



The New Application of UHPLC-DAD-TOF/MS in Identification of Inhibitors on β -Amyloid Fibrillation From *Scutellaria baicalensis*

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Literary evidence depicts that aggregated β -amyloid (A β) leads to the pathogenesis of Alzheimer's disease (AD). Although many traditional Chinese medicines (TCMs) are effective in treating neurodegenerative diseases, there is no effective way for identifying active compounds from their complicated chemical compositions. Instead of using a traditional herbal separation method with low efficiency, we herein apply UHPLC-DAD-TOF/MS for the accurate identification of the active compounds that inhibit the fibrillation of A β (1-42), via an evaluation of the peak area of individual chemical components in chromatogram, after incubation with an A β peptide. Using the neuroprotective herbal plant *Scutellaria baicalensis* (SB) as a study model, the inhibitory effect on A β by its individual compounds, were validated using the thioflavin-T (ThT) fluorescence assay, biolayer interferometry analysis, dot immunoblotting and native gel electrophoresis after UHPLC-DAD-TOF/MS analysis. The viability of cells after A β (1-42) incubation was further evaluated using both the tetrazolium dye (MTT) assay and flow cytometry analysis. Thirteen major chemical components in SB were identified by UHPLC-DAD-TOF/MS after incubation with A β (1-42). The peak areas of two components from SB, baicalein and baicalin, were significantly reduced after incubation with A β (1-42), compared to compounds alone, without incubation with A β (1-42). Consistently, both compounds inhibited the formation of A β (1-42) fibrils and increased the viability of cells after A β (1-42) incubation. Based on the hypothesis that active chemical components have to possess binding affinity to A β (1-42) to inhibit its fibrillation, a new application using UHPLC-DAD-TOF/MS for accurate identification of inhibitors from herbal plants on A β (1-42) fibrillation was developed.

Keywords: Alzheimer's disease, β -amyloid, fibrillation inhibitors, *Scutellaria baicalensis*, UHPLC-DAD-TOF/MS

INTRODUCTION

Population aging is becoming a major demographical and health issue in the 21st century. Consistently, the number of people with aged-related neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD) are accelerating rapidly (Sheikh et al., 2013). The number of people with AD, the most common type of dementia (Fang et al., 2017), is expected to reach 74.7 and 131.5 million globally by 2030 and 2050, respectively (Alzheimer's Disease International, 2018). The extracellular plaque of the A β peptide, and the neurofibrillary tangles (NFTs) composed of the microtubule associated filamentous tau protein in the brain, are two important pathological hallmarks of AD (Bloom, 2014). The accumulation of A β in neurons is recognized as the initiation step of AD progression, with the induction of oxidative stress, inflammation, and cell death (Di Bona et al., 2010). Although it remains controversial, A β is considered as one of the key targets for the treatment of AD (Yoshiike et al., 2003). Considering the fact that many natural small molecules have been proven to inhibit A β fibrillation and reduce the cytotoxicity of neurons, recent studies have focused on the screening of natural compounds with anti-AD effects in TCMs. For example, natural polyphenols such as curcumin can bind A β (1–42) and block its fibril formation and aggregation (Yang et al., 2005); resveratrol is capable of changing the oligomeric conformation and attenuate the cytotoxicity of A β (Feng et al., 2009); epigallocatechin-3-gallate is able to reduce the level of A β in brain and protect the mitochondrial function of neurons in the amyloid precursor protein (APP) and presenilin-1 (PS1) double transgenic AD mice (Dragicevic et al., 2011).

Scutellaria baicalensis (SB) (Huangqin) is a widely used TCM (Mehlhorn et al., 2014), which was firstly described in “Shen Nong Ben Cao Jing” (Yuan et al., 2015). Modern pharmacological studies have depicted its positive effects in neuroprotection (Yune et al., 2009; Miao et al., 2014), anti-cancer (Ye et al., 2002; Sato et al., 2013), anti-inflammation (Huang et al., 2006; Kim et al., 2009; Yoon et al., 2009), anti-oxidation (Gabrielska et al., 1997; Huang et al., 2006; Wang et al., 2014), anti-bacteria, and anti-virus (Zandi et al., 2013; Shi et al., 2016). Flavonoids including baicalin, baicalein, wogonin, oroxylin A-7-O-glucuronopyranoside, and oroxylin A are the major chemical components in SB (Lee et al., 2014). While baicalin, baicalein and wogonin were reported to inhibit fibrillation of A β (Heo et al., 2004; Zhu and Wang, 2015), total flavonoids from the stem and leaf of SB might improve learning or memory impairment and attenuate neuronal loss induced by the A β peptide in rats (Wang et al., 2013). However, the traditional bioactivity guided separation of active compounds from TCMs, requires repeated rounds of isolation and bioactivity validation before a single active fraction or chemical component can be identified. Due to the disadvantages of being time-consuming and laborious, the traditional way of identifying novel inhibitors on fibrillation of A β from TCMs is ineffective with slow progress (Gerardo Castillo et al., 2007; Liu et al., 2011; Kandasamy et al., 2012).

With the recent advances in the development of chemical analysis technologies, such as HPLC coupled with

ultraviolet (UV) or fluorescence detector, mass spectrometry (MS) and nuclear magnetic resonance (NMR), identification of the bioactive component through the analysis of chromatograms, has become more prevalent (Li and Lurie, 2014; Wu et al., 2015; Bertini et al., 2017). Recent literature has reported a novel method on the structure-based discovery of fiber-binding compounds using computational docking, which was then validated by NMR for their binding affinity to both A β (16–21) and A β (1–42). The results confirmed that compounds with a binding affinity to A β , decreased toxicity of fiber by increasing its stability (Jiang et al., 2013). Consistently, a model using NMR for the validation of the binding affinity of non-natural peptides to tau fibers, showed a diminished ^1H NMR spectrum of the effective peptides (Sievers et al., 2011). Based on these observations, we hypothesized that chemical components would show a reduction in the UHPLC-DAD-TOF/MS chromatogram peak area upon effective A β binding. To validate the hypothesis, SB was selected as the study subject for the evaluation of method accuracy. Our results confirmed that 13 major chemical components were identified from the total ethanol extract of SB (SB-TEE). Among them, two of the chemical components, baicalin and baicalein showed a significant decrease of the peak area in the extracted-ion chromatogram (EIC) as evaluated by UHPLC-DAD-TOF/MS. Consistently, SB-TEE, baicalin and baicalein possess an inhibitory effect on the A β (1–42) fibril formation as validated by bilayer interferometry analysis, ThT fluorescence detection assay, native gel and dot blot analysis. The anti-fibrillation effect on A β was further confirmed by the decrease in its cytotoxicity after treatment with baicalin and baicalein. The current study presents a new approach using the UHPLC-DAD-TOF/MS system for the accurate screening or the quality control of anti-fibrillation compounds from TCMs, which may facilitate the drug discovery of potential anti-AD agents in the future.

MATERIALS AND METHODS

Chemicals and Reagents

Baicalin, wogonoside and baicalein ($\geq 98\%$ purity, HPLC) were obtained from Chengdu MUST Bio-technology Company Ltd. (Chengdu, China). SB with the place of origin in Ji Lin province of China was purchased from Beijing Tong Ren Tang Zhuhai Pharmacy Co., Ltd. (Guangdong, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT) and ThT were purchased from Sigma (St. Louis, MO, United States). Milli-Q water was prepared by the Milli-Q integral water purification system (Millipore, Billerica, MA, United States) in our laboratory. Acetonitrile was purchased from Anaqua Chemicals Supply (Houston, TX, United States). A β (1–42) was obtained from the China Peptides Co., Ltd. (Shanghai, China). EZ-Link NHS-LC-LC-Biotin was obtained from Thermo Scientific Waltham (MA, United States). Super Streptavidin (SSA) biosensors were purchased from FortéBIO, PALL Life Sciences (Port Washington, NY, United States). The Annexin V staining kit was purchased from BD Biosciences (San Jose, CA, United States). The native PAGE Bis-Tris Gel (4–16%), running buffer (20X) and the native

PAGE sample buffer were obtained from Invitrogen (Carlsbad, CA, United States). The PVDF membrane was obtained from PALL Life Sciences (Port Washington, NY, United States).

Preparation of SB-TEE

25 gram of the SB plant was smashed into powder and extracted with 10 times its volume of 75% ethanol for 2 h, by refluxing two times. The extracted solution was then filtered, concentrated and dried with a rotary evaporator under reduced pressure to produce the final SB-TEE. The dried ethanol extracts were re-dissolved in DMSO at a suitable concentration for further use.

Instrument and Chromatographic Conditions

UHPLC (Agilent Technologies 1290 Series), equipped with the time of flight (TOF) MS (Agilent Technologies 6230) with a jet stream ion source, was operated in negative and positive ion modes during the UHPLC analysis. All samples were analyzed using the Agilent Eclipse Plus C-18 column (100 \times 2.1 mm) with a particle size of 1.8 μ m at a flow rate of 0.35 mL/min. The separation was conducted in accordance with the gradient elution program, comprised of mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in ACN): 0–2 min, 2% B; 2–5 min, 2–10% B; 5–15 min, 10–50% B; 15–18 min, 50–80% B; 18–20 min, 80–100% B; 20–22 min, 100% B; 22.1–25 min, 2% B. For UHPLC-DAD-TOF-MS analysis, the data was acquired using the UV detector with the detection wavelength at 254 nm and in the scan mode with a m/z value from 100 to 1700 Da by 2.0 spectra/s. Data were analyzed using Agilent MassHunter Workstation software B.01.03.

A β (1–42) Peptide Preparation

1 mg of A β peptide (1–42) was dissolved in 400 μ L of hexafluoroisopropanol (HFIP; Sigma) and subjected to ultrasonic for 15 min. A β peptide solution was aliquoted into a 1.5 mL tube (100 μ L/tube) and dried under a stream of nitrogen gas to produce a peptide film which was stored at -80° C. A β (1–42) was re-dissolved in 10 μ L of DMSO (Sigma, United States) and an appropriate volume of PBS (pH = 7.4) to acquire the final concentration before use. The A β (1–42) peptide was then incubated at 37° C for 5 days to form the aggregated form of A β for all biological and chemical assays.

Biolayer Interferometry Analysis

200 μ L of solution containing 100 μ g of the A β peptide was incubated at 37° C for 5 days. EZ-Link NHS-LC-LC-Biotin (Thermo Scientific, United States) was dissolved in DMSO to a concentration of 10 mM. A β (1–42) was biotinylated in a 1:0.5 molar ratio of biotin reagent and incubated for 30 min at room temperature before being added into a 96-well plate (Greiner Bio-One, PN:655209). Biotinylation was ascertained by loading the mixture onto super streptavidin (SSA) capacity tips (ForteiBIO, Menlo Park, CA, United States) and detected by the ForteiBIO Octet Red instrument. Additionally, SSA biosensors were pre-wetted with PBS for the recording of baselines. Successful biotinylated A β (1–42) solution was collected and immobilized

onto SSA tips overnight at 4° C. Compounds (baicalin and baicalein) dissolved in DMSO were diluted to an appropriate concentration with PBS to a final volume of 200 μ L/well. Control wells were added with an equal amount of DMSO. All experiments consisted of repeated cycles of four major steps: wash (300 s), baseline (120 s), association (120 s), and dissociation (120 s). The results including the association and dissociation plot and kinetic constants were analyzed with ForteiBIO data analysis software.

Thioflavin-T (ThT) Fluorescence Assay

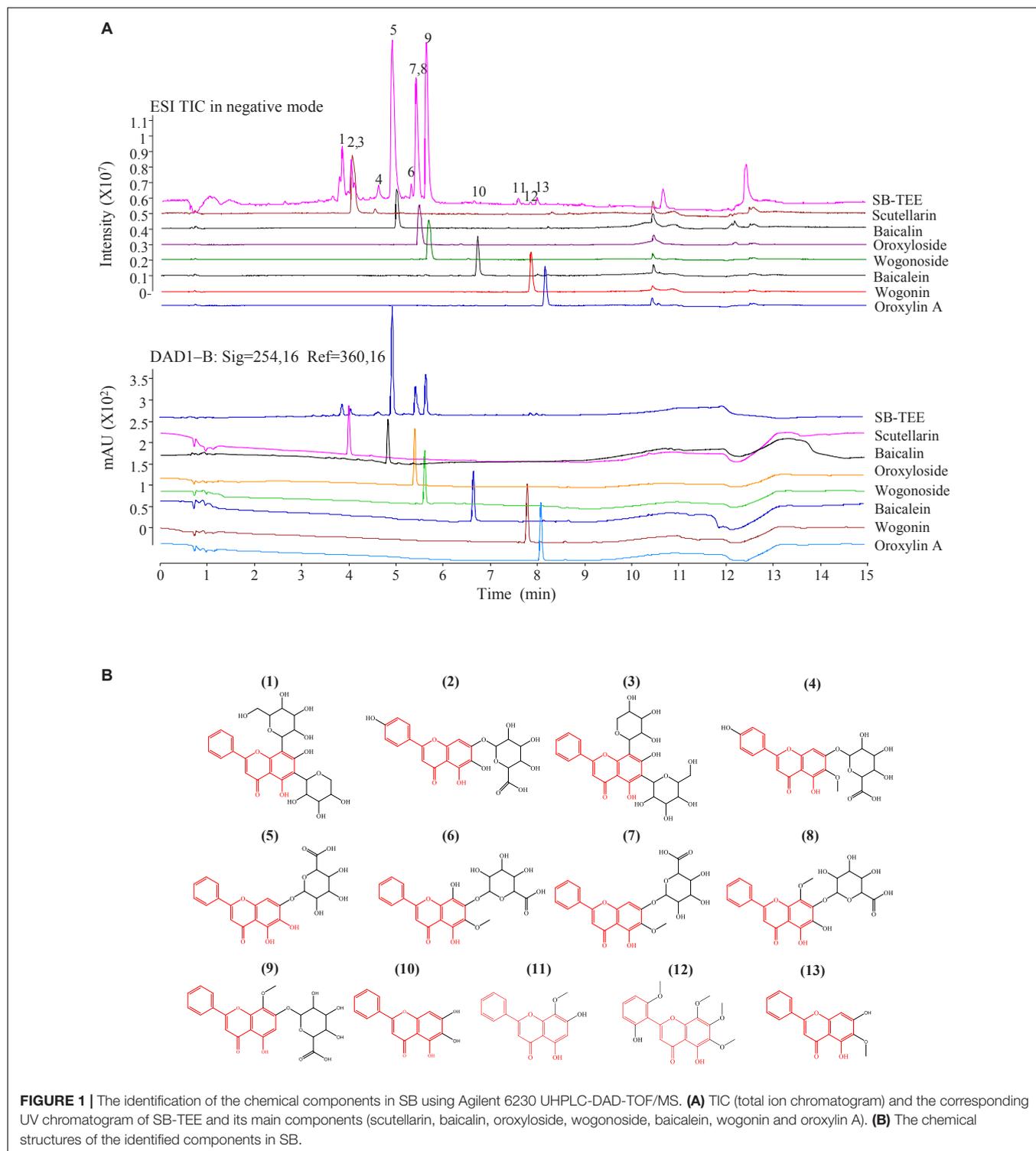
20 μ L of A β (1–42) (100 μ M) was diluted with PBS or the tested SB compounds, to a final volume of 100 μ L with 5 days of incubation at 37° C. A ThT fluorescence assay was performed as described in the previous report (Lu et al., 2011). Briefly, ThT was dissolved with PBS (pH = 7.4) at a final concentration of 20 μ M and was kept away from light. 10 μ L of aggregated A β with or without the tested compounds and 190 μ L of ThT solutions were added into a black 96-well-plate and incubated for 1 h. Fluorescence measurements were carried out using the microtiter plate reader (SpectraMax Paradigm, Molecular Devices, United States) with excitation at 450 nm and emission at 490 nm. Background fluorescence was measured in the control sample containing PBS and 0.02% of DMSO.

Dot Blot Assay

20 μ M of A β (1–42) was incubated with SB-TEE or SB single compounds for 5 days at 37° C. 4 μ L of each incubated solution was spotted onto the methanol pre-activated PVDF membrane, which was then blocked with 5% non-fat dried milk in Tris-buffered saline and Tween 20 (TBST) for 1 h. The membrane was incubated with primary anti-amyloid fibril antibody [mOC87] (1:1000) (Abcam, Cambridge, MA, United States) overnight at 4° C, followed by an incubation with HRP-conjugated secondary antibody. Protein bands were detected using ultra signal sensitive ECL Western Blotting detection reagent (4A Biotech Co., Ltd, Beijing, China) and visualized using gel imaging equipment (Amersham Imager 600, GE, Tokyo, Japan). Band intensity was quantified using the software ImageJ (National Institutes of Health, Bethesda, MD, United States).

Native Gel Electrophoresis

After the incubation of A β (1–42) with SB-TEE or SB single compounds at 37° C for 5 days, with A β (1–42) alone set as the control group, the incubated solutions were centrifuged at 10,000 g for 10 min. The supernatant was collected for the detection of soluble A β (1–42) oligomers by native gel electrophoresis. In brief, samples in the Novex native PAGE sample buffer were loaded into the pre-casted native PAGE gels for electrophoresis in 1X of Novex Native PAGE running buffer. The proteins on the gel were then transferred to a PVDF membrane. The membrane was blocked with 5% non-fat milk in TBST and then immunoblotted with an antibody against amyloid fibril [mOC87] (1:1000) (Abcam, Cambridge, MA, United States) overnight at 4° C. After an incubation with HRP-conjugated secondary antibody, protein bands were detected and visualized as described in the previous “Dot Blot Assay” section.



Cell Viability

PC-12 cells were cultured with DMEM (Gibco, Grand Island, NY, United States) containing 10% horse serum (Gibco, Grand Island, NY, United States), 5% fetal bovine serum (FBS, PAN Biotech, Germany) and 1% penicillin and streptomycin, in a humidified

incubator with 5% CO₂ at 37°C. Cell viability of PC-12 cells was measured using MTT method (Wong et al., 2005). In brief, PC-12 cells plated on 96-well plates were incubated with A β (1–42) alone, A β (1–42) with SB-TEE or A β (1–42) with single compounds from SB, respectively. After 48 h of

TABLE 1 | The retention time, formula, chemical name and the mass of the chemical components in negative and positive ion modes.

Peak No.	Retention time (min)	Formula	Chemical name	[M-H] ⁻ (m/z)		ppm	[M+H] ⁺ (m/z)		ppm
				Calculated	Measured		Calculated	Measured	
1	3.91	C26H28O13	Chrysin -6-C-arabinose-8-C-glucose	547.1457	547.1474	3.11	549.1603	549.1641	6.92
2	4.081	C21H18O12	Scutellarin	461.0725	461.033	1.74	463.0871	463.0911	8.64
3	4.223	C26H28O13	Chrysin -6-C-glucose-8-C-arabinose	547.1457	547.1485	5.12	549.1603	549.1647	8.01
4	4.78	C22H20O12	Hispidulin 7-glucuronide	475.0882	475.0899	3.58	477.1028	477.105	4.6
5	5.046	C21H18O11	Baicalin	445.0776	445.0809	7.41	447.0922	447.0959	8.24
6	5.62	C22H20O12	5,7,8-Tetrahydroxyflavone; 6-Me ether, 7-O- β -D-glucuronopyranoside	475.0882	475.0917	7.37	477.1028	477.1063	7.34
7	5.562	C22H20O11	Oroxyloside	459.0933	459.097	8.06	461.1078	461.1116	8.24
8	5.656	C22H20O12	5,6,7-Tetrahydroxyflavone; 8-Me ether, 7-O- β -D-glucuronopyranoside	475.0882	475.0862	-4.2	477.1028	477.1035	1.46
9	5.762	C22H20O11	Wogonoside	459.0933	459.0975	9.15	461.1078	461.1109	6.72
10	6.748	C15H10O5	Baicalein	269.0455	269.0469	5.2	271.0601	271.0622	7.75
11	8.024	C16H12O5	Wogonin	283.0612	283.0614	0.71	285.0757	285.078	8.07
12	8.149	C19H18O8	Skullcapflavone II	373.0929	373.0929	0	375.1074	375.1103	7.73
13	8.291	C16H12O5	Oroxylin A	283.0612	283.0629	6	285.0757	285.0776	6.66

treatment, 10 μ L of MTT solution (Sigma, United States) was added to cells in each well and further incubated for 4 h at 37°C. The incubation medium was then removed and 150 μ L of DMSO was added to cells to dissolve the formazan. Absorbance (OD) of each well was then detected by spectrophotometer at the wavelength of 490 nm. The percentage of cell viability was calculated using the formula: cell viability (%) = cells number_(treated)/cells number_(DMSO control) \times 100 %. Data were obtained from 3 independent experiments.

Flow Cytometry Analysis

Cell viability of PC-12 cells was further evaluated by flow cytometry using the annexin V staining kit (BD Biosciences, San Jose, CA, United States). In brief, PC-12 cells seeded in a 6-well-plate were treated with A β (1–42) with or without the addition of SB-TEE or its single compounds, for 48 h. After treatments, the cells were trypsinized and centrifuged. The cells pellets were re-suspended with 250 μ L of PBS and then stained with 2 μ L of propidium iodide and 1 μ L of FITC (BD Biosciences, San Jose, CA, United States) for 15 min. The cells were then analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, United States). Data acquisition and analysis were performed using the Flowjo 7.6.1 (TreeStar, San Carlos, CA, United States).

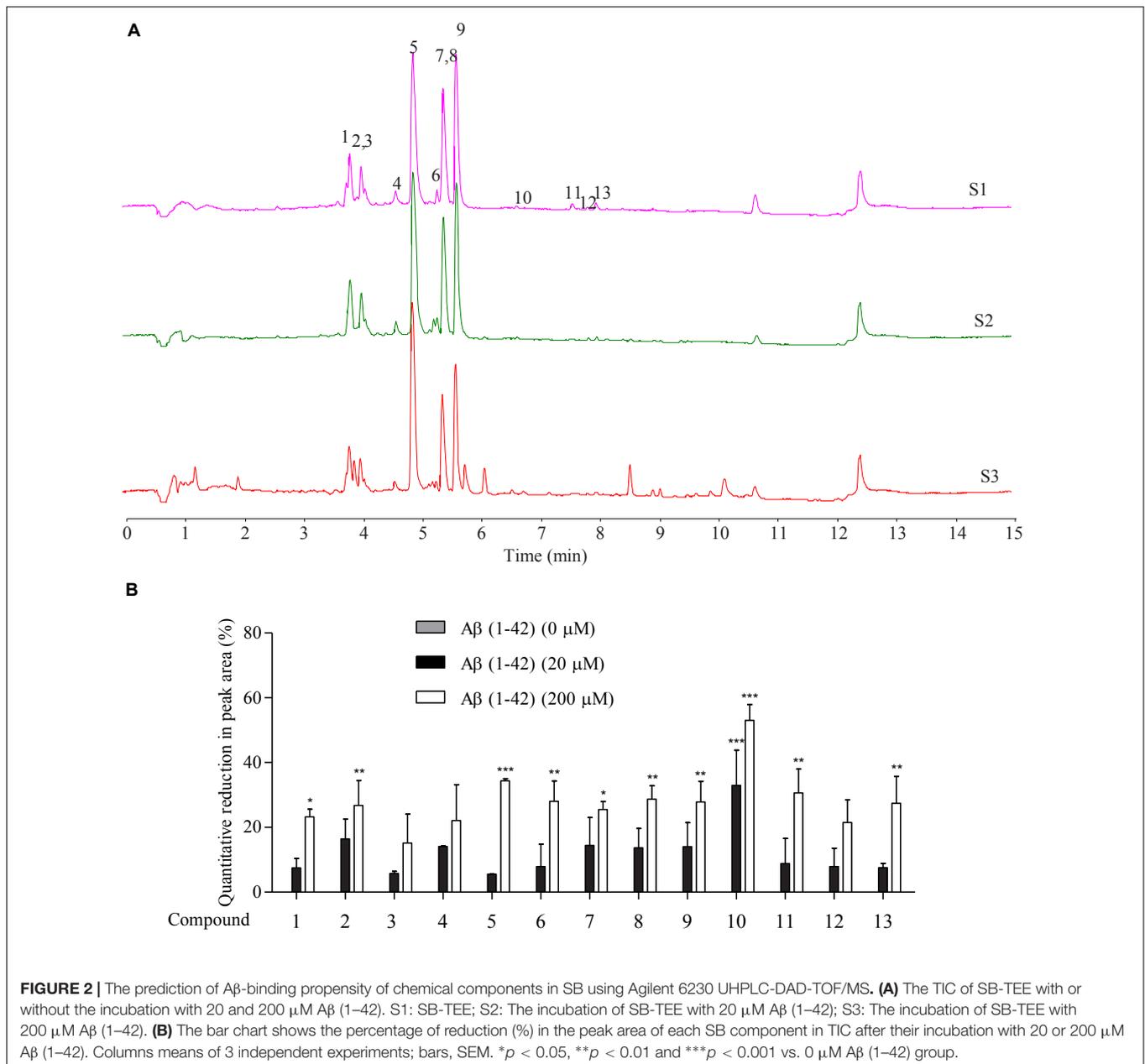
Statistical Analysis

All analyses were performed using the GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA, United States). All data were presented as means \pm SEM. The difference was considered to have statistical significance if $p < 0.05$. Student's t -test or one-way ANOVA was applied for statistical analysis to compare all the different groups in the current study.

RESULTS

Characterization of the Chemical Components in the Total Ethanol Extract of *Scutellaria baicalensis*

SB is a rich source of polyphenols, especially flavonoids, which are a major class of bioactive components in SB (Zhou et al., 2015). Up to now, more than 100 polyphenols were identified from the genus *Scutellaria* (Sripathi and Ravi, 2017). Recent studies have reported the neuroprotective effects of SB and its flavonoids in anti-A β fibrillation and improvement of cognitive function (Heo et al., 2004; Yune et al., 2009; Wang et al., 2013). With its well-known neuroprotective effects, SB was selected as the subject of our study. To begin, the chemical components of SB were characterized using UHPLC-DAD-TOF/MS in negative or positive ion mode and UV at 254 nm. As shown in the total ion chromatogram (TIC) and UV chromatogram of SB-TEE in **Figure 1A**, a total of 13 peaks were observed and marked. Among them, the identities of 7 flavonoids including scutellarin (#2), baicalin (#5), oroxyloside (#7), wogonoside (#9), baicalein (#10), wogonin, (#11) and oroxylin A (#13) from SB-TEE, were confirmed with reference to the chromatograms of their corresponding standard pure compounds analyzed under the same chromatographic conditions. The identities of the remaining six components (#1, #3, #4, #6, #8, and #12) were confirmed by comparing their accurate mass and retention features on the C₁₈ column, to the reported literature. With their common nuclear structure labeled in red, 13 major peaks were identified, with their chemical structures shown in **Figure 1B**. The retention time, formula, chemical name and the accurate MS in negative mode or positive mode are listed in **Table 1**. All the characterizations of the major chemical components in SB were confirmed before being further subjected to biological evaluation.



Prediction of A β (1-42) Peptide-Binding Propensity of Selected SB Chemical Components by UHPLC-DAD-TOF-MS

The A β peptide, which constitutes the amyloid plaques in the brains of AD patients, has long been identified as the major cause of AD. Upon sequential cleavage by the γ -secretase and β -secretase, A β can be produced from the APP with the major cleavage sites of γ -secretase at the positions 40 and 42 of the A β peptide. With its soluble toxic nature and high propensity to fibrillate and form amyloid plaques (Yiannopoulou and Papageorgiou, 2013; Xiao et al., 2015; Walti et al., 2016), accumulation of A β (1-42) is highly correlated with the pathogenesis of AD, with a cascade of cellular responses such

as inflammation, oxidative damage and neurotoxicity (Graham et al., 2017). Therefore, therapeutic approaches that target the accumulated aggregation and fibrillation of A β with antibodies, peptides, or chemical molecules has intensively been investigated (Cho and Kim, 2011). To predict the peptide-binding propensity of the above 13 identified chemical components in SB, SB-TEE was incubated with 20 and 200 μ M of A β (1-42), respectively. With a blank solution and SB-TEE without A β (1-42) incubation working as the control, all reaction product mixture was analyzed using UHPLC-DAD-TOF/MS under the same chromatographic conditions. **Figure 2A** shows the TIC of SB alone (S1), and SB-TEE incubated with 20 μ M (S2) or 200 μ M (S3) of A β (1-42), respectively. With the peak area of all the identified components calculated and analyzed, **Figure 2B** and **Table 2**

show the percentage (%) of decrease in the peak area of all the identified chemical components in TIC. Among them, baicalin (peak #5) and baicalein (peak #10) showed the highest reduction in peak area (34.38 and 53.03%) after incubation with A β (1–42) (200 μ M).

Validation of A β (1–42) Peptide-Binding Propensity of Selected SB Chemical Components by ThT Assay

To further validate the peptide-binding propensity of selected SB compounds, baicalin (peak #5) and baicalein (peak #10), which showed the highest percentage of peak area reduction in TIC, were subjected to further validation with the negative control, wogonoside (peak #9), which showed no significant change in the peak area (Table 3). As shown in Figures 3A,B, the peak areas of baicalin and baicalein in TIC were negatively correlated to the concentrations of A β . Upon binding to amyloid fibrils, benzothiazole fluorescent dye (ThT) can produce a strong fluorescence signal at the wavelength of 482 nm (Xue et al., 2017), therefore, the widely used ThT is applied to further monitor and confirm the inhibition of A β fibril formation *in vitro*. As shown in Figure 3C, fluorescence intensity indicated that the concentration of A β fibril was decreased upon treatment with baicalin and baicalein, suggesting the positive role of baicalin and baicalein in anti- A β fibril formation.

Direct Binding Measurement of Baicalin and Baicalein to A β (1–42) by Biolayer Interferometry Analysis

To determine the binding affinity of the selected SB chemical components to A β (1–42) fibril, the label-free biolayer interferometry assay which measures the biomolecular interaction was used. It is performed by monitoring the binding of a ligand immobilized on the tip surface of a biosensor to a

TABLE 3 | The percentage (%) of reduction in the peak areas of baicalin, baicalein and wogonoside in TIC after incubation with 20 or 200 μ M A β (1–42).

Compound	Quantitative reduction of peak area (%)	
	With 20 μ M A β (1–42)	With 200 μ M A β (1–42)
Baicalin (50 μ M)	16.96 \pm 1.28	44.07 \pm 9.03*
Baicalein (50 μ M)	32.52 \pm 34.67	70.28 \pm 23.84**
Wogonoside (50 μ M)	9.49 \pm 8.81	15.77 \pm 8.84

Note: * $p < 0.05$ and ** $p < 0.01$ vs. 0 μ M A β (1–42) group.

specific analyte in the tested solution. Upon the association, the optical thickness at the biosensor tip will be increased, leading to a real time shift in wavelength ($\Delta\lambda$) due to a change in the thickness of the biological layer. These Interactions will then be quantitated to provide accurate data on binding specificity and the association or dissociation rates. To begin, increasing concentrations of baicalin (25, 50, 100, 200, 400, 800 μ M) and baicalein (12.5, 25, 50, 100, 200, 400 μ M) were applied for the real-time monitoring of their direct association with the biotinylated A β (1–42). As shown by the association/dissociation binding curves of the 2 compounds in Figures 4A,B, there is a dose dependent increase in the optical thickness (nm) of the sensor layer, suggesting the direct binding of baicalin and baicalein to A β . Table 4 showed the kinetic constants calculated using the ForteBio data analysis software. The results showed that the K_D value of baicalin and baicalein were 242 and 170 μ M, respectively, suggesting the direct and reversible interaction of baicalin and baicalein with A β (1–42).

In vitro Inhibitory Effect of SB-TEE, Baicalin, Baicalein and Wogonoside on A β (1–42) Fibrillation

Dot immunoblot, utilizing the strong A β -binding capacity of PVDF membrane, was used for further validation of the *in vitro* anti-A β (1–42) fibrillation effect of SB-TEE, baicalin, baicalein and wogonoside. To begin, incubation of 20 μ M A β (1–42) with or without SB-TEE, baicalin, baicalein or wogonoside for 5 days at 37°C was performed. Equal amount of each incubated sample spotted onto the PVDF membrane was then subjected to immunoblotting with antibodies specific for detection and quantitation of amyloid fibrillation (Gong et al., 2003). As shown in Figures 5A,B, while baicalein possesses the highest potency in anti-fibrillation of A β , wogonoside was not effective in inhibiting the fibrillation of A β . To further confirm the anti-fibrillation effect of the selected compounds, non-denaturing native gel electrophoresis was applied for the effective detection of fibrillary A β . Consistently, baicalein showed the highest potency in anti-fibrillation of A β , as shown in Figure 5C. These data have confirmed the feasibility of our proposed new application of using a UHPLC-DAD-TOF/MS based detection system for precise identification of β -amyloid fibrillation inhibitors from TCMs that contain multiple chemical components.

TABLE 2 | Quantitative reduction (%) of peak area of each SB component in TIC after their incubation with 20 or 200 μ M A β (1–42).

Compounds in SB	Quantitative reduction of peak area (%)	
	With 20 μ M A β (1–42)	With 200 μ M A β (1–42)
1	7.53 \pm 4.05	23.24 \pm 3.33*
2	16.41 \pm 8.71	26.78 \pm 10.92**
3	5.79 \pm 0.94	15.2 \pm 12.59
4	14.11 \pm 0.15	22.1 \pm 15.63
5	5.58 \pm 0.06	34.38 \pm 0.96***
6	7.87 \pm 9.84	28.04 \pm 8.81**
7	14.40 \pm 12.19	25.51 \pm 3.51*
8	13.72 \pm 8.38	28.69 \pm 5.85**
9	14.03 \pm 10.43	27.8 \pm 9.05**
10	32.94 \pm 15.38***	53.03 \pm 6.93***
11	8.81 \pm 10.94	30.61 \pm 10.39**
12	7.89 \pm 8.06	21.47 \pm 9.9
13	7.59 \pm 1.89	27.4 \pm 11.81**

Note: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. 0 μ M A β (1–42) group.

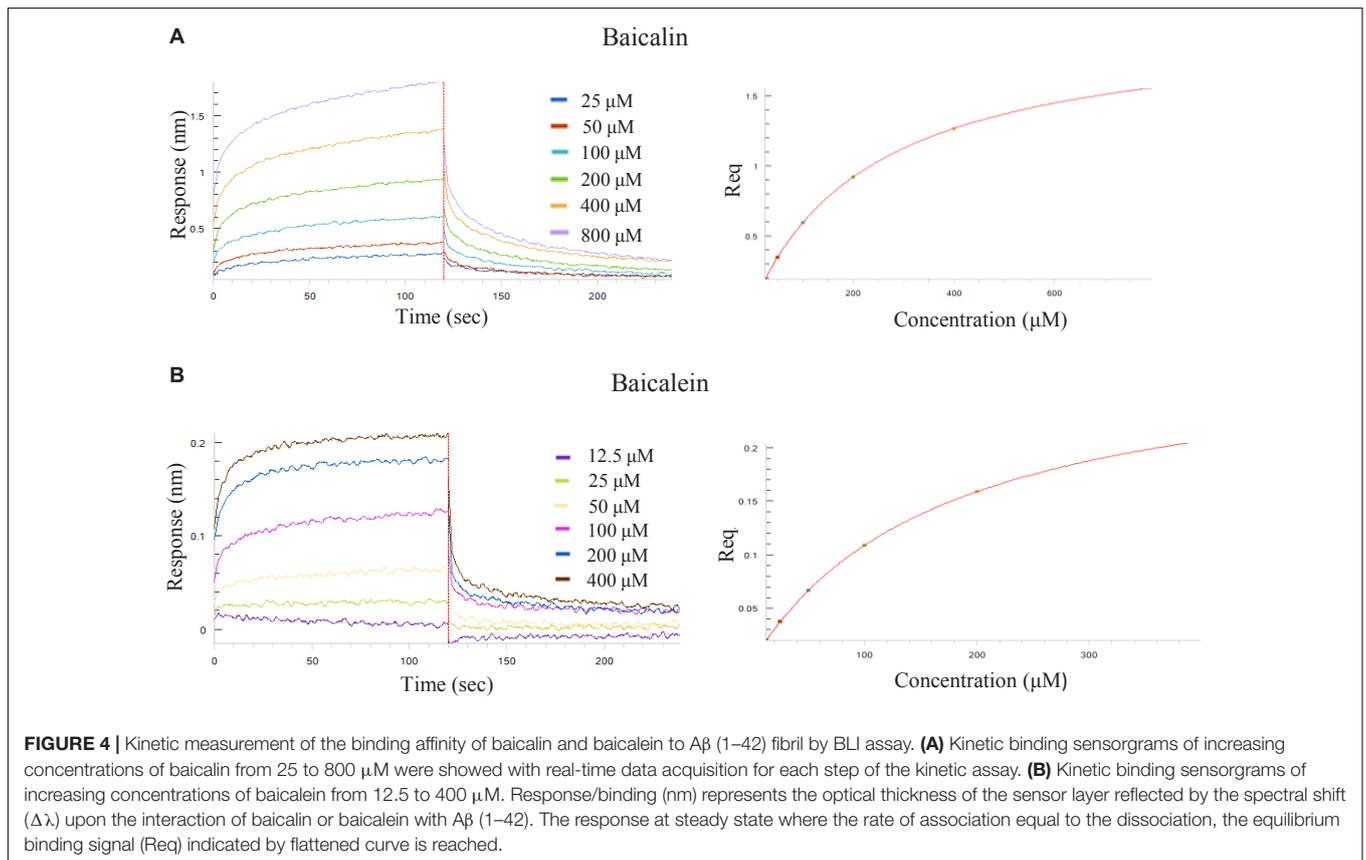
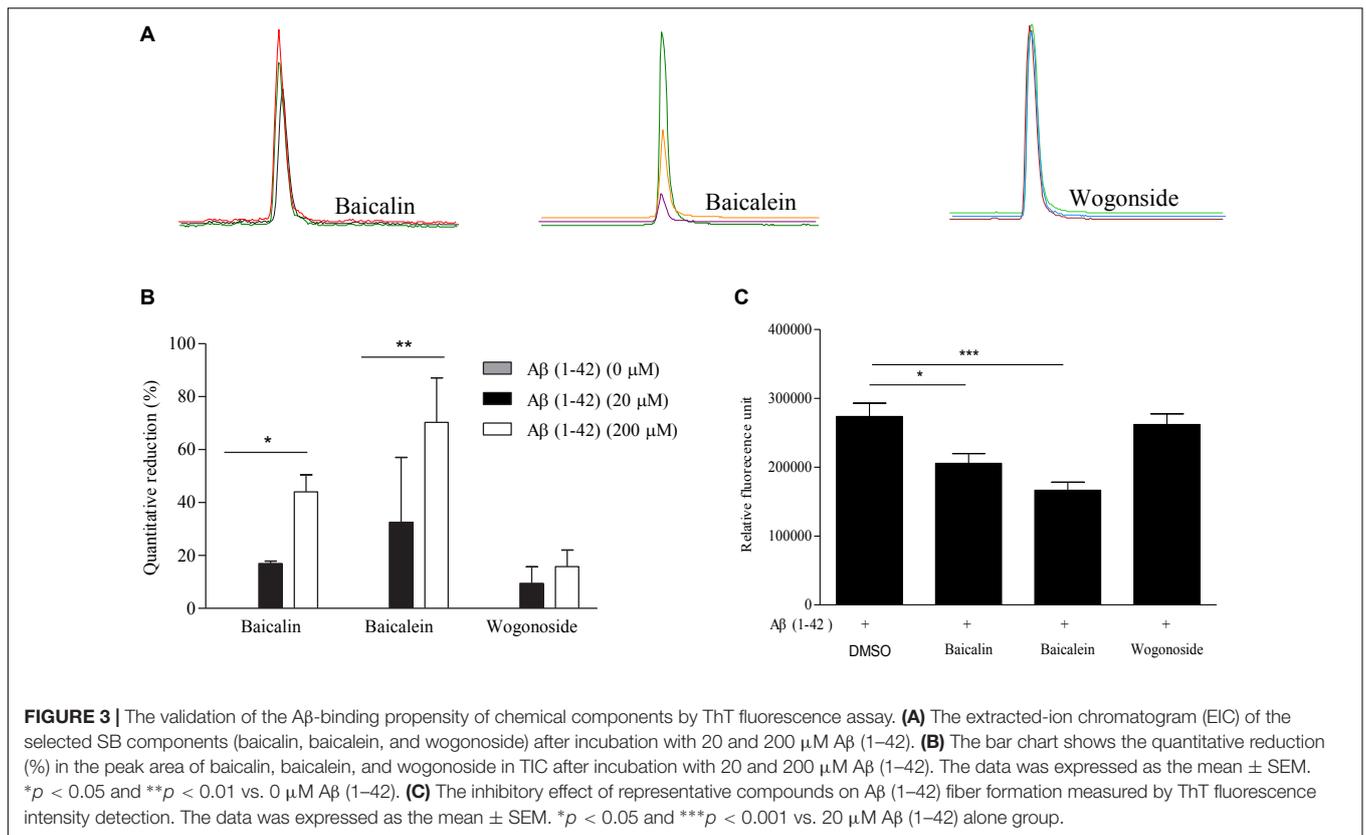


TABLE 4 | The binding affinity (KD), association rate constant (K_{on}) and dissociation rate constant (K_{dis}) of baicalin and baicalein to $A\beta$ (1–42).

	KD (μ M)	Kon (1/Ms)	Kdis (1/s)
Baicalin	242	$1.66 \times 10^{+02}$	4.01×10^{-02}
Baicalein	170	$9.22 \times 10^{+02}$	1.57×10^{-01}

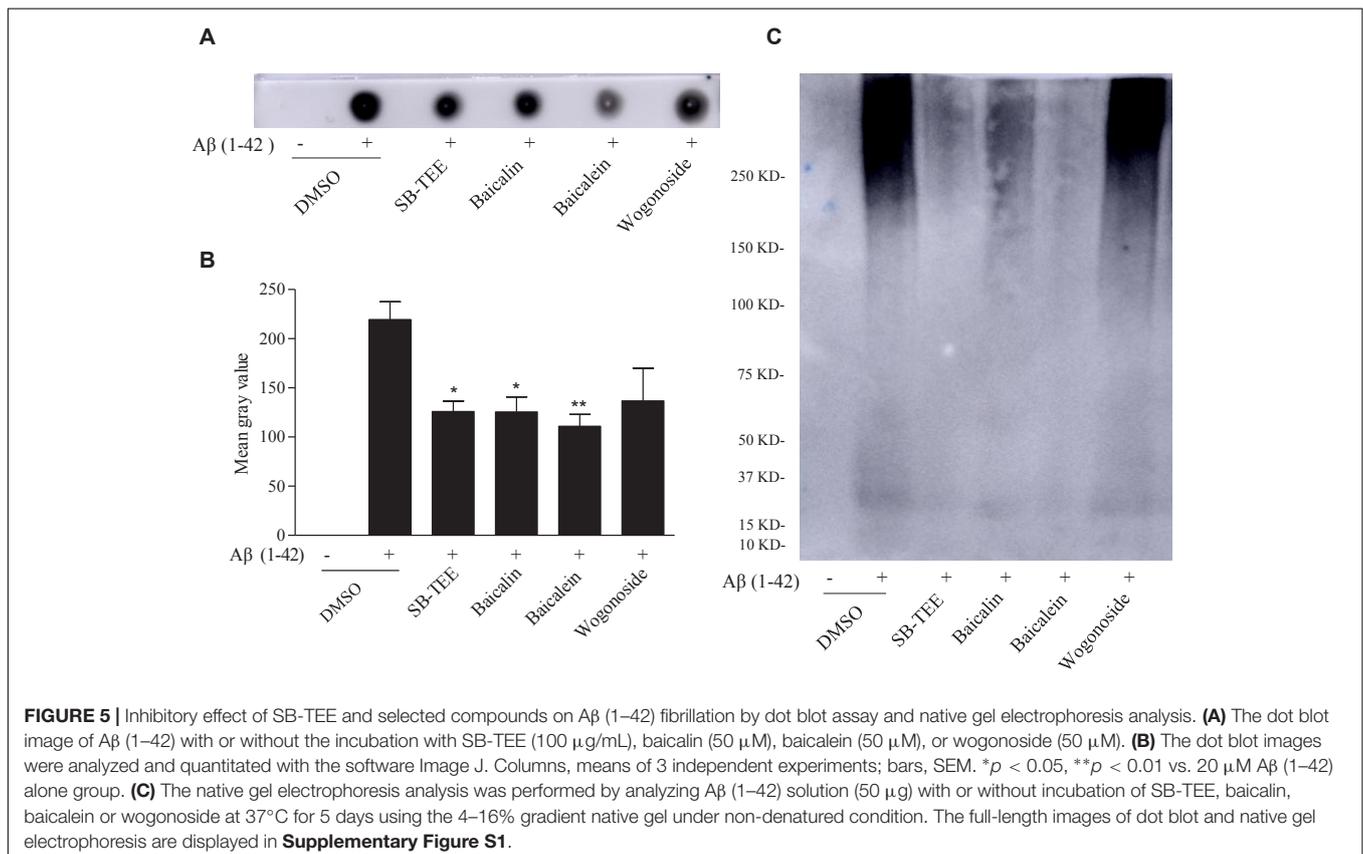
SB-TEE Alleviates the Cytotoxicity of $A\beta$ (1–42) in PC-12 Cells

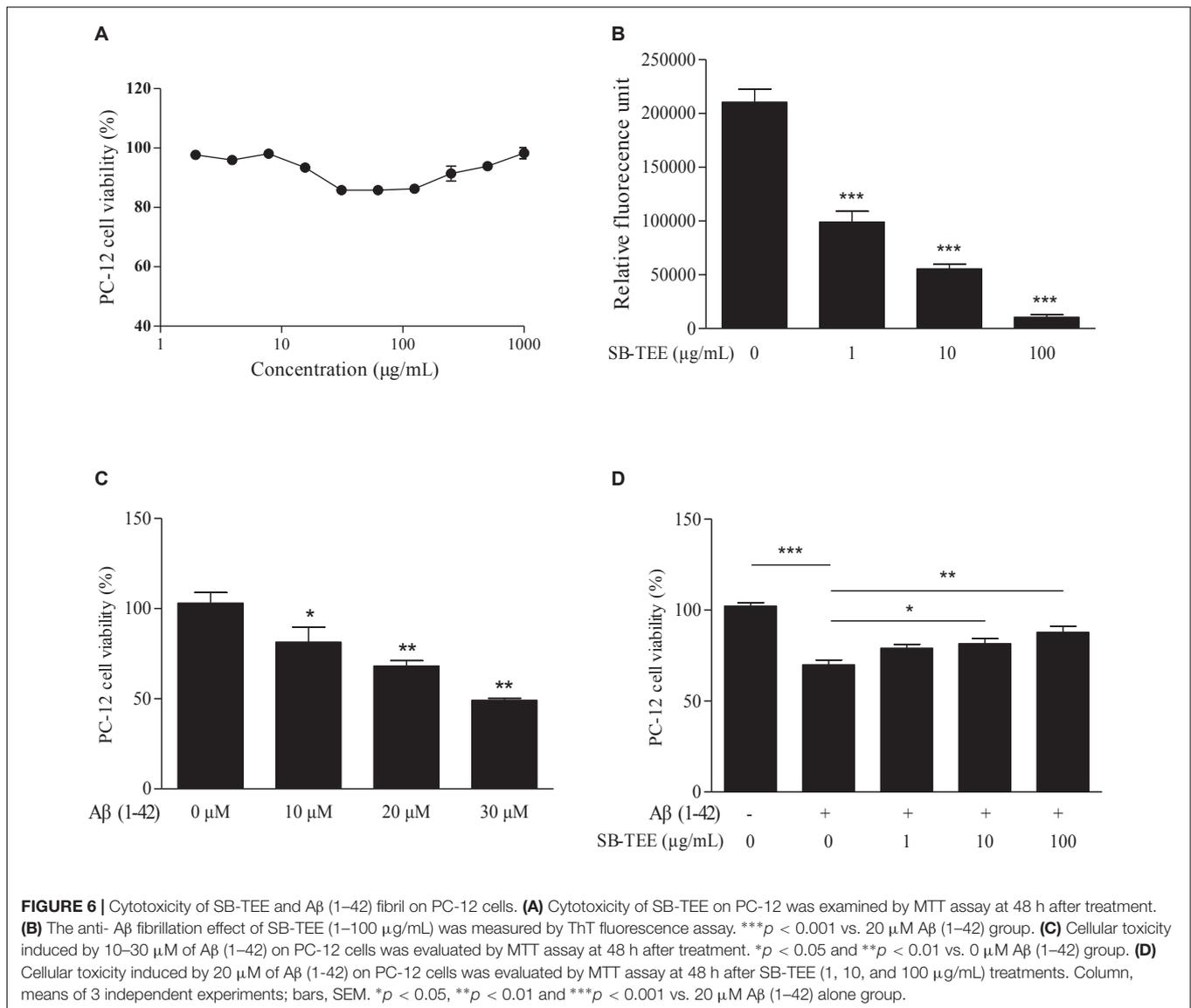
To further validate the correlation between the anti-fibrillation effect and the functional role of SB, the effect of SB-TEE on $A\beta$ (1–42)-induced cell death was evaluated. To begin, the viability of PC-12 cells upon SB-TEE treatment, from 0 to 1000 μ g/mL, was evaluated by MTT assay. As shown in **Figure 6A**, no obvious cellular toxicity was observed within the tested concentration range. Furthermore, fibrillation of $A\beta$ (1–42) was performed in the presence of PBS or SB-TEE with 5 days of incubation at 37°C. A ThT fluorescence assay confirmed that the fibrillation of $A\beta$ decreased with an increasing concentration of SB-TEE from 1 and 100 μ g/mL, as shown in **Figure 6B**. To further elucidate the anti- $A\beta$ fibrillation effect of SB-TEE in cells, viability of PC-12 cells incubated with an increasing concentration of $A\beta$ (1–42), from 10 to 30 μ M, was evaluated by MTT assay as shown in **Figure 6C**. With an incubation concentration of 20 μ M $A\beta$ (1–42), **Figure 6D** confirmed that an increasing

concentration of SB-TEE decreased the toxicity of $A\beta$ (1–42), which is correlated with the anti- $A\beta$ fibrillation effect of SB-TEE. Our results have confirmed the protective role of SB-TEE on $A\beta$ -induced toxicity in cells, which further validated the accuracy and precision of applying the UHPLC-DAD-TOF/MS detection system for the identification of $A\beta$ fibrillation inhibitors, from analytical chemistry to cellular functional level.

Protective Role of Baicalin, Baicalein and Wogonoside in the Cytotoxicity of $A\beta$ (1–42) in PC-12 Cells

$A\beta$ (1–42)-induced oxidative stress and neurotoxicity have been implicated in the pathogenesis of AD (Butterfield, 2002). With the protective effect of SB-TEE in alleviating the cytotoxicity of $A\beta$ (1–42) in PC-12 cells, the role of the selected potential anti- $A\beta$ fibrillation herbal compounds, baicalin and baicalein, identified by UHPLC-DAD-TOF/MS were further evaluated by MTT and flow cytometry analysis. As shown by the MTT results in **Figure 7A**, baicalin, baicalein and wogonoside possess very low or no cytotoxicity in PC-12 cells at concentrations ranging from 0 to 100 μ M. Viability of PC-12 cells incubated with $A\beta$ (1–42) (20 μ M) with or without the presence of baicalin (50 μ M), baicalein (50 μ M) or wogonoside (50 μ M) were evaluated by MTT assay. As shown in **Figure 7B**, while both baicalin and baicalein were able to rescue cells from $A\beta$ (1–42)-induced cell death, wogonoside showed no effect in the restoration of



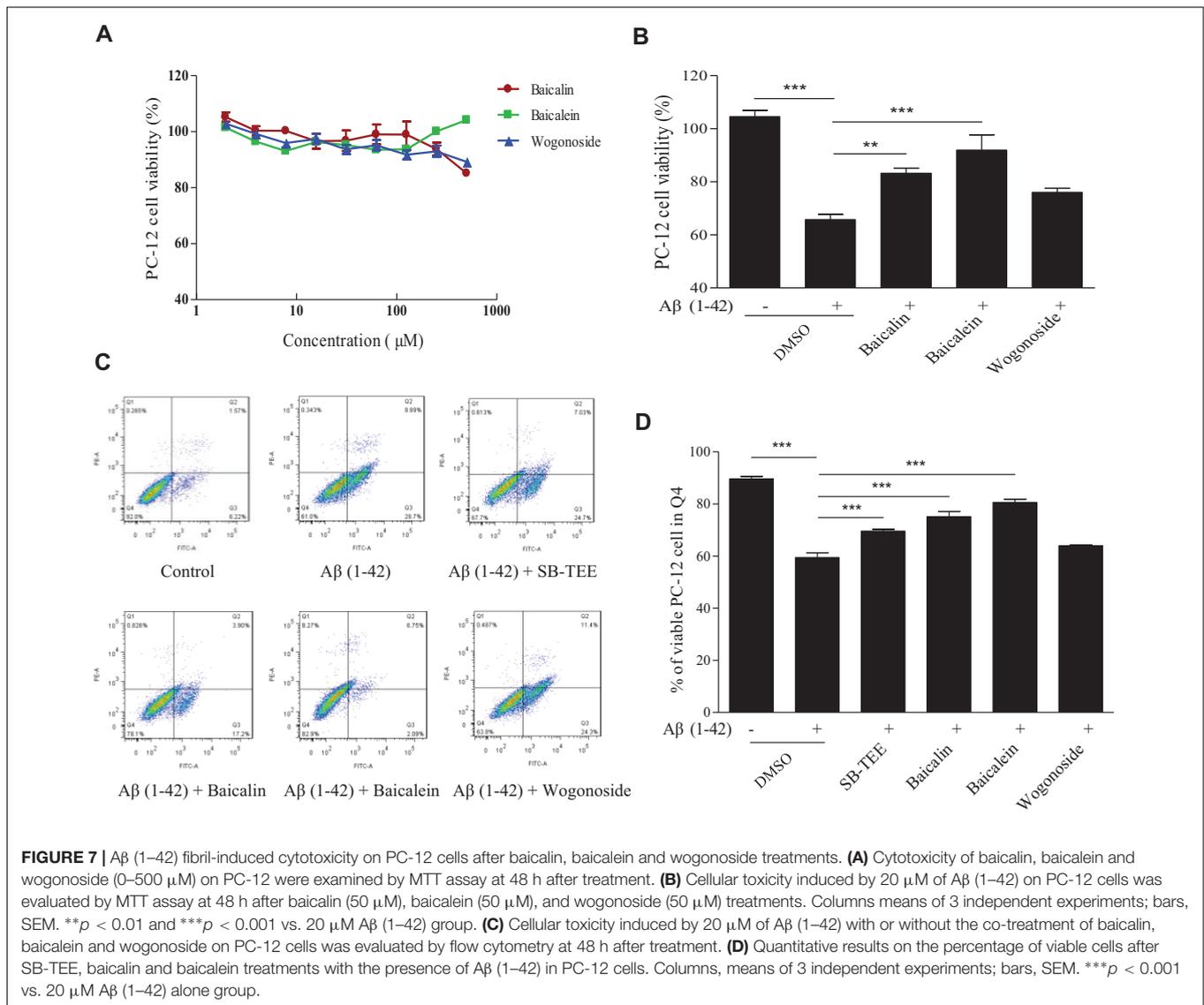


cell viability. The MTT result was further confirmed by flow cytometry analysis using annexin V staining. As shown by the increased percentage of viable cells in **Figures 7C,D**, quantitated flow cytometry results confirmed the protective role of baicalin and baicalein in A β (1–42)-induced cell death. All these data have further supported the notion of applying UHPLC-DAD-TOF/MS as a system for accurate and rapid detection of anti- A β fibrillation inhibitors from TCMs.

DISCUSSION

Accumulation of A β in senile plaques and hyperphosphorylated tau in NFTs are the two main pathological characteristics in the brain of AD patients. Experimental evidence has suggested that A β is an important biomarker for diagnosis and drug target of AD, based on the amyloid hypothesis of AD (Thakur

et al., 2009; Selkoe and Hardy, 2016; Tamagno et al., 2017). The A β (1–42) peptide derived from APP through the cleavage of γ -secretases and β -secretases, is the predominant aggregated and neurotoxic form of A β found in the brain of AD patients due to its two additional hydrophobic amino acids (Butterfield et al., 2013; Xiao et al., 2015). The excessive formation and failure on the clearance of A β , can lead to the formation of self-aggregated A β in different forms such as oligomers and neuritic plaques, which possess neurotoxicity and can affect axon function via oxidative stress (Lecanu et al., 2006), altered electrochemical signaling (Walsh et al., 2002) or *N*-methyl-D-aspartate (NMDA) receptor-mediated excitotoxicity (You et al., 2012), which can lead to impaired cognitive and memory functions (Palop and Mucke, 2010). Current pharmacological interventions to AD include the use of cholinesterase inhibitors, NMDA receptor antagonists and neurotrophic factors (Masters et al., 2015). In addition, inhibitors targeting the different

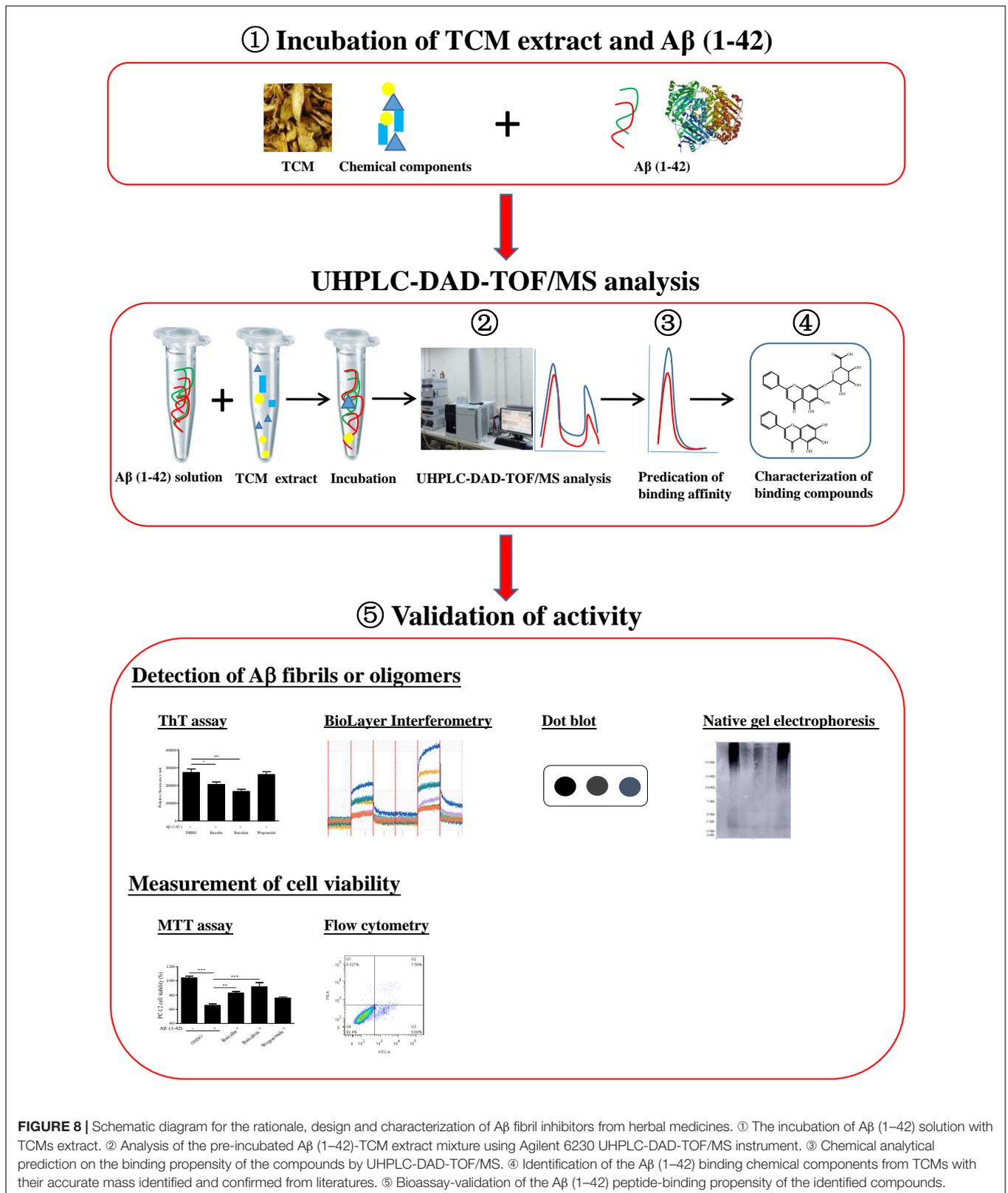


structures of $A\beta$ have also been widely studied (Kepp, 2012; Doig and Derreumaux, 2015). For example, carnosine (β -alanyl-L-histidine) can interact with $A\beta$ (1-42) monomer and inhibit $A\beta$ aggregation (Attanasio et al., 2013). As an inhibitor of $A\beta$ oligomers, both curcumin and resveratrol can bind to the N-terminus (residues 5–20) of $A\beta$ 42 monomers (Fu et al., 2014), while BAN2401 (humanized version of mAb158) decreases the level of soluble $A\beta$ protofibrils (Lannfelt et al., 2014). A recent report has suggested that the human innate immune peptide LL-37 can bind to $A\beta$ and modulate the formation of the $A\beta$ fibril (De Lorenzi et al., 2017). However, current available drugs or treatments are only symptomatic which mainly target the modulation of cognitive ability or neuropsychiatric symptoms.

As amyloid deposition starts early, before the onset of dementia symptoms (Lannfelt et al., 2014), preventive drugs are particularly important in AD treatment. With the advantages

of multi-targets and high drug safety, TCMs are commonly used as a preventive prescription for aged-related degenerative diseases, therefore, identifying potential active compounds from TCMs has become an important strategy for AD therapy (Pan et al., 2013). TCMs have been used for the treatment of AD for a long time in China. For example, huperzine A, a lycopodium alkaloid isolated from *huperzia serrata*, could improve AD symptoms through inhibiting acetylcholinesterase (AChE) activity (Zhu et al., 2010). The leaf extract of Ginkgo biloba (EGb 761) could protect hippocampal neurons against cytotoxicity induced by $A\beta$ fragments (Bastianetto et al., 2000; Wu et al., 2011). However, potential drug discoveries from TCMs still encounters challenges in the quick and precise identification of bioactive components from its complex herbal formulation (Liu et al., 2017).

SB has been reported for its protective roles in the improvement of learning ability (Heo et al., 2009) and brain



injury (Hwang et al., 2011; Lin et al., 2011) possibly via its anti-oxidation, -inflammation (Yune et al., 2009) and -apoptosis properties (Jeong et al., 2011). Although a previous study has reported only the moderate neuroprotectivity of SB extract for A β insult (Kim et al., 2007), possibly due to the low testing concentration, other studies have confirmed that flavonoids from SB play a protective role in the recovery of neurological functions and in the prevention of AD (Gasiorowski et al., 2011; Gao et al., 2013; Vauzour, 2014). For example, total flavonoids from SB can protect neuronal damage and improve memory deficits induced by cerebral ischemia in rats (Shang et al., 2006; Cao et al., 2016). Additionally, a flavonoid composition (UP326) containing baicalin can help to maintain memorizing and processing abilities of aged animals (Yimam et al., 2016). As the major type of flavonoids in SB, baicalein and baicalin ameliorated cognitive impairment in AD animal models (Wei et al., 2014; Chen et al., 2015). In fact, the recent development of plant-derived drugs has received considerable attention in the treatment of AD (Kumar and Nisha, 2014), therefore, based on the previous literature on SB, the development of accurate detection methods of active components from herbal plants is performed in this study by choosing baicalein and baicalin as the study subjects.

It is noteworthy that traditional bioactivity guided purification of single active compounds from TCMs is time-consuming, laborious, costly and has low sensitivity. Although ThT fluorescence dye is a commonly used fluorescent probes for A β fibril detection, it possesses the disadvantages of low specificity and sensitivity (Roberti et al., 2009; Kitts et al., 2011; Li et al., 2013). Cell-based detection methods have been developed by expressing the GFP-A β 42 fusion protein in *E. coli*, which allows the rapid high throughput screening of inhibitors on A β aggregation (Kim et al., 2006). Furthermore, the advances in the use of affinity chromatography in conjunction with highly sensitive detection technology has also facilitated the detection of bioactive compounds from TCMs. For example, biolayer interferometry accurately measures the real-time biomolecular interactions in the cells-free system, which has been used to identify D-enantiomeric peptide D3 and its derivatives as A β oligomers inhibitors (Concepcion et al., 2009; Klein et al., 2016). Nuclear magnetic resonance spectroscopy (NMR), was used to identify inhibitors on A β fibrillation from a compound library with high sensitivity and speed (Sievers et al., 2011). Furthermore, mass spectroscopy coupled with liquid chromatography (LC) was used for rapid detection of small molecules such as epigallocatechin gallate (EGCG), hemin and tramiprosate that could bind to A β precursors (Young et al., 2015). Although some of the current detection methods show high sensitivity and analytical speed, these methods work best only in targeting single compounds, but not to extract of TCMs that contain multiple chemical components.

UHPLC-DAD-TOF-MS is a common analytical equipment that provides accurate mass analysis including profiling, characterization, identification and quantification of a mixture of chemical molecules, based on their molecular weights, with highly sensitive. Therefore, it serves as a useful tool for

the identification of single components from complicated chemical compositions like the extract of TCMs (Hossain et al., 2010). We have previously reported the successful identification of the natural methylglyoxal (MGO) scavenger from *Polygonum cuspidatum* and the autophagy inducer from *Radix polygalae* using UHPLC-DAD-MS and UHPLC-DAD-TOF-MS, respectively (Tang et al., 2013; Wu et al., 2013). According to the published reports, the peak area of the bioactive inhibitors including small molecule and peptide in the NMR spectrum decreased significantly upon their binding to the A β or tau fiber (Sievers et al., 2011; Jiang et al., 2013). Based on the above observation, we have therefore hypothesized and confirmed that the components in TCMs, with an inhibition effect on A β fibrillation, must possess binding affinity to the A β fibril, and these bioactive components can be successfully detected using UHPLC-DAD-TOF-MS via chromatogram analysis. In the present study, although the method was successfully applied on SB-TEE and its major known chemical components, further verification on different TCMs along with a biological evaluation is required to further validate accuracy. In addition to MS, which provides information on the possible potency and accurate identifies of the herbal chemicals, combinational use of column chromatography and NMR technology, for the analytical isolation and identification of novel or low abundance active compounds presented in SB or other herbs, are required.

CONCLUSION

In conclusion, up to now, there are still no effective and reliable detection methods for the inhibitors of β -amyloid fibrillation from complicated chemical composition such as TCMs. As shown in **Figure 8**, based on the fact that the peak area of the compounds on the chromatogram are reduced upon their binding with A β fibril, we hypothesized that the components in TCMs, that have binding propensity for A β , can be identified using UHPLC-DAD-TOF/MS. Through the effective and accurate isolation of the active compounds from TCMs, UHPLC-DAD-TOF-MS has therefore proved to be a useful method for identifying the bioactive compounds in TCMs with anti-A β fibrillation effects. In the near future, the method proposed in this study may also be applied for the precise screening of inhibitors on other AD-related proteins such as tau, which may facilitate AD drug discovery from TCMs.

AUTHOR CONTRIBUTIONS

LY and A-GW conducted the experiments and drafted the manuscript. A-GW and WZ conducted the chemical analysis experiments. LY, L-QQ, BT, NZ, and H-MW conducted the bioassay experiments. V-WW and D-LQ conceived and designed the experiments. BL revised the whole manuscript. QW and BL conceived the idea and designed the experimental plan. All authors reviewed the above manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2019.00194/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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