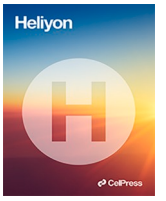
Heliyon 10 (2024) e39159

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Neuroprotective effect against amyloidogenic transthyretin aggregates - Induced cytotoxicity on human neuroblastoma cell by phenolic-rich *Centella asiatica* extract

Fredrick Nwude Eze [a,b], Apinna Bunyapongpan [c], Porntip Prapunpoj [c,*]

[a] *Drug Delivery System Excellence Center, Prince of Songkla University, Hat Yai, Songkhla, 90110, Thailand*
[b] *Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, 90110, Thailand*
[c] *Department of Biochemistry, Division of Health and Applied Sciences, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, 90110, Thailand*

ARTICLE INFO

Keywords:
Protein aggregates
Transthyretin amyloidosis
Neuroprotection

ABSTRACT

Transthyretin (ATTR) amyloidosis is a progressive and life-threatening neurodegenerative disease caused by aggregation of the plasma transport protein, transthyretin, for which treatment is rare and cure unavailable. *Centella asiatica* is a small edible herb with a long history of neurological application in ethnomedicine. This work investigated whether hydrophilic extract of *C. asiatica* (CAB) could suppress the toxic effects of transthyretin amyloid aggregate (TTRa) in cell model derived from the same *in vivo* target. TTRa was prepared via thermal-induced aggregation. Chemical cross-linking and Tricine-SDS-PAGE, Thioflavin-T fluorescence, and TEM analyses confirmed that TTRa matched the profile of TTRL55P nonfibrillar amyloid aggregates. PrestoBlue cell viability assay revealed that exposure of IMR-32 human neuroblastoma cells to TTRa (2–8 μM) resulted in significant cytotoxicity. Conversely, exposure of IMR-32 cells to CAB did not adversely affect their viability. In addition, when IMR-32 cells were co-treated with TTRa and varied concentrations of CAB, the toxic effect of TTRa was significantly ($p < 0.01$) inhibited dose-dependently. The extract was found to possess potent radical scavenging effects, and quantitative RP-HPLC analysis showed that asiaticoside and phenolics were its main components. The cyto-protective effect against TTRa, antioxidant property, and good safety profile collectively suggest that CAB could be applied in the development of nutraceuticals or therapeutics against trans-thyretin amyloidosis.

1. Introduction

Transthyretin amyloidosis is a spectrum of progressive, debilitating and life-threatening disorders associated with the deposition of misfolded and/or aggregated transthyretin in the body. Transthyretin (ATTR) amyloidosis affects several tissues and organs including the peripheral nervous system, lungs, eyes, gut, heart, and kidneys [1]. Without effective therapeutic intervention, the average life-span of ATTR amyloidosis patient is often less than eleven years from the onset of clinical manifestations [2]. Current treatment options include orthotopic liver transplantation, which is associated with high risk of complications, as well as extremely costly disease modifying agents, namely Tafamidis, Patisiran and Inotersen. These therapeutics are not accessible to many and still pose long term

* Corresponding author.
E-mail addresses: fredrickeze10@gmail.com (F.N. Eze), porntip.p@psu.ac.th (P. Prapunpoj).

https://doi.org/10.1016/j.heliyon.2024.e39159
Received 14 December 2023; Received in revised form 8 October 2024; Accepted 8 October 2024
Available online 9 October 2024
2405-8440/© 2024 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

safety concerns. Thus, the search for novel, effective, safe and affordable therapeutic alternatives remains a research imperative.

Transthyretin (TTR) is a homotetrameric protein of hepatic origin and mainly present in human serum where it mediates the transport of thyroxine and holo-RBP. Biophysical evidence had convincingly shown that the most crucial event in the pathophysiology of ATTR amyloidosis is the dissociation of the native tetramer into misfolded monomers. Subsequently, the misfolded monomers misassemble as ATTR aggregates and are deposited in several tissues leading to organ dysfunction. Deposition of wild type or mutant ATTR aggregates in cardiac tissues leads to ATTR cardiomyopathy which causes congestive heart failure and death. Deposition of amyloidogenic mutant TTR in autonomic and peripheral nerves leads to autonomic and peripheral neuropathy (ATTR polyneuropathy). ATTR amyloid deposition also affects other tissues including the gut, eyes, lungs, kidneys [3]. As seen in carpal tunnel syndrome, tissue damage ensues upon the accumulation of large amounts of fibrillar deposits. Nonetheless, growing lines of evidence strongly indicate that the major damage in ATTR amyloidosis precedes the accumulation of fibrillar species and is mainly perpetrated by small, diffusible, amorphous, non-fibrillar oligomers [4,5]. This is supported by reports of ATTR oligomer-induced neurotoxicity in cell models, such as neuroblastoma or Schwannoma cells [6], and animal models such as *C. elegans* or Drosophila [7,8]. Preventing the toxic effects of the ATTR oligomers presents an attractive therapeutic approach to ameliorate or delay disease progression.

Although tremendous progress had been achieved in the treatment of ATTR amyloidosis, current therapeutic options are still very limited and rare. Liver transplantation is still the predominant treatment for hereditary transthyretin polyneuropathy. The highly invasive nature, safety issues and the fact that not all patients are suitable for this procedure are limitations associate with liver transplantation. On the other hand, pharmacotherapeutic interventions are not only highly expensive, such as Tafamidis (USD 225,000), Inotersen and Patisiran (USD 450,000), but also raise long term safety concerns [9]. Thus, the use of various botanicals have been suggested as potentially viable therapeutic alternatives. This notion is bolstered by increasing evidence demonstrating the inhibition of native TTR tetramer dissociation by natural compounds such resveratrol, quercetin, curcumin, EGCG, amongst others [10]. Also many plant extracts have demonstrated remarkable ability to suppress TTR amyloidogenesis, including *Bacopa monnieri*, *Juglans mandshurica* var *cordiformis*, Green tea, and *Centella asiatica* [11–14].

C. asiatica is a small succulent edible plant widely consumed in Asia and several parts of the world. It has a rich ethnopharmacological history. For several millennia it was used in Ayurveda as a nootropic and for the mitigation of cognitive impairment, boosting memory, and wound healing. *C asiatica* has demonstrated potent neuroprotective property in diverse preclinical models. In Tg2576 mice, a model for Alzheimer's disease that accumulates amyloid-beta, *C. asiatica* water extract (CAW) improved learning and memory [15]. It was later revealed that the neuroprotective effect of CAW against amyloid-β-induced cell death, alteration in Tau expression and phosphorylation in MC65 and SH-SY5Y neuronal cells was due to caffeoylquinic acids [16,17]. Also, it was shown that CAW promoted mitochondrial biogenesis, potentiated intracellular antioxidant response and normalized calcium homeostasis, which together mediated its neuroprotective function against amyloid-β toxicity [18]. The extract also reduced the effects of rotenone-induced parkinsonism in Zebrafish [19] and rats [20]. And previously, it was revealed in a cell-free mechanistic study that *C. asiatica* hydrophilic fraction inhibited the dissociation of native TTR into amyloid competent species [14]. Nonetheless, the neuroprotective effect of *C. asiatica* against amyloidogenic TTR in cell models is yet to be investigated. The objective of this study was to determine whether *C. asiatica* could counter the toxic effects of exogenous amyloidogenic variant TTRL55P aggregates in IMR-32 human neuroblastoma cells. IMR-32 neuroblastoma cells is a well-established cell model for studying the inhibitory effects of compounds on ATTR amyloidosis [21] while TTRL55P is the most pathogenic variant causing the disease [22]. The findings from this work could have beneficial implications in the management of ATTR polyneuropathy and other amyloid-related diseases.

2. Materials and methods

2.1. Expression and purification of recombinant TTRL55P

Recombinant TTRL55P was produced using a *Pichia pastoris* expression system as described earlier [13,23]. TTRL55P was purified from the concentrated culture supernatant using preparative discontinuous native-PAGE performed on BIO-RAD Model 491 Prep Cell, Bio-Rad Laboratories (Hercules, CA, USA). Protein solutions were collected as fractions in test tubes. Silver staining was used to determine fractions which contained only TTRL55P. These fractions were subsequently combined and concentrated. The concentration of purified TTRL55P was determined by Bradford assay using bovine serum albumin as standard [24]. Purified TTRL55P was stored at −20 °C for future use.

2.2. Preparation of amyloid TTRL55P aggregates (TTRa)

TTRa was prepared using a thermal denaturation method previously described by Ref. [25]. Briefly, soluble TTRa was obtained by incubation of native L55PTTR solution (1 μg/μL in PBS buffer pH 7.4) at 80 °C for 4 h. Formation of transthyretin aggregates was confirmed via glutaraldehyde chemical cross-linking assay and Tricine-SDS-PAGE.

2.3. Structural and morphological characterization of TTRa

2.3.1. Chemical cross-linking

The quaternary structure of TTRL55P after thermal-induced aggregation was determined by chemical cross-linking using glutaraldehyde followed by Tricine-SDS-PAGE. Aliquot of the protein solution was diluted with 100 mM phosphate buffer, pH 7.4. Glutaraldehyde was added to the protein solution to a final concentration of 2.5 %. After 4 min of cross-linking, the reaction was

stopped by 7 % $NaBH_4$ in 0.1 M NaOH. SDS sample buffer was immediately added to the mixture (final SDS concentration of 2 %) and boiled for 10 min before electrophoresis on 10 % Tricine-SDS-PAGE gel. Protein bands were detected using Coomassie brilliant blue staining.

2.3.2. Thioflavin-T assay

The presence of transthyretin prefibrillar oligomeric species or aggregates in the protein solution subjected to aggregation was determined by thioflavin-T assay performed according to previous report [26]. Aliquot (3 μL) of transthyretin solution was added to 450 μL of 50 mM glycine-NaOH buffer, pH 9 containing 25 μM thioflavin-T dye and briefly mixed by using a vortexed mixer. Fluorescence emission was recorded at 485 nm upon excitation at 450 nm using JASCO-FP-8200 (Easton, USA) spectrophotometer.

2.3.3. Transmission electron microscopy (TEM)

TEM analysis of TTRa was performed as previously described [27]. The protein solution was diluted with ultrapure water (1:100), and 10 μL of the diluted solution was spotted onto a TEM grid for 2 min. Excess protein solution was removed by touching the edge of the grid with a cut filter paper. Thereafter, the grid was rinsed twice with ultrapure water (10 μL each time) followed by staining with uranyl acetate for 3 min. Excess uranyl acetate was removed and the grid was left to dry at room temperature in the dark. Electron micrographs were obtained using JEOL JEM-2100 Plus electron microscope (JEOL Ltd., Tokyo, Japan) at 100 kV.

2.4. Preparation of hydrophilic fraction of C. asiatica (CAB)

Fresh *C. asiatica* was obtained locally in Hat-Yai, Thailand and CAB was prepared as previously described [14].

2.5. Quantitative RP-HPLC analysis of CAB

CAB powder was dissolved in methanol and filtered through a 0.22 μm nylon membrane before analysis. The following phenolic and terpenoid compounds were quantified in CAB: gallic acid, catechin, quercetin, asiaticoside, madecassic acid and asiatic acid based on a previous report [28]. Quantitative HPLC analysis was performed on Hewlett-Packard 1100 series high-performance liquid chromatography (HPLC) system (Palo Alto, USA) equipped with a diode array detector and autosampler. Separation was achieved with an Agilent Hypersil 5 ODS 250 × 5 mm column at 27 °C. The mobile phase was composed of eluent A (0.3 % phosphoric acid in Milli-Q water) and eluent B (100 % acetonitrile). The gradient elution profile was 0 min, 20 % B; 15 min, 45 % B, 35 min, 60 % B; 37 min, 80 % B; 40 min, 20 % B. The flow rate was set at 0.9 mL/min over a 55 min run time including 15 min for re-equilibration. The sample injection volume was 10 μL and detection was at a wavelength of 210 nm for all compounds. The linear equations were $Y = 44.38215X+223.66131$ ($r2 = 0.99900$), $Y = 69.97551X+1.74832$ ($r2 = 0.9965$), $Y = 32.43422 + 121.67322$ ($r2 = 0.99970$), $Y = 1.88648X+24.2944$ ($r2 = 0.99902$), $Y = 4.63774X+32.25217$ ($r2 = 0.99962$), $Y = 5.06898X+32.78553$ ($r2 = 0.99969$) for gallic acid, catechin, quercetin, asiaticoside, madecassic acid, and asiatic acid, respectively.

2.6. Antioxidant activity of CAB

2.6.1. ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) radical scavenging activity

The *anti*-$ABTS^+$ activity of CAB was determined as previously reported [29]. The $ABTS^+$ stock solution was generated from combining an equal volume of 7 mM ABTS [2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] and 2.45 mM potassium persulfate followed by incubation for 12–16 h in the dark. The solution of the radical reagent was obtained by diluting the stock solution to an absorbance of 0.70 ± 0.01 at 734 nm. Subsequently, the assay was performed by adding the $ABTS^+$ reagent (270 μL) into 96-well plate followed by 30 μL of CAB solution (5–100 μL). Ascorbic acid was used as a positive control. Absorbance of the samples was read at 734 nm after incubating for 6 min. The percentage inhibition of $ABTS^+$ was calculated as follows:

Inhibition (%) = [(OD control - OD sample) / OD control] * 100

The IC_{50} values (i.e., the concentration of CAB or ascorbic acid capable of scavenging 50 percent of the radical species) were obtained from plots of inhibition (%) vs. concentration using GraphPad prism software ver. 7, GraphPad Software (San Diego, CA, USA).

2.6.2. Hydroxyl radical scavenging

The hydroxyl radical scavenging activity of CAB was performed according to Ref. [30]. CAB solution (150 μL, 5–100 μg/mL) was mixed with 1.5 mM $FeSO_4$ (25 μL). Salicylic acid (10 μL of 10 mM) dissolved in ethanol was added to the reaction mixture followed by 6 mM H_2O_2 (15 μL). After incubation at 37 °C for 1 h, the absorbance was read at 520 nm. The radical scavenging activity was calculated as follows:

Inhibition (%) = {1 – [(Ai – Aii) / Aiii]} * 100

Where, Ai, Aii and Aii represent the absorbance of the sample, blank and negative control, respectively. The IC_{50} values were obtained as described above in ABTS assay.

2.6.3. Superoxide anion radical scavenging

Briefly, 2.52 mM nitroblue tetrazolium chloride (50 μL) 624 mM NADH (50 μL) were mixed to generate the superoxide anion. CAB solution (50 μL; 5–100 μg/mL) was added to the radical solution. To initiate the reaction, 120 μg/mL of phenazine methosulfate solution (50 μL) was added to the reaction mixture. After 5 min of incubation at room temperature, absorbance of the solutions was recorded at 560 nm [31]. The radical scavenging activity was calculated as follows:

Inhibition (%) = [(OD control-OD sample)/OD control] * 100

The IC_{50} values were obtained as described above in ABTS assay.

2.6.4. Hydrogen peroxide scavenging

The H_2O_2 scavenging activity of CAB was carried out by mixing 40 mM H_2O_2 solution (0.6 mL) constituted in phosphate buffer, pH 7.4 with CAB solution (5–100 μg/mL). The reaction mixture was incubated at room temperature for 10 min and the absorbance was read at 230 nm. The percentage H_2O_2 scavenging activity of CAB was calculated thus:

Inhibition (%) = [(OD control – OD sample)/ OD control] * 100

Where, control was the solution with only H_2O_2, and sample was the solution of H_2O_2 with CAB. The IC_{50} values were obtained as described above in ABTS assay.

2.6.5. Nitic oxide radical (NO) scavenging

The anti-NO· activity of CAB was performed as previously described [32]. Briefly, sodium nitroprusside prepared in phosphate buffered saline, pH 7.4 was mixed with CAB (5–100 μg/mL). The mixture was incubated at room temperature for 2.5 h. Thereafter 1.0 mL of premixed Griess reagent was added to the reaction mixture. Absorbance was read at 540 nm and the percentage NO· inhibition was calculated thusly:

Inhibition (%) = [(Ai – Aii)/ Ai] * 100

Where, Ai was the absorbance before the reaction, and Aii was the absorbance after the reaction with Griess reagent had occurred [32]. Decrease in absorbance was an indication of NO· scavenging effect. The IC_{50} values were obtained as described above in ABTS assay.

2.7. Cell culture

IMR-32 human neuroblastoma cells were obtained from American Type Culture Collection (ATCC, USA) and nurtured in RPMI 1640 medium supplemented with 10 % fetal bovine serum. The cells were maintained at 37 °C in a humid CO_2 incubator. To determine the impact of CAB on neuronal cell viability, the cells were treated with CAB at concentrations that ranged from 50 to 1600 μg/mL for 48 h. To determine the effect of aggregated TTRa on cell viability, the cells were challenged with TTRa (4 and 8 μM) for 48 h. To ascertain the neuroprotective effect of CAB on IMR-32 cells, TTRa at a concentration capable of inducing cytotoxicity was used as a stressor. The TTRa was pre-incubated with different concentrations of CAB. The IMR-32 cells were then subjected to TTRa with or without CAB for 48 h. IMR-32 human neuroblastoma cells seeded at 5000 cells/well and grown to about 80 % confluence were used for these assays.

2.8. Cell viability assay

IMR-32 neuroblastoma cell viability was measured using PrestoBlue™ Cell Viability Reagent from Life Technologies Corporation (Oregon, USA) according to the manufacturer's instructions. Room temperature pre-warmed cell viability reagent (11 μL) was added to IMR-32 cells in culture medium (100 μL). Sodium dodecyl sulfate (3 %, 50 μL) was added into each well and the plate was incubated for 90 min at 37 °C in the cell culture incubator in the dark. Thereafter, fluorescence intensity was recorded using Synergy HT microplate reader (BioTek Instruments, Winooski, USA) at an excitation wavelength of 530/25 nm and emission wavelength of 590/35 nm. Cell viability was obtained from:

(Mean fluorescence intensity of treatment / Mean fluorescence intensity of control) * 100

Cell viability experiments were performed in triplicates and every experiment was repeated at least twice.

2.9. Statistical analysis

Statistical analysis was performed using one-way ANOVA using GraphPad Prism 7 for Microsoft windows (GraphPad Software, San Diego CA, USA). Tukey post-hoc test was used for multiple comparisons of means. Statistical significance was defined as $p < 0.001$, $p < 0.01$ or $p < 0.05$.

3. Results and discussion

Transthyretin amyloidoses is characterized by destruction of peripheral and autonomic nerves (ATTR polyneuropathy) as well as

dysfunction in other organs such as the gut, eyes, lungs, heart, and kidneys [33]. *Centella asiatica* is known to modulate the adverse cellular effects of amyloidogenic proteins/peptides associated with many neurodegenerative diseases [16,19,34]. This work presents the first direct evidence of the cytoprotective role of *C. asiatica* against TTRL55P amyloid aggregates (TTRa) – induced toxicity in IMR-32 human neuroblastoma cells. IMR-32 cells were exposed to TTRa amounts within the confines of physiological concentrations (pre-aggregation) and very similar to previous reports known to exert cytotoxicity in various relevant cell lines [6,21,35]. Exposure to TTRa caused substantial cell death in IMR-32 cells; however, upon cotreatment with CAB, the adverse effect induced by TTRa was mitigated.

3.1. Morphological characterization of transthyretin amyloid species (TTRa)

Native TTRL55P was purified by preparative discontinuous native-PAGE as previously reported [13]. In order to induce aggregation, the protein sample in phosphate buffered saline, pH 7.4 was subjected to thermal denaturation [25]. Although TTRL55P can form aggregates under physiological pH and temperature, in our experience this requires days. The thermal induction ensured that protein aggregation was expedited from days to only few hours. Subsequently, quaternary structural transformation of the protein samples before and after thermal denaturation was evaluated using Tricine-SDS-PAGE of the protein solutions. Prior to the gel electrophoresis, cross-linking the protein samples with glutaraldehyde was performed. This was necessary to preserve the quaternary structure of the proteins in solution. It is important to stress that glutaraldehyde cross-linking followed by Tricine-SDS-PAGE is a well-established approach for elucidating protein quaternary structure [36–38]. Native human TTR in its tetrameric form has a molecular weight of 55 kDa. We observed that the protein band of the cross-linked native TTRL55P (Lane 3, Fig. 1A) was located around 53–76 kDa, which is consistent with the expected molecular weight of the native tetramer [36]. This indicated that the prepared TTRL55P was actually in its native quaternary form. In contrast, TTRL55P subjected to thermal denaturation featured protein bands that were larger than 220 kDa (Lane 4, Fig. 1A). In fact, the protein species barely entered the resolving and stacking layers of the gel (Fig. S1), indicating that the proteins were in aggregated form (TTRa). To verify whether the protein aggregates featured in Lane 4 (Fig. 1A) i.e., TTRa, were actually in the amyloidogenic pathway, thioflavin-T assay was performed. Thioflavin-T is a benzothiazole dye with a strong affinity for cross-β sheet-rich structures which are predominant in amyloid proteins [39]. When bound to amyloid aggregates or fibrils, thioflavin-T dye produces enhanced fluorescence intensity. On the other hand, when free in solution or bound to native proteins, the fluorescence quantum yield from the dye is unremarkable. Based on the data obtained in this study (Fig. 1B), there was no marked change in the thioflavin-T fluorescence intensity of native TTRL55P (nTTR) relative to the buffer (noTTR). In contrast, the TTRa sample produced a significant increase in thioflavin-T fluorescence intensity compared to both the native protein and buffer solution ($p < 0.001$), confirming the presence of amyloid species in TTRa.

It was important to determine the actual morphology of TTRa. This is because thioflavin-T fluorescence assay is incapable of discriminating between mature fibrillar and amorphous non-fibrillar amyloid species, and the preponderance of evidence pointing to the fact that cytotoxicity and pathogenicity is mainly due to the latter [40]. The morphology of the TTR species was examined by transmission electron microscopy (TEM) after negative staining. The electron micrographs the native proteins revealed that they were entirely soluble as evinced by the absence of any detectable oligomeric or aggregate species (Fig. 2A). Conversely, post-incubation, an abundance of non-fibrillar amorphous aggregate species could be seen on the micrographs (Fig. 2B). Considering the Tricine-SDS-PAGE profile, ThT fluorescence as well as the morphology of TTRa, it can be deduced that thermal treatment of native TTRL55P successfully converted the native proteins into amyloidogenic aggregates.

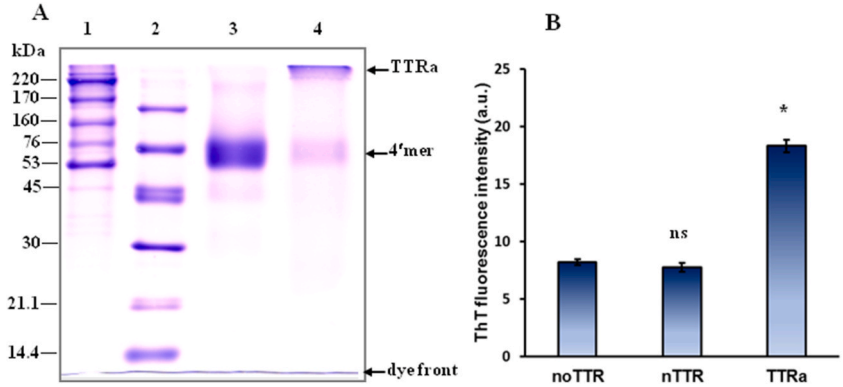


Fig. 1. (A) Tricine-SDS-PAGE image of TTRL55P after glutaraldehyde cross-linking. Lanes 1 and 2 represent high and low molecular weight protein markers, respectively; Lanes 3 and 4 represent TTRL55P before and after incubation at 80 °C for 4 h, respectively. TTRa and 4′mer represent aggregated and tetrameric forms of TTRL55P, respectively (for the full-length gel image, see Fig. S1). (B) Thioflavin-T fluorescence of native TTRL55P (nTTR) and TTRL55P aggregates (TTRa). Asterisks represent statistical significance at $p < 0.001$ while ns denotes not significant, i.e., $p > 0.05$.

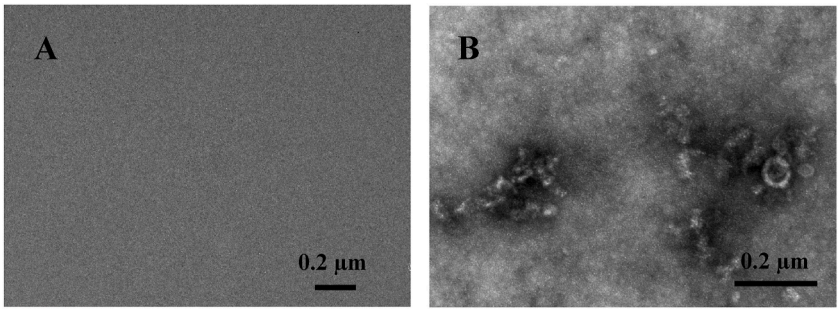


Fig. 2. Transmission electron micrographs of TTRL55P protein. (A) Native TTRL55P (nTTR) that has not been subjected to thermal denaturation. (B) TTRL55P aggregates (TTRa) prepared by thermal denaturation method.

3.2. Chemical characterization of CAB

Reverse-phase HPLC with ultraviolet detection at 210 nm was used to determine the content of CAB. HPLC chromatograms of CAB as well as that of six standard compounds, vis: gallic acid, catechin, asiaticoside, quercetin, madecassic acid and asiatic acid are presented in Fig. 3. The triterpenes, asiatic acid and madecassic acid were not detected in CAB. This is consistent with previous report by Soumyanath et al., who also noted the absence of the triterpenoids in the aqueous extracts of *C. asiatica* [15].

Our quantitative HPLC analysis revealed that asiaticoside constituted 2.73 % of CAB while catechin, gallic acid and quercetin together accounted for less than 0.18 % (Table 1). The content of asiaticoside in CAB was almost three times the amount previously reported in the aqueous extract of *C. asiatica* (CAW) [17] but similar to that obtained under microwave-assisted extraction using methanol as solvent [41]. Several factors may account for this disparity in phytochemical content, such as growth conditions, genetic factors, plant collection and method of preparation. And as shown by Puttarak and Panichayupakaranant, there can still be substantial variation in the content of bioactive compounds in *C. asiatica* cultivated even within a narrow geographical region [42].

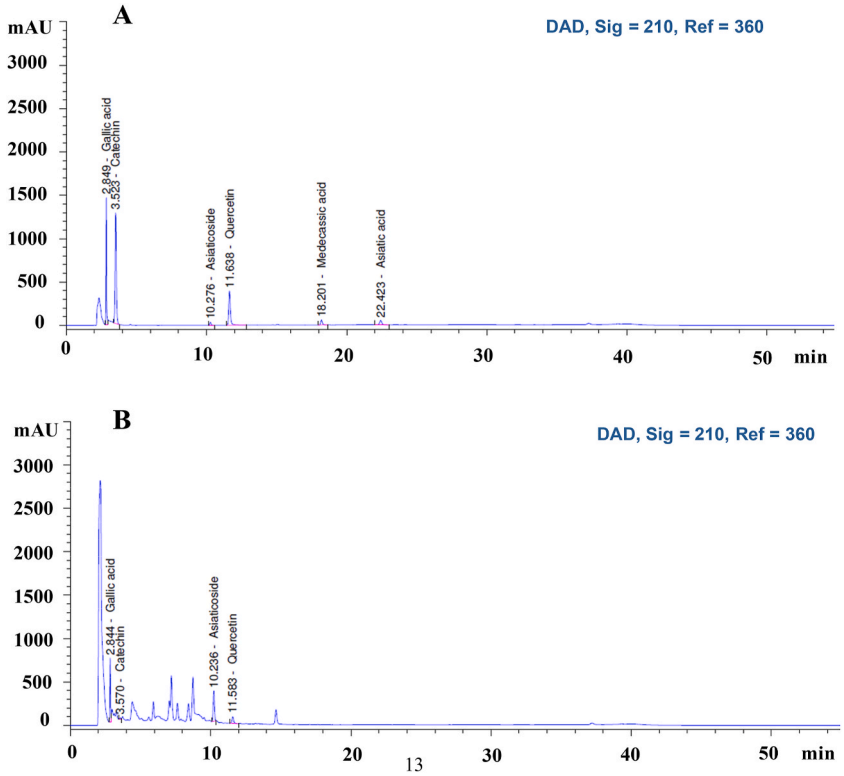


Fig. 3. HPLC chromatograms of standard (A) and CAB (B) detected at a wavelength of 210 nm.

Table 1
The percent composition of some phenolics and triterpenoids in CAB determined by RP-HPLC-DAD analysis at 210 nm.

Compound	CAB composition (%)
Catechin	2.20×10^{-03}
Gallic acid	0.13
Quercetin	0.04
Asiaticoside	2.73
Asiatic acid	Not detected
Madecassic acid	Not detected

3.3. *In vitro* antioxidant activity of CAB

In view of the widespread involvement of free radical species in normal physiological processes, and more importantly, in the development and exacerbation of neurodegeneration, evaluating the radical scavenging properties of botanicals for potential therapeutic application has become widespread. Although there have been several reports on the antioxidant effects of *C. asiatica*, variation in the origin of plant material, biotype or extraction methodology could impact the ultimate bioactive properties of the extract. The *in vitro* antioxidant properties of CAB was profiled using $ABTS^{+}$, superoxide anion, hydroxyl, nitric oxide and hydrogen peroxide radicals. A diversity of assays was adopted given the multiplicity of radical scavenging mechanisms employed by phytoconstituents. In all the radical scavenging assays the extract was evaluated at concentration of 5–100 μg/mL, while ascorbic acid was used as standard. In both CAB and ascorbic acid, plots of the radical scavenging activity against various concentrations indicated a concentration-dependent inhibition of all the radicals evaluated (Fig. S2). Plots of CAB or ascorbic acid inhibition (%) of the free radical versus concentration of the antioxidant were used to estimate the IC_{50} values. Results of the antioxidant activity of both CAB and ascorbic acid are presented in Table 2, with lower IC_{50} values denoting greater radical scavenging effect. The IC_{50} value is widely regarded as a more reliable yardstick for evaluating and comparing the antioxidant activity of bioactive compounds and extracts regardless of their source, mode of preparation, etc. [41,43,44].

The IC_{50} values presented in Table 2 are the means ± SD (standard deviation) of triplicates. The IC_{50} values were obtained from plots of radical scavenging activity or inhibition (%) vs. concentration as depicted in Fig. S2. Mean values within the same row but with different superscript letters (a, b, or c) are significantly different from each other ($p < 0.05$).

From Table 2 it can be seen that CAB had an IC_{50} of 36.55 μg/mL against $ABTS^{+}$ which is higher than that of vitamin C (14.32 μg/mL), but still quite low for a plant extract. The low IC_{50} of CAB against $ABTS^{+}$ is indicative of its potent antioxidant capacity. Other authors had previously observed that *C. asiatica* methanol extract, fraction enriched with terpenoids and fraction free of terpenoids displayed ABTS antiradical activity with IC_{50} values of 50.86 μg/mL, not active, and 94.75 μg/mL [43]. Similar ABTS radical scavenging effect was reported for *C. asiatica* extracts prepared using different solvents by heat reflux (IC_{50} 46.58–125.21 μg/mL), maceration (IC_{50} 39.52–129.16 μg/mL), microwave (IC_{50} 26.38–135.06 μg/mL), and ultrasonication (IC_{50} 40.34–153.44 μg/mL) [41]. It is clear from the IC_{50} values that CAB does not only compete favorably with *C. asiatica* extracts from the other studies reported in literature, but also outperforms most of them in terms of ABTS radical scavenging activity. The antioxidant activity of CAB can be credited to its rich content of phenolic compounds which could participate in single electron transfer, hydrogen atom transfer or radical chain-breaking reactions. ABTS is a synthetic reagent and one that is not encountered in physiological environments. Thus, reactive species that are more commonly found in normal biological milieu in cells and tissues and which have been implicated in various disease were also matched against CAB. The radical scavenging effect of CAB was high as revealed by the relatively low IC_{50} values against superoxide anion radical, hydroxyl radical, nitric oxide and hydrogen peroxide. The superoxide anion radical (O_2^{-}) is a reasonably strong oxidant. It can cause damage to proteins such as iron and sulfur group of enzymes, DNA as well as cells, leading to various disorders. Also, superoxide radical can be rapidly converted into even more toxic reactive oxygen species such as hydroxyl radicals [45]. CAB is capable of scavenging superoxide radical, but to a lesser extent relative to ascorbic acid (Fig. S2B and Table 2), which is a highly potent O_2^{-} scavenger [46]. Compared to other natural antioxidant agents reported in literature such as the triterpenoid ketone compound, friedelin (O_2^{-} IC_{50} 9.34 mg/mL) [47] and ethanol extract of *Stachytarpheta angustifolia* leaves (O_2^{-} IC_{50} 64.68 μg/mL) [46], CAB (O_2^{-} IC_{50} 29.33 μg/mL) could be seen to have markedly higher antioxidant activity. The antioxidant property of CAB was further confirmed by its ability to inhibit hydroxyl radical (˙OH). In this regard, CAB demonstrated superior inhibitory effect relative to ascorbic acid (Fig. S2C), with ˙OH IC_{50} value of 68.25 μg/mL vs. 76.10 μg/mL for ascorbic acid (Table 2). Meanwhile, nitric oxide is an endogenous radical which is important for signal transduction processes and modulation of many other physiological

Table 2
Antioxidant properties of CAB.

Antioxidant activity	CAB IC_{50} [μg/mL]	Vit. C IC_{50} [μg/mL]
ABTS radical scavenging	$36.55^{a} \pm 1.78$	$14.32^{b} \pm 0.81$
Superoxide scavenging	$29.33^{b} \pm 2.19$	$7.47^{a} \pm 0.30$
Hydroxyl radical scavenging	$68.25^{a} \pm 5.02$	$76.10^{c} \pm 4.85$
Nitric oxide scavenging	$34.01^{c} \pm 1.22$	$22.84^{b} \pm 0.95$
Hydrogen peroxide scavenging	$25.43^{a} \pm 2.01$	$7.68^{b} \pm 0.53$

functions at low concentrations. However, higher concentrations of nitric oxide could induce reactions leading to protein oxidative damage and lipid peroxidation. This can be perilous to normal cellular functions. Results of nitric oxide scavenging activity indicated that CAB is efficacious at suppressing the level of this free radical in a concentration-dependent manner (Fig. S2D), exhibiting an IC_{50} value of 34.01 μg/mL. Similar observations were made by Arora et al., who noted that methanol extract and the triterpenoid free fraction of *C. asiatica* were capable of scavenging nitric oxide with IC_{50} values of 13.02 μg/mL and 34.46 μg/mL, respectively [43]. In addition, CAB also exhibited a considerable hydrogen peroxide scavenging activity (Fig. S2E) with IC_{50} value of 25.43 μg/mL. Under normal physiological conditions, endogenous hydrogen peroxide plays several valuable functions, especially when the level is properly modulated. Aberrant high levels of hydrogen peroxide leads to oxidative stress and, has been implicated in the development of many pathologies. Indeed, previous studies have shown that the cytotoxic effect of TTR aggregates is linked to the ability of the aberrant proteins to significantly increase hydrogen peroxide production, upend redox balance and induce oxidative stress in the cells [48]. Beyond their direct role as kinetic stabilizers, exogenous antioxidant compounds such as carvedilol, curcumin, resveratrol, EGCG amongst others have been shown to ameliorate the neurotoxic effects of amyloid transthyretin in several studies [49–51]; as such the potent radical scavenging activity of CAB is suggestive of its potential neuroprotective function.

3.4. Impact of CAB on IMR-32 human neuroblastoma cell viability

The effect of CAB on the viability of IMR-32 cells was evaluated using PrestoBlue Cell Viability assay. The PrestoBlue protocol is based on the reduction of its active ingredient-resazurin. Resazurin is a nontoxic, non-fluorescent and cell-permeable reagent. Upon its uptake into metabolically active viable cells, resazurin is immediately reduced via electron acceptance from cytochromes, NADH, NADPH, FADH, and FMNH into resorufin – a highly fluorescent compound. The fluorescence is proportional to the number of viable cells [52]. It was found that treatment of IMR-32 human neuroblastoma cells with CAB (50–800 μg/mL) did not induce any considerable cell death (Fig. 4A). The viability of the treated cells at the highest CAB concentration was 91.04 % which was not significantly different from the untreated control ($p > 0.01$). These results are actually not surprising given that *C. asiatica* has been consumed for several ages as food, beverage, spices, in folk medicine amongst others, and had been generally considered as safe. Nonetheless, determining its safety profile on the neuroblastoma cells was important because CAB, unlike the form of *C. asiatica* extract typically consumed as food and beverage, is far richer in the amount of phytoconstituents. In fact, similar to the results obtained in this study, Omar et al., previously observed that raw hydroethanolic extract of *C. asiatica* was safe on human mesenchymal stem cells *in vitro* up to a concentration of 1200 μg/mL. However, they also observed that the extract became highly cytotoxic at concentrations higher than 1200 μg/mL [53]. Thus, it could be conceivable that even though CAB might not be entirely free of the potential to elicit cytotoxicity at very high concentrations, at the concentrations tested in this study (i.e., up to 800 μg/mL) the extract was non-toxic to and compatible with IMR-32 human neuroblastoma cells.

3.5. Effect of TTRa on IMR-32 human neuroblastoma cell viability

In order to ascertain the potential of TTRa to induce cell damage, the impact of TTRa on the viability of IMR-32 cells was assessed using PrestoBlue cell viability assay. The cells were exposed to 2–8 μM of TTRa (pre-aggregation concentration of native tetramer) for 48 h. These TTR concentrations cover the range found in human serum (3.6–7.2 μM) [54]. As shown in Fig. 4B, there was a substantial decrease in the number of viable cells in the samples treated with TTRa relative to the untreated control ($p < 0.01$). And this decrease was in a dose-dependent manner. The viability of neuroblastoma cells decreased from 100 % (untreated) to 72.73, 44.76, and 32.87 % upon exposure to 2, 4, and 8 μM of TTRa, respectively. In other words, exposure of IMR-32 cells to TTRa for 48 h led to massive cell death, indicating that the amyloidogenic protein aggregates were indeed cytotoxic and detrimental to the cells. The observed toxic effect can be attributed to the nature as well as the morphology of the amyloid aggregates. TTRL55P is the most aggressive form of variant transthyretin involved in familial transthyretin amyloidosis. In an earlier analogous study by Koch, exogenous aggregated TTRL55P was shown to instigate toxic effect on human cardiomyocytes [25]. Meanwhile, in the highly regarded work by Sousa et al., the authors examined the toxic effect of TTRL55P at different stages in the amyloidogenic pathway, viz; soluble native proteins,

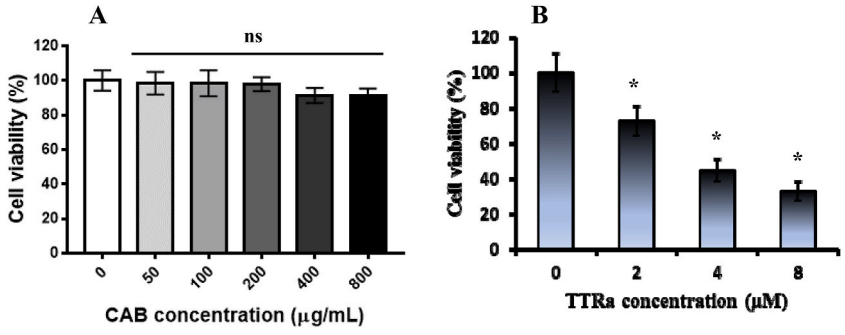


Fig. 4. Influence of CAB (A) and TTRo (B) on viability of IMR-32 human neuroblastoma cells. Asterisks represent statistical significance at $p < 0.05$ while ns denotes not significant, i.e., $p > 0.05$.

nonfibrillar aggregates, and mature fibrillar amyloid species of TTRL55P on Schwannoma cell line via caspase-3 activation assay. The authors observed that as opposed to the soluble native proteins and mature fibrillar species, only the nonfibrillar aggregates were actually toxic to the cells [55]. As deduced from the TEM analysis, the TTRL55P sample employed in the current study consisted of nonfibrillar aggregates. The substantial toxic effect exerted by TTRa against IMR-32 confirmed its viability as a neurotoxic agent, consistent with the aforementioned reports.

3.6. *Influence of CAB on TTRa – induced toxicity on IMR-32 human neuroblastoma cells*

In an attempt to determine whether CAB could counter the toxic effect induced by TTRa, the neuroblastoma cells were challenged with 4 μM TTRa. It is clear from the preceding subsection that at this concentration, TTRa was capable of eliciting substantial cytotoxicity. IMR-32 cells were also treated with both TTRa and CAB (100 and 200 μg/mL) for 48 h. The viability of TTRa-treated cells with or without CAB was ascertained. Fig. 5 shows that in the absence of CAB, TTRa-treated neuronal cells experienced significant decrease in viability relative to non-treated control cells ($p < 0.01$). Conversely, in the presence of CAB, the viability of the TTRa-treated cells was substantially higher compared to cells in the absence of CAB. The relative increase in viability of TTRa-treated cells by CAB reflected a concentration-dependence. This result indicated that CAB had a cytoprotective effect against TTRa-induced toxicity on IMR-32 neuroblastoma cells.

Although a complete picture of the precise molecular mechanisms underlying TTR aggregate mediated tissue damage is still emerging, what is undisputed is the fact that early events of this process is preceded by deposition of the amyloidogenic nonfibrillar TTR aggregates [55]. The involvement of oxidative stress occasioned by deposition of transthyretin amyloid species both at the cellular and tissue levels has been well documented [5,6,50,51,56]. Interestingly, oxidation has also been implicated in promoting the process of transthyretin aggregation [57,58], and thereby facilitate the adverse cellular effects triggered by the protein amyloid aggregates. This cross-talk between oxidation and protein aggregation highlights the potential role of antioxidant agents in the modulation of neuronal damage caused by accumulation of protein aggregates [51]. This was evinced in a previous study by Kugimiya et al., who demonstrated that by virtue of its antioxidant property, human serum albumin was able to effectively attenuate the formation of TTR amyloid species [59]. In plasma, albumin is not only the predominant protein, but also the major component responsible for plasma antioxidant property. Human mercaptoalbumin (reduced form, with anti-oxidative property) and human nonmercaptoalbumin (oxidized form) are the two forms of albumin in plasma. The authors reported that a significant loss in functional albumin (i.e., human mercaptoalbumin) was observed in plasma of FAP patients as the disease progressed compared to normal age-matched control. Also, with the loss of functional albumin, deposition of transthyretin was accelerated in analbuminemic (without albumin) transgenic rats with human mutant *TTR* gene compared to control rats wit albumin [59]. In an analogous work, Choi et al., found that antioxidant flavonoids such as quercetin, catechin, kaempferol, EGCG, luteolin, rutin, myricetin, etc., were capable of inhibiting neuronal damage induced by β-amyloid-beta peptide (a major component of the amyloid protein deposit present in Alzeimer's disease). According to the authors, the standard antioxidant compounds (Trolox and ascorbic acid) as well as the afore-mentioned flavonoids with potent antioxidant capacity (DPPH IC_{50} values $\leq$ 34.4 μM) inhibited Aβ peptide-induced damage in mouse cortical neuronal cells. In contrast, the flavonoid without antioxidant property failed to prevent neurotoxicity in the cells caused by Aβ peptide [60], underscoring the essential role of anti-oxidative of property of the agent in countering oxidative damage occasioned by the amyloid protein. In the present study, it is apparent on the basis of the data obtained that CAB possesses a strong antioxidant property as evidenced by its scavenging activity against many radical species. Taken together, the cytoprotective effect displayed by CAB against TTRa induced neuronal loss can be attributed to the direct effect of the extract in inhibiting the formation of cytotoxic aggregates as well as the antioxidant property of the extract. In essence, both of these effects are ascribable to the composition of CAB. CAB is rich in phenolic compounds such as gallic acid, catechin and quercetin. These compounds have been shown to inhibit aggregation of amyloidogenic proteins, including transthyretin [61,62]. For example, quercetin binds to the thyroxine binding sites of transthyretin tetramer and as a

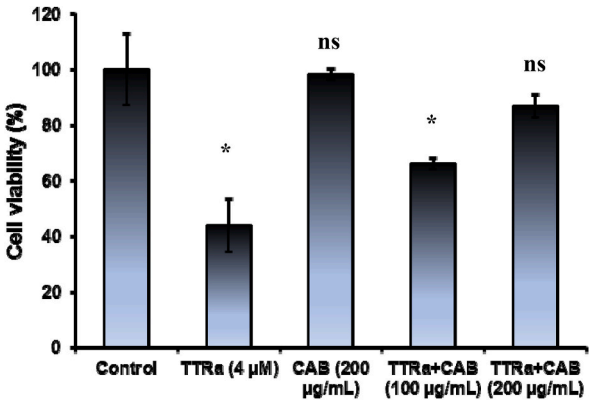


Fig. 5. Effect of CAB on TTRa-treated IMR-32 human neuroblastoma cells. Cell viability was ascertained relative to the untreated control. Asterisk represents statistical significance at $p < 0.01$ while ns indicates not significant ($p > 0.05$).

consequence improves the stability of the native protein. Within the binding pocket of the protein, the benzopyran ring of the flavonoid is oriented outwards facing the solvent, whereas the dihydroxyl phenyl ring is oriented towards the inner cavity of the protein. Both hydroxyl groups of the phenyl ring can form hydrogen bond with the hydroxyl group of Ser 117 residue. There is also an interaction between the hydroxyl group of the benzopyran ring and the O^{γ} atom of the Thr 119 residue [63]. These interactions facilitate ligand binding stabilization of the native protein. The enhancement in tetramer stability greatly suppresses the tendency of the native tetramer to dissociate and aggregate. In fact, Trivella et al. noted that quercetin is capable of inhibiting acid-induced aggregation of human TTRWT with an IC_{50} value of 13.34 μM and TTRV30M with and IC_{50} value of 13.34 μM [61]. Also, these compounds are well-known antioxidants, and their antioxidative property was reportedly vital in countering amyloid-induced neuronal damage caused by oxidative stress [64]. Interestingly, we found that asiaticoside, the only triterpenoid detected in CAB and a major component of the extract, was not capable of stabilizing the native protein tetramers (data not shown). However, it is equally germane to mention that other authors have found this triterpenoid to be a strong contributor to the antioxidant property of *C. asiatica* [65]. This seems to suggest that it is not just the individual compounds, but their combination, for example, as found in CAB that contributes to the observed beneficial neuroprotective effect. In fact, a similar notion had been previously presented by Chaudhary et al., in their study on the TTR amyloid fibril disrupting activity of Juglan plants. The authors noted that ethyl acetate and n-butanol fractions of *Juglan mandshurica* var. *cordiformis* fruit extract showed fibril disrupting activity; however, when the major compound in both fractions was isolated, it failed to display the observed effect [12].

The use of botanicals with antioxidant and neuroprotective properties such as CAB could be beneficial for countering ATTR amyloidosis. Although pharmacotherapeutics that stabilize TTR tetramer such as diflunisal had been shown to improve the quality of life in familial amyloid polyneuropathy patients by reducing the rate of neurological impairment [66], as a non-steroidal anti-inflammatory drug (NSAID), potential NSAID-associated increase in gastrointestinal bleeding risk and decrease in renal blood flow are serious concerns [67]. Tafamidis, another kinetic stabilizer, had been shown to improve outcomes in patients with cardiac failure and polyneuropathy; however, the associated benefits are only obtained at early stages of the disease even though in most cases successful diagnosis of the disease occur at a later stage. In addition, the drug is extremely expensive. In recognition of the fact that organ impairment caused by deposition of TTR amyloid species can be irreversible, early interventions including the use of drugs or supplements had been proposed [68]. *C. asiatica* continues to be consumed in many parts of the world without any obvious adverse effects. CAB is easy to prepare and could be further developed as an inexpensive nutraceutical or supplement to counter ATTR amyloidosis.

4. Conclusions

Hereditary transthyretin polyneuropathy is a rapidly progressive and devastating neurodegenerative condition. The main culprit behind this disorder is amyloidogenic mutant transthyretin, of which TTRL55P is the most pathogenic variant. In this work, we have demonstrated that hydrophilic extract from *C. asiatica* (CAB) is capable of preventing cell death caused by TTRL55P aggregates in IMR-32 neuroblastoma cells, thus providing the first direct evidence for the neuroprotective role of *C. asiatica* against amyloidogenic transthyretin-induced cytotoxicity. In addition to multiple previous studies both *in vitro* and *in vivo* highlighting the neuroprotective effects of *C. asiatica* in diseases, such as Alzheimer's and Parkinson's, the present preclinical investigation revealed that *C. asiatica* also holds potential for countering amyloid transthyretin-related neuropathies. These findings therefore supports further research of CAB on relevant animal models of transthyretin amyloidosis to delineate its efficacy, selectivity, bioavailability, and safety.

CRediT authorship contribution statement

Fredrick Nwude Eze: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Apinna Bunyapongpan:** Investigation, Formal analysis, Data curation. **Porntip Prapunpoj:** Visualization, Supervision, Project administration, Methodology, Formal analysis, Conceptualization.

Data availability

Data included in article/supp. material/referenced in article.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Porntip Prapunpoj reports equipment, drugs, or supplies was provided by the National Science, Research and Innovation Fund (NSRF). If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was financially supported by the National Research Council of Thailand, the National Science, Research and Innovation Fund (NSRF), and Prince of Songkla University (Grant No. SCI6405036S, SCI6601080c).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e39159.

References

[1] Y. Sekijima, Transthyretin (ATTR) amyloidosis: clinical spectrum, molecular pathogenesis and disease-modifying treatments, J. Neurol. Neurosurg. Psychiatry 86 (2015) 1036–1043, https://doi.org/10.1136/jnnp-2014-308724.

[2] Y. Parman, D. Adams, L. Obici, L. Galán, V. Guergueltcheva, O.B. Suhr, T. Coelho, European Network for TTR-FAP (ATTReuNET), Sixty years of transthyretin familial amyloid polyneuropathy (TTR-FAP) in Europe: where are we now? A European network approach to defining the epidemiology and management patterns for TTR-FAP, Curr. Opin. Neurol. 29 (Suppl 1) (2016) S3–S13, https://doi.org/10.1097/WCO.0000000000000288.

[3] A.P. Barreiros, P.R. Galle, G. Otto, Familial amyloid polyneuropathy, Dig. Dis. 31 (2013) 170–174, https://doi.org/10.1159/000347214.

[4] N. Reixach, S. Deechongkit, X. Jiang, J.W. Kelly, J.N. Buxbaum, Tissue damage in the amyloidoses: transthyretin monomers and nonnative oligomers are the major cytotoxic species in tissue culture, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 2817–2822, https://doi.org/10.1073/pnas.0400062101.

[5] M.M. Sousa, R. Fernandes, J.A. Palha, A. Taboada, P. Vieira, M.J. Saraiva, Evidence for early cytotoxic aggregates in transgenic mice for human transthyretin Leu55Pro, Am. J. Pathol. 161 (2002) 1935–1948, https://doi.org/10.1016/S0002-9440(10)64469-0.

[6] V.H. Fong, A. Vieira, Pro-oxidative effects of aggregated transthyretin in human Schwannoma cells, Neurotoxicology 39 (2013) 109–113, https://doi.org/10.1016/j.neuro.2013.08.013.

[7] R.B. Ibrahim, S.-Y. Yeh, K.-P. Lin, F. Ricardo, T.-Y. Yu, C.-C. Chan, J.-W. Tsai, Y.-T. Liu, Cellular secretion and cytotoxicity of transthyretin mutant proteins underlie late-onset amyloidosis and neurodegeneration, Cell. Mol. Life Sci. 77 (2020) 1421–1434, https://doi.org/10.1007/s00018-019-03357-1.

[8] K. Madhivanan, E.R. Greiner, M. Alves-Ferreira, D. Soriano-Castell, N. Rouzbeh, C.A. Aguirre, J.F. Paulsson, J. Chapman, X. Jiang, F.K. Ooi, C. Lemos, A. Dillin, V. Prahlad, J.W. Kelly, S.E. Encalada, Cellular clearance of circulating transthyretin decreases cell-nonautonomous proteotoxicity in Caenorhabditis elegans, Proc. Natl. Acad. Sci. USA 115 (2018) E7710–E7719, https://doi.org/10.1073/pnas.1801117115.

[9] N.M. Fine, Disease-modifying therapy for transthyretin amyloidosis: where to start? Where to stop? JACC (J. Am. Coll. Cardiol.): Case Reports 2 (2020) 2128–2130, https://doi.org/10.1016/j.jaccas.2020.10.004.

[10] L. Ciccone, N. Tonali, S. Nencetti, E. Orlandini, Natural compounds as inhibitors of transthyretin amyloidosis and neuroprotective agents: analysis of structural data for future drug design, J. Enzym. Inhib. Med. Chem. 35 (2020) 1145–1162, https://doi.org/10.1080/14756366.2020.1760262.

[11] F. aus dem Siepen, R. Bauer, M. Aurich, S.J. Buss, H. Steen, K. Altland, H.A. Katus, A.V. Kristen, Green tea extract as a treatment for patients with wild-type transthyretin amyloidosis: an observational study, Drug Des. Dev. Ther. 9 (2015) 6319–6325, https://doi.org/10.2147/DDDT.S96893.

[12] N. Chaudhary, R. Sasaki, T. Shuto, M. Watanabe, T. Kawahara, M.A. Suico, T. Yokoyama, M. Mizuguchi, H. Kai, H.P. Devkota, Transthyretin amyloid fibril disrupting activities of extracts and fractions from Juglans mandshurica maxim. Var. cordiformis (makino) kitam, Molecules 24 (2019), https://doi.org/10.3390/molecules24030500.

[13] F.N. Eze, K. Ingkaninan, P. Prapunpoj, Transthyretin anti-amyloidogenic and fibril disrupting activities of Bacopa monnieri (L.) wettst (brahmi) extract, Biomolecules 9 (2019), https://doi.org/10.3390/biom9120845.

[14] F.N. Eze, L. Leelawatwattana, P. Prapunpoj, Structural stabilization of human transthyretin by Centella asiatica (L.) urban extract: implications for TTR amyloidosis, Biomolecules 9 (2019) 128, https://doi.org/10.3390/biom9040128.

[15] A. Soumyanath, Y.-P. Zhong, E. Henson, T. Wadsworth, J. Bishop, B.G. Gold, J.F. Quinn, Centella asiatica extract improves behavioral deficits in a mouse model of Alzheimer's disease: investigation of a possible mechanism of action, Int. J. Alzheimer's Dis. 2012 (2012) 381974, https://doi.org/10.1155/2012/381974.

[16] N.E. Gray, J.A. Zweig, C. Murchison, M. Caruso, D.G. Matthews, C. Kawamoto, C.J. Harris, J.F. Quinn, A. Soumyanath, Centella asiatica attenuates Aβ-induced neurodegenerative spine loss and dendritic simplification, Neurosci. Lett. 646 (2017) 24–29, https://doi.org/10.1016/j.neulet.2017.02.072.

[17] N.E. Gray, J. Morré, J. Kelley, C.S. Maier, J.F. Stevens, J.F. Quinn, A. Soumyanath, Caffeoylquinic acids in Centella asiatica protect against β-amyloid toxicity, J Alzheimers Dis 40 (2014) 359–373, https://doi.org/10.3233/JAD-131913.

[18] N.E. Gray, H. Sampath, J.A. Zweig, J.F. Quinn, A. Soumyanath, Centella asiatica attenuates amyloid-β-induced oxidative stress and mitochondrial dysfunction, J Alzheimers Dis 45 (2015) 933–946, https://doi.org/10.3233/JAD-142217.

[19] H. Khotimah, M. Ali, S.B. Sumitro, M.A. Widodo, Decreasing α-synuclein aggregation by methanolic extract of Centella asiatica in zebrafish Parkinson's model, Asian Pac. J. Trop. Biomed. 5 (2015) 948–954, https://doi.org/10.1016/j.apjtb.2015.07.024.

[20] N. Teerapattarakan, H. Benya-aphikul, R. Tansawat, O. Wanakhachornkrai, M.H. Tantisira, R. Rodsiri, Neuroprotective effect of a standardized extract of Centella asiatica ECa233 in rotenone-induced parkinsonism rats, Phytomedicine 44 (2018) 65–73, https://doi.org/10.1016/j.phymed.2018.04.028.

[21] N. Reixach, S.L. Adamski-Werner, J.W. Kelly, J. Koziol, J.N. Buxbaum, Cell based screening of inhibitors of transthyretin aggregation, Biochem. Biophys. Res. Commun. 348 (2006) 889–897, https://doi.org/10.1016/j.bbrc.2006.07.109.

[22] H.A. Lashuel, C. Wurth, L. Woo, J.W. Kelly, The most pathogenic transthyretin variant, L55P, forms amyloid fibrils under acidic conditions and protofilaments under physiological conditions, Biochemistry 38 (1999) 13560–13573, https://doi.org/10.1021/bi991021c.

[23] P. Prapunpoj, L. Leelawatwatana, G. Schreiber, S.J. Richardson, Change in structure of the N-terminal region of transthyretin produces change in affinity of transthyretin to T4 and T3, FEBS J. 273 (2006) 4013–4023, https://doi.org/10.1111/j.1742-4658.2006.05404.x.

[24] O. Ernst, T. Zor, Linearization of the Bradford protein assay, J. Vis. Exp. (2010), https://doi.org/10.3791/1918.

[25] C. Koch, Characterizing the effect of transthyretin amyloid on the heart. https://open.bu.edu/handle/2144/16195, 2015. (Accessed 13 May 2021).

[26] Y. Misumi, Y. Ando, N.P. Gonçalves, M.J. Saraiva, Fibroblasts Endocytose and Degrade Transthyretin Aggregates in Transthyretin-Related Amyloidosis, vol. 93, Laboratory Investigation, 2013, pp. 911–920, https://doi.org/10.1038/labinvest.2013.83.

[27] S. Feng, X.-H. Song, C.-M. Zeng, Inhibition of amyloid fibrillation of lysozyme by phenolic compounds involves quinoprotein formation, FEBS Lett. 586 (2012) 3951–3955, https://doi.org/10.1016/j.febslet.2012.09.037.

[28] A. Alqahtani, W. Tongkao-on, K.M. Li, V. Razmovski-Naumovski, K. Chan, G.Q. Li, Seasonal variation of triterpenes and phenolic compounds in Australian Centella asiatica (L.) urb, Phytochem. Anal. 26 (2015) 436–443, https://doi.org/10.1002/pca.2578.

[29] D. Zhang, Y. Wang, H. Liu, Corn silk extract inhibit the formation of Nε-carboxymethyllysine by scavenging glyoxal/methyl glyoxal in a casein glucose-fatty acid model system, Food Chem. 309 (2020) 125708, https://doi.org/10.1016/j.foodchem.2019.125708.

[30] R. Fu, Y. Zhang, Y. Guo, F. Liu, F. Chen, Determination of phenolic contents and antioxidant activities of extracts of Jatropha curcas L. seed shell, a by-product, a new source of natural antioxidant, Ind. Crop. Prod. 58 (2014) 265–270, https://doi.org/10.1016/j.indcrop.2014.04.031.

[31] A. Sannasimuthu, V. Kumaresan, M. Pasupuleti, B.A. Paray, M.K. Al-Sadoon, J. Arockiaraj, Radical scavenging property of a novel peptide derived from C-terminal SOD domain of superoxide dismutase enzyme in Arthrospira platensis, Algal Res. 35 (2018) 519–529, https://doi.org/10.1016/j.algal.2018.09.028.

[32] S. Kumari, R. Elancheran, J. Kotoky, R. Devi, Rapid screening and identification of phenolic antioxidants in Hydrocotyle sibthorpioides Lam. by UPLC–ESI-MS/MS, Food Chem. 203 (2016) 521–529, https://doi.org/10.1016/j.foodchem.2016.02.101.

[33] M.D. Benson, J.N. Buxbaum, D.S. Eisenberg, G. Merlini, M.J.M. Saraiva, Y. Sekijima, J.D. Sipe, P. Westermark, Amyloid nomenclature 2020: update and recommendations by the International Society of Amyloidosis (ISA) nomenclature committee, Amyloid 27 (2020) 217–222, https://doi.org/10.1080/13506129.2020.1835263.

[34] C.-L. Chen, W.-H. Tsai, C.-J. Chen, T.-M. Pan, Centella asiatica extract protects against amyloid β1–40-induced neurotoxicity in neuronal cells by activating the antioxidative defence system, Journal of Traditional and Complementary Medicine 6 (2016) 362–369, https://doi.org/10.1016/j.jtcme.2015.07.002.

[35] P. Manral, N. Reixach, Amyloidogenic and non-amyloidogenic transthyretin variants interact differently with human cardiomyocytes: insights into early events of non-fibrillar tissue damage, Biosci. Rep. 35 (2015) e00172, https://doi.org/10.1042/BSR20140155.

[36] L.Z. Robinson, N. Reixach, Quantification of quaternary structure stability in aggregation-prone proteins under physiological conditions: the transthyretin case, Biochemistry 53 (2014) 6496–6510, https://doi.org/10.1021/bi500739q.

[37] S.C. Penchala, S. Connelly, Y. Wang, M.S. Park, L. Zhao, A. Baranczak, I. Rappley, H. Vogel, M. Liedtke, R.M. Witteles, E.T. Powers, N. Reixach, W.K. Chan, I. A. Wilson, J.W. Kelly, I.A. Graef, M.M. Alhamadsheh, AG10 inhibits amyloidogenesis and cellular toxicity of the familial amyloid cardiomyopathy-associated V122I transthyretin, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 9992–9997, https://doi.org/10.1073/pnas.1300761110.

[38] C.E. Tagoe, C.E. Tagoe, N. Reixach, C.E. Tagoe, N. Reixach, L. Friske, D. Mustra, D. French, G. Gallo, J.N. Buxbaum, In vivo stabilization of mutant human transthyretin in transgenic mice, Amyloid 14 (2007) 227–236, https://doi.org/10.1080/13506120701464396.

[39] K.G. Malmos, L.M. Blancas-Mejia, B. Weber, J. Buchner, M. Ramirez-Alvarado, H. Naiki, D. Otzen, ThT 101: a primer on the use of thioflavin T to investigate amyloid formation, Amyloid 24 (2017) 1–16, https://doi.org/10.1080/13506129.2017.1304905.

[40] K. Sörgjerd, T. Klingstedt, M. Lindgren, K. Kågedal, P. Hammarström, Prefibrillar transthyretin oligomers and cold stored native tetrameric transthyretin are cytotoxic in cell culture, Biochem. Biophys. Res. Commun. 377 (2008) 1072–1078, https://doi.org/10.1016/j.bbrc.2008.10.121.

[41] P. Mohapatra, A. Ray, S. Jena, S. Nayak, S. Mohanty, Influence of extraction methods and solvent system on the chemical composition and antioxidant activity of Centella asiatica L. leaves, Biocatal. Agric. Biotechnol. 33 (2021) 101971, https://doi.org/10.1016/j.bcab.2021.101971.

[42] P. Puttarak, P. Panichayupakaranant, Factors affecting the content of pentacyclic triterpenes in Centella asiatica raw materials, Pharmaceut. Biol. 50 (2012) 1508–1512, https://doi.org/10.3109/13880209.2012.685946.

[43] R. Arora, R. Kumar, A. Agarwal, K.H. Reeta, Y.K. Gupta, Comparison of three different extracts of Centella asiatica for anti-amnesic, antioxidant and anticholinergic activities: in vitro and in vivo study, Biomed. Pharmacother. 105 (2018) 1344–1352, https://doi.org/10.1016/j.biopha.2018.05.156.

[44] M. Lesjak, I. Beara, N. Simin, D. Pintać, T. Majkić, K. Bekvalac, D. Orčić, N. Mimica-Dukić, Antioxidant and anti-inflammatory activities of quercetin and its derivatives, J. Funct.Foods 40 (2018) 68–75, https://doi.org/10.1016/j.jff.2017.10.047.

[45] M. Takei, T. Kuda, M. Eda, A. Shikano, H. Takahashi, B. Kimura, Antioxidant and fermentation properties of aqueous solutions of dried algal products from the Boso Peninsula, Japan, Food Biosci. 19 (2017) 85–91, https://doi.org/10.1016/j.fbio.2017.06.006.

[46] F.M. Awah, P.N. Uzoegwu, J.O. Oyugi, J. Rutherford, P. Ifeonu, X.-J. Yao, K.R. Fowke, M.O. Eze, Free radical scavenging activity and immunomodulatory effect of *Stachytarpheta angustifolia* leaf extract, Food Chem. 119 (2010) 1409–1416, https://doi.org/10.1016/j.foodchem.2009.09.020.

[47] C. Sunil, V. Duraipandiyan, S. Ignacimuthu, N.A. Al-Dhabi, Antioxidant, free radical scavenging and liver protective effects of friedelin isolated from *Azima tetracantha* Lam. leaves, Food Chem. 139 (2013) 860–865, https://doi.org/10.1016/j.foodchem.2012.12.041.

[48] V.H. Fong, A. Vieira, Cytotoxic effects of transthyretin aggregates in an epidermoid cell line, PAT 84 (2017) 218–222, https://doi.org/10.1159/000466701.

[49] N. Ferreira, S.A.O. Santos, M.R.M. Domingues, M.J. Saraiva, M.R. Almeida, Dietary curcumin counteracts extracellular transthyretin deposition: insights on the mechanism of amyloid inhibition, Biochim. Biophys. Acta (BBA) - Mol. Basis Dis. 1832 (2013) 39–45, https://doi.org/10.1016/j.bbadis.2012.10.007.

[50] V.-H. Fong, A. Vieira, Transthyretin aggregates induce production of reactive nitrogen species, NDD 11 (2013) 42–48, https://doi.org/10.1159/000338153.

[51] B. Macedo, J. Magalhães, A.R. Batista, M.J. Saraiva, Carvedilol treatment reduces transthyretin deposition in a familial amyloidotic polyneuropathy mouse model, Pharmacol. Res. 62 (2010) 514–522, https://doi.org/10.1016/j.phrs.2010.08.001.

[52] M. Xu, D.J. McCanna, J.G. Sivak, Use of the viability reagent PrestoBlue in comparison with alamarBlue and MTT to assess the viability of human corneal epithelial cells, J. Pharmacol. Toxicol. Methods 71 (2015) 1–7, https://doi.org/10.1016/j.vascn.2014.11.003.

[53] N. Omar, Y. Lokanathan, Z.R. Mohd Razi, R. Bt Haji Idrus, The effects of Centella asiatica (L.) Urban on neural differentiation of human mesenchymal stem cells in vitro, BMC Compl. Alternative Med. 19 (2019) 167, https://doi.org/10.1186/s12906-019-2581-x.

[54] J. Buxbaum, I. Anan, O. Suhr, Serum transthyretin levels in Swedish TTR V30M carriers, Amyloid 17 (2010) 83–85, https://doi.org/10.3109/13506129.2010.483118.

[55] M.M. Sousa, I. Cardoso, R. Fernandes, A. Guimarães, M.J. Saraiva, Deposition of transthyretin in early stages of familial amyloidotic polyneuropathy : evidence for toxicity of nonfibrillar aggregates, Am. J. Pathol. 159 (2001) 1993.

[56] M.L. Fiszman, M. Di Egidio, K.C. Ricart, M.G. Repetto, L.N. Borodinsky, S.F. Llesuy, R.D. Saizar, P.L. Trigo, S. Riedstra, P.P. Costa, A.M. Villa, N. Katz, J. C. Lendoire, R.E.P. Sica, Evidence of oxidative stress in familial amyloidotic polyneuropathy type 1, Arch. Neurol. 60 (2003) 593–597, https://doi.org/10.1001/archneur.60.4.593.

[57] S. Saito, Y. Ando, M. Nakamura, M. Ueda, J. Kim, Y. Ishima, T. Akaike, M. Otagiri, Effect of nitric oxide in amyloid fibril formation on transthyretin-related amyloidosis, Biochemistry 44 (2005) 11122–11129, https://doi.org/10.1021/bi050327i.

[58] T. Nakanishi, M. Yoshioka, K. Moriuchi, D. Yamamoto, M. Tsuji, T. Takubo, S-sulfonation of transthyretin is an important trigger step in the formation of transthyretin-related amyloid fibril, Biochim. Biophys. Acta Protein Proteonomics 1804 (2010) 1449–1456, https://doi.org/10.1016/j.bbapap.2010.03.010.

[59] T. Kugimiya, H. Jono, S. Saito, T. Maruyama, D. Kadowaki, Y. Misumi, Y. Hoshii, M. Tasaki, Y. Su, M. Ueda, K. Obayashi, M. Shono, M. Otagiri, Y. Ando, Loss of Functional Albumin Triggers Acceleration of Transthyretin Amyloid Fibril Formation in Familial Amyloidotic Polyneuropathy, vol. 91, Laboratory Investigation, 2011, pp. 1219–1228, https://doi.org/10.1038/labinvest.2011.71.

[60] S.-M. Choi, B.C. Kim, Y.-H. Cho, K.-H. Choi, J. Chang, M.-S. Park, M.-K. Kim, K.-H. Cho, J.-K. Kim, Effects of flavonoid compounds on β-amyloid-peptide-induced neuronal death in cultured mouse cortical neurons, Chonnam Med J 50 (2014) 45–51, https://doi.org/10.4068/cmj.2014.50.2.45.

[61] D.B.B. Trivella, C.V. dos Reis, L.M.T.R. Lima, D. Foguel, I. Polikarpov, Flavonoid interactions with human transthyretin: combined structural and thermodynamic analysis, J. Struct. Biol. 180 (2012) 143–153, https://doi.org/10.1016/j.jsb.2012.07.008.

[62] N. Ferreira, A. Pereira-Henriques, M.R. Almeida, Transthyretin chemical chaperoning by flavonoids: structure–activity insights towards the design of potent amyloidosis inhibitors, Biochem Biophys Rep 3 (2015) 123–133, https://doi.org/10.1016/j.bbrep.2015.07.019.

[63] M. Cianci, C. Folli, F. Zonta, P. Florio, R. Berni, G. Zanotti, Structural evidence for asymmetric ligand binding to transthyretin, Acta Crystallogr D Biol Crystallogr 71 (2015) 1582–1592, https://doi.org/10.1107/S1399004715010585.

[64] Y.-L. Li, H. Guo, Y.-Q. Zhao, A.-F. Li, Y.-Q. Ren, J.-W. Zhang, Quercetin protects neuronal cells from oxidative stress and cognitive degradation induced by amyloid β-peptide treatment, Mol. Med. Rep. 16 (2017) 1573–1577, https://doi.org/10.3892/mmr.2017.6704.

[65] H.Y. Shin, H. Kim, S. Jung, E.-J. Jeong, K.-H. Lee, Y.-J. Bae, H.J. Suh, K.-I. Jang, K.-W. Yu, Interrelationship between secondary metabolites and antioxidant capacities of Centella asiatica using bivariate and multivariate correlation analyses, Applied Biological Chemistry 64 (2021) 82, https://doi.org/10.1186/s13765-021-00656-9.

[66] J.L. Berk, O.B. Suhr, L. Obici, Y. Sekijima, S.R. Zeldenrust, T. Yamashita, M.A. Heneghan, P.D. Gorevic, W.J. Litchy, J.F. Wiesman, E. Nordh, M. Corato, A. Lozza, A. Cortese, J. Robinson-Papp, T. Colton, D.V. Rybin, A.B. Bisbee, Y. Ando, S. Ikeda, D.C. Seldin, G. Merlini, M. Skinner, J.W. Kelly, P.J. Dyck, Diflunisal Trial Consortium, Repurposing diflunisal for familial amyloid polyneuropathy: a randomized clinical trial, JAMA 310 (2013) 2658–2667, https://doi.org/10.1001/jama.2013.283815.

[67] F.J. Tsai, L.T. Nelson, G.M. Kline, M. Jäger, J.L. Berk, Y. Sekijima, E.T. Powers, J.W. Kelly, Characterising diflunisal as a transthyretin kinetic stabilizer at relevant concentrations in human plasma using subunit exchange, Amyloid 30 (2023) 220–224, https://doi.org/10.1080/13506129.2022.2148094.

[68] H. Matsushita, A. Isoguchi, M. Okada, T. Masuda, Y. Misumi, C. Tsutsui, N. Yamaguchi, Y. Ichiki, J. Sawashita, M. Ueda, M. Mizuguchi, Y. Ando, Glavonoid, a possible supplement for prevention of ATTR amyloidosis, Heliyon 7 (2021) e08101, https://doi.org/10.1016/j.heliyon.2021.e08101.