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ORIGINAL ARTICLE

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Medicinal cannabis extracts are neuroprotective against $A\beta_{1-42}$-mediated toxicity in vitro

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Abstract

Background: Phytocannabinoids inhibit the aggregation and neurotoxicity of the neurotoxic Alzheimer's disease protein β amyloid (Aβ). We characterised the capacity of five proprietary medical cannabis extracts, heated and non-heated, with varying ratios of cannabidiol and Δ^9-tetrahydrocannabinol and their parent carboxylated compounds to protect against lipid peroxidation and Aβ-evoked neurotoxicity in PC12 cells.

Methods: Neuroprotection against lipid peroxidation and $A\beta_{1-42}$-induced cytotoxicity was assessed using the thiazolyl blue tetrazolium bromide (MTT) assay. Transmission electron microscopy was used to visualise phytocannabinoid effects on $A\beta_{1-42}$ aggregation and fluorescence microscopy.

Results: Tetrahydrocannabinol (THC)/tetrahydrocannabinolic acid (THCA)-predominant cannabis extracts demonstrated the most significant overall neuroprotection against $A\beta_{1-42}$-induced loss of PC12 cell viability. These protective effects were still significant after heating of extracts, while none of the extracts provided significant neuroprotection to lipid peroxidation via *t*bhp exposure. Modest inhibition of $A\beta_{1-42}$ aggregation was demonstrated only with the non-heated BC-401 cannabis extract, but overall, there was no clear correlation between effects on fibrils and conferral of neuroprotection.

Conclusions: These findings highlight the variable neuroprotective activity of cannabis extracts containing major phytocannabinoids THC/THCA and cannabidiol (CBD)/cannabidiolic acid (CBDA) on Aβ-evoked neurotoxicity and inhibition of amyloid β aggregation. This may inform the future use of medicinal cannabis formulations in the treatment of Alzheimer's disease and dementia.

KEYWORDS
amyloid β, cannabis, lipid peroxidation, neuroprotection, phytocannabinoids

Plain English Summary

With access to approved pathways increasing globally, medicinal cannabis formulations are increasingly being used to treat neuropsychiatric conditions. With laboratory and animal studies now showing benefits of cannabis and cannabinoids in treating neurodegenerative diseases, this study investigated whether whole cannabis extracts could protection neuronal cells against the toxicity of a signature Alzheimer's disease protein, beta (β) amyloid.

We found that cannabis extracts afforded neuronal cells protection against amyloid β toxicity, mostly in extracts with the major phytocannabinoid, Δ^9-THC, or its parent compound, Δ^9-THC-COOH. These results suggest that medicinal cannabis may have potential in the further treatment of dementia.

1 | INTRODUCTION

Cannabis sativa (*C. sativa*) has garnered considerable interest for its potential therapeutic benefits in a variety of disease contexts.[1–3] *Cannabis* is known for its rich biochemical diversity and is the source of hundreds of structurally diverse compounds, including various terpenes, flavonoids and phytocannabinoids (pCBs).[4] To date, more than 540 individual compounds have been extracted and isolated from cannabis, with more than 120 of these considered to be pCBs.[5] The classical phytocannabinoids are a group of C_{21} terpeno-phenolic compounds found in all major species of cannabis that share several common structural features, including a dibenzopyran ring and an alkyl side chain.[6,7] These pCBs also vary in their structure in many ways, including differences in alkyl side chain length, degree of aromatisation and the nature of the dibenzopyran B ring, whether open, as in cannabidiol (CBD), or closed, as in Δ^9-tetrahydrocannabinol (Δ^9-THC) (Figure 1). Moreover, it is currently believed that all phytocannabinoids originate as carboxylated precursors which then undergo decarboxylation to "neutral" forms in a predominantly non-enzymatic manner via exposure to light, heat and/or atmospheric oxygen during preparation and the storage process.[5,8] These structural changes account, at least in part, for the structural diversity of the known phytocannabinoids (Figure 2).

Phytocannabinoids such as CBD and Δ^9-THC have been shown to inhibit the neurotoxicity associated with β-amyloid (Aβ), the hallmark protein associated with Alzheimer's disease (AD).[2,9] These phytocannabinoids exert their effects via their actions at numerous receptors and pathways, including activation of PPARγ, CB_1 and CB_2 receptors, inhibition of caspase-3 and reductions in oxidative stress.[10–13] However, although there is a wealth of literature regarding the neuroprotective potential of specific phytocannabinoids, both in isolation and in combination, far less research has been conducted to determine the possible therapeutic benefits of whole botanical extracts in the context of AD and neurodegenerative diseases more generally.

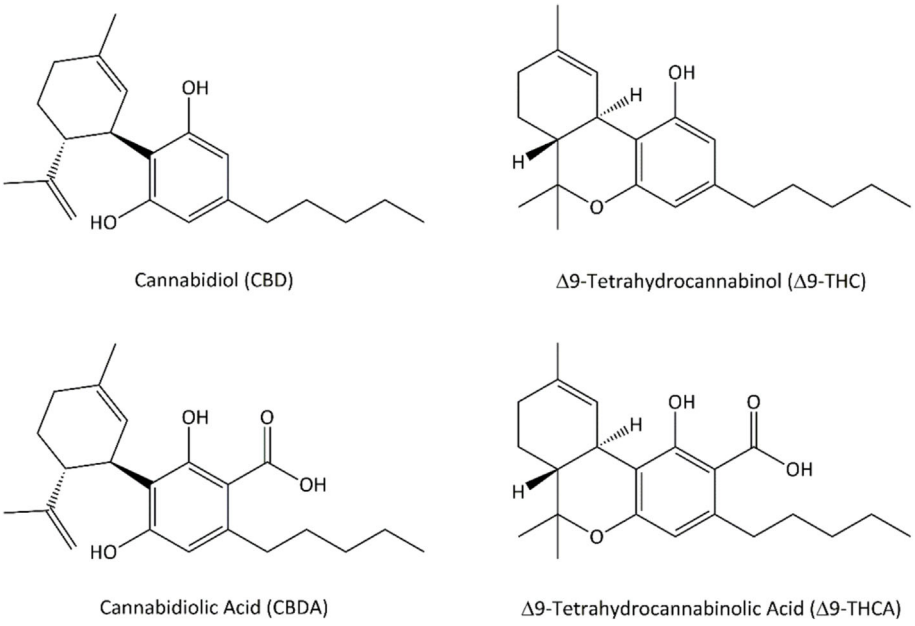


FIGURE 1 Structures of CBD and Δ^9-THC and their carboxylic acid precursors, CBDA and THCA. CBD, cannabidiol; CBDA, cannabidiolic acid; Δ^9-THC, Δ^9-tetrahydrocannabinol; Δ^9-THCA, Δ^9-tetrahydrocannabinolic acid.

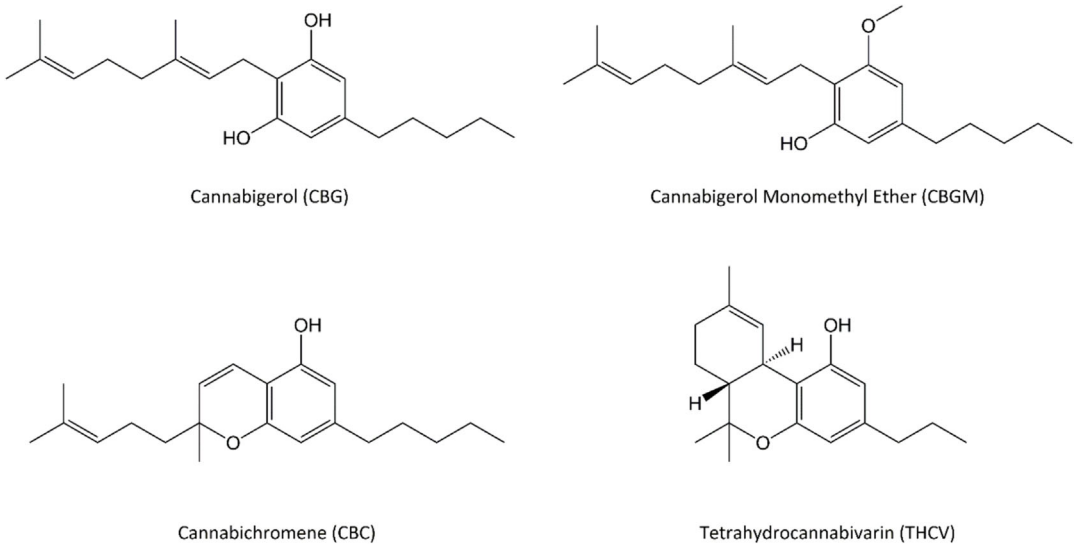
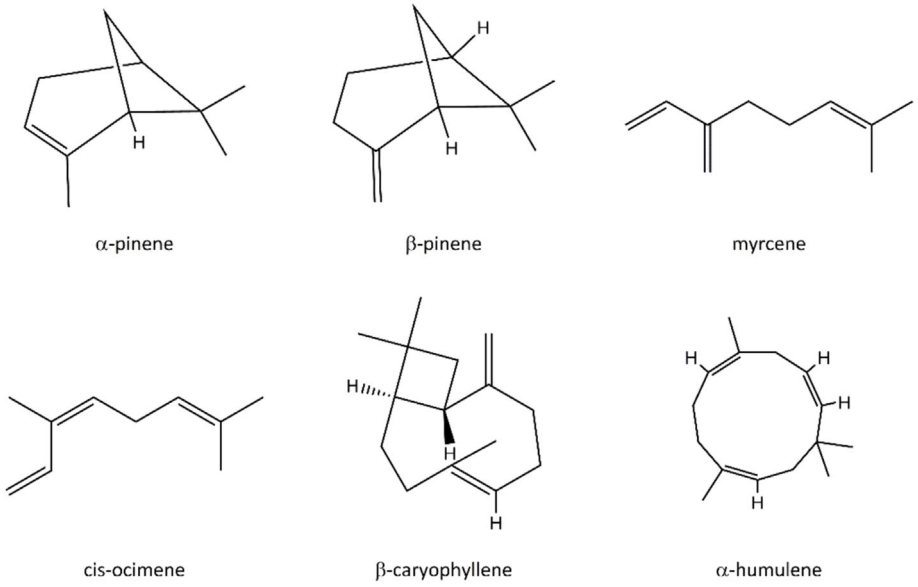


FIGURE 2 Structures of other phytocannabinoids present in the Bedrocan products used in this study, including the non-classical phytocannabinoids CBG, CBGM and CBC. CBG, cannabigerol; CBC, cannabichromene; CBGM, cannabigerol monomethyl ether; THCV, tetrahydrocannabivarin.

FIGURE 3 Structures of the primary terpenes found in each of the Bedrocan products used in this study.



While specific phytocannabinoids have been shown to possess considerable therapeutic potential in isolation, it has been suggested that these compounds may be more effective when used in combination with one another in the form of whole botanical extracts.[14,15] This concept is termed the "entourage effect" and refers to the manner in which combinations of phytocannabinoids, various cannabis terpenes and other compounds such as cannabis flavonoids work in concert with one another to provide a greater degree of protection than that afforded by the use of phytocannabinoids in isolation.[16,17] Interestingly, previous research in other neurological disease contexts has suggested that CBD-rich extracts may possess greater therapeutic potential than purified CBD, likely through the actions of this entourage effect.[18] Furthermore, selected terpenes found in cannabis (Figure 3) have been ascribed cannabimimetic properties with the ability to selectively enhance cannabinoid activity in vivo.[19] Whole botanical extracts have been ascribed neuroprotective capacity, with the ability to attenuate oxidative stress in neuronal cell lines.[20] Moreover, chemovars of various types have also been shown to reduce cytokine expression and alter Aβ processing in transgenic mouse models of AD, highlighting their therapeutic potential in the treatment of this disease.[21] Sativex, a cannabis extract containing both Δ^9-THC and CBD, has been

effective in the symptomatic treatment of other neurodegenerative diseases including spasticity control in patients with multiple sclerosis.[22] However, there is a considerable lack of data available on the general neuroprotective properties of whole botanical cannabis extracts, highlighting the need to identify and develop novel candidate chemovars.

Cannabis chemovars may be differentiated by their relative phytocannabinoid content, with type I chemovars characterised by the predominance of Δ^9-THC, type II containing both Δ^9-THC and CBD and type III being CBD-predominant.[23] Variations in the method of cultivation of medicinal cannabis chemovars are largely responsible for their diverse phytcoannabinoid and terpene profiles.[23] It has previously been shown that heat exposure in cannabis extracts results in altered cannabinoid content due to the thermo-chemical conversion of phytocannabinoid acids to their neutral bioactive forms; heating may also affect the pharmacokinetic and metabolic profile of these extracts.[24,25] Moreover, heating has been shown to significantly reduce the terpene content of cannabis, given their volatile and less stable nature.[26] However, very little research has been performed investigating the effects of heat exposure on the bioactivity of cannabis extracts in the context of neuroprotection.

In the present study, we characterised the protective capacity of extracts of five proprietary cannabis chemovars in an in vitro model of *tert*-butyl hydroperoxide (*t*bhp)–evoked oxidative damage and Aβ-induced neurotoxicity, as well as comparing the effect of heat exposure on the bioactivity of each extract. In addition, we compared the in silico binding profiles of the predominant phytocannabinoids in these medicinal cannabis formulations, Δ^9-THC and CBD and their carboxylated precursors tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA), with the Aβ protein. This study presents the first characterisation of these proprietary medicinal cannabis formulations in the context of Aβ-evoked neuronal cell damage and provides a comparative insight into the variable bioactivity of these botanical extracts.

2 | MATERIALS AND METHODS

This study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.[27]

2.1 | Materials and reagents

Cannabis chemovars used in this study were sourced from Bedrocan International (Veendam, Groningen, Netherlands) and are commercially available (see Section 2.2 for more product details). Human $A\beta_{1-42}$ protein was purchased from rPeptide (Bogart, GA, USA) with thiazolyl blue tetrazolium bromide (MTT), trypan blue, *tert*-butyl hydroperoxide (*t*bhp), Roswell Park Memorial Institute 1640 (RPMI-1640) medium, uranyl acetate, phosphate-buffered saline (PBS), non-essential amino acids (NEAA), penicillin/streptomycin, 1× Trypsin EDTA and foetal bovine serum (FBS) obtained from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was obtained from Chem-Supply (Gillman, SA, Australia).

2.2 | Cannabis extract and $A\beta_{1-42}$ preparation

Lyophilised human $A\beta_{1-42}$ was prepared by dissolving in 100% DMSO to a concentration of 3.8 mM. This stock solution was then diluted to 100 μM in sterile PBS, dispensed into aliquots and frozen at −80°C until required. Cannabis from five commercial chemovars arrived in either flos or granulate form and underwent ethanolic extraction prior to use in the study. Briefly, 1 g dry, macerated and powdered biomass was added per 10 ml ethanol and vortexed thoroughly before being placed on a platform roller for 30 min. This mixture was then centrifuged for 5 min and decanted to remove remaining biomass; the final solution was then filtered into aliquots using a 0.45-μm filter. The Δ^9-THC:CBD ratio varied between chemovars: Bedrocan® (BC-101, *sativa*), 22%:<1%; Bedrobinol® (BC-201, *sativa*), 13.5%:<1%; Bediol® (BC-301, *sativa*), 6.3%:8%; Bedica® (BC-401, *indica*), 14%:<1%; and Bedrolite® (BC-501, *sativa*), < 1%:9% (see Table 1); the full terpene profile for each chemovar is listed in Table 2 and is also available on the manufacturer's website (https://bedrocan.com/). To determine the effect of heat exposure, 5 ml of each chemovar was heated at 130°C for 10 min to ensure that any residual carboxylated phytocannabinoid acid precursors in the dried flos or granulate were converted to neutral, decarboxylated phytocannabinoids.[28,29]

2.3 | Neuronal cell culture

Mouse neuroblastoma × spinal cord cells (NSC-34) were cultured and maintained in complete medium (Dulbecco's Modified Eagles Medium, supplemented with 10% foetal bovine serum, 1% non-essential amino acids and 1% penicillin/streptomycin) and subcultured every 2–3 days as necessary. We have previously

TABLE 1 Manufacturer-reported percentages for the phytocannabinoids present in each of the Bedrocan products used in this study.

Phytocannabinoid	BC-101 (∼%)	BC-201 (∼%)	BC-301 (∼%)	BC-401 (∼%)	BC-501 (∼%)
Tetrahydrocannabivarin (THCV)	0.5	0.5	–	0.5	–
Cannabidiol (CBD)	0.5	0.5	8	0.5	9
Cannabichromene (CBC)	0.5	0.5	1	0.5	1
Cannabigerol monomethyl ether (CBGM)	–	–	0.5	–	0.5
Δ^9-tetrahydrocannabinol (Δ^9-THC)	22	13.5	6.3	14	0.5
Cannabigerol (CBG)	2	0.5	0.5	1	0.5

TABLE 2 Manufacturer-reported terpene profiles for each of the Bedrocan products used in this study.

Terpene	BC-101 (∼mg/g)	BC-201 (∼mg/g)	BC-301 (∼mg/g)	BC-401 (∼mg/g)	BC-501 (∼mg/g)
α-2-pinene	0.75	2	0.75	3.5	0.35
β-2-pinene	1.5	0.75	0.35	1	0.35
Myrcene	5	10	7.5	17.5	1.5
α-phellandrene	0.35	–	–	–	–
Delta-3-carene	0.35	–	–	–	–
R-limonene	1.5	0.2	0.35	–	0.2
Cis-ocimene	3.5	0.5	0.5	2	0.2
γ-terpinene	0.2	–	–	–	–
Terpinolene	5	–	0.75	–	0.35
(−)linalool	0.2	–	–	0.75	–
β-fenchol	0.1	–	–	–	–
Camphor	–	–	0.2	–	0.2
Borneol	0.75	–	–	–	–
α-terpineol	–	–	0.5	0.2	0.1
β-caryophyllene	1.5	1	0.75	1.5	0.75
Trans-bergamotene	0.35	–	0.2	0.2	0.1
α-guaiene	0.35	–	0.35	–	0.1
α-humulene	0.5	0.35	0.35	0.5	0.35
Trans-β-farnesene	0.35	–	0.35	0.1	0.35
γ-selinene	0.5	0.2	0.35	0.35	–
γ -cadinene	0.35	0.35	0.2	0.2	–
Eudesma-3,7(11)-diene	0.35	0.35	–	0.75	–
γ-elemene	1	0.35	0.2	0.5	–
β-caryophyllene oxide	–	–	–	–	0.2
Guaiol	–	–	–	0.5	–
γ-eudesmol	–	0.1	–	0.5	–
β-eudesmol	–	–	–	0.35	–
α-bisabolol	–	–	–	1	–

established the use of these cells in an in vitro model of Alzheimer's disease.[30] For cell viability measurements, cells were seeded in 96-well plates at a density of 3×10^5 cells/well and left to equilibrate for 24 h at 37°C and 5% CO_2 prior to treatment.

2.4 | Transmission electron microscopy

Transmission electron microscopy (TEM) was used to directly visualise changes in $A\beta_{1-42}$ aggregate morphology after treatment with each extract. Samples were

prepared by incubating native $A\beta_{1-42}$ (10 μM) in PBS, alone or in the presence of the extracts (10 μM principal component) for a period of 48 h at 37°C and 5% CO_2. After incubation, a 5-μl sample of each solution was added onto a 400-mesh formvar carbon-coated nickel electron microscopy grid (Proscitech, Kirwan, QLD, Australia) and left for 1 min. Samples were then blotted off, and 10 μl of a contrast dye containing 2% uranyl acetate was added. After 1 min, the contrast dye was blotted off and sample loaded into an FEI Tecnai G2 Spirit Transmission Electron Microscope (FEI, Milton, QLD, Australia). Each grid was scanned extensively for the presence of the target peptide, and representative images were taken at 18 500× magnification.

2.5 | MTT assay of cell viability

Cell viability was determined using the MTT assay. After equilibration, each well was treated with cannabis extracts at 10 μM principal component or a vehicle control and left to equilibrate for a further 15 min before being treated with $A\beta_{1-42}$ (0, 0.05, 0.1, 0.2, 0.5 and 1 μM) or *t*bhp (0–150 μM). After treatment, the plates were incubated for 48 h at 37°C and 5% CO_2. After incubation, culture medium was removed from each well and replaced by serum-free medium containing 0.25 mg/ml MTT. Plates were then incubated for a further 2 h, after which MTT solution was aspirated and cells were lysed with DMSO. Absorbance readings were taken at 570 nm using a Synergy MX microplate reader (BioTek, Bedfordshire, UK). Cell viability was determined on a sample size of four to five independent experiments (plates).

2.6 | Computational modelling of extract principal component binding with $A\beta_{1-42}$

Molecular modelling simulations were conducted as described previously.[31] Briefly, equilibrium geometries for the principal components of each cultivar, namely THC, THCA, CBD and CBDA, were optimised using density functional theory (DFT) utilising the Becke-Lee-Yang-Parr three-parameter hybrid functional, commonly referred to as B3LYP.[32] A large basis set, aug-cc-pVDZ was used to approximate molecular orbitals in optimised geometry for each compound, with all computations carried out using the Gaussian 09 package of codes. Optimised structures were then modelled for their binding affinity with both the $A\beta_{1-42}$ monomer (PDB ID: 1IYT) and an $A\beta_{17-42}$ oligomer composed of parallel β-sheet structures (PDB ID: 2BEG), using CLC Drug Discovery Workbench v2.4.1. Since $A\beta_{1-42}$ contains no specific pharmacophore, a large search space covering the entire peptide was used in each case; each ligand was also kept flexible to allow for rotation of bonds during docking simulations. Docking simulations were repeated five times with 1000 iterations per simulation, and the binding pose with the highest overall docking score for each ligand was selected for representative imaging.

2.7 | Statistical analysis

Cell viability data from the MTT assay was analysed via a two-way ANOVA using Dunnett's multiple comparisons test to determine significance *versus* treatment ($A\beta_{1-42}$ or *t*bhp). Modelling data was analysed by one-way ANOVA with significance determined by Tukey's multiple comparisons test. All data is presented as mean ± SD, with results deemed significant at $p < 0.05$. Summary ANOVA results for all analyses are presented in Supplementary Data (Tables S1–S2). Data analysis was performed using GraphPad Prism v.9.0.0 for Windows (GraphPad Software, San Diego, USA).

3 | RESULTS

3.1 | The THC-dominant chemovars BC-101 and BC-201 provide the greatest degree of neuroprotection against $A\beta_{1-42}$ in NSC-34 cells; heat exposure generally decreases the efficacy of cannabis extracts

Incubation with $A\beta_{1-42}$ (0–1.5 μM) over 48 h resulted in a concentration-dependent reduction in NSC-34 cell viability to a maximum of 75.38% initial viability (****$p < 0.0001$) (Figure 4). Of the five unheated extracts screened in this study (Figure 4, panel 1), the Δ^9-THC-dominant chemovars BC-101 and BC-201 consistently provided the greatest degree of neuroprotection across the full range of $A\beta_{1-42}$ concentrations, maintaining cell viability at 86.27% and 85.96%, respectively, at 1.5 μM $A\beta_{1-42}$ (****$p < 0.0001$). The Δ^9-THC-dominant BC-401 extract also significantly inhibited the cytotoxicity of $A\beta_{1-42}$ at 0.5, 1 and 1.5 μM, maintaining cell viability at 88.26%, 83.36% and 83.93% initial, respectively (*$p = 0.0268$, **$p = 0.0097$ and ****$p < 0.0001$ *versus* control). The BC-301 extract exhibited similar efficacy as an inhibitor of $A\beta_{1-42}$ cytotoxicity, maintaining cell viability at 88.69%, 84.34% and 82.95% initial at 0.5, 1 and 1.5 μM $A\beta_{1-42}$, respectively (*$p = 0.0139$, **$p = 0.0018$ and ***$p = 0.0004$ *versus* control), while the CBD-dominant BC-501 extract displayed only modest protection, with significance seen only at the highest concentration of $A\beta_{1-42}$ (80.5% initial, *$p = 0.0263$).

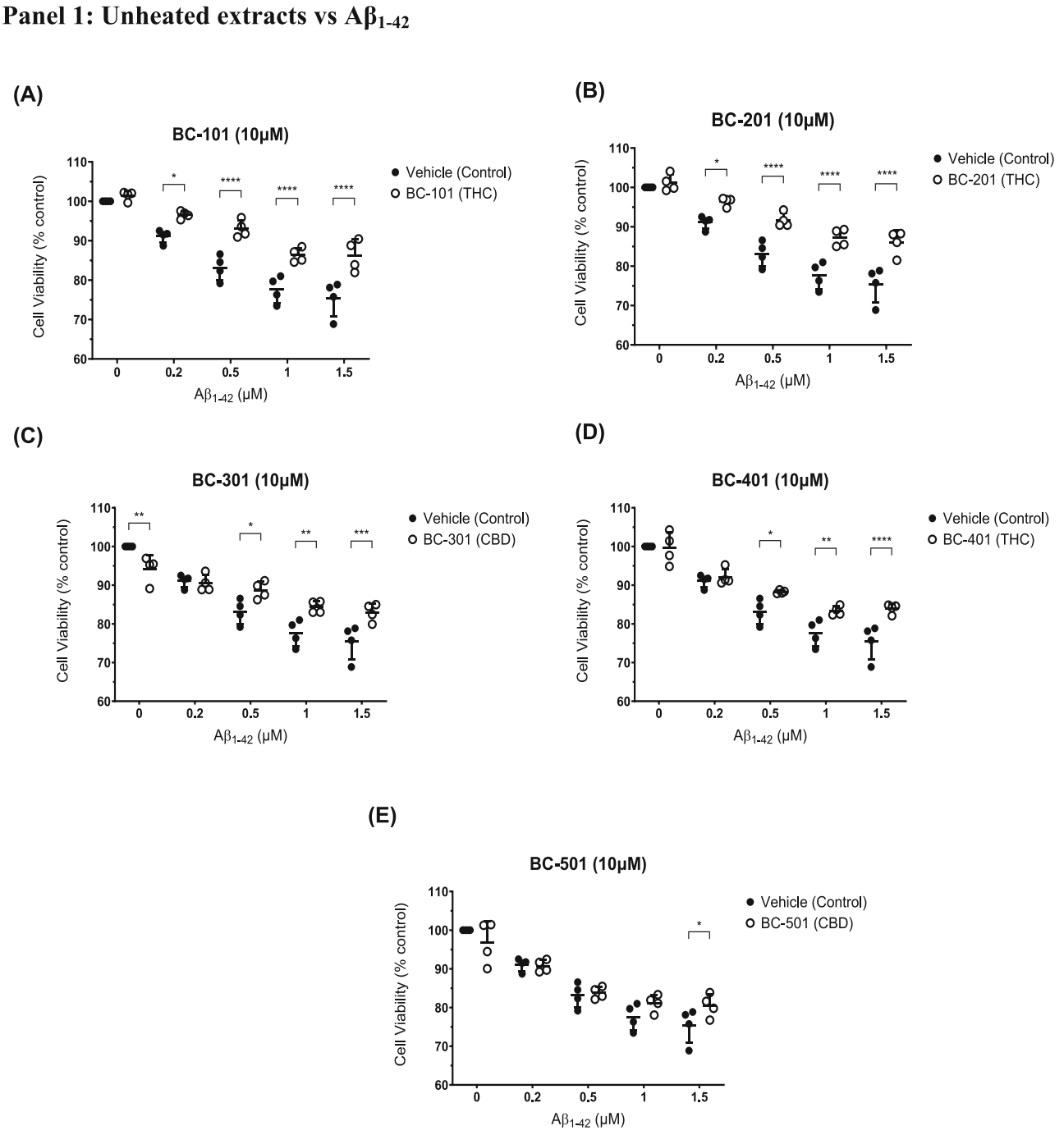


FIGURE 4 MTT assay of cell viability after 48 h incubation with $A\beta_{1-42}$ (0–1.5 μM) alone and in the presence of unheated (panel 1) and heated (panel 2) cannabis extracts (10 μM PC): (A) BC-101/H-BC-101, (B) BC-201/H-BC-201, (C) BC-301/H-BC-301, (D) BC-401/H-BC-401, (E) BC-501/H-BC-501. Mean (SD), $n = 4$ extract. Mean (SD), $n = 5$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ *versus* vehicle. ANOVA Summary Table: Supplementary Data, Table 1. CBD, cannabidiol; THC, tetrahydrocannabinol; Aβ, β amyloid.

The neuroprotective profile of each extract displayed similar trends in efficacy after heat exposure (Figure 4, panel 2). H-BC-101 extract displayed the greatest neuroprotective capacity of all heated extracts, significantly inhibiting $A\beta_{1-42}$ cytotoxicity at each amyloid concentration. Significant neuroprotection was also observed after treatment with H-BC-201 extract, with cell viability maintained at 89.25%, 84.5% and 84.85% initial at 0.5, 1 and 1.5 μM $A\beta_{1-42}$, respectively (**$p = 0.0019$, ****$p < 0.0001$ and ****$p < 0.0001$ *versus* control). H-BC-401 extract provided significant protection at 0.5, 1 and 1.5 μM $A\beta_{1-42}$, maintaining NSC-34 cell viability at 87.75%, 83.39% and 82.95%, respectively (*$p = 0.0378$, ***$p = 0.0002$ and ***$p = 0.0001$ *versus* control). The

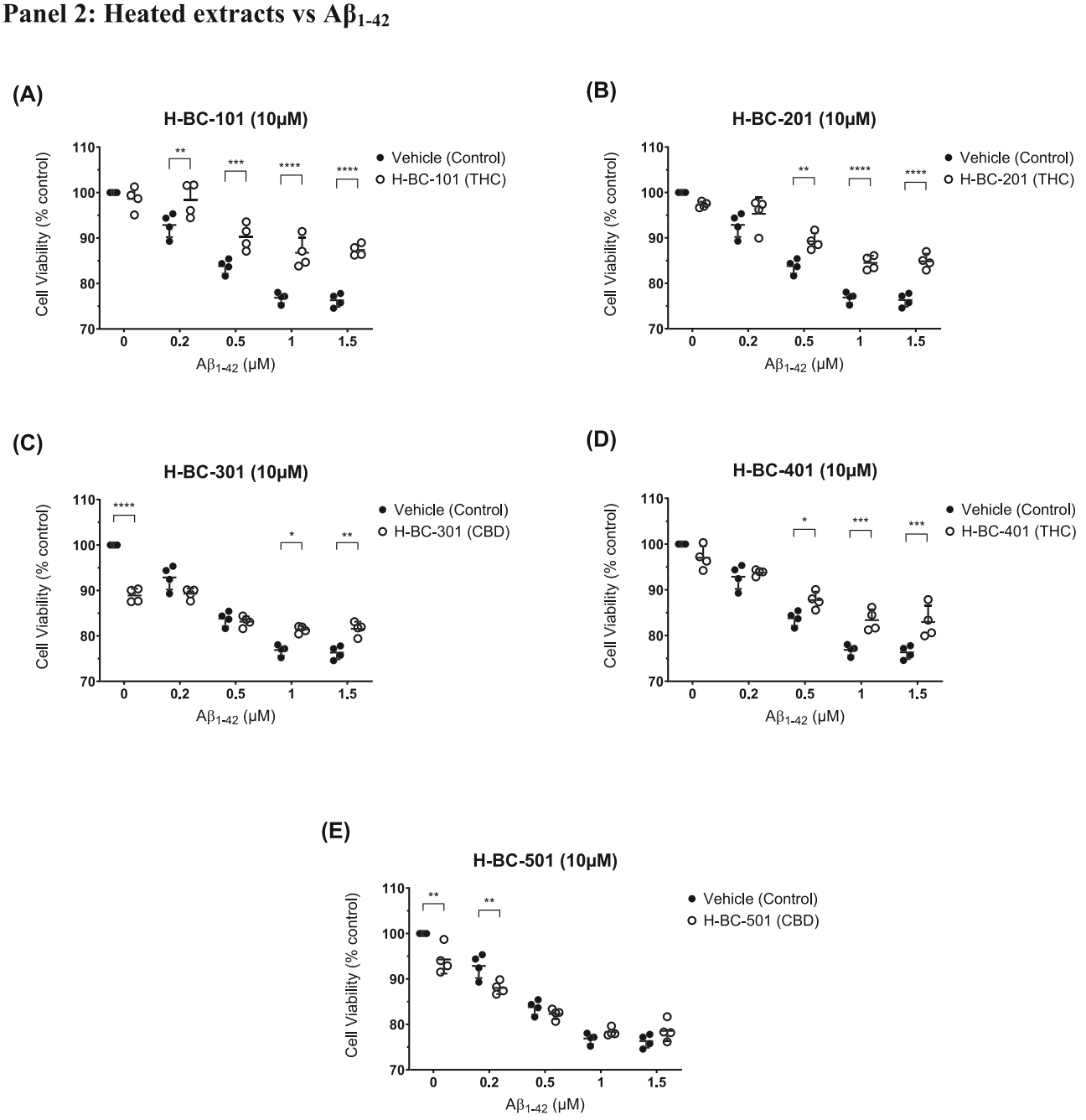


FIGURE 4 (Continued)

CBD-dominant H-BC-301 extract provided significant protection only at 1 and 1.5 μM $A\beta_{1-42}$, maintaining cell viability at 81.4% and 81.6%, respectively (*$p = 0.014$ and **$p = 0.003$ *versus* control); H-BC-501 extract displayed no significant neuroprotection. Interestingly, a significant decrease in NSC-34 viability was seen after treatment with H-BC-301 extract alone (88.89% initial, ****$p < 0.0001$); this was also observed at 0 and 0.2 μM $A\beta_{1-42}$ in the H-BC-501 extract (94.3% and 88.0% initial, **$p = 0.0011$ and **$p = 0.0076$).

3.2 | Cannabis extracts provide no significant protection against *t*bhp-induced neurotoxicity in NSC-34 cells; heat exposure marginally increased the cytotoxicity of CBD-dominant chemovars

Incubation with *t*bhp (0–150 μM) over 24 h resulted in a concentration-dependent decrease in cell viability to a maximum of 9.4% initial viability (****$p < 0.0001$) (Figures 5 and 6). None of the chemovars tested provided

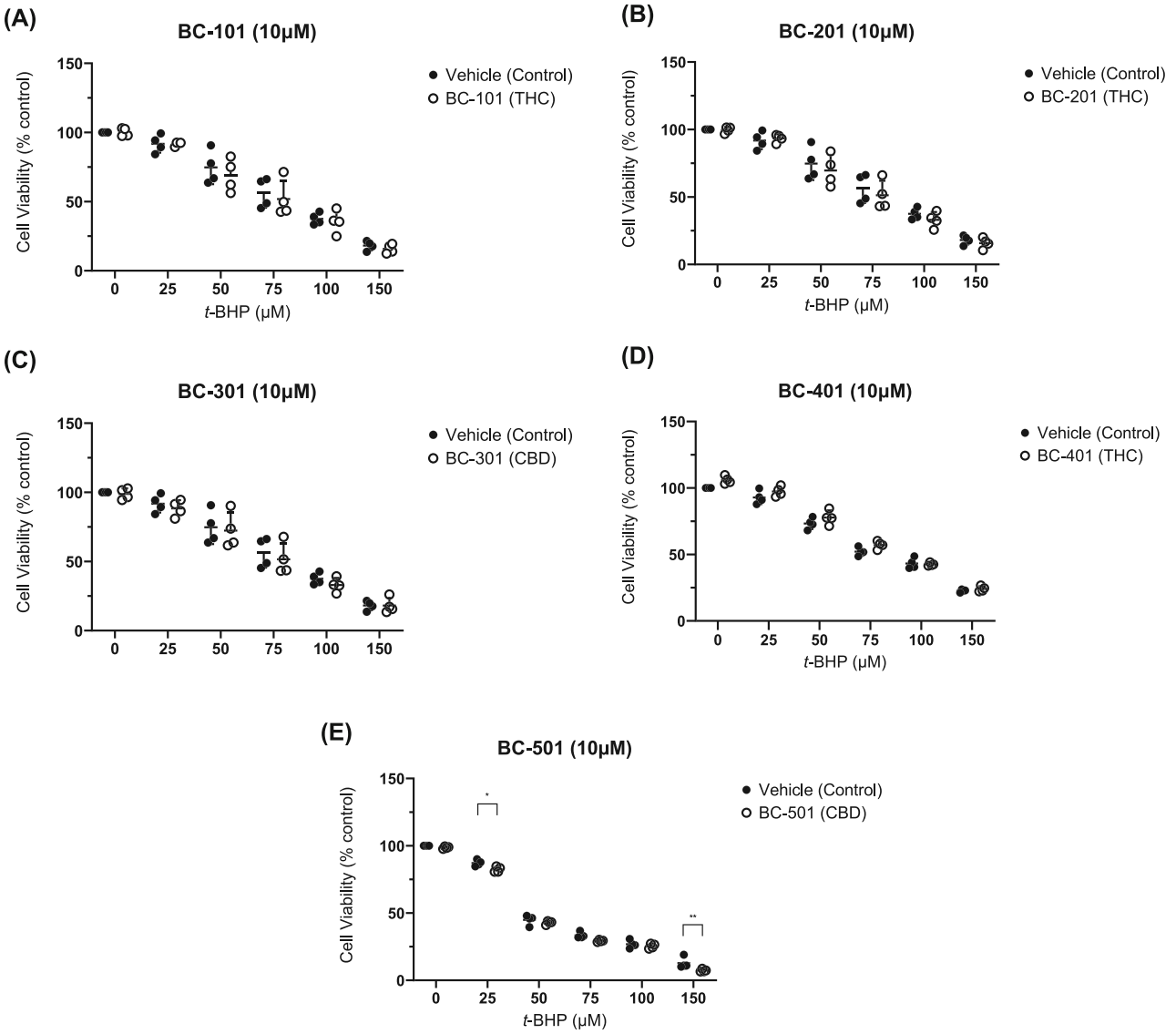


FIGURE 5 MTT assay of cell viability after 24 h incubation with *tert*-butyl hydroperoxide (*t*bhp) (0–150 μM) alone and in the presence of non-heated cannabis extracts (10 μM PC): (A) BC-101, (B) BC-201, (C) BC-301, (D) BC-401, (E) BC-501. Mean (SD), $n = 4$. *$p < 0.05$, **$p < 0.01$ *versus* vehicle. ANOVA Summary Data: Supplementary Data, Table 2. CBD, cannabidiol; THC, tetrahydrocannabinol; *t*bhp, *tert*-butyl hydroperoxide.

any significant protection against *t*bhp-induced cytotoxicity. However, significant increases in toxicity were observed at 25 μM (82.36% initial *versus* 87.13%, *$p = 0.0305$) and 150 μM *t*bhp (7.3% initial *versus* 12.74%, *$p = 0.01$) after treatment with the CBD-dominant BC-501 extract (Figure 5e). Heat exposure resulted in a notable increase in the cytotoxicity of CBD-dominant chemovars. H-BC-301 extract displayed intrinsic toxicity while also increasing the toxicity of 25 μM *t*bhp (86.95% initial *versus* control, ****$p < 0.0001$ and 75.02% initial *versus* 87.84%, ****$p < 0.0001$, respectively) (Figure 6c). A similar pattern was observed in H-BC-501-treated cells, which also displayed intrinsic toxicity (88.24% initial *versus* control, ***$p = 0.0002$) as well as reducing cell viability to 83.74%, 65.66% and 35.32% initial at 25, 50 and 100 μM *t*bhp, respectively (**$p = 0.0038$, *$p = 0.0192$ and *$p = 0.0145$).

3.3 | Cannabis extract's effects on $A\beta_{1-42}$ aggregation: Modest effects on aggregate density and overall morphology from extract BC-401

The cannabis extracts screened in this study generally exerted little effect on the overall morphology of $A\beta_{1-42}$

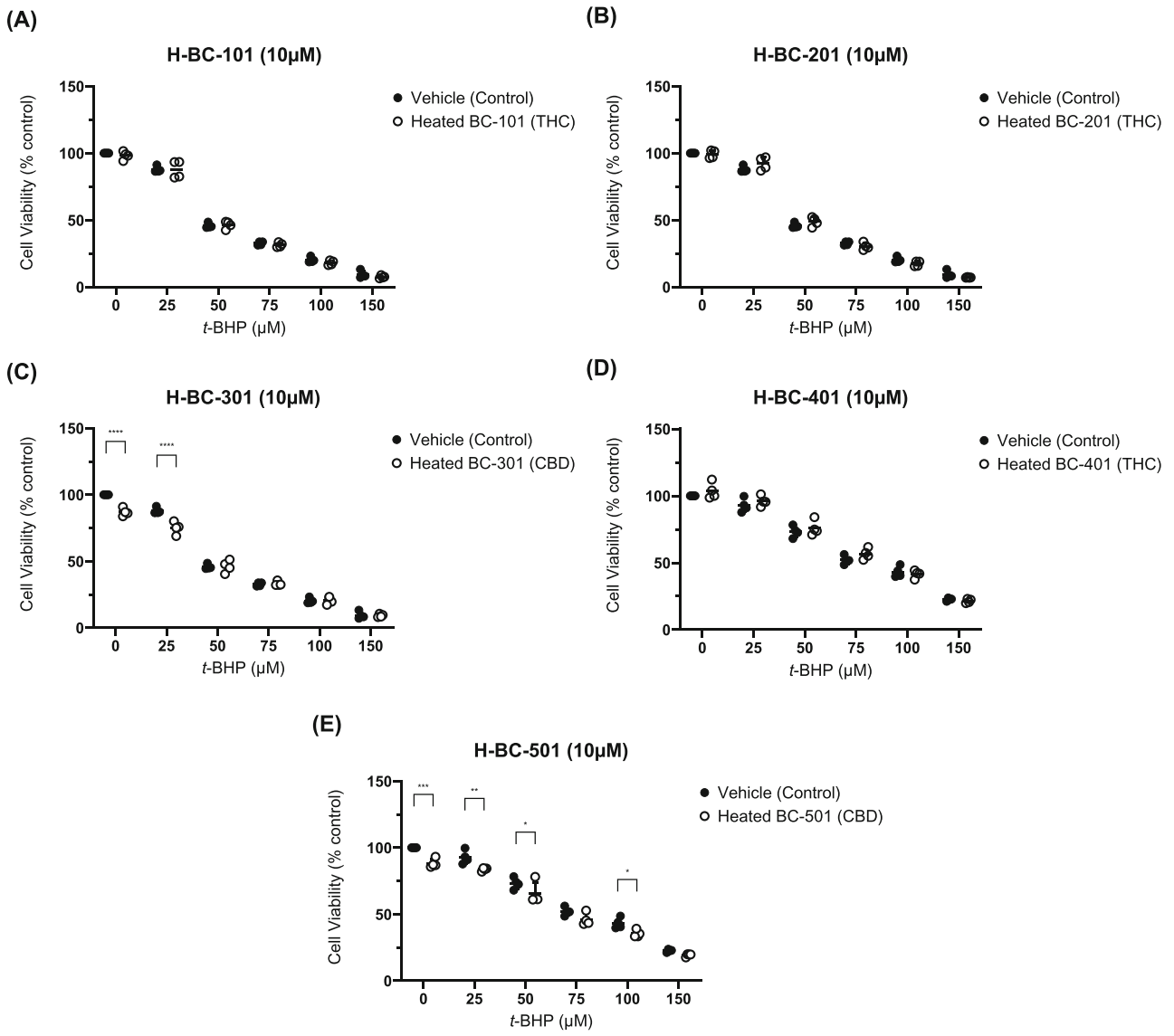


FIGURE 6 MTT assay of cell viability after 24 h incubation with *tert*-butyl hydroperoxide (*t*bhp) (0–150 μM) alone and in the presence of various heated cannabis extracts (10 μM PC): (A) H-BC-101, (B) H-BC-201, (C) H-BC-301, (D) H-BC-401, (E) H-BC-501. Mean (SD), $n = 5$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ *versus* vehicle. ANOVA Summary Table: Supplementary Data, Table 2. CBD, cannabidiol; THC, tetrahydrocannabinol.

(Figure 7). However, some qualitative differences were observed with unheated extract BC-401, which effectively reduced the overall density of amyloid β fibrils and aggregates (Figure 7e).

3.4 | Comparative molecular modelling of binding interactions between carboxylated and decarboxylated phytocannabinoids and the Aβ protein

The amyloid binding characteristics of the phytocannabinoids Δ^9-THC and CBD were compared with their carboxylated precursors THCA and CBDA (Figures 8, 9 and 10). In the $A\beta_{1-42}$ monomer, both Δ^9-THC and CBD bound towards the centre of the protein, interacting with the key Lys16 residue via hydrogen bonding (Figure 9a and c), although Δ^9-THC displayed slightly greater binding affinity over CBD ($^{*}p = 0.0147$) (Figure 8a [i]). A similar relationship was observed between Δ^9-THC and THCA, whereby both molecules bound towards the centre of the $A\beta_{1-42}$ monomer, although THCA bound at slight opposition to Δ^9-THC and did not form hydrogen bonds with Lys16; no significant difference was observed between the docking scores of Δ^9-THC and THCA. CBD possessed greater affinity for the $A\beta_{1-42}$ monomer than

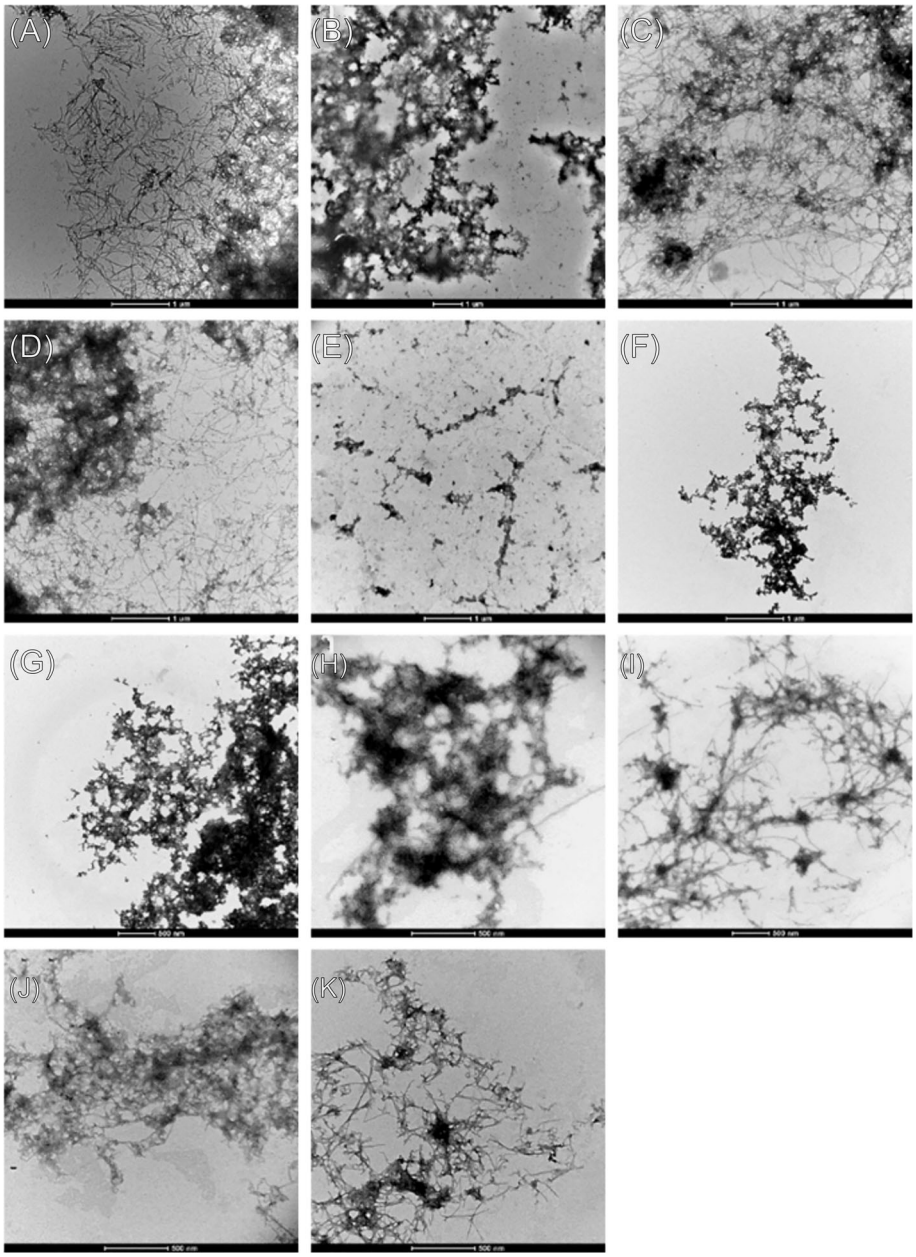


FIGURE 7 Transmission electron micrographs demonstrating the effect of unheated (b-f) and heated (g-k) cannabis extracts (10 μM PC) on the aggregation and morphology of $A\beta_{1-42}$ (10 μM). (A) Control, (B) BC-101, (C) BC-201, (D) BC-301, (E) BC-401, (F) BC-501, (G) H-BC-101, (H) H-BC-201, (I) H-BC-301, (J) H-BC-401, (K) H-BC-501.

CBDA (**$p = 0.0011$) (Figure 8a [i]), despite the similarity in binding pose and position (Figure 9c and d). Of particular note was the relationship between THCA and CBDA, whereby THCA displayed significantly greater affinity for the $A\beta_{1-42}$ monomer (****$p < 0.0001$) (Figure 8a [i]), perhaps due primarily to differences in steric interactions (*$p = 0.0483$) (Figure 8a [iii]).

In the pentamer, Δ^9-THC displayed far greater affinity than both CBD and THCA (****$p < 0.0001$), with no significant difference observed between CBD and CBDA nor between THCA and CBDA (Figure 8b [i]). The carboxylated phytocannabinoids THCA and CBDA displayed significantly greater hydrogen bonding potential than their decarboxylated counterparts (****$p < 0.0001$), while Δ^9-THC displayed great hydrogen bonding than CBD (****$p < 0.0001$) and THCA significantly greater than CBDA (****$p < 0.0001$) (Figure 8b [ii]). Δ^9-THC also displayed considerably greater steric interaction scores than all other phytocannabinoids screened (****$p < 0.0001$), likely contributing to its relative binding potency (Figure 8b [iii]). Each phytocannabinoid bound in a similar position within the hydrophobic groove of the Aβ pentamer, with all but CBD displaying hydrogen-bonding interactions.

4 | DISCUSSION

The results of this study highlight the novel neuroprotective effects of ethanolic extracts of the type I cannabis

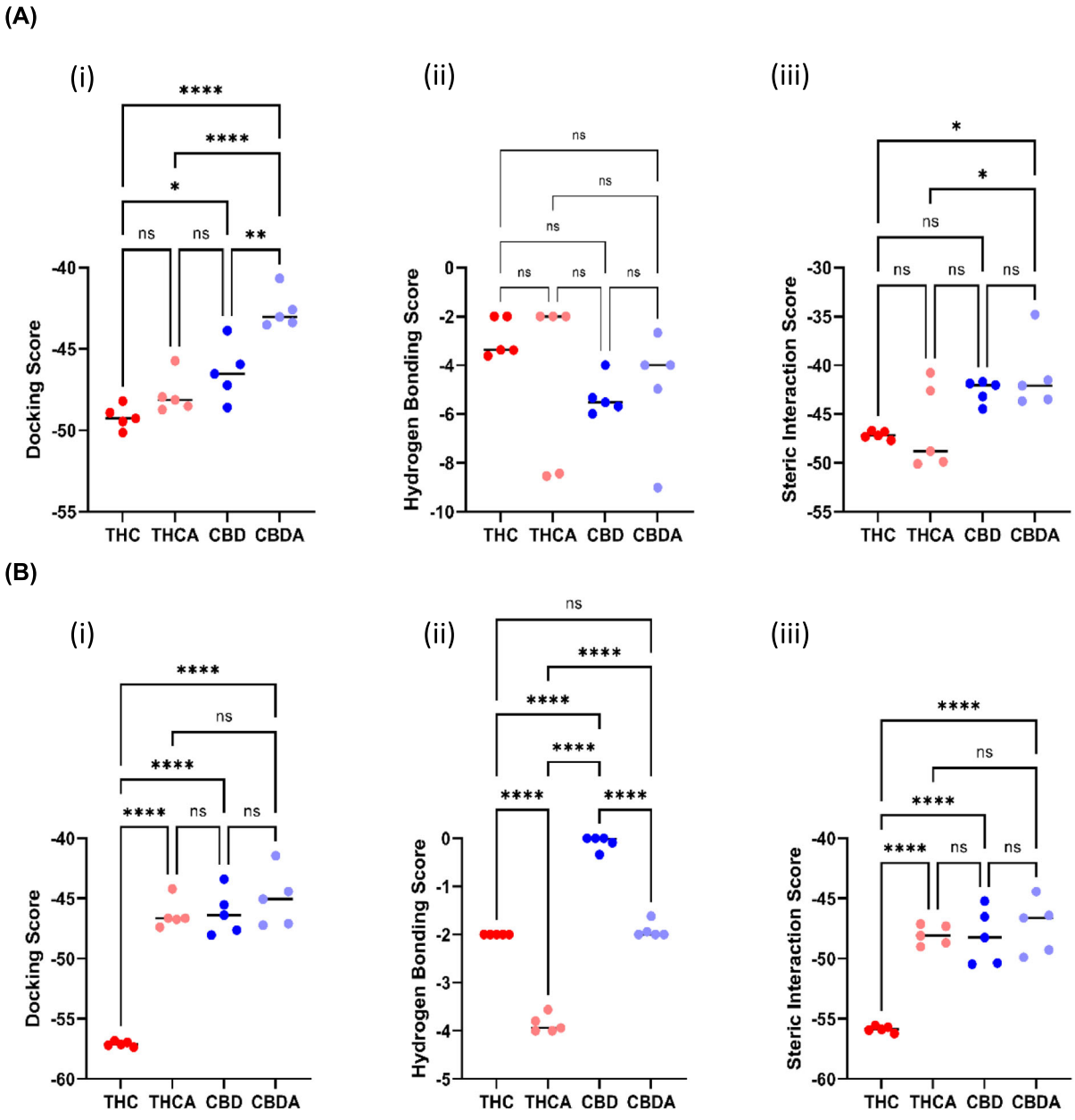


FIGURE 8 In silico modelling of binding interactions between Δ9-THC, THCA, CBD and CBDA with $A\beta_{1-42}$ (A) monomer (PDB ID: 1IYT) and (B) pentamer (PDB ID: 2BEG). Data presented as median (i) best docking score and the corresponding (ii) hydrogen bonding score and (iii) steric interaction score from $n = 5$ independent simulations. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. ANOVA data summary: Table 3, Supplementary Data. CBD, cannabidiol; CBDA, cannabidiolic acid; THC, tetrahydrocannabinol; THCA, tetrahydrocannabinolic acid; ns, not significant.

chemovars BC-101, BC-201 and BC-401 against $A\beta_{1-42}$-evoked neurotoxicity. No significant protective effect was observed in *t*bhp-treated cells after co-incubation with any of the cannabis extracts screened in this study, suggesting that this neuroprotection is likely independent of antioxidant capacity. With the exception of BC-401, these extracts exerted minimal qualitative effects on the fibrillization and aggregation of Aβ, indicating that this protective effect is likely independent of direct interactions with the amyloid protein. This study presents the first characterisation of these five proprietary cannabis chemovars in the context of neuronal cell-based models of Alzheimer's disease neurotoxicity.

The Δ^9-THC-predominant chemovars BC-101, BC-201 and BC-401 significantly inhibited the neurotoxicity of $A\beta_{1-42}$ in NSC-34 cells, with the type II chemovar BC-301 providing a lesser degree of protection; no significant neuroprotection was observed after treatment with the type III chemovar BC-501. This pattern suggests that Δ^9-THC-predominant cannabis extracts may provide a

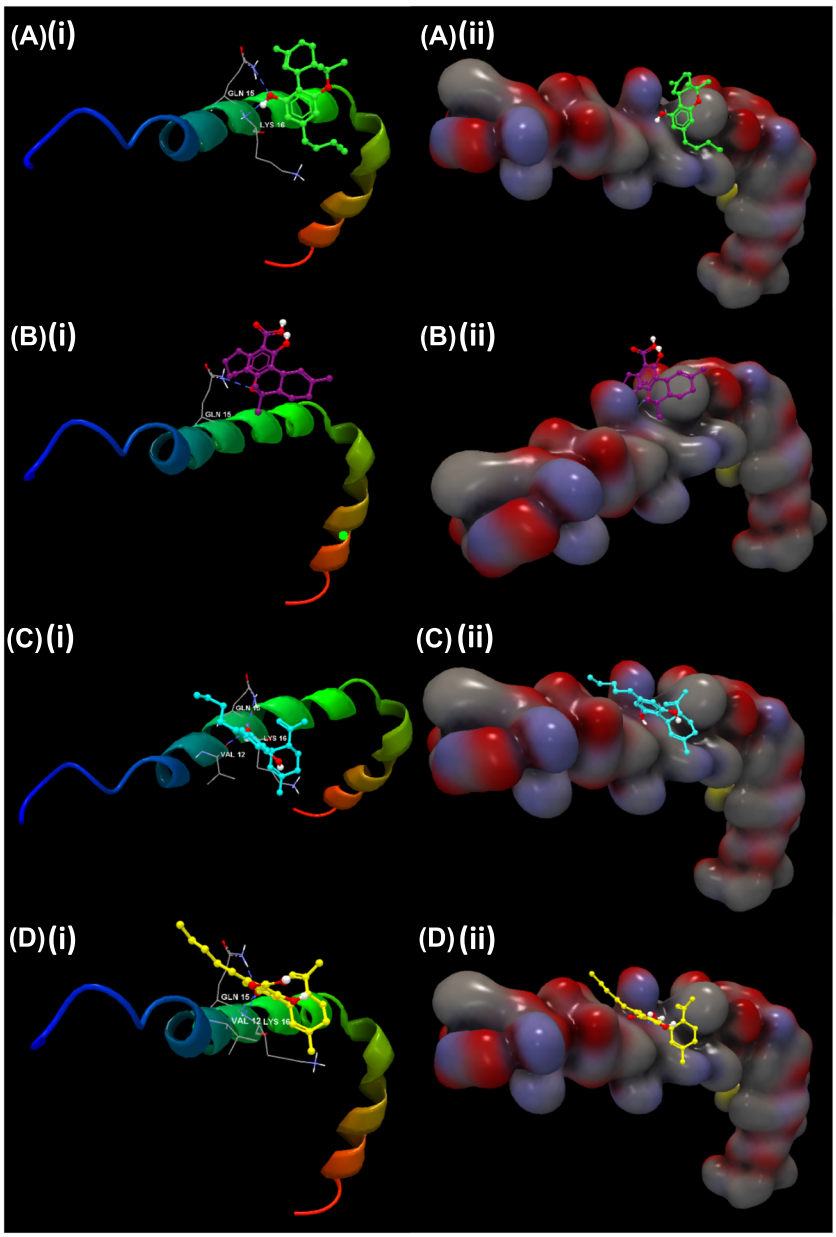


FIGURE 9 Representative images of the most favourable binding interactions between (A) Δ9-THC, (B) THCA, (C) CBD and (D) CBDA with $A\beta_{1-42}$ monomer (PDB ID: 1IYT); images presented with (i) ribbon structure and (ii) bubble.

greater degree of neuroprotection against Aβ-evoked toxicity than CBD-predominant extracts. This finding is in line with previous studies from our laboratory, which have demonstrated no significant neuroprotective capacity for CBD against $A\beta_{1-42}$.[33,34] Interestingly, none of the extracts screened in this study provided any protection against the lipid peroxidising agent *t*bhp in NSC-34 cells. This is in contrast to the findings of a previous study which highlighting the particular efficacy of THC-dominant extracts in reducing H_2O_2-induced reactive oxygen species (ROS) generation.[20] This difference may, in part, be attributed to the different cell types used in these studies, as well as the different pro-oxidant stressors used, where hydrogen peroxide acts within the cytosol and *t*bhp more so in the lipid membrane. However, the authors noted the particular lack of efficacy of CBD-dominant extracts as antioxidants, a trend that was observed in the present study. Interestingly, previous research from our laboratory has shown that CBD possesses the ability to protect neuronal PC12 cells from *t*bhp-evoked cytotoxicity, while affording no significant neuroprotection against $A\beta_{1-42}$, suggesting either possible differences in prooxidant resilience between these phenotypically distinct neuronal cell lines or the differential expression of targets or operant signalling pathways for CBD-based neuroprotection, whereby the definitive pharmacological target(s) for CBD's action remains unclear.

While unheated extracts would be expected to contain predominantly acidic carboxylated forms of THC (THCA) and CBD (CBDA), previous research on the composition

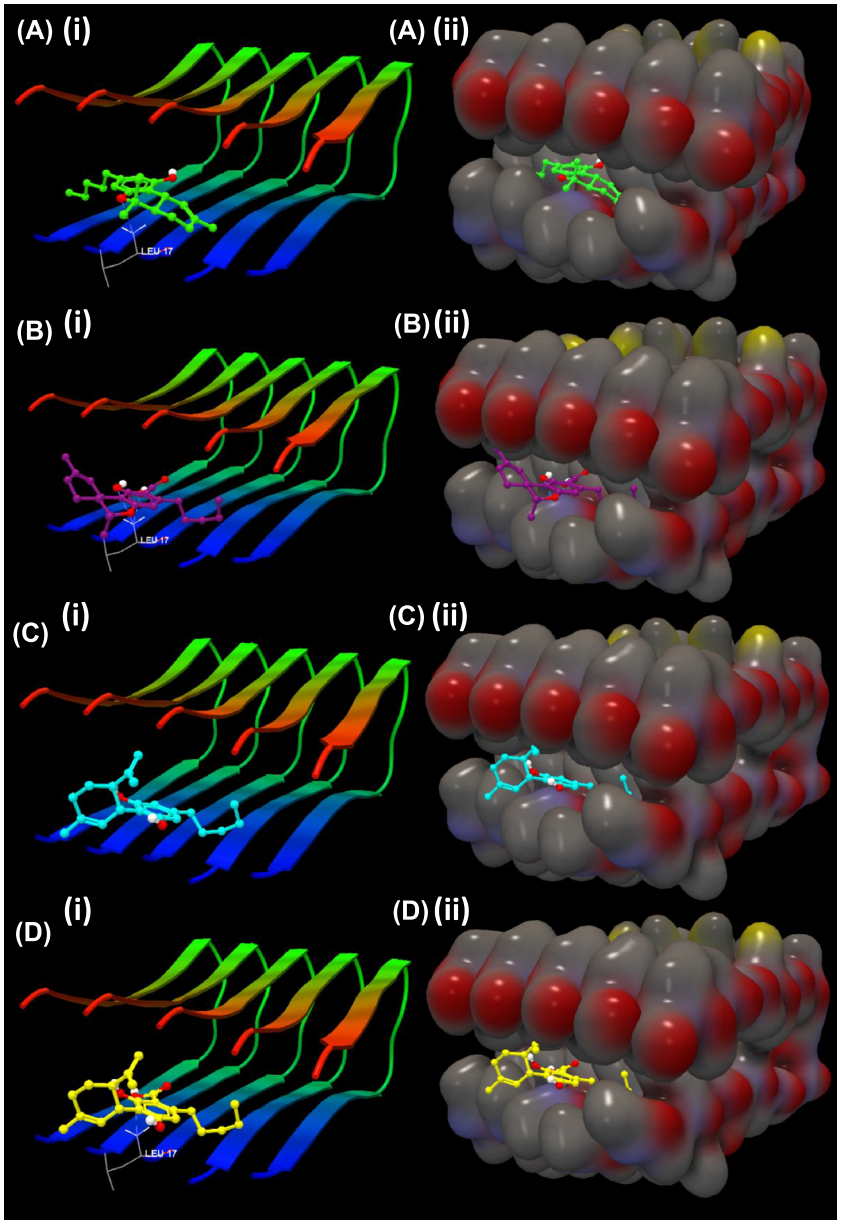


FIGURE 10 Representative images of the most favourable binding interactions between (A) Δ9-THC, (B) THCA, (C) CBD and (D) CBDA with $A\beta_{17-42}$ pentamer (PDB ID: 2BEG); images presented with (i) ribbon structure and (ii) bubble.

of dried medicinal cannabis plant material has demonstrated the presence of both carboxylated and neutral major phytocannabinoids,[35] as THCA and CBDA may decarboxylate under varying drying techniques and ambient storage conditions.[28] We did not separately quantify the relative levels of carboxylic parent forms and neutral phytocannabinoids individually in each extract, so the total major phytocannabinoid levels are the sum of the carboxylated and neutral phytocannabinoid forms. However, in the context of neurobiological activity, THCA and CBDA have also recently been ascribed neuroprotective activity, suggesting that they may share similar properties or operant pathways for activity in the brain to THC and CBD.[36] Future studies aimed at an individual determination of THCA and CBDA neuroprotective bioactivity will delineate the comparative effects *versus* THC and CBD, which may have substantive implications for the manufacture, extraction and preparation of medicinal cannabis formulations for neurological and neuroprotective applications, notwithstanding that many other components such as the minor phytocannabinoids and other non-cannabinoid phytochemicals may contribute to the neuro-bioactivity of cannabis.

Given their lack of efficacy generally in the inhibition of both amyloid β aggregation and *t*bhp-induced cytotoxicity, it is clear that these extracts mediate their protective effects via other mechanisms. Both Δ^9-THC and CBD have been shown to exert their neuroprotective effects via the activation of numerous receptor targets, including cannabinoid receptor 1 and 2 subtypes (CB_1 and CB_2) for THC and for CBD, a somewhat promiscuous pharmacology directed at peroxisome proliferator-activated receptor

gamma (PPAR-γ) receptors, TRPA/V channels, serotonergic, GABAergic and a range of orphan G protein–coupled receptors.[10,37,38] Interestingly, the non-selective cannabinoid receptor agonist WIN55212-2 has been shown to reduce Aβ-evoked neuroinflammation via the activation of both CB_1 and CB_2 receptors, as well as increased PPAR-γ signalling.[39] Moreover, activation of the CB_2 receptor has been associated with the reversal of AD-associated memory impairment, with CB_2 receptor deficiency being shown to worsen the pathological progression of AD in vivo.[40,41] Such studies highlight the nominal importance of cannabinoid and PPAR-γ receptors as targets in AD, noting however that the full spectrum cannabis extracts used in this study contain a multitude of other bioactive phytochemicals such as minor phytocannabinoids, terpenes, flavonoids and stilbenoids[42] that may act at many cellular targets aside from just cannabinoid and PPAR-γ receptors, making discerning any discreet pharmacological basis to such bioactivity challenging.

A general trend was observed, whereby heating of each extract reduced their overall neuroprotective effects, with heating even increasing the toxicity of CBD-dominant extracts. One possible explanation for this effect is the possible decrease in terpenoids in the heated extracts, as heating of cannabis has been shown to reduce terpene retention, given their volatile nature.[26] Moreover, heat exposure may result in the formation of oxidation products such as hydroperoxides (e.g. limonene hydroperoxide) which have been associated with ROS-induced cytotoxicity via lipid peroxidation.[43] This would be consistent with the prevailing theory of the entourage effect, whereby the presence of such terpenoids may act to synergistically enhance the protective effects of cannabinoids.[16] This effect highlights the potential therapeutic benefits of using whole (full-spectrum) botanical cannabis extracts over phytocannabinoids in isolation. It is particularly interesting to note the potential role of such cannabis terpenes in the neuroprotection observed in this study. Terpenes such as myrcene have been shown to possess neuroprotective qualities in in vivo models of oxidative neuronal damage and AD, with the terpenes α-bisabolol, myrcene and β-caryophyllene providing significant neuroprotection in both *in vitro* and in vivo models.[30,44,45] Additionally, both α-pinene and β-pinene have been ascribed neuroprotective properties,[46] with α-pinene providing in vivo neuroprotection via the suppression of the TNFα/NF-κB pathway.[47,48] Interestingly, it has been suggested that cannabis terpenes including α-humulene, linalool and β-pinene have cannabimimetic properties, with the ability to produce cannabinoid tetrad behaviours in mice, suggestive of an ability to enhance cannabinoid activity.[19] Each of these terpenes is found in the chemovars used in this study, suggesting they may play a role in the observed neuroprotection, albeit the extract with the highest neuroprotective candidate levels, BC-401, was seemingly unaffected in its neuroprotective effects by heating. Future in vivo studies may consider using standardised combinations of these terpenes in the presence and absence of Δ^9-THC and CBD in order to further assess their therapeutic potential in AD, as well investigating any possible therapeutic synergism.

Computational modelling with Aβ revealed that, despite binding at similar positions, Δ^9-THC possesses significantly greater binding affinity for both the monomer and pentamer than both THCA and CBD. We have previously shown that Δ^9-THC possesses greater binding affinity for both the Aβ monomer and pentamer than does CBD.[49] It was observed that THCA and CBDA possess significantly lower binding affinity for the Aβ protein than their decarboxylated counterparts. Although we did not quantify residual THCA or CBDA in extracted cannabis samples, the in silico data predicts that unheated extracts, which may contain a greater proportion of THCA and CBDA, should be less efficacious as inhibitors of amyloid aggregation than heated extracts. However, an interesting lack of correlation was observed between this modelling data and the morphological data obtained via TEM. Only the unheated BC-401 inhibited $A\beta_{1-42}$ aggregation and morphology, an effect which was lost after heat exposure. As this extract contains more bioactive terpenoids than any of the others used in thus study, it may be that such compounds as myrcene and α-bisabolol collectively contribute to anti-aggregatory effects,[30] noting also the relatively high levels of pinenes associated with neuroprotection and anti-aggregatory effects against amyloid β.[46] In any case, it is clear that the neuroprotection against $A\beta_{1-42}$ observed in this study is not directly correlated with any direct interactions of one compound with the $A\beta_{1-42}$ protein. This is consistent with a previous study which found both Δ^9-THC and CBD to have negligible effects on $A\beta_{1-42}$ aggregation.[9]

In conclusion, this study demonstrated the novel neuroprotective capacities of the proprietary type I chemovars BC-101, BC-201 and BC-401 against Aβ-evoked neurotoxicity, an effect that was reduced after heat exposure, is independent of antioxidant capacity and is unrelated to direct interactions with the $A\beta_{1-42}$ protein. Given the protective effect observed in this study and the considerable interest surrounding medicinal cannabis and the therapeutic use of whole botanical extracts, further research into the neuroprotective efficacy of such cannabis extracts is warranted. As our understanding of medicinal cannabis and the entourage effect continues to grow, so too does the need for further in vivo studies of whole botanical extracts in the context of Alzheimer's disease

and related neurodegenerative conditions. Such studies may provide considerable insight into the true therapeutic potential of medicinal cannabis, presenting an important avenue for future exploration.

AUTHOR CONTRIBUTIONS

Dylan T. Marsh conducted all cell viability experiments, transmission electron microscopy and data analysis, data visualisation and writing of the manuscript. Mayu Shibuta assisted with the transmission electron microscopy and interpretation of molecular modelling. Scott D. Smid and Ryuji Kato were responsible for overall project administration, supervision and funding acquisition, as well as the editing and writing of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

DECLARATIONS

We confirm that the study has been performed in accordance with the BCPT policy for experimental and clinical studies.[27]

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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