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Manuscript Title: Inhibition of β-Amyloid Aggregation by Albiflorin, Aloeemodin and Neohesperidin and their Neuroprotective Effect on Primary Hippocampal Cells against β-Amyloid Induced Toxicity

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Abstract:
Being one of the hallmarks of Alzheimer’s disease, β-amyloid (Aβ) aggregates induce complicated neurotoxicity. Evidences show that the underlying mechanism of neurotoxicity involves a glutamate receptor subtype, N-methyl-D-aspartate (NMDA) receptor, an increase in intracellular calcium(II) ion loading as well as an elevation in oxidation stress. In this work, among the 35 chemical components of Chinese herbal medicines (CHMs) being screened for inhibitors of Aβ aggregation, four of them, namely albiflorin, aloeemodin, neohesperidin and physcion, were found for the first time to exhibit a potent inhibitory effect on Aβ1-40 and Aβ1-42 aggregation. Their neuroprotective capability on primary hippocampal neuronal cells was also investigated by MTT assay, ROS assay and intracellular calcium(II) ion concentration measurement. It was interesting to find that physcion was rather toxic to neuronal cells while albiflorin, aloeemodin and neohesperidin reduced the toxicity and ROS induced by both monomeric and oligomeric Aβ species. In addition, albiflorin was particularly powerful in maintaining the intracellular Ca²⁺ concentration.

Keywords
amyloid aggregation, inhibitors, neuroprotection, calcium influx, herbs, reactive oxygen species.
INTRODUCTION

According to the Alzheimer’s Disease Association, there were 36 million people who suffered from dementia worldwide in 2010. This number is expected to be 115 million by 2050 for the global aging population.[1] Therefore there is an urgent need to develop effective drugs that can prevent, slow down the disease progression and hopefully cure the disease. β-Amyloid (Aβ) peptides i.e., Aβ1-40 and Aβ1-42, are the principal protein components of Aβ plaques found in the brains of Alzheimer’s disease (AD) patients by autopsy. The occurrence of the Aβ plaques, considered as a cardinal feature of AD, provides the only confirmed diagnosis of the disease. Extensive research in past decades has evidenced that the Aβ peptides play a central role in the disease progression, in which Aβ peptides assemble into aggregates that exert cytotoxic effects towards neurons and initiate the pathogenic cascade, i.e. the amyloid cascade hypothesis.[2; 3] Recent studies showed that oligomeric, prefibrillar and diffusible assemblies of Aβ peptides are particularly deleterious.[4] The underlying mechanism of Aβ neurotoxicity however is very complex. Evidences show that it involves a glutamate receptor subtype, glutamate release, N-methyl-D-aspartate (NMDA) receptor, an increase in intracellular Ca2+ ion concentration as well as an elevation in oxidative stress. Thus, compounds that can (i) inhibit Aβ aggregation formation; (ii) protect cells against reactive oxygen species toxicity and; (iii) reduce Ca2+ ion influx induced by Aβ species are promising approaches to prevent and treat AD. Chinese herbal medicines (CHMs) have been used to treat AD patients for a long time although the efficacy and safety of its formulation is still not conclusive. Various types of compounds have been identified, such as oligopeptides, polyphenols, anti-oxidants and anti-inflammatory drugs, and are reported to be Aβ-aggregation inhibitors that provide neuroprotection.[5-11] While this thematic issue reported other two kinds of curcumin derivatives [12-13] and iridium complex [14] and rhodium complex [15] as effective inhibitors of Aβ aggregation.

CHMs could be a good source of single components for the therapeutic treatment of AD. As inflammation and oxidation stress are shown to be highly related to AD, it would be interesting to investigate the roles of CHMs that are known to have anti-inflammatory and anti-oxidant effect, in AD treatment. For examples, albiflorin is one of the natural components found in Paeoniae radix,[16] which is an important crude drug used in traditional Chinese medicines (TCMs) for treating abdominal pain and wounds.[17] Aloeemodin is an anthraquinone derivative that is extracted from the root and rhizome of Rheum palmatum. Its anti-cancer effect has been demonstrated.[18] Neohesperidin obtained from Poncirus trifoliata shows protection on gastric cancer cells.[19] Physcion is an ingredient found in the
roots of the Chinese rhubarb named Rheum officinale Baill which was shown to have anti-
microbial activity. [20] In this study, we have screened more than 35 single compounds
extracted from CHMs for inhibition of Aβ aggregation and investigated the potential of the
four potent inhibitors for AD prevention and treatment. We studied (i) their inhibitory effect
on Aβ aggregation by fluorescence microscopy, Thioflavin T fluorescence assay and circular
dichroism measurement; (ii) their neuroprotective effect on primary hippocampal cells; (iii)
their influence on ROS generation induced by the monomeric and oligomeric Aβ species; and
(iv) the change in the intracellular Ca^{2+} ion concentration in response to Aβ_{1-42}.

MATERIALS AND METHODS

Animals
Pregnant SD rats were purchased from the Laboratory Animal Services Center, the Chinese
University of Hong Kong, Hong Kong. The animals were fed with a standard rodent diet ad
libitum with free access to water and were housed in rooms maintained at 22 ± 1 °C with a 12
hour light/dark cycle (lights on 6:00-18:00). The pregnant rats were acclimated to the facility
for 1 week before giving birth. The Animal Ethics Committee of Hong Kong Baptist
University, approved all experimental protocols, in accordance with “Institutional Guidelines
and Animal Ordinance” from Department of Health of Hong Kong Special Administrative
Region.

Chemicals and reagents
Compounds of CHMs screened in this study including albiflorin, aloeemodin, amygdalin,
astragaloside IV, atractylenolide III, baicalin, benzoylgomisin Q, calcosin,
calcosin-7-Abeta-D-glucoside, chrysophanol, dehydrocostus lactone, deoxyschizandrin,
emodin, formononetin, gallic acid, geniposide, gomisin M2, hesperidin, honokiol,
isoimperatorin, liquiritin, magnolol, naringin, neohesperidin, ononin, paeoniflorin, paeonol,
physcion, progomisin, quercetin, rhein, schisantherin A, schizandrin, γ-schizandrin were
purchased from Shenzhen Chemstrong Scientific Co., Ltd. (Shenzhen, China) and used
without further purification. Other chemicals and reagents were purchased from Sigma-Aldrich unless specified.

Preparation of β-amyloid solution
Monomeric β-amyloid (1-40) (Aβ_{1-40}) and β-amyloid (1-42) (Aβ_{1-42}) were purchased from
Invitrogen (USA) and used without further purification. Stock solutions were prepared by
dissolving 1 mg of the monomeric Aβ in 400 μL of 0.02 % filtered ammonium solution and
stored at −20 °C prior to use. The Aβ_{1-40} fibril seed solution was prepared as reported elsewhere.[21; 22] Briefly, 5 μL of stock monomeric Aβ_{1-40} solution was diluted to 50 μM with filtered phosphate buffer (50 mM sodium phosphate, 100 mM NaCl) and incubated at 37 °C with gentle shaking for 20 hours. The resultant fibrils were sonicated for 3s trice and used as seeding for seed-mediated fibrillation study. Preparation of Aβ_{1-42} oligomer. The oligomer solution of Aβ_{1-42} was prepared by diluting the monomeric Aβ_{1-42} stock solution to 100 μM with phosphate buffer and incubated at 37 °C with gentle shaking for 72 hours.

Aβ_{1-40} samples for TIRFM
Stock monomeric Aβ_{1-40} solution was diluted to 50 μM with phosphate buffer; a final concentration of 10 μg/mL seed solution was added to speed up the fibrillation process. For inhibition study, CHMs of 50 μM were added to the mixture and incubated at 37 °C for an hour. The fibrils were then diluted to 10 μM with thioflavin T (ThT) in a ratio of ThT: monomeric Aβ_{1-40} 1:1. The ThT labeled fibrils were then visualized under the home-built prism-type total internal reflection fluorescence microscopic (TIRFM) imaging system. In general, an Olympus IX-71 inverted microscope (Japan) was equipped with an oil-type high numerical-aperture 60 × objective. For imaging, the sample was placed between the objective and the fused silica isosceles Brewster prism (CVI Laser, USA). A 455 nm of 50 mW diode laser (Newport, USA) was used as an excitation source to excite the ThT labeled Aβ_{1-40}. The incident angle of the laser beam was set as ~ 70 ° to cause the total internal reflection and generate the evanescent field for the sample excitation. A band pass filter HQ 480/40 (Chroma Tech. Corp., USA) was used. The fluorescent image of the sample was captured by an electron-multiplying charge coupled device (EMCCD) (Princeton Instrument, USA) coupled with a Uniphase mechanical shutter (Vincent Associates, USA) and a driver (Vincent Associates, USA) for external synchronization and frame-transfer mode. The exposure time was set as 100 ms for both EMCCD and shutter; the multiplication gain of the EMCC and the delay time of the shutter were set as 4000 and 100 ms respectively. Images were obtained with WinSpec/32 software (Princeton Instruments, USA).

Transmission electron microscopy (TEM) imaging
Sample solution of 5 μL was applied on a carbon-coated copper grid (Electron Microscopy Science, USA) and dried at room temperature. The sample was then negative stained with 0.2 % uranyl acetate. The TEM imaging of the dried sample were recorded by a Tecnai G2 20 S-TWIN Transmission Electron Microscope (FEI, USA) with an acceleration voltage of 200
kV.

**Thioflavin T Fluorometric Assay**

Stock monomeric $\text{A} \beta_{1-42}$ solution was diluted to 200 μM in phosphate buffer. For inhibition study, CHMs of 200 μM was added to the monomeric $\text{A} \beta_{1-42}$ solution. The sample solutions were then incubated at 37 °C for 0, 4, 7, 15, 19, 22, 39, 43 hours respectively. The resulting samples were then labeled with ThT ($\text{A} \beta_{1-42}$:ThT, 1:2 in molar ratio). The emission spectrum of the sample was measured by PTI TimeMaster C720 Spectrometer.

**Circular Dichroism (CD) Measurement**

The CD spectra of 50 μM monomeric $\text{A} \beta_{1-42}$ solution in the presence or absence of the CHMs were obtained by a Jasco J-810 Circular Dichroism Spectropolarimeter in a spectral range of 180-260 nm using a 1-mL quartz cell. The bandwidth and the scan speed were set at 1 nm and 200 nm/min respectively. Each spectrum was an average of three individual measurements. Spectra were smoothed with Savitzky-Golay smoothing by OriginPro 8 software.

**Primary Cell Isolation and Culture**

Seven day-old SD rats were euthanized with CO$_2$, and the hippocampus tissues were harvested quickly. Neuronal cells were isolated as described previously with modification. [23] Briefly, the hippocampus tissues were washed with DMEM/F12 medium (Gibco, USA) with 10 % fetal bovine serum (Gibco Life, USA) for three times and then digested in DMEM/ F12 medium containing 2 mg/mL papain (SIGMA, USA) at 37 °C for 20 min under gentle shaking. The tissues were then dispersed using 3 mL transfer pipette (SIGMA, USA) and the solution was filtered by a 0.4 μm nylon filter (BD falcon). The filtrate was further subjected to centrifugation at 1000 rpm for 5 min. The pellet containing the dissociated neurons was resuspended in neurobasal medium (Gibco, USA) with 2 % B27 (B-27® Supplement 50X, Gibco, USA), 0.25 % Penicillin-Streptomycin-Neomycin (PSN, Gibco, USA) and 0.25 % glutamax (GlutaMAX-I 100×, Gibco, USA) and rinsed for 3 times. The cells were plated at a density of $5 \times 10^4$ cells/well on poly-D-lysine (Sigma, USA) coated 96-well plates (Nunc, USA) and incubated at 37 °C, under 80 % humidity and 5 % CO$_2$. Cytosine of 1 μM was added into the culture medium on the day after cell isolation and removed the next day by changing medium. Culture medium was changed every 3 days, and the cells were treated with designated drugs after 7 days in culture.
Cell line
Cell line SH-SY5Y (Cat. Number CRL2266, ATCC, USA) were cultured in DMEM/F12 supplemented with 10% fetal bovine serum and 0.25% PSN. The cells were incubated in humidified incubator at 37 °C with 5% CO₂.

Cytotoxicity and viability assays
Viability of SH-SY5Y cells was determined by a methylthiazole tetrazolium ([3-(4,5)-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, MTT) assay with some modifications.[24] Briefly, SH-SY5Y cells were cultured in 96-well plates at 3 × 10⁴ cells/well for 1 day before treatment. For CHM toxicity, cells were incubated with different concentrations of CHM (0, 0.1, 0.5, 1, 5, 10 and 50 μM) for 24 hours. At the end of each treatment, the culture medium was replaced with fresh medium containing 0.5 mg/mL MTT for 3 hours at 37 °C under 5% CO₂ and 80% humidity. After incubation, the medium was replaced by dimethyl sulfoxide (DMSO) to dissolve the formazan crystals. The plate was shaken gently. The optical density (OD) was measured by a Universal Microplate Reader (Elx 800, Bio-TEK instruments Inc., USA) at 540 nm (690 nm as a reference). The data were expressed as mean values of the OD_{herbal}/OD_{control} and error bars show the standard deviation of three trials.

Neuroprotection on hippocampal cells
Hippocampal cell viability was determined by a modified MTT assay.[25] Briefly, hippocampal neurons were cultured in 96-well plates at 5 × 10⁴ cells/well for 7 days before treatment. Cells were incubated with different neurotoxic Aβ of 10 μM, including the monomer and oligomer forms of Aβ₁₋₄₀ and Aβ₁₋₄₂ separately as controls. The herbals (aloemodin, albiflorin and neo hesperidin) were added into the wells in a 1:1 molar ratio of each Aβ species. The cells were placed in humidified incubator at 37 °C under 5% CO₂ for 24 hour. The culture medium was replaced with fresh medium containing 0.5 mg/mL MTT for further incubation for 3 hours at 37 °C. The medium was replaced by DMSO. The optical density (OD) at 540 nm and 690 nm was measured. The neuroprotection effect was calculated as the mean value of OD_{CHM treated with Aβ}/OD_{Aβ} and error bars show the standard deviation of three trials.

Measurement of reactive oxygen species (ROS) generation
The effect of each herbal compound on intracellular ROS formation was evaluated by the 2′,7′-dichlorofluorescein (DCF) fluorescence assay.[26; 27] Briefly, primary hippocampal
cells were cultured in 96-well plates, cells were loaded with the fluorescent dye DCFH-DA of
congcentration 100 μM (Molecular Probes, life technologies, USA) and incubated in dark at 37
° C under 5 % CO₂ for 30 minutes. The DCFH-DA medium solution was removed and the cell
culture was washed with phosphate buffer saline (PBS, Gibco, USA) twice. Cells were treated
with different peptides, Aβ1-40 monomer, Aβ1-40 oligomer, Aβ1-42 monomer and Aβ1-42 oligomer
of final concentration 10 μM in HBSS as controls. Alloemodin, albiflorin and hesperidin were
mixed with Aβ peptides in1:1 molar ratio and used to treat the cells at a final concentration of
10 μM. The cells were further incubated for 24 hours. The fluorescence intensities were
detected at excitation and emission wavelengths of 485 nm and 530 nm respectively by a
microplate reader (Tecan, Switzerland). The data were expressed as a mean value of ODCHM
treated with Aβ/ODAβ and error bars show the standard deviation of three trials.

**Laser confocal fluorescence imaging**

Calcium influx induced by Aβ1-42 monomer was measured by Ca²⁺ ion imaging with fluo-4
AM.[28] Hippocampus neuronal cells were seeded into a 35 mm confocal culture dish at
8000 cells/well and cultured for 7 days before treatment. The cells were pre-treated with
the three CHMs, albiflorin, neohesperidin and alloemodin of final concentration 10 μM
separately for 30 min. Then the cells were incubated with 3 μM fluo-4 AM for 30 min at 37
° C in the dark. The cells were further incubated with fresh serum free medium for another 30
min. The medium was replaced by HBSS before calcium measurement. The Ca²⁺ fluxes
induced by Aβ1-42 monomer on treated and non-treated cells were monitored by Leica
confocal laser scanning microscope (TCS SP8) with a 20× water-oil objective (HC PL APO
20×/0.75 Imm). The samples were excited at 488 nm and emission peak at 506 nm was
measured. The Ca²⁺ fluxes were monitored for 15 min in 1 frame/3s collecting by Leica
Application Suite X (LAS X).

**RESULTS**

![Molecular structures of the four compounds isolated from Chinese herbal medicines.](image-url)
Inhibition study on Aβ1-40 aggregation

The inhibitory effect of 35 chemical components isolated from Chinese herbal medicines (CHMs) including albiflorin, neohesperidin, aloemodin and physcion on Aβ1-40 fibrillation was studied by monitoring the seed-mediated growth of Aβ1-40. The molecular structures of the four CHMs which were found for the first time to be Aβ effective aggregation inhibitors in our study are shown in Figure 1. Total internal reflection fluorescence microscopy (TIRFM) was applied to visualize and estimate the population of the resulted fibrils. The CHMs were co-incubated with monomeric Aβ1-40 at 1:1 molar ratio by the seed-mediated growth method. The resulting fibrils were then labeled with the conventional Aβ fibril labeling dye, Thioflavin T (ThT), which is a cationic benzothiazole dye that is highly specific to Aβ aggregates. The excitation and emission peaks of ThT shift to 450 nm and 482 nm respectively upon binding with Aβ aggregates. As shown in Figure 2, the length of the resulting fibrils with CHM treatment is remarkably shorter with smaller population as compared with the control (without CHMs) under same conditions. This indicated that these four CHMs were capable of slowing down the elongation process of Aβ1-40. Among them, neohesperidin and aloemodin exhibited a stronger inhibitory effect on fibril formation as compared to physcion and albiflorin. Based on these results, the inhibition potency of these compounds on Aβ1-42 and their biological properties were further investigated.

Fig. (2). TIRFM images of Aβ1-40 fibrils formed after incubation with the CHMs of seed-mediated growth Aβ1-40 at 37 °C for an hour. Scale bar = 20 μm.

Inhibition effect of CHMs on Aβ1-42 aggregation

Aggregates of Aβ1-42, particularly oligomers are shown to be more neurotoxic than aggregates of Aβ1-40. The inhibitory effect of the CHM compounds on the formation of Aβ1-42 aggregates was studied by a ThT aggregation assay. As demonstrated in Figure 3a, the fluorescence signal of the samples in the presence of these CHM compounds remained the same after 40
hours of incubation, whereas the fluorescence intensity of the control experiment dramatically increased after 20 hours. These results indicated that $A\beta_{1-42}$ monomers readily aggregated by themselves whereas no $A\beta_{1-42}$ aggregates formation occurred in the presence of the compounds from CHMs. In order to further confirm the inhibition properties of these compounds, $A\beta_{1-42}$ monomer was incubated with aloemodin for 2 days and visualized by TEM. The TEM images (Figure 3b) further revealed that neither $A\beta_{1-42}$ aggregates nor fibrils was formed in the presence of aloemodin. These results consistently revealed the capability of these compounds to prevent the formation of toxic $A\beta_{1-42}$ aggregates.
Fig. (3). (a) ThT fluorescence assay of Aβ_{1-42} aggregation demonstrated the inhibition effect of the CHM compounds on Aβ_{1-42} aggregate formation. (b) TEM images of Aβ_{1-42} aggregates grown in the absence (left) and presence (right) of aloemodin after 48-hour incubation at 37 °C. Scale bar = 500 nm. (c) Circular dichroism spectra of monomeric Aβ_{1-42} in the presence of the CHMs, which was mixed with Aβ at the molar ratio of 1:1.

Circular dichroism studies

In order to gain insight into the mechanism of the inhibitory effect of these compounds, circular dichroism (CD) spectra were measured to investigate the conformational changes of monomeric Aβ_{1-42} upon mixing with these compounds. The process of Aβ aggregation is believed to result from the conformation change of Aβ peptides from a random coil α-helix conformer to the amyloidogenic β-sheet conformer that causes the favorable self-assembly of the peptides to neurotoxic intermediates and Aβ plaques. The CD spectra of the Aβ_{1-42} peptides with or without the CHMs were shown in Figure 3c. In the absence of the CHM compounds, monomeric Aβ_{1-42} showed a negative CD signal at 219 nm which is the characteristic wavelength of the β-sheet conformation.[29] Upon addition of the CHM components, the β-sheet character peak was significantly diminished. The peak shifted from 219 to 225 nm for albiflorin, aloemodin and physcion while the peak for neohesperidin further shifted to 230 nm. The alterations in peak amplitude and position indicated that the population of β-sheet conformer was dramatically reduced and meanwhile a new conformation, Aβ-CHM complex, was established.
Cytotoxicity of CHMs by MTT assays
The cytotoxicity of the compounds from CHMs were determined by a MTT assay on human SH-SY5Y neuroblastoma cells. Cell cultures were treated with CHMs in a concentration range from 0.1 to 50 μM for 24 hours before the MTT assays. As depicted in Figure 4, these four compounds are generally of low toxicity in the micro-molar range. Physcion is relatively toxic among the four selected compounds. The survival of the cells dropped to about 55 % at 10 μM of physcion. However, the cell mortality was less than 15 % for the remaining three compounds at a concentration of 10 μM. Therefore, albiflorin, aloeemodin and neohesperidin being non-toxic to neuronal cells were further investigated for their clinical potential using primary hippocampal cells.

**Fig. (4).** Toxicity of albiflorin, aloeemodin, neohesperidin and physcion on human SH-SY5Y neuroblastoma cells as determined by an MTT assay. Cells were exposed to CHMs of various concentrations for 24 hours.

Neuroprotection on primary hippocampal cells
The toxicity of the monomeric and oligomeric Aβ species on primary hippocampal cells was measured by an MTT assay after incubation of 24 hours. Consistent with the literature, it was found and that the toxicity of Aβ1-42 was higher than that of Aβ1-40, and that the oligomeric form was more toxic than the monomeric form. Among the four potent inhibitors, albiflorin, aloeemodin and neohesperidin were chosen for an investigation of neuroprotection effect because of their low cytotoxicity. The primary hippocampal cells were co-incubated with each of these compounds and the monomeric Aβ in a 1:1 molar ratio (10 μM). The
mortality of the cells in the presence of both these inhibitors and $A\beta$ species relative to that treated by $A\beta$ species alone is depicted in Figure 5a. It was found that all three CHM compounds provided protection against monomeric $A\beta$-induced toxicity. In another similar experiment, the primary hippocampal cells were incubated for 24 hours with the oligomeric form of $A\beta_{1-40}$ and $A\beta_{1-42}$, with and without the three CHM compounds. The MTT results are depicted in Figure 5b. It is worthy to note that aloeemodin is the most potent compound at protecting neuronal cells against both oligomeric $A\beta_{1-40}$- and $A\beta_{1-42}$-induced neurotoxicity.
Fig. (5). The neuroprotection effect shown by relative % MTT (Monomer/CHM-Monomer) after 24 hour co-incubation with aloeemodin, neohesperidin and albiflorin on hippocampal neuron cells (A). The neuroprotection effect shown by relative % MTT (Oligomer/CHM-Oligomer) after 24 hour co-incubation with aloeemodin, neohesperidin and albiflorin on hippocampal neuron cells (B).

**Reactive oxygen species (ROS) measurement**

It has been demonstrated that one of the toxic effects induced by $A\beta$ species is the production of intracellular reactive oxygen species (ROS). The effect of the neuroprotective inhibitors, albiflorin, aloeemodin and neohesperidin was determined by ROS measurement with the 2',7'-dichlorofluorescein (DCF) fluorescence assay. Primary hippocampal cells were incubated with monomeric $A\beta$ peptides with and without the three compounds from CHMs for 24 hours. The final concentration of the $A\beta$ peptides and the CHMs was kept as 10 µM. As illustrated in Figure 6A, aloeemodin showed consistent attenuation against $A\beta$ induced ROS generation on primary hippocampal cells while albiflorin and neohesperidin did not. These results implied that these three compounds from various CHMs protect the hippocampal neuron cells through different pathways. Similarly, primary hippocampal cells were incubated with preformed oligomeric $A\beta_{1-40}$ and $A\beta_{1-42}$ in the presence and absence of
the three compounds for 24 hours. The fluorescence intensity of the ROS indicator, DCF, in the presence of the preformed oligomeric $A\beta$ was doubled compared to the control cells (without treatment) indicating the oxidative stress induced by $A\beta$ aggregates. However, the ROS production was obviously suppressed by the three compounds of CHMs. As shown in Figure 6B, albiflorin and neohesperidin were able to cut down the ROS level by about 50%. These results suggest that these two compounds are powerful in protecting neuronal cells by reducing the ROS level as induced by the oligomeric $A\beta$ species.
**Fig. (6).** The change of ROS level is shown by a relative % ROS (CHM-Monomer/Monomer) after 24-hour co-incubation with aloemodin, neohesperidin and albiflorin on hippocampal neuron cells (a). The change of ROS level is shown by a relative % ROS (CHM-Oligomer/Oligomer) after 24-hour co-incubation with aloemodin, neohesperidin and albiflorin on hippocampal neuron cells (b).

**Calcium(II) ion imaging**

The neurotoxic $A\beta_{1-42}$ induces overloading of intracellular $\text{Ca}^{2+}$ and thus could result in cell death.[28] To explore the influence of the three neuroprotective compounds from CHMs on the calcium influx induced by $A\beta_{1-42}$, the primary hippocampal cells were pre-treated with the CHMs for 30 min before the addition of $A\beta_{1-42}$. The calcium concentration was then monitored by fluo-4 AM. As depicted by Figure 7, the intracellular $\text{Ca}^{2+}$ concentration rapidly increased upon the treatment of 10 $\mu$M $A\beta_{1-42}$, and it showed higher fluorescence intensity compared with those pretreated with the compounds. For those primary hippocampal neuron cells treated with aloemodin and neohesperidin, the increase of $\text{Ca}^{2+}$ influx was gentle and ended with a lower peak intensity. Albiflorin-treated cells showed the lowest intensity (about half of the control) suggesting that the calcium influx induced by $A\beta_{1-42}$ was the most strongly attenuated by albiflorin.
Fig. (7). (a) Traces representing the \( \text{Ca}^{2+} \) ion influx induced by \( A\beta_{1-42} \) monomer in the presence of CHMs was measured by confocal imaging. (b) Confocal images of calcium influx of hippocampal neuron cells induced by \( A\beta_{1-42} \) without or with pretreatment of CHMs. Albiflorin showed the highest effectiveness on maintaining the \( \text{Ca}^{2+} \) level of hippocampal neuron cells. Each trace was an average of at least three independent measurements.

**DISCUSSION**

We have demonstrated for the first time that four compounds isolated from Chinese herbal medicines, namely albiflorin, aloemodin, neohesperidin and physcion exhibit remarkable inhibitory effect against \( A\beta_{1-40} \) and \( A\beta_{1-42} \) aggregation. Albiflorin, aloemodin and neohesperidin also show the ability to reduce neurotoxicity induced by \( A\beta \) species on primary hippocampal neuronal cells. To obtain insight into the AD disease mechanism, the influence of these three neuroprotective CHM compounds on the oxidative stress and intracellular \( \text{Ca}^{2+} \) ion concentration were investigated. The compounds were found to be able to effectively attenuate ROS generation induced by \( A\beta \) species. These findings are attributed to the fact that
these CHM compounds are anti-oxidant and anti-inflammatory in nature. Moreover, albiiflorin can remarkably maintain intracellular \( \text{Ca}^{2+} \) ion level upon A\( \beta \)\(_{1-42} \) attack. Thus, these three active and non-toxic CHM compounds that can (i) inhibit the A\( \beta \) aggregation formation; (ii) protect cells against toxicity induced by reactive oxygen species and; (iii) reduce \( \text{Ca}^{2+} \) ion influx induced by A\( \beta \) species are promising candidates to prevent and treat AD. Our study here strongly supports further in-vivo investigation on the clinical potential of these three CHM compounds, in particular their brain bioavailability, pharmacokinetics and capability in cognitive improvement in AD models.

**CONFLICT OF INTEREST**

None

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