Study Protocol and Statistical Analysis Plan

Study title: Enhancement of Calmness and Mood Following Supplementation with Lemon Balm During Periods of Cognitive Demand in an Adult Population (Lemcog)

Acronym: Lemcog

Date: 30th October 2023

1. **Sample Size Calculation**
   An a priori power analysis in G* power with an effect size of 0.5 (Kennedy et al., 2003; Kennedy et al., 2004) indicated that a sample of 130 participants (65 per group, placebo and treatment respectively) with a 10% attrition rate would be necessary to achieve a power level of 0.80 at a significance level of 0.05. Similar trials investigating mood and cognition have shown a change from baseline following an acute dose of lemon balm beginning with changes first observed after 1h and maintained throughout the test day, in samples as small as 18 participants (Kennedy et al., 2004).

2. **Recruitment and screening**
   Participants will be recruited using opportunity sampling. The study will be advertised through email distribution and with posters across the University of Reading and on online social media platforms. Before interested participants are enrolled in the trial, a brief pre-screen video call will be undertaken alongside completion of an entry stress test (PSS). Once pre-screened, participants will be invited to a screening session to determine if they meet additional eligibility criteria regarding habitual diet and general health and lifestyle (examples include willingness to not consume alcohol or coffee prior to the test session and on the test day, or regular exercise levels deemed as excessive, any restrictive diet, no smoking or consuming nicotine-based products 7 days prior to the test session). The visit will also provide an opportunity to become familiar with the mood and cognitive test batteries to be completed at specific timepoints (baseline (0h), 1h, 3, and 5h timepoints) on the test day. If eligible, participants will be invited to attend a test visit the following week. Participants will be compensated with £75 for completing all phases of the study.

3. **Randomisation and Blinding Process**
   A researcher not involved in recruitment or data collection will used blocked randomization with random permuted blocks of two and six individuals to form the allocation list for the two comparison groups of equally balanced males and females weighted across placebo and intervention conditions. The allocation sequence will be concealed to the researchers enrolling and assessing participants. These randomisation codes will be pre-labelled onto opaque containers containing identical pink coloured capsules (intervention and placebo, respectively) and prepared by researcher personnel not involved in the conduct of the trial. On the test day, the investigator running the visit will administer a capsule from the correct container in accordance with the randomisation schedule.

4. **Testing protocol**

   <u>Test visits</u>
   - Participants will be asked to follow a low polyphenol diet for 48hours and restrict caffeine and alcohol intake 24 hours prior to test visits.
   - At the screening visit participants will have a chance to complete the various cognitive and mood tasks that will be delivered at various timepoints on the test day.
   - On arrival of the acute test day, participants will be randomly assigned to treatment: 300mg lemon balm or placebo condition.
   - Adverse events and vital signs (bp, HR) will be recorded to estimate tolerance.

- Participants will consume a standardised breakfast (2 croissants and a glass of water) and complete the first block of cognitive and mood tasks between 8am-9am.
- After the baseline (0h), participants will complete the same cognitive and mood tasks at specific timepoints (1h following supplementation, 3h and 5h) and will receive a standardised lunch (cheese sandwich with a packet of crisps).

The screening session should take no longer than 3 hours, and the acute test day should take no longer than 6 hours spread out over an 8-hour window (8am-4pm). Participants may withdraw at any time without giving any reason. In addition, a participant will be withdrawn from the study if they request discontinuation, exhibit a serious adverse event to any component of the test product, the participant significantly violates the exclusion or inclusion criteria, an illness emerges and/or opinion is that withdrawal is appropriate.

5. Statistical Analysis Plan

Main aim: The primary objective is to investigate the acute effects of a single dose of 300mg lemon balm on calmness and mood during cognitive overload in adults with subjective stress complaints.

Baseline characteristics will be homogenous across the two groups, placebo and intervention, respectively, as identified by pre-screening and screening criteria. The determined criteria include PSS scores between 14 to 26 to indicate stress problems, physical and emotional health, gender, age and lifestyle factors such as intake of daily fruit and vegetables (no more than 3 portions), no excessive caffeine or alcohol consumption, no excessive exercise, adequate performance on the cognitive tasks, and not on any restrictive diet (i.e., vegan/vegetarian) or medication.

Primary Outcome Variable

To determine the effect of 300 mg lemon balm on ratings of calmness following a period of cognitive demand, measured using a 9-point Likert Scale, at baseline (0h) and 5 hours post administration. This Calmness 9-point Likert scale is scored from 1, “Not at all Calm” to 9, “Extremely Calm” Calmness Anchor points.

Exploratory Outcome Variables

- To determine the effects of 300mg lemon balm on various mood measures (domains including fatigue and relaxation) measured at baseline (0h), 1h, 3h and 5h post administration.
- To determine the effects of 300mg lemon balm on cognition (specifically, executive function and memory) measured at baseline (0h), 1h, 3h and 5h post administration.
- Heart rate and aortic blood pressure will be taken in triplicate where averaged scores will be assessed at pre-(before 0h block) and post-test (after 5h block) to estimate tolerance to intervention versus placebo.

Analysis

Before analysis, all data will be checked for assumptions of parametric testing, including Normal distribution, then assessed with boxplots to screen for any outliers. On recommendation of a statistician, all repeated measures data will be analysed using Linear Mixed Modelling (LMM) in RStudio v4.0. This technique can be used to model variance relating to both fixed parameters such as experimental doses and random parameters such as individual differences between subjects, within multiple layers of the same model. Models will be adjusted for potential predictive factors, such as sex, diet quality, physical activity level and severity of baseline stress complaints and/or baseline performance on cognitive tasks where the inclusion of these covariates improves the fit of the model, as long as assumptions are met. Subjects will be included as a random factor as a way of controlling for non-independence of data from the same subjects. The models will be applied in a systematic way to retain consistency between analyses. Where possible, an unstructured covariance matrix will be specified for the repeated observations on a subject within the same period. As LMMs do not require balanced data, subjects with missing data points will not be excluded from the analyses. Post-hoc Bonferroni-corrected parametric tests such as independent t-tests will be performed to estimate between-group differences between placebo and intervention groups, or within-group differences between time points, where appropriate.