Pharmacokinetics of Centella asiatica in the Elderly

NCT03929250

STUDY PROTOCOL INCLUDING STATISTICAL ANALYSIS PLAN

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A. OBJECTIVES

A.1. Purpose

The purpose of this pilot study is to measure the oral bioavailability and pharmacokinetics of known bioactive compounds from a standardized *Centella asiatica* water extract product (CAP) in cognitively healthy elders. Compound levels will be measured in human plasma and urine over 12 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, as well as the rate of clearance (t ½) of the known compounds, will be identified. These data will be used to inform decisions on the dosage and dosing frequency for future phase 1 and phase 2 clinical trials.

A.2. Primary goal

To determine the oral bioavailability and pharmacokinetics of known compounds from two single administrations of a *Centella asiatica* product in non-demented elders.

A.3. Endpoints

- The primary endpoints for this study are the maximum concentration (C_{max}), the area under the curve (AUC), and the time of maximum concentration (t_{max}) of known compounds from *Centella asiatica* (triterpenes and caffeoylquinic acids and their metabolites) in human plasma.
- A secondary endpoint is the half-life ($t_{1/2}$) of the known bioactive compounds and their metabolites to help determine dosage intervals.
- Additional secondary endpoints include temporal changes in ferric reducing ability of plasma (FRAP) as an indicator of antioxidant potential over time, tolerability and detection of acute adverse events through monitoring by participant interviews, biometrics, vital signs and questionnaires.

B. SPECIFIC AIMS

Aim 1: To assess the bioavailability and rate of clearance of *Centella asiatica* derived compounds in cognitively healthy elders 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 cognitively healthy elders.

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

C.BACKGROUND

C.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.

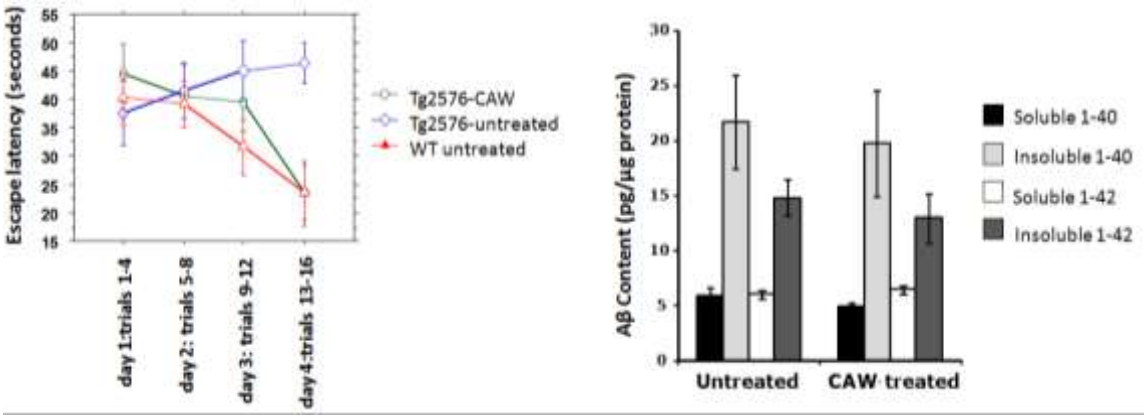
Beyond cognitive impairment, Alzheimer's disease also has significant comorbidities including insomnia, depression[11] and anxiety.[12] Multiple interventions are often needed to manage these symptoms affecting patient compliance and safety. This warrants further investigation into effective, inexpensive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

C.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]

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).



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.

C.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.

C.4. Safety

Centella asiatica is an edible plant and the Botanical Safety Handbook[49] 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.[35,50-53] There have also been several clinical studies in which multi-component products containing *Centella asiatica* herb or extracts were found to be well tolerated.[54-60] 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.[61] Two clinical studies on polyherbal preparations containing *Centella asiatica* (albeit in small quantities) also reported no changes in safety laboratory results.[54,57]

TTFCA (a purified triterpene mixture), was well-tolerated at all of the following doses in microcirculation studies: 30 or 60mg daily for 7 days[62]; 30 or 60 mg administered twice daily for 6 months[63]; and 60mg twice daily for 12 months.[64] Two studies on femoral and carotid plaque formation respectively[65,66] both used TTFCA at 180mg daily for 12 months. Again, TTFCA was well tolerated at this highest reported dose.

C.4.1. 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[67] 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.[68] 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[69] 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,[68] and the presence of additional ingredients, low *Centella asiatica* dose and concomitant use of lymecycline in the second report[69] 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[35,70] noted decreases in systolic and/or diastolic blood pressure, and one also reported improved sleep patterns among participants.[70] 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*.[61] Topical administration of CA extracts and triterpenes is reported to cause contact dermatitis in some individuals.[71-73]

C.4.2. Non-clinical pharmacology/toxicology

Rats administered dried Centella asiatica herb:[74] 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 microscopical 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":[75] 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 subchronic 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.[76]

Mice administered Centella asiatica extract:[77] 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:[78] 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.[79] 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.[80] 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.[81] 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[20,30,82] showed no obvious adverse effects. These rodent doses are comparable, by interspecies scaling[83,84] to the proposed 2g and 4g per day human doses in this study.

C.4.3. Influence of CA 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.[85] 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.[86,87] 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.[88] Extracts of *Centella asiatica* were inhibitory to CYP 1A2 and CYP2C9[89] CYP3A4 and CYP 2D6[90] 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.[91] The effect of a water extract of *Centella asiatica* on these Phase I and Phase II enzymes is yet to be determined.

C.4.4. 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,[92-94] 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 cause any change in liver enzymes.[61] The total triterpenes (approximately 4% w/w) delivered by our proposed CAP doses is expected to be 80, 160 and 320 mg/day. The lower doses are comparable to the TTFCA and CAST studies described earlier where these doses were well tolerated. 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[83,84] 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.

D. 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[95] 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.[96-98] *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 (Section C.2.). 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.

E. 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*.[99] Similarly, the caffeoylquinic acids metabolize to caffeic acid, ferulic acid, isoferulic acid, and their dihydroderivatives.[100,101] 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).

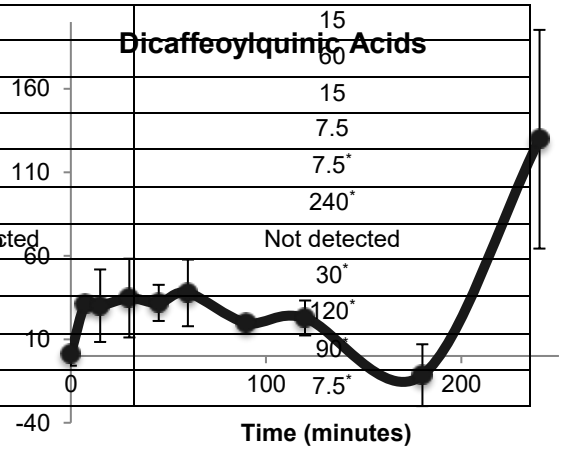
Previous pharmacokinetic literature in rodents and dogs has reported maximum concentrations of the polar caffeoylquinic acid compounds within one hour of ingestion[101,102] and the less polar triterpenes at 4-5 hours.[103] We have performed a pharmacokinetic study in wild-type mice on a low phytochemical 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). 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.

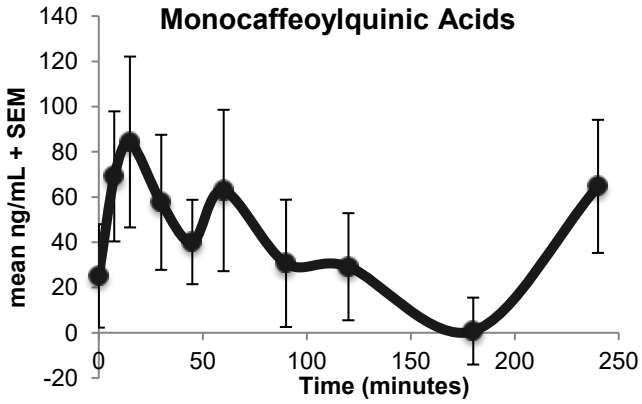
Table 1. Pharmacokinetic parameters of caffeoylquinic acids and metabolites in mouse plasma after single oral administration of *Centella asiatica* water extract.

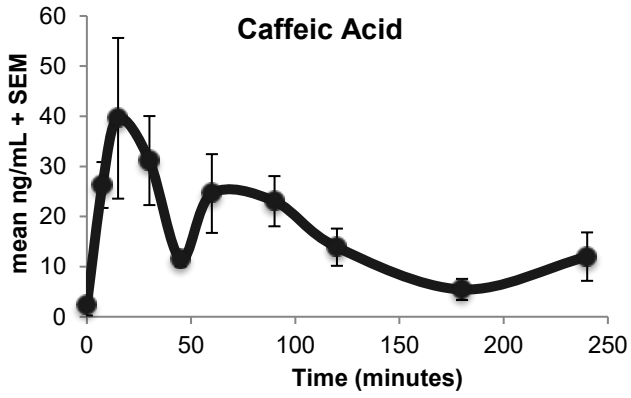
Analyte	C_{max} (ng/mL)	t_{max} (min)

Monocaffeoylquinic acids	84.3	15
Dicaffeoylquinic acids	37.8	60
Caffeic acid	39.6	15
Ferulic acid	15.3	7.5
Isoferulic acid	2.5	7.5*
Dihydrocaffeic acid	13.4	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







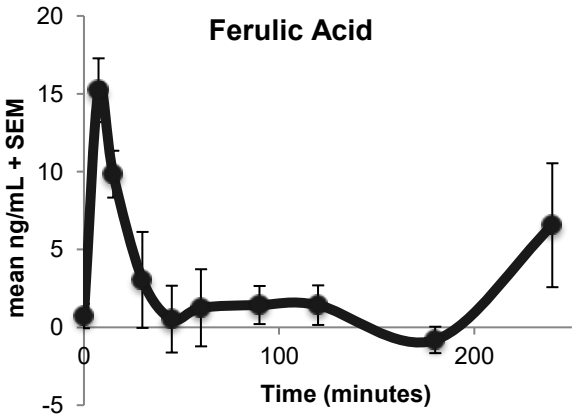


Figure 2. Pharmacokinetic profile of monocaffeoylquinic acids, dicaffeoylquinic acids and metabolites caffeic acid and ferulic acid in wild-type female mice. Six-month-old female mice (n=39) were administered *Centella asiatica* water extract (200 mg/kg) via oral gavage. Four mice were euthanized per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

F. RESEARCH DESIGN AND METHODS

F.1. Study design

This is a phase 1 pilot clinical study using a blinded randomized crossover design. Randomization will use an arm equivalence design to promote equal numbers of participants for each order schema. We will dispense a single administration of a *Centella asiatica* water extract

product to the research participants at two doses (2g and 4g) 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 (Section I.4.). We will provide participants a handout at screening outlining the diet with meal examples. Serial blood samples (2 teaspoons; 10 mL/sample) will be obtained through a peripheral intravenous catheter, once prior to *Centella asiatica* consumption and then over a 12-hour post-administration period (15, 30, 45, 60, 90, 120, 180, 240, 360, 480, 600, and 720 min). Participants will also perform a 12-hour urine collection at each study visit to assess for excretion of *Centella asiatica* metabolites. 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).

F.2. Study Population

F.2.1. Number of participants

Eight non-demented elderly volunteers (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 expect a 10% drop out rate. We are still powered to see changes from baseline and differences between doses with a 25% drop out rate resulting in 6 subjects.

F.2.2. Inclusion and exclusion criteria

Inclusion criteria:

1. Age 65-85, male and female
2. Sufficient English language skills to complete all tests
3. Sufficient vision and hearing to complete all tests
4. No known allergies to *Centella asiatica*, **(excipients redacted)** or any of their derivatives
5. Willingness to discontinue all botanical dietary supplements for one week prior to and during each study visit
6. Willingness to comply with a 48-hour low plant diet for each study visit
7. Absence of significant depression symptoms (Geriatric Depression Scale-15 score of <12)
8. Body Mass Index (BMI) greater than 17 and less than 35 at screening
9. Non-demented, defined as Clinical Dementia Rating (CDR) score of zero and Mini Mental State Examination (MMSE) score >28
10. General health status that will not interfere with the ability to complete the study

Exclusion criteria:

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. Diseases associated with dementia such as Alzheimer's disease, vascular dementia, normal pressure hydrocephalus or Parkinson's disease with a CDR score $\geq$0.5 and MMSE score <28
12. Current drug or alcohol use or dependence that, in the opinion of the site investigator, would interfere with adherence to study requirements.
13.

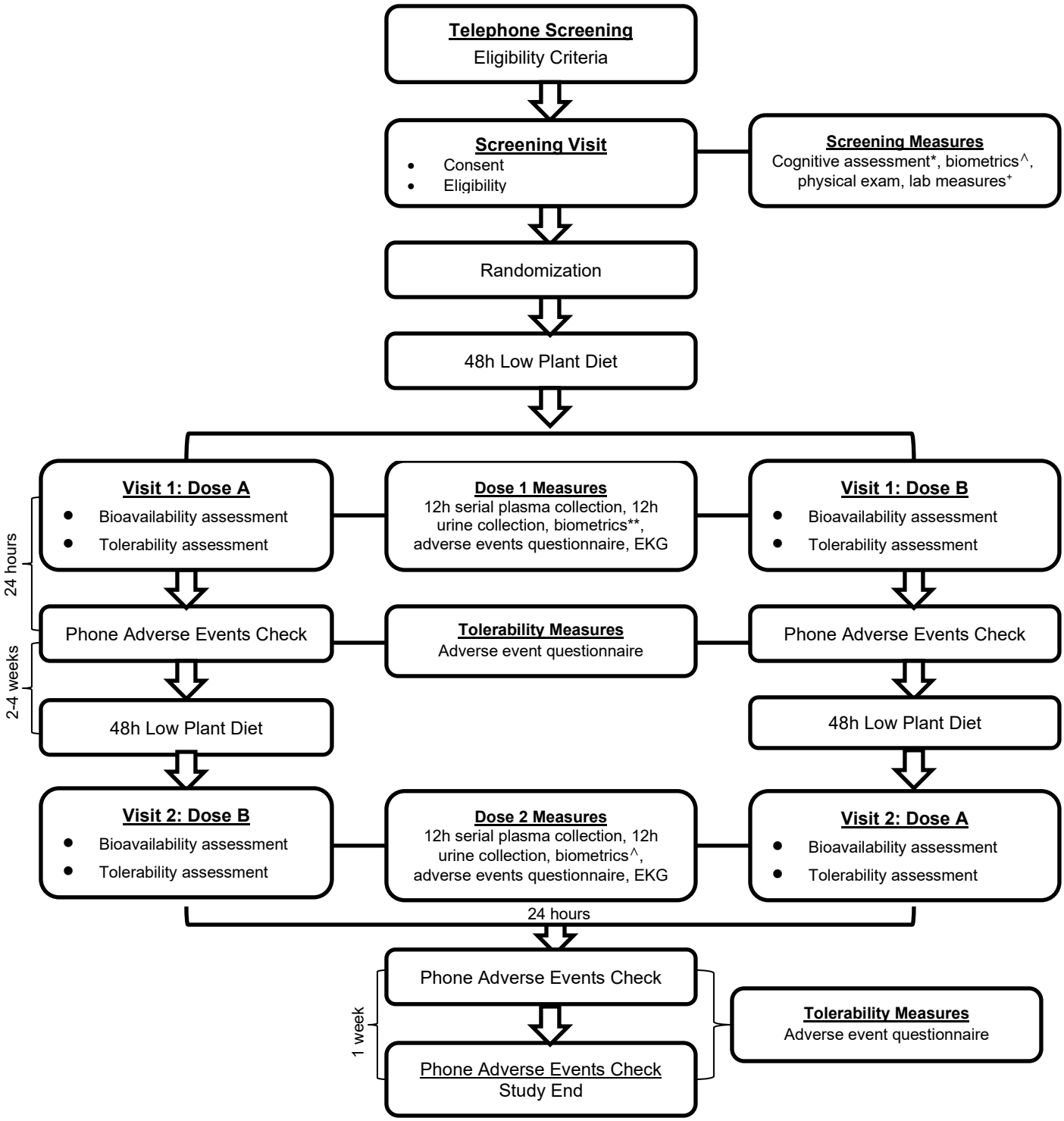


Figure 3. Study design. *Cognitive assessments: Clinical Dementia Rating (CDR), Mini Mental State Examination (MMSE), Geriatric Depression Scale (GDS); ^Biometrics: weight, height, body mass index (BMI), age, blood pressure, pulse rate, temperature; +Lab measures: complete blood count (CBC), comprehensive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

F.2.2.1. Concomitant interventions

F.2.2.1.1. 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

F.2.2.1.2. Required interventions: none

F.2.2.1.3. 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

F.2.3. Vulnerable populations

Based upon the age range and cognitive status needed in our study population, children and decisionally impaired adults 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.

F.3. 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.

F.3.1. 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.

G. RECRUITMENT

Participants will be enrolled on a rolling basis until the desired number of participants is achieved. We will recruit from the Oregon Clinical and Translational Research Institute's volunteer repository, with over 40 qualifying participants identified with preliminary searches, the National Institute of Neurological Disorders and Stroke (NINDS) NeuroNext database, and the Oregon Alzheimer Disease Center's participant database. Potential participants available through the above databases 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 OHSU clinics and 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. It will be emphasized that participation in the study is completely voluntary. Copies of all advertisements have been submitted with this application for IRB approval .

G.1. 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 (section I.4.).

H. INTERVENTION

H.1. *Centella asiatica* intervention (Figure 4) (information on the preparation method is confidential/proprietary): *Centella asiatica* dried herb (aerial parts) has been 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 **(matrix, redacted)** 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 matrix, coloring agent and flavoring agent (excipients) was blended in appropriate ratios so that each dose contains 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 eight 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 4°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. Investigational New Drug (IND) approval has been obtained from the FDA.The drug will be used in compliance with the requirements of HRP-815.

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[104] 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. Following study completion, the remaining intervention and placebo sachets will be provided to Dr. Soumyanath for destruction.

H.2. 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 8-12 ounces of warm water and provide it to the participant for oral consumption. One dose will be administered at each study visit in a random order.

H.3. 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).

Figure 4: Redacted

H.4. 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:

<u>Administrative:</u>
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 (Section N).

I. SCREENING AND STUDY ACTIVITIES

I.1. 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 has been submitted for IRB approval. The telephone screening will take approximately 10-15 minutes. Potential participants that meet eligibility criteria (Section F.2.2) over the phone will be scheduled for a screening visit.

I.2. Consent process

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 CTRC outpatient room. At the in-person screening visit, a member of the study team will review the consent/HIPAA form and then will ask the participant to describe key points in the form (e.g. purpose of study, dietary requirements, length of study) before signing. After the participant signs the consent, the participant will continue with screening assessments and baseline measurements. 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 will be called three days prior to their second study visit to ask if they wish to continue their participation in the study. At this time, participants will also be reminded of their upcoming study visit, to stop necessary supplements, and begin the necessary dietary changes (Section I.4.). 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.

I.3. Screening visit

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 2). A

member of the study staff with venipuncture training or CTRC nursing personnel will collect approximately two teaspoons (10 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. Participants will be provided with a handout describing the “low plant diet” (Section I.4.) 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.

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. 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.

I.4. Study visits 1 and 2

Visit timing: Visit 1 will occur a minimum of 72 hours following the screening visit to ensure adequate time for the low plant diet (Section I.4.), 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. This has been submitted with this protocol for approval.

Sample collection: Participants will be asked to fast for 10-12 hours beginning at 9:00 PM the day 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 plasma 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

teaspoons (10 mL)/sample) will be obtained through the peripheral intravenous catheter using a syringe over a 12-hour post-administration period (15, 30, 45, 60, 90, 120, 180, 240, 360, 480, 600, and 720 min) (Table 2). Participants will collect their urine for the duration of the study visit (12-hours) to assess for excretion of *Centella asiatica* metabolites.

Tolerability and safety: We will record weight, height, body mass index, age, blood pressure, temperature and pulse rate at each study visit to monitor for adverse events and asymptomatic illnesses that may impact study results (Figure 3, Table 2). 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 and has been included in this IRB submission for approval.

I.5. 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 2). 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.

I.6. 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 2).

Table 2. Schedule of study visits and assessments.

	Screening	**Visit 1**	**Visit 1 + 1 day**	**Visit 2**	**Visit 2 + 1 day**	**Visit 2 + 7 days**
Event		Study visit	Phone visit	Study visit	Phone visit	Phone visit
Informed consent	•					
Vitals**+**	•	•		•		
Health screening, biometric assessment	•	•		•		
Electrocardiogram (EKG)		•		•		
Blood draw (CBC, CMP*) and urinalysis	•					
Low plant diet (starting 48h prior to visit)		•		•		
Single dose of intervention		•		•		
Blood collected at intervals (0-12hr) for bioavailability and antioxidant assessment	•^	•		•		
12-hour urine collection, baseline urinalysis		•		•		
Adverse events (AE) questionnaire		•	•	•	•	•

Vitals include blood pressure, temperature and pulse rate
* CBC = Complete Blood Count with differential; CMP = Comprehensive Metabolic Panel
^ Single time point

I.7. 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.

J. 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 3).

Table 3. 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												

K. 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.

K.1. Data collection methods

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. 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.

K.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 and 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.

K.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). We will also conduct additional targeted and untargeted analysis to fingerprint the *Centella asiatica* derived compounds found in plasma and urine samples.

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).

Additional targeted and untargeted analysis of *Centella asiatica* derived compounds: Frozen aliquots of de-identified plasma and urine samples will be shipped on dry ice to Dr. Claudia Maier, Director of the Mass Spectrometry Center at Oregon State University according to OHSU biological materials shipping procedures. Targeted or untargeted high-performance liquid chromatography coupled to high resolution tandem mass spectrometry (HPLC-MS) analysis will be performed to fingerprint the *Centella asiatica* derived compounds present in the samples. The analytical method applied will be based on a method published in Alcazar Magana et al as described below.[105]

Samples will undergo chromatographic separation using an Intersil Phenyl-3 column with an ejection volume of 10µL. Triplicate analysis will be performed. Gradient elution will be performed using a mobile phase consisting of solvent A (water containing 0.1% v/v formic acid) and solvent B (methanol containing 0.1% v/v formic acid), with a flow rate at 0.4mL/min. HPLC-MS analysis will be performed using a Shimadzu Nexera UHPLC system connected to an AB SCIEX TripleTOF® 5600 mass spectrometer equipped with a Turbo V ionization source operated in the electrospray ionization (ESI) mode will be used. Data-dependent acquisitions (DDAs) will be conducted to obtain precursor and fragment ion information using the negative (ESI-) and positive ionization (ESI+) modes. Level 1 annotations we will be based on accurate mass, fragment ion spectral similarity, and retention time co-elution based on authentic commercially available standards. Putative Level 2 annotations will be based on exact mass, isotopic pattern, and MS/MS spectral data.

K.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.

K.5. 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) 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. As there will be no additional laboratory assessments with clinical implications beyond the screening visit, no results from the study will be shared with the research participant or their provider(s).

K.6. 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 or shipped to collaborators at Oregon State University for future analysis of 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.

L. DATA ANALYSIS

L.1. Specimen analysis

Each analyte of interest from *Centella asiatica* will be identified by its retention time and characteristic mass spectral fragmentation pattern using selected reaction monitoring (MS/MS). 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 profiling will use non-compartmental analysis to calculate common pharmacokinetic parameters such as peak concentration (C_{max}), the time at peak concentration (t_{max}) and the total 12-hour bioavailability represented by the total area under the curve (AUC_{0-12}). These will be compared to our mouse data in order to assess appropriateness of dosing level. Other calculable non-compartmental analysis metrics will also be determined, such as the elimination half-life ($t_{1/2}$) calculated from the profile tails and the mean residency time of the compounds in plasma. Descriptive assessment will be done at each of the doses to give basic pharmacokinetic summarization of the various *Centella asiatica* compounds and total antioxidant compounds measured by ferric reducing ability of plasma. Dose dependence of the study outcomes will be determined by evaluation of the within-subject differences in study outcomes between the different doses. For all continuous variables, dose effects will be evaluated both naively using paired t-tests and with ordinary least-squares regression with correction for commonly cited confounders including but not limited to baseline age and gender. 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 participants but the effect of removing clearly obvious outliers will be evaluated. Although all *Centella asiatica* compounds of interest and pharmacokinetic parameters are defined *a priori*, multiple comparison correction to p-values will be applied as necessary, with final two-tail significance defined at $p<0.05$.

L.2. Sample size

Power analysis and sample size estimation for the dose-dependent pharmacokinetics of *Centella asiatica* analytes were derived from the literature, specifically the plasma dose response analysis of the triterpene asiatic acid by Grimaldi et. al.[45] after single acute administration of 30 mg and 60 mg of a *Centella asiatica* triterpene fraction. In a repeated measures cohort of 12 males, they found significant differences ($p<0.05$) in asiatic acid's peak plasma concentration (C_{max}) (30 mg: 0.70 µg/ml ± 0.38; 60 mg: 1.36 µg/ml ± 0.45; d=1.57) and 24-hour total bioavailability (AUC_{0-24}) (30 mg: 4.16 ± 3.81; 60 mg: 9.39 ± 3.53; d=1.42) between the two doses. This paper was chosen as an analog for the current study since a proportionally equimolar dose response shift in the pharmacokinetic profile of asiatic acid can be expected of the proposed *Centella asiatica* product doses. Under that assumption, a significant within-group dose response at $\alpha=0.05$ with at least 80% power would be found for C_{max} with six participants and for AUC_{0-12} with seven participants. Even if the inclusion of additional compounds in the

water extract reduces the dose effect anticipated in the current study, a significant difference in the pharmacokinetic outcomes would still be seen with eight participants. We therefore, propose a cohort of four men and four women, which will enable dose responses to be seen within each gender individually, and allow for evaluation of an attenuated response by pooling gender or provide protection against study dropout.

L.3. Data quality and management

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.

M. PRIVACY, CONFIENTIALITY, 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.

N. PROVISIONS TO MONITOR THE DATA TO ENSURE THE SAFETY OF SUBJECTS

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.

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. Severe AEs (SAEs) are defined as events that are life-threatening or result in hospitalization, permanent disability, cancer, death, or another significant outcome.

N.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, Dr. Quinn and Dr. Wright, 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.

N.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 natural supplement research will chair the IMC and the other two members will be a statistician and a second clinician. 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.

N.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 the protocol be amended as a result of data review, the OHSU IRB and monitoring body will be notified 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 OHSU IRB and IMC will be notified directly after protocol amendment implementation.

O. RISKS AND BENEFITS

O.1. Risks to subjects

Potential harm to participants will be minimized by excluding participants with the conditions detailed in section F.2.2. 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[49] 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* (Appa Rao, Srinivasan et al. 1973, Tiwari, Singh et al. 2008, Dev, Mohamed et al. 2009, Wattanathorn, Mator et al. 2008, Roushan, Tiwari et al. 2013)[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). See section C.4 above for further information.

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)[106] 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 section M of 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.

O.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 trials of CAP in patients with mild cognitive decline or Alzheimer's disease.

P. 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.

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