

Herbal extracts alter amyloid beta levels in SH-SY5Y neuroblastoma cells

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SUMMARY

Alzheimer's disease (AD) is a type of dementia that affects more than 5.5 million Americans. It is characterized by progressive memory loss and impairment of other cognitive abilities that affect daily life. Unfortunately, there are no approved treatments that can delay the advancement of the disease. However, it is known that factors such as amyloid beta ($A\beta$) plaques and tau neurofibrillary tangles disrupt connections between neurons, leading to the eventual death of neurons that are responsible for memory. For this investigation, we focused on the neurotoxic $A\beta_{1-40}$ peptide, which is formed by the amyloidogenic cleavage and processing of amyloid precursor protein (APP), a crucial component in the development of AD. Neuroinflammatory cytokines have also been shown to reduce the efflux transport of $A\beta$ from the brain, leading to increased $A\beta$ concentrations. The objective of the experiment was to test the effects of various herbal extracts (bugleweed, hops, sassafras, and white camphor) on $A\beta_{1-40}$ peptide levels in human neuroblastoma cells that were transfected to overexpress APP. Due to the herbal extracts' common anti-inflammatory property, the experiment determined whether or not this property had the potential to change $A\beta_{1-40}$ concentrations. Prior to the quantification of $A\beta_{1-40}$ peptide with an enzyme-linked immunosorbent assay (ELISA), we determined the cytotoxicity of the extracts using an MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, assay to discern whether decreases in $A\beta_{1-40}$ concentrations were the result of cell death. The results indicated that white camphor was toxic to neuroblastoma cells and resulted in decreased $A\beta_{1-40}$ levels; sassafras was not toxic and resulted in slightly elevated $A\beta_{1-40}$ levels; hops was not toxic and resulted in increased $A\beta_{1-40}$ levels; and bugleweed was not toxic, yet resulted in decreased $A\beta_{1-40}$ levels. Thus, only bugleweed may have the potential to reduce $A\beta_{1-40}$ levels through its anti-inflammatory properties.

INTRODUCTION

Characterized by gradual memory loss and impairment of cognitive abilities, Alzheimer's disease (AD) is a type of dementia that affects more than 5.5 million Americans and

interferes with their daily lives [1-2]. Although there are no approved treatments for AD, it is known that amyloid-beta ($A\beta$) plaques and tau neurofibrillary tangles disrupt connections between neurons, leading to the neuronal loss that is responsible for memory [2-3].

This investigation focused on the neurotoxic $A\beta_{1-40}$ peptide, which accumulates between neurons in the hippocampus and forms amyloid plaques, one of the hallmarks of AD [3]. The sequential processing of amyloid precursor protein (APP) through the amyloidogenic pathway is significant in the production of cytotoxic $A\beta$ [4]. APP is a transmembrane protein that is processed in two distinct pathways: non-amyloidogenic and amyloidogenic (Figure 1). In the non-amyloidogenic pathway, APP is cleaved by α -secretase and γ -secretase. In contrast, in the amyloidogenic pathway, APP is cleaved by β -secretase and γ -secretase, releasing an $A\beta$ peptide. Due to its inclination to aggregate, $A\beta$ forms oligomers that are cytotoxic, disrupting cell function and leading to cell death [5]. Hence, inhibiting enzymes involved in amyloidogenic APP processing and promoting degradation of amyloid plaques are crucial goals among researchers looking to find treatments for AD [4]. For example, many clinical trials study inhibition of β -site amyloid precursor protein cleaving enzyme 1 (BACE1), which is a β -secretase enzyme that is thought to

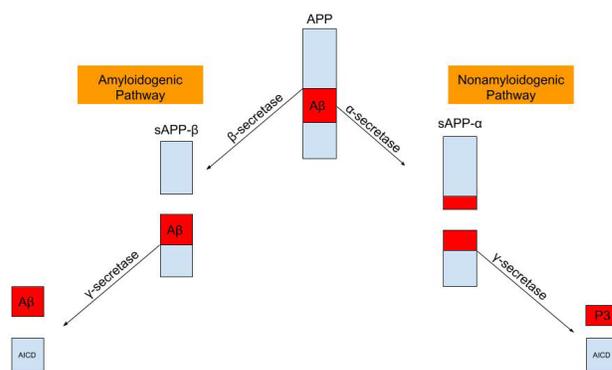


Figure 1. Amyloid precursor protein (APP) is a transmembrane protein that is processed in two distinct pathways: non-amyloidogenic (right) and amyloidogenic (left). In the non-amyloidogenic pathway, APP is sequentially cleaved by α -secretase and γ -secretase to form sAPP- α , $A\beta_{1-40/42}$ (P3) peptide, and APP intracellular domain (AICD) peptides. In contrast, in the amyloidogenic pathway, APP is cleaved by β -secretase and γ -secretase, creating sAPP- β , $A\beta$, and AICD peptides. This releases the cytotoxic $A\beta$ peptide that aggregates to form amyloid plaques, one of the major characteristics of Alzheimer's disease.

play a crucial role in the beginning stages of AD [6]. In theory, inhibition of this enzyme should prevent amyloidogenic APP processing and reduce A β plaque formation, but these clinical trials have met various challenges over the past years. In a study done in mice, BACE1 inhibition interfered with adult neurogenesis regulation in the hippocampus [7]. Despite reducing A β plaque formation, BACE1 inhibitors also caused side effects during chronic administration, including seizures, neurodegeneration, and hypomyelination [6]. Thus, more research into the appropriate level of BACE1 inhibitor needed for treatment and the stage of AD at which the inhibitor should be administered will be needed to attain optimal efficacy.

In our investigation, we wanted to screen the effects of various herbal extracts on A β ₁₋₄₀ peptide concentrations. Previous studies have shown that some herbs or herbal formulations can offer cognitive benefits in treating AD. For instance, there is evidence that ginseng's main active ingredient, panaxsaponin, can enhance psychomotor and cognitive performance in those with AD [8]. Another Japanese-Chinese traditional medicine, Kihito, has been shown in mice to ameliorate A β -induced impairments in memory and object recognition [9]. The extracts we used (bugleweed, hops, sassafras, and white camphor) were selected by virtue of availability, on the condition that they possessed anti-inflammatory properties. Past studies have shown that neuroinflammatory cytokines reduced the efflux transport of A β from the brain, resulting in increased A β concentrations [10]. Thus, compounds that reduce inflammation may, in theory, reduce A β levels. An immortalized line of human neuroblastoma cells (SH-SY5Y) was chosen for this experiment because they divide rapidly and give rise to neurons, which AD primarily affects. Cells were transfected to overexpress APP prior to the extract treatments so that measurable amounts of A β ₁₋₄₀ peptide would be present during the experiment. After treating our cells with the various herbal extracts, we measured changes in extracellular A β ₁₋₄₀ peptide concentrations. To ensure that decreases in A β ₁₋₄₀ concentration were not due to cell toxicity, we also used an MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, assay to measure the viability of the cells, because MTT can readily penetrate viable eukaryotic cells in comparison with compounds used in other cytotoxicity assays [11]. All the herbal extracts used in our study have anti-inflammatory properties, which are known to decrease A β ₁₋₄₀ peptide levels. Thus, we predicted that all treatment groups would have decreased A β ₁₋₄₀ levels, with the possible exception of white camphor, because even small doses of this extract are known to be highly cytotoxic [12].

RESULTS

We chose SH-SY5Y cells for this investigation because they are part of an immortalized cell line that can divide indefinitely and are often used as an *in vitro* model to study neurodegenerative diseases, as well as neuronal function and differentiation [13]. We engineered these neuroblastoma

cells to overexpress APP (see Methods and Materials). After incubating the cells overnight to allow for sufficient APP production, we exposed them to a 1% herbal extract solution. We retrieved the supernatants from these cell cultures and measured the extracellular A β ₁₋₄₀ peptide concentrations by a sandwich ELISA. We could determine the effects of each extract on the extracellular A β ₁₋₄₀ peptide concentrations using the ELISA, but a decrease in A β ₁₋₄₀ could be a result of an extract's cytotoxicity, destroying cellular metabolism. Thus, we also measured the cell's viability in the overnight treatment using an MTT assay.

Addition of the control solution (1% ethanol) resulted in an extracellular A β ₁₋₄₀ peptide concentration of 26.3 fmol/mL (Figure 2). A β ₁₋₄₀ levels were largely unaffected by sassafras (concentrations were 30.1 fmol/mL, or 114.45% of the concentration measured for the control). SH-SY5Y cells exposed to bugleweed and white camphor treatments had diminished A β ₁₋₄₀ peptide concentrations of 11.1 fmol/mL and 0.1 fmol/mL, or 42.21% and 0.38% of the control value, respectively. On the other hand, the hops extract drastically increased A β ₁₋₄₀ concentrations to 95.4 fmol/mL, 362.74% of the control value.

We set the MTT assay cell survival cutoff value to 50% of the control value; previous studies have also used this cutoff to define certain compounds as irritants that would prevent accurate data collection of A β ₁₋₄₀ peptide concentrations [14]. The cell viability rates of bugleweed, hops, sassafras, and white camphor were, respectively, 98.99%, 81.94%, 70.18%, and 11.48% of the control value. Therefore, only the white camphor treatment caused the cell survival rate to fall below the 50% cutoff (Figure 3).

Taken together, the results demonstrate that bugleweed had the greatest effect of lowering A β ₁₋₄₀ peptide levels, because while cell survival rates remained relatively similar

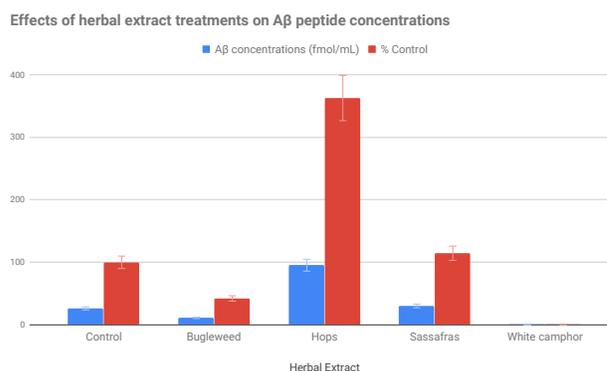


Figure 2. Comparing the effects of the various treatments on the A β ₁₋₄₀ peptide concentrations in fmol/mL and as a percent of control. The averages of the triplicate A β ₁₋₄₀ peptide concentrations in the control, bugleweed, hops, sassafras, and white camphor treatments were 26.3 fmol/mL, 11.1 fmol/mL, 95.4 fmol/mL, 30.1 fmol/mL, and 0.1 fmol/mL, respectively. As percentages of the control value, these levels were 42.21%, 362.74%, 114.45%, and 0.38%, respectively. The error bars represent 10% deviations from the measured results.

Effects of herbal extract treatments on cell viability

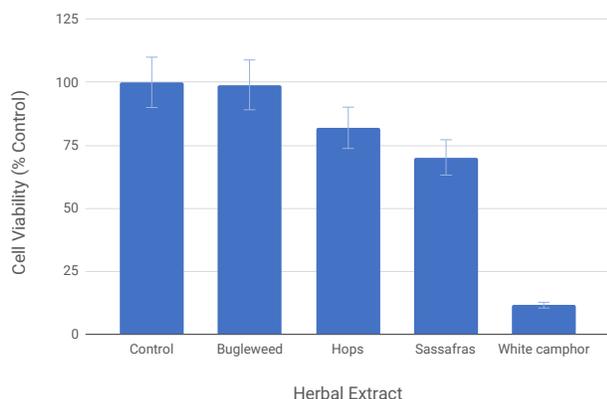


Figure 3. Comparing the effects of the various treatments on cell viability as a percent of control. The average survival rates of the triplicate bugleweed, hops, sassafras, and white camphor treatments were 98.99%, 81.94%, 70.18%, and 11.48%, respectively. The cutoff mark for cell toxicity was 50% cell survival, so only the white camphor extract was deemed toxic to the cells. The error bars represent 10% deviations from the measured results.

to those of the control (98.99%), bugleweed treatment caused $A\beta_{1-40}$ levels to decrease by 42.21%. Similarly, white camphor treatment caused $A\beta_{1-40}$ peptide levels to decrease by 0.38% (a 41.83% drop from the bugleweed treatment), but the cytotoxicity assay suggested that the decreased $A\beta_{1-40}$ peptide levels were due to cell toxicity, rather than a true effect on $A\beta$ peptide processing or degradation. White camphor's cytotoxicity limited our ability to assess the compound's effects on $A\beta$ processing or degradation due to inflammation. On the other hand, hops drastically increased $A\beta_{1-40}$ levels despite cell survival rates that decreased by 18.06% in comparison to the control. In fact, hops-treated cells showed a 322.51% increase in survival in comparison to bugleweed treatment. Furthermore, sassafras did not induce significant cell death, and $A\beta_{1-40}$ levels increased by 14.45% in comparison to control levels (a 72.24% increase compared to bugleweed treatment). While none of the effects observed in this experiment reached statistical significance, the dramatically different effects induced by each herbal compound tested was nevertheless striking. However, bugleweed's ability to greatly reduce $A\beta_{1-40}$ levels may be attributed to its anti-inflammatory property.

DISCUSSION

Excessive accumulation of $A\beta$ peptides leads to the characteristic amyloid plaques of AD. Thus, finding potential drug targets that lower these concentrations has been a major goal of various clinical trials. Although we observed changes in $A\beta_{1-40}$ peptide levels across different experimental groups, a mechanistic explanation for these effects is outside the scope of this study. Decreases may have been due to inhibition of secretases that cleave APP and release $A\beta$. We could test this hypothesis in the future by using a western blot

to compare the amounts of each protein fragment produced after the cleavage of APP with β -secretase and γ -secretase. Similarly, an increase in $A\beta_{1-40}$ levels could have resulted from over-stimulation of these secretases. Other ways these extracts could have decreased $A\beta_{1-40}$ levels are by degrading the peptide faster than normal or modulating the production of APP through mechanisms not explored in this experiment. Moreover, we measured $A\beta_{1-40}$ levels by the amounts of peptide found in the extracellular media. Future experiments could analyze intracellular peptide concentrations or the peptide aggregates that remain adhered to the cells when the media is removed. Comparing this data to that found in this experiment could offer further insight into the compound's effects and may help to determine whether these extracts also impact plaque formation. For example, increased $A\beta_{1-40}$ concentrations in media could result from the ready dissociation of peptides from the cell membranes, and changes due to extract treatments could represent potential drugs that prevent $A\beta$ peptide formations.

In this investigation, the only viable candidate for reducing amyloid plaques was bugleweed, because it was the sole compound that lowered $A\beta_{1-40}$ peptides levels without causing cell viability to fall lower than the 50% cutoff. Bugleweed is currently used as a thyrostatic, as it can reduce the symptoms of hyperthyroidism by blocking thyroid-stimulating hormone (TSH) production, inhibiting the binding of stimulating antibodies of Graves' disease to thyroid cells, and inhibiting iodine metabolism [15-16]. Several studies also indicate that bugleweed treats malaria, insomnia, and hypoglycemia [16-17]. In AD, microglial cells can become chronically activated to clear $A\beta$ peptides via phagocytosis. As a result, these cells release cytokines that result in inflammation [18]. Currently, any agents that can assuage neuro-inflammation are prime candidates for the treatment of AD. Although bugleweed has not been tested in clinical trials, it could be a potential drug, especially with its uses in treating other inflammatory disorders, including hepatitis, pneumonia, and bone disease [19]. Nonetheless, further *in vivo* experiments and clinical trials will be needed to determine the efficacy of bugleweed in both reducing $A\beta$ concentrations and reducing inflammation. For instance, because this experiment was performed on neuroblastoma cells, the experimental results do not necessarily translate to other cell models, such as neurons and glial cells, that are more directly related to AD. Going forward, experimentation on these cell lines will offer more definitive results on bugleweed's potential uses in AD treatment.

Further, the herbal extract packaging was not sterile or airtight, so the purity of each compound could not be ensured. However, alcohol-based extracts were used to minimize the chances of bacterial and fungal growth, and the precaution was taken to view the extracts under a microscope prior to treatment to ensure that there was no microscopic growth. Regardless, the extracts may still have contained other compounds or contaminants.

The differential growth of the cells in separate wells after overnight incubation may have also affected the $A\beta_{1-40}$ levels measured. The experiment assumed that the number of cells per well was the same, but this is likely not true due to the stochastic nature of cell culture. Higher counts of cells in certain wells would result in higher $A\beta_{1-40}$ production relative to that in other wells. To prevent large deviations in the data, variables, such as the amount of media and the sizes of the wells, were kept constant and each control and treatment group was cultured in triplicate. Additionally, future experiments can test the concentration of another protein, whose concentration is not expected to change. That value can then be normalized as a control for cell number to more accurately calibrate $A\beta_{1-40}$ production.

The MTT reagent used in the cytotoxicity assay is also slightly toxic to cells. To lessen the possible detrimental effects, the cells were only incubated with the reagent for two hours to prevent overexposure that would affect the cell survival.

While the herbal extracts chosen were not known to have properties that would decrease the amount of amyloid plaques, these tests simulate drug screening conducted in research laboratories and pharmaceutical companies. Because there is currently no approved treatment for AD, extensive research is being undertaken in order to discover the basis of neuronal loss that leads to cognitive decline and memory loss. Many clinical trials are focusing on drugs that will prevent the production and aggregation of $A\beta$, such as the inhibition of secretase enzymes that cleave APP. Others focus on removing $A\beta$ plaques post-formation through antibody targeting [20]. Unfortunately, these trials have failed to reduce the load of toxic plaques or improve cognition; however some recent studies with an $A\beta$ -targeted antibody therapy may show promise [21]. These unfavorable results may force researchers to explore new avenues to understand the molecular basis of the disease and find treatments for AD. Nonetheless, more information will be needed to produce appropriate drugs, and drug screenings similar to this experiment would be employed to determine the compounds' efficacy in treating AD.

MATERIALS AND METHODS

Transfection

For SH-SY5Y transfection, NanoJuice reagent (EMD Chemicals) was used according to the manufacturer's suggestions. A transfection enhancer with a DNA ratio of 2:1 was added to the media, and the cells were incubated for 72 hours prior to experimentation.

Cell culture

The SH-SY5Y neuroblastoma cells reached a state of confluency after being incubated at 37°C. In order to lift and seed these cells, the media was removed, and the cells were washed with 5 mL of phosphate buffered saline (PBS). After removing the PBS, 1 mL of trypsin was introduced into the

flask to break down the proteins that allow the cells to adhere to the flask. After incubating the cells with trypsin at 37°C for 5 minutes, 2 mL of media was added to dilute the trypsin solution. Then, the cells were gently pipetted up and down to break up cell clumps. Using a hemocytometer, an average of six cells was calculated per quadrant; after accounting for the trypan blue 1:2 dilution, the concentration of the cells was calculated as 5.75×10^5 cells/mL. The cells were diluted to a 4 mL cell to 11 mL media ratio, and 100 μ L per well of the cell suspension was plated in a 96 well plate to incubate overnight.

Herbal extract dilution

The herbal extracts were bought from Herb Pharm. Because they were alcohol-based to prevent bacterial and fungal growth, 1% dilutions were made to prevent the alcohol from killing the SH-SY5Y cells. After transferring 100 μ L of each compound into individual eppendorf tubes, the extracts were centrifuged to precipitate any debris. Next, the compounds were diluted in a 96 well plate by adding 198 μ L of media to 2 μ L of extract. The same was done for the control by adding 198 μ L of media to 2 μ L of ethanol.

Cell treatment

After overnight incubation of the SH-SY5Y cells, the media was aspirated without disturbing the cells. 100 μ L of the diluted extracts and control were added to the cells in triplicate wells and incubated overnight.

MTT assay

An MTT assay was used to assess cell viability after treatment. Because this assay involves a colorimetric reaction that can measure mitochondrial respiration, it indirectly assesses cellular energy capacity. All the media, which contains extracellular $A\beta_{1-40}$ peptide, was removed with a multichannel pipette and transferred to a blank 96 well plate (to be used in the ELISA). 100 μ L of MTT reagent diluted to a concentration of 0.25 mg/mL was added to each of the wells that contained the remaining cells. The plates were incubated at 37°C for two hours to prevent excessive toxicity. After two hours, the yellow tetrazole found within the MTT reagent was reduced by living cells to purple formazan crystals. Dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals and form a homogenous solution whose absorbance can be read with a spectrophotometer. Based on the wavelength absorbance, the survival rates of each of the treatments were measured as a percent of control. The cutoff mark for cell toxicity in the experiment was 50% cell survival.

Sandwich enzyme-linked immunosorbent assay (ELISA)

The wells were first coated with human $A\beta_{1-40}$ capture antibody. Human $A\beta_{1-40}$ 33.1.1 was diluted in PBS to 50 μ g/mL, and 100 μ L of the antibody was added to each well in the blank 96 well plate. The plate was then incubated overnight. After overnight incubation of the treated cells with capture

antibody, the capture antibody was discarded and 300 μ L of blocking solution (Block Ace) was added to block nonspecific binding sites. The plate was left at room temperature for 3 hours before blocking solution was discarded and wells were washed two times with 300 μ L of PBS. Afterwards, 80 μ L of electrical conductivity (EC) buffer and 20 μ L of the samples (set aside while performing the MTT assay) were added to each well for a total volume of 100 μ L in each well.

Next, a standard curve using A β ₄₀ (Thermo Fisher) was created using two-fold serial dilutions, the first one containing only EC buffer and the following having the concentrations of 3.125 fmol/mL, 6.25 fmol/mL, 12.5 fmol/mL, 25 fmol/mL, 50 fmol/mL, 100 fmol/mL, 200 fmol/mL, 400 fmol/mL, and 800 fmol/mL. 100 μ L of each standard was added in triplicate to wells that did not contain the samples. This plate was put in the refrigerator overnight to allow the samples and standards to bind to the capture antibody. After removing the samples and standards, the plates were washed two times with 300 μ L of PBS. The C-terminal A β ₄₀ 13.1.1 horseradish peroxidase (HRP) conjugated detection antibody was diluted at a 1 to 2000 ratio in Buffer C (Thermo Fisher). 100 μ L of the detection antibody was added to each well and incubated at room temperature for two hours.

After two hours, the detection antibody was removed by washing the wells two times with 300 μ L of PBS-Tween, and 100 μ L of a combination of TMB (3,3',5,5'-tetramethylbenzidine) substrate and HRP was added to each well. After 15 minutes, blue color changes in the wells were evident and analyzed under a spectrophotometer. Once the 800 fmol/mL standard reached an optical density (OD) of 2.0, 100 μ L of stop solution (sulfuric acid) was added. The solution turned yellow and the plate was read under the spectrophotometer again. This data was recorded, and the A β ₁₋₄₀ peptide levels were calculated as a percent of the control value.

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