Safety, tolerability, and pharmacokinetics of an oral *Withania somnifera* product in older adults

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Title: Safety, tolerability, and pharmacokinetics of an oral *Withania somnifera* product in older adults

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A. OBJECTIVES

A.1. Purpose

The purpose of this pilot study is to measure the oral absorption and pharmacokinetics (PK) of compounds from two doses of a standardized *Withania somnifera* extract product (Shoden®) in healthy older adults. Compound levels will be measured in human plasma and urine over 48 hours after acute oral administration and after four weeks' use of Shoden®. Peak exposure and time, total exposure, half-life, and urinary excretion of compounds will be calculated. 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 PK of withanolides from two doses of a *Withania somnifera* product (Shoden®) in healthy older adults following single administration and after four weeks' use.

A.3. Endpoints

- Primary endpoints:
  - Peak exposure and time (C_{max}, T_{max}) and total exposure (AUC_{0-t}, $AUC_{0-\infty}$) of withanolides from Shoden® in human plasma after a single administration.
- Secondary endpoints:
  - Half-life ($t_{1/2}$) of withanolides from Shoden® to help determine dosage intervals.
  - Urinary excretion of withanolides from Shoden®
  - Steady-state levels of withanolides after four weeks' use.
  - Tolerability and safety of Shoden® following single administration and after four weeks' use.
  - Feasibility of assessing sleep- and stress-related outcomes remotely using REDCap surveys.

B. SPECIFIC AIMS

Aim 1: Evaluate the oral absorption and PK of selected withanolides from Shoden® in older adults.

- **Hypothesis**: Selected withanolides will be detectable in the plasma and urine at both doses of Shoden®, with significantly greater plasma levels achieved at the 480 mg dose.

Aim 2: Assess the safety and tolerability of Shoden® in older adults.

- **Hypothesis:** A single administration and four weeks' use of Shoden® at 240 and 480 mg per day will be well tolerated and produce no severe adverse events.

Aim 3: Assess the feasibility of remotely measuring sleep- and stress-related outcomes.

- **Hypothesis**: Remotely measuring sleep- and stress-related outcomes using REDCap surveys will be feasible, as defined by at least 80% of all questionnaires completed by the study population.

C. BACKGROUND

C.1. Botanical dietary supplements

The use of botanical dietary supplements in the US has exploded in recent years, with sales exceeding $10 billion for the first time in 2020,[1] and further increasing to $12 billion in 2021.[2] The popularity of these products has increased despite a relative lack of evidence to support their widespread use,[3] along with significant questions regarding product quality and variability[4] and safety.[5] Rigorous efficacy trials would support the evidence-based use of botanical dietary supplements, but key preliminary data from early-phase clinical research are required first in order to optimize these efficacy trials. The National Center for Complementary and Integrative Health (NCCIH) has established a stepwise approach for conducting natural products research, which includes collecting human PK data of putative active compounds from a natural product and demonstrating mechanism-based biological signatures in humans prior to efficacy trials.[3,6]

C.2. Withania somnifera (WS)

C.2.1. Overview

Withania somnifera (L.) Dunal (WS; common name: ashwagandha) is an example of a botanical for which key preliminary data from early-phase clinical studies are lacking, limiting the impact of efficacy trials using WS products. WS enjoys a formidable reputation in Ayurvedic medicine as a rasayana herb, i.e., one that can rejuvenate the body and promote the health of all the tissues.[7] WS is also classified as an adaptogen, an agent that promotes homeostasis of the whole body not by one specific pharmacological mechanism, but by eliciting complex responses.[8] Traditionally, WS preparations have been used for conditions such as arthritis, asthma, goiter, and ulcers, as well as anxiety, insomnia, and neurological disorders. These uses are linked to the botanical's reputed adaptogenic, anti-stress, and anti-inflammatory properties.[9,10] Numerous WS commercial products are now readily available to the public. In the United States (US), WS products are classified as "botanical dietary supplements" by the Food and Drug Administration. According to the National Institutes of Health Office of Dietary Supplements, there are currently more than 1,300 products on the US market that contain WS.[11] From 2018 to 2021, yearly sales of WS botanical dietary supplements increased from $7.5 million to over $92 million, making it the 7th best-selling botanical dietary supplement in the US.[2] This dramatic rise in WS's popularity, which has caught the attention of mainstream news outlets,[12,13] has been driven in part by its status as an adaptogen.[14] Clinical trials of varying rigor have investigated WS for conditions including stress[15-20] and WS is actively being studied at OHSU's NIH-funded BENFRA Center.[15,21-23] Despite significant research interest in WS, human PK data are scant, and yet they are needed to support rigorous biological signature studies and efficacy trials of this popular botanical.

C.2.2. Phytochemistry

Earlier studies have examined the PK of WS compounds in silico[24,25] and in mice[26-28] or rats.[29-35] All these studies have focused on withanolides, a group of steroidal lactones relatively unique to WS,[36,37] which many pre-clinical studies support as the main bioactive compounds in WS.[38,39] Several withanolides have been investigated for their therapeutic potential, with withaferin A being most well-studied.[40,41] Figure 1 shows the chemical structures for commonly studied withanolides. To our knowledge, only two human PK studies of WS products have been published. A PK study in osteosarcoma patients[42] found that a WS root extract did not produce detectable plasma levels of withaferin A as measured by liquid chromatography (LC) coupled to UV detection, a less sensitive method than LC coupled to multiple reaction monitoring mass spectrometry (LC-MRM-MS). Another PK study comparing two WS root extracts detected the

withanolides 12-deoxywithastramonolide and withanolide A (using LC-MRM-MS) from both products in the plasma of healthy men aged 18-45.[43] These studies are helpful, but limited in relevance due to their use of root only extracts (WS leaves are also used commercially, and contain higher levels of withanolides[15]), the small number of withanolides targeted, the specificity of the data to the products tested, and the lack of female or older participants. The present study will be the most robust PK study to-date for WS, generating PK data, including steady state levels after four weeks' use, for a wide variety of withanolides at two doses of a commercially available WS product using a randomized crossover design.

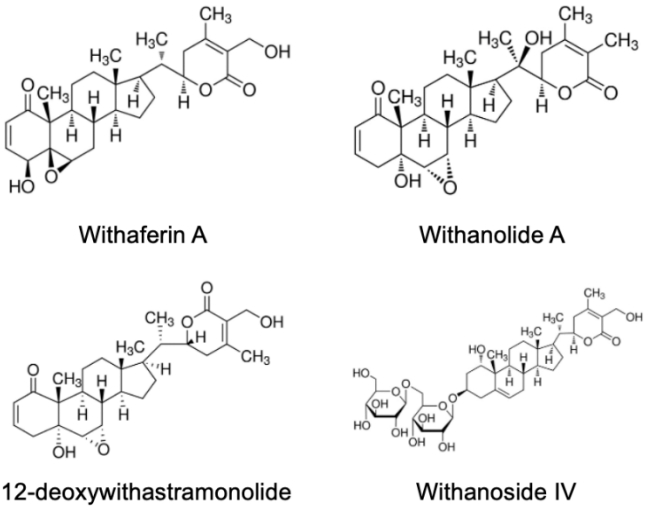

Figure 1. Chemical structures of commonly studied withanolides.

C.2.3. Safety

In animals, WS extracts have also been shown to be safe in toxicity studies. Acute and sub-acute toxicity of a hydroalcoholic root extract of WS was investigated in female Wistar rats.[44] No behavioral signs of toxicity or gross pathological changes were observed with acute doses up to 2000 mg/kg. Similarly, no signs of subacute toxicity were observed in rats given 500, 1000 or 2000 mg/kg of the extract daily for 28 days. In a separate study, a hydroalcoholic root extract of WS was used to assess prenatal developmental toxicity in rats at doses of 500, 1000 and 2000 mg/kg daily for 28 days.[45] No signs of toxicity, gross pathology changes, or mortality were observed in the pregnant rats or fetuses. Further studies are required to examine the safety of WS extracts prepared using other extraction methods and plant parts.

In humans, the widespread use of WS as a traditional medicine and dietary supplement, as well as current scientific literature, support its general safety. In a review of clinical trials that used root preparations of WS for a wide variety of conditions, the authors noted that reasonable safety outcomes were seen with no serious adverse events or changes in vital signs or hematological and biochemical parameters.[46] Mild to moderate transient adverse events including somnolence, giddiness, vertigo, drowsiness, and gastrointestinal effects (e.g., nausea, epigastric pain, loose stools, constipation, decreased appetite) were reported in some studies, while other studies involving adults and children did not report any adverse events.[46] Similarly, an earlier review reported that WS has been used in all age groups and in both sexes, even during pregnancy, without any reported side effects.[10] One study formally measured the tolerability of a WS root extract using the Patient's Global Assessment of Tolerability to Therapy and found it to have a high tolerability score.[47] Rare, serious adverse events have been reported, however. Isolated cases of mild to severe liver toxicity have occurred, with three deaths in patients who had underlying liver disease.[48-50] A case report has also been published detailing a kidney transplant rejection due to WS.[51] Liver and renal abnormalities will be assessed at the beginning and end of each four-week study period.

There are also reported effects of WS for which safety implications are unclear. Several studies have reported increased testosterone levels in males taking WS,[52-55] but not in females.[16,53] Elevated DHEA-S levels have been observed In a study of 50 aging, overweight males, with no related side effects,[52] and a case report of a female patient with X-linked heterozygous adrenoleukodystrophy taking WS. However, other clinical trials that included female participants have found either *decreased* levels of DHEA-S[53] or no effect on DHEA-S.[56] DHEA is a precursor

to testosterone. Testosterone will be assessed at the beginning and end of each four-week study period. Potentially related side effects will be assessed with an adverse events questionnaire every two weeks. The risk of taking WS while pregnant or breast-feeding is unknown and therefore, it is recommended to avoid WS during pregnancy and breast-feeding.

The intervention used in this study, Shoden®, is commercially available and has been studied in randomized, double-blind, placebo-controlled clinical trials, where it has been well-tolerated with no significant adverse events reported.[52,53,57] The doses of Shoden® being used in this study are 240 and 480 mg. In a previous clinical trial of 60 participants, 240 mg Shoden® once daily for 60 days was not associated with any significant adverse events or laboratory abnormalities.[53] The higher dose of 480 mg was studied in a recently completed study of Shoden®, where it was well-tolerated after a single administration in 16 participants.[58] We anticipate that 480 mg of Shoden® will also be safe and well-tolerated over four weeks, but will closely monitor participants for potential adverse events, as described in Section N.

While these studies suggest that WS is generally safe, the possibility of adverse events arising from herb-drug interactions must be considered.[59] A study of 50 participants with subclinical hypothyroidism found that a WS root extract modulated thyroid indices,[20] suggesting that caution is advised in patients with thyroid disease. Shoden® has been found to increase testosterone levels in men in two separate trials, by 11%[53] and 14.7%[52], indicating a need to avoid WS in patients with a history of prostate cancer. A small human study (n=6) found that a root extract of WS had hypoglycemic effects "comparable to that of an oral hypoglycemic drug[60]," suggesting caution in diabetic patients.[56,57,61,62] Pharmacodynamic interactions between WS and other drugs may exist as well, as evidenced by additive effects seen in rodent studies between WS and the drugs imipramine,[63-65] diazepam,[66] and fluoxetine.[67] The GABA-mimetic and serotonergic activities seen with some WS extracts in rodents would suggest caution when co-administering WS with conventional drugs that work by similar mechanisms.

Pharmacokinetic interactions may also occur when botanicals alter the activity of drug transporters or drug metabolizing enzymes.[68] It has been suggested that an IC_{50} of less than 100 µg/mL for extracts or 100 µM for active constituents should be classified as potent inhibition that could lead to undesirable herb-drug interactions.[69,70] Various root extracts of WS had IC_{50} values greater than 100 µg/mL for the cytochrome P450 isoenzymes CYP3A4, CYP2D6, CYP1A2, and CYP2C9 in human liver microsomes, while withaferin A and withanolide A did not inhibit these enzymes at doses up to 50 µM.[71,72] An aqueous extract of WS did not inhibit human recombinant CYP3A4 at doses as high as 1000 µg/mL.[73] In a detailed biopharmaceutical study of withanone, the compound was found to have IC_{50} values of >100 µM for CYP 2C9/11 in rat and human liver microsomes and IC_{50} values between 28.5 and 80 µM for other CYP isoenzymes, the lowest being 28.5 µM for CYP3A4 in human liver microsomes.[74] The clinical relevance of these findings will depend on the systemic concentrations of this compound achieved during regular use of WS. Further studies are required to fully evaluate this important aspect of the use of WS.

D. SIGNIFICANCE

This study will be the most thorough investigation of the oral absorption and PK of withanolides to date, which is needed given the widespread use of WS. These PK data will support biological signature research and provide a rationale for mechanistic studies focusing on orally absorbed withanolides. PK studies of WS are important to guide clinical herb drug interaction (HDI) studies, per the recommendations of the Natural Products-Drug Interaction (NAPDI) Center.[75,76] This is particularly important given the high rate of polypharmacy in older adults.[77]

E. PRELIMINARY DATA

E.1. Rationale for the choice of Shoden®

Shoden® is a commercially available 70% ethanolic extract of WS root and leaves, standardized to 35% withanolide glycosides. This product was chosen for use in the current PK

study due to its high content of withanolides compared to other products on the market and its use in multiple earlier studies. The effects of Shoden® have been investigated in one pre-clinical study and three randomized controlled trials. In vitro, Shoden® upregulated gene expression levels of $GABA_A\rho1$ receptors and histamine H3 receptors compared to control in *Rattus norvegicus* glioblastoma (C6) cells, suggesting a potential role of Shoden® for sleep.[78] In a mouse model of pentobarbital-induced sleep, Shoden® decreased time to sleep onset and increased total sleep duration.[78] Shoden® also increased nonrapid eye movement (NREM) sleep in Wistar rats.[78] In clinical trials, Shoden® has been studied for its effects on sleep-, stress-, and vitality-related outcomes. In a randomized, double-blind, placebo-controlled, crossover study of 50 aging, overweight males, a dose of Shoden® equivalent to 21 mg withanolide glycosides per day for eight weeks significantly increased levels of DHEA-S and testosterone compared to placebo, but did not affect cortisol, estradiol, fatigue, vigor, or sexual well-being.[52] In a randomized, double-blind, placebo-controlled study of 60 adults (37 males, 23 females) reporting high stress however, 240 mg Shoden® daily for 60 days significantly *reduced* morning cortisol and *increased* DHEA-S compared to placebo.[53] Shoden® was also associated with a significant reduction in anxiety scores, as measured by the Hamilton Anxiety Rating Scale.[53] Finally, in a randomized, double-blind, placebo-controlled study of 150 healthy adults (72 males, 78 females), 120 mg Shoden® once daily for six weeks significantly improved subjective sleep quality and objective measures of sleep as measured by a wrist actigraphy watch (e.g., sleep efficiency, total sleep time, sleep latency, and wake after sleep onset) compared to placebo.[57] Based on these studies suggesting likely biological efficacy, Shoden® is an ideal product for which to examine withanolide plasma levels and biological signatures.

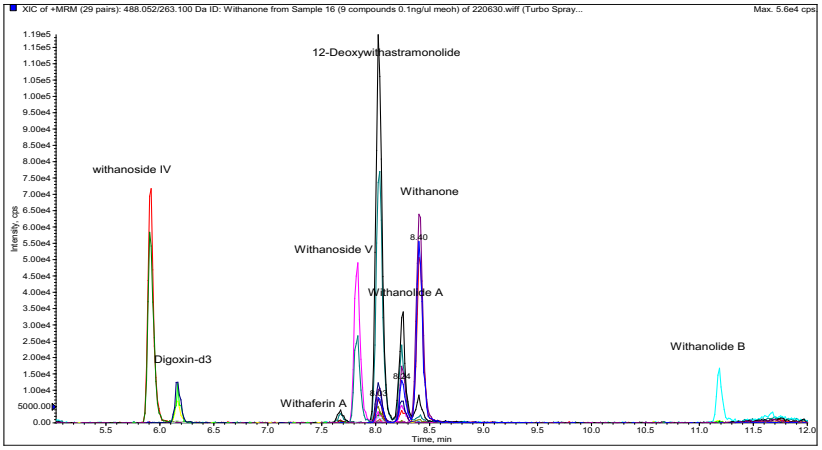

Figure 2. LC-MRM-MS chromatogram of selected withanolides. Separation was achieved using a Kinetex 2.6um Phenyl-Hexyl 100A 50x2.1mm column and a gradient of (A) water with 10 mM aqueous ammonium and (B) acetonitrile both containing 0.1% formic acid.

E.2. PK of withanolides in Shoden®:

An unpublished clinical trial conducted in India of Shoden® (CTRI/2020/10/028397) evaluated the PK of three withanolides (withanolide A, withaferin A, and withanoside IV) in 16 healthy adults, aged 18-45 years. All three withanolides were detected by LC-MRM-MS in plasma (data provided by Arjuna LTD under a NDA).[79] This study was limited in scope due to the dose (480 mg), which was higher than that used in clinical trials involving Shoden®,[52,53,57] the lack of older participants, and the limited range of withanolides targeted.

E.3. LC-MS detection of withanolides

As part of ongoing research at the BENFRA Center, Dr. Soumyanath and the BSR/PKCore at OHSU have been developing LC-MS methods of detecting withanolides in mouse plasma, which will be optimized for human plasma and urine as part of the study. Figure 2 demonstrates good separation for several of the withanolides that will be analyzed in this study.

F. RESEARCH DESIGN AND METHODS

F.1. Study design

The proposed study is a randomized, double-blind, crossover trial evaluating (a) the PK of withanolides, from two doses of a commercially available WS root and leaf extract (Shoden®), (b) safety and tolerability of these doses and (c) the feasibility of remotely measuring sleep- and stress-related outcomes in older adults. Participants will be randomized to one of two dose sequence groups. There will be two 4-week study periods separated by a 2- to 4-week washout period. During each study period, participants will attend a 13-hour baseline PK study visit and return for 24- and 48-hour blood and urine collections. After the 48-hour visit, they will continue taking Shoden® at the administered dose (240 or 480 mg) for four weeks, at which time they will return for a follow-up visit. A flow chart of the study is provided in Figure 3.

Study Design Diagram

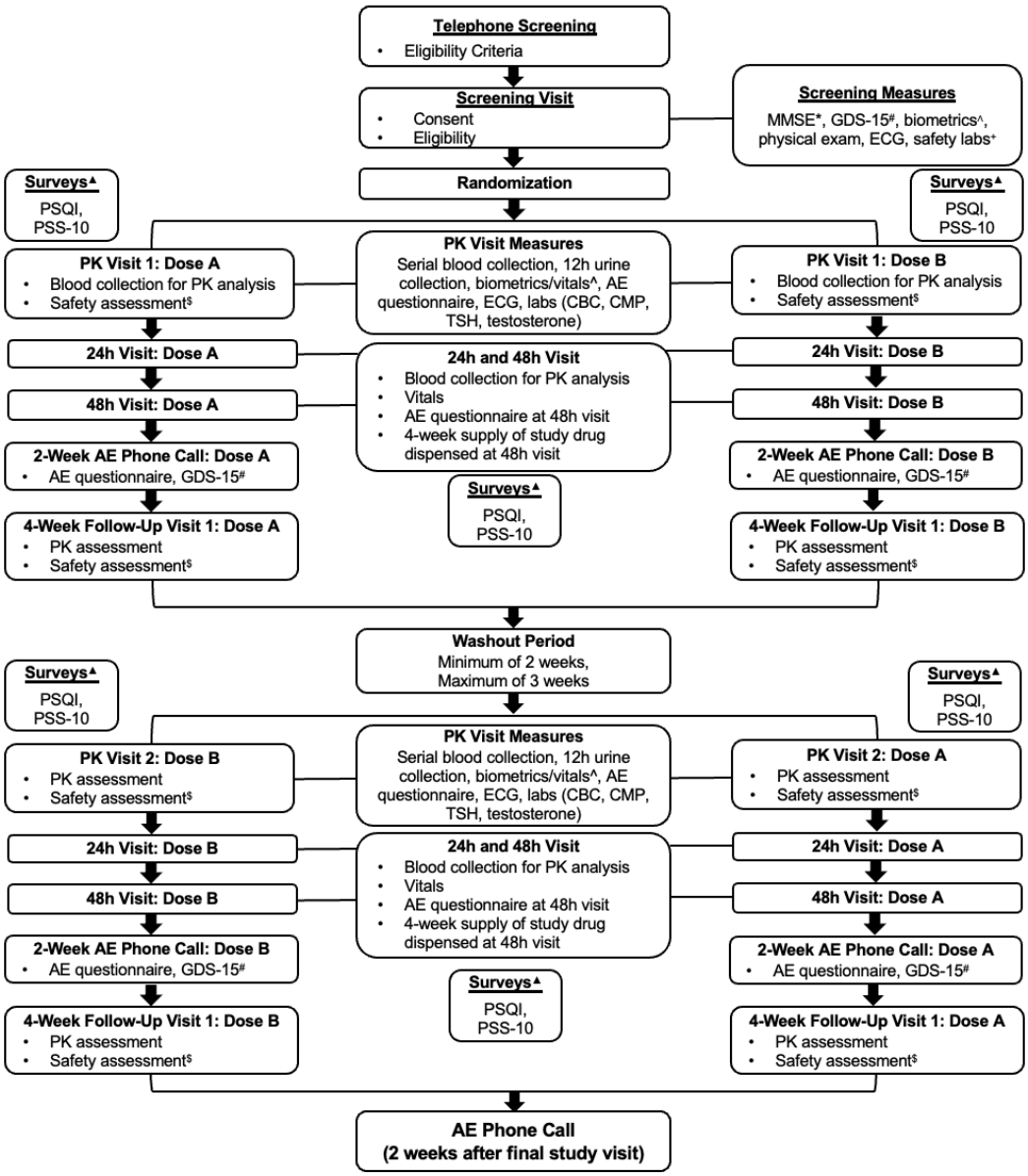

Figure 3. Study design. *Mini Mental State Examination (MMSE), #Geriatric Depression Scale (GDS); ^Biometrics: weight, height, body mass index (BMI), age, blood pressure, pulse rate, temperature; +Lab measures: complete blood count (CBC), comprehensive metabolic panel (CMP), urinalysis, and TSH.' ▲ REDCap surveys delivered to and completed by participants in the week prior to each PK visit and 4-Week Follow-Up Visit. $Safety assessment at study visits includes safety labs (CBC, CMP, TSH, testosterone, UA), GDS-15, ECG, and AE questionnaire.

F.2. Study Population

F.2.1. Number of participants

Twelve non-demented older volunteers (6 female, 6 male) 65 years of age or older will be screened and enrolled in the study. We estimate that we will need to screen approximately twenty potential participants to meet the twelve volunteers needed to complete the study. We expect two dropouts, for a total of 10 participants completing the study.

F.2.2. Inclusion and exclusion criteria

Inclusion criteria:

1. Age 65 and older, male and female
2. Body Mass Index (BMI) greater than 17 and less than 35 at screening
3. Sufficient vision and hearing to complete all tests
4. Willingness to discontinue all botanical supplementation for one week prior to and throughout study
5. No known sensitivity to *Withania somnifera* or any of its derivatives
6. Normal or clinically not significant 12-lead electrocardiogram (ECG) recording
7. Hepatic (ALT, AST, bilirubin), renal (creatinine, estimated GFR), and TSH parameters within normal range
8. Hemoglobin ≥13.0 g/dL or hematocrit ≥39% (males) OR hemoglobin ≥12.5 g/dL or hematocrit ≥38% (females), per FDA recommendations on blood donation
9. General health status that will not interfere with the ability to complete the study
10. Willingness to attend all study visits
11. Willingness to avoid caffeine and xanthine-containing foods or beverages (e.g., coffee, tea, chocolate, caffeine-containing sodas, colas, etc.), as well as grapefruit juice and poppy-containing foods for 48 hours prior to baseline visits
12. Willingness to adhere to special diet (no dairy, grapefruit products, poppy-containing foods, high-fat meals, caffeine, or xanthine-containing foods or beverages) during baseline visits and until after 24-hour visit
13. Mini-Mental State Exam (MMSE) score ≥27

Exclusion criteria:

1. Current smoking, alcohol, or substance abuse according to DSM-V criteria
2. Participants who are currently pregnant, actively trying to conceive a child, or planning to within three months of study completion
3. Severe aversion to venipuncture
4. Donation of blood within 90 days of screening
5. Participation in drug research study within 90 days of screening
6. Serious health condition (i.e., illness, injury, impairment, or physical or mental condition which requires a) overnight hospitalization or b) continuing treatment that may cause episodic periods of incapacity of more than 3 consecutive days) within 30 days of screening
7. Allergy to nightshade plants (*Solanaceae* family)
8. Abnormal labs indicating symptomatic and untreated urinary tract infection
9. History of prostate cancer
10. History of kidney transplant
11. Cancer within the last five years, with the exception of non-metastatic skin cancers
12. Comorbid conditions requiring medication such as diabetes, kidney failure, liver failure, hepatitis, blood disorders, hypotension, thyroid disease, respiratory disorders, or cardiovascular disease
13. Presence of sleep apnea, moderate to severe restless leg syndrome, major circadian rhythm changes, or narcolepsy
14. Significant disease of the Central Nervous System (CNS) such as brain tumor, seizure disorder, subdural hematoma, cranial arteritis, or clinically significant stroke

15. Diagnosis of major depression, schizophrenia, bipolar disorder, or other major psychiatric disorder as defined by DSM-V criteria
16. Diseases associated with dementia such as Alzheimer's disease, vascular dementia, normal pressure hydrocephalus or Parkinson's disease

F.2.2.1. Allowable exceptions to inclusion/exclusion criteria:

1. Depression: Participants who report a diagnosis of depression, but (a) have a Geriatric Depression Scale score <5 at the screening visit (b) do not exhibit suicidal ideation, and (c) have been on a stable anti-depressant medication dose for at least two months prior to enrollment may be enrolled at the discretion of the clinical investigator.

F.2.2.2. Concomitant interventions

Allowed interventions*:* probiotic supplements, non-plant-based laxatives such as magnesium citrate or milk of magnesia, gastroesophageal reflux disorder medications such as antacids, dietary supplements that do not contain plant-based extracts, and non-narcotic analgesics

Prohibited interventions: thyroid medications, anticoagulants (e.g., Warfarin), anticonvulsants, neuroleptics, anti-Parkinsonian agents, hypoglycemics, narcotic analgesics, sedatives (barbiturates, benzodiazepines, opioids, sleep-inducing drugs), nicotine (tobacco, patches, gum, lozenges, etc.), glucocorticoids, immunosuppressants, cannabis products (herb or edibles), investigational drugs used within five half-lives of baseline PK visit, systemic corticosteroids used within five half-lives of baseline PK visit, and any over the counter or prescribed products containing plant extracts such as herbal supplements, plant-based over-the-counter vitamin supplements, or 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 exclude men who are trying to father a child. We will 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) or the Center for Health & Healing, Building 1 (CHH1). Screening visits, baseline visits, 24- and 48-hour visits, and 4-week follow-up visits will occur in the CTRC. All plasma and urine storage will occur in a -80°C freezer 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.

G. RECRUITMENT

Potential participants will be identified through multiple sources, including the Oregon Clinical and Translational Research Institute's volunteer repository and the Oregon Center for Aging and Technology (ORCATECH) Life Lab. We will also query the electronic health records system using OHSU approved tools, such as Cohort discovery, Research Data Warehouse (RDW), and

ACTNOW! Research Contact and Health Information Registry (IRB#: 11606). We will request patient lists that meet the eligibility criteria and medical record numbers, to perform subsequent chart review. Potential participants will be mailed or securely emailed an advertisement

describing the study and requesting they call if they are interested in participation. IRB-approved study flyers will be posted with permission in OHSU clinics and local retirement communities/elder care facilities. The study will be advertised online through the March Wellness “March Stride” e-mail newsletter, OHSU’s StudyPages platform, and the NIH Clinical Trials website. All study advertisements will be submitted to the OHSU IRB before recruitment begins and all study materials will emphasize that participation is completely voluntary.

G.1. Incentives

Participants will be given the option of complimentary transportation by taxi to and from their study visits 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 $200 for completion of the study via the ClinCard. Payments will be prorated, $100 for each completed baseline PK visit. While not a direct incentive, participants will be provided with three complete meals and snacks during each study visit that will not interfere with the PK analysis.

H. INTERVENTION

H.1. Shoden® powder, a commercial, dried 70% ethanolic extract of *Withania somnifera* (ashwagandha, WS) root and leaf.

Shoden® powder will be prepared from WS root and leaf by the company Arjuna Natural, using their standard procedures in GMP-compliant facilities. WS root and leaf will be purchased from “qualified” suppliers, i.e., suppliers who have previously supplied raw herbs that have passed Arjuna’s standards for identity, and pesticide and heavy metal levels. Every batch of plant material will be examined by the following tests: macrosocopic examination for verification of identity, thin layer chromatography (TLC) to verify the presence of characteristic withanolides, impurity testing to confirm that pesticide and heavy metal tests (lead, arsenic, cadmium, and mercury) remain below specified limits, and high-performance liquid chromatography (HPLC) analysis to confirm the presence of acceptable levels of total withanolide glycosides. WS root and leaf raw materials will then be subjected to two-stage extraction with 70% ethanol. After each extraction, the extract will be examined by thin layer chromatography (TLC) to verify the successful extraction of withanolides and concentration during the second stage. The extract will be dried by spray drying and the dried extract will undergo impurity testing, microbial testing, and HPLC analysis to confirm the presence of acceptable levels of total withanolide glycosides. A dossier with a paper trail from the raw material to finished product will be maintained by the company and a Certificate of Analysis will be provided for each batch of extract detailing analytical results.

The PI will ensure that the Shoden® powder provided by Arjuna undergoes the following tests through three collaborating institutions of the NIH-funded Botanical Research Core of the BENFRA Botanical Dietary Supplements Research Center:

1. High-performance TLC (HPTLC) of the Shoden® extract will be performed in Dr. Soumyanath’s laboratory at OHSU. Using HPTLC chromatographic methods described in the United States Pharmacopoeia monograph for ashwagandha extract, the presence of sitosterol and withanolide A (characteristic of WS root) and withaferin A (characteristic of WS leaf) in Shoden® will be verified.
2. The Shoden® sample will be analyzed at Oregon State University Mass Spectrometry Center (OSMSC) using methods they have developed for BENFRA Center research. Liquid chromatography coupled to multiple reaction monitoring mass spectrometry (LC-MRM-MS) will be used for targeted quantification of several withanolides for which commercial reference standards are available. LC coupled to high resolution tandem mass spectrometry (LC-HRMS/MS) will be used to record a fingerprint of all detectable components in the extract.
3. The Shoden® sample will be analyzed at or through the QC laboratory at the dietary supplement company, Oregon’s Wild Harvest (OWH), for pesticides, heavy metals (lead,

arsenic, cadmium, and mercury) and microbial content (coliforms, pathogenic E.coli 0157, Salmonella, yeast, mold, and total aerobic plate count).

Arjuna Natural will custom prepare capsules containing two dose levels (120 mg or 240 mg/capsule) from the same batch of Shoden® powder for which they provided samples for characterization studies. The capsules will also contain a standard mixture of inactive excipients (microcrystalline cellulose (filler) and vegetable cellulose (capsules)). The weight of excipients will be adjusted so that capsules of the two doses have the same final total weight to preserve blinding. The capsules will be provided in sufficient quantities for the entire clinical trial as well as QC and stability tests to be conducted. Voucher specimens of the Shoden® powder and capsules (120 mg and 240 mg) will be maintained in Dr. Soumyanath's laboratory at -80 ˚C.

Randomization and blinding will be done by the OHSU research pharmacy. A single dose of Shoden® will be dispensed by the research pharmacy to a member of the study team just prior to the participant's baseline PK visit for each study period. Participants will receive two single administration doses of Shoden® (240 and 480 mg). The order in which they receive these doses will be random to prevent a tolerance effect. The OHSU Research Pharmacy will randomize participants to one of two dose sequence groups, stratified by sex. Participants will consume Shoden® in the presence of a study team member following baseline measurements in order to ensure adherence to the study protocol. Prior to the 48-hour visit for each study period, a 35-day supply of Shoden® (240 or 480 mg per day) will be dispensed to a study team member. Whenever the study intervention is dispensed, the OHSU research pharmacy will record the date, participant ID number, and dose for accountability.

H.2. Administration

A single dose (240 or 480 mg) of the study intervention will be administered in an outpatient setting at each baseline PK visit. The dose sequence will be randomized. The research pharmacy will dispense one dose of Shoden® to a member of the study team, who will provide it to the participant for oral consumption. At the 48-hour visit for each study period, participants will be given a 35-day supply of the study intervention.

H.3. Criteria for intervention discontinuation and stopping guidelines

Participants will be advised during the informed consent process that they have the right to permanently discontinue Shoden® 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

<u>Adverse event (AE)/experience:</u>

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 serious adverse events. Severe adverse events will be reviewed immediately by the PI to determine if severity and relatedness warrant termination of the study (Section N).

I. SCREENING AND STUDY ACTIVITIES

A schedule of study visits and assessments is shown in Table 1.

I.1. Phone screening

Potential participants will be screened over the phone by the study team, prior to attending an in-person screening visit. An IRB-approved telephone script will be used. During the phone screen we will ask participants to self-report that they are not are currently pregnant, breast feeding, or actively trying to conceive a child. The telephone screening will take approximately 10-15 minutes. Eligibility will be documented and potential participants that meet the eligibility criteria 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 or securely emailed a copy of the IRB approved consent 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. For participants with limited English proficiency a qualified interpreter will be present for screening, the consent discussion, and the subsequent study visits. As this study does not specifically target participants with limited English proficiency, the short consent form in the language needed will be submitted to the IRB prior to use. 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 form and then assess their understanding by asking the participant to describe key points of the study (e.g., purpose of study, number of visits, length of study) before signing. After the participant signs the consent, screening assessments and baseline measurements will start. A copy of their signed consent form will be provided to each participant. To ensure ongoing consent, participants will be asked if they wish to continue their participation in the study at the beginning of every in-person study visit and phone visit.

I.3. Screening visit

After the consent form is signed the participant's health status will be evaluated using a biometric assessment (height, weight, and body mass index), vital signs (blood pressure, pulse rate, temperature, and respiration rate), a screening physical exam, a mood assessment (Geriatric Depression Scale-15), and cognitive assessment (Mini-Mental State Examination). A non-fasting plasma and urinary laboratory evaluation (complete blood count (CBC), comprehensive metabolic panel (CMP), thyroid-stimulating hormone (TSH), and urinalysis) will be performed and submitted to the OHSU clinical core laboratory to screen for abnormalities that could impact study results. Study staff will collect approximately three teaspoons (15 ml) of venous blood by peripheral venipuncture and the participant will collect 20 ml of urine via a clean-catch method for analysis. A screening electrocardiogram (ECG) will also be taken at the screening visit. The visit is expected to take 60 to 90 minutes.

The study clinician (Dr. Quinn) will review screening and laboratory results with the candidate prior to participant enrollment. Participants will be contacted by phone and told whether they qualify for the study or not. Participants who meet all final eligibility criteria will be invited to participate in the intervention stage of the study. The OHSU research pharmacy will randomize

each participant into one of two dose sequence groups. Any participant 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. PK Visits 1 and 2

1.4.1. Baseline Visit (0-12hr)

Visit Timing: For the first study period, Baseline Visit 1 will occur a minimum of three days after the screening visit to allow for review of laboratory results and scheduling of appropriate facilities. For the second study period, Baseline Visit 2 will occur a minimum of two weeks (and not more than three weeks) after the four-week follow-up visit for the first study period.

Sample collection: Participants will be asked to fast for 10-12 hours, excluding water, the night before each baseline visit in an attempt to standardize gastrointestinal transit time and minimize delayed absorption of the study intervention due to the presence of food. Participants will be reminded during the pre-visit reminder call to arrive at the visit well-hydrated to facilitate blood collections. Study staff will place a peripheral intravenous catheter in the participant's arm or hand to allow for serial plasma specimen collection. Prior to administration of Shoden®, a baseline plasma sample of two teaspoons (10 ml) will be collected through the catheter using a syringe and transferred immediately into a heparinized Vacutainer tube. The participant will then be administered two capsules of Shoden® (240 or 480 mg total) on an empty stomach in the presence of study staff to ensure compliance. Participants will be asked to abstain from food and drink (excluding water) for the first two hours following consumption of Shoden® capsules to allow for transition into the small intestine and absorption. If signs of hypoglycemia are present, participants will be allowed to consume a high glycemic food and monitored for improvement. Participants will be provided water at the visit and will be encouraged to drink four 16-ounce cups of water over the 12 hours to maintain good hydration status. Serial blood samples (two teaspoons (10 ml) per sample) will be collected through a peripheral intravenous catheter, at 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 300, 360, 540, and 720 minutes after dosing. A baseline urine sample and a 12-hour pooled urine collection will be obtained to measure excretion of WS metabolites.

Tolerability and safety: Participants' weight, height, body mass index, age, blood pressure, temperature, respiration rate, and pulse rate will be recorded at each study visit to monitor for adverse events and asymptomatic illnesses that may impact study results. Vitals will be collected at baseline, 7 hours post-Shoden administration, and at the end of each baseline visit. At the beginning of each baseline visit, participants will complete an electrocardiogram (ECG) and the Geriatric Depression Scale-15. Four teaspoons of blood (20 ml) will be collected for safety labs (CBC, CMP, urinalysis, TSH, serum total testosterone). ECG will be repeated 7 hours post-intervention to assess for cardiac changes. One teaspoon (5 ml) will be collected for a repeat CMP at 10 hours post-intervention to evaluate kidney and liver function. Participants will be interviewed and administered a standardized multi-system adverse events questionnaire at the beginning and end of each baseline visit. This visit is expected to take 13 hours a total of 175 mL of blood will be collected.

If the baseline CBC shows hemoglobin or hematocrit levels below what is recommended by the FDA for blood donation, the visit will be halted immediately. Participants will be asked to stay for 2.5 hours after the study agent has been administered to assess for acute side effects. Two and a half hours was chosen because common withanolides have reached their C_{MAX} by this time based on a previous PK study using Shoden®.[80] The end-of-visit adverse events questionnaire will be administered by phone and missed safety evaluations (7-hour ECG and vitals, 10-hour CMP, 12-hour vitals) will be collected at the 24-hour visit. After the 24-hour visit, the participant will be withdrawn from the study.

If study staff have difficulties collecting blood samples for any reason, the decision may be made to halt the visit. Participants will be asked to stay for 2.5 hours after the study agent has been administered to assess for acute side effects. If the visit ends before the 7-hour mark, the end-of-visit adverse events questionnaire will be administered by phone and missed safety evaluations (7-hour ECG and vitals, 10-hour CMP, 12-hour vitals) will be collected at the 24-hour visit. Participants will be allowed to remain on the study if difficulties with blood sample collection were the only reason for halting the study visit early.

I.4.2 24- and 48-Hour Visits

Participants will return at 24 hours and 48 hours for a single blood collection (two teaspoons (10 ml)) and urine collection. Vitals will be collected. A CMP and ECG may be collected at the 24-hour visit if the baseline visit was halted prior to 7 hours. At the 48-hour visit, the adverse events questionnaire will be administered as the baseline measure for the subsequent four-week period. After the 48-hour visit, participants will be issued a 35-day supply of the appropriate dose of Shoden® (240 or 480 mg), which they will begin taking before bed every day for four weeks. These visits are expected to take 20 minutes. After analyzing the plasma and urine samples for the first two participants, a determination will be made as to whether the 48-hour PK visit is needed for future participants. If no withanolides are detected in plasma or urine at 48-hours, this time point will be eliminated to avoid undue participant burden.

1.5. Two-Week Adverse Event Phone Visit

The study team will call the participant two weeks after the baseline visit during each study period to administer an adverse events questionnaire and the Geriatric Depression Scale-15, and schedule the 4-week follow-up visit. To ensure ongoing consent, participants will be asked if they want to continue participation in the study. This visit is expected to take 15-30 minutes.

1.6. Four-Week Follow-Up Visit

Participants will be asked to fast for 10-12 hours, excluding water, the night before each four-week follow-up visit in order to collect fasting glucose as part of the CMP. A single blood sample (two teaspoons (10 ml)) and a urine sample will be collected to assess steady state plasma levels and excretion of WS compounds. Returned capsules will be counted to assess for adherence. Participants will have an ECG and blood and urine samples will be sent for safety labs (CBC, CMP, urinalysis, TSH, serum total testosterone, serum DHEA-S). The Geriatric Depression Scale-15 will be administered. At the four-week follow-up visit for the second study period, participants and investigators will be queried as to their perception of the dose sequence administered. This visit is expected to take 45-60 minutes.

1.7. Final Phone Visit

Two weeks following the four-week follow-up visit for the second study period, participants will be telephoned to assess for delayed adverse events using interviews and a standardized multi-system adverse events questionnaire. The Geriatric Depression Scale-15 will be administered. Participants will be instructed to contact the study team if they become pregnant or father a child after they have completed the study.

1.8. REDCap Surveys

Participants will be emailed a link to electronic surveys in REDCap at four time points during the study: during the week prior to each PK visit and during the week prior to each four-week follow-up visit. Participants will complete two surveys at each time point: Pittsburgh Sleep Quality Index (PSQI) and the Perceived Stress Scale (PSS).

I.9. Washout Period

The two study periods will be separated by a 2- to 4-week washout period where the participant will not take the study agent. If the washout period is longer than 21 days, participants will be called at two weeks to assess for delayed side effects.

I.10. Subject participation duration

Including the screening visit, a two to four-week washout period between doses, and an exit phone interview for adverse event monitoring, it will take participants 13 to 14 weeks to complete the study.

Table 1. Schedule of study visits and assessments.

	Screen	PK visit			2-week f/u	4-week f/u		PK visit			2-week f/u	4-week f/u	Final visit +2 weeks
Study Period		1			1	1		2			2	2	
Event	Clinic Visit	0 to 12h	24h	48h	Phone visit	Clinic visit		0 to 12h	24h	48h	Phone visit	Clinic visit	Phone visit
Informed consent	X												
Vitals and biometrics	X	X[+]	X	X		X		X[+]	X	X		X	
Health screening	X												
Electrocardiogram (ECG)	X	X[$]	X[°]			X		X[$]	X[°]			X	
CBC*, CMP*, TSH[▲], and UA*	X	X[^]	X[°]			X		X[^]	X[°]			X	
Serum testosterone		X				X		X				X	
Single dose of intervention		X						X					
Single-dose PK blood draws		X	X	X				X	X	X			
Issue 35-day supply of Shoden®				X[#]						X[#]			
Steady state PK blood draw						X						X	
12-hour urine collection		X						X					
Single urine collection		X[¶]	X	X		X		X[¶]	X	X		X	
Collect and count unused capsules						X						X	
Adverse events questionnaire		X[★]		X	X	X		X[★]		X	X	X	X
Cognitive screen (MMSE)	X												
Depression screen (GDS)	X	X[◆]			X	X		X			X	X	X
REDCap surveys		X[▼]				X[▼]		X[▼]				X[▼]	

Washout Period

f/u – follow-up
[$] ECG collected prior to Shoden® administration and at 7 hours post-Shoden® administration
* CBC = Complete Blood Count with differential; CMP = Comprehensive Metabolic Panel; UA = Urinalysis
[^] CMP collected prior to Shoden® administration and at 10-hours post-Shoden® administration
[▲] TSH = Thyroid-stimulating hormone
[#] This will take place at the 24-hour visit if 48-hour visit is eliminated, as described in Section I.4.
[¶] Aliquot of baseline urine prior to administration of Shoden®
[★] Adverse events questionnaire administered at beginning and end of baseline visits
[◆] If PK Visit 1 is ≥14 days since the screening visit
[▼] REDCap surveys will be delivered to participants' email during the week prior to each study visit.
[°] An ECG and CMP will be collected at the 24hr visit if the baseline visit was halted prior to the 7hr ECG and CMP being collected.

J. STUDY DURATION

Recruitment and data collection is expected to begin upon approval from the IRB and continue for nine months. We estimate a total of six months for final data analysis. Total estimated study duration is 21 months (Table 2).

Table 2. Study timeline.

	Month																							
Activity	1-3			4-6			7-9			10-12			13-15			16-18			19-21			22-24		
Characterization studies																								
Product quality control																								
LC/MS Method Optimization																								
Recruitment/enrollment																								
Data collection																								
Plasma/urine LC/MS analysis																								
PK 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 case report forms for this study include a telephone screening script, a medical history form, a cognitive screening questionnaire (Mini Mental State Examination), a mood screening questionnaire (Geriatric-Depression Scale-15), a stress questionnaire (Perceived Stress Scale (PSS)), a sleep questionnaire (Pittsburgh Sleep Quality Index (PSQI)), an adverse events questionnaire, and a 2-week follow-up phone script. Other data collection methods include administering an electrocardiogram, urine collection, and blood collection. The PSS and PSQI will be delivered to participants' emails via REDCap.

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. Blood will be centrifuged 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 Soumyanath Lab (Richard Jones Hall, OHSU). The plasma and a 50mL aliquot of the urine specimens will be stored in labeled boxes in Dr. Soumyanath's -80°C freezer.

Electronic data from case report forms and specimen analysis will be entered in REDCap. Analyzed data will be stored in an OHSU approved cloud storage (OneDrive). Only study team members will be granted access to the REDCap database or OneDrive with access specific to their role in the project. Paper records including participant contact information, telephone screening responses, consent forms, electrocardiogram reports, lab results, and paper case report forms will be stored in participant-specific binders, kept in a locked cabinet at the Parkinson's Center of Oregon office suite (Sam Jackson Hall), where Dr. Speers is located.

K.3. Sharing of results with participants

All screening laboratory results, performed by the CLIA-certified OHSU Core Laboratory, will be shared with the participant through their medical record. 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 (ECGs) will be performed at the screening visit, PK visits, and four-week follow-up visits. If the ECGs 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.4. Data, Specimen banking, and Repository Procedures

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 project; however, data, plasma and urine specimens will be stored for future analyses in a repository included in this protocol. Specimens and data from other studies will not be accepted in this repository. For future analyses, plasma and urine specimens will be stored in a locked -80˚C freezer in Dr. Soumyanath's laboratory, access to which is badge-restricted. All specimens will be coded with each participant's unique numeric ID code, visit number, and data of collection. The key for the coded specimens will be stored on OneDrive, and access to the key will be limited to the PI and the Repository Guardian. As this study is NIH funded, a Certificate of Confidentiality was issued.

Participants will sign a consent form stating that specimens and data will be stored for future use. The PI will notify the Repository Guardian promptly if consent has been withdrawn for future use of specimens or data. If consent is withdrawn, the Repository Guardian or designee will remove identifiers from the samples and data, but the material will not be destroyed, and we will continue to use it for research.

The repository guardian will be Alex Speers. For repository requests, the repository guardian or designee will review the requestor's IRB approval memo, protocol, and repository sharing agreement before samples/data are released. Separate IRB approval/determination will be required for each specific human subject research activity that uses coded data/specimens from the repository. The Guardian or designee will check for genetic opt out status, withdrawn consent for data/samples, and limitations on future use of data/samples. A signed Repository Sharing Agreement will be collected before data or samples are released. The Repository Guardian will ensure that material transfer agreements for the transfer of biological materials and data use agreements for data shared outside of OHSU are executed as applicable. Data and specimen releases will be documented on the repository tracking sheet. Data will be released using OHSU approved cloud storage or secure email. Samples will be hand delivered or mailed in accordance with OHSU dangerous goods shipping policies. Specimens and data will be coded with the participant's unique ID code, visit number, and date of collection. The key for requested specimens and data will be provided separately and with the appropriate IRB approval.

L. DATA ANALYSIS

L.1. Specimen analysis (Aim 1)

Eleven withanolides (withanolide A, withanolide B, withaferin A, withanone, withanoside IV, withanoside V, 12-deoxywithastramonolide, sominone, viscosalactone B, 4-oxo withaferin A, and 2,3-dihydro-3β-methoxy withaferin-A) have been initially selected based on their availability as reference standards. Final selection of withanolides to be included in the PK analysis will be based on product characterization studies conducted at the Oregon State Mass Spectrometry Center. Only withanolides that are confirmed to be in the test product, or an acid hydrolysate of the test product (mimicking deglycosylation), will be included in the PK analysis.

Targeted analysis of the selected withanolides in plasma and urine samples will be performed using LC-MRM-MS instrumentation at OHSU's BSR/PKCore. The BSR/PKCore has developed

a 15-minute LC-MRM-MS method for the BENFRA Center for the analysis of seven withanolides using digoxin-d_3 as an internal standard (section E.3, Figure 2). The BENFRA center (through Dr. Soumyanath) is working with the BSR/PKCore lab to include the four additional withanolides in the method and optimize mouse plasma work-up methods for the analysis. These methods will be adapted for human plasma and urine in this study, using commercially available human plasma and urine spiked with reference withanolides under the guidance of Dr. Soumyanath and Dr. Andrea DeBarber, director of the BSR/PKCore.

Each withanolide analyte will be identified by its retention time and characteristic mass spectral fragmentation pattern using selected reactions within a multiple-reaction monitoring paradigm. The peak area ratio to the appropriate internal standard will be identified and evaluated against the standard calibration curve of each analyte prepared by spiking known concentrations into blank plasma. Each analyte's concentration in participant plasma will be used to generate a concentration-time curve for pharmacokinetic profiling. The primary endpoints are the maximum concentration (C_{max}) and its corresponding timepoint (t_{max}) of each withanolide analyte. To determine the rate of elimination (k), linear regression of the plasma concentration against time will be used. This value will be used to determine the elimination half-life ($t_{1/2}$) using the formula $t_{1/2}$=0.693/k. Area under the concentration-time curve from dosing (time 0) to time *t* (AUC_{0-t}) will be calculated using the linear-log trapezoidal method. Area under the concentration-time curve from dosing (time 0) extrapolated to infinite time ($AUC_{0-\infty}$) will be calculated using the equation $AUC_{0-t} + AUC_{t-\infty}$, where $AUC_{t-\infty} = C_{last}/k_e$. C_{last} is the last observed concentration and k_e is the elimination rate constant. Differences in in C_{max}, AUC_{0-t}, $AUC_{0-\infty}$, and $t_{1/2}$ for the two doses of Shoden® will be evaluated using paired t-tests or the non-parametric equivalent Wilcoxon paired test, while differences in t_{max} will be compared between dose groups using chi-square tests of distribution. With numerous analytes of interest, post-hoc multiple comparison adjustments in p-values will be applied using the Holm-Sidak stepwise correction. All final statistical tests will be two-tailed and considered significant at α=0.05.

Peak area for each withanolide analyte and internal standard will be obtained using Analyst software from Sciex Technologies (version 1.7.1). The Core lab will provide and set up LC-MRM-MS instrumentation. The applicant will prepare the plasma and urine samples for analysis in Dr. Soumyanath's lab and retrieve and analyze the LC-MRM-MS data generated at the Core Lab to obtain concentrations of each withanolide in each sample. The applicant will work with Dr. Munar to obtain PK curves and parameters.

L.2. Safety analysis (Aim 2)

Changes in laboratory outcomes from the baseline visit to the four-week follow-up visit for each study period will be presented using descriptive statistics (e.g., means and standard deviations, frequency and percent). Within group differences for laboratory outcomes at each dose will be evaluated for significance using paired t tests. Data will also be presented visually using histograms and line graphs. Treatment-emergent AEs will be organized by body system and summarized in table format by dose, with AE severity and possible relation to study drug reported.

L.3. Feasibility analysis (Aim 3)

The feasibility of administering REDCap surveys will be assessed by calculating the percentage of administered questionnaires that are returned and fully completed by participants, with feasibility defined as at least 80% of all administered questionnaires returned and completed. For each of the four timepoints (prior to PK Visit 1 and 2, prior to 4-Week Follow-Up Visit 1 and 2), the percentage of administered questionnaires returned and the percentage of returned questionnaires that are fully completed will also be reported.

L.4. Sample size

Based on a paired sample t-test with alpha=0.05 and a sample size of 10 (anticipating two dropouts), we will have 95% power to detect an effect size of 1.3 (Cohen's d). Based on plasma levels of withaferin A, withanoside IV, and withanolide A observed in an unpublished PK study of 480 mg Shoden® in 16 healthy adults,[79] this effect size would be a difference in C_{max} between the two doses of about 6.5 ng/ml for withaferin A, 2.2 ng/ml for withanoside IV, and 1.17 ng/ml for withanolide A. We expect to see much bigger differences between the two doses based on previous botanical studies where half doses resulted in C_{max} values that were 35-50% lower than the full dose.[81-84]

L.5. Data quality and management

The Principal Investigator will oversee study progress including recruitment, retention, demographics, and study participant status. 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. Data will be input into REDCap promptly after the participant's visit and double-checked 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. Study personnel will calculate PK outcomes using the Phoenix WinNonlin program and results will be double-checked by Dr. Munar to ensure accuracy.

M. PRIVACY, CONFIENTIALITY, AND DATA SECURITY

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. During screening and study visits, the investigators will ensure physical privacy by conducting interviews and examinations in a closed room. Participant data and specimens will be labeled with their participant ID. 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.

Data for this project will be stored in OCTRI's installation of REDCap, a highly secure and robust web-based research data collection and management system. Features of REDCap that protect participants' privacy and data security include:

- Physical Security: OCTRI's REDCap software is housed on servers located in ITG's Advanced Computing Center providing locked physical security
- Electronic Security: The REDCap servers are housed behind both the OHSU firewall and a second ACC firewall. All web-based data transmissions are encrypted with industry-standard SSL methods.
- Controlled User Access: REDCap is employs a robust multi-level security system that enables researchers to easily implement "minimum necessary" data access for their research staff, including specification of data fields that are identifiers. This feature includes "single click" ability to provide completely deidentified (removing all identified data fields and shifting dates) for analysis or other purposes. User activities are logged to enable auditing of all data access. Access is integrated with OHSU's network such that users who are also OHSU employees are authenticated against their OHSU network credentials.
- Data Integrity: REDCap is jointly managed in accordance with OHSU Information Security Directives by ACC staff and members of OCTRI's Biomedical Informatics Program, ensuring fidelity of database configuration and back-ups. User activities are logged to enable auditing of all data changes.

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

To ensure participant safety, we will monitor for adverse events (AE) with AE questionnaires, ECGs, and laboratory tests. During the baseline visit and four-week follow-up visit for each study period, participants will undergo an ECG and laboratory testing (CBC, CMP, TSH, testosterone, and urinalysis). At baseline visits, ECG will be repeated 7 hours post-Shoden® administration to assess for cardiac changes and CMP will be repeated 10 hours post-Shoden® administration to evaluate liver and kidney function. Participants whose ECGs and/or lab results reflect abnormalities will be further screened for potential causes of the abnormalities as determined by Dr. Quinn. We will notify the participant and the participants' primary care physician if blood and urine safety parameters fall outside the normal range or if abnormalities are seen on ECG, and Dr. Quinn will undertake the necessary steps to ensure appropriate clinical care.

Although assessment of AEs will also include solicitation of non-specific changes (e.g., "do you feel different since beginning the treatment?"), a multisystem checklist covering all major organ systems will be included to prevent missing any AEs. The standardized adverse event questionnaire will be administered to participants at the beginning and end of each baseline visit and four-week follow-up visit. In addition, the AE questionnaire will be administered by phone two weeks after the baseline visit for each study period, and two weeks after the final study visit. The nature of each AE, such as severity, likelihood of relationship to the study intervention, duration, and necessary palliative treatments will be recorded.

Participants will be reminded and encouraged during clinic visits and telephone calls to contact a member of the study team if a moderate or serious AE occurs. Participants will be given a telephone number and email address they can contact to report potential adverse effects. The participant will also be advised to go to their primary care physician or emergency room as warranted for treatment of AEs.

The principal investigator will do real-time review of AEs and protocol deviations to determine if the event meets the OHSU IRB's definition of reportable new information. All AEs and protocol deviations will be reported to the IRB at the time of continuing review.

Adverse events will be organized based on System Organ Class (SOC), based on the highest level of the MedDRA system (https://evs.nci.nih.gov). Within each SOC, events will be defined using the same criteria held by the National Cancer Institute's Common Terminology Criteria for Adverse Events (CTCAE). Severity classification will similarly follow the five-point grading scale for each defined event as described under the CTCAE. Events will be classified as severe for any grade 3 (severe), 4 (life-threatening) or 5 (resultant death) and marked for immediate reporting to OHSU's IRB. Severe AEs (SAEs) are defined as events that are life-threatening or resulted in hospitalization, permanent disability, cancer, death, or other significant outcome. All SAEs will be reported promptly to the PI and clinician Co-Investigator.

To reduce pregnancy risk, participants will be instructed at the Exit Phone Call to contact the study team if they believe they are pregnant or have fathered a child post-study. A records review will be conducted by a member of the study team one month after each participant has completed the study to check for reports and outcome of pregnancy.

N.1. Attribution to intervention

Items identified on the Adverse Event Monitoring Form will be compared to baseline levels to determine if the symptom was preexisting. 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, the co-investigators and the PI 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. Plan for review and reporting

The study team will submit continuing review questionnaires to the OHSU IRB per institutional policy. The PI will review protocol deviations, reports, and AEs in real time to determine if they must be reported as Reportable New information (RNI). Events that are RNI will be reported to the OHSU IRB per RNI policy. All other events will be summarized and reported at continuing review.

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.

Study Diet: The study diet requires that participants avoid any caffeine for 48 hours prior to each baseline visit. If participants regularly drink caffeinated beverages like coffee, they may experience symptoms of caffeine withdrawal, including headache, fatigue, decreased energy/activeness, decreased alertness, drowsiness, decreased contentedness, depressed mood, difficulty concentrating, irritability, and feeling foggy/not clearheaded.[85]

Shoden® extract: Shoden® is a 70% ethanolic extract of *Withania somnifera* root and leaves, standardized to 35% withanolide glycosides and produced in a good manufacturing practice (GMP)-certified facility. We will also conduct independent analyses of impurities (pesticides, heavy metals, microbes) through Oregon's Wild Harvest (Redmond, OR) and confirm dose levels through analysis at Oregon State University (Corvallis, OR).

Shoden® is commercially available and has been studied in randomized, double-blind, placebo-controlled clinical trials, where it has been well-tolerated with no significant adverse events reported.[52,53,57] The doses of Shoden® being used in this study are 240 and 480 mg. In a previous clinical trial of 60 participants, 240 mg Shoden® once daily for 60 days was not associated with any significant adverse events or laboratory abnormalities.[53] The higher dose of 480 mg was studied in a recently completed study of Shoden®, where it was well-tolerated after a single administration in 16 participants.[79] We anticipate that 480 mg of Shoden® will also be safe and well-tolerated over four weeks, but will closely monitor participants for potential adverse events, as described in Section N. The widespread use of WS as a herbal dietary supplement[1] and reviews of clinical studies using WS[46,86] supports its general safety. When side effects have been reported in clinical trials with other WS products, they have been mild to moderate transient effects including somnolence, giddiness, vertigo, drowsiness, and GI effects (e.g., nausea, epigastric pain, loose stools, constipation, decreased appetite).[46] There have also been rare reports of mild to severe liver toxicity for other WS products[48,49] and a case of kidney transplant rejection in a patient using WS.[51] There is also evidence that WS may modulate thyroid indices[20] and testosterone.[52-55] Liver and renal abnormalities, thyroid function, and

testosterone will be assessed at the beginning and end of each four-week study period. The risk of taking WS while pregnant or breast-feeding is unknown and therefore, it is recommended to avoid WS during pregnancy and breast-feeding.

Treatment-emergent suicidal ideation and behavior have been identified as a concern for a number of drugs and drug classes. There is currently no published evidence that WS increases suicidal ideation and behavior.[15] Suicide rates increase with age,[87] however, and because safety information for ashwagandha in older adults is limited, a prospective assessment for suicidal ideation and behavior will be monitored. Heisel et al. (2011)[88] 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. Specifically, items 3, 7, 11, 12 and 14 are used to screen for suicidal ideation. We are using the GDS as part of study procedures, and if the participant has a total score of 2 or more on the five items listed, it is considered indicative of possible suicidal ideation. This participant will then be administered the Columbia-Suicide Severity Rating Scale (C-SSRS), which determines suicidal risk severity. If the scale reveals active suicidal ideation with some intent to act but without a specific plan, active suicidal ideation with a specific plan and intent, or suicidal behavior, a study clinician or neuropsychologist will call the participant and assess. Participants with high risk as determined by the clinician will be excluded from the study and referred for mental health intervention.

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 attend an in-person screening visit plus eight additional study visits, including a baseline PK visit during each study period where participants will remain in the Clinical and Translational Research Center for 13 hours. To compensate participants for their time, they will be remunerated in a prorated fashion ($100 per completed study period) for their participation. To offset travel costs, $1500 is set aside in the project budget to pay for transport to and from study visits for participants when needed. To mitigate the burden associated with bringing food that doesn't interfere with the PK analysis, participants will be provided with three meals and snacks at each baseline PK visit at no expense.

O.2. Potential benefits to subjects

There is no direct benefit to participation in the study.

P. IMPORTANCE OF KNOWLEDGE GAINED

WS is an Ayurvedic herb commonly used for stress relief and stress-related neuropsychiatric disorders (e.g., anxiety, depression, and insomnia).[15] Despite being one of the most popular herbal dietary supplements in the United States,[2] there is a lack of data on the oral absorption and PK of WS compounds in humans. This study will assess the oral absorption and PK of eleven withanolides from Shoden® in healthy older adults. Compared to previous PK studies using WS, this study will a) include several additional withanolides, b) measure withanolide levels in urine as well as plasma, c) measure steady state levels after 4 weeks of consumption, and d) provide PK data in an important population (i.e., older adults) where dietary supplement use is common and risk of HDIs is higher. The data on dosing and tolerability will be directly applicable to future trials of Shoden® in family caregivers of people with dementia**.** The data generated from this study will also inform biological signature studies, mechanism-based research on WS compounds, and HDI trials.

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