Treatment of Bace-1 $^{-/-}$ Alzheimer's disease mice with Senegalese traditional herbal medicaments

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Class of 2016



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A Senior Comprehensive Project in Partial Fulfillment of the Requirements for a Bachelor of Science Degree from Allegheny College

I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code and to maintain the integrity of both myself and the College community as a whole.

_____
Pledge

Approval by the Senior Thesis Committee:

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Dr. French

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Dr. Mumme

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Abstract

Botanical medicine is a main component of Eastern traditional medicine. Alternative medical approaches are of increasing interest in the Western world. Therefore, there is a growing need for scientific based evidence of effectiveness and safety of botanical medicine treatments. To elucidate whether an herbal Alzheimer's disease (AD) protocol from a Traditional Hospital in Dakar, Senegal minimizes symptoms and signs of AD, I ran Bace 1 $^{-/-}$ Alzheimer's mice and C57 wild type mice through the Morris Water Maze and Y-maze. Animals were divided into four groups: Bace 1$^{-/-}$ mice receiving herbal medicaments, C57 mice receiving treatment, Bace 1 $^{-/-}$ mice receiving tap water, and C57 mice receiving tap water. Dosages for treatment were calculated based on average water consumption. Mice were treated and tested for a period of four weeks, and a second period that lasted eight weeks. There was a noteworthy decline in all Bace 1 $^{-/-}$ animals' performance in the MWM demonstrating AD signs were present. The herbal treatment had a negative effect on the mice decreasing the performance in the MWM in both the treated Bace 1$^{-/-}$ and C57 mice. The Y-maze test did not show any significant differences among groups. The AD protocol as a whole did not appear to be effective, however, individual plant extracts within the protocol have potential therapeutic treatment to lessen deterioration of the AD brain.

Introduction

L'Hôpital Traditionnelle de Keur Massar in Senegal, Africa is a unique and prestigious hospital in West Africa. Yvette Parés, a French researcher and student studying in Dakar, Senegal, founded the hospital in 1980. Her research on leprosy, a chronic, and now curable, disease affecting the skin and nerves, and her professors guided her to traditional, natural African medicine as a solution for leprosy. During her exploration she met Dadi Diallo and Yoro Ba who helped her create the leprosy clinic in Keur Massar, which became *L'Hôpital Traditionnelle* (Hôpital Traditionnelle de Keur Massar [Internet]).

Today their products are widely used along the West African coast and throughout Europe. The composition of the medicine is regulated by the director of the hospital, Djibril Ba, son of Yoro Ba and the Saltigués, a group of individuals that practice and study divination ideology, which is the practice of predicting future events or outbreaks. To create a new treatment, the hospital identifies a common disease in the community and assesses the signs and symptoms. Next both Djibril and the Saltigués identify possible solutions via online research, books, and ancestral knowledge. Finally, solutions are compared and the product is created and distributed (D. Ba, email correspondence; unreferenced).

The effectiveness of medications is based on patients' responses to the treatments. Plants are harvested from the on-site botanical garden and then mixed with other plants and extracts to create different medicines, which treat individual or multiple ailments. For common, chronic diseases such as cancer, AIDS, or

fibromyalgia there are established medicinal protocols, as is the case with Alzheimer's disease.

The Alzheimer's disease (AD) protocol contains a variety of different medications. Some key plants in the protocol are *Tamarindus indica (*tamarind)*,* *Moringa oleifera* (horseradish tree)*,* and *Mentha spicata* (spearmint). Seed coat extracts of tamarind have demonstrated neuritogenic and neuroprotective abilities, which could be useful in treating neurodegenerative disorders like AD (Tadtong et al., 2013). *Moringa oleifera* is known to decrease lipid levels, in response to a high cholesterol and fatty diet, and regulate blood glucose levels, both of which can be correlated to the reduction of brain metabolism in AD (Mehta et al., 2003 and Mbikay, 2012). Spearmint, which is also used in Danish folk medicine, exhibits moderate inhibition of acetylcholinesterase and reveals effective antioxidant activity (Mata et al., 2007 and Adsersen et al., 2006). The antioxidant activity of *Mentha spicata* may improve axonal degeneration, based on the idea that oxidative stress can lead to axonal degeneration in neurodegenerative disorders (Galea et al., 2012).

Creating medicines from the plants and using them in a protocol to treat Alzheimer's disease has proven to be successful for the hospital. Patients have reported elimination of nervous tension and some improvement of memory after receiving just the AD protocol. However, the improvement of memory is strongly correlated with the immune response of each individual. Thus, after the AD protocol is complete, the hospital prescribes the Immune Support Protocol in hopes to further improve a patient's memory (D. Ba, email correspondence). The protocol for Alzheimer's disease is located in the appendix.

As mentioned, the effectiveness of medications at L*'Hopital Traditionnel* is based on patients' feedback. This has proven to be a trustworthy system for the hospital, but many consumers from different cultures and countries would like to know more specific effects the herbal medications have in curing the patients, and at which stage of Alzheimer's disease they are most effective, if at all effective. More frequently individuals in Western society are turning to complementary and alternative medicines (CAM) due to a steady increase in chronic diseases and resistance to pharmaceuticals. Providing scientific evidence that CAMs cure or prevent disease would confirm their effectiveness and further promote alternative medical options.

AD in humans is categorized into several stages based on memory deficits and a patient's behavior (Wu et al., 2014). However, it has been observed that patients showing signs of dementia or early onset Alzheimer's already have accumulation of amyloid beta and tau protein in the brain, which are the physical changes that, for the moment, are irreversible (Oddo et al. 2003). In AD, the protein amyloid beta forms extracellular plaques in the brain while the hyperphosphorylated tau protein creates neurofibrillary tangles (Laferla et al., 2007). Amyloid beta 42, a form of amyloid beta most prone to oligomerization (LaFerla et al., 2007) in mouse models, first accumulates in the cortex and then proceeds to the hippocampus where tau pathology is initiated (Oddo et al., 2003). Overall, it is assumed that formation of neurofibrillary tau tangles is influenced by intracellular amyloid beta accumulation (Oddo et al., 2003; Billings et al., 2005).

In 4-month-old triple transgenic (3xTg-AD) mice, intraneuronal accumulation of amyloid beta is also correlated with an inability to retain information (Billings et

al., 2005). As time progresses, the intraneuronal amyloid beta deposits outside the cell form plaques that are seen in 6-month-old 3xTg-AD mice who have short-term memory impairment (Billings et al., 2005). When treated with human specific tau antibody (HT7), which acts as a marker, the tau protein pathology of 3xTg-AD mice at 6 months appears to be present, but is not hyperphosphorylated until the mice are 15 months old (Oddo et al., 2003). The early onset stage of AD in a human patient is equivalent to mice at 6 months of age (Billings et al., 2005).

Following the retention deficit of episodic memory, or the ability to recall past experiences, a decline in spatial orientation further limits a patient's memory (Billings et al., 2005). Spatial orientation can be tested in mice using the Morris Water Maze (MWM) (Billings et al., 2007). To complete the MWM task, the mouse is placed in a pool of water with a platform covered with approximately 1 cm of water. The mouse must escape the water by finding the platform and using four different spatial cues attached to the pool (Billings et al., 2007). Spatial orientation encompasses learning and memory. It allows animals to recall certain places, find objects, and discover the world independently (Astur et al., 2002). Therefore, it is used on a day-to-day basis. In humans, this is comparable to following directions based on landmarks.

The Y-maze is another behavioral test that is used to assess short-term memory of AD mice. The Y-maze consists of three connected paths formed in the shape of the letter "Y". The first trial consists of familiarizing the mouse with one arm. In the second trial a wall is removed to reveal another arm, which is considered the novelty arm. This maze differs from the MWM because instead of long-term recall, it measures short-term working memory and novelty interest by recording the amount of

time spent in the novelty arm of the "Y". Novelty interest is considered a normal and accepted trait in mice. The amount of time the mouse rests in the novel arm demonstrates the mouse has retained a recent memory and exhibits typical mice behavior (Wheelan et al., 2015).

The AD mice used in this study are homozygous Bace1 knock out (-/-) mice. The Bace 1 gene codes for a beta-secretase enzyme that cleaves at the beta-site of the amyloid precursor protein (APP). Specifically, it is the main beta-secretase that cleaves APP at the +1 and +11 sites. The +11 cleavage site appears to be the major splice site for the Bace 1 enzyme to form oligomerized amyloid beta plaques. Creating Bace 1 $^{-/-}$ mice diminishes amyloid beta plaques, because the enzyme is not present to cleave APP abnormally (Cai et al., 2011). Therefore, Bace 1 $^{-/-}$ mice could be used as a therapy for AD. Despite the reduction of amyloid beta plaques in Bace 1 $^{-/-}$ mice, they still express signs of AD (Wang et al., 2014). His team revealed that in the hippocampus where the mossy fiber (MF), a bundle of axons, innervates the Cornu Ammonis area 3 (CA3) region there is a synaptic dysfunction affecting the postsynaptic targets (CA3 pyramidal cells) (Wang et al, 2014). This finding demonstrates that the amyloid beta plaques are a sign of AD and not necessarily the cause. Wang et al. suggests the issue could be related to synaptic plasticity where the MF innervates the CA3 region (2014).

Research has been conducted on Bace 1 $^{-/-}$ mice in both the Y-maze and the MWM. In the Y-maze, Bace 1 $^{-/-}$ mice demonstrated increased exploration, but impaired spontaneous alteration behavior (Ohno et al., 2004). Laird et al. tested 3-6 month old Bace 1 -/- mice in the Y-maze, and found they crossed the threshold to

enter different arms the same number of times as other Bace1 mice (+/-, +/+), yet favored two of the arms demonstrating significantly less arm alteration. This behavior suggests early cognitive deficits in spatial working memory (Laird et al., 2005; Ohno et al., 2004). Also in this study 16-18-month-old and 3-6-month-old Bace1$^{-/-}$ mice completed the MWM task with probe trials, which measured the percentage of time spent in different quadrants, swimming speed, and time spent in the area near the platform. At 3-months-old there was no significant difference between Bace1$^{-/-}$ mice, other Bace1 mice (+/-, +/+), and the controls. However, 16-month-old Bace1$^{-/-}$ mice demonstrated significant decline in spatial reference memory during probe trials compared to controls, concluding that there is an age-dependent deficit in spatial reference memory for Bace1$^{-/-}$ mice (Laird et al., 2005).

The MWM is applicable to human spatial orientation. The hippocampus controls spatial memory, and its physiology is believed to be altered in patients with Alzheimer's disease by amyloid beta and tau tangles (Oddo et al., 2003; Astur et al., 2002). With this in mind, Astur et al. performed an experiment with patients who received unilateral hippocampus resections. A computerized MWM was created for humans. They found that humans, like mice, have spatial memory and learning impairments in the MWM, justifying the comparison of mice to humans (Astur et al., 2002). Also similar to humans, Billings et al. observed that practice improves performance when testing the 3xTg-AD mice in the MWM between the ages of 2 - 12 months (Billings et al. 2007). To test an additional factor, such as an herbal medicine, it was necessary to control that mice were equally trained prior to commencing the study to control for the effect of practice.

Traditional herbs from the AD protocol from *L'Hopital Traditionnel* were fed to homozygous Bace 1 $^{-/-}$ mice. Their spatial orientation was tested and compared with that of non-treated homozygous Bace 1 $^{-/-}$ mice in the MWM and Y-maze to see an equivalent performance to that of the healthy wild type mice. This study explored the value of the treatment of Bace1 $^{-/-}$ mice with Senegalese herbal medicaments to present their potential effectiveness in lessening side effects of AD.

Methods

Mice. All mice were housed individually, kept on a 12 hour light/dark cycle, and given access to food and water *ad libitum*. C57BL/6J.000664 wild type mice and B6.129-Bace1.004714 mice were the mouse strains tested (Jackson Laboratory, 2015).

Bace1 $^{-/-}$ mice. The homozygous strain of Bace1 null mice were bred by Elizabeth Caskey in the Steffee Hall Mouse Colony. The original mice were purchased from The Jackson Laboratory (Jackson Laboratory, 2015). The mice do not produce detectable amounts of oligomerized amyloid beta 1-40/42 or amyloid beta 11-40/42 plaques, and have fairly normal physical and behavioral tendencies. AD symptoms are displayed in these mice; the observations include: slower swimming speed, abnormal-spatial learning, spatial reference memory, and spatial working memory (Jackson Laboratory, 2015). Bace-1 null mice demonstrate these traits due to synaptic dysfunctions located where the mossy fiber innervates the pyramidal cells in the CA3 region of the hippocampus (Wang et al., 2014).

Treatments. Three different forms of medicaments were administered: powder, dry leaves, and liquid. The powder and dry leaf medications were seeped in boiling water for approximately 12 hours and then cooled for mice to drink. The liquid medication was added to the tea after it cooled. Treated mice consumed the tea concoction instead of their regular drinking water. The treatment commenced seven days prior to testing. Only ten of the twenty mice were treated (Figure 1). To determine the proper medicinal dosage for mice, water consumption was measured over a period of four days, averaged, and based on that average dosages were calculated. Please see appendix for calculated dosages and specific concoctions.

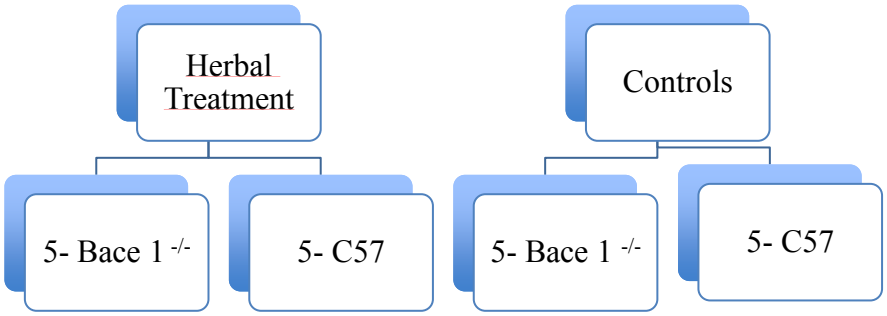


Figure 1. Pictorial representation of mice groups tested in this study.

Morris Water Maze (MWM). This procedure was adopted from Robinson, 2015. A platform with a 3.5 inch diameter was placed in a 17 gallon multi-purpose metal tub with a 2 foot diameter. The tub was filled with room temperature water, to minimize stress levels in mice, and it covered the platform by about 1-2 centimeters. Four

spatial cues were placed in four different quadrants. Mice were placed at the same starting position in the water for each trial. A timer was set for one minute after the mice were placed in the water. Their objective was to escape from the water by finding the platform in the shortest amount of time. If the mice did not find the platform after one minute they were placed on the platform for 10 seconds to orient themselves. Each mouse completed three trials every test day and was returned to its cage in between each trial. Any strange behavior or abnormal motor activities were recorded.

Y-maze. This procedure was adopted from Robinson, 2015. The apparatus of the Y maze is 11'x 2.8'x 5.5' and made up of three enclosed, black, metal arms to eliminate outside spatial cues or distractions. In the maze there were three pieces of yellow tape placed in each arm equidistant from each other. At the end of each arm were pictorial cues to further allow the mouse to orient itself. In trial one, the novel arm of the maze was blocked and the mouse was placed in the starting arm for two minutes to familiarize itself with the maze. After two minutes the mouse was removed and the Y-maze was sprayed with ethanol to remove the mouse's scent. For trial 2, the novel arm was revealed to each mouse. In a period of two minutes it was recorded how many times the mouse entered the novel arm (the one previously blocked off), the old arm, and the start arm, which is indicated when the mouse crosses the yellow tape. In between trial 1 and 2 mice had an 18-minute rest period in which all other mice completed trial 1. During all testing days mice were placed at the same starting arm. Any strange behavior or abnormal motor activities were recorded.

Analysis. Data were collected to determine escape latency time in the MWM and the percentage of time spent in the novelty arm of the Y-maze. At the end of each experiment an ANOVA was conducted with the statistical software package, JMP, to analyze overall results, and Graph Builder was used to create a pictorial form of the data. P values less than 0.05 were considered significant. A pie chart was completed via Microsoft Excel for each week of experiment 2 to determine the percentage of time spent in each arm in the Y-maze. Number of entries into each arm was divided by the total amount of entries for that week.

Results

Two experiments were completed in this study. Each experiment tested mice in the MWM and Y-maze, with half of the mice being treated for AD. Experiment 1 lasted for 4 weeks, and tested half of the AD protocol. Experiment 2 lasted for 8 weeks, and the AD protocol was completed.

Experiment 1. Over the four-week period results in the MWM and Y-maze were fairly consistent for the Bace 1 $^{-/-}$ mice tested: treated Bace 1$^{-/-}$ mice would escape the MWM faster than the control mice. In the Y-maze, control Bace 1 $^{-/-}$ mice explored the novel arm more than the treated Bace 1 $^{-/-}$ mice (Fig 2A). The treated Bace 1 $^{-/-}$ mice continuously were slower to escape the water in the MWM than control mice (Fig2 B). The wild type C57 mice, both the treated and the control, did not demonstrate any consistent patterns, suggesting the herbal treatment did not have an effect on the mice

(Fig 2 C, D). Overall, when the mice were tested at an age of 6-months-old no significant differences were found between treatment and control.

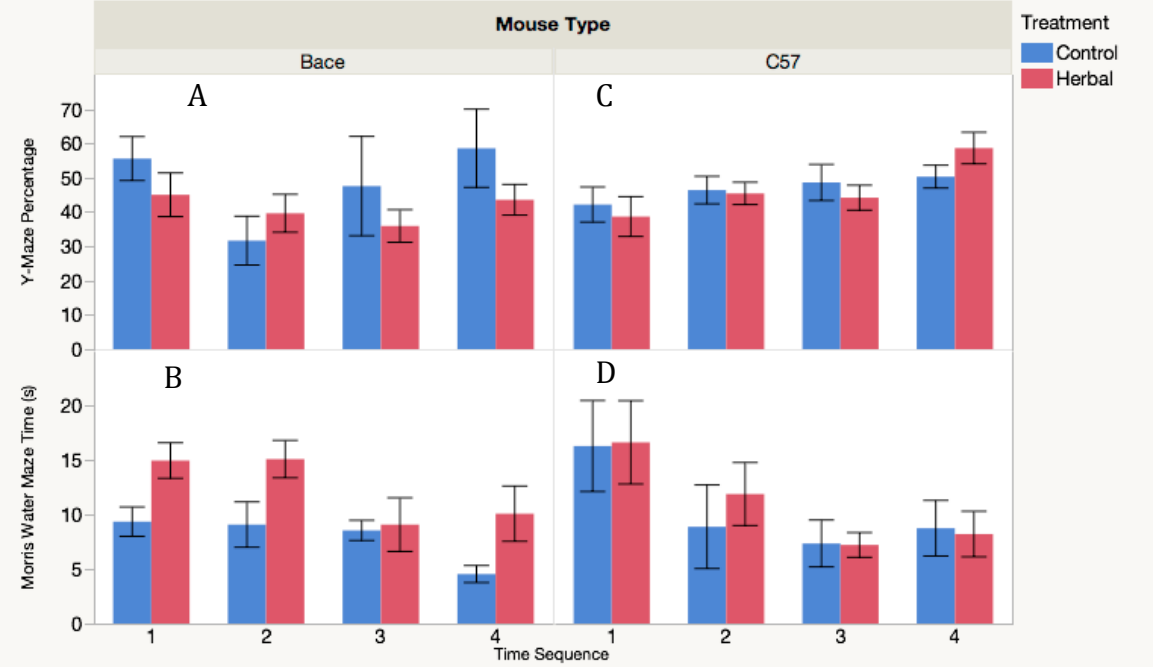


Figure 2 In experiment 1 mice are compared in the two behavioral tests: the MWM and Y-maze, over a period of four-weeks. A) The average time spent exploring the novel arm in the Y-maze is compared between control Bace1 $^{-/-}$ mice and Bace 1 $^{-/-}$ mice treated with herbal medicaments. B) Treated Bace1 $^{-/-}$ mice time in the MWM is compared to control mice. C) Treated and control C57 wild type mice performance in the Y-maze is compared. D) Average escape time of herbal treatment and control C57 wild type mice in the MWM is compared. N=20 mice with 5 mice in each group. Each error bar is constructed using 1 standard error from the mean.

Experiment 2. Previous research concludes that Bace 1 $^{-/-}$ mice demonstrate an age-dependent decline in spatial reference memory, which becomes apparent around 16-months-old (Laird et al., 2005). In experiment 2, mice were approximately 9-months-old, which led me to hypothesize that Bace 1 $^{-/-}$ mice not treated with herbal medicaments would begin to demonstrate a decline in both mazes. During experiment 2, the entire herbal protocol for AD was completed. During the first four weeks of the

protocol mice were tested every week, whereas in weeks 5-8 mice were tested once every other week, hence there are 6 weeks represented on the x-axis (Fig 3).

A

B

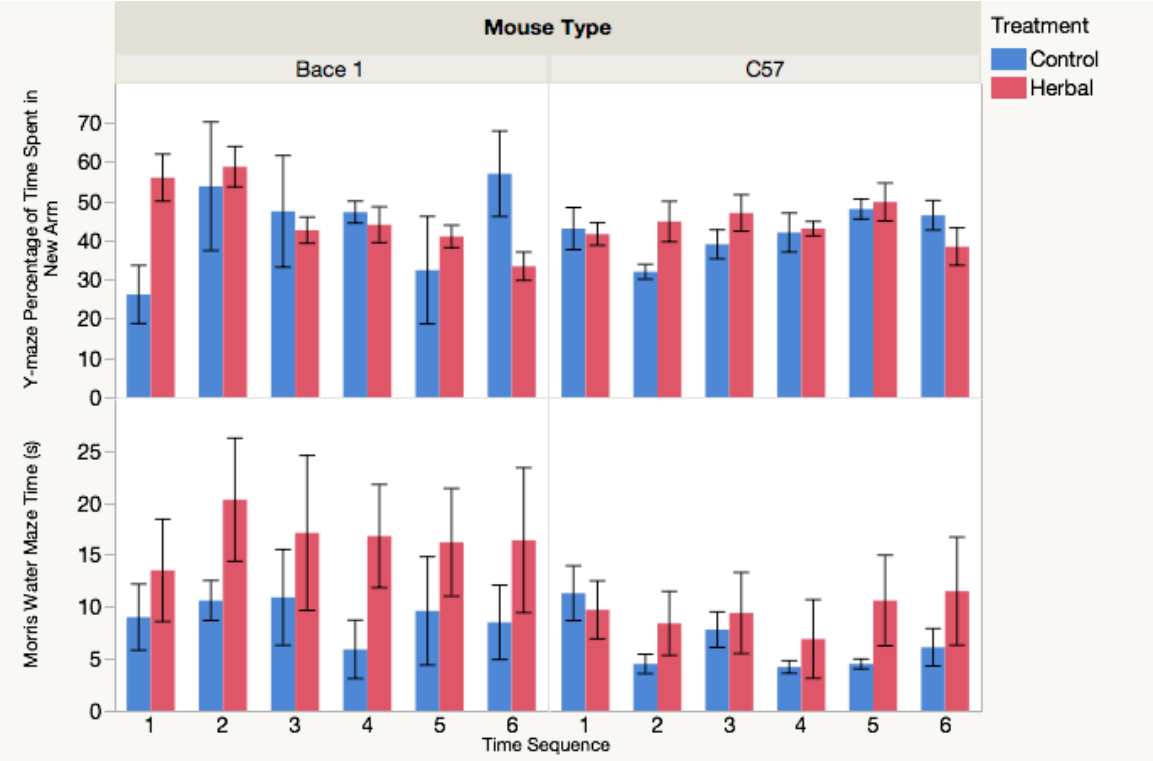


Figure 3. Experiment 2 tested mice at the age of 9 months. The results of the Bace 1 $^{-/-}$ control and treated groups, are on the left, while C57, control and treated, mice's results are on the right. The percentage of time spent in the novel arm in the Y-maze is shown (A). The average time it took mice to escape the water in the MWM is demonstrated (B). N=20 mice with 5 mice in each group. Each error bar is constructed using 1 standard error from the mean.

Y-maze. All Bace 1 $^{-/-}$ mice explored the novel arm for almost an equal percentage of time, except for week one and week five. Each of these weeks the treated Bace 1$^{-/-}$ mice showed greater exploratory behavior than their control counterparts (Fig 3A). As in experiment one, the C57 mice demonstrated almost equivalent exploratory behavior each week (Fig 3A). There was no difference in Y-maze performance among any of the treatment groups and mouse strains. However, in week 5 of testing in experiment 2 treated mice, Bace 1 $^{-/-}$ and C57, demonstrated a larger percentage of time spent in

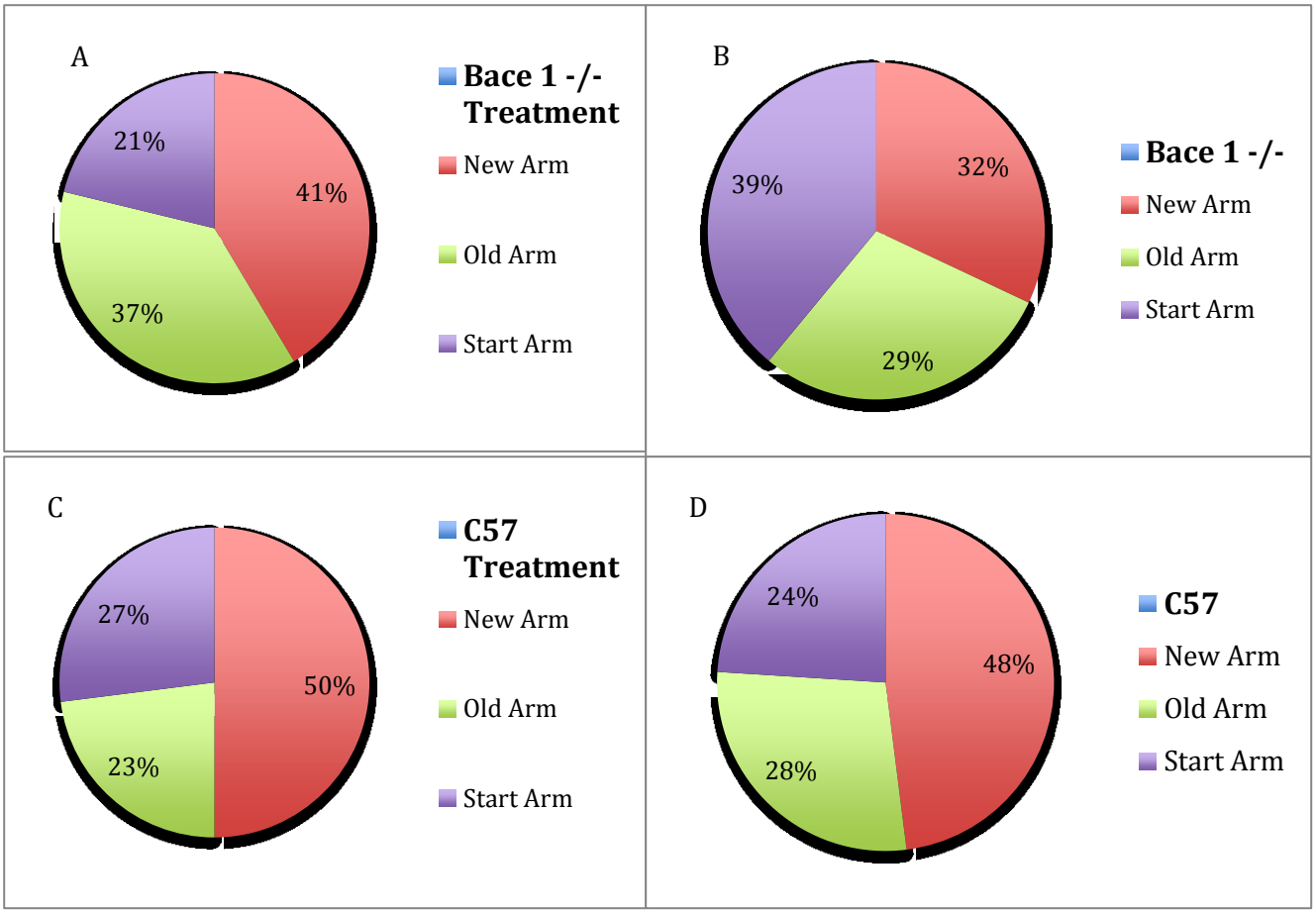


Figure 4 (below). Mean percentage times of week 5 results from the Y-maze for experiment 2 are depicted as pie charts. Treated Bace 1 $^{-/-}$ mice spent 9% more time in the new arm than the control Bace 1$^{-/-}$ group. (A,B). Wild type mice demonstrated accepted normal behavior of mice by spending a majority of time in the novel arm. (C,D)

the novel arm compared to the controls, revealing a greater exploratory behavior than the control mouse types in response to herbal medicaments (Fig 4).

MWM. Overall, the treated mouse types had a consistently longer escape latency time than the control mice, except for week one for the C57 mice (Fig 3B). The average amount of time it took each mouse type to escape the water revealed no statistical differences (Fig 3B). When average escape latency time is summed over all six weeks, herbal treatment of the Bace $^{-/-}$ 1 mice demonstrated a slower escape time compared to control Bace 1 $^{-/-}$ mice. The same phenomenon occurs in C57 mice but with overlapping standard error values (Fig 5).

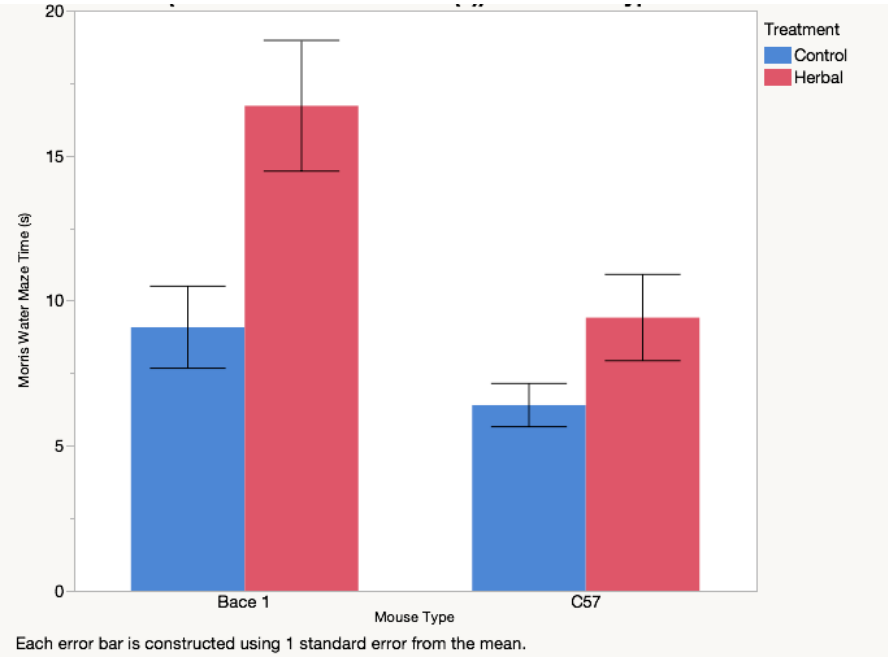


Figure 5 The mean escape latency time, in the MWM, for all mouse types is summed over all six weeks of testing in experiment 2. N=20 mice with 5 mice in each group.

In previous studies completed with 16-month-old Bace 1 $^{-/-}$ mice, probe trials revealed impaired spatial memory. However, during platform trials these same mice were able to develop strategies to find the hidden platform (Laird et al., 2005). Interestingly, in my study, at only 9-months-old Bace 1 $^{-/-}$ mice demonstrated a slight deficit in spatial reference memory compared to control wild type mice during platform trials (Fig 6).

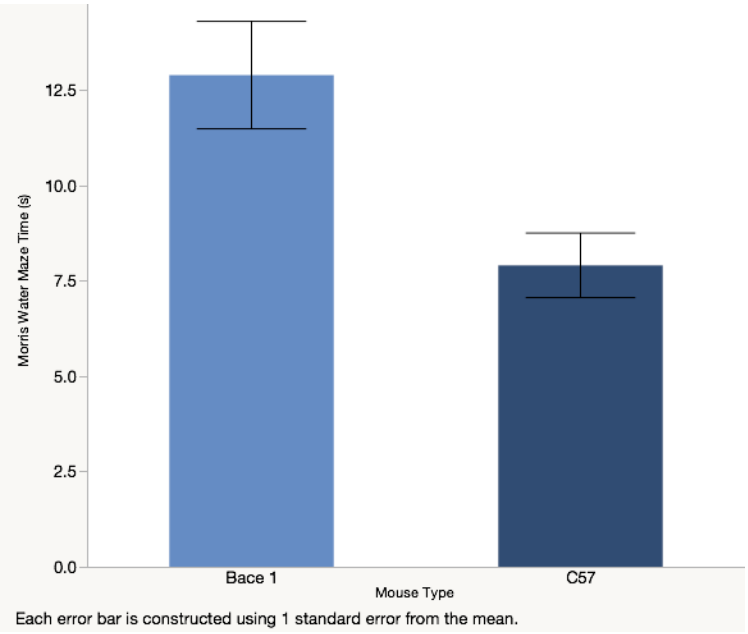


Figure 6. All mice, treated and control groups, are represented in the bar graph. The average escape time in the MWM for Bace 1 $^{-/-}$ mice was slower than C57 wild type mice, revealing a decline in spatial reference memory for Bace 1 $^{-/-}$ mice at 9-months-old. N=20 mice with 10 mice in each group.

Discussion

The present study showed that Bace 1 $^{-/-}$ mice ages 9-10 months old began to show a decline in spatial orientation, which is associated with memory recall. The treatment of Bace 1 $^{-/-}$ mice with an herbal AD protocol for eight weeks revealed no significant effects, however mice began to demonstrate positive reception to the treatment as they aged, specifically week 5 of experiment 2 around the age of 10 months old (fig 6).

Opposing my hypothesis, the treated Bace 1 $^{-/-}$ mice consistently demonstrated a slower escape latency time than the control Bace 1 $^{-/-}$ mice suggesting that the herbs had a negative effect on the animal's memory (fig 2). In addition, the treated wild type mice in experiment 2 were overall slower to escape the maze. The slower time could be correlated to the herbs acting as a relaxant. To further explore this theory

researchers could test the mice in the EPM (elevated plus maze). The EPM tests anxiety and consists of two closed arms, two open arms, and is elevated above the ground. Less time spent in the open arms correlates with an increase in anxiety (Ward et al., 2002). Previous research has revealed that a popular herb used to treat memory decline, *Ginkgo biloba,* has acted as an antianxiety buffer when mice were tested in the EPM (Ward et al., 2002). Qualitative analysis of treated Bace 1 $^{-/-}$ and treated C57 mice in my study demonstrated decreased levels of anxiety when placed in the MWM, in comparison to the mice not receiving treatment. Untreated mice demonstrated signs of anxiety including fast swimming, clawing the edge of the basin, and resistance when entering MWM and Y-maze, respectively. In addition, mice tended to be more jumpy in their cages during water changes and cleaning cages. I would predict that the treated mice from my study would act in a similar fashion to the mice used in the Ward et al. study, that is mice had decreased anxiety as determined by the EPM in response to *G. biloba* (2002).

Three herbs were suggested to be the main components of the protocol: *Tamarindus indica*, *Moringa oleifera,* and *Mentha spicata.* There is research supporting each plant's potential role in diminishing the progression of AD. The phenolic and flavonoid content of tamarind has been suggested to be a potential supplement for AD. Tadtong et al. (2013) tested tamarind extract on cultured neurons. Researchers focused on neurite growth and the antioxidant ability of tamarind. The tamarind extracts significantly enhanced neurite growth and neuronal differentiation. They also prevented toxicity from a free oxygen radical of H_2O_2. In conjunction with other research, this study concluded tamarind activated the mitogen-activated protein

(MAP) kinase, which promotes cell proliferation and prevents apoptosis, led to the neuroprotective behavior more so than the antioxidant effect (Tadtong et al., 2013). Moreover, Yang et al. (2008) concluded that stimulating different kinases, namely extracellular signal-regulated kinase (ERK) in the MAPK pathway, the outgrowth of neurites from neurons would be observed (Fig 7.). This research further confirms that tamarind, which activates the MAP kinase pathway, could demonstrate neuroprotective ability in the AD brain (Yang et al., 2008).

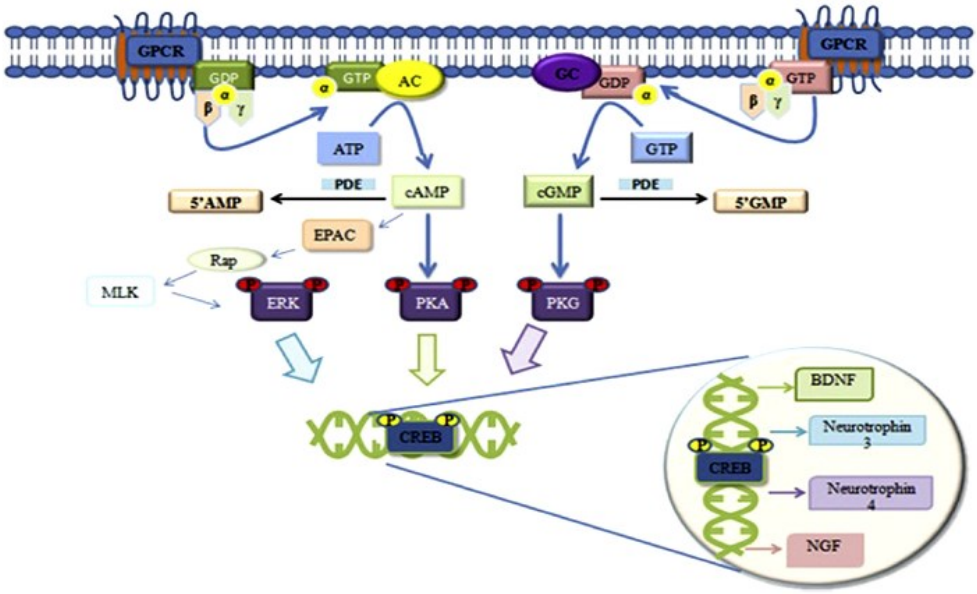


Figure 7. Cyclic nucleotide CREB activation is displayed. CREB transcribes for growth factors NGF and BDNF. ERK is the final kinase associated in the MAPK pathway and it activates CREB. Kumar et al., 2015.

Neurofibrillary tau tangles are found in the extracellular fluid of patients diagnosed with AD. Tau protein in a healthy patient binds to microtubules, promotes microtubule accumulation, and stabilizes them via specific phosphorylation sites. The AD brain consists of abnormally, or hyperphosphorylated tau (A68) that is phosphorylated at different sites than normal adult tau and is considered unhealthy. When one of these sites, serine396, is phosphorylated abnormally it most likely interferes with tau A68

protein's ability to bind with microtubules. Normally tau is able to stabilize microtubules, but in AD patients tau cannot bind to microtubules, which leads researchers to believe that the destabilization of microtubules results in neuronal degeneration (Bramblett et al., 1993). Tamarind activates the mitogen-activated protein kinase (MAPK) pathway, which can activate CREB via ERK phosphorylation (Fig 7). CREB transcribes DNA to produce growth factors like nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) (Kumar et al., 2015 and Pende et al.,1997). Furthermore, NGF studied by Tadtong et al. (2013) is known to induce microtubule formation and increase tau levels (Azad et al., 1994). The increase in tau levels restores MAPK activation (Azad et al., 1994). Thus, since tamarind activates MAPK, which results in production of NGF, then the final result is healthier neurons with increased amounts of microtubules and healthy tau protein. With increased microtubules and healthy tau, there is a greater chance that microtubules will remain stabilized and not result in neuronal degeneration. Moreover, tamarind should allow mice to retain long term and short term working memory tested in the MWM and Y-maze.

Since tamarind is involved in the NGF production pathway, it could be a potential candidate as a phosphodiesterase (PDE) inhibitor. PDE inhibitors are proposed to lead to a pathway, which could reduce amyloid beta plaque formation (Kumar et al., 2015). Cyclic nucleotides (cAMP and cGMP) are important for regulating cellular function and are closely correlated with cognitive function. Specifically, reports suggest that the activation of cAMP response element binding protein's (CREB) transcriptional activity enhances synaptic plasticity, neuronal growth, and development. CREB could

also enhance other growth factors, such as NGF, which have neuroprotective abilities. Phosphodiesterases (PDE) degrade cyclic nucleotides. Therefore, inhibiting PDE could increase cyclic nucleotide levels, and overall improve cognitive function. One study looked at various plants with putative cAMP or cGMP specific PDE inhibitory activity (Kumar et al., 2015). Future studies testing tamarind as a PDE inhibitor could determine whether or not tamarind shares this inhibitory behavior.

Another herb, *Moringa oleifera*, has a couple noteworthy characteristics that could play a role in the pathology of AD. *M. oleifera* can regulate blood glucose levels (Mbikay, 2012). This is important because AD has been proposed as type-3 diabetes (Steen et al., 2005). AD represents a neuroendocrine disorder similar to type 2 diabetes mellitus. Type 2 diabetes is caused from hyperglycemia. In a normal functioning patient, the hormone insulin will act as a transporter for sugar to be brought in the cell and stored as energy. However, in hyperglycemic patients the excessive amount of sugar in the blood causes insulin receptors on cells to become unresponsive to insulin. Thus the pancreas pumps out more insulin to bind to the free glucose creating a vicious cycle. Eventually, this leads to type 2 diabetes (Perlmutter, 2013). In 2005, researchers uncovered that reduced utilization of glucose and energy metabolism are a common pattern observed in AD patients. In addition, the expression levels of insulin, insulin-like growth factors (I and II), insulin receptor substrate (IRS) mRNA, tau mRNA, and amyloid precursor protein's mRNA were all reduced (Steen et al., 2005). Thus suggesting a weakened ability for the brain to consume glucose as energy. In 2013, researchers completed a statistical analysis of 2,067 individuals without dementia, and confirmed, based on glucose levels and

incidence of dementia diagnoses over several years, that a risk factor for dementia is diabetes (Crane et al., 2013). Since the early stages of AD and glucose levels are closely correlated with each other, *M. oleifera* could act as a potential treatment for early AD. Diabetic Wistar rats treated with *M. oleifera* leaf extract had decreased fasting plasma glucose levels and increased glucose tolerance compared to their controls (Mbikay, 2012). The study suggests that *M. oleifera* could enhance the weakened ability for the brain to consume glucose in a "pre-AD" state by increasing plasma glucose storage or usage and minimizing insulin resistance.

In addition to reducing glycemic levels, several experiments have demonstrated *Moringa oleifera* as a treatment for dyslipidemia (Mbikay, 2012). *M. oleifera* is known to decrease lipid levels in response to a high cholesterol and fatty diet, which could lead to more cell destruction via degradation of the phospholipid bilayer (Mehta et al., 2003 and Mbikay, 2012). Fruit extract and leaf extract of *M. oleifera* decreased lipid levels in rabbits fed a high cholesterol diet compared to their controls. The total cholesterol, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) levels were about 50% lower than the controls, the triglycerides were 75% lower, and the amount of carotid plaque build-up in arteries decreased by 97% (Mehta et al., 2003). Lipids are important for a brain to function accurately. A report published by the National Institute of Health compared memory function to cholesterol levels, and found that higher levels of cholesterol and LDL were associated with better memory function (West et al., 2008). LDLs transport cholesterol into the neuron to perform important functions (Perlmutter, 2008). When LDLs are damaged via oxidation or sugar binds to it accelerating oxidation, then the LDL can no longer enter the neuron.

Therefore, the brain does not receive the proper dosages of cholesterol necessary to function (Perlmutter, 2008). Furthermore, fatty acids, the building blocks of lipids, from fish oil are effective in improving memory (Languille et al., 2012 ; Serini et al., 2012 ; Hooijmans et al., 2007). The omega-3 fatty acids of fish oil increase cell membrane strength by resting in the hydrophobic fluid in the phospholipid bilayer of a cell (Melissa. Dengler, ND, Vitality Offices, Meadville, PA). Since *M. oleifera* is known to reduce lipid levels it could be associated with a negative effect on cell membrane degradation, and memory loss, hence agreeing with my findings that treated Bace $1^{-/-}$mice demonstrated decreased spatial reference memory more so than the control Bace $1^{-/-}$ mice in the MWM.

The third potentially significant herb in the AD protocol is *Mentha spicata*, commonly known as spearmint. A notable quality of *M. spicata* is its antioxidant activity (Brahmi et al., 2016). Oxidative damage is one of the proposed pathways leading to a decline in memory for AD patients (Mecocci et al., 1994). Oxidation precedes axonal degeneration, which results in inflammation and then extracellular amyloid beta plaque formation follows. A proposed connection between oxidation and axonal degeneration is the increase in reactive oxygen species (ROS), which damages the mitochondria and thus decreases levels of ATP. The decreased levels of ATP leads to axonal degeneration (Galea et al., 2012). Specifically axonal degeneration begins when a thiol-based reduction-oxidation (redox) sensor is altered by ROS. The damaged thiol redox sensor alters transcription factors, which affects cell signaling and leads to neuronal inflammation (Rosales-Corral et al., 2010). Neuroinflammation is a build up of localized immunoreactive Bace 1 and amyloid beta deposits in the

presynaptic terminals, which eventually causes dystrophy and apoptosis resulting in amyloid-beta entering the extracellular space (Zhang et al., 2009). Apoptosis has been shown to be a result of the activation of the enzyme ZNRF1 (zinc- and RINGfinger-1) by ROS, specifically NADPH oxidase (Wakatsuki et al. 2016). In a normal functioning neuron, Nrf2 (erythroid 2-related factor 2), a transcription factor, is activated when oxidative stress is detected in the cell. Nrf2 activates transcription of antioxidant enzymes. Scientists have not had success in treating oxidative damage in AD patients with antioxidant compounds. However, researchers hypothesize that increasing supplement antioxidants, like *M. spicata,* in addition to activating Nrf2 to transcribe antioxidant enzymes could reduce oxidative damage and decreases the incidence of apoptosis (Prasad et al., 2016).

Despite research providing examples that some herbs, which are included in the AD protocol, could be an effective supplement for AD, my results have shown no significant effect. This result echoes studies done on a well-known herbal supplement to treat memory loss, *Gingko biloba*. The Chinese tree has been cultivated and used for its various therapeutic abilities for thousands of years. Most relevant, *G. biloba* is used to treat vertigo, short-term memory, and lack of attention (Ward et al., 2002). Literature supporting this herb is similar to the present study, because *G. biloba* when tested provides inconsistent results, in that some clinical trials demonstrate improved memory, while other studies do not show similar improvement. Such was the case in the Ward et al. study, which tested the same wild type mice used in my study, C57 (2002). These mice were treated with Zingicomb (*Gingko biloba* extract and ginger) and were tested in the MWM. There were no significant differences between treated

and untreated mice (Ward et al., 2002). Another study using the same treatment as Ward et al. revealed Zingicomb enhanced spatial learning in rats tested in the MWM (Topic et al., 2002). These results contradict each other, yet *G. biloba* is used in humans as a treatment for cognitive therapy and has been used for many years. Researchers concluded that when *G. biloba* is tested in human trials, the improvement in memory observed was minimal, suggesting that *G. biloba* has an indirect effect on cognitive function. Therefore, spatial reference memory improvement would not be demonstrated in mice in a MWM because there are more factors involved that may not be present in the mouse brain (Ward et al., 2002). Perhaps this is the case with the AD protocol, where patients do report improvement in memory, but my study did not support that improvement. In addition to the herbal AD protocol *L'Hôpital Traditionnelle de Keur Massar* prescribes patients an immune protocol following the completion of the AD protocol, to ensure the patient's immune response is fully supported and functioning to its best ability and possibly increases neuroprotection in the brain. More commonly, patients of the hospital have reported stabilized memory and decrease in anxiety after taking the AD protocol and the immune support protocol one after the other rather than only taking the AD protocol. However, this is very subjective because each patient's immune system varies greatly (D. Ba, email correspondence; unreferenced).

The newer Bace 1 $^{-/-}$ mouse model could also be a point of question in this study. Few studies have demonstrated exactly when AD becomes apparent in these specific mouse models. One study reported that a decrease in performance was not observed until mice were 15 months old in both the Y-maze and the MWM (Laird et al., 2005).

Still, most researchers report that removing the Bace 1 gene is a potential therapeutic treatment for AD (Ohno et al., 2004; Farah et al., 2011). However, my research demonstrates a trend suggesting otherwise (fig 5). Cai et al. (2001) have demonstrated that Bace 1 is the main beta secretase that cleaves amyloid precursor protein (APP) at the +1 and +11 sites to create amyloid beta. The +11 cleavage site could be species specific and appears to be the major splice site for Bace 1. It is possible to create Bace 1 null mice that will reduce amyloid beta deposits in the extracellular fluid. Yet, there is still a Bace 2 beta secretase that cleaves APP (Cai et al., 2001). Based on this study, I would suggest that removing Bace 1 lessens disease, but Bace 2 still causes it at a slower pace. This hypothesis correlates well with my study and Laird et al. (2005) where mice began to show memory deficits around the ages of 10 months and 15 months, respectively. In addition, more recent studies suggest the Bace 1 $^{-/-}$ mice still experience AD because there is an issue with the postsynaptic pyramidal cells where the mossy fiber innervates the CA3 region in the hippocampus (Wang et al., 2014). Wang et al. suggests that Bace 1 is not a cause of AD, but simply in the pathway leading to amyloid beta formation, and the mouse model will still develop AD.

In this study treatment began during the learning phase of the MWM in experiment 1, and therefore their memory recall was indirectly tested. In order to directly test the effects of the herbal protocol on memory it is necessary to test after the mice are trained. Shif et al. (2006) directly tested the memory of male Sprague-Dawley rats treated with *Ginkgo biloba* in the MWM and found no significant results in the improvement of long-term spatial memory. However, rats treated with *Ginkgo biloba* during the learning phase demonstrated improved spatial orientation (Shif et al.,

2006). Perhaps the reverse could be true for the herbal protocol. Instead of treating mice throughout learning process, treatment could begin after training in the MWM to observe a direct effect of the herbal treatment rather than an indirect effect.

To enhance the results and conclusion of my study, analyzing cross-sections of mouse brains after the complete treatment of the herbal protocol would be beneficial. Based on our knowledge of tamarind's neuroproctective ability, a section could be analyzed for neurite growth or neuronal differentiation. Most studies measure growth and differentiation via PC12 cultures (cells derived from the adrenal medulla of rats) by measuring the longest neuron of the cell culture and counting cells (Yang et al., 2008). Another option could be to focus on the NGF, which binds to the tyrosine kinase receptor and leads to stabilization of microtubules and long-term neurite outgrowth (Yang et al., 2008). Perhaps by injecting an antibody of the receptor into the mouse, staining for that antibody on brain slices, and using a confocal microscope one could determine the prevalence of the receptor, and thus the potential for neurite growth.

Further experimentation could utilize a neuronal cell culture called, "Alzheimer's in a dish" created by Boston Massachusetts General Hospital. The culture is a three dimensional representation of human neuronal pathogenesis of Alzheimer's disease (Collins, 2014). In this culture method, amyloid beta precursor protein (APP) and prenesilin-1 create amyloid beta plaques and neurofibrillary tau tangles in the extracellular fluid. Ideally this model is used to discover effects of drugs on AD and neuronal mechanisms that could lead to AD, such as the known pathway of beta secretase cleaving APP to create amyloid beta (Choi et al., 2014). Testing the herbs

individually would be more effective to completely understand individual therapeutic potential of each herb. The culture could be an excellent template to directly test extracts of the herbs. For example, future researchers could extract flavonoids or phenols from each herb and observe their effects on the pathogenesis of AD (Kumar et al., 2015).

The present study provided additional evidence that Bace $1^{-/-}$ mice exhibit long-term spatial memory impairment. The herbal protocol from the *L'Hôpital Traditionnelle de Keur Massar,* Senegal did not demonstrate any significant effects on the AD brain of C57 and Bace $1^{-/-}$ mice, but contains several herbs with therapeutic potential which can be analyzed in future studies to diminish the progression of AD.

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Appendix

Protocol Alzheimer from *L'Hôpital Traditionnelle de Keur Massar*
1 period = 2 weeks
1 cc = 1mL

1ere période

- Poudre G
  Composition : phillantus acidus, citrus sp, combretum glutionosum
  Mode d'emploi : 1 cc de poudre dans un verre d'eau chaude. Boire le matin
  Quantité : 1 sachet
- Tisane G
  Composition : carica papaya, moringa oleifera, phillantus acidus
  Mode d'emploi : 1cc de plantes dans un verre d'eau chaude. Boire le soir
  Quantité : 1 sachet
- Extrait TOM
  Composition : saba senegalensis, landolphia heudelotti, morinda citrifolia,
  Mode d'emploi : 1cc de la solution diluiée dans un peu d'eau ou jus de fruit. 2 fois par jour
  Quantité : 2 flacons de 60ml

2eme période

- Extrait cactus
  Composition : opuntia tuina, moringa oleifera, aloe vera
  Mode d'emploi : 1cc de la solution diluiée dans un peu d'eau ou jus de fruit. 2 fois par jour
  Quantité : 2 flacons de 60ml
- Poudre MSS
  Composition : Tamarindus indica, ziziphus mauritioana, capparis sp
  Mode d'emploi : 1 cc de poudre dans 1 verre d'eau chaude. Boire le matin
  Quantité : 1 sachet
- Tisane MEM
  Composition : combretum micranthum, scoparia dulcis, mentha sp
  Mode d'emploi : 1 cc de plantes dans un v erre d'eau chaude. Boire le soir
  Quantité : 1 sachet

3eme période

- Poudre MSS
  Composition : Tamarindus indica, ziziphus mauritioana, capparis sp
  Mode d'emploi : 1 cc de poudre dans 1 verre d'eau chaude. Boire le matin
  Quantité : 1 sachet
- Extrait MTK
  Composition : Balanites aegyptiaca,
  Mode d'emploi : 1 cc de la solution dans un peu d'eau ou jus de fruits. Boire le matin
  Quantité : 1 flacon de 60ml
- Tisane MEM
  Composition : combretum micranthum, scoparia dulcis, mentha sp
  Mode d'emploi : 1 cc de plantes dans un verre d'eau chaude. Boire le soir

Quantité : 1 sachet

- Extrait NDR

Composition : cyperus esculentus, dispyros mespilliformis
Mode d'emploi : 1 cc de la solution dilué dans un peu d'eau ou de jus de fruits. Boire le soir
Quantité : 1 flacons de 60ml

4eme période

- Poudre MSS

Composition : Tamarindus indica, ziziphus mauritioana, capparis sp
Mode d'emploi : 1 cc de poudre dans 1 verre d'eau chaude. Boire le matin
Quantité : 1 sachet

- Extrait FDB

Composition : Fagara sp, securidaca longepedunculata, ziziphus mucronata
Mode d'emploi : 1 cc de la solution dilué dans un peu d'eau ou de jus de fruits. Boire le matin
Quantité : 1 flacon de 60ml

- Tisane MEM

Composition : combretum micranthum, scoparia dulcis, mentha sp
Mode d'emploi : 1 cc de plantes dans un verre d'eau chaude. Boire le soir
Quantité : 1 sachet

- Extrait DAK

Composition : Tamarindus indica, dialium senegalensis,
Mode d'emploi : 1 cc de la solution dilué dans un peu d'eau ou de jus de fruits. Boire le soir
Quantité : 1 flacon de 60ml

Dosages

*mice drink 5-8 mL/day avg: 6.5mL/day

Name	Dosages (based on average water consumption)	Dosages for a 7-day week
	1 cc = 1mL	
Powder G	1 cup*(8 oz/1cup)*(29.6 mL/ 1oz) = 237 mL 1mL/237mL = x/6.5mL x=0.0274 mL/day	0.0274 mL/day * 7 = 0.1918 mL
Tisane G	0.0274 mL/ day	0.1918mL
Extract TOM	0.5 cup*(8oz/ 1cup)* (29.6 mL/ 1oz) = 118.4mL 1mL/118.4 mL =x/6.5mL x=0.0550mL/day *2xday = 0.110mL/day	0.110 mL /day *7 = 0.77mL
Cactus Extract	0.110 mL/day	0.77 mL
Powder MSS	0.0274 mL/ day	0.1918mL
Tisane MEM	0.0274 mL/ day	0.1918mL

Powder MSS	0.0274 mL/ day	0.1918mL
Extract MTK	0.0550 mL /day	0.0550mL/day * 7 = 0.385mL
Tisane MEM	0.0274 mL/ day	0.1918mL
Extract NDR	0.0550 mL /day	0.385mL
Powder MSS	0.0274 mL/ day	0.1918mL
Extract FDB	0.0550 mL /day	0.385 mL
Tisane MEM	0.0274 mL/ day	0.1918mL
Extract DAK	0.0550 mL /day	0.385 mL