Do Terpenes Play a Role in the Stress-reducing Effects of a Forest Bathing Intervention?

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1. Exclusion criteria:

Potential participants were screened via a phone call and excluded if they were pregnant, smoking, and/or had a current or prior diagnosis of neurologic, hypertensive, psychiatric, respiratory disorder, or anosmia/hyposmia. Participants were also excluded if they were prescribed a short list of prescription medications known to influence terpene metabolic pathways and short-term inflammatory biomarkers including beta-blockers, antibiotics, statins, hypertension medications, steroid medications, and diabetes control medications. Eligible participants were asked to avoid certain foods, beverages, cleaning products, and supplements that contain terpene compounds in the 24 hours leading up to their forest sitting experience.

Additionally, the clinically validated University of Pennsylvania Smell Identification Test (UPSIT) (Sensonics International, Haddon Heights, NJ) was administered to determine whether participants had anosmia/hyposmia. The UPSIT is a 40-item, self-administered "scratch-and-sniff" test that uses microencapsulated odorants that are released by scratching designated spaces on a paper test booklet. Summed scores were used to evaluate olfactory function and identify/exclude participants with undiagnosed smell loss or total anosmia (summed UPSIT score $\leq$18).

2. Specific Aims

2. 1. Aim 1. Assess whether VOC inhalation regulates stress reduction and affective outcomes of the terpenes-on vs. terpenes-off sessions.

Primary physiological outcome: <u>increases</u> in the HF (ms^2) component of HRV.

Secondary outcomes: <u>decreases</u> in blood pressure, heart rate, self-reported stress, negative affect, and levels of inflammatory cytokines (IL-6, TNF-alpha, CRP) and cortisol in serum, and <u>increases</u> in positive affect, measured via mobile physiology equipment and blood using standard clinical methods.

Hypothesis: VOC inhalation will regulate increases in the HF (ms^2) component of HRV and decreases in blood pressure, heart rate, self-reported stress, and levels of inflammatory cytokines in serum as secondary outcomes.

2. 2. Aim 1a. Assess the degree of association of absorbed dose of seven forest-derived VOCs in serum (i.e., α- pinene, β-pinene, β-myrcene, Δ 3- carene, limonene, β- carophyllene, α-humulene) with these outcomes.

Hypothesis: Absorbed dose of seven forest-derived VOCs in serum will be associated with stress reduction and affective outcomes.

3. Statistical Analysis

3.1 Exploratory Analysis

Histograms and descriptive statistics of the study data were plotted/calculated for the manuscript. Histograms and boxplots of UPSIT scores, time in nature, time in nature setting, frequency in nature, PSS

scores, PROMIS (4-item Anxiety Scale) scores, and PANAS scores were plotted. Spaghetti plots of HF HRV, SCL, SF-PANAS scores, systolic and diastolic blood pressure, heart rate, and self-reported stress were plotted. Boxplots of inflammatory biomarkers were plotted.

Primary outcome: High Frequency Heart Rate Variability

- Check for missing or out-of-range values
- We applied a natural logarithmic transformation to 5-minute averages of HF HRV (hereafter called ln-HF HRV), following established precedent in prior literature (Shaffer and Ginsberg, 2017).

Secondary outcomes: Blood pressure, heart rate, self-reported stress, SF-PANAS positive affect, SF-PANAS negative affect, and levels of inflammatory cytokines (IL-6, TNF-alpha, CRP) and cortisol in serum, measured via mobile physiology equipment and blood using standard clinical methods.

- Check for missing or out-of-range values
- Explore imputation approaches for missing PANAS items
- One CRP observation was right-censored. The observation was set to the limit of quantitation (35.98 mg/L) and CRP observations were log transformed.
- As the machine values for left-censored IL-6 observations are closest to the true values, machine values will be used for censored observations for primary analysis.

3.2 Primary Analysis: Linear Mixed Effect Model

We will calculate the main effect of the filter on the difference between baseline measurements and time point 2 (T2) for ln-HF HRV, skin conductance levels, self-reported stress, positive and negative affect levels (SF-PANAS) and timepoint 4 (T4) for systolic blood pressure, diastolic blood pressure, heart rate, inflammatory biomarkers, and cortisol using a mixed effect model. Systolic blood pressure, diastolic blood pressure, heart rate, inflammatory biomarkers and cortisol data were collected at T4, but not T2. As the machine values for left-censored IL-6 observations are closest to the true values, machine values will be used for censored observations for the primary analysis. Sensitivity analyses will be performed for the IL-6 analysis by comparing model output using machine values to model output using limit of detection and zero and a logistic regression using a detect/non-detect variable. If using a different approach for imputing IL-6 changes the results substantially, then our result is not easily interpretable. Participants who completed only one session will also be included in analysis. Analyses will be conducted in R (version 4.3.1; R Core Team, 2023) using the lme4 R package (Bates et al., 2015). We will present the main effect results and associated statistics, including effect sizes and confidence intervals.

For ln-HF HRV, skin conductance levels, self-reported stress, positive and negative affect levels (SF-PANAS):

$$Y_{is} = \beta_0 + \beta_1 baseline_{is} + \beta_2 filter_{is} + \alpha_i + \varepsilon_{is}$$

Where:

- Y_{is} is the observed average outcome for individual *i* at T2, and session *s* where *s* is session 1 or session 2

- β_0 is the average Y_{is} for the study population at T2 when they were assigned the A filter, adjusted for baseline.
- β_1 is the estimated difference in Y_{is} at T2 between two groups differing in baseline by 1 unit.
- β_2 is our primary coefficient of interest and is the estimated difference in Y_{is} between the A filter and the B filter at T2, adjusted for baseline.
- α_i is the random intercept for subjects to account for repeated measures within individuals
- ε_{is} is the observation-specific error

For systolic blood pressure, diastolic blood pressure, heart rate, inflammatory biomarkers, and cortisol:

- Y_{is} is the observed outcome for individual *i*, at time point *t* where *t* is T4, and session *s* where *s* is session 1 or session 2
- β_0 is the average Y_{is} for the study population at T4 when they were assigned the A filter, adjusted for baseline.
- β_1 is the estimated difference in Y_{is} at T4 between two groups differing in baseline by 1 unit.
- β_2 is the average difference in Y_{is} between the A filter and the B filter, adjusted for baseline.
- α_i is the random intercept for subjects to account for repeated measures within individuals
- ε_{is} is the observation-specific error

For positive and negative affect levels (SF-PANAS):

$$Y_{is} = \beta_0 + \beta_1 baseline_{is} + \beta_2 filter_{is} + \beta_3 missingscaleitem_{is} + \alpha_i + \varepsilon_{is}$$

Where:

- Y_{is} is the observed average outcome for individual *i* at T2, and session *s* where *s* is session 1 or session 2
- β_0 is the average Y_{is} for the study population at T2 when they were assigned the A filter, adjusted for baseline and missing scale items.
- β_1 is the estimated difference in Y_{is} at T2 between two groups differing in baseline by 1 unit.
- β_2 is our primary coefficient of interest and is the estimated difference in Y_{is} between the A filter and the B filter at T2, adjusted for baseline and missing scale items.
- $\beta_3$is the estimated difference in Y_{is} between two groups differing in missing affect scale items by 1 item, adjusted for baseline.
- α_i is the random intercept for subjects to account for repeated measures within individuals
- ε_{is} is the observation-specific error

3.2 Secondary Analysis: Nested Mixed Effect Models

We will calculate the effect of the filter on the difference between time point 1(T1) and time point 2 (T2), time point 3 (T3), and time point 4 (T4) to see how early any effect appears and characterize the temporal pattern of the response.

For ln-HF HRV, skin conductance levels, and self-reported stress:

Null model:

$$Y_{its} = \beta_0 + \beta_1 baseline_{is} + \beta_2 T2_{is} + \beta_3 T3_{is} + \beta_4 T4_{is} + \alpha_i + \varepsilon_{its}$$

- Where:
  - Y_{its} is the observed average outcome for individual *i*, at time point *t* where *t* is T1, T2, T3, or T4, and session *s* where *s* is session 1 or session 2
  - β_0 is the value of Y_{its} at T1 and adjusted for baseline.
  - β_1 is the estimated difference in Y_{its} at T1, T2, T3, or T4 between two groups differing in baseline by 1 unit.
  - β_2 is the difference between Y_{its} comparing T1 and T2, adjusted for baseline.
  - β_3 is the difference between Y_{its} comparing T1 and T3, adjusted for baseline.
  - β_4 is the difference between Y_{its} comparing T1 and T4, adjusted for baseline.
  - α_i is the random intercept for subjects to account for repeated measures within individuals
  - ε_{its} is the observation-specific error

Full model:

$$Y_{its} = \beta_0 + \beta_1 baseline_{is} + \beta_2 T2_{is} + \beta_3 T3_{is} + \beta_4 T4_{is} + \beta_5 filter_{is} + \beta_6 T2_{is} * filter_{is} + \beta_7 T3_{is} * filter_{is} + \beta_8 T4_{is} filter_{is} + \alpha_{is} + \varepsilon_{its}$$

- Where:
  - Y_{its} is the observed average outcome for individual *i*, at time point *t* where *t* is T1, T2, T3, or T4, and session *s* where *s* is session 1 or session 2
  - β_0 is the average Y_{its} at T1 and adjusted for baseline for the study population when they were assigned the A filter.
  - β_1 is the estimated difference in Y_{its} at T1, T2, T3, or T4 between two groups differing in baseline by 1 unit.
  - β_2 is the average difference in Y_{its} comparing T1 and T2 for the study population when they were assigned the A filter, adjusted for baseline.
  - β_3 is the average difference in Y_{its} comparing T1 and T3 for the study population when they were assigned the A filter, adjusted for baseline.

- β_4 is the average difference in Y_{its} comparing T1 and T4 for the study population when they were assigned the A filter, adjusted for baseline.
- β_5 is the average difference between Y_{its} at T1 when the study population was assigned the A filter and Y_{its} at T1 when they were assigned the B filter, adjusted for baseline.
- β_6 is the average difference between Y_{its} at T2 when the study population was assigned the A filter and Y_{its} at T2 when they were assigned the B filter, adjusted for baseline.
- β_7 is the average difference between Y_{its} at T3 when the study population was assigned the A filter and Y_{its} at T3 when they were assigned the B filter, adjusted for baseline.
- β_8 is the average difference between Y_{its} at T4 when the study population was assigned the A filter and Y_{its} at T4 when they were assigned the B filter, adjusted for baseline.
- α_i is the random intercept for subjects to account for repeated measures within individuals
- ε_{its} is the observation-specific error

For positive and negative affect levels (SF-PANAS):

Null model:

$$Y_{its} = \beta_0 + \beta_1 baseline_{is} + \beta_2 T2_{is} + \beta_3 T3_{is} + \beta_4 T4_{is} + \beta_5 missingscaleitems_{is} + \alpha_i + \varepsilon_{its}$$

- Where:
  - Y_{its} is the observed average outcome for individual *i*, at time point *t* where *t* is T1, T2, T3, or T4, and session *s* where *s* is session 1 or session 2
  - β_0 is the value of Y_{its} at T1 and adjusted for baseline and missing scale items.
  - β_1 is the estimated difference in Y_{its} at T1, T2, T3, or T4 between two groups differing in baseline by 1 unit, adjusted for missing scale items.
  - β_2 is the difference between Y_{its} comparing T1 and T2, adjusted for baseline and missing scale items.
  - β_3 is the difference between Y_{its} comparing T1 and T3, adjusted for baseline and missing scale items.
  - β_4 is the difference between Y_{its} comparing T1 and T4, adjusted for baseline and missing scale items.
  - β_5 is the estimated difference in Y_{its} between two groups differing in missing affect scale items by 1 item, adjusted for baseline.
  - α_i is the random intercept for subjects to account for repeated measures within individuals
  - ε_{its} is the observation-specific error

Full model:

$$Y_{its} = \beta_0 + \beta_1 baseline_{is} + \beta_2 T2_{is} + \beta_3 T3_{is} + \beta_4 T4_{is} + \beta_5 filter_{is} + \beta_6 T2_{is} * filter_{is} + \beta_7 T3_{is} * filter_{is} + \beta_8 T4_{is} filter_{is} + \beta_9 missingscaleitems_{is} + \alpha_{is} + \varepsilon_{its}$$

- Where:
    - Y_{its} is the observed average outcome for individual *i*, at time point *t* where *t* is T1, T2, T3, or T4, and session *s* where *s* is session 1 or session 2
    - β_0 is the average Y_{its} at T1 and adjusted for baseline and missing scale items for the study population when they were assigned the A filter.
    - β_1 is the estimated difference in Y_{its} at T1, T2, T3, or T4 between two groups differing in baseline by 1 unit, adjusted for missing scale items.
    - β_2 is the average difference in Y_{its} comparing T1 and T2 for the study population when they were assigned the A filter, adjusted for baseline and missing scale items.
    - β_3 is the average difference in Y_{its} comparing T1 and T3 for the study population when they were assigned the A filter, adjusted for baseline and missing scale items.
    - β_4 is the average difference in Y_{its} comparing T1 and T4 for the study population when they were assigned the A filter, adjusted for baseline and missing scale items.
    - β_5 is the average difference between Y_{its} at T1 when the study population was assigned the A filter and Y_{its} at T1 when they were assigned the B filter, adjusted for baseline and missing scale items.
    - β_6 is the average difference between Y_{its} at T2 when the study population was assigned the A filter and Y_{its} at T2 when they were assigned the B filter, adjusted for baseline and missing scale items.
    - β_7 is the average difference between Y_{its} at T3 when the study population was assigned the A filter and Y_{its} at T3 when they were assigned the B filter, adjusted for baseline and missing scale items.
    - β_8 is the average difference between Y_{its} at T4 when the study population was assigned the A filter and Y_{its} at T4 when they were assigned the B filter, adjusted for baseline and missing scale items.
    - β_9 is the estimated difference in Y_{its} at T1, T2, T3, or T4 between two groups differing in missing affect scale items by 1 item, adjusted for baseline.
    - α_i is the random intercept for subjects to account for repeated measures within individuals
    - ε_{its} is the observation-specific error

3.3 Absorbed Dose Association with Outcomes

We will calculate the association of absorbed dose (difference between baseline and T4 serum terpene levels) and the difference between baseline measurements and time point 4 (T4) for ln-HF HRV, skin conductance levels, self-reported stress, positive and negative affect levels (SF-PANAS), systolic blood pressure, diastolic blood pressure, heart rate, inflammatory biomarkers, and cortisol using a mixed effect model. Participants who completed only one session will also be included in analysis.

For ln-HF HRV, skin conductance levels, self-reported stress, systolic blood pressure, diastolic blood pressure, heart rate, inflammatory biomarkers, and cortisol:

$$Y_{is} = \beta_0 + \beta_1 baseline_{is} + \beta_2 absorbeddose_{is} + \alpha_i + \varepsilon_{is}$$

Where:

- Y_{is} is the observed average outcome for individual *i* at T4, and session *s* where *s* is session 1 or session 2
- β_0 is the average Y_{is} for the study population at T4, adjusted for baseline.
- β_1 is the estimated difference in Y_{is} at T4 between two groups differing in baseline by 1 unit, adjusted for absorbed dose.
- β_2 is our primary coefficient of interest and is the estimated difference in Y_{is} between two groups differing in absorbed dose by 1 unit, adjusted for baseline.
- α_i is the random intercept for subjects to account for repeated measures within individuals
- ε_{is} is the observation-specific error

For positive and negative affect levels (SF-PANAS):

$$Y_{is} = \beta_0 + \beta_1 baseline_{is} + \beta_2 absorbeddose_{is} + \beta_3 missingscaleitem_{is} + \alpha_i + \varepsilon_{is}$$

Where:

- Y_{is} is the observed average outcome for individual *i* at T2, and session *s* where *s* is session 1 or session 2
- β_0 is the average Y_{is} for the study population at T2, adjusted for baseline, missing scale items, and absorbed dose.
- β_1 is the estimated difference in Y_{is} at T2 between two groups differing in baseline by 1 unit, adjusted for missing scale items and absorbed dose.
- β_2 is our primary coefficient of interest and is the estimated difference in Y_{is} between the two groups differing in absorbed dose by 1 unit, adjusted for baseline and missing scale items.
- $\beta_3$is the estimated difference in Y_{is} between two groups differing in missing affect scale items by 1 item, adjusted for baseline and absorbed dose.
- α_i is the random intercept for subjects to account for repeated measures within individuals
- ε_{is} is the observation-specific error

4. Data Exclusions

4.1 Handling Missing Data

We assessed missing data after obtaining a blinded dataset but before being unblinded to the actual treatment per session. Unexpected missing data emerged in the blinded dataset, resulting in the absence of a predefined approach to address this issue prior to data collection. Primary analysis will use a complete case dataset, except for the positive and negative affect outcome. 16 (3.9%) of the 414 questionnaires

were missing 1-2 items at one of the time points, and the impact of missing items will be adjusted for using the number of missing items variable in the model.

We expect the small amount of missingness to have a minimal impact on our results, however we will assess this impact with sensitivity analyses. For comparison, we will perform the analysis on an imputed dataset. We expect this to narrow confidence intervals without greatly impacting the coefficient.

We will be imputing some missing values for two types of data, 1) missing questionnaire items (relevant to secondary outcomes) and 2) missing baseline ln-HF HRV (relevant to primary outcome). The PANAS positive affect (PA) and negative affect (NA) outcomes are scored by summing 5 individual PA and NA items. 16 (3.9%) of the 414 questionnaires were missing 1-2 items at one of the time points, though the data were typically complete for other time points. Because answers for the same item are modestly correlated between timepoints from the same day (mean $R^2 = 0.58$), but still performed better than other imputation approaches (random PA and NA item from the day, mean PA or NA score, and regression imputation), we will use the mean available item value for missing items. This methodology is similar to the mean imputation approach demonstrated in Shrive et al. (2006), but employs individual item row mean imputation, a single item imputation approach that can be used for longitudinal data (Engels and Diehr, 2003).

Brief State Rumination Inventory (BSRI) is a sum of 8 individual items. 6 (4.3%) of the 138 questionnaires were missing 1-2 items. The answers for the same item were correlated with the other time point for the same day (mean $R^2 = 0.83$).

For ln-HF HRV, there were 5 participants whose ln-HF HRV was partially captured both before and during exposure, but missing during the window of our standard baseline time (i.e., B1).

Additional 5-minute periods of ln-HF HRV were calculated using continuous data from before treatment exposure, including 10-5 minutes before B1 (R1), 5-0 minutes before B1 (R2), 0-5 minutes into the venipuncture procedures (V1), 5-10 minutes into the venipuncture procedure (V2), 0-5 minutes into the drive up to the forest site (D1), and 5-10 minutes into the drive up to the forest site (D2).

Correlations between B1 ln-HF HRV and each additional time point prior to exposure were assessed using a complete case data set.

Time points with the highest correlations (R1, R2, V1, D1) were included in single linear regression models to predict B1 values using a complete case data set. One participant did not have any additional timepoints available, so we used a regression model with the B1 measurement from the other session as a predictor (s2_b1_hrv ~ s1_b1_hrv).

Each single linear regression model (b1_hrv ~ r1_hrv, b1_hrv ~ r2_hrv, b1_hrv ~ v1_hrv, b1_hrv ~ d1_hrv, s2_b1_hrv ~ s1_b1_hrv) was cross validated using a complete-case dataset to assess R^2 values (0.92, 0.95, 0.98, 0.85, and 0.83, respectively).

Models were preferentially ranked based on R^2 values and predicted values for B1 will be imputed depending on R1, R2, V1, D1, and S1B1 (session 1 baseline 1) timepoint availability. Of the missing

baseline observations, there is one person for whom we would use V1 to predict B1, three people for whom we would use D1 to predict B1, and one person for whom we would use S1B1 to predict S2B1.

4.2 Data Exclusion Criteria

Five participants were removed from analysis: two participants who were recommended for exit by IRB staff following responses to study protocols, one participant who withdrew mid-session, and two participants who were found to meet exclusion criteria following their first session.

5. References

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