Clinical Intervention Study Protocol

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Biological Signatures in the Cognitive Effects of *Centella asiatica*:

Protocol for Aim 1

A pharmacokinetic study of a *Centella asiatica* product (CAP) in elderly participants with mild cognitive impairment receiving cholinesterase inhibitor therapy

NCT# 03937908

BIOLOGICAL SIGNATURES OF THE COGNITIVE EFFECTS OF *CENTELLA ASIATICA:*

A pharmacokinetic study of a *Centella asiatica* product (CAP) in elderly participants with mild cognitive impairment receiving cholinesterase inhibitor therapy

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IND #136099

STUDY TEAM ROSTER

Table 1. Study team roster

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PARTICIPATING STUDY SITES

This is a single site study, which will take place at the Marquam Hill Campus and Center for Health and Healing at Oregon Health and Science University, 3181 SW Sam Jackson Park Road, Portland OR, 97239.

PRÉCIS

Study title

Biological signatures in the cognitive effects of *Centella asiatica*: A pharmacokinetic study of a *Centella asiatica* product (CAP) in elderly participants with mild cognitive impairment receiving cholinesterase inhibitor therapy

Objectives

- *Primary objective:* To determine the oral bioavailability and pharmacokinetics of compounds from a single administration of CAP in demented elderly humans receiving cholinesterase inhibitor treatment.
    - The primary endpoints for this study are the maximum concentration (C_{max}) and the area under the curve (AUC) of known compounds from *Centella asiatica* (triterpenes and caffeoylquinic acids and their metabolites) in human plasma.
    - Secondary endpoints are the time of maximum plasma concentration (t_{max}) and half-life ($t_{1/2}$) of the known bioactive compounds and their metabolites to help determine dosage intervals.
    - Another secondary endpoint will be temporal changes in ferric reducing ability of plasma (FRAP) as an indicator of antioxidant potential over time.
    - An additional endpoint will be the levels of triterpenes, caffeoylquinic acids, and their metabolites in a pooled urine sample collected over 10 h after CAP administration.

- *Secondary objectives*:
  - To assess the tolerability of acute administration of CAP in demented elderly humans. Additional secondary endpoints include tolerability and detection of acute adverse events through monitoring by participant interviews, biometrics, electrocardiography, laboratory assessment of liver and kidney function, vital signs and questionnaires.
  - To investigate the activation of the NRF2 antioxidant response element pathway in peripheral blood mononuclear cells (PBMC) over 6 hours following CAP administration.

Design and outcomes
This is an outpatient open-label phase 1 pilot clinical study using a blinded randomized crossover design of two doses (n=8) of a *Centella asiatica* water extract product (CAP). This study is to determine bioavailability, safety and acute tolerability of two doses of CAP in mildly cognitively impaired elders aged 65-85 on cholinesterase inhibitor therapy.

Outcomes:
- *Primary outcome:*
  - A change in pharmacokinetic parameters (C_{max} and AUC_{0-10}) of plasma analytes after treatment with a product made from a water extract of *Centella asiatica* (CAP) with a detectable difference between doses (2g and 4g).
- *Secondary outcomes:*
  - The time of maximum concentration (t_{max}) and half-life ($t_{1/2}$) of the known bioactive compounds and their metabolites to help determine dosage intervals
  - Temporal changes in ferric reducing ability of plasma (FRAP) as an indicator of antioxidant potential over time
  - Safety and tolerability of single doses of CAP determined by participant interviews, biometrics, electrocardiography, laboratory assessment of liver and kidney function, vital signs and questionnaires
  - The level of triterpenes, caffeoylquinic acids, and their metabolites in a pooled urine sample collected over 10 h after CAP administration.
  - Activation of the NRF2 antioxidant response element pathway in peripheral blood mononuclear cells (PBMC) over 6 hours following CAP administration.

Intervention and duration
This is a study using a product composed of a water extract of the botanical *Centella asiatica*. The C_{max}, T_{max} and other pharmacokinetic parameters and tolerability assessments of two doses of CAP (2g and 4g) will be compared to one another. Participants will be in the study for up to two months. The minimum time a participant could be on study would be six weeks.

Sample size and population
The target population is elders aged 65-85 years old with a diagnosis of mild cognitive impairment for which they are currently on cholinesterase inhibitor therapy. There will be 4 male and 4 female participants in the study. This is a randomized crossover study, so no stratification will be implemented in randomization.

1. STUDY OBJECTIVES
1.1. Purpose
The purpose of this study is to measure the oral bioavailability and pharmacokinetics of known bioactive compounds from a standardized *Centella asiatica* water extract product (CAP) in

mildly demented elders on cholinesterase inhibitor therapy. Compound levels will be measured in human plasma and urine over 10 hours after acute oral administration of two doses of the botanical extract product. The dose giving maximum plasma levels (C_{max}) closest to those observed in our mouse studies, the area under the curve (AUC_{0-10}), as well as the rate of clearance ($t_{½}$) of the known compounds and time of maximum concentration (t_{max}), will be identified. These data will be used to inform decisions on the dosage and dosing frequency for future clinical trials.

1.2. Primary goal

To determine the oral bioavailability and pharmacokinetics of known compounds from two single administrations of two different doses of a *Centella asiatica* product in mildly demented elders on cholinesterase inhibitor therapy.

1.3. Endpoints/objectives

- *Primary objective:* To determine the oral bioavailability and pharmacokinetics of compounds from a single administration of CAP in demented elderly humans receiving cholinesterase inhibitor treatment.
    - The primary endpoints for this study are the maximum concentration (C_{max}) and the area under the curve (AUC) of known compounds from *Centella asiatica* (triterpenes and caffeoylquinic acids and their metabolites) in human plasma.
    - Secondary endpoints are the time of maximum plasma concentration (t_{max}) and half-life ($t_{1/2}$) of the known bioactive compounds and their metabolites to help determine dosage intervals.
    - Another secondary endpoint will be temporal changes in ferric reducing ability of plasma (FRAP) as an indicator of antioxidant potential over time.
    - An additional endpoint will be the levels of triterpenes, caffeoylquinic acids, and their metabolites in a pooled urine sample collected over 10 h after CAP administration.
- ***Secondary objectives*:**
    - To assess the tolerability of acute administration of CAP in demented elderly humans. Additional secondary endpoints include tolerability and detection of acute adverse events through monitoring by participant interviews, biometrics, vital signs, electrocardiography, laboratory assessment of liver and kidney function, and questionnaires.
    - An exploratory end point will be the activation of the NRF2 antioxidant response element pathway in peripheral blood mononuclear cells (PBMC) over 6 hours following CAP administration.

1.4. Specific aims

Aim 1: To assess the bioavailability and rate of clearance of *Centella asiatica* derived compounds in mildly demented elders on cholinesterase inhibitor therapy through a pharmacokinetic study.

- **Hypothesis**: We hypothesize that the triterpene and caffeoylquinic acid components from *Centella asiatica* will be bioavailable, and a dose giving similar plasma levels to those associated with cognitive effects in mice can be achieved.

Aim 2: To determine the acute tolerability of a *Centella asiatica* product in mildly demented elders on cholinesterase inhibitor therapy.

- **Hypothesis:** We hypothesize that acute usage of the *Centella asiatica* product will be well tolerated and produce no severe adverse events.

Aim 3: To investigate whether activation of the NRF2 antioxidant pathway can be detected in PBMC of mildly demented elders on cholinesterase inhibitor therapy following ingestion of a *Centella asiatica* product.

- **Hypothesis:** We hypothesize that activation of the NRF2 pathway can be measured as increased NRF2 gene expression and/or nuclear protein levels in PBMC during a 6 hour period following ingestion of the *Centella asiatica* product.

2. BACKGROUND

2.1. Alzheimer's Disease

Alzheimer's disease is a severe form of cognitive impairment and one of the most expensive and debilitating conditions known to modern medicine. The National Institute of Aging suggests that greater than five million Americans may have Alzheimer's disease, making it the sixth leading cause of death in the United States.[1] In 2016 alone, government health agencies (Medicare and Medicaid) spent an estimated $160 billion on Alzheimer's disease with a projected 365% increase by 2050.[2]

The pathogenesis of Alzheimer's disease is highly complex. Current evidence suggests it is a combination of genetics, environment and lifestyle factors making the development of treatments very challenging. Its most striking pathological feature is the accumulation of β-amyloid (Aβ) plaques within the brain.[3] Current pharmaceutical investigation is aimed at preventing the accumulation of, or promoting the clearance of these neurotoxic plaques (anti-amyloid immunotherapy)[4,5]; however, recent trials of such agents have failed to produce a significant clinical effect on Alzheimer's disease.[6,7] Drugs currently FDA-approved for the symptomatic treatment of Alzheimer's disease are either cholinesterase inhibitors[8] or act at the N-methyl-D-aspartate (NMDA) receptor.[9,10] Unfortunately, these treatments do not influence disease progression, and their effectiveness is highly variable.

Beyond cognitive impairment, Alzheimer's disease also has significant comorbidities including insomnia, depression[11] and anxiety.[12] Multiple interventions are often needed to manage these symptoms affecting patient compliance and safety. This warrants further investigation into effective, inexpensive and well-tolerated treatments for Alzheimer's disease and its comorbidities.

Gender differences have been observed in the incidence, development and progression of Alzheimer's disease, and in response to interventions targeting cognitive impairment. Women develop cognitive disability more rapidly compared to men.[13,14] Previous studies of intranasal insulin for cognition showed functional abilities were better preserved in women compared to men.[15] These differences highlight the importance of evaluating responses to any agent in both males and females. As part of this proposed project we will evaluate potential gender differences in bioavailability and pharmacokinetics.

2.2. *Centella asiatica*

Centella asiatica is a highly regarded botanical reputed in Eastern medicine to increase intelligence and memory.[16] In Western countries, it is sold as the dietary supplement "gotu kola"[17] for use in improving brain health and cognitive function. Preclinical studies have shown that *Centella asiatica* extracts have biological effects of relevance to memory, learning, aging, mood and potentially disease progression in Alzheimer's disease.[18] Water extracts of *Centella asiatica* have improved learning and memory in wild-type rats,[19] wild-type mice,[20,21] and rats subjected to central nervous system toxicity.[22,23] In addition, similar extracts have been shown to modify the brain structure by increasing hippocampal neuron dendritic arborization in adult

rats,[24] neonatal rats,[25] and neonatal mice,[21] potentially contributing to the observed cognitive changes. With regards to mood and behavior, ethanol extracts given to mice subjected to chronic and acute stress[26] and sleep deprivation[27] demonstrated anxiolytic properties. Similar ethanol extracts revealed antidepressant properties in rats.[28]

With applications specific to Alzheimer's disease, one study showed that long-term usage of a *Centella asiatica* extract (six months) reduced β-amyloid plaque burden in a mouse model of Alzheimer's disease[29]; however, studies performed at Oregon Health and Science University (OHSU) suggest that water extracts of *Centella asiatica* may to achieve its cognitive effects without a direct action on β-amyloid (Figure 1).[30] Rather, *Centella asiatica* appears to affect downstream targets protecting neurons from β-amyloid induced neurotoxicity.[20,31,32] This suggests that *Centella asiatica* may be able to limit disease progression even when β-amyloid deposition has already taken place, thereby representing a novel treatment mechanism, potentially complementary to the medications in development.

Human studies, although limited, support *Centella asiatica's* ability to affect cognition and mood. Placebo-controlled trials showed that herbal extracts, or dried herb, improved cognitive function in healthy elderly[33] and middle-aged[34] volunteers. In elderly participants with mild cognitive impairment, investigators found improvements in cognitive test results (Mini Mental State Examination) following use of dried *Centella asiatica* for six months[35]; however, no control group was incorporated. Unfortunately, these studies used highly variable *Centella* preparations that were poorly characterized and were either performed in cognitively normal individuals thereby limiting clinical applicability, or were not placebo-controlled. This warrants a robust, randomized, double blind, placebo-controlled study in cognitively impaired humans to examine the effects of a chemically well-characterized *Centella asiatica* extract on cognition.

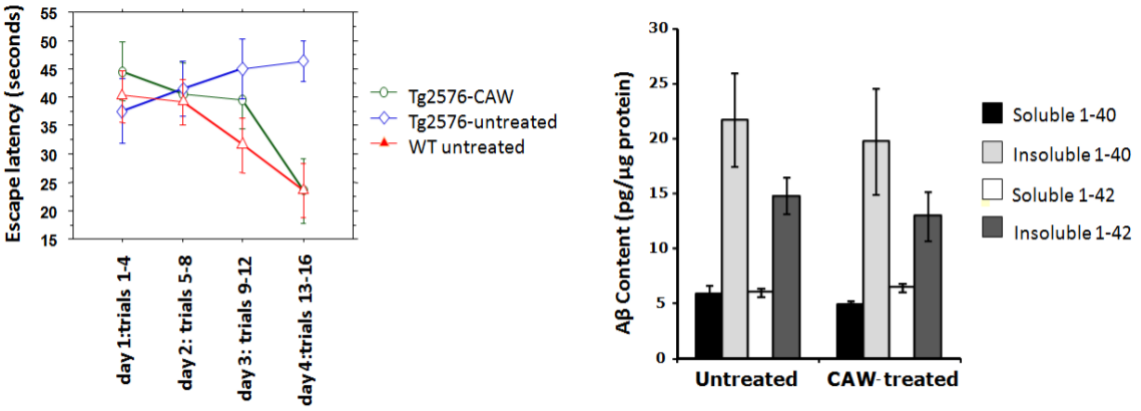

Figure 1. *Centella asiatica* water extract improves learning and memory in aged female Tg2576 mice without altering β-amyloid accumulation. Tg2576 mice (20 month old) were exposed to *Centella asiatica* water extract (200mg/kg/day) in their drinking water for 2 weeks before and during testing (total 5 weeks). Left – Morris water maze escape times. Right – Soluble and insoluble fractions of Aβ were extracted from cortical tissue and analyzed via ELISA (bars represent means +/- standard error).

2.3. Phytochemistry

Botanical agents are complex mixtures that show considerable variability due to growth conditions, geography, the parts utilized and preparation method.[36,37] Limited regulation of the production of these agents makes the efficacy of commercial products unpredictable. To this end, it is imperative that pharmacokinetic and clinical studies of botanicals use botanically

authenticated, chemically well-characterized, stable products that are standardized to a specific content of active ingredients.

Chemical analyses of *Centella asiatica* performed by our group and others have identified unique substances known as triterpenoid saponins (asiatic acid, madecassic acid, asiaticoside and madecassoside),[38,39] which are believed to be associated with *Centella asiatica's* neuroprotective and neurotropic effects.[40-43] Previous pharmacokinetic studies of *Centella* products have focused primarily on these compounds (most specifically asiatic acid) in plasma using purified extracts or synthesized compounds.[44-46] Our group has found, using high performance liquid chromatography coupled to mass spectral detection (HPLC-MS), additional active phenolic components known as caffeoylquinic acids in water extracts of Centella.[31] These acids have been shown to protect against excitotoxic and hypoxic damage[47] and β-amyloid toxicity in primary neurons.[31,48] To date, there are no pharmacokinetic studies of caffeoylquinic acids and triterpenes from water extracts of *Centella asiatica* in humans. This project aims to investigate the bioavailability of all of the aforementioned compounds from a water extract of *Centella asiatica* in human plasma and urine.

2.4. Absorption, distribution, metabolism and elimination (ADME) of *Centella asiatica's* active compounds

The complex composition of botanical materials renders ADME studies of their multiple biologically active compounds in humans or animal models particularly challenging. To date, *in vivo* ADME studies of *Centella asiatica* have primarily focused on the *Centella*-specific triterpenoids asiaticoside, madecassoside and their aglycones asiatic acid and madecassic acid. No reports were found of studies examining the ADME of caffeoylquinic acids (CQAs) or flavonoids after administration of *Centella asiatica*; however, there is literature on the ADME of these latter groups of compounds derived from other botanical sources.

Triterpenes: In humans, bioavailability studies of *Centella asiatica* have focused on asiaticoside and asiatic acid. PK studies have reported the time to reach the maximum plasma concentration (t_{max}) for asiatic acid was 4.0-4.6 hours post-ingestion of either purified asiatic acid or the triterpene mixture, TTFCA.[45,49] Rush et al.[50] found that the separate administration of equimolar oral doses of asiatic acid (12mg) and asiaticoside (24mg) in humans resulted in a similar total concentration (area under the curve) of plasma asiatic acid over 12 hours. Of note, peak plasma concentration was attained earlier when asiatic acid was administered compared to asiaticoside. The asiatic acid plasma profile also demonstrated more of a typical "sawtooth pattern" compared to asiaticoside suggestive of more immediate availability, compared to the delay required for *in vivo* hydrolysis of asiaticoside by intestinal enzymes.[49] This is also seen in a separate human study,[51] where there was no observed difference in the area under the curve of asiatic acid after the administration of pure asiatic acid and asiaticoside independently. In this study, the investigators also compared a single administration to a seven-day course of treatment, in which they found the area under the curve over 24 hours was much greater for chronic treatment than single treatment despite the short half-life. They propose this is due to slow metabolism of asiaticoside from previous doses, either because of delayed absorption or delayed metabolism.

Since most *Centella asiatica* preparations are not composed of purified compounds, further studies are needed to understand the pharmacokinetics of triterpenes when present in complex extracts. One study has been conducted in a dog model using an encapsulated water extract of *Centella asiatica* standardized to the amount of asiaticoside. In this study, the peak concentration of asiatic acid was observed at 2.7 hours after consumption.[46] As with the human study, this model demonstrated little asiaticoside within the plasma after oral administration due

to a proposed complete biotransformation into asiatic acid and its glucuronide and sulphate conjugates.[46,49]

Studies in rats have shown the absolute oral bioavailability of *Centella*'s triterpenoids is 16-30% for the glycosides and 50% for the acids[44,52,53] with primary elimination via the feces.[54] These low levels are proposed to be due to poor solubility, slow absorption, dissolution within the gastrointestinal tract, and/or variations in gastrointestinal transit time.[44,52] Bioavailability studies in rats using pure triterpenoid compounds, or ethanolic extracts of *Centella asiatica* aerial parts, have revealed similarities in the observed pharmacokinetic profile of madecassoside [54-56], madecassic acid,[56] and asiatic acid.[44,57] Most of the studies reported a rapid time to maximum concentration (T_{max}= 0.5-1.3h) and a delayed second maximum concentration peak in plasma levels and/or tissue distribution due to a proposed enterohepatic recirculation and/or selective and differential absorption from the gastrointestinal tract.[54-57] Notably, unlike the human studies on asiaticoside described earlier,[45,49] the glycoside madecassoside was reported to be present in the plasma of rats following oral administration.[54-56] Of these studies, Wang et al. also found the amount detected in plasma was affected by the disease state. In their rat model of arthritis, they noted higher levels of madecassic acid and lower levels of madecassoside in rats with induced arthritis, compared to non-arthritic rats.[56] They propose this may be due to a wider distribution of the madecassoside and decreased elimination *in vivo* due to the disease.

Xia et al. used the well-established zebrafish model and LC/IT (ion trap)-MS^n to identify any downstream metabolites from the aglycones. Using this sensitive methodology, they detected ten different phase one metabolites from asiatic acid and nine from madecassic acid, derived from hydroxylation and /or dehydrogenation reactions *in vivo*.[58] It is hypothesized that these metabolites may be more bioactive compared to their parent compounds. The occurrence and biological activity of these metabolites in human and other mammalian systems requires further study.

Few studies have looked at the tissue distribution of the triterpenes following oral administration, in particular brain levels of these compounds. One study detected asiatic acid and madecassic acid within the brains of mice.[59] Another focused only on madecassoside levels, which were reported to be below the lower limit of quantification in the brains of rats.[54] Given the previously discussed observed cognitive changes and brain biochemical effects seen in animal models with *Centella asiatica* administration, further investigation is needed to understand the brain bioavailability of these compounds and their metabolites.

Caffeoylquinic acids (CQAs): There have been no reported studies on the ADME of CQAs following oral administration of *Centella asiatica*, but there are multiple studies on the fate of these compounds when derived from other botanical sources.[60] Coffee, in particular, is a major source of CQAs, being rich in 3-, 4- and 5- CQA as well as the 3,4-, 3,5- and 4,5- diCQA isomers. Controversial data exists on the oral absorption of the CQAs.[60] In humans, high plasma levels of intact mono and diCQAs were reported following coffee consumption.[61,62] Both 1,5-diCQA and its methylated metabolite, 1,5-diferuloylqunic acid, were measured in human plasma following oral administration of 1,5-diCQA.[63] DiCQAs were also found in rat plasma after oral administration of *Ainsliaea fragrans* extract containing 1,5-, 3,4-, 3,5- and 4,5 diCQA.[64] By contrast, intact CQAs were not seen in the plasma after oral administration of artichoke extracts known to contain both mono and di-CQAs.[65] It has been suggested that absorption is dose dependent; at higher doses, a proportion of the CQAs may escape metabolism or hydrolysis due to enzyme saturation[66] facilitating their appearance in the plasma. It has also been noted, that mono and di-CQAs can isomerize *in vivo*.[67,68] A comparison of bioavailability and PK data

for CQAs and metabolites from multiple studies[60] identifies several other discrepancies and highlights the need for dose response studies.

The metabolism of CQAs is well documented[60,66,69,70] and includes hydrolytic cleavage of caffeic acid moieties from the quinic acid group, Phase I methylation of caffeic acid to ferulic and isoferulic acid, reduction of the double bond in hydroxycinnamic acids by colonic microflora to produce dihydrocaffeic, dihydroferulic and dihydroisoferulic acids, Phase II sulfation or glucuronidation of the hydroxyl groups of any these compounds, and glycine conjugation with the free carboxylic acid group of the hydroxycinnamic acids. Lactone forms of mono CQAs have also been identified.[69] Further breakdown of caffeic acid to ethyl, methyl and vinyl catechols by gut microflora has been proposed.[71] Urinary excretion products include the CQA metabolites rather than the CQAs themselves, mostly as sulfate and glucuronide conjugates.[69] Total urinary metabolites corresponded to approximately 30% of the CQAs intake from coffee.[69]

An LC-MS^n method capable of quantifying 56 compounds that may be derived from CQA metabolism in human plasma has been reported.[72] Using this method, Scherbel et al.[73] reported the pharmacokinetics in humans of monoCQAs and various metabolite groups listed earlier following consumption of coffee. Considerable inter-subject variation was noted in PK parameters. Peak plasma levels for the mono CQAs and hydroxycinnamic acid metabolites were generally rapidly attained ($t_{max} \leq 1$ hour) suggesting their absorption from the upper gastrointestinal tract, whereas the dihydro metabolites of the hydroxycinnamates peaked significantly later (5.5 to 8.5 hours), supporting their later formation in the colon. Similar results were found in other studies on CQAs from coffee[69] and artichoke extract.[74] Peak urinary excretion of CQA metabolites were reported in the 6-12 hour[73] or 5-8 hour[69] sample collections.

A PK and tissue distribution study of mono and diCQAs from *Ainsliaea fragrans* in rats[64] reported mono CQAs were undetectable in the plasma, but that diCQA levels peaked once at less than 30 minutes and again at around four hours, possibly due to enterohepatic circulation. Mono and diCQAs and caffeic acid were detected in multiple organs (lung, liver, muscle, brain, spleen, kidney and heart). Peak brain levels of individual diCQAs ranged from 25 ng/g to 20 µg/g following oral administration of the *A. fragrans* extract (46 mg/kg b.wt total diCQAs). Based on this data, the *Centella asiatica* oral doses (2.5 mg/kg b. wt. total diCQAs) used in our *in vivo* studies[30 20] could potentially give brain levels around1000 ng/g of individual diCQAs. This is comparable to the minimum neuroprotective concentration (500 ng/ml) we have observed *in vitro* for these compounds.[75] Caffeic acid and ferulic acids levels in the brain were significantly increased after injection of an intravenous bolus of these compounds to rats.[76] Ferulic acid but not caffeic acid was also found in rat brains after oral administration of *Portulaca oleracea* extract, which contains both compounds.[77] The detection of these CQAs and metabolites in the brain supports their potential to penetrate the blood brain barrier after administration of *Centella asiatica*, and suggests that *in vitro* effects seen for some of these compounds on neuronal cultures may be recapitulated *in vivo.*

2.5. Safety

Centella asiatica is an edible plant and the Botanical Safety Handbook[78] classifies it as a Class 1 herb that can safely be consumed when used appropriately. The widespread use of *Centella* as a dietary supplement and the available human studies support its safety.[17,33-35] The recommended dose of this herb, which is consumed in India and Sri Lanka as a healthy addition to the diet, is between 0.5 and 1.5 g dried leaf daily.[16,17] In multiple human studies using the fresh or dried whole herb, or in many clinical studies using extracts of *Centella asiatica*, no adverse effects were reported.[35,79-82] There have also been several clinical studies in which multi-component products containing *Centella asiatica* herb or extracts were found to be well

tolerated.[83-89] One study in which elderly participants consumed dried herb (1000 mg per day for 6 months) found no change in their liver enzymes (Serum glutamic oxaloacetic transaminase (SGOT) or serum glutamic-pyruvic transaminase (SPGT)).[35] In another study, administration of a concentrated extract at 750mg or 1000 mg per day daily for six weeks caused no change in liver enzymes Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) levels.[90] Two clinical studies on polyherbal preparations containing *Centella asiatica* (albeit in small quantities) also reported no changes in safety laboratory results.[83,86]

TTFCA (a purified triterpene mixture), was well-tolerated at all of the following doses in microcirculation studies: 30 or 60mg daily for 7 days[51]; 30 or 60 mg administered twice daily for 6 months[91]; and 60mg twice daily for 12 months.[92] Two studies on femoral and carotid plaque formation respectively[93,94] both used TTFCA at 180mg daily for 12 months. Again, TTFCA was well tolerated at this highest reported dose.

2.5.1. Reports of adverse effects in humans: It is noteworthy that despite its presence in dietary supplements in the United States, there are no reports of adverse events associated with *Centella asiatica* found in the FDA CFSAN Adverse Event Reporting System (CAERS accessed 8/08/2017). A recent review[95] of hepatotoxicity linked to herbs or dietary supplements lists two reports associated with *Centella asiatica*. In the first report, hepatotoxicity was seen in three women following ingestion of *Centella asiatica* products.[96] The publication does not give any details of the products (dose, whole herb or extract, etc), nor does it mention whether the products contained other ingredients in addition to *Centella asiatica*. Email correspondence between Dr. Amala Soumyanath (Principal Investigator on the present study) and the author (Dr. Oliver Jorge) established that the products contained "extracts" of *Centella asiatica*, and these were not the *Centella asiatica* mixed triterpene products similar to CAST known as TTFCA or TECA. The second report[97] was of a 15-year-old girl who experienced acute hepatitis after taking a product containing "several ingredients, one of them Gotu kola (*Centella asiatica*) 20mg for 6 weeks". The patient had also been on lymecycline for 8 weeks. While these reports are concerning, the lack of detailed information on the products used in the first report,[96] and the presence of additional ingredients, low *Centella asiatica* dose and concomitant use of lymecycline in the second report[97] make it unclear what role (if any) *Centella asiatica* played in the hepatotoxicity seen in these individuals.

In a recent clinical trial conducted at OHSU (unpublished), one female participant receiving CAST (a product similar to TTFCA) at 240mg per day withdrew due to abnormal liver enzyme levels, which returned to normal on stopping CAST. However, CAST was well tolerated by other participants who completed the 52-week study (dose escalated from 120mg and180 mg during weeks 1-8, to 240mg CAST daily for weeks 9-52). In this study, 29 of 43 randomized subjects (67%) experienced at least one adverse event (AE). The proportion of patients who experienced at least one AE in the Placebo (56%) and in the CAST (80%) groups was not significantly different (p-value = 0.1). AEs included transient abnormal liver and kidney function or gastrointestinal symptoms, which resolved on their own. Abnormal electrocardiograms (ECGs) were noted in some subjects and were linked to either pre-existing conditions, or returned to normal on subsequent tests. All AEs were graded as minor.

Two studies where *Centella asiatica* herb or extract was taken for six months[35,98] noted decreases in systolic and/or diastolic blood pressure, and one also reported improved sleep patterns among participants.[98] Due to their relatively mild nature, these effects were mentioned as potentially beneficial rather than noted as adverse effects of the intervention. In a study involving 48 subjects, one case each of constipation, abdominal bloating and itchiness were reported following 750mg or 1000mg daily of a hydroethanolic extract of *Centella asiatica*.[90]

Topical administration of *Centella asiatica* extracts and triterpenes is reported to cause contact dermatitis in some individuals.[99-101]

2.5.2. Non-clinical pharmacology/toxicology

Rats administered dried Centella asiatica herb:[102] Oral administration of dried *Centella asiatica* aerial parts to rats at 250, 500, and 1000 mg/kg body weight over 30 days did not produce any clinical signs of toxicity, morbidity or mortality or show any behavioral effects. However, significant increases in serum levels of liver enzymes (ALT and AST) and kidney markers (BUN and creatinine) were observed, mostly in a dose dependent manner, suggesting effects on both liver and kidney. No gross pathological lesions were seen in any organs after necropsy; however, histopathology revealed tissue alterations on microscopic examination in liver, kidney, and spleen particularly at the highest dose. A decrease in viable cell count of the liver was seen particularly with the highest dose. Spleen weight was increased by *Centella asiatica* treatment; however, the statistical comparisons given in this paper are not clear.

Rats administered Centella asiatica extract "INDCA":[103] For acute toxicity studies, INDCA* was administered orally to male and female rats at a single oral dose of 2000mg/kg body weight. No deaths, weight loss or treatment-related gross pathological changes occurred in the 14 days following treatment. This suggested that the LD50 of INDCA was >2000mg/kg body weight. For sub-chronic studies, INDCA was given orally at 250, 500 or 1000 mg/kg body weight for 90 days. No deaths were observed, nor were any clinical signs of toxicity observed over this period. While some significant differences to vehicle control were seen in hematological and biochemical parameters, these were not dose-dependent and the authors conclude that these were not indicative of an adverse effect. Mild focal lymphocytic infiltration and focal necrosis were seen in both liver and kidneys of INDCA treated animals. In this study, INDCA did not exhibit mutagenic activity in an Ames test either before or after metabolic activation. *INDCA was prepared by initial isopropyl alcohol extraction of dried *Centella asiatica* followed by concentration of the compounds of interest by solvent:solvent partition to yield a product containing 45.75% of the triterpene asiaticoside.[104]

Mice administered Centella asiatica extract:[105] The title and some sections of this paper state "acetone leaf extract" or "acetone extract", while the method describes the *Centella asiatica* extract as being made with 50% ethanol. The extract is stated as 5.75% of the original dried plant material. For the acute study, mice were administered 100, 500, 1000, 2000, or 4000mg/kg body weight of extract by gastric intubation. No deaths occurred over a 24 hour observation period after administration of the extracts, showing that the LD50 is > 4000 mg/kg. For subacute toxicity, animals were treated with 500, 1000, 2000 or 4000 mg/kg/day of extract for 15 days. No significant adverse effects were observed in these animals, nor were any gross pathological changes to organs seen at necropsy. The liver showed a significant decrease in weight in animals treated with 2000 or 4000 mg/kg/day. Hematological parameters were unaltered, and liver enzymes (ALT and AST) were not significantly different from control at any dose. Creatinine showed a significant decrease from control at the 1000 mg/kg/day dose only. The authors conclude that *Centella asiatica* is "destitute of toxic effects".

Effect on reproductive system in rats:[106] An extract of *Centella asiatica* (solvent could not be verified as the original paper was not available) was administered to male rats at 100, 200 or 300 mg/kg body weight for 42 days. All treatment groups showed some degeneration of spermatogenic cells, reduction of sperm count, and reduced testosterone levels compared to control rats.

Other studies: A manufacturer's brochure on *Centella asiatica* selected triterpenes (CAST; Indena SpA) refers to two studies, which report that asiaticoside was not toxic at up to 1g/kg on oral administration to rabbits, whereas toxicity was seen at 50-50mg/kg given intramuscularly. Rabbits given a standardized extract of *Centella asiatica* were reported not to show any teratogenic effects. These data could not be verified. We have ordered the two original publications and hope to include more details in our formal FDA IND application that is currently being composed. Tests in guinea-pigs show that the three triterpenes in CAST are very weak sensitizers.[107] The author concludes that the risk of developing contact sensitivity to the plant or its constituents is low. An increased incidence of skin papillomas compared to controls was observed in hairless mice painted for about 20 months with asiaticoside (0.1%) dissolved in benzene compared to benzene painted controls.[108] The authors conclude that asiaticoside is a weak tumor promoter in this model, its effects only appearing with repeated applications over a long period. A number of Internet sites erroneously quote a report of an increase in blood glucose in animals administered *Centella asiatica* citing a paper by Ramaswamy et al, 1970.[109] Careful reading of the original paper revealed no mention of this effect. Rats administered an ethanol extract of *Centella asiatica* (300-330mg/kg per day for 18 days) or water extract of *Centella asiatica* (200 mg/kg per day for 5 weeks) in our preclinical studies[20,30,110] showed no obvious adverse effects. These rodent doses are comparable, by interspecies scaling[111,112] to the proposed 2g and 4g per day human doses in this study.

2.5.3. Influence of *Centella asiatica* on drug metabolizing enzymes: *In vitro* studies suggest that *Centella asiatica* may contain compounds that are weak inhibitors of Phase I drug metabolizing CYP450 isoenzymes. These need to be evaluated *in vivo*. In studies on Phase I metabolic reactions, *Centella asiatica* methanolic extract and asiaticoside were reported to weakly inhibit recombinant human Phase I metabolizing isoenzymes CYP3A4, CYP2D6, CYP2C9 and CYP1A2 compared to standard inhibitors of these isoforms.[113] In another study, *Centella asiatica* was sequentially extracted with hexane, dichloromethane, ethanol and water. When tested against human CYP isoforms expressed by *E. Coli*, the ethanol and dichloromethane extracts showed a higher inhibitory effect on CYP2C19, CYP2C9, CYP2D6 and CYP3A4 compared to the water or hexane extracts. Madecassic and asiatic acid were stronger inhibitors compared to asiaticoside.[114,115] A standardized *Centella asiatica* extract (ECa223) containing mostly the triterpenes madecassoside (43%) and asiaticoside (39%) inhibited purified CYP3A4, CYP2C19 and CYP2B6, but did not affect CYP1A2, CYP2C9, CYP2D6 and CYP2E1.[116] Extracts of *Centella asiatica* were inhibitory to CYP 1A2 and CYP2C9[117] CYP3A4 and CYP 2D6[118] in human liver microsomes. When ECa223 was administered to rats at 10, 100, and 1000 mg/kg/d for 90 days, there was no significant effect on the Phase II metabolizing enzymes UDPGT, SULT, GST, and NQOR.[119] The effect of a water extract of *Centella asiatica* on these Phase I and Phase II enzymes is yet to be determined.

2.5.4. Conclusions: Based on the extensive use of *Centella asiatica* herb globally as a food, tea, traditional medicine or dietary supplement, and the lack of serious adverse effects in clinical studies of TTFCA, CAST or concentrated extracts of *Centella asiatica*, we expect that the product to be used in our trial (CAP) will be well-tolerated and safe. In particular, since hot water extracts of *Centella asiatica* are consumed regularly in teas,[120-122] it appears that harmful components are not extracted by hot water. However, no human safety data is available on concentrated water extracts at the doses (2g and 4g) proposed in this study. There is some concern about possible hepatotoxicity, but a study in which 1g per day of concentrated hydroalcoholic extract of *Centella asiatica* was administered for six weeks, did not cause any change in liver enzymes.[90] The total triterpenes (approximately 4% w/w) delivered by our proposed CAP doses is expected to be 80 and 160 mg/day. The lower doses are comparable to the TTFCA and CAST studies described earlier where these doses demonstrated potential liver

toxicity, which will be monitored with laboratory assessments in this study. We are also encouraged by the fact that no obvious adverse effects have been observed in our preclinical studies where mice were administered *Centella asiatica* water extract at 200mg/kg/day[20,30] or 500 mg/kg/day (unpublished) for five weeks. These murine doses are comparable, by interspecies scaling[111,112] to the proposed 2g and 4g per day human doses in this study. This study aims to provide preliminary safety data on this water extract product.

3. STUDY RATIONALE AND SIGNIFICANCE

Alzheimer's disease is a severe form of memory loss, initially manifesting as mild cognitive impairment and followed by a decline in cognitive function. There is an acknowledged need[123] to develop novel disease modifying agents to prevent progressive cognitive decline as patients and health providers are currently without viable pharmaceutical options for this debilitating disease. Concurrently, an interest in alternative therapies has increased substantially over the past two decades, especially among the elderly.[124-126] *Centella asiatica* may offer an effective way to bridge this gap, providing a botanical-based therapy to reduce cognitive impairment in Alzheimer's disease.

Centella asiatica shows remarkable cognitive enhancing and neuroprotective properties in extensive preclinical research performed by our group and by others. The small number of human clinical studies favors the use of *Centella asiatica* as a dietary supplement to improve cognitive function and brain health; however, limited regulation of these agents makes efficacy of commercial products unpredictable. Our ultimate goal is to develop an FDA-approved, standardized, botanical extract of *Centella asiatica* for evidence-based treatment of mild cognitive impairment and Alzheimer's disease.

This is the first human pharmacokinetic study of a standardized crude water extract of *Centella asiatica*. Previous pharmacokinetic studies have used refined combinations of *Centella's* unique triterpenes.[44-46] Use of such extracts eliminates possible chemical interactions and bioactive synergistic compounds that may result from crude extract preparation. Mouse studies have shown improvements in cognition in wild-type and Alzheimer's disease models using crude water extracts with or without triterpenes[20,30] suggestive of other neuroactive ingredients within *Centella asiatica* water extract. These appear to include caffeoylquinic acids and potentially other unidentified compounds. This study will preserve possible synergistic effects by using a crude water extract, but also standardize the product to the known bioactive constituents, which has not been done in previous human trials. In addition to studying the triterpenes, this study will investigate the pharmacokinetics and bioavailability of *Centella* derived caffeoylquinic acids and their metabolites, which has not been previously evaluated. The observed pharmacokinetic profile will inform dosage decisions and dosing frequency for future human clinical trials.

3.1. Preliminary data

Centella asiatica water extract is comprised of 0.94-2.41% triterpenes and 0.01-0.46% caffeoylquinic acids.[31] The abundant triterpenes asiaticoside and madecassoside are known to metabolize to the neuroactive metabolites asiatic acid and madecassic acid *in vivo*.[49] Similarly, the caffeoylquinic acids metabolize to caffeic acid, ferulic acid, isoferulic acid, and their dihydroderivatives.[127,128] Working with OHSUs' Bioanalytical Shared Resource/Pharmacokinetic Core (BSR/PKC) we have developed a sensitive methodology using HPLC-MS/MS to separate and analyze the triterpenes, caffeoylquinic acids, and their further metabolites within a human plasma matrix. Linear calibration curves have been obtained for each of these analytes (25–2000 ng/mL for asiatic acid, 5-2000 ng/mL for madecassic acid, and 5-100 ng/mL for several caffeoylquinic acids and their metabolites).

Previous pharmacokinetic literature in rodents and dogs has reported maximum concentrations of the polar caffeoylquinic acid compounds within one hour of ingestion[128,129] and the less polar triterpenes at 4-5 hours.[130] We have performed a pharmacokinetic study in wild-type mice on a low phytochemical diet to identify the C_{max} and time to reach C_{max} (t_{max}) of *Centella's* known bioactive compounds using the standardized water extract. We were able to detect the bioactive caffeoylquinic acids and their metabolites in plasma following oral administration of the extract using reversed phase HPLC-MS/MS (Figure 2, Table 2). Maximum plasma concentrations (C_{max}) of CQAs and metabolites (15 – 2350 ng/ml) primarily occurred within 45 minutes of oral administration (Figure 2) and there were no observable adverse events after extract administration. These results demonstrate *Centella asiatica* water extract is orally bioavailable and well-tolerated in mice warranting further evaluation in humans. They also confirm the proposed metabolism of caffeoylquinic acids from *Centella asiatica* water extract into their respective metabolites. To assess for changes in the bioavailability of the standardized manufactured product to be used for this study (CAP), we will perform another mouse bioavailability study.

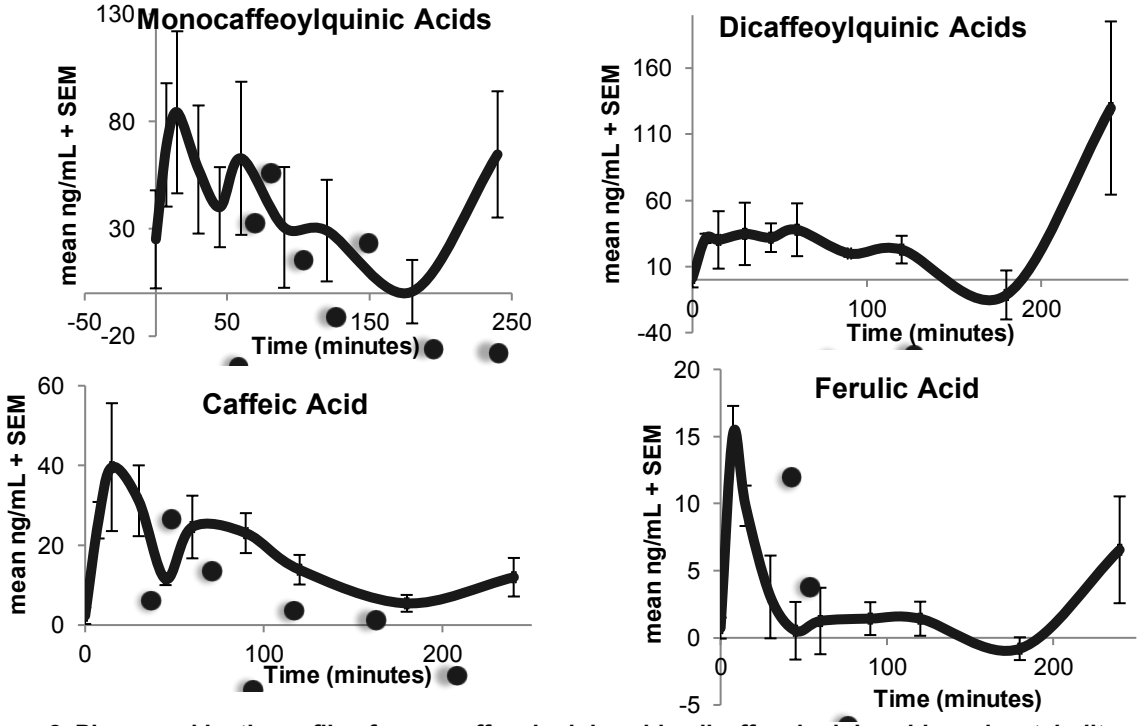

Figure 2. Pharmacokinetic profile of monocaffeoylquinic acids, dicaffeoylquinic acids and metabolites caffeic acid and ferulic acid in wild-type female mice. Six-month-old female mice (n=39) were administered *Centella asiatica* water extract (200 mg/kg) via oral gavage. Four mice were euthanized per time point and blood collected via cardiac puncture for analysis with HPLC-MS/MS.

Table 2. Pharmacokinetic parameters of caffeoylquinic acids and metabolites in mouse plasma after single oral administration of *Centella asiatica* water extract.

Analyte	C_{max} (ng/mL)	t_{max} (min)
Monocaffeoylquinic acids	84.3	15
Dicaffeoylquinic acids	37.8	60
Caffeic acid	39.6	15
Ferulic acid	15.2	7.5
Isoferulic acid	2.5	7.5*
Dihydrocaffeic acid	13.1	240*

Dihydroferulic acid	Not detected	Not detected
Dihydroisoferulic acid	124	30*
4-methyl catechol	4.9	120*
4-ethyl catechol	0.94	90*
3-(3-hydroxyphenyl)propionic acid	84.5	7.5*

* Inconsistent time curve

3.2 Rationale for exploration of NRF2 activation by CAP in PBMCs

The rationale for this investigation is provided in Appendix 2 of this protocol.

4. RESEARCH DESIGN AND METHODS

4.1. Study design

This is an outpatient open-label phase 1 pilot clinical study using a blinded randomized crossover design of two doses (n=8). Randomization will use an arm equivalence design to promote equal numbers of participants for each order schema. We will dispense a single administration of two different doses (2g and 4g) of a *Centella asiatica* water extract product (CAP) to the participants on two separate occasions two to four weeks apart (Figure 3). Participants will be required to adhere to a "low phytochemical diet" known as a "low plant diet" designed by the Oregon Clinical and Translational Research Institute's (OCTRI) Bionutrition Department for 48 hours immediately preceding each study visit and for the duration of each study visit. We will provide participants a handout at screening outlining the diet with meal examples. Serial blood samples (2-3 teaspoons; 10 -15 mL/sample) will be obtained through a peripheral intravenous catheter, once prior to *Centella asiatica* consumption and then over a 10-hour post-administration period (15, 30, 45, 60, 90, 120, 150, 180, 240, 360, 480, and 600, and min). Participants will also perform a 10-hour urine collection at each study visit to assess for excretion of *Centella asiatica* metabolites. EKG, measurements as well as kidney and liver function tests will be performed as described in section 6. Adverse events and tolerability will be assessed with questionnaires in-person at each study visit, by phone 24 hours after each study visit, and one week following study completion (Figure 3).

4.2. Outcomes

- Primary outcome:
  - A change in pharmacokinetic parameters (C_{max} and $AUC_{0\text{-}10}$) of plasma analytes after treatment with a product made from a water extract of *Centella asiatica* (CAP) with a detectable difference between doses (2g and 4g).
- Secondary outcomes:
  - The time of maximum concentration (t_{max}) and half-life ($t_{1/2}$) of the known bioactive compounds and their metabolites to help determine dosage intervals
  - Temporal changes in ferric reducing ability of plasma (FRAP) as an indicator of antioxidant potential over time
  - Safety and tolerability of acute doses of CAP determined by participant interviews, biometrics, electrocardiography, laboratory assessments for liver and kidney function, vital signs and questionnaires
  - The level of triterpenes, caffeoylquinic acids, and their metabolites in a pooled urine sample collected over 10 h after CAP administration.
  - Activation of the NRF2 antioxidant response element pathway in peripheral blood mononuclear cells (PBMC) over 6 hours following CAP administration.

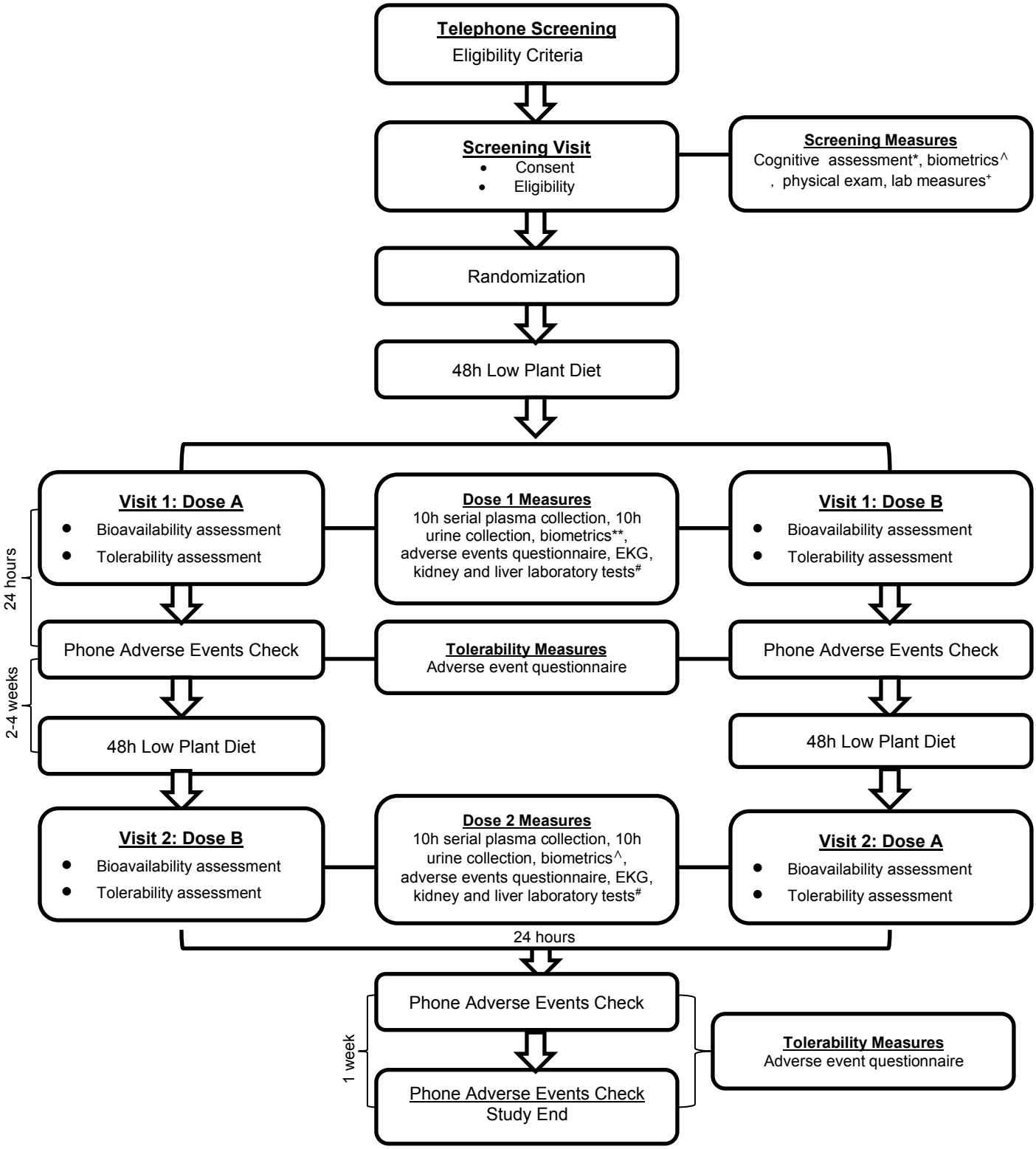

Figure 3. Study design. *Cognitive assessments: Clinical Dementia Rating (CDR), Mini Mental State Examination (MMSE), Geriatric Depression Scale (GDS); ^Biometrics: weight, height, body mass index (BMI), age, blood pressure, pulse rate, temperature; +Lab measures: complete blood count (CBC), comprehensive metabolic panel (CMP), urinalysis, # comprehensive metabolic panel

4.3. Study Population

4.3.1. Number of participants

Eight mildly demented elderly volunteers on cholinesterase inhibitor therapy (n=4 each female and male) 65-85 years of age will be screened and enrolled in the study. We estimate that we will need to screen approximately twelve potential participants to meet the eight volunteers needed to complete the study. We anticipate a possible dropout rate of 1 participant (14%) However, we are still powered to see differences between doses with a 25% drop out rate resulting in 6 remaining subjects.

4.3.2. Inclusion and exclusion criteria

Inclusion criteria: Participants must meet all of the inclusion criteria below at screening to participate in this study.

1. Age 65-85, male and female
2. Meet the National Institute of Aging – Alzheimer's Association core clinical criteria for mild cognitive impairment or probable Alzheimer's disease dementia with a Clinical Dementia Rating (CDR) score of 0.5-1 and MMSE score of 20-28
3. Report a history of subjective memory decline with gradual onset and slow progression over the last one year before screening; MUST be corroborated by an informant
4. On cholinesterase inhibitor therapy for Alzheimer's disease (AD) and must be on a stable dose for at least 12 weeks prior to baseline
5. Caregiver/study partner that can accompany participant to all study visits
6. Sufficient English language skills to complete all tests
7. Sufficient vision and hearing to complete all tests
8. No known allergies to *Centella asiatica*, Maltrin-100,coconut sugar, citric acid, malic acid, caramel color or any of their derivatives
9. Willingness to discontinue all botanical dietary supplements for one week prior to and during each study visit
10. Willingness to comply with a 48-hour low plant diet for each study visit
11. Absence of significant depression symptoms (Geriatric Depression Scale-15 score of <12)
12. Body Mass Index (BMI) greater than 17 and less than 35 at screening
13. General health status that will not interfere with the ability to complete the study

Exclusion criteria: All candidates meeting any of the exclusion criteria below at baseline will be excluded from study participation.

1. Current smoking, alcohol or substance abuse according to DSM-V criteria
2. Women who are pregnant, planning to become pregnant or breastfeeding
3. Men who are actively trying to conceive a child or planning to within three months of study completion
4. Severe aversion to venipuncture
5. Abnormal laboratory evaluation indicating asymptomatic and untreated urinary tract infection
6. Cancer within the last five years, with the exception of localized prostate cancer (Gleason Grade <3) and non-metastatic skin cancers
7. Comorbid conditions such as diabetes mellitus, kidney failure, liver failure, hepatitis, blood disorders, clinical symptomatic orthostatic hypotension, and unstable or significantly symptomatic cardiovascular disease
8. Significant disease of the central nervous system such as brain tumor, seizure disorder, subdural hematoma, cranial arteritis, or clinically significant stroke
9. Major depression, schizophrenia, or other major psychiatric disorder defined by DSM-V criteria

10. Medications: sedatives (except those used occasionally for sleep), central nervous system active medications that have not been stable for two months (including beta blockers, cimetidine, SSRIs, SNRIs), anticoagulants (i.e. Warfarin), investigational drugs used within five half-lives of baseline visit, systemic corticosteroids, neuroleptics, anti-Parkinsonian agents, narcotic analgesics, nicotine (tobacco, patches, gum, lozenges, etc.), *Cannabis sativa* (herb or edibles)
11. Non-Alzheimer dementia such as vascular dementia, normal pressure hydrocephalus or Parkinson's disease
12. Mini Mental State Examination (MMSE) score of <20 or >28 or CDR score >1 or zero
13. Unwilling to maintain stable dosage of AD medications throughout study duration
14. Inability or unwillingness of individual or legal guardian/representative to give written informed consent.
15. Current drug or alcohol use or dependence that, in the opinion of the site investigator, would interfere with adherence to study requirements.

4.3.3. Vulnerable populations

Our study population includes mildly demented elders. The signing of the consent form will follow guidelines from sections of the Code of Federal Regulation regarding protocols that involve decisional impaired adults. Whenever possible, consent will be obtained from the subject, assent must always be obtained directly from the subject. We will have an IRB approved plan (a Decisionally Impaired Adult Supplement) to utilize surrogate consent by a legally authorized representative (LAR) if the subject gives assent but is not capable of signing consent. The LAR may be different from the study partner. In accordance with OHSU POLICY HRP-021, the following individuals may serve as an LAR for an adult subject who lacks capacity to consent, in order of priority:

1) Health care representative who is legally authorized by a valid advance directive or health care power of attorney
2) Court-appointed guardian
3) If the above two do not exist or cannot be located with reasonable effort, another surrogate who knows and can represent the previously expressed wishes of the potential subject, in the following order of preference:

a) Spouse or registered domestic partner
b) Adult child
c) Either parent
d) Adult sibling
e) Adult designated by others on this list, if no one on the list objects
f) Other adult relative or friend who has an established relationship with the potential subject

A signature line has been included in the participant consent form for the LAR. The LAR will co-sign consent for subjects capable of signing consent at the start of the study, in case the subject's cognitive impairment worsens over the course of the study. If the subject is not capable of signing consent, the LAR will sign the consent form only after the subject gives assent for participation.

Based upon the age range needed in our study population, children will be excluded. We will exclude women who are pregnant, planning to become pregnant or breast-feeding. We will also exclude prisoners. We will not exclude any ethnic group.

4.4. Setting

All participants will be enrolled at Oregon Health and Science University (OHSU) and the OHSU Institutional Review Board (IRB) will oversee the study. There will be no additional sites for this

study. All screening and study visits will be conducted at the Oregon Clinical and Translational Research Institute's Clinical and Translational Research Center (CTRC). Screening visits will occur in the equipped outpatient rooms and the study visits will occur in the infusion suite of the CTRC. All plasma and urine storage will occur in a -70°C freezer in Dr. Quinn's laboratory in the Biomedical Research Building and processing will occur in Dr. Soumyanath's laboratory in Richard Jones Hall at OHSU. All sample analysis will occur at the Bioanalytical Shared Resource/Pharmacokinetic Core (BSR/PK) in Richard Jones Hall at OHSU and/or the mass spectrometry service at Oregon State University.

4.4.1. Data management

We will use the Oregon Clinical and Translational Research Institute's installment of REDCap to manage the data and prevent data corruption or loss. All analyzed data will be stored in a password-protected folder on OHSU's X-drive. All paper case report forms, consent forms and questionnaires will be scanned into the participant's REDCap entry. Only people directly involved with the study will be granted access to the REDCap database and X-drive with access specific to their role in the project as determined by the Principal Investigator.

4.5. Institutional Review Board (IRB) review

This protocol and the informed consent document and any subsequent modifications will be reviewed and approved by the IRB or ethics committee responsible for oversight of the study.

5. INTERVENTION

5.1. *Centella asiatica* water extract product (Figure 4) (information on the preparation method is confidential/proprietary): *Centella asiatica* dried herb (aerial parts) was purchased through Oregon's Wild Harvest (OWH; Redmond, OR) and authenticated by organoleptic analysis, thin layer chromatography (TLC), and high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Heavy metal testing of the raw herb and a pilot hot water extraction at OHSU was performed to ensure low levels of all heavy metals and compliance with the Food and Drug Administration (FDA) and American Public Health Association's (APHA) limits. Pharmachem Laboratories (PCL: Kearny, NJ), a Good Manufacturing Practice (GMP) certified facility, performed a large-scale hot water extraction of *Centella asiatica* using a standardized protocol. The water extract was spray dried onto a food grade maltodextrin matrix at PCL and then shipped to OWH for blending with a food-grade sweetening and coloring agent before packaging into individual dose sachets. To yield different doses (equivalent to 2g and 4g of water extract) the maltodextrin matrix, coloring agent and flavoring agent (excipients) were blended in appropriate ratios so that each dose will contain the same amount of the excipients but different amounts of the water extract. The final product is to be referred to as *Centella asiatica* product (CAP) and is to be reconstituted in ten to twelve ounces of room temperature water and consumed by mouth once daily. Samples of the raw herb, extract and product have been retained for fingerprinting using high-performance liquid chromatography-mass spectrometry (HPLC-MS) and voucher samples deposited in Dr. Soumyanath's (PI) laboratory and the Oregon State University Herbarium.

OWH will send the sachets of intervention to the OHSU Research Pharmacy for storage at in the freezer (-20°C) and dispensing following the Pharmacy's applicable policies and procedures. Dr. Soumyanath, members of the study team, and the mass spectrometry service at Oregon State University will perform quality control and stability assessments throughout the study. The study has received Investigational New Drug (IND) approval from the FDA (136099). The drug will be used in compliance with the requirements of HRP-815.

5.2. Dispensing

Each sachet will be dispensed to a member of the study team just prior to the participant's study visit and recorded by the Pharmacy staff including the date, participants ID number and dosage for accountability. Participants will receive two single administration doses of the *Centella asiatica* product equivalent to 2g and 4g of *Centella asiatica* water extract. The order in which they receive these doses will be random to prevent a tolerance effect. The doses selected are based on allometric scaling[131] from previous murine studies, which showed that 1.4 g/day of *Centella asiatica* water extract for a 70 kg human was equivalent to the 200 mg/kg/day estimated intake in mice. Participants will consume CAP in the presence of a member of the research team following baseline measurements, in order to ensure adherence to the study protocol.

5.3. Destruction

Following study completion, the remaining intervention sachets will be provided to Dr. Soumyanath for destruction.

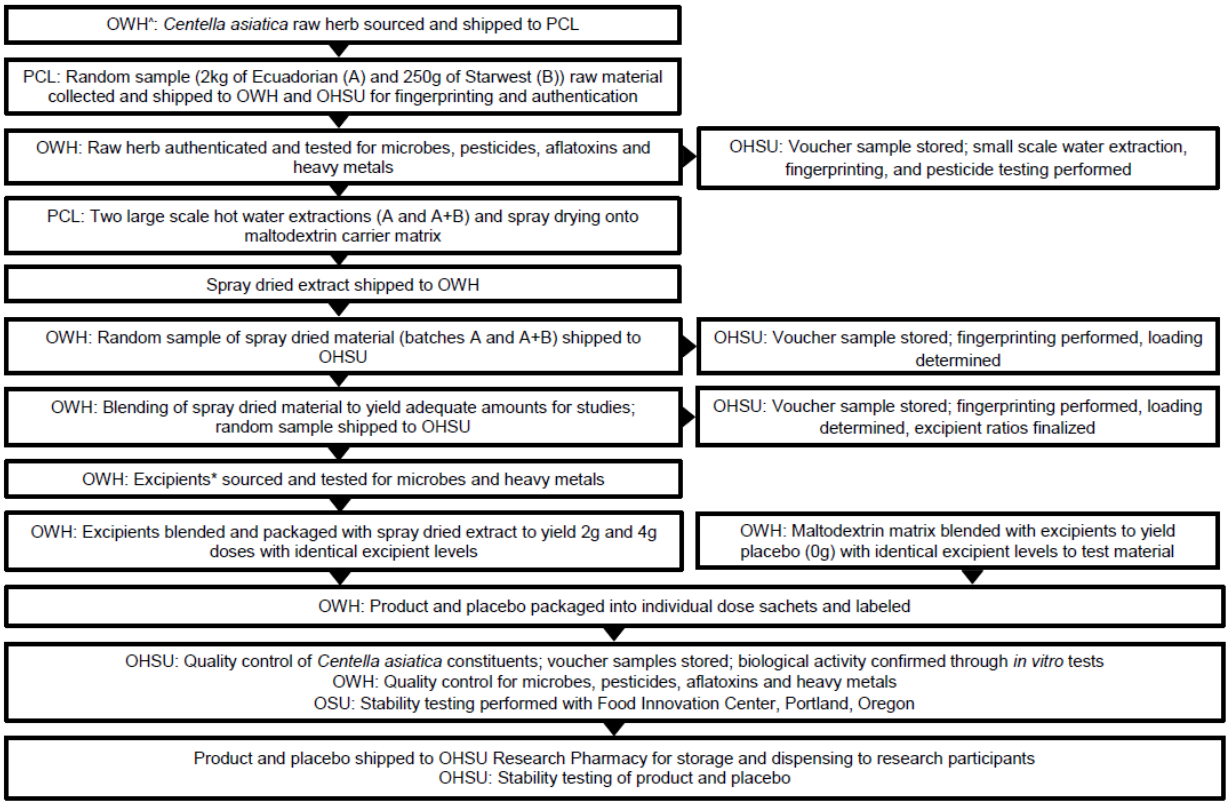

Figure 4. Process of product and placebo manufacture.
^OWH = Oregon's Wild Harvest (Redmond, OR); PCL = Pharmachem Laboratories (New Kearny, NJ); OHSU = Oregon Health and Science University (Portland, OR); OSU = Oregon State University; *Excipients = additional maltodextrin, coconut sugar, malic acid, citric acid, caramel color.

5.4. Accountability

The OHSU Research Pharmacy will dispense following the Pharmacy's applicable policies and procedures. Each dose and dispensing will be documented in the Pharmacy's electronic database, which utilizes a time and signature stamp. See Manual of Operations for further instructions on appropriate accountability documentation methods.

5.5. Adherence assessment
Participants will consume single doses of CAP in the presence of a member of the research team following baseline measurements, in order to ensure adherence to the study protocol.

5.6. Randomization, blinding and stratification
Randomization: Randomization will use an arm equivalence design to promote equal numbers of participants for each order schema. Randomization will occur within 72 hours of completion of the screening and baseline assessments collected at the screening visit and 72 hours prior to study intervention initiation. The order in which the doses are administered will be randomized so that some data will be available for both doses in case of dropouts after the first visit.

Blinding: All participants will be blinded to the dose they will be receiving at each visit. We have developed a formulation that tastes and looks very similar at each dose. The investigator administering the intervention and the analyst performing the LC-MS analysis of collected plasma will be blinded as to the dose of the product administered. The LC-MS analyst will also be blinded as to the time point of collection. The principal investigator, Dr. Soumyanath, is authorized to break the blind.

Stratification: Participants will be stratified by gender so that each dose level (2g or 4g) is administered to equal numbers (n=8) of each gender. Although exploratory in nature, contrasts between gender and cognitive impairment status will be calculated for powering and pilot data purposes.

5.7. Administration
The study intervention will be administered in an outpatient setting. The research pharmacy will dispense one dose of CAP to a member of the study team, who will reconstitute the CAP powder in 10-12 ounces of room temperature water and provide it to the participant for oral consumption. One dose will be administered at each study visit in a random order.

5.8. Modification to study intervention
There will be no modifications to the study intervention during the study unless there are adverse events are noted and recommendations are made by the study's Independent Monitoring Committee (IMC).

5.9. Criteria for intervention discontinuation and stopping guidelines
Participants will be advised during the informed consent process that they have the right to permanently discontinue the *Centella asiatica* product and/or withdraw from the study at any time without negative repercussions. Participants will also be counseled during the consent process that they may be withdrawn from the study at the discretion of the Principal Investigator or designees. A participant may discontinue and/or withdraw and/or be withdrawn from the study for the following reasons:

Administrative:
1) Withdrawal of participant consent
2) Request of site investigator or designees
3) Request of primary care physician
4) Non-compliance
5) Protocol deviation
6) Premature termination of the study

Adverse event (AE)/experience:

1) Worsening of pre-existing disease
2) Intercurrent illness
3) Death
4) Major/clinically significant alteration in laboratory values or biometric assessments after beginning product
5) Other AE
6) Other reasons concerning the participant's health or well-being

The study will be terminated if two participants terminate due to intervention-related adverse events. Severe adverse events will be reviewed immediately by the Principal Investigator, clinical investigators and the Independent Monitoring Committee (IMC) to determine if severity and relatedness warrant termination of the study.

5.10. Concomitant interventions

5.10.1. Allowed interventions*:* diphenhydramine, temporary use of sedatives for sleep (such as Ambien), probiotic supplements, non-plant based laxatives such as magnesium citrate or milk of magnesia, gastroesophageal reflux disorder medications such as antacids (non-flavored), central nervous system active medications that have been stable for greater than two months, analgesics

5.10.2. Required interventions: cholinesterase inhibitors therapy for the treatment of cognitive decline. The dose needs to be stable for a minimum of 12 weeks prior to study enrollment.

5.10.3. Prohibited interventions: sedatives (except those used occasionally for sleep), central nervous system active medications that have not been stable for two months (including beta blockers, cimetidine, SSRIs, SNRIs), anticoagulants (i.e. Warfarin), investigational drugs used within five half-lives of baseline visit, systemic corticosteroids, neuroleptics, anti-Parkinsonian agents, narcotic analgesics, nicotine (tobacco, patches, gum, lozenges, etc.), *Cannabis sativa* (herb or edibles), any over the counter or prescribed products containing plant extracts such as herbal supplements, plant based over the counter vitamin supplements, plant based over the counter protein powder supplements

6. SCREENING AND STUDY ACTIVITIES

6.1. Candidate identification

We require equal numbers of male and female elderly subjects (age 65-85). These would include 8 individuals with amnestic mild cognitive impairment (MCI) or mild Alzheimer's disease (AD).

We will utilize the following resources available at OHSU to identify potential subjects:

- Oregon Alzheimer Disease Center (OADC) registry of subjects enrolled in a longitudinal study;
- NeuroNEXT database (an NINDS initiative to conduct/foster exploratory trials in neurological conditions). Dr. Joseph Quinn (Co-PI) has access;
- OHSU's patient database EPIC using the "Cognos" searching tool.

The table below gives the numbers of subjects that *prima facie* would qualify for our study from these sources.

Table 4. Recruitment resources

Resource	**Number of mild AD subjects Age 65-85**
OCTRI research volunteer repository Research Match	0 with known MCI/AD
OADC subjects registered in an NIA funded longitudinal study and who have consented to being approached for clinical trials. Mild AD or MCI (age 65-85; CDR 0.5-1 and MMSE 20-28): 38 women, 20 men, of which 13 women, 11 men are OHSU patients.	38 women and 20 men, of which 10 women and 6 men are already OHSU patients. CDR 0.5 to 1 MMSE 20-25
NIH/NINDS Neuronext database of subjects interested in taking part in dementia research	11 women and 10 men who registered with clinically confirmed diagnoses of MCI
Data from Cognos search of EPIC Feasibility of in-clinic recruitment for OHSU's Layton Center for Alzheimer's and Aging based on visits over the last 12 months	40 women and 37 men with confirmed MCI were seen at OHSU in the last 12 month
Totals assuming no overlap between recruitment tools	89 women, and 67 men

If we require additional subjects beyond the number we can recruit from these sources, we will use additional methods. For example, the Oregon Clinical and Translational Research Institute at OHSU also provides other recruitment tools such as "Research Data Warehouse" and "Cohort Discovery".

6.2. Recruitment

Participants will be enrolled on a rolling basis until the desired number of participants is achieved. Potential participants available through the above sources will be mailed a study advertisement describing the study and requesting they call if they are interested in participation. The study will be advertised using fliers in out-patient OHSU clinics, other regional hospitals within traveling distance to OHSU, local retirement communities/elder care facilities and an online posting in the March Wellness "March Stride" e-mail newsletter. In addition, it will be advertised using the NIH Clinical Trials website, local newspapers and radio announcements. We will also advertise through online social media platforms, such as targeted Facebook ads. We may also recruit by obtaining physician referrals from providers outside of OHSU using IRB approved recruitment materials. It will be emphasized that participation in the study is completely voluntary. Copies of all advertisements and non-OHSU physician referral request letters will be submitted for IRB approval.

6.3. Incentives

Participants will be given the option of complimentary transportation by taxi to and from their study visits (up to $50 per visit) or a parking permit for each study visit. They will not be provided with transportation for screening visits. To compensate them for their time and effort, participants will receive $80 for completion of the study via the ClinCard. Prorated amounts of $40 per study visit will be implemented for participants who do not complete the study and provided using the above card at the end of the first study visit. While not a direct incentive, participants will be provided with three complete meals and snacks during each study visit that comply with a low plant diet.

6.4. Phone screening

Potential participants will be screened over the phone by a member of the study team, primarily the study coordinator, prior to coming in for the in-person screening visit. A standardized

telephone script will be used and will be submitted for IRB approval. The script will ask the study participant basic questions about eligibility and provide necessary details about the study purpose, duration of visits, number of visits and major procedures. Study personnel conducting the phone screening will then ask to talk to the participant's designated study partner to discuss the study in more detail and ask detailed eligibility questions including medical history. The telephone screening will take approximately 10-15 minutes. Potential participants that meet eligibility criteria over the phone will be scheduled for a screening visit with their study partner.

6.5. Consent process

Participants: To ensure full comprehension of all aspects of their participation in the study, all potential participants will be mailed a copy of the IRB approved consent and HIPAA form prior to their in-person screening visit. They will be instructed to review the form ahead of time but not to sign it until the visit. All in-person screening visits and consenting will occur in a Clinical and Translational Research Center's outpatient room. At the in-person screening visit, a study clinician will review the single consent/HIPAA form that describes both the screening and study procedures and then will ask the participant and ARR to describe key points in the form (e.g. purpose of study, dietary requirements, length of study) before signing. A physical copy of their consent form will be provided to each participant and kept in the affiliated study folders to be made available as necessary to an Independent Monitoring Committee or the OHSU IRB. To ensure ongoing consent, participants and their LAR will be called three days prior to their second study visit to ask if they wish to continue their participation in the study. This will be documented in the participant's instance of REDCap. Appropriate language will be in included in the consent form to avoid coercion. We will underscore that they can decline to participate at any point in the study, even if they have signed the consent form, and it will not impact their participation in future research trials or health care.

Study partner: To ensure full comprehension of all aspects of their participation as a study partner in the study, all potential study partners will be consented in the screening visit with their participant. All in-person screening visits and consenting will occur in a Clinical and Translational Research Center's outpatient room. At the in-person screening visit, a study clinician will review the single consent/HIPAA form that describes both the screening and study procedures and then will ask the study partner to describe key points in the form (e.g. purpose of study, dietary requirements, length of study) before signing. A physical copy of their consent form will be provided to each study partner and kept in the affiliated study folders to be made available as necessary to an Independent Monitoring Committee or the OHSU IRB. To ensure ongoing consent, the study partner will be called three days prior to the second study visit to ask if they wish to continue their participation in the study. This will be documented in the participant's instance of REDCap. Appropriate language will be in included in the consent form to avoid coercion. We will underscore that they can decline to participate at any point in the study, even if they have signed the consent form, and it will not impact their participation in future research trials or health care.

6.6. Screening visit

After the participant signs the consent, the participant will continue with screening assessments and baseline measurements. The participant's health status will be evaluated by a study clinician using a biometric assessment (blood pressure, height, weight and body mass index), vital signs (pulse rate and temperature), a screening exam, a mood assessment (Geriatric Depression Scale-15) and cognitive assessments (Clinical Dementia Rating and Mini Mental State Examination). A non-fasting plasma and urinary laboratory evaluation (complete blood count, comprehensive metabolic panel and urinalysis) will be performed and submitted to the OHSU clinical core laboratory to identify asymptomatic illnesses that could impact study results

(Table 5). A member of the study staff with venipuncture training or CTRC nursing personnel will collect approximately three teaspoons (15 mL) of venous blood by peripheral venipuncture and the participant will collect half a cup of urine (20 mL) via a clean-catch method for analysis. . An aliquot of urine (15 mL) and of blood (3 mL; spun down to plasma) will be frozen for later LC-MS analysis for the bioactive compounds of interest (see section 6.9). One aliquot of blood (5 mL) will be used to prepare PBMC for evaluation of NRF2 gene expression and/or protein levels. Blood and urine collected for safety assessments will initially be treated per standard laboratory processing methods in the CTRC laboratory and transported to OHSU Hatfield Lab Services (Hatfield Research Building) for further processing and analysis. These labs will need to be collected within two weeks of consent. Participants will be provided with a handout describing the "low plant diet" that they are to follow prior to each study visit, should they qualify for the study. The visit is expected to take 45-60 minutes. Study assessments will only be started after the subject and study partner (ARR) have signed the consent form.

A study clinician will review screening and laboratory results prior to participant enrollment. Participants will be contacted by phone and told whether they qualify for the study or not within one week of study enrollment. Participants meeting all final eligibility criteria will be invited to participate in the intervention phase of the study and will be scheduled for Study Visit 1. Any participants with abnormal health screening or laboratory evaluation will be directed to their primary care physician for treatment and excluded from the study. In the event that a participant does not qualify for the study, their data will be destroyed at the end of the study.

6.7. Documentation of ineligibility or non-participation of eligible candidates

A participant's qualification for participation in the intervention phase of the study will be documented on the medical history case report form collected at screening and initialed by the study personnel who determined eligibility. It will be documented on this same form if an eligible participant elects not to participant. If a participant elects to drop out of the study, this will be documented on their phone follow-up script and input into their instance of REDCap.

6.8. Enrollment

Enrollment will be defined as the date of randomization. Randomization will occur once the individual agrees to participate and all screening criteria have been met. This should occur between 72 hours and two weeks following their screening visit.

6.9. Study visits 1 and 2

Visit timing: Visit 1 will occur a minimum of 72 hours following randomization and the screening visit to ensure adequate time for the low plant diet the screening laboratory results and for scheduling of appropriate facilities. Visit 2 will occur approximately 2-4 weeks following Visit 1 to allow for complete wash out of the study intervention (Figure 3).

Low phytochemical "plant" diet: Due to the nature of the bioactive compounds from *Centella asiatica*, participants will be required to adhere to a "low plant diet" designed by the Oregon Clinical and Translational Research Institute Bionutrition Department at OHSU. They will comply with this diet for 48 hours prior to each study visit, for which they will be given a handout at the screening visit. The diet is essentially limiting intake of fruits, vegetables, coffee, whole grains, chocolate and tea, which may contain compounds similar to those found in *Centella asiatica*. Participants will be provided with food conforming to this diet for the duration of each study visit including three full meals and snacks as needed. To assess for success of the diet, a small amount of the blood obtained at the screening visit will be analyzed for the presence of the bioactive compounds while the participant is following their normal diet and then compared to baseline levels at each study visit. To monitor for dietary compliance, a 48-hour diet diary will be

provided to each participant to be filled out each day and brought to each study visit. The diary will also include areas for documenting supplement and medication usage.

Sample collection: Participants will be asked to fast for 10 hours prior to each study visit, excluding water, in an attempt to standardize gastrointestinal transit time and minimize delayed absorption of the study intervention due to the presence of food. A study clinician or CTRC nursing staff member will place a peripheral intravenous catheter in the participant's arm or hand to allow for serial plasma specimen collection. Prior to administration of the *Centella asiatica* product, a baseline blood sample of one tablespoon (15 mL) will be collected through the catheter using a syringe and transferred immediately into a heparinized Vacutainer tube. The participant will be asked to consume by mouth one of the two doses of *Centella asiatica* product dissolved in 8-12 ounces of warm water, on an empty stomach in the presence of study personnel to ensure compliance. Participants will be asked not to consume food for two hours following consumption of the study intervention to allow for transition into the small intestine and absorption. If signs of hypoglycemia present, participants will be allowed to consume a high glycemic food and monitored for improvement. Serial blood samples (two or three teaspoons (10 -15 mL)/sample) will be obtained through the peripheral intravenous catheter using a syringe over a 10-hour post-administration period (15, 30, 45, 60, 90, 120, 150, 180, 240, 360, 480, and 600min) (Table 4). The 15 mL samples will be collected at 60, 120, 180, 240 and 360 mins to allow for 5 mL to be processed for PBMC isolation. Participants will collect their urine for the duration of the study visit (10 hours) to assess for excretion of *Centella asiatica* metabolites.

Tolerability and safety: We will record weight, height, body mass index, and age at baseline and blood pressure, temperature and pulse rate at baseline and ten hours post-administration at each study visit to monitor for adverse events and asymptomatic illnesses that may impact study results (Figure 3, Table 5). We will also collect serum kidney and liver function markers at each study visit (a comprehensive metabolic panel) at baseline and ten hours post-administration (Table 5). Participants will have an electrocardiogram (EKG) at the beginning of each study visit and six hours after intervention administration. Participants will be interviewed and administered a standardized multi-system adverse events questionnaire at the beginning and end of each study visit. This questionnaire has been adapted from the National University of Natural Medicine approved questionnaire (Appendix).

Table 5. Schedule of study visits and assessments.

Event	**Screening**	**Visit 1**	**Visit 1 + 1 day**	**Visit 2**	**Visit 2 + 1 day**	**Visit 2 + 7 days**
		Study visit	Phone visit	Study visit	Phone visit	Phone visit
Informed consent	•					
Vitals[+]	•	•		•		
Health screening	•					
Cognitive assessment[#]	•					
Biometric assessment	•	•		•		
Electrocardiogram (ECG)		•		•		
Blood draw (CBC, CMP*) and urinalysis	•	•[V, U]		•[V, U]		
Low plant diet (starting 48h prior to visit)		•		•		
Single dose of intervention		•		•		
Blood collected at intervals (0-10hr) for bioavailability, antioxidant and NRF2 activation assessment	•[^]	•		•		

10-hour urine collection, baseline urinalysis		•		•		
Adverse events (AE) questionnaire		• [Q]	•	• [Q]	•	•

[+] Vitals include blood pressure, temperature and pulse rate; measured once at screening, and at baseline and ten hours post administration at study visits; * CBC = Complete Blood Count with differential; CMP = Comprehensive Metabolic Panel, [^] Single time point, [#]Mini Mental State Exam, Clinical Dementia Rating, Geriatric Depression Scale-short form, [V]CMP only collected at baseline and ten hours post administration, [U]- urinalysis at baseline only; ECG collected at baseline and six hours post-administration; [Q] – adverse event questionnaire administered at baseline and end of visit.

Baseline measurements at each visit:

- Plasma and urinary levels of bioactive compounds (triterpenes and caffeoylquinic acids) derived from the diet.
  - Plasma and urine collected in the screening visit will be analyzed to assess for success of the low phytochemical diet
- NRF2 gene expression and/or nuclear protein levels in PBMC at baseline.
  - PBMC obtained from screening visit sample will be compared to assess possible changes due the the low phytochemical diet.
- Electrocardiogram (EKG)
- Comprehensive metabolic panel (CMP)

Evaluation of Blinding Success:
At study visit 2, after administration of CAP, the participant will be asked which of the two doses, lower (2g) or higher (4g) they thought they received at this visit. The study team member will also record their opinion on which of the 2 doses they thought was administered at this visit. This information will be used to evaluate blinding success after unblinding.

6.10. Tolerability and safety assessments

Visit 1 and 2 tolerability phone assessment: Participants will be telephoned the day following each study visit to assess for delayed adverse events and/or complications from the intravenous catheter placement using interviews and a standardized multi-system adverse events questionnaire (Figure 3, Table 5). Participants will be instructed to contact a study clinician if they experience any reactions following the phone assessment, and prior to their second study visit or one-week exit interview.

Exit interview tolerability assessment: Participants will be telephoned one week following the completion of their second study visit, or first study visit if they elect to drop out of the study, to assess for delayed adverse events and/or complications from the intravenous catheter placement using interviews and a standardized multi-system adverse events questionnaire (Figure 3, Table 5).

6.11. Study duration

Recruitment and data collection is expected to begin upon approval from the IRB and FDA and continue for seven months. We estimate a total of four months for final data analysis. Total estimated study duration is eleven months (Table 6).

6.11.1. Subject participation duration

Including the screening visit, a two to four-week interval between doses, and an exit phone interview for adverse event monitoring, it will take a participant six to eight weeks to complete the study.

Table 6. Study timeline.

Month / Activity	1-3			4-6			7-9			10-12		
Finalize product manufacture/quality control												
Recruitment/enrollment												
Data collection												
Data analysis/summary												
Reports, manuscripts												

7. PROVISIONS TO MONITOR THE DATA TO ENSURE SUBJECT SAFETY

Adverse event (AE) monitoring will occur in "real time" using a standardized Adverse Event Monitoring Form and reviewed weekly by the principal (Soumyanath) and clinical (Quinn) investigators. This form is a ninety-one point multi-system questionnaire that assesses all organ systems (eyes/ears/nose/throat, gastrointestinal, neurological/ musculoskeletal, psychological/general, cardiopulmonary, skin, genitourinary and whole body systems). It will be completed at the screening visit in order to collect data on any/all pre-existing symptoms and their severity, and will serve as baseline data to determine attribution to the study intervention. The Adverse Event Monitoring Form will be administered in person at the beginning and end of Visits 1 and 2, via telephone the day following Visit 1 and 2, and by telephone one week after Visit 2, to assess for acute and delayed adverse events. Electrocardiograms will be performed just prior to administration of *Centella asiatica* product and six hours after intervention to monitor for adverse events. This time point has been selected based on prior pharmacokinetic literature on *Centella asiatica* triterpenes and from our murine studies which suggest that at 6 hours, the bioactive triterpenes should have reached maximum concentration and the caffeoylquinic acids are beyond their time of maximum concentration. This is also to allow for the collection of the ECG to be feasible given the frequent timing and short intervals between blood sample collection earlier in the experiment. A comprehensive metabolic panel (CMP) will be measured at baseline of each study visit and at 10 hours after CAP administration to assess for liver or kidney toxicity. This time point was selected to allow for markers of toxicity to develop if present, and for logistical reasons to allow adequate time for sample collection, processing and transfer to the OHSU Core Laboratory for analysis within the 10 hour time frame of the experiment.

Participants will be reminded and encouraged during clinic visits to contact a study clinician if a moderate or serious AE occurs. Participants will be given a telephone number they can call at any time to report potential adverse effects. All severe AEs will immediately be reported to the Independent Monitoring Committee (IMC) and the OHSU IRB by the study principal investigator.

An **adverse event (AE)** is defined as any unfavorable and unintended diagnosis, symptom, sign (including an abnormal laboratory finding), syndrome or disease which either occurs during the study, having been absent at baseline, or if present at baseline, appears to worsen. Adverse events are to be recording regardless of their relationship to the study intervention.

A **serious adverse event (SAE)** is defined as any untoward medical occurrence that results in death, is life threatening, requires inpatient hospitalization or prolongation of existing hospitalization, results in persistent or significant disability/incapacity, or is a congenital anomaly.

7.1. Attribution to intervention

At each administration, items identified on the Adverse Event Monitoring Form will be compared to baseline levels to determine if the symptom was pre-existing. If the symptom was pre-existing at the same or a previously greater severity, the symptom will *not* be considered attributable to the study intervention. If the symptom was pre-existing at lesser severity (or was not previously present), the symptom may be considered attributable to the study intervention. If the intervention is considered attributable, a study clinician, in consultation with the Principal Investigator, will determine if an alternative clinical explanation exists for the adverse event. If an alternative explanation does not exist, the adverse event *will* be considered attributable to the study intervention. If an alternative explanation does exist, the adverse event will *not* be considered attributable to the study intervention.

7.2. Independent Monitoring Committee (IMC)

Data monitoring and safety oversight is to be carried out via an independent monitoring committee (IMC) composed of at least three members beyond the principal study team. A clinician with experience in relevant disease research will chair the IMC and the other two members will be a statistician and a second clinician with human subject research monitoring expertise. An IMC is planned over a Data Safety Monitoring Board (DSMB) based on the National Institute of Health (NIH) and National Center for Complementary and Integrative Health (NCCIH) listed criteria with respect to data safety and monitoring. This study is single-site and phase I trial in scope. In addition, *Centella asiatica* is expected to be largely safe based on pre-clinical work and its long-standing position and extensive usage in herbal medicine. However, the use of blinded randomization and an elderly study cohort predicates the use of a multi-person committee and not just an independent monitor. See IMC charter.

7.3. Plan for review and reporting

The IMC will meet biannually to study un-blinded AE data coded by organ system, and on an ad-hoc basis as necessary, based on the cited reporting of severe AEs and continuing reviews to OHSU's IRB. Should it be determined that the protocol need to be amended as a result of data review, the principal investigator will submit a protocol amendment to the sponsor (NCCIH) for approval. Once approval is obtained from the sponsor, the amendment will be submitted to the OHSU IRB and monitoring body and the amendment approved prior to study amendment implementation, unless immediate implementation is required to protect the safety of the study participants. In such a case, the protocol amendment will be immediately implemented and the sponsor, OHSU IRB and IMC will be notified directly after protocol amendment implementation.

8. RISKS AND BENEFITS

8.1. Risks to subjects

Potential harm to participants will be minimized by excluding participants with the conditions detailed in section 4.3.2. The risks to the subjects participating in this proposed research project are mainly those associated with venous access, potential toxicity of the test material, and time commitment.

Venipuncture: The potential risks to participants are minimal and reversible. Likely risks include pain and bruising from the intravenous catheter or venipuncture needle insertion. Occasional risks include feeling lightheaded or faint during or immediately after a blood sample is collected. Unlikely risks include swelling, infection, transient hematomas, transient thrombophlebitis, a fractured catheter embolus, or an allergic reaction to the adhesives or sterilization materials used at the venous access point. To minimize risk, catheter insertions and blood draws will be conducted in a seated position and using aseptic technique by trained personnel.

Centella asiatica product: *Centella asiatica* is an edible plant. The Botanical Safety Handbook[78] classifies *Centella asiatica* as a Class 1 herb, i.e. one that can safely be consumed when used appropriately. The widespread use of *Centella* as a dietary supplement and human studies performed with *Centella asiatica*[33-35,80,98,12] support its safety. At the doses given in this study, the risks are expected to be minimal and unlikely. The lowest dosage is based upon allometric scaling from mouse studies in which no toxicity was observed; however, the present product is a concentrated extract, and it is important to establish its safety, particularly in a vulnerable elderly population. We do not anticipate a great increase in adverse effects with the higher dosage, but are aware that they may occur. In particular, we anticipate potential transient gastrointestinal side effects such as nausea, gastric reflux/discomfort or diarrhea. Due to unknown interactions with pharmaceutical medications, we will exclude those on blood thinning medications (e.g. warfarin). We will also exclude those with hepatitis and/or liver disease, as there is potential for *C. asiatica* to harm the liver, and will closely monitor those on drugs with hepatotoxic potential (e.g., acetaminophen, methotrexate, pravastatin). *Centella asiatica* may cause sleepiness and drowsiness in large amounts, so individuals taking sedatives (besides occasionally for sleep) and Central Nervous System (CNS) depressants are likewise excluded, as the study drug could increase these side effects. Additional risks include allergic reaction to the intervention, which will be managed by oral or intravenous diphenhydramine depending on the severity.

Treatment-emergent suicidal ideation and behavior have been identified as a concern for a number of drugs and drug classes. Because of these concerns, a prospective assessment for suicidal ideation and behavior will be monitored. Heisel et al. (2011)[132] have identified a "suicide ideation" subscale in the Geriatric Depression Scale (GDS). We are using the GDS as part of study procedures, and will pay special attention to the identified subscale questions to assess potential risk of suicidal ideation or behaviors.

Risks associated with a breach of confidentiality: There is a small risk that information about a study participant could be inadvertently disclosed to non-study personnel. Procedures to minimize this risk have been described in this protocol.

Other risks/discomforts associated with participation: The participants will experience a time burden by participation in the research study, as they will need to remain within the Clinical and Translational Research Center for 13 hours at each study visit (total 26 hours). Participants will be remunerated in a prorated fashion ($40 per visit) for their participation. They will also be asked to follow a low plant diet, which may present an economic burden. To mitigate the burden associated with bringing food that conforms to the diet or purchasing food during their study visits, participants will be provided with three meals and snacks that conform to the diet for each study visit at no expense. As this study is being conducted only at the OHSU Marquam Hill campus, there are travel and parking expenses associated with study participation. To address this expense, participants will be provided with an option of a round trip taxi trip to their home (up to $50) or a validated parking pass for each study visit at no expense.

8.2. Potential benefits to subjects

As this is a pilot pharmacokinetic study with single administrations of the intervention, it is unlikely participants will experience any direct benefits. The data on dosing and tolerability will be extrapolated to longer trials of CAP in patients with mild cognitive decline or Alzheimer's disease.

9. DATA AND SPECIMENS

The principal investigator will oversee the management of the participants, intervention, and analysis, which will be carried out by the clinical investigators, research statistician, and research assistant. Data entry, quality control and preparation, and participant management will be ongoing throughout the study.

9.1. Data collection methods

Information will be collected from each participant and their representative by a study clinician and/or research assistant. These personnel will be blinded to the participant's dose. The procedures for obtaining research material will include a telephone screening form, a medical history form, cognitive screening questionnaires (Mini Mental State Examination and Clinical Dementia Rating), a mood-screening questionnaire (Geriatric-Depression Scale-15), a standardized adverse event questionnaire, a diet diary, electrocardiogram, urine collection and blood collection. The following will be recorded directly on the case report forms (CRFs) telephone screening form, a medical history form, cognitive screening questionnaires (Mini Mental State Examination and Clinical Dementia Rating), a mood-screening questionnaire (Geriatric-Depression Scale-15), and a standardized adverse event questionnaire. This data will them be input into the participant's instance of REDCap. Data will be collected directly from study participants specifically for research purposes. All blood collection will be performed at the CTRC. All data used for this project will be obtained only after receiving participant informed consent.

9.2. Handling of data and specimens

Upon enrollment, each participant will obtain a unique identification number. This number will be associated with all specimens and data collected from that participant. Following the collection of whole blood from the participant, a trained member of the study team will transfer the blood to the specified Vacutainer tubes. Vacutainers containing a 5 mL aliquot of blood for NRF2 analysis from the screening (6.6) and study visits (6.9) will be transferred to Dr Joseph Quinn's laboratory for isolation of PBMC and measurement of gene expression and/or protein levels related to NRF2 activation (in collaboration with Dr Nora Gray, OHSU). For analysis of CA compounds, study staff will process the blood by centrifugation in the CTRC's laboratory to separate the plasma for analysis. The isolated plasma will be transferred into labeled tubes and placed on ice until transported by a member of the study team via a sealed Styrofoam cooler labeled with biohazard to The Oregon Alzheimer Disease Center Biomarker Core Lab (Biomedical Research Building, OHSU). The plasma and a 50mL aliquot of the urine specimens will be stored in labeled boxes in Dr. Quinn's -70°C freezer for up to five years. All written data will be stored in a locked cabinet in Dr. Soumyanath's office (Richard Jones Hall, OHSU) for up to five years. All computerized data will be stored in OHSU's installment of REDCAP and in a password protected folder on the OHSU X-drive for up to five years.

9.3. Detection of *Centella asiatica* derived compounds in plasma and urine

Biological levels of *Centella asiatica* triterpenes, caffeoylquinic acids, and their metabolites will be assessed in both plasma and urine using high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). Plasma calibration curves will be made using pooled baseline human plasma from the study participants (one teaspoon (5 mL)/participant) separated by gender. The blank plasma will be spiked with increasing doses of commercially available reference triterpenes, caffeoylquinic acids and known metabolites, as well as appropriate internal standards (see below). If the blank plasma is found to contain traces of the analytes, we will calculate the true concentration by back extrapolation using the method of standard addition. A pooled baseline urine sample taken from participants (separated by gender) will be used as a "blank" urine sample for spiking with standards.

Triterpenes; Plasma (200 µl) and urine samples (3000 µl) will be mixed with phosphoric acid and internal standard (chrysin) before incubation with *E. coli* glucuronidase and *S. aerogene* sulfatase to release the analytes of interest from glucuronide and sulfate conjugates. Incubated samples are passed through a Supelco Supelclean™ C8 column, washed with dilute aqueous acetic acid to remove salts, and eluted with ammoniacal methanol. The eluant is vacuum dried, reconstituted in 200 µl of 1:1 methanol: ammonium acetate and filtered through a 0.2 µM spinfilter to remove residual particles before analysis. HPLC-MS/MS will be performed on an Applied Biosystems Q-Trap 4000 LC-MS instrument. The analytical method to be used is a modification of that described by Nair et al.(Nair, Menon et al. 2012) Chromatographic separation will be achieved using a Poroshell 120 EC18 column and methanol:ammonium acetate gradient. Triterpenes will be detected as their ammonium adducts with positive ion mode electrospray ionization using the following MS/MS transitions: AA (506/453) and MA (522/451), and chrysin will be detected as the molecular ion (255/255).

Caffeoylquinic acids and metabolites: Plasma (200 µl) and urine samples (3000 µl) will be mixed with ascorbic acid and isotopic internal standards ($^{13}C_9$-caffeic acid, $^{13}C_3$-ferulic acid, and d_3-isoferulic acid) before incubation with *E. coli* glucuronidase and *S. aerogene* sulfatase to release the analytes of interest from glucuronide and sulfate conjugates. Incubated samples will be applied to a Phree® column along with acidified acetonitrile (1 mL) and passed through by centrifugation, precipitating proteins and removing lipids. The eluant will be vacuum dried, reconstituted in 200 µl of 66% aqueous acetonitrile with 0.66% formic acid and filtered through a 0.2µM spinfilter to remove residual particles. LC-MS/MS will be performed on an Applied Biosystems 5500 QTRAP HPLC-MS instrument. Chromatographic separation will be achieved using a C8 reversed-phase column and acidified acetonitrile:water gradient. Caffeoylquinic acids (CQAs), their metabolites and internal standards will be detected using negative ion mode electrospray ionization and the following MS/MS transitions: mono-CQAs (353/191); di-CQAs (515/353; 515/191); caffeic acid (179/135); ferulic acid and isoferulic acid (193/134); dihydrocaffeic acid (181/109); 4-methylcatechol (123/108); 4-ethylcatechol (137/122); $^{13}C_9$-caffeic acid (188/143); $^{13}C_3$-ferulic acid (196/136); d_3-isoferulic acid (196/134).

9.4. Analysis of total antioxidant compounds

Total antioxidant compounds in plasma will be measured at each time point using the OxiSelect™ kit (Cellbiolabs Inc.) and following the manufacturer's instructions. The assay, performed in 96-well plates, is a quantitative assay for measuring antioxidant potential within plasma samples. Following the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) by antioxidants in the sample, the kit colorimetric probe develops a blue color that is read at 540-600 nm. The antioxidant potential of samples is determined based on an iron standard curve and results are calculated at Fe^{2+} equivalents (µM) or FRAP value.

9.5. Investigation of blood biomarkers of NRF2 activation

These methods are described in Appendix 2.

9.6. Sharing of results with participants

All screening laboratory results, performed by the CLIA certified OHSU Core Laboratory, will be provided to the participant. If a screening result is found abnormal (an incidental finding), the participant will be instructed to share their result with their primary care provider. Electrocardiograms (EKGs) and comprehensive metabolic panels (CMPs) will be performed at each study visit. If the EKGs are found to be abnormal, the participant will be instructed to share their result with their primary care provider.

9.7. Data and specimen banking

All data obtained from this study will be used for research purposes only and will comply with HIPAA regulations. No genetic research will be performed on these specimens. The specimens and data collected relate specifically to this research project; however, plasma samples may be stored at -70°C in Dr. Quinn's laboratory for future analysis of newly identified bioactive compounds of *Centella asiatica*. No specimens will be sent to or used in a repository. All specimens will be labeled with each participant's unique numeric ID code but will not contain any identifiable participant information, and will be maintained at OHSU.

10. DATA ANALYSIS

10.1. Study outline

This is a phase 1 pilot clinical study using a blinded randomized crossover design. Participants will consume one of two single doses of CAP (2g or 4g) on two separate occasions at least 2 weeks apart. After each administration, blood and urine samples will be collected over a 10 hour period, and an analyzed for compounds derived from CAP. The order in which the doses are administered will be randomized.

10.2. Reason for randomization

The order in which the doses are administered will be randomized so that some data will be available for both doses in case of dropouts after the first visit. It is also randomized to prevent a tolerance effect.

10.3. Justification of two-week washout

The minimum two-week period was chosen based on a balance between participant convenience (allowing sufficient time between the lengthy study visits), and the need to complete the study in a timely manner. An additional factor is that the compounds of interest, particularly the caffeoylquinic acids (CQAs), may also be derived from the diet. For this reason, participants will undertake a low phytochemical diet 48h before and during each study visit. As described below, we predict based upon the reported half-life ($t_{½}$) values of some compounds found in *Centella asiatica*¸ that 48h on the restricted diet will be sufficient to clear 97% of the starting levels of the compounds. We will compare baseline plasma levels of the compounds of interest prior to administration of each *Centella asiatica* dose (i.e. after 48h on low phytochemical diet and before CAP administration) to examine for sufficient washout between doses. The triterpenes (asiatic acid and madecassic acid, and their glycosides) will be most useful in this regard, as they have a narrower distribution in plants than the CQAs, and are less likely to be ingested from the general diet in the period intervening study visits.

10.4. Primary and secondary hypotheses

- The primary hypothesis is that that the triterpene and caffeoylquinic acid components in CAP will be orally bioavailable, such that administration of CAP will result in elevations from baseline values of the plasma concentration of *Centella* triterpenes, caffeoylquinic acids (CQAs), and CQA metabolites, and that the magnitude of this elevation will be higher for the 4g dose of CAP than the 2g dose.
- A secondary hypothesis is that absorption of compounds from CAP will result in an increase in antioxidant potential of the plasma.
- An additional secondary hypothesis is that consumption of a single dose of CAP will be safe and well tolerated.
- An additional secondary hypothesis is that consumption of a single dose of CAP will result in activation of the NRF2 antioxidant pathway over a 6 hour period.

10.5. Primary and secondary outcome measures

- *Primary outcome:*
  - A change in pharmacokinetic parameters (C_{max} and AUC_{0-10}) of plasma analytes after treatment with a product made from a water extract of *Centella asiatica* (CAP) with a detectable difference between doses (2g and 4g).
- *Secondary outcome:*
  - The time of maximum concentration (t_{max}) and half-life ($t_{1/2}$) of the known bioactive compounds and their metabolites to help determine dosage intervals
  - Temporal changes in ferric reducing ability of plasma (FRAP) as an indicator of antioxidant potential over time
  - Safety and tolerability of acute doses of CAP determined by participant interviews, biometrics, electrocardiography, laboratory assessments of liver and kidney function, vital signs and questionnaires
  - The levels of triterpenes, caffeoylquinic acids, and their metabolites in a pooled urine sample collected over 10 h after CAP administration
  - Activation of the NRF2 antioxidant response element pathway in peripheral blood mononuclear cells (PBMC) over 6 hours following CAP administration.

10.6. Specimen analysis

Each CAP derived analyte will be identified by its retention time and characteristic mass spectral fragmentation pattern using selected reaction monitoring. The peak area ratio to the appropriate internal standard will be identified and evaluated against the standard calibration curve of each analyte. Each analyte's concentration will be used to generate a concentration-time curve for pharmacokinetic profiling. Pharmacokinetic (PK) profiling of the triterpenes, CQAs and their metabolites will use non-compartmental analysis (NCA) to calculate common PK parameters such as peak concentration (C_{max}), the time at peak concentration (t_{max}) and the total 10 hour bioavailability represented by the total area under the curve (AUC_{0-10}). These will be compared to the mouse data in order to assess appropriateness of dosing level. Other calculable NCA metrics will also be determined, such as the elimination half-life ($t_{1/2}$) calculated from the profile tails and the mean residency time (MRT) of the compounds in plasma. Descriptive assessment will be done at each of the doses to give basic PK summarization of the various CA compounds and total antioxidant compounds measured by FRAP. NRF2 antioxidant pathway related gene and/or protein levels will be compared in PBMCs from the screening visit, the baseline study visit sample and hourly up to 6h following CAP administration. Dose dependence of the study outcomes will be determined by evaluation of the within-subject differences in study outcomes between the 2g and 4g CAP doses.

Assumptions of normality will be confirmed using outcome density distributions alongside utility from formal tests, such as the Anderson-Shapiro. In instances of normality violation, statistical transformation such as natural logs and square roots will be considered as will non-parametric equivalent tests such as the paired Wilcoxon rank-sum test. Standard diagnostics will be very conservative given the limited number of available subjects but clearly obvious outliers will be removed as necessary. Non-continuous variables such as t_{max} will have frequency distributions compared between doses using chi-square testing. Although all CA compounds of interest and PK parameters are defined a priori, multiple comparison correction to p-values will be applied as necessary, with final two-tail significance defined at $p<0.05$.

Incidences of adverse events, both overall and within distinct organ systems, will be compared across treatment arms using Fisher's Exact test. Pairwise examination will test for significant differences in rates between each CAP dose..

10.7. Interim analysis
There will be not any interim analysis performed in this study.

10.8. Sample size
Sample size was calculated based on the primary outcome measures of C_{max} and AUC. Changes in the response values from the literature were taken from Grimaldi et. al.[45] for two outcomes, peak concentration (C_{max}) and bioavailability/area-under-the-curve (AUC), at two treatment doses. With respect to C_{max}, estimations from G*Power indicated a sample size of 6 would be sufficient to detect the established criterion difference (critical t=2.57).

The post-treatment mean difference in C_{max} from the literature was found to be 0.66 and was replicated during simulation with confidence intervals indicating a significant difference {0.36, 0.96}. Both the observed mean and median test statistics was found to be greater than the critical t (mean t=4.23, median t=3.88). Mean power was found to be slightly lower than expected (84% versus 86%) although median power was significantly larger (92%). Accordingly, six subjects would be sufficient to detect a difference in C_{max} between two doses of CAP. With the enrollment of 8 subjects, the study will be sufficiently powered even with a dropout rate of 25%.

Similar results were found for bioavailability, as measured as AUC, although at an attenuated effect, with an indicated increase in subject count to a minimum of 7 (critical t=2.45). Simulation verified a mean difference of 5.25 that would be expected to be significant based on the simulated confidence intervals {2.43, 8.09}. AUC was also found to be similarly powered, with a mean observed power of 83% and a median power of 93%. Accordingly, a sample size of seven is more than sufficient to meet the proposed criterion for CAP analyte bioavailability. With the enrolment of 8 subjects, the study will be sufficiently powered with 1 dropout (14%). Given the short duration of the study, we expect that a dropout rate of only 1 subject is reasonable. Eight total subjects were proposed for the dose comparison of Aim 1, which would allow for reductions in the detectable shifts of both peak concentration (37% of literature effect; ΔC_{max}=0.42) and bioavailability (31% of literature effect; ΔAUC=3.63) allowing for even greater powering to detect a dose effect of CAP.

Based on previously described bench results, gender is anticipated to potentially play a role in CAP metabolism, which may limit the ability to pool results. Maintaining the variances used in the calculations detailed above, the study would still be powered to see the anticipated dose effects within either gender with reduced powers of 75% and 67% for C_{max} and AUC bioavailability respectively.

10.9. Data quality and management
The principal investigator will oversee the management of the participants, intervention, and analysis, which will be carried out by the clinical investigators, research statistician, and research assistant. Data entry, quality control and preparation, and participant management will be ongoing throughout the study.

The principal investigator will oversee study progress including recruitment, retention, demographics, study participant status and error rates pertaining to inclusion/exclusion criteria and the study protocol. Tabulation of study data will have both physical and digital formats available. A member of the study team will record data from each study visit and phone visit on paper case report forms. Physical copies of the case report forms will be scanned into the participant's REDCap encounter, kept in participant-specific binders and kept available for comparison against the final study database. Digital documentation will use the REDCap

database system, which will maintain quality of integrity using prescribed logic rules inherent to the system. Data will be input immediately upon the participant's visit and double-checked within 48 hours for accuracy by a separate member of the study team or the Principal Investigator. This will include limited options for responses, protection against redundancy and double-data entry, and locking of the database for information entry upon study completion. This combination of steps will promote cohesiveness of study outcomes from study visit to data entry while maintaining the necessary protection of participant privacy. All plasma concentration calculations will be performed using a Microsoft Office Excel database by study personnel and double-checked within one week by a second member of the study team to ensure accuracy. The computer files will be protected with a password and only appropriate study personnel will have access to the database.

11. PRIVACY, CONFIDENTIALITY, AND DATA SECURITY

All participant information, and even the fact that an individual is in the study, is considered confidential. Confidentiality will be assured in this study through several mechanisms. Individuals interested in participation in the study will call the study coordinator's personal office telephone for a phone screening. The potential participant's contact information (phone number, name, and address), telephone screening responses, and any other paper records will be documented in a secured binder that is kept in a secured and locked area when not in use and only accessible to study staff. During screening and study visits, the investigators will ensure physical privacy by conducting interviews and examinations in a closed room. After enrollment, each participant will be assigned an anonymous study ID number, which will be used on all case report forms, computerized data sheets and specimen containers. In the case of computerized information, access to the study data on computers will be password protected. Finally, participants will not be identified by name in any reports or publications, nor will data be presented in such a way that the identity of individual participants can be inferred. All staff are trained and annually re-certified regarding these procedures in Responsible Conduct of Research and HIPAA.

12. IMPORTANCE OF KNOWLEDGE GAINED

Alzheimer's disease is a debilitating disease with very limited successful treatments. Due to a lack of effective interventions, physicians are looking to alternative therapies that can address comorbidities and cognitive changes. *Centella asiatica* water extract has shown promising changes in preclinical models warranting further robust investigation in humans. Because there are no previous studies of a well-characterized *Centella asiatica* product in Alzheimer's disease, it is an important public health issue to scientifically evaluate this therapy to be able to perform further studies investigating its effect on cognition. This pharmacokinetic study of *Centella asiatica* will provide the bioavailability data for the selection of dosing and dosage intervals for future clinical trials. It will also provide preliminary acute safety and tolerability data on an extract of *Centella asiatica* that has not been previously investigated in humans.

13. REFERENCES CITED

1. National Institutes of Health NIoA. The Alzheimer's Disease Education and Referral Center Alzheimer's Disease Fact Sheet. August 18, 2016; https://www.nia.nih.gov/alzheimers/publication/alzheimers-disease-fact-sheet. Accessed November 1, 2016, 2016.
2. Association As. Factsheet. March 2016; http://act.alz.org/site/DocServer/2012_Costs_Fact_Sheet_version_2.pdf. Accessed November 1, 2016, 2016.

3. Jack CR, Knopman DS, Jagust WJ, et al. Tracking pathophysiological processes in Alzheimer's disease: an updated hypothetical model of dynamic biomarkers. *Lancet Neurol.* 2013;12(2):207-216.
4. Company ELa. A study of semagacestat for Alzheimer's patients (Identity XT)). Septebmer 22, 2014; http://clinicaltrials.gov/ct2/show/NCT01035138. Accessed November 15, 2016, 2016.
5. Panza F, Frisardi V, Imbimbo BP, et al. Bapineuzumab: anti-β-amyloid monoclonal antibodies for the treatment of Alzheimer's disease. *Immunotherapy.* 2010;2(6):767-782.
6. Wasilewski A. Johnson & Johnson Announces Discontinuation of Phase 3 Development of Bapineuzumab Intravenous (IV) in Mild to Moderate Alzheimer's Disease. http://www.jnj.com/connect/news/all/johnsonand johnsonannouncesdiscontinuationofphase3developmentofbapineuzumabintravenousivin mildtomoderatealz heimersdisease%3E. Accessed October 22, 2012.
7. Announcement ELM. Eli Lilly and Company Announces TopLine Results on Solanezumab Phase 3 Clinical Trials in Patients with Alzheimer's Disease. http://newsroom.lilly.com/releasedetail.cfm?releaseid=702211%3E. Accessed October 22, 2012.
8. Tariot PN, Federoff HJ. Current treatment for Alzheimer disease and future prospects. *Alzheimer Dis Assoc Disord.* 2003;17 Suppl 4:S105-113.
9. Tariot PN, Farlow MR, Grossberg GT, et al. Memantine treatment in patients with moderate to severe Alzheimer disease already receiving donepezil: a randomized controlled trial. *JAMA.* 2004;291(3):317-324.
10. Reisberg B, Doody R, Stöffler A, et al. Memantine in moderate-to-severe Alzheimer's disease. *N Engl J Med.* 2003;348(14):1333-1341.
11. Migliorelli R, Tesón A, Sabe L, Petracchi M, Leiguarda R, Starkstein SE. Prevalence and correlates of dysthymia and major depression among patients with Alzheimer's disease. *Am J Psychiatry.* 1995;152(1):37-44.
12. Ferretti L, McCurry SM, Logsdon R, Gibbons L, Teri L. Anxiety and Alzheimer's disease. *J Geriatr Psychiatry Neurol.* 2001;14(1):52-58.
13. Sinforiani E, Citterio A, Zucchella C, et al. Impact of gender differences on the outcome of Alzheimer's disease. *Dement Geriatr Cogn Disord.* 2010;30(2):147-154.
14. Gambassi G, Lapane KL, Landi F, Sgadari A, Mor V, Bernabie R. Gender differences in the relation between comorbidity and mortality of patients with Alzheimer's disease. Systematic Assessment of Geriatric drug use via Epidemiology (SAGE) Study Group. *Neurology.* 1999;53(3):508-516.
15. Claxton A, Baker LD, Wilkinson CW, et al. Sex and ApoE genotype differences in treatment response to two doses of intranasal insulin in adults with mild cognitive impairment or Alzheimer's disease. *J Alzheimers Dis.* 2013;35(4):789-797.
16. Kapoor L. *Handbook of Ayurvedic Medicinal Plants.* Boca Raton: CRC Press; 1990.
17. Newall C, Anderson L, Phillipson J. *Herbal Medicines. A Guide for Healthcare Professionals.* London: Pharmaceutical Press; 1996.
18. Lokanathan Y, Omar N, Ahmad Puzi NN, Saim A, Hj Idrus R. Recent Updates in Neuroprotective and Neuroregenerative Potential of Centella asiatica. *Malays J Med Sci.* 2016;23(1):4-14.
19. Veerendra Kumar MH, Gupta YK. Effect of different extracts of Centella asiatica on cognition and markers of oxidative stress in rats. *J Ethnopharmacol.* 2002;79(2):253-260.
20. Gray NE, Harris C, Quinn J, Soumyanath A. Centella asiatica modulates antioxidant and mitochondrial pathways and improves cognitive function in mice. *Journal of Ethnopharmacol.* 2016;180:78-86.

21. Rao SB, Chetana M, Uma Devi P. Centella asiatica treatment during postnatal period enhances learning and memory in mice. *Physiol Behav.* 2005;86(4):449-457.
22. Veerendra Kumar MH, Gupta YK. Effect of Centella asiatica on cognition and oxidative stress in an intracerebroventricular streptozotocin model of Alzheimer's disease in rats. *Clin Exp Pharmacol Physiol.* 2003;30(5-6):336-342.
23. Gupta YK, Veerendra Kumar MH, Srivastava AK. Effect of Centella asiatica on pentylenetetrazole-induced kindling, cognition and oxidative stress in rats. *Pharmacol Biochem Behav.* 2003;74(3):579-585.
24. Gadahad MR, Rao M, Rao G. Enhancement of hippocampal CA3 neuronal dendritic arborization by Centella asiatica (Linn) fresh leaf extract treatment in adult rats. *J Chin Med Assoc.* 2008;71(1):6-13.
25. Mohandas Rao KG, Muddanna Rao S, Gurumadhva Rao S. Centella asiatica (L.) leaf extract treatment during the growth spurt period enhances hippocampal CA3 neuronal dendritic arborization in rats. *Evid Based Complement Alternat Med.* 2006;3(3):349-357.
26. Wanasuntronwong A, Tantisira MH, Tantisira B, Watanabe H. Anxiolytic effects of standardized extract of Centella asiatica (ECa 233) after chronic immobilization stress in mice. *J Ethnopharmacol.* 2012;143(2):579-585.
27. Chanana P, Kumar A. Possible Involvement of Nitric Oxide Modulatory Mechanisms in the Neuroprotective Effect of Centella asiatica Against Sleep Deprivation Induced Anxiety Like Behaviour, Oxidative Damage and Neuroinflammation. *Phytother Res.* 2016;30(4):671-680.
28. Selvi P, Kumar M, Rajesh R, Kathiravan T. Antidepressant activity of ethanolic extract of leaves of *Centella asiatica* Linn by *in vivo* methods. *Asian J Res Pharm Sci.* 2012;2(2):76-79.
29. Dhanasekaran M, Holcomb LA, Hitt AR, et al. Centella asiatica extract selectively decreases amyloid beta levels in hippocampus of Alzheimer's disease animal model. *Phytother Res.* 2009;23(1):14-19.
30. Soumyanath A, Zhang Y, Henson E, et al. Centella asiatica Extract Improves Behavioral Deficits in a Mouse Model of Alzheimer's Disease: Investigation of a Possible Mechanism of Action. . *Int J Alzheimers Dis,.* 2012:381974.
31. Gray NE, Morre J, Kelley J, et al. Caffeoylquinic acids in Centella asiatica protect against amyloid-beta toxicity. *J Alzheimers Dis.* 2014;40(2):359-373.
32. Gray NE, Sampath H, Zweig JA, Quinn JF, Soumyanath A. Centella asiatica Attenuates Amyloid-beta-Induced Oxidative Stress and Mitochondrial Dysfunction. *J Alzheimers Dis.* 2015;45(3):933-946.
33. Wattanathorn J, Mator L, Muchimapura S, et al. Positive modulation of cognition and mood in the healthy elderly volunteer following the administration of Centella asiatica. *J Ethnopharmacol.* 2008;116(2):325-332.
34. Dev RDO, Mohamed S, Hambali Z, Samah BA. Comparison on cognitive effects of *Centella asiatica* in healthy middle age female and male volunteers2009:553-656, European Journal of Scientific Research.
35. Tiwari S, Singh S, Patwardhan K, Ghlot S, Gambhir I. Effect of *Centella asiatica* on mild cognitive impairment (MCI) and other common age-related clinical problems2008:215-220, Digst Jounral of Nanomaterials and Biostructures.
36. <Appa Rao 1973.pdf>.
37. Forte J, Raman A. Regulatory issues related to herbal products part 3: Quality and its determination. *J Med Food.* 2000;3(1):59-70.
38. Brinkhaus B, Lindner M, Hentschel C, et al. Centella asiatic*a in traditional* and modern phytomedicine - A pharmacological and clinical profile - part I; Botany, chemistry, preparations. *Perfusion.* 1998;11(11):466-474.

39. James JT, Dubery IA. Pentacyclic triterpenoids from the medicinal herb, Centella asiatica (L.) Urban. *Molecules.* 2009;14(10):3922-3941.
40. Soumyanath A, Zhong YP, Gold SA, et al. Centella asiatica accelerates nerve regeneration upon oral administration and contains multiple active fractions increasing neurite elongation in-vitro. *J Pharm Pharmacol.* 2005;57(9):1221-1229.
41. Lee MK, Kim SR, Sung SH, et al. Asiatic acid derivatives protect cultured cortical neurons from glutamate-induced excitotoxicity. *Res Commun Mol Pathol Pharmacol.* 2000;108(1-2):75-86.
42. Jew SS, Yoo CH, Lim DY, et al. Structure-activity relationship study of asiatic acid derivatives against beta amyloid (A beta)-induced neurotoxicity. *Bioorg Med Chem Lett.* 2000;10(2):119-121.
43. Mook-Jung I, Shin JE, Yun SH, et al. Protective effects of asiaticoside derivatives against beta-amyloid neurotoxicity. *J Neurosci Res.* 1999;58(3):417-425.
44. Yuan Y, Zhang H, Sun F, Sun S, Zhu Z, Chai Y. Biopharmaceutical and pharmacokinetic characterization of asiatic acid in Centella asiatica as determined by a sensitive and robust HPLC-MS method. *J Ethnopharmacol.* 2015;163:31-38.
45. Grimaldi R, De Ponti F, D'Angelo L, et al. Pharmacokinetics of the total triterpenic fraction of Centella asiatica after single and multiple administrations to healthy volunteers. A new assay for asiatic acid. *J Ethnopharmacol.* 1990;28(2):235-241.
46. Zheng XC, Wang SH. Determination of asiatic acid in beagle dog plasma after oral administration of Centella asiatica extract by precolumn derivatization RP-HPLC. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2009;877(5-6):477-481.
47. Kim J, Lee H, Hwang B, Kim S, Yoo J, Seong Y. Neuroprotection of Ilex latifolia and caffeoylquinic acid derivatives against excitotoxic ad hypoxic damage of cultured rat cortical neurons. *Arch Pharm Res.* 2012;35:1115-1122.
48. Xiao H, Cao X, Wang L, et al. 1,5-dicaffeoylquinic acid protects primary neurons from amyloid B 1-42- induced apoptosis via PI3K/Akt signaling pathway. *Chin Med J (Engl).* 2011;124:2628-2635.
49. Rush WR, Murray GR, Graham DJ. The comparative steady-state bioavailability of the active ingredients of Madecassol. *Eur J Drug Metab Pharmacokinet.* 1993;18(4):323-326.
50. Rush WR, Murray GR, Graham DJ. The comparative steady-state bioavailability of the active ingredients of Madecassol. *European Journal of Drug Metabolism & Pharmacokinetics.* 1993;18(4):323-326.
51. Grimaldi R, De Ponti F, D'Angelo L, et al. Pharmacokinetics of the total triterpenic fraction of Centella asiatica after single and multiple administrations to healthy volunteers. A new assay for asiatic acid. *Journal of Ethnopharmacology.* 1990;28(2):235-241.
52. Chassaud LF, Fry BJ, Hawkins DR, et al. The metabolism of asiatic acid,-madecassic acid and asiaticoside in the rat. *Arzneimittelforschung.* 1971;21(9):1379-1384.
53. Vogel H, De Souza N, D'Sa A. Effect of terpenoids isolated from centella asiatica on granuloma tissue. Vol 16. Acta Therapeutica1990:285-298.
54. Leng DD, Han WJ, Rui Y, Dai Y, Xia YF. In vivo disposition and metabolism of madecassoside, a major bioactive constituent in Centella asiatica (L.) Urb. *Journal of Ethnopharmacology.* 2013;150(2):601-608.
55. Han WJ, Xia YF, Dai Y. Development and validation of high-performance liquid chromatography/electrospray ionization mass spectrometry for assay of madecassoside in rat plasma and its application to pharmacokinetic study. *Biomed Chromatogr.* 2012;26(1):26-32.
56. Wang T, Leng DD, Gao FF, Jiang CJ, Xia YF, Dai Y. A LC-ESI-MS method for the simultaneous determination of madecassoside and its metabolite madecassic acid in rat

plasma: Comparison pharmacokinetics in normal and collagen-induced arthritic rats. *Chinese Journal of Natural Medicines.* 2014;12(12):0943-0951.
57. Nair SN, Menon S, Shailajan S. A liquid chromatography/electrospray ionization tandem mass spectrometric method for quantification of asiatic acid from plasma: application to pharmacokinetic study in rats. *Rapid Commun Mass Spectrom.* 2012;26(17):1899-1908.
58. Xia B, Bai L, Li X, Xiong J, Xu P, Xue M. Structural analysis of metabolites of asiatic acid and its analogue madecassic acid in zebrafish using LC/IT-MSn. *Molecules.* 2015;20(2):3001-3019.
59. Yin MC, Lin MC, Mong MC, Lin CY. Bioavailability, distribution, and antioxidative effects of selected triterpenes in mice. *J Agric Food Chem.* 2012;60(31):7697-7701.
60. Williamson G, Dionisi F, Renouf M. Flavanols from green tea and phenolic acids from coffee: Critical quantitative evaluation of the pharmacokinetic data in humans after consumption of single doses of beverages. *Molecular Nutrition and Food Research.* 2011;55(6):864-873.
61. Farah A, Monteiro M, Donangelo CM, Lafay S. Chlorogenic acids from green coffee extract are highly bioavailable in humans. *Journal of Nutrition.* 2008;138(12):2309-2315.
62. Monteiro M, Farah A, Perrone D, Trugo LC, Donangelo C. Chlorogenic acid compounds from coffee are differentially absorbed and metabolized in humans. *Journal of Nutrition.* 2007;137(10):2196-2201.
63. Liu J, Dou G, Dong X, et al. An improved LC-MS/MS method for simultaneous determination of 1,5-dicaffeoylquinic acid and its active metabolites in human plasma and its application to a pharmacokinetic study in patients. *Biomedical Chromatography.* 2010;24(9):935-940.
64. Su D, Huang J, Song Y, Feng Y. Comparative pharmacokinetics and tissue distribution study of mono-, and di-caffeoylquinic acids isomers of Ainsliaea fragrans Champ by a fast UHPLC-MS/MS method. *Fitoterapia.* 2014;99(1):139-152.
65. Wittemer SM, Ploch M, Windeck T, et al. Bioavailability and pharmacokinetics of caffeoylquinic acids and flavonoids after oral administration of Artichoke leaf extracts in humans. *Phytomedicine.* 2005;12(1-2):28-38.
66. Erk T, Williamson G, Renouf M, et al. Dose-dependent absorption of chlorogenic acids in the small intestine assessed by coffee consumption in ileostomists. *Molecular Nutrition & Food Research.* 2012;56(10):1488-1500.
67. Basavarajappa BS, Nagre NN, Xie S, Subbanna S. Elevation of endogenous anandamide impairs LTP, learning, and memory through CB1 receptor signaling in mice. *Hippocampus.* 2014;24(7):808-818.
68. Kahle K, Huemmer W, Kempf M, Scheppach W, Erk T, Richling E. Polyphenols are intensively metabolized in the human gastrointestinal tract after apple juice consumption. *Journal of Agricultural and Food Chemistry.* 2007;55(26):10605-10614.
69. Stalmach A, Mullen W, Barron D, et al. Metabolite profiling of hydroxycinnamate derivatives in plasma and urine after the ingestion of coffee by humans: Identification of biomarkers of coffee consumption. *Drug Metabolism and Disposition.* 2009;37(8):1749-1758.
70. Wong CC, Meinl W, Glatt HR, et al. In vitro and in vivo conjugation of dietary hydroxycinnamic acids by UDP-glucuronosyltransferases and sulfotransferases in humans. *J Nutr Biochem.* 2010;21(11):1060-1068.
71. Senger DR, Li D, Jaminet SC, Cao S. Activation of the Nrf2 Cell Defense Pathway by Ancient Foods: Disease Prevention by Important Molecules and Microbes Lost from the Modern Western Diet. *PlosOne.* 2016;Feb 17(PLOS ONE | DOI:10.1371/journal.pone.0148042):1-40.
72. Marmet C, Actis-Goretta L, Renouf M, Giuffrida F. Quantification of phenolic acids and their methylates, glucuronides, sulfates and lactones metabolites in human plasma by

LC-MS/MS after oral ingestion of soluble coffee. *Journal of Pharmaceutical and Biomedical Analysis.* 2014;88:617-625.
73. Scherbl D, Renouf M, Marmet C, et al. Breakfast consumption induces retarded release of chlorogenic acid metabolites in humans. *European Food Research and Technology.* 2016:1-16.
74. Wittemer SM, Veit M. Validated method for the determination of six metabolites derived from artichoke leaf extract in human plasma by high-performance liquid chromatography-coulometric-array detection. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences.* 2003;793(2):367-375.
75. Gray NE, Morré J, Kelley J, et al. Caffeoylquinic Acids in Centella asiatica Protect against Amyloid-β Toxicity. *J Alzheimer's Dis.* 2014;40:359–373.
76. Gasperotti M, Passamonti S, Tramer F, et al. Fate of Microbial Metabolites of Dietary Polyphenols in Rats: Is the Brain Their Target Destination? *ACS Chemical Neuroscience.* 2015;6(8):1341-1352.
77. Zhang W, Li D, Leng A, et al. Tissue distribution and excretion of the five components of Portulaca oleracea L. extract in rat assessed by UHPLC. *Brazilian Journal of Pharmaceutical Sciences.* 2015;51(3):643-652.
78. *Botanical Safety Handbook.* American Herbal Products Association. CRC Press, Boca Raton, USA1997.
79. Liyanage C, Goonaratna C, Thabrew I. Iron absorption from a traditional Sri Lankan weaning food and the enhancing effect of ascorbic acid in adult male volunteers. *The Ceylon medical journal.* 1996;41(4):135-140.
80. Rao MA, Srinivasan K, Rao To. The effect of Mandookaparni (*Centella asiatica*) on the general mental ability (Medhya) of mentally retarded children1973:9-16, *Jour. Res. Ind. med*.
81. Bradwejn J, Zhou Y, Koszycki D, Shlik J. A double-blind, placebo-controlled study on the effects of Gotu Kola (Centella asiatica) on acoustic startle response in healthy subjects. *J Clin Psychopharmacol.* 2000;20(6):680-684.
82. Dev RDO, Mohamed S, Hambali Z, Samah BA. Comparison on cognitive effects of Centella asiatica in healthy middle aged female and male volunteers. *European Journal of Scientific Research.* 2009;31(4):553-565.
83. Banerjee P, Maity S, Das T, Mazumder S. A double-blind randomized placebo-controlled clinical study to evaluate the efficacy and safety of a polyherbal formulation in geriatric age group: A phase IV clinical report. *Journal of Ethnopharmacology.* 2011;134(2):429-433.
84. Carlson JJ, Farquhar JW, DiNucci E, et al. Safety and Efficacy of a Ginkgo Biloba-Containing Dietary Supplement on Cognitive Function, Quality of Life, and Platelet Function in Healthy, Cognitively Intact Older Adults. *Journal of the American Dietetic Association.* 2007;107(3):422-432.
85. Harris E, MacPherson H, Vitetta L, Kirk J, Sali A, Pipingas A. Effects of a multivitamin, mineral and herbal supplement on cognition and blood biomarkers in older men: A randomised, placebo-controlled trial. *Human Psychopharmacology.* 2012;27(4):370-377.
86. Katz M, Levine AA, Kol-Degani H, Kav-Venaki L. A compound herbal preparation (CHP) in the treatment of children with ADHD: A randomized controlled trial. *Journal of Attention Disorders.* 2010;14(3):281-291.
87. Lewis JE, Melillo AB, Tiozzo E, et al. A double-blind, randomized clinical trial of dietary supplementation on cognitive and immune functioning in healthy older adults. *BMC Complementary and Alternative Medicine.* 2014;14.
88. Sarokte AS, Rao MV. Effects of Medhya Rasayana and Yogic practices in improvement of short-term memory among school-going children. *Ayu.* 2013;34(4):383-389.

89. Udani JK. Effects of superulam on supporting concentration and mood: A randomized, double-blind, placebo-controlled crossover study. *Evidence-based Complementary and Alternative Medicine.* 2013;2013.
90. Farhana KM, Malueka RG, Wibowo S, Gofir A. Effectiveness of Gotu Kola Extract 750 mg and 1000 mg Compared with Folic Acid 3 mg in Improving Vascular Cognitive Impairment after Stroke. *Evid Based Complement Alternat Med.* 2016;2016:2795915.
91. Cesarone MR, Incandela L, De Sanctis MT, et al. Evaluation of treatment of diabetic microangiopathy with total triterpenic fraction of Centella asiatica: A clinical prospective randomized trial with a microcirculatory model. *Angiology.* 2001;52:S49-S54.
92. Incandela L, Belcaro G, Cesarone MR, et al. Treatment of diabetic microangiopathy and edema with total triterpenic fraction of Centella asiatica: A prospective, placebo-controlled randomized study. *Angiology.* 2001;52:S27-S31.
93. Incandela L, Belcaro G, Nicolaides AN, et al. Modification of the echogenicity of femoral plaques after treatment with total triterpenic fraction of Centella asiatica: A prospective, randomized, placebo-controlled trial. *Angiology.* 2001;52:S69-S73.
94. Cesarone MR, Belcaro G, Nicolaides AN, et al. Increase in echogenicity of echolucent carotid plaques after treatment with total triterpenic fraction of Centella asiatica: A prospective, placebo-controlled, randomized trial. *Angiology.* 2001;52:S19-S25.
95. Brown AC. Liver toxicity related to herbs and dietary supplements: Online table of case reports. Part 2 of 5 series. *Food and Chemical Toxicology.* 2017;107:472-501.
96. Jorge OA, Jorge AD. Hepatotoxicity associated with the ingestion of Centella asiatica. *Revista Espanola de Enfermedades Digestivas.* 2005;97(2):115-124.
97. Dantuluri S, North-Lewis P, Karthik SV. Gotu Kola induced hepatotoxicity in a child - need for caution with alternative remedies. *Digestive & Liver Disease.* 2011;43(6):500.
98. Rakesh R, Sushma T, Sangeeta G, Gambir I. Response of *Centella asiatica* in the management of age related problems among elderly with special reference to cognitive problems as per Prakriti2013:163-167, Int. J. res. Ayurveda Pharm.
99. Danese P, Carnevali C, Bertazzoni MG. Allergic Contact-Dermatitis due to Centella-Asiatica Extract. *Contact dermatitis.* 1994;31(3):201-201.
100. Izu R, Aguirre A, Gil N, Diazperez JL. Allergic Contact-Dermatitis from a Cream Containing Centella-Asiatica Extract. *Contact dermatitis.* 1992;26(3):192-193.
101. Eun HC, Lee AY. Contact dermatitis due to madecassol. *Contact Dermatitis.* 1985;13(5):310-313.
102. Oruganti M, Roy BK, Singh KK, Prasad R, Kumar S. Safety assessment of *Centella asiatica* in albino rats. *Pharmacognosy Journal.* 2010;2(16):5-11.
103. Deshpande P, Mohan V, Thakurdesai P. Preclinical safety assessment of standardized extract of Centella asiatica (L.) urban leaves. *Toxicology International.* 2015;22:10.
104. Kalshetty P, Aswar U, Bodhankar S, Sinnathambi A, Mohan V, Thakurdesai P. Antidepressant effects of standardized extract of *Centella asiatica L* in olfactory bulbectomy model. *Biomedicine & Aging Pathology.* April-June 2012;2(2):48-53.
105. Chauhan PK, Singh V. Acute and Subacute Toxicity study of the Acetone Leaf extract of Centella asiatica in Experimental Animal Models. *Asian Pac J Trop Biomed.* 2012;2(2 SUPPL.):S511-S513.
106. Yunianto I, Das S, Mat Noor M. Antispermatogenic and antifertility effect of Pegaga (Centella asiatica L) on the testis of male Sprague-Dawley rats. *Clinica Terapeutica.* 2010;161(3):235-239.
107. Hausen BM. Centella-Asiatica (Indian Pennywort), an Effective Therapeutic but a Weak Sensitizer. *Contact dermatitis.* 1993;29(4):175-179.
108. Laerum OD, Iversen OH. Reticuloses and epidermal tumors in hairless mice after topical skin applications of cantharidin and asiaticoside. *Cancer Res.* 1972;32(7):1463-1469.

109. Ramaswamy AS, Periyasamy, S.M., and Basu, N. Pharmacological studies on Centella asiatica Linn (Brahma Manduki) (N.O. Umbelliferae). *Journal of Research in Indian Medicine.* 1970;4(2):160-173.
110. Soumyanath A, Zhong YP, Gold SA, et al. Centella asiatica accelerates nerve regeneration upon oral administration and contains multiple active fractions increasing neurite elongation in-vitro. *Journal of Pharmacy & Pharmacology.* 2005;57(9):1221-1229.
111. Clymer J. Javascript Allometric Scaling calculator. Vol October 18, 2016.
112. Nair AB, Jacob S. A simple practice guide for dose conversion between animals and humans. 2016;7:27-31. J Basic Clin Pharma. *Journal of Basic & Clinical Physiology & Pharmacology.* 2016;7:27-31.
113. Kar A, Pandit S, Mukherjee K, Bahadur S, Mukherjee PK. Safety assessment of selected medicinal food plants used in Ayurveda through CYP450 enzyme inhibition study. *Journal of the Science of Food and Agriculture.* 2017;97(1):333-340.
114. Pan Y, Abd-Rashid BA, Ismail Z, et al. In vitro modulatory effects of Andrographis paniculata, Centella asiatica and Orthosiphon stamineus on cytochrome P450 2C19 (CYP2C19). *Journal of ethnopharmacology.* 2011;133(2):881-887.
115. Pan Y, Abd-Rashid BA, Ismail Z, et al. In vitro modulatory effects on three major human cytochrome P450 enzymes by multiple active constituents and extracts of Centella asiatica. *Journal of ethnopharmacology.* 2010;130(2):275-283.
116. Seeka P, Niwattisaiwong N, Warisnoicharoen W, Winitthana T, Tantisira MH, Lawanprasert S. Effects of the standardized extract of Centella asiatica ECa233 on human cytochrome P450. *Thai J Pharm Sci.* 2012;36(1):30-37.
117. Savai J, Varghese A, Pandita N, Chintamaneni M. In vitro assessment of CYP1A2 and 2C9 inhibition potential of Withania somnifera and Centella asiatica in human liver microsomes. *Drug Metabolism and Personalized Therapy.* 2015;30(2):137-141.
118. Savai J, Varghese A, Pandita N, Chintamaneni M. Investigation of CYP3A4 and CYP2D6 interactions of withania somnifera and centella asiatica in human liver microsomes. *Phytotherapy Research.* 2015;29(5):785-790.
119. Seeka P, Niwattisaiwong N, Tantisira MH, et al. Effects of the standardized extract of Centella asiatica ECa233 on hepatic Phase II drug-metabolizing enzymes in rats. *Thai J Pharm Sci.* 2017;41(2):41-46.
120. Hashim P. Centella asiatica in food and beverage applications and its potential antioxidant and neuroprotective effect. *International Food Research Journal.* 2011;18(4):1215-1222.
121. Li DL, Zheng XL, Duan L, et al. Ethnobotanical survey of herbal tea plants from the traditional markets in Chaoshan, China. *Journal of Ethnopharmacology.* 2017;205:195-206.
122. Naithani V, Nair S, Kakkar P. Decline in antioxidant capacity of Indian herbal teas during storage and its relation to phenolic content. *Food Research International.* 2006;39(2):176-181.
123. Cummings J, Aisen PS, DuBois B, et al. Drug development in Alzheimer's disease: the path to 2025. *Alzheimers Res Ther.* 2016;8:39.
124. Flaherty JH, Takahashi R. The use of complementary and alternative medical therapies among older persons around the world. *Clin Geriatr Med.* 2004;20(2):179-200, v.
125. Flaherty JH, Takahashi R, Teoh J, et al. Use of alternative therapies in older outpatients in the United States and Japan: prevalence, reporting patterns, and perceived effectiveness. *J Gerontol A Biol Sci Med Sci.* 2001;56(10):M650-655.
126. Astin JA, Pelletier KR, Marie A, Haskell WL. Complementary and alternative medicine use among elderly persons: one-year analysis of a Blue Shield Medicare supplement. *J Gerontol A Biol Sci Med Sci.* 2000;55(1):M4-9.

127. Booth AN, Emerson OH, Jones FT, Deeds F. Urinary metabolites of caffeic and chlorogenic acids. *J Biol Chem.* 1957;229(1):51-59.
128. Wittemer SM, Ploch M, Windeck T, et al. Bioavailability and pharmacokinetics of caffeoylquinic acids and flavonoids after oral administration of Artichoke leaf extracts in humans. *Phytomedicine.* 2005;12(1-2):28-38.
129. Erk T, Williamson G, Renouf M, et al. Dose-dependent absorption of chlorogenic acids in the small intestine assessed by coffee consumption in ileostomists. *Mol Nutr Food Res.* 2012;56(10):1488-1500.
130. Pagano I, Piccinelli AL, Celano R, et al. Chemical profile and cellular antioxidant activity of artichoke by-products. *Food Funct.* 2016;7(12):4841-4850.
131. Nair AB, Jacob S. A simple practice guide for dose conversion between animals and human. *J Basic Clin Pharm.* 2016;7(2):27-31.
132. Heisel MJ, Duberstein PR, Lyness JM, Feldman MD. Screening for Suicide Ideation among Older Primary Care Patients. *Journal of the American Board of Family Medicine : JABFM.* 2010;23(2):260-269.

14. APPENDIX 1: LIST OF CASE REPORT FORMS TO BE USED IN THIS STUDY

Name of form:	Completed during:	Completed by:
Informed consent (participant and LAR)	Screening visit	Participant and LAR
Informed consent (Study partner)	Screening visit	Study partner
Clinical Dementia Rating Worksheet (CDR)	Screening visit	Study team
Mini Mental State Examination (MMSE)	Screening visit	Study team
Geriatric Depression Scale (GDS)	Screening visit	Study team
Diet and Medication Diary	Two day period prior to study visits 1 and 2	Participant with help of study partner
Standardized Adverse Events Questionnaire: Multi-system	study visits 1 and 2, at baseline and again at end of visit. Phone calls on the days following study visits 1 and 2. Phone call on 7th day following study visit 2.	Study team
Medical History Screening Questionnaire (health screening)	Screening visit	Study team

Study visit case report form	**Biometrics (height, weight, body mass index)**	**Screening visit, Study Visits 1 and 2 at baseline**	**Study team**
	Vitals (heart rate, blood pressure, temperature)	**Screening visit, Study Visits 1 and 2 at baseline and end of visit.**	
	Electrocardiogram interpretation	**Screening visit, Study Visits 1 and 2 at baseline and 6 hours.**	
	Safety tests CBC, CMP, Urinalysis test result and interpretation	**Screening visit, Study Visits 1 and 2 at baseline (all tests) and 10 hours (CMP only)**	

15. APPENDIX 2: Measurement of NRF2 activation in PBMC of study participants.

Background and rationale:
NRF2 expression in peripheral blood mononuclear cells (PBMC's) has been used as a biomarker of NRF2 activation in several previous clinical trials. NRF2 expression was found to be elevated in patients with type 2 diabetes treated with resveratrol [1] as well as in obese subjects taking grape powder [2]. NRF2 levels were increased in nuclear extracts of PBMCs of subjects following ingestion of a coffee bean extract [3]. We and others have shown that the constituent compounds from CAW, including AA and CQAs, can activate NRF2 ([4-8], suggesting that monitoring NRF2 expression in the blood could be a good marker for target engagement.

The studies mentioned above suggest that dietary polyphenols can influence NRF2 expression. The present study population is therefore ideal to examine the possible effects of CAW on NRF2 expression since they will have been on a low phytochemical diet for 48 hours prior to ingesting CAP containing CAW 2g or 4g. This will reduce any dietary influences on NRF2 levels. We will compare the NRF2 expression in blood taken at the screening visit (participants would have been on their usual diet) with that taken at baseline during the 2 study visits to see if reducing fruit and vegetables in the diet has an effect. We will then explore the time course of any change in NRF2 expression over 6 hours following CAW ingestion, taking samples at 1, 2, 3, 4 and 6 hours.

Protocol modification needed from V10: In order to measure NRF2 activation, it will be necessary to draw an additional 5 mL at the screening visit and at study visits 1 and 2 at baseline, and 1, 2, 3, 4 and 6 hours after CAP administration.

Method:
PBMC will be isolated from 4.5 mL of blood. NRF2 gene expression will be measured by qPCR analysis of RNA isolated from the PBMC. We will also attempt to measure NRF2 protein by ELISA of nuclear extracts from 0.5mL of blood [3].

Other considerations:

- The Red Cross recommends a maximum blood draw of 470 mL every 56 days. The total blood drawn during the 6 weeks of this study will be 335 mL, consisting of screening visit (15 mL) + study visit 1 (160 mL) + study visit 2 (160 mL).
- A limitation is the absence of a placebo comparison which would have allowed an assessment of the role of CAP excipients on NRF2 activation. However a comparison of NRF2 activation by CAP 2g and 4g (which have equal amounts of excipients) would provide information on the effect of CAW vs excipients in CAP.

Literature cited:

1. Seyyedebrahimi, S., et al., *The effects of resveratrol on markers of oxidative stress in patients with type 2 diabetes: a randomized, double-blind, placebo-controlled clinical trial.* Acta Diabetol, 2018. **55**(4): p. 341-353.
2. Bardagjy, A.S., et al., *Effects of grape consumption on biomarkers of inflammation, endothelial function, and PBMC gene expression in obese subjects.* Arch Biochem Biophys, 2018. **646**: p. 145-152.

3. Reyes-Izquierdo T., et al., *Stimulatory effect of acute single dose of dried whole coffee cherry powder on NRF2 activity in freshly isolated blood cells. A single-blind, placebo controlled cross-over pilot clinical study.* J Aging Res Clin Practice 2016;5(3):120-127.
4. Gray, N.E., et al., *Caffeoylquinic acids in Centella asiatica protect against amyloid-beta toxicity.* J Alzheimers Dis, 2014. **40**(2): p. 359-73.
5. Fan, J., et al., *Asiatic acid ameliorates CCl4-induced liver fibrosis in rats: involvement of Nrf2/ARE, NF-kappaB/IkappaBalpha, and JAK1/STAT3 signaling pathways.* Drug Des Devel Ther, 2018. **12**: p. 3595-3605.
6. Jiang, J.Z., et al., *Asiaticoside Mitigates the Allergic Inflammation by Abrogating the Degranulation of Mast Cells.* J Agric Food Chem, 2017. **65**(37): p. 8128-8135.
7. Liu, S., et al., *Madecassoside ameliorates lipopolysaccharide-induced neurotoxicity in rats by activating the Nrf2-HO-1 pathway.* Neurosci Lett, 2019. **709**: p. 134386.
8. Yang, B., et al., *Madecassic Acid protects against hypoxia-induced oxidative stress in retinal microvascular endothelial cells via ROS-mediated endoplasmic reticulum stress.* Biomed Pharmacother, 2016. **84**: p. 845-852.