% [v/v] horse serum) for 2 h at room temperature.

Conditioned medium or $\text{A}\beta$ standards were added to the plates and incubated overnight at 4°C. Following 3 washes, biotinylated antibody, 4G8 (0.5 $\mu\text{g}/\text{mL}$ in PBS with 1% [w/v] BSA) was added to the plates and incubated for 2 h at room temperature. After 5 washes, streptavidin-horseradish peroxidase (1:200 dilutions in PBS with 1% BSA) was added to the 96-wells for 30 min at room temperature.

Tetramethylbenzidine (TMB) substrate was added to the plates and incubated for 15 minutes at room temperature. A 50 μL aliquot of stop solution (2 N N_2SO_4) was added to each well of the plates to top the reaction. The optical density of each well was determined immediately on a microplate reader at O.D. 450 nm. The $\text{A}\beta$ levels were expressed as a percentage of control (conditioned medium from untreated N2a SweAPP cells).

Interaction Matrices

Interaction matrices were designed following the methods of Delaney *et al.* [43] to address the possible antagonistic, synergistic and/or additive effects of the different extracts and the individual curcuminoids when combined with HSS-888. Matrices included a range of concentrations of extracts and the curcuminoids that were combined in equal portions ranging from 0 amounts of each to amounts that exceed the IC_{100} values. The combination matrix was analyzed using the IC_{50} values obtained for *in vitro* and combinations of the extracts and the curcuminoids as described previously [44].

RESULTS

Standardized Turmeric Extracts Have Defined Bioactive Compositions

Extracts HSS-888, HSS-838 and HSS-848

The three standardized turmeric extracts were fingerprinted using DART TOF-MS (Fig. (1)). The distribution by mass (m/z $[\text{M}+\text{H}]^+$) of the curcuminoids and turmerones in the different extracts are shown in Fig. (1) with their relative abundances. Both HSS-888 and HSS-838 possessed over 120 chemical entities each in the m/z $[\text{M}+\text{H}]^+$ range of 100.2 to 996.0, and ca. 85-90 were identified in each extract using a proprietary database. The unidentified species include some MS-generated fragments and isotopes of parent compounds, as well as novel-compounds. Extracts HSS-888 and HSS-838 were chosen because they represent extracts with the greatest difference in the ratios of curcuminoids to turmerones (see also Table 1). HSS-888 is enriched in the four curcuminoids, Cur, DMC, BDMC and THC in an approximate ratio of 20:4:1:0.01. This extract contains 72% curcuminoids and 28% turmerones based on DART TOF-MS composition. In contrast, HSS-838, which had a distinct fingerprint from HSS-888 (see Fig. (1)), lacks detectable THC, and possessed about 22% curcuminoids (primarily Cur, DMC and BDMC) and 78% turmerones. Extract HSS-848, derived from a neat ethanolic extract of HSS-838, is highly enriched in turmerones (> 97%; See Fig. (1)), and contains very low levels (< 2%) of Cur, DMC and BDMC. Table 1 summarizes the key curcuminoids and turmerones present in these extracts. The turmerones, xanthorrhizol, ar-turmerone, and zingiberene were particularly abundant in HSS-838. Extract HSS-888, enriched in curcuminoids, also has significant amounts (1-10% composition) of the three turmerones, xanthorrhizol, ar-turmerone, and zingiberene.

HSS-888, Curcumin, Demethoxycurcumin, Bisdemethoxycurcumin, and Tetrahydrocurcumin Inhibit $\text{A}\beta_{1-42}$ Aggregation *In vitro*

The $\text{A}\beta$ aggregation assays were carried out with the synthetic $\text{A}\beta_{1-42}$ peptide incubated with the extracts (HSS-888, HSS-838, and HSS-848) or the curcuminoid standards (Cur, DMC, BDMC, and THC) at varying concentrations from 0 to 30 $\mu\text{g}/\text{mL}$ at 120 hours (Fig. (2)) with aggregation being monitored by the thioflavin T method. The thioflavin T method detects mainly mature β -pleated sheet amyloid fibers. Fig. (2) shows that HSS-888, Cur, THC, BDMC, and DMC were all effective inhibitors of $\text{A}\beta_{1-42}$ aggregation as compared to HSS-838. The 50% inhibition (IC_{50}) values ranged from 5-10 $\mu\text{g}/\text{mL}$ at 20 μM $\text{A}\beta_{1-42}$ concentration. Among the curcuminoids, however, DMC was the least effective in inhibiting $\text{A}\beta_{1-42}$ aggregation.

HSS-888 and Curcumin Inhibit $\text{A}\beta_{1-42}$, 1-40 Generation from SweAPP N2a Cells

In order to compare the effects of turmeric extracts (HSS-888, HSS-838, and HSS-848), and the curcuminoid standards (Cur, DMC, BDMC and THC) on APP (Amyloid Precursor Protein) cleavage, the SweAPP N2a cells were treated with a concentration-range of 3-30 $\mu\text{g}/\text{mL}$ of each compound or extract for 12 hours (Fig. (3)). The $\text{A}\beta_{1-40}$, $\text{A}\beta_{1-42}$ peptides were

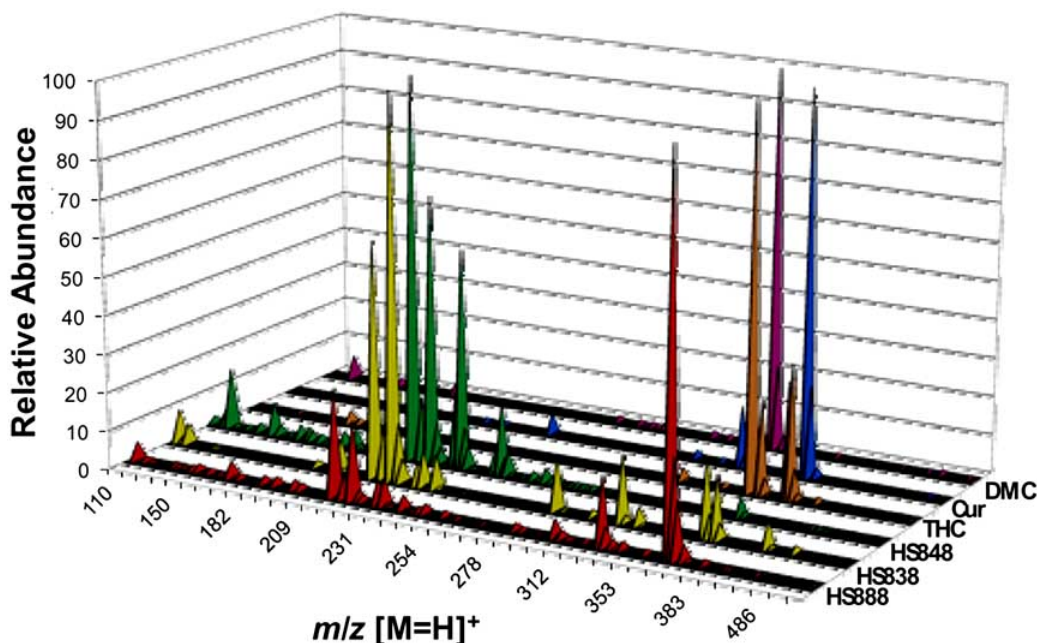


Fig. (1). DART TOF-MS of turmeric extracts HSS-888, HSS-838, and HSS-848, and curcumin (Cur), demethoxycurcumin (DCM), and tetrahydrocurcumin (THC). The extracts and curcuminoid standards were analyzed in positive ion mode by DART-MS. The parent ions for Cur ($m/z = [M+H]^+ = 369.1$); DMC ($m/z = [M+H]^+ = 339.1$); THC ($m/z = [M+H]^+ = 373.3$).

Table 1. Interaction Matrices Between HSS-888 and Extracts HSS-838, HSS-848 and Curcuminoid Standards. The Individual (extract or standard alone), calculated theoretical, and experimental (HSS-888 plus interaction extract or standard) IC_{50} values ($\mu g/ml$) are provided.

Extract	IC_{50} ($\mu g/ml$)			Effect
	Individual	Theoretical	Experimental	
HSS-888 vs.HSS-838	40.0	4.6	5.4	Additive
HSS-888 vs.HSS-848	1682.0	5.1	3.5	Additive
HSS-888 vs.Cur	41.4	4.6	4.9	Additive
HSS-888 vs.DMC	575.2	4.6	6.7	Additive
HSS-888 vs.BDMC	8.7	4.6	3.7	Additive
HSS-888 vs.THCH	35.5	4.6	4.9	Additive

The individual (extract or standard alone), calculated theoretical, and experimental (HSS-888 plus interaction extract or standard) IC_{50} values ($\mu g/ml$) are provided.

analyzed in conditioned media from SweAPP N2a cells by ELISA ($n = 3$ for each condition). Data are represented as percentage of $A\beta_{1-40, 42}$ peptides secreted in 12 hours after turmeric extracts or the curcuminoid standards were added relative to a control (128pg of $A\beta_{1-40, 42}$ peptides, secreted by untreated SweAPP N2a cells). As shown in Fig. (3), extract HSS-888 and Cur significantly reduce $A\beta$ generation (both $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides) in SweAPP N2a cells in a concentration-dependent manner.

In contrast HSS-848 which is essentially turmerones (>97%) and two curcuminoids, DMC and BDMC, showed only low inhibition of $A\beta$ generation (ca. 10%), while HSS-838 which is enriched in turmerones over HSS-888, showed no inhibition. Interestingly, THC stimulated $A\beta$ secretion from SweAPP N2a cells .

HSS-888, Curcumin, and Tetrahydrocurcumin Reveal Additive Inhibition of $A\beta_{1-42}$ Aggregation *In vitro*

Synergy matrices were designed following the methods of Delaney *et al.* [43] to address the possible antagonistic, synergistic and additive effects between extract HSS-888 and the other extracts and the individual curcuminoids on inhibition of $A\beta_{1-42}$ aggregation. The matrices were set-up to cover concentrations of 0 $\mu g/mL$ to levels in excess of the IC_{100} of the two components in the matrix, and the components were mixed to give all the concentration combinations in the matrices. These combinations were then evaluated in the *in vitro* $A\beta_{1-42}$ aggregation assay, and experimental and theoretical IC_{50} values were determined. If the experimental IC_{50} values in the combined samples decreased beyond a simple additive effect reflected in the theoretical IC_{50} value, the combined effects were synergistic, and if the IC_{50} values

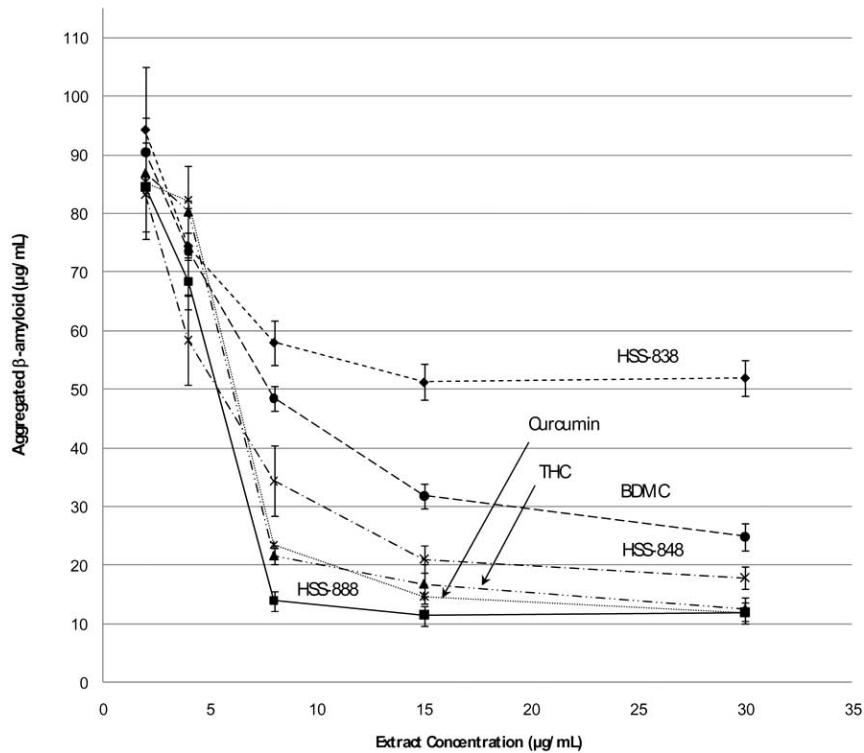


Fig. (2). Effects of turmeric extracts and the curcuminoid standards on $A\beta_{1-42}$ aggregation as determined by the thioflavin T assay. Ninety-five $\mu\text{g/mL}$ of $A\beta_{1-42}$ peptide ($25 \mu\text{M}$) were incubated at 37°C alone and in the presence of turmeric extracts (HSS-888, HSS-838, and HSS-848), and the curcuminoid standards at varying concentrations as indicated for 120 hours. All experiments were carried out in Tris-HCL buffer (pH 7.4). Data are represented as concentration of aggregated $A\beta_{1-42}$ determined on the relative fluorescence units of $95 \mu\text{g/mL}$ ($25 \mu\text{M}$) $A\beta_{1-42}$ peptide incubated alone ($n=3$). The error bars represent the standard deviations for each data point.

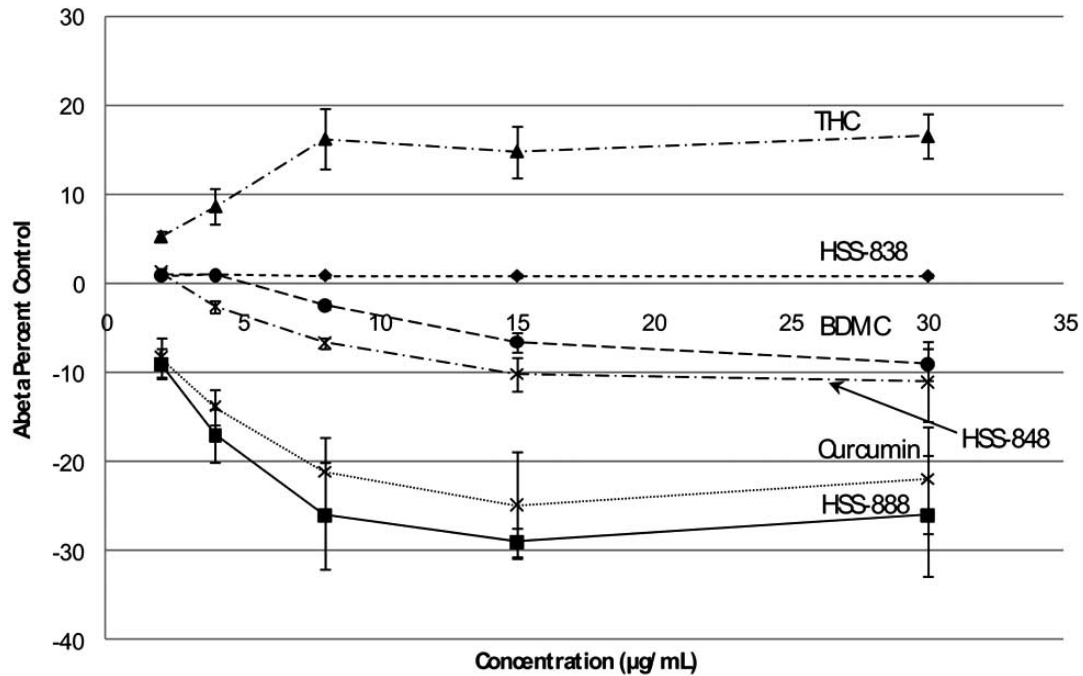


Fig. (3). Turmeric extract HSS-888 and Curcumin inhibit $A\beta$ generation in cultured neuronal cells. Both turmeric extract HSS-888 and Curcumin inhibit $A\beta$ generation in cultured neuronal cells, however, tetrahydrocurcumin increased $A\beta$ generation while extract HSS-838 displayed no significant effect. $A\beta_{1-40, 42}$ peptides were analyzed in conditioned media from SweApp N2a cells by ELISA ($n=3$ per condition). Data are represented as percentage of $A\beta_{1-40, 42}$ peptides secreted 8 hours after extracts HSS-888, HSS-838, and HSS-848, and the curcuminoid standards relative to control (128pg of $A\beta_{1-40, 42}$ peptides, generated by non treated SweApp N2a). The error bars represent the standard deviations for each data point.

Table 2. Chemical Composition of Turmeric Extracts and the Curcumin Standard. The chemical class, measures molecular mass (DART TOF-MS) and normalized relative abundances of the curcuminoids and turmerone chemistries present in the turmeric extracts HSS-888, HSS-838, and HSS-848 as well as a Curcumin standard. Note that the Curcumin standard is a mixture of curcuminoids and contains traces of turmerones.

Chemical Name	Chemical Class	Measured Mass	Normalized Abundance (%)			
			Curcumin Std	HSS-888	HSS-838	HSS-848
Curcumin	curcuminoid	369.1	81.2	66.8	17.3	0.6
Demethoxycurcumin	curcuminoid	339.1	12.5	11.9	3.5	NP
Bisdemethoxycurcumin	curcuminoid	309.1	1.9	3.3	1.1	NP
Tetrahydrocurcumin	curcuminoid	373.2	NP	0.5	NP	NP
ar-Turmerone	turmerone	217.2	2.0	10.1	40.1	49.3
Xanthorrhizol	turmerone	219.2	2.3	6.7	34.6	38.7
Zingiberene	turmerone	205.2	NP	0.7	3.5	11.4

The chemical class, measures molecular mass (DART TOF-MS) and normalized relative abundances of the curcuminoids and turmerone chemistries present in the turmeric extracts HSS-888, HSS-838, and HSS-848 as well as a Curcumin standard. Note that the Curcumin standard is a mixture of curcuminoids and contains traces of turmerones.

increased the combined effects were antagonistic. We found the effects of addition of the individual curcuminoids to HSS-888 to be additive in all cases as summarized in Table 2. The greatest reductions in experimental IC₅₀ values for A β aggregation were observed when HSS-888 was added to HSS-848, the extract rich in turmerones (>450 fold decrease in IC₅₀) and DMC (>110-fold decrease in IC₅₀; Table 2). Extract HSS-838 and Cur, THC and BDMC showed only ca. 2-8-fold enhancements in the inhibition activity of HSS-888. Since the effects were only slightly additive, even at concentrations as high as 30 μ g/mL of each of the curcuminoids, indicates that none of these compounds are particularly more effective in blocking A β aggregation *in vitro* than HSS-888.

DISCUSSION

Standardized extracts of turmeric prepared using super critical CO₂ and affinity chromatography yield defined chemical profiles that are enriched in key AD bioactives such as the 4 known major curcuminoids, Cur, THC, DMC and BDMC, as well as the known turmerones. DART TOF-MS allowed for a rapid chemical characterization of the extracts and revealed a high molecular complexity of the extracts. Only 5% of the compounds present in the extracts were known chemicals, already identified in existing natural products and other chemical databases, the remainder (95%) are novel and as such, are a rich source for drug discovery for AD and other therapeutic targets [24]. Through the use of super-critical carbon dioxide and affinity extraction technologies, extracts with differing bioactive profiles were generated possessing very different activities as assessed by *in vitro* A β ₁₋₄₂ aggregation and A β _{1-42, 1-40} generation from SweAPP N2a cells. Extract HSS-888 (enriched in curcuminoids) was highly active in both assays while extract HSS-838 (enriched in turmerones) and extract HSS-848 (>97% turmerones), were inactive in the assays, consequently indicating that turmerones play little, if any role, in the generation or aggregation of A β *in vitro*. The interaction matrix analyses strongly supported this conclusion; in fact, the addition of extracts rich in turmerones did not significantly in-

crease the A β aggregation inhibition activity of HSS-888. When HSS-888 was evaluated in combinations with the 4 known curcuminoids (Cur, THC, DMC or BDMC) to examine the potential for synergy, only simple additive effects on A β ₁₋₄₂ aggregation inhibition was observed ranging from 2 to 8-fold increases in activity with the exception of BDMC which showed >100-fold increase.

Of the four major curcuminoids, curcumin (Cur) is arguably the most efficacious for reducing A β generation and aggregation *in vitro* though not significantly better than the HSS-888 extract based on findings here. Previous studies have shown that Cur is active in these processes and in animal models [24, 45, 46]. Its activity is often ascribed to its role in ROS scavenging and reduction in neurotoxicity. Curcumin used here and in most other studies generally contains DMC and BDMC, with these two curcuminoids constituting up to 18% of the curcumin standard (see Table 1). Therefore, the effectiveness of Cur observed here most likely results from additive interactions between the three curcuminoids (Cur, DMC, and BDMC) present in this standard, as BDMC alone was equally as active as Cur and HSS-888 in inhibiting A β aggregation in the current studies. While highly purified and micronized forms of Cur have been investigated in pre-clinical studies and are currently being employed in pilot clinical trials for AD [47], it is clear that enriched turmeric extract like HSS-888 could be just as or more effective than this compound alone. Extract HSS-888 contains 82% of the major curcuminoids (67% Cur, 12% DMC, and 3% BDMC) and its activity is significantly greater than Cur alone (81% Cur, 13% DMC, and 2% BDMC), indicating that pure Cur (diferuloylmethane) and the other 3 major curcuminoids cannot account for the full activity of this extract. Therefore, other bioactive compounds must be present at low levels in HSS-888 that function in reducing A β generation and aggregation *in vitro*. This possibility is being actively investigated. Interestingly, in the present study, THC increased A β secretion in SweAPP N2a cells. Though there is no precedent for this observation, it is possible that THC might influence cel-

lular process or cytoskeleton activities that would favor production and/or release of A β in SweAPP N2a cells.

Other phenolic compounds in addition to the curcuminoids were identified in HSS-888 and the major ones include eugenol and ferulic acid (unpublished data). Recent studies have shown that eugenol inhibits A β -induced excessive influx of calcium ion into neurons that causes neuronal death [47] and that the ethyl ester of ferulic acid protects against A β -induced oxidative stress [48]. Phenolics like resveratrol have received recent attention for their possible role in AD [49]. Though not present in turmeric (unpublished data), resveratrol has been shown to reduce secreted and intracellular levels of A β through a mechanism that likely involves enhanced intracellular amyloid degradation [41]. In addition the neuroprotective role of resveratrol and other phenolics is well known [6-10, 49].

Also key for CNS active extracts or compounds, is their ability to cross the blood-brain barrier [47] and their bioavailability. The known curcuminoids possess low bioavailability [50]. As extracts like HSS-888 are characterized in more chemical detail and key bioactives are identified the physicochemical properties of bioactives can be better understood to meet these requirements. A highly standardized extract as demonstrated in this study will be required for FDA approval of a turmeric derived botanical formulation.

Although it is clear from this study that turmerones have little anti-amyloidogenic effects *in vitro*, at present it is unclear whether these compounds possess other important disease modifying properties. Because turmerones have been reported to have anti-platelet [51], COX-2 inhibitory [52], hypoglycemic [53], and cytotoxic activities [54], it may be desirable to remove certain or all turmerones from a turmeric extract designed for the treatment of AD. *In vivo* studies with HSS-888 and other extracts are currently underway by our group to address these and other questions.

In summary, these results provide *proof-of-concept* that an optimized turmeric extract can be produced in a dose-reliable fashion that demonstrates an effective preclinical therapeutic profile against key early steps in the Alzheimer's disease.

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DISCLOSURE STATEMENT

P.C.B. and P.R.S. are founders of and R.D.S. and J.T. are consultants of Natura Therapeutics, Inc., a USF-spin out company. BR and RSA are employees of HerbalScience Group. RCF is currently an employee of HerbalScience Group.

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