Effects of Botanical Microglia Modulators in Gulf War Illness

Study Protocol & Statistical Analysis Plan

NCT02909686

10/11/2022

Jarred Younger, Professor, Principal Investigator
University of Alabama at Birmingham
Birmingham, AL 35294

A Placebo-Controlled, Pseudo-Randomized, Crossover Trial of Botanical Agents for Gulf War Illness: Curcumin (Curcuma longa), Boswellia (Boswellia serrata), and French Maritime Pine Bark (Pinus pinaster)

2. Materials and Methods

The full study was a pseudo-randomized, placebo-controlled, crossover clinical trial testing the effects of nine different anti-inflammatory botanical compounds on symptoms of GWI. The list of agents tested in the larger study were curcumin, boswellia, maritime pine, epimedium, fisetin, luteolin, reishi mushroom, resveratrol, and stinging nettle. Participants trialed the botanicals serially, with each participant receiving up to three botanical agents. Outcome measures were obtained daily throughout the entire duration of participation. The University of Alabama at Birmingham (UAB) Institutional Review Board first approved the study protocol (F150318011) on 30 June 2015, and the study was registered on ClinicalTrials.gov (NCT02909686) on 21 September 2016. Participants were recruited to the study through public flyers and online postings. All participants were given a written description of the study and provided written informed consent. Participants were randomized to botanicals so that each condition was completed by at least 10 individuals.

2.1. Participants

Men were considered for inclusion in the study if they were aged 37–65 and able to attend up to 11 study visits every 30 +/− 3 days. Participants were required to meet Kansas GWI case definition criteria [36], with the exception that (1) participants were permitted to have a comorbid diagnosis of diabetes mellitus type 2 if controlled with medications and if having a hemoglobin A1C ≤ 9% and (2) one individual with a remote history of cancer (Hodgkin's lymphoma in remission for 20 years) was included. Participants must have been present in the Persian Gulf between August 1990 and August 1991.

Exclusionary criteria included current opioid, daily anti-inflammatory, nitroglycerine, or lithium medication use; history of anaphylaxis to study botanical compounds; presence of severe depressive symptoms as indicated by a Hospital Anxiety and Depression Scale (HADS [37]) depression subscale score ≥16; presence of a blood or clotting disorder; hypotension (under 90/60 mmHg) or history of cardiovascular disease; diagnosed rheumatologic or autoimmune disease; and acute infection (body temperature over 100.4 °F). Baseline laboratory values of erythrocyte sedimentation rate (ESR) > 40 mmHr, positive rheumatoid factor (RF), CRP > 10.0 mg/L, or positive antinuclear antibody (ANA) were also exclusionary. Participants could also not have current Posttraumatic Stress Disorder (PTSD). PTSD was initially screened with the PTSD Checklist—Military Version (PCL-M [38]), and individuals with PCL-M scores ≥50 were given the Clinician Administered PTSD Scale (CAPS-5 [39]) for a final determination.

2.2. Botanicals

The botanical compounds were sourced from a university-approved vendor, Pure Encapsulations (Sudbury, MA, USA), prior to being sent to a compounding pharmacy for re-encapsulation. Botanical compounds were re-encapsulated in size 0 or size 00 blue gelatin capsules by Double Oak Mountain Pharmacy in Birmingham, AL. Capsules were placed in standardized QUBE Weekly (28 cavity) Cold Seal Compliance Blister packs (Pharmacy Automation Supplies, Romeoville, IL, USA).

Curcumin was obtained as Pure Encapsulation's CurcumaSorb product (SKU#: MCU1), which contains the trademarked Meriva® turmeric phytosome (Indena, S.p.A., Milan, Italy). Curcumin was administered at 1000 mg/day (lower dosage condition) and 4000 mg/day (higher dosage condition). Boswellia was obtained as Pure Encapsulation's Boswellia product (SKU# BW31) and was administered at 400 mg/day and 800 mg/day. Maritime pine bark extract was obtained as Pure Encapsulation's Pycnogenol (pine bark extract) product (SKU# PY16) which contains the trademarked Pycnogenol (Horphag Research, Hoboken, NJ, USA). Maritime pine was administered at 200 mg/day and 400 mg/day. All botanicals were administered twice per day (morning and evening), with the total daily dosage being split evenly between the morning and evening doses.

2.3. Study Protocol

All participants were assessed for initial eligibility for the study using an online prescreening questionnaire, followed by a phone interview. Individuals who met initial inclusion criteria after the online screening and phone interview were given an in-person screening at UAB's Center for Clinical and Translational Science Clinical Research Unit (CCTS CRU).

At the in-person screening visit, participants provided written informed consent as well as blood samples to test for exclusionary lab values. Participants also completed baseline study measures on a tablet device and were loaned a tablet for completion of daily symptom reporting during the study. Following the screening visit, participants reported symptoms every day (in the evening) during a one-month (30 +/− 3 days) baseline period, which served as a habituation period and a means to assess whether participants would reliably complete their daily symptom reports. Participants were allowed to continue with study participation if they completed at least 80% of baseline symptom reports.

Participants were then pseudo-randomized to receive up to three out of the nine botanical compounds, in the design presented in **Figure 1**. Botanical assignments were pseudo-randomized so that (1) approximately equal numbers of participants would take each botanical and (2) to ensure there were no drug interactions that contraindicated the use of the botanicals assigned to participants. There were no contraindications used for curcumin or boswellia. Individuals with prediabetes, evidence of diabetes, or diabetic medications were excluded from taking maritime pine due to its lowering effects on blood glucose [40]. Contraindications were handled by a pharmacist with no other connections to the study so that all research personnel could remain blinded to the botanical being assigned. After completion of a full protocol of three botanical assignments, participants were offered the opportunity to re-enroll into the study protocol to receive up to three more botanicals.

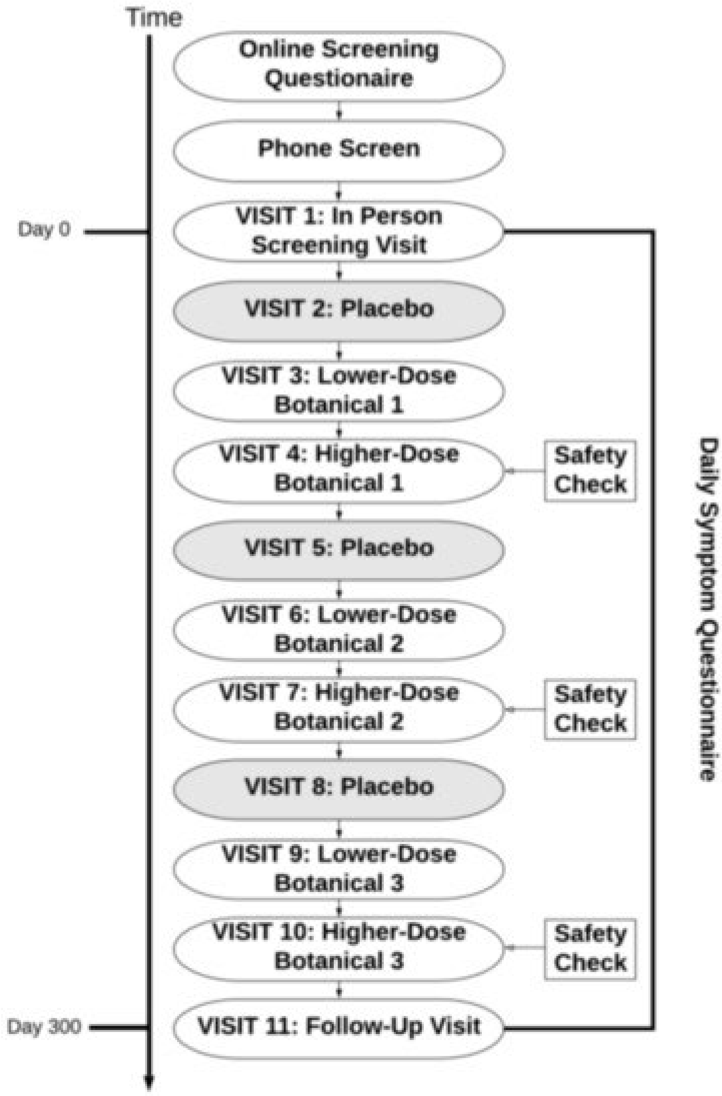

Figure 1. Study protocol. Each participant completed testing of up to three botanicals. Some participants opted to re-enroll in the study protocol after completion, resulting in a maximum of six botanical assignments. For each botanical, there was a placebo condition, followed by lower-dose botanical and higher-dose botanical conditions. The period of time between visits was 30 +/− 3 days.

After the baseline period, participants returned once monthly (every 30 +/− 3 days) for up to 10 additional visits to the CCTS CRU for dispensation of their placebo or botanical capsule kits. At visits 4, 7, and 10, participants received additional blood draws to monitor liver and kidney function (sodium, potassium, chloride, bicarbonate, anion gap, glucose, blood urea nitrogen (BUN), creatinine, calcium, phosphorus, albumin, total protein, total bilirubin, direct bilirubin, indirect bilirubin, alkaline phosphatase, aspartate aminotransferase (AST), and alanine aminotransferase (ALT)). Participants were compensated for each laboratory visit.

For each botanical, participants followed the same protocol: one month (30 +/− 3 days) of placebo, one month (30 +/− 3 days) of lower-dose botanical, and one month (30 +/− 3 days) of higher-dose botanical. Study participants were blinded to both the assigned botanicals and administration protocol (placebo, lower-dose, higher-dose). This administration order was chosen for safety reasons, i.e., adverse effects could be detected in a lower-dose condition before a higher-dose, with possibly greater adverse effects, would be administered. Research personnel were blinded to the assigned botanicals but not to the administration protocol. The study pharmacist was unblinded to both administration protocol and assigned botanicals but had no contact with study participants. Adherence to botanicals was checked at each study visit when participants returned their used blister packs. If participants had missed doses, study staff reminded them of the importance of a regular dosing schedule.

2.4. Screening Measures

The Kansas GWI case definition [36] was used to determine if participants met criteria for GWI. Exclusionary criteria for the definition included certain chronic conditions (heart disease, stroke, lupus, multiple sclerosis, cancer (other than skin cancer), melanoma, and liver disease) not associated with service in the Gulf War, as well as certain conditions that could impact participants' ability to report their symptoms (bipolar disorder or manic depression, schizophrenia, or recent hospitalization for alcohol or drug dependence, depression, or PTSD). Inclusionary criteria for the definition required that participants report presence of symptoms that began during or after service in the Gulf War. Each symptom was scored on a severity scale of 0 to 3, with 0 = none and 3 = severe. Veterans with a score of 2 (at least one moderate symptom or two mild symptoms) or greater in at least three out of six symptom domains (fatigue, pain, neurological/cognitive/mood, skin, gastrointestinal, and respiratory) met criteria for GWI.

The HADS [37] was used to screen for severe depressive symptoms. Potential participants completed the questionnaire via the Qualtrics Research Suite Online Application as part of the online prescreening process. The HADS consists of 14 items divided into two, seven-item subscales: Anxiety (HADS-A) and Depression (HADS-D). Respondents rate items on a 0 to 3 scale, with higher ratings indicating greater presence of the symptoms. Five of the 14 items are reverse scored. The total score is calculated by summing all items and ranges from 0–42. Scores of 16 or greater on the HADS-D subscale during prescreening were exclusionary for participation in the study.

To screen for severe PTSD symptoms, the PCL-M [38] was used. Potential participants completed this questionnaire via the Qualtrics Research Suite Online Application as part of the online prescreening procedures. The PCL-M corresponds to criteria according to the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV [41]) and consists of 17 questions referring to military experiences and symptoms of stress and trauma (e.g., repeated, disturbing memories, thoughts, or images of a stressful military experience) answered on a rating scale of 1 (not at all) to 5 (extremely). A score greater than or equal to 50 indicates a probable diagnosis of PTSD according to DSM-IV criteria [42].

If potential participants scored 50 or greater on the PCL-M during online prescreening, the past-month version of the CAPS-5 [39] was performed at the in-person screening visit by a member of the UAB Office of Psychiatric Clinical Research. The 30-item structured interview evaluates PTSD diagnostic status and symptom severity by assessing onset and duration of symptoms, levels of distress, changes in social and occupational functioning, response validity, and symptoms of the dissociative subtype of PTSD. Each CAPS-5 item is rated on frequency and intensity, which is then combined into a single severity score for that symptom. A severity rating of 2 (moderate/threshold) indicates a symptom that meets diagnostic threshold for current PTSD [43]. If a potential participant met criteria for current PTSD, they were excluded from the study.

2.5. Main Outcome Measures

The Qualtrics Research Suite Offline Application (Qualtrics, Provo, UT, USA) was used for daily symptom reporting. As part of the daily report, participants scored symptoms on a 0–100 digital visual analog scale (VAS). GWI symptom severity, the primary outcome variable of interest, was assessed by asking, "Overall, how severe have your symptoms been today?" anchored on the left by, "No symptoms at all," and on the right by, "Severe symptoms." The single-item, overall GWI severity measure was chosen as the primary outcome, because of the multisymptom and idiosyncratic nature of the condition's manifestation in affected individuals. Veterans living with GWI may primarily experience pain, fatigue, gastrointestinal distress, or any of several other common symptoms. The overall severity measure was determined to be a way to provide the most universal assessment of GWI symptom severity across participants with varying symptom presentations. Participants were instructed to use the GWI symptom severity measure to include whichever specific symptoms they attributed to GWI.

2.6. Secondary Outcome Measures

Several specific symptoms were assessed with similar one-item measures. These symptoms included pain, fatigue, cognitive dysfunction, depressed mood, dermatologic complaints, respiratory problems, and gastrointestinal distress. Of these, two (pain and fatigue) were endorsed by a sufficient number of participants to allow for statistical analysis. These two symptoms were measured with two separate 0–100 VAS items. Pain was assessed by asking, "Overall, how severe is your pain?" anchored on the left by, "No pain at all," and on the right by, "Severe pain." Fatigue was assessed by asking, "How fatigued have you felt today?" anchored on the left by, "Not fatigued at all," and on the right by, "Severely fatigued."

2.7. Statistical Analyses

Analyses were conducted using SPSS Statistics for Windows, version 24 (IBM Corp., Armonk, NY, USA). Effects of botanicals on symptom severity were tested using separate two-level linear mixed models (LMM). LMMs are designed for nested data structures and allow for the dependence between observations typically seen with repeatedly measured variables. In this study, longitudinal outcome assessments are nested within individuals and conditions, violating the assumption of independence between observations. For each botanical, three models were built with condition (baseline, placebo, lower-dose, and higher-dose) entered as a fixed factor and symptom severity (GWI, pain, or fatigue) as the dependent variable, resulting in a total of nine models. Subject ID was used as the subject identifier, and the day in the study was the repeated measures index. A compound symmetry repeated measures covariance structure was selected, as it improved the Bayesian information criterion (BIC) and Akaike information criterion (AIC) for model performance beyond the AR (1) autoregressive covariance structure. For main analyses, daily GWI symptom severity was entered as the dependent variable. For all analyses, the outcome variable was derived by taking a mean of the last 14 days of daily symptom severity reports for each participant during each condition (placebo, lower dose, higher dose). This was consistent with the registered analysis plan, in order to allow the botanicals time to exert clinical effects. A restricted maximum likelihood (REML) estimation approach was used. Post hoc contrasts were carried out with the least-squares differences method. Significance for all tests was set at $p < 0.05$.

Sample size was determined with a-priori power analyses conducted in G*Power 3 [44]. The botanical trials were powered to detect a medium effect (Cohen's d = 0.5) with 0.99 power at a $p = 0.05$ level of significance. Ten individuals were needed to reach 0.99 power, due to the large number of repeated outcome assessments (56 per participant, repeated measures correlation of 0.5). The trials were not powered to detect small (Cohen's d = 0.25) effects, with only 0.42 predicted power. Small effects, however, were not expected to have important clinical impacts on GWI.

A Placebo-Controlled, Pseudo-Randomized, Crossover Trial of Botanical Agents for Gulf War Illness: Resveratrol (Polygonum cuspidatum), Luteolin, and Fisetin (Rhus succedanea)

2. Materials and Methods

The three agents discussed in this report were part of a larger program of study that investigated the effects of nine anti-inflammatory botanicals on GWI symptoms. The study protocol was approved by the University of Alabama at Birmingham (UAB) Institutional Review Board on 30 June 2015 (F150318011), and this study was registered on ClinicalTrials.gov on 21 September 2016 (NCT02909686). Individuals were recruited via radio, print, and online advertisements. All participants provided written informed consent. Participants were randomized to receive up to three botanical compounds in total over the course of the study, such that each botanical was trialed by at least ten individuals. Participants recorded symptoms daily throughout the baseline, placebo, and treatment periods.

2.1. Participants

Inclusion criteria included the following: male sex; age 37–65; presence in the Persian Gulf region between August 1990 and August 1991; ability to come to the study site for 11 monthly visits; ability to receive a venous blood draw; and successful completion of ≥80% of daily symptom reports over the baseline period. All participants had to fulfill Kansas Gulf War Illness case criteria [33], with an exception for well-controlled diabetes mellitus type 2 (A1C ≤ 9%) and in one case for an individual with a history of cancer outside of the last 5 years (Hodgkin's lymphoma in remission for 20 years). In such cases of unclear decisions for inclusion, the author of the Kansas GWI case criteria was consulted for guidance.

Individuals were excluded from study participation for the following: current daily use of opioid or anti-inflammatory medications; use of nitroglycerine or lithium; history of anaphylaxis to any botanical used in the study; hypotension (<90/60 mmHg) or history of cardiovascular disease; diagnosis of rheumatologic or autoimmune disease; blood or clotting disorder; current litigation of worker's compensation claim; and inability to read and understand English. Baseline exclusionary criteria also included acute infection (body temperature above 100.4 °F), positive rheumatoid factor (RF), positive antinuclear antibody (ANA), erythrocyte sedimentation rate (ESR) > 40 mm/hr, and CRP > 10.0 mg/L. Individuals with current severe depressive symptoms as suggested by a depression subscale score ≥ 16 on the Hospital Anxiety and Depression Scale (HADS; [34]) were excluded. Current Posttraumatic Stress Disorder (PTSD) was also exclusionary. Individuals with scores ≥ 50 on the PTSD Checklist–Military Version (PCL-M; [35]) were assessed for the presence of current PTSD with the Clinician Administered PTSD Scale (CAPS-5; [36]).

2.2. Treatments

Resveratrol was sourced from Pure Encapsulations (Sudbury, MA, USA) and was standardized to contain 20% trans resveratrol. Luteolin was obtained from A.P.I. Solutions (Daphne, AL, USA), and fisetin was sourced from VitaCost (Boca Raton, FL, USA). All botanicals were sent to Double Oak Mountain Pharmacy in Birmingham, AL, USA, where they were re-encapsulated in size 0 or 00 opaque blue gelatin capsules. Microcrystalline cellulose was used as filler for the placebo treatment, with the same blue covering capsules as the botanical compounds, so that all treatments appeared identical. All capsules were placed in standardized QUBE Weekly (28 cavity) Cold Seal Compliance Blister Packs (Pharmacy Automation Supplies, Romeoville, IL, USA).

Treatments were administered twice a day. The lower dose for resveratrol was 200 mg/day (200 mg in morning and 0 mg in evening), and the higher dose for resveratrol was 600 mg/day (400 mg in morning; 200 mg in evening). For luteolin, the lower dose was 200 mg/day, and the higher dose was 400 mg/day. Fisetin was administered at 200 mg/day for the lower dose and 800 mg/day for the higher dose. For both luteolin and fisetin, total dosage was evenly split between morning and night doses.

2.3. Study Protocol

The study protocol is depicted in **Figure 1**. Interested individuals completed an online pre-screening questionnaire prior to a phone interview with research personnel in order to assess for initial eligibility. For individuals who met initial inclusion criteria, an in-person screening visit was held at the UAB Center for Clinical and Translational Science Clinical Research Unit (CRU). All participants provided written informed consent at the in-person screening. Baseline study questionnaires were administered on a tablet device, and vital signs and venous blood samples were collected by CRU staff to assess for exclusionary laboratory values. Participants were also provided with computer tablet devices to take home with them in order to complete once daily symptom reports during the entire study period. A one-month baseline symptom reporting period began immediately following the in-person screening visit. Participants were required to complete at least 80% of daily symptom reports during this period to remain eligible for the study.

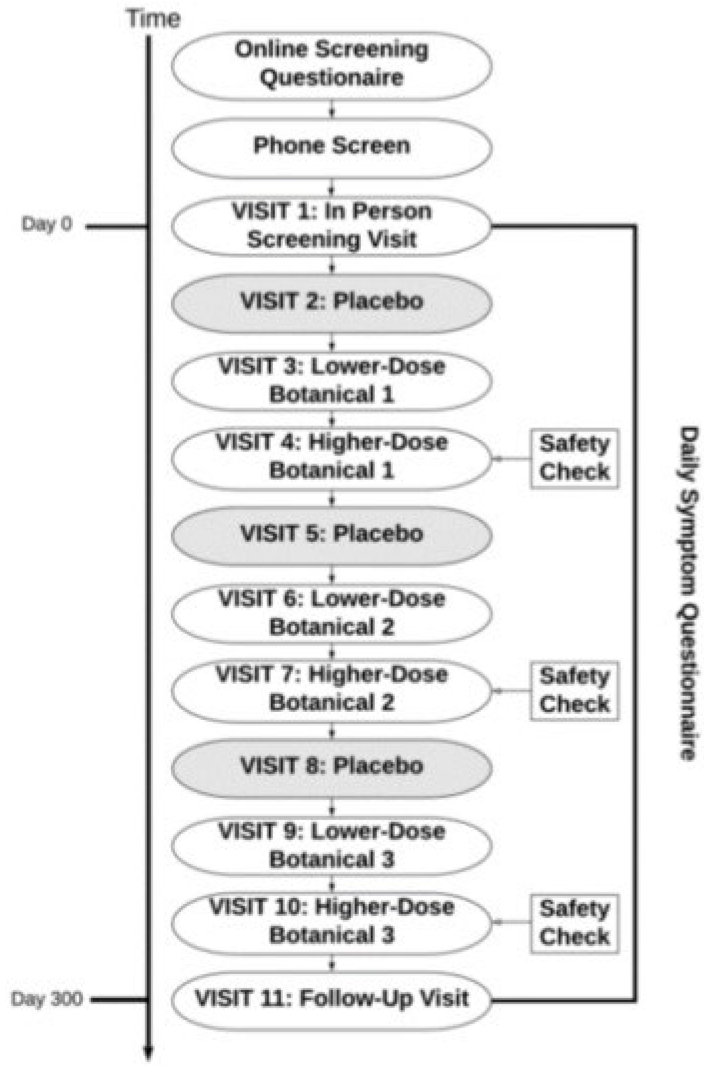

Figure 1. Study protocol. Each participant completed testing of up to three botanicals. For each botanical, there was a placebo condition, followed by lower-dose botanical and higher-dose botanical conditions. The period of time between visits was 30 ± 3 days. Some participants re-enrolled in the study after completion, receiving up to a maximum of six botanicals.

Pseudo-randomization was then performed so that participants were assigned to receive up to three out of the nine botanical compounds, as shown in Figure 1. Pseudo-randomization was done in such a way to prevent drug interactions that contraindicated the use of the botanical in a given participant. There were no contraindications for the use of luteolin. Because blood glucose may be lowered by resveratrol [37] and fisetin [38], individuals taking medications for diabetes, or with evidence of prediabetes or diabetes, were excluded from taking these agents. Additionally, individuals with clotting disorders, hypotension, or those taking anticoagulants or antihypertensive medications were excluded from taking resveratrol, due to its effects on blood pressure [39,40]. Prophylactic use of 81 mg aspirin daily was permitted.

All study visits were held at the CRU and were conducted once a month for 10 months after completion of the baseline period. Participants received kits at each visit containing a one-month supply of either placebo or botanical capsules. Kidney and liver function were monitored with blood draws conducted at visits 4, 7, and 10. The following laboratory values were tested at those visits: sodium, potassium, chloride, bicarbonate, anion gap, glucose, blood urea nitrogen, creatinine, calcium, phosphorus, albumin, total protein, total bilirubin, direct bilirubin, indirect bilirubin, alkaline phosphatase, aspartate aminotransferase (AST), and alanine aminotransferase (ALT).

The same protocol (one month of placebo, one month of lower-dose botanical, and one month of higher-dose botanical) was completed for each botanical. For safety reasons, lower-dose conditions always preceded higher-dose conditions, allowing adverse effects to be detected before a higher dose of the agent was used. Only the study pharmacist was unblinded to the assigned botanicals. All other study staff were aware of the design condition order, but were blinded to the specific botanical taken by each participant. Participants were blinded to both the administration order (placebo, lower-dose, and higher-dose) and the botanical compounds.

Treatment adherence was monitored at each study visit. Each month, participants were instructed to return their blister packs. Study staff checked for any missed doses and discussed with participants the importance of adhering to the administration regimen. Participants were provided with the option of re-enrolling in the study after completing the protocol, such that they could be assigned up to a total of six out of the nine compounds.

2.4. Screening Measures

Participants were screened for GWI using the Kansas Gulf War Illness (GWI) case definition [33], in which symptoms are rated in severity from 0 (none) to 3 (severe). Inclusion criteria were met if an individual obtained a score of 2 or greater (indicating at least one moderate symptom or two mild symptoms) across three or more of six symptom domains (pain, fatigue, neurological/cognitive/mood, gastrointestinal, respiratory, and skin). Symptoms were required to have begun during or after Gulf War service. Chronic medical conditions not associated with Gulf War military service (including heart disease, stroke, lupus, multiple sclerosis, cancer [other than skin cancer], melanoma, and liver disease) were exclusionary for case definition. Due to the possibility of impacting a participant's ability to report symptoms, the following other conditions were exclusionary per case criteria: bipolar or manic depression, schizophrenia, and recent hospitalization for alcohol or drug dependence, depression, or PTSD.

Participants were screened for symptoms of severe depression with the HADS [34]. This questionnaire was part of the online pre-screening process and was completed using the Qualtrics Research Suite Online Application. The 14 items of the HADS contain subscales for Anxiety (HADS-A) and Depression (HADS-D), each with 7 items. Items are rated from 0 to 3, and reverse scoring is used for 5 of 14 total items. Higher ratings suggest greater symptomatology. All ratings are summed for a total score ranging from 0 to 42.

Participants were also screened for severe symptoms of PTSD using the PCL-M [35]. Like the HADS, the PCL-M was completed during the online pre-screening process using the Qualtrics Research Suite Application. The 17-item PCL-M reflects PTSD diagnostic criteria of the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV; [41]). Each item inquires about trauma and stress symptoms associated with military experiences (e.g., Repeated, disturbing memories, thoughts, or images of a stressful military experience?) and is rated on a Likert scale from 1 (not at all) to 5 (extremely). Scores of 50 or greater suggest an increased likelihood of meeting DSM-IV diagnostic criteria for PTSD [42].

If a PCL-M score of 50 or above was obtained, potential participants were evaluated further for current PTSD symptoms using the last month version of the CAPS-5 [36]. Personnel of the UAB Office of Psychiatric Clinical Research administered the CAPS-5 at the CRU during the in-person screening visit. The CAPS-5 is a structured interview consisting of 30 items assessing PTSD symptom severity and diagnostic status (symptom duration and onset, distress levels, impact on social and occupational functioning, response validity, and dissociative subtype symptoms). Items are rated with a severity score based on frequency and intensity of symptoms. A symptom that meets the threshold for diagnosis of current PTSD is suggested by a severity rating of 2 ("Moderate/threshold").

2.5. Main Outcome Measures

Participants reported their symptoms once daily in the evening via the Qualtrics Research Suite Offline Application (Qualtrics, Provo, UT, USA). The reports consisted of visual analog scale (VAS) ratings of symptoms scored from 0 to 100. Given that GWI consists of multiple idiosyncratic symptoms, a single-item severity score was chosen as the primary outcome to provide a common measure for GWI symptom severity. The main outcome was assessed with the following question, "Overall, how severe have your symptoms been today?" with "Not severe at all" fixed on the far left and "Extremely severe" fixed on the far right. Research personnel instructed participants to rate their daily overall GWI symptom severity based on their particular GWI symptoms, encompassing all possible six GWI domains.

2.6. Secondary Outcome Measures

In addition to the GWI symptom severity item, participants responded to several other daily single-item measures. These items assessed specific symptoms including pain, fatigue, cognitive dysfunction, depressed mood, skin problems, respiratory complaints, and gastrointestinal issues. A sufficient number of participants endorsed the pain and fatigue items so that statistical analyses could be applied. Participants responded to pain and fatigue questions using two separate 0–100 VAS ratings. To assess pain, participants were asked, "Overall, how severe is your pain?", with "No pain at all" fixed on the left and "Severe pain" on the right. The item regarding fatigue asked, "How fatigued have you felt today?", with "Not fatigued at all" fixed on the left and "Severely fatigued" on the right.

2.7. Statistical Analyses

SPSS Statistics for Windows, version 24 (IBM Corp., Armonk, NY, USA) was used to conduct analyses. Linear mixed models (LMM) were utilized to test differences in self-reported symptom severity between the four conditions. A separate model was tested for each of the three botanicals. Subject ID was entered as the subject identifier, with all individual longitudinal data nested within-person. The participant's day in the study was entered as the index variable for repeated measures. Compound symmetry was selected as the repeated measures covariance type. The AR (1) autoregressive covariance type was also considered, but it did not improve the Akaike information criterion (AIC) or Bayesian information criterion (BIC) for model performance. The dependent variable was daily GWI symptom severity (0–100). Treatment condition (baseline, placebo, lower-dose, and higher-dose) was entered as a fixed factor. The last 14 days of each condition were selected for analyses to permit time for clinical effects of the treatments to occur. A restricted maximum likelihood (REML) estimation approach was used. Statistical significance was set at $p < 0.05$ for all tests. The secondary analyses were conducted using the same approach.

We performed a-priori power analyses using G*Power 3 [43] to estimate sample size. Each of the nine botanical trials was powered to detect a medium effect (Cohen's d = 0.5) at 0.99 power with $p = 0.05$ threshold for significance. Given 56 repeated outcome measurements per participant (with a repeated measures correlation of 0.5), each trial required 10 individuals to obtain 0.99 power. Our studies were not powered (0.42 predicted power) to detect small effects (Cohen's d = 0.25).

A Placebo-Controlled, Pseudo-Randomized, Crossover Trial of Botanical Agents for Gulf War Illness: Reishi Mushroom (Ganoderma lucidum), Stinging Nettle (Urtica dioica), and Epimedium (Epimedium sagittatum)

2. Materials and Methods

This report is part of a larger clinical trial in which nine botanical agents were tested for effects in GWI. A pseudo-randomized, placebo-controlled, crossover design was used. Curcumin, boswellia, maritime pine, epimedium, fisetin, luteolin, reishi, resveratrol, and stinging nettle were tested in the larger study. In this paper, the results of reishi, stinging nettle, and epimedium are discussed. All procedures were approved by the Institutional Review Board of the University of Alabama at Birmingham (UAB: F150318011) in June 2015, and the trial was registered on **ClinicalTrials.gov** (NCT02909686) accessed on 21 September 2016.

2.1. Participants

Individuals screened for the study were men between the ages of 44 and 65 who met the Kansas GWI case criteria [34] and were deployed to the Persian Gulf region between 1990 and August 1991. As participants must have been at least 18 years old during the Persian Gulf War, the youngest possible age at the study start in 2016 was 44 years. All inclusion and exclusion criteria in the Kansas GWI criteria were utilized, with the exception that individuals with diabetes type 2 could participate. Individuals with diabetes could participate only if the disease was medically controlled, with hemoglobin A1C below 9%.

Individuals were excluded from participation if they were taking opioid analgesics, anti-inflammatory medications, nitroglycerine, or lithium, or if they reported allergies to any study compounds. Participants could not have blood/clotting disorders, hypotension (below 90/60 mmHg), cardiovascular disease, rheumatologic disorders, autoimmune conditions, or acute infection with a body temperature over 100.4°F. Individuals could not participate if their laboratory blood values showed an erythrocyte sedimentation rate greater than 40.0 mm/Hr, a C-reactive protein value greater than 10.0 mg/L, or positive rheumatoid factor or antinuclear antibody.

Individuals were also excluded if they scored 16 or greater on the Hospital Anxiety and Depression Scale (HADS; [35]) depressive subscale by summing the depression items rated on a 0 to 3 scale. Significant posttraumatic stress disorder (PTSD) was identified if individuals scored equal or greater than 50 on the 17-item PTSD Checklist—Military Version (PCL-M; [36]). Individuals with possible ongoing PTSD were subsequently assessed by a member of UAB's Office of Psychiatric Clinical Research, using the Clinician Administered PTSD Scale (CAPS-5; [37]). The 30-item structured interview was used to make a final determination of current PTSD, using established criteria [38]. Individuals meeting criteria for current PTSD were excluded from the study.

2.2. Botanicals

Reishi was sourced from JHS Natural Products, Mushroom Science (Eugene, OR, USA) as a hot water and alcohol extract with 12% polysaccharides and 4% triterpene. Stinging nettle was procured from Nature's Way (Green Bay, WI, USA) as pure nettle leaf. Epimedium was from Barlowe's Herbal Elixirs (Palm Beach, FL, USA) as a 20% icariin extract. Procured agents were sent to a compounding pharmacy (Double Oak Mountain Pharmacy, Birmingham, AL, USA) for re-encapsulation. Materials were put in size 0 or 00 blue gelatin capsules and organized in weekly Cold Seal Compliance blister packs (Pharmacy Automation Supplies, Romeoville, IL, USA). Participants and research personnel were blinded to the botanical agent assignments.

Reishi was administered at 1600 mg (lower dosage) and 3200 mg (higher dosage) per day. Epimedium was given at 500 mg and 1000 mg per day (100 mg and 200 mg of icariin, respectively). Stinging nettle was given at 435 mg and 1305 mg per day. All botanicals were administered twice per day (morning and evening), with the total daily dosage being split evenly between the morning and evening doses. Placebo was administered in identical gelatin capsules with microcrystalline cellulose filler.

Individuals on any antihypertensive medications were not allowed to take reishi or epimedium, even if their blood pressure was well-controlled. Individuals were not assigned to take stinging nettle if they had any signs of diabetes, pre-diabetes, or were taking medications for diabetes. Individuals could participate in the study despite being excluded from taking one or more of the botanicals, as the entire list of nine botanicals had several contraindications. As many individuals with GWI also suffer from conditions such as high blood pressure and diabetes, excluding these individuals would have created a sample that is not representative of the general patient population.

2.3. Study Protocol

Individuals were screened via an online questionnaire and phone interview, followed by an in-person screening conducted at UAB's Clinical Research Unit (CRU). Informed consent was obtained at the in-person visit, and blood samples were collected for further screening. As the screening involved blood tests, individuals provided informed consent before being determined eligible for participation. Therefore, several individuals who provided consent were not eligible to participate in the study. Consented individuals received a tablet device for completing daily symptom reports each evening for the duration of their study participation.

Before beginning capsules, all participants completed a baseline period of 30 days. This period served as the baseline for all botanicals taken by the participant. Participants then were pseudo-randomized to receive up to three of the nine study compounds, as seen in **Figure 1**. A pseudo-randomized procedure was used to avoid contraindications with the botanicals. Randomization was conducted by a pharmacist. The pharmacist in charge of blinding and randomization was not otherwise involved in the study. The research personnel were blinded to the botanicals assigned to each participant. For each botanical, participants completed 30 days of placebo capsules, 30 days of lower-dose botanical, and 30 days of higher-dose botanical. Participants were blinded to all aspects of the study (the protocol and botanicals assigned). Research personnel were blinded to the botanicals assigned, though they were aware that placebo occurred first, followed by lower-dose and higher-dose treatment.

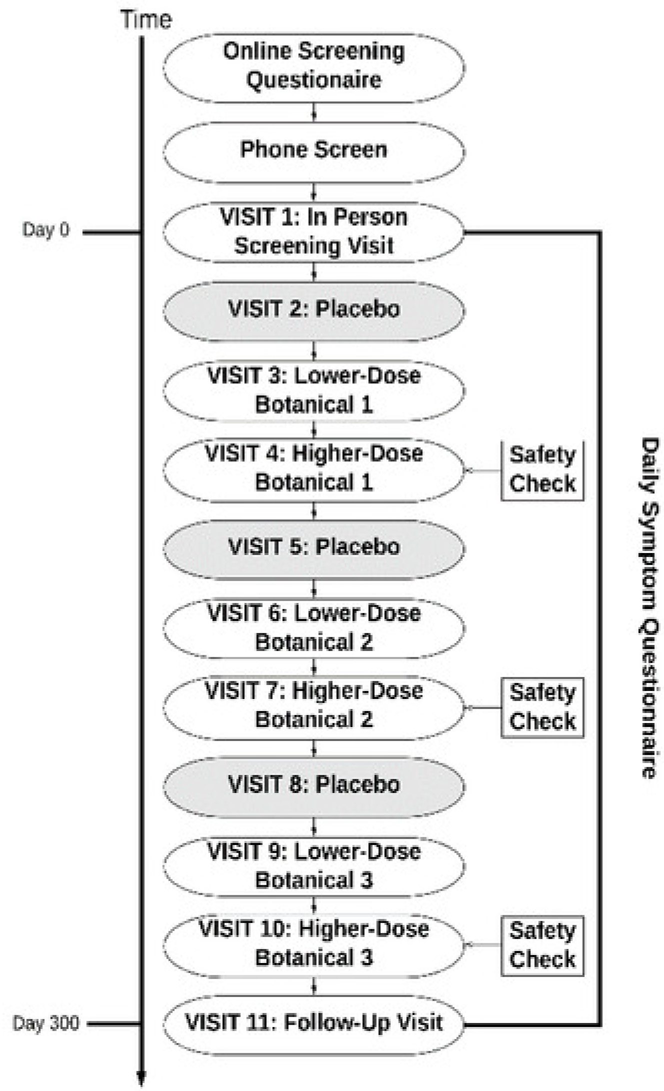

Figure 1. Study event flow. Participants took three of the tested compounds in the following order: placebo, lower-dose botanical, and higher-dose botanical.

Throughout their involvement with the study, participants returned to the CRU every month. At these visits, participants would obtain a new supply of study capsules. Blood samples were also obtained at visits 4, 7, and 10 to conduct safety tests. Standard clinical renal and hepatic panels were performed. Participants first took the lower dosage and then the higher dosage of the botanical so that any renal or hepatic issues could be detected before the participant took the larger dosage. Participants received compensation during each visit. The participants received a total of $1500 for attending all 11 of the study visits and completing the protocol. Participants who successfully completed all three botanical assignments were allowed to enroll in the study a second time, where they would be assigned new botanicals.

2.4. Outcome Measures

Participants completed self-reported symptom severity measures every evening during the study, using the Qualtrics Research Suite (Qualtrics, Provo, UT, USA) on a tablet. The primary outcome used in this clinical trial was a 0–100 item assessing overall GWI severity. The item read, “Overall, how severe have your symptoms been today?” The zero response was anchored by “No symptoms at all” and 100 was labelled as “Severe symptoms”. This generic symptom response was chosen because GWI sufferers can have a wide range of principal complaints.

2.5. Secondary Outcome Measures

To further explore the impact of botanicals on common GWI complaints, we also measured changes in pain and fatigue severity. Both outcomes were measured on a 0–100 scale. Pain was measured by the item, “Overall how severe is your pain?” from “No pain at all” to “Severe pain”. Fatigue was measured by “How fatigued have you felt today?” from “Not fatigued at all” to “Severely fatigued”. Other GWI symptom domains such as respiratory and skin issues were rarely endorsed by participants and were not analyzed.

2.6. Statistical Analyses

All analyses were conducted in SPSS version 24 (IBM Corp., Armonk, NY, USA). For each botanical, a linear mixed model (LMM) was created to test changes in the primary outcome. LMMs were used to accommodate the repeated outcome assessments in each condition, nested in each participant. Subject ID was the repeated-measures nesting variable, the repeated-measures index was the day in the study, and the repeated measures covariance type was compound symmetry. The predictor was study condition, which could take four values (baseline, placebo, lower-dose, and higher dose botanical). The last 14 days of each condition were included in the models. The final 14 days of the baseline condition at the beginning of participation were used as the baseline for all tested botanicals. Post hoc tests were conducted using least-squares differences. A $p < 0.05$ was used for all tests. The same statistical approach was used to test the secondary outcomes of pain and fatigue. Nine total models are presented in this report (three botanicals tested for the outcomes of GWI severity, pain and fatigue).

If a participant did not complete the protocol, any valid data were still used in statistical tests. However, data imputation of missing values was not used, and participants were not included if they never took the assigned botanical. Therefore, we did not use a complete intent-to-treat method for analysis.

The three trials were powered to detect within-person fixed effects for study condition (baseline, placebo, lower dosage and higher dosage). Detection of a medium effect size (Cohen’s d = 0.5) was targeted at a threshold of $p < 0.05$. With 56 repeated outcome assessments per participant, and a repeated measures correlation of 0.5, it would require 10 individuals to achieve 0.99 power for the main effect test.