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SYNOPSIS

Name of the Sponsor: Water and Light Applications India Private Limited	Individual study table referring to the part of the dossier Volume: Page :	*(For national authority use only)*
Name of the finished product: Analemma Water		
Name of the active ingredient: Analemma Water		

Study Title:

A double blinded, balanced, randomized, two treatment, two period, preliminary microbiome study of Analemma Water and assess the NAD+ assay from whole blood pre and post treatment of Analemma Water in normal, healthy, adult, human subjects

Study Design:

A double blinded*, balanced, randomized, two treatment, two period, preliminary microbiome study and assess the NAD+ assay.

*Person involved in product handling, dispensing and dosing were not involved in particular assessment which causes biasness. e.g., AE monitoring and management. Subject participating in the study were also blinded about the treatment (Test/ Placebo) to be administered.

Investigators:

Clinical Investigator: Dr. Yashvant Khaire, M.B.B.S., Diploma in Anesthesia	**Raptim Research Pvt. Ltd.,** PAP-213, PAP-A-218 and PAP-A-219 (Screening Facility) A-226 (Clinical Unit); A-242 (Biostatistical Unit); T.T.C., Industrial Area, Mahape M.I.D.C., Navi Mumbai - 400 710, India. Tel. No.: +91 22 27781889 Fax No.: +91 22 27781884
Clinical Co-Investigator: Dr. Yagnesh Tadvi, M.B.B.S. Dr. Raviraj Jagdhani, M.B.B.S, M.D (Pharmacology)	
Bioanalytical Investigator Dr. Milind Bagul, Ph.D.	
Chief Controller Data Processing Services Dr. Chandrashankar Gupta, M. Sc., Ph.D. (Statistics)	
Head of Quality Assurance: Ms. Usha Ramakrishnan, B. Pharm.	

Study Center(s): **Screening and Clinical Facility and Statistical Facility (NAD study):** Raptim Research Pvt. Ltd., PAP-A-218 and PAP-A-219 (Screening Facility) A-226 (Clinical Unit); A-242 (Biostatistical Unit); T.T.C., Industrial Area, Mahape M.I.D.C., Navi Mumbai - 400 710, India. Tel. No.: +91 22 27781889 Fax No.: +91 22 27781884	**Bioanalytical Facility (Stool Sample Analysis) and Statistical Facility (Microbe Analysis):** Decode Age, No. 7, 1, Haudin Rd, Halasuru, Yellappa Chetty Layout, Sivanchetti Gardens, Bengaluru, Karnataka 560042 Centenarians Life Sciences Pvt Ltd, No.7, 1, Haudin Rd, Yellappa Chetty Layout Sivanchetti Gardens, Halasuru, Bengaluru, Karnataka 560042

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Publications: None

Study Period:

Phase		***Initiation***	***Completion***
Screening		05/06/24	08/06/24
Clinical		08/06/24	13/09/24
Bioanalysis	Day 00	09/06/24	
	Day 96	13/09/24	
Statistical analysis		09/10/24	09/10/24

Study Objectives:

Primary Objective:

To ascertain the preliminary microbiome study of Analemma Water on human health.

To assess the NAD+ assay from whole blood pre and post treatment of Analemma Water in comparison with Placebo drinking water.

Secondary Objective:

- To assess safety of the Analemma Water in comparison with the placebo drinking water.

Methodology:

Based on pre-study examinations performed within 21 days prior to check-in and verification of compliance with the inclusion and exclusion criteria, eligible subjects were checked into the clinical pharmacology unit (CPU) and were provided Analemma Water or Placebo drinking water.

Baseline Day -1 Activities

The activities including biometric identification, obtaining study specific written informed consent (Only during baseline day 0 activities), medical and medication history, urine alcohol test, urine screen for drugs of abuse, physical examination, vital signs measurements (blood pressure, pulse rate, body temperature and respiratory rate), assessing general well-being since last visit and evaluation of inclusion and exclusion criteria were performed one-day prior (Day 0) to consumption of Analemma Water or Placebo drinking water.

Blood and Stool sample were collected from each subject for preliminary microbiome study and NAD+ assay.

Subjects were report one day prior to consumption of Analemma Water or Placebo drinking water.

Following were the study subject visit details:

Period	Day	Visit No.	Activity
Period I	Day 0	01	▪ Blood sample were collected ▪ Stool sample were collected ▪ Sufficient volume of Analemma Water or Placebo drinking water was provided to the subjects to consume daily from day 1 to day 30.

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	Day 10	02	▪ Water consumption compliance check ▪ Safety Monitoring
	Day 20	03	▪ Water consumption compliance check ▪ Safety Monitoring
	Day 31	04	▪ Water consumption compliance check ▪ Safety Monitoring ▪ Blood sample were collected ▪ Stool sample were collected. ▪ Remaining quantity of Analemma Water or Placebo drinking water was taken back from the subject.
Wash-out Period	Day 31 to Day 35		
Period II	Day 36	05	▪ Blood sample were collected ▪ Stool sample were collected ▪ Safety Monitoring ▪ Sufficient volume of Analemma Water or Placebo drinking water was provided to the subjects to consume daily from day 36 to day 96.
	Day 46	06	▪ Water consumption compliance check ▪ Safety Monitoring
	Day 56	07	▪ Water consumption compliance check ▪ Safety Monitoring
	Day 66	08	▪ Water consumption compliance check ▪ Safety Monitoring
	Day 76	09	▪ Water consumption compliance check ▪ Safety Monitoring
	Day 86	10	▪ Water consumption compliance check ▪ Safety Monitoring
	Day 96	11	▪ Water consumption compliance check ▪ Safety Monitoring ▪ Blood sample were collected ▪ Stool sample were collected. ▪ Remaining quantity of Analemma Water or Placebo drinking water was taken back from the subject. ▪ Post-study Safety Assessments

Period I: From day 1 to day 30, sufficient volume of placebo drinking water was provided to the subjects. Subject was instructed to consume minimum 1.5-liter water daily as per the randomization schedule.

Subjects were instructed to record the details (Quantity, date and time) of water consumption in the subject diary from day 01 to day 30.

Period II: From day 36 to day 96, sufficient volume of Analemma water was provided to the subjects as per the randomization schedule. Subject was instructed to consume minimum 1.5 liter water daily.

Subjects were instructed to record the details (Quantity, date and time) of water consumption in the subject diary from day 36 to day 96.

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For appropriate use of Analemma Water below mentioned Procedure was followed for appropriate use of Analemma Water.

Before you start using this product:
After receipt first checked this product for any fractures. Assured that the Water Tube was totally undamaged before every use and Never use the Water Tube.

Drinking the coherent water:
Subject instructed to use the Water Tube exclusively to produce coherent drinking water for human consumption: water of good quality that has already been filtered and purified and that is free from chemical and/or biological pollution.

All subjects were visited to facility for water consumption compliance check on Day 10, Day 20 and Day 31 during period I and on Day 36, Day 46, Day 56, Day 66, Day 76, Day 86 and Day 96 during period II. Subjects were allowed to visit the facility ± 2 days from above scheduled days.

Blood Sample Collection
A total of 4 blood samples (5.0 mL each) per participant were collected in pre-labeled vacutainers containing K_3EDTA as an anticoagulant during the study.

1. First Baseline Blood Sample was collected at Baseline day (Day 0) during period I.
2. Second Blood Sample was collected on Day 31 during period I.
3. First Baseline Blood Sample was collected at Baseline day (Day 36) during period II.

Second Blood Sample was collected on Day 96 during period II.

Total blood loss for a subject during the study did not exceed 40.0 mL for male subjects and 44.0 mL for female subjects.

Stool Sample Collection
The sample was collected and stored in a Stool DNA stabilizer solution tube by lnvitek
A total of 4 stool samples per participant were collected during the study.

1. First Baseline Sample was collected at Baseline day (Day 0) during period I.
2. Second Sample was collected on Day 31 during period I.
3. First Baseline Sample was collected at Baseline day (Day 36) during period II.
4. Second Sample was collected on Day 96 during period II.

For Women
Collected before or after your menstrual period to prevent potential contamination.

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After Returning to Normal Routine
If any of the above conditions apply, wait until your gut microbiome stabilizes post-recovery or post-exposure. Then you can proceed with the test.

Preventing Sample Contamination
Before collecting the stool sample, subjects empty their bladder. Urine or blood In the stool sample can lead to inaccurate test results.

Stool sample was shipped at 2°C to 8°C to Centenarians Life Sciences Pvt Ltd. for Microbe Analysis.
• After registration, we'll schedule a pickup.
• Place the tube in the test box, put it in the return cover and seal it.

Metagenomic sample processing at Decode Age laboratory:

The human microbiome was tested here using Shotgun metagenomic sequencing. The samples and DNA were stored in -20°C to ensure no more microorganism growth in the samples.

Methodology
Processing of Collected Stool Sample
Detailed protocol for processing collected stool samples, including any extraction, preservation, or preparation steps

Sample receiving: When the samples were received in the lab, its weight, colour was noted along with date and time of receiving. If there is any sign of contamination or leak and less quantity, it was rejected.

DNA extraction: DNA extracted using Qiagen Fast Stool Mini kit was used to extract DNA according to the manufacturer's protocol.

Quality analysis: Nanodrop, qubit and gel electrophoresis was conducted on the eluted DNA using standard procedure.

Library preparation: Sample library was prepared using Native Barcoding Kit 96 V14 by attaching unique barcodes and pooling into batches.

Nanopore sequencing: The DNA library was loaded onto nanopore flowcell (v: R10.4.1).

The stool sample processing here in the lab was divided into two major steps. First step was DNA extraction where the stool sample was processed aseptically through Qiagen Fast stool mini kit. The steps were followed as manufacturer’s instructions given in the kit protocol. The quality of the DNA was checked with nanodrop reading using standard Nanodrop, qubit and ratios of 260/280 (1.7 to 2.0) and 260/230 (2.0-2.2) were observed to ensure good quality DNA goes for sequencing.

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Once we have a good quality DNA per sample, it was then processed for the library preparation step for long read sequencing. The standard steps involved in this process were DNA end preparation, barcode ligation and library pooling. The samples were labelled with a unique barcode to ensure that it was identified during analysis when mixed more samples together during sample pooling. The prepared and pooled library then was loaded onto the flow cell for sequencing. The most common run time for a sequencing machine was around 12 hours depending upon the number of samples and data needed per sample.

Stool samples were stored in a -20 deep freezer after processing.
The data was transferred to the bioinformatics department for further analysis.

Blood Sample Processing

Collected whole blood sample were divided into three aliquots as mentioned below into pre-labeled polypropylene tubes, as early as possible and were stored in the deep freezer maintained at -2°C to 8°C immediately after sample collection:
Aliquot 1: 2.0 mL of whole blood sample was transferred into aliquot 1.

Aliquot 2 (Analytical): 1.5 mL of whole blood sample was transferred into aliquot 2.
Aliquot 3 (Replicate): 1.5 mL of whole blood sample was transferred into aliquot 3.
Transfer of Aliquots:
Aliquot 1 was shipped at 2°C to 8°C to the Pathology Lab of Raptim Research Pvt. Ltd. (For RBC count)
Aliquot 2 & 3 was shipped at 2°C to 8°C to the bioanalytical facility of Raptim Research Pvt. Ltd. (A-242). (NAD+ assay)
Note:

- Aliquot 2 & 3 were transferred on the same day as collection for fresh analysis to the bioanalytical facility. In case fresh analysis was not possible on the same day, it was not postponed beyond 1 day.

RBC count provided prior to sample transfer to the bioanalytical facility.

Number of subjects planned and analyzed:

A total of 10 normal, healthy, adult human subjects were planned and enrolled in the study. Out of these, 09 subjects completed the study.
Subject No.03 withdraw from the study due to personal reason from period I.
Samples of 08 subjects were considered for Test Product and Samples of 09 subjects were considered for Placebo of NAD+ study.
Samples of 09 subjects were considered for Microbiome Analysis

Main Inclusion Criteria:

Normal, healthy, adult, human subjects, 25 to 40 years (both inclusive) of age with a body mass index (BMI) in the range of 18.50 to 29.99 kg/m^2 (both inclusive).

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Identity of Investigational Products (IPs):

Parameter	Test Product (A)	Placebo (B)
Product name	Analemma Water (As per protocol)	Placebo drinking water
Manufactured by	Water and Light Applications India Private Limited (As per protocol)	Water and Light Applications India Private Limited (As per protocol)

Study Duration:

The duration of the subject participation was 98 days including washout period of 05 days between consecutive dosing.

Statistical Analysis (NAD+ study):

Applied the Change from baseline approach on the respective parameters data and presented accordingly.

Appropriate statistical test were performed on all dependent variables data (i.e., test parameters data) to test significance among the before and after consumption of the coherent water.
P values greater than 0.05 were considered statistically non-significant.

Safety Analysis Criteria:

Safety and tolerability was assessed in terms of adverse events (AEs), serious adverse event (SAE) if any, or any illness requiring administration of other medication(s) during the study, vital signs and laboratory assessments were performed during the entire course of the study. Adverse events were evaluated based on frequency, severity grades, causality and outcome.

Results of Total NAD assessment based on whole blood concentration data of Analemma water are summarized below in Table 2.1

Table 2.1: Mean Summary table of Analemma Water

Mean ±SD (CV %)			
Placebo (C)		Test Product (D)	
Day 0 (N=10)	Day 31 (N=09)	Day 36 (N=09)	Day 96 (N=08)
3044.49 ± 463.27 (15.22)	2559.30 ± 1843.43 (72.03)	8372.76 ± 1026.64 (12.26)	10033.75 ± 1956.77 (19.50)

N- Number of evaluated subjects;

For checking normality we used Shapiro-Wilk Test:

1. **Period 1 (Day 0 vs. Day 31)**:

   - Day 0 concentration data (baseline) follows a normal distribution ($p = 0.8777$).
   - Day 31 concentration data does not follow a normal distribution ($p < 0.0001$).
   - As a result, the baseline-corrected concentration data for the placebo (Day 31 - Day 0) also does not follow a normal distribution ($p < 0.0001$).

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2. **Period 2 (Day 36 vs. Day 96)**:

   - Both Day 36 (baseline) and Day 96 concentration data follow a normal distribution (p = 0.1116 and p = 0.4408, respectively).
   - Therefore, the baseline-corrected concentration data for the test water (Day 96 - Day 36) follows a normal distribution (p = 0.5120).

For Period 1 (Day 0 vs. Day 31) data does not follow normal distribution. So, we used Wilcoxon signed-rank test and For Period 2 (Day 36 vs. Day 96) data follow normal distribution. So, we used Paired T-test and the same results mentioned below:

1. **Placebo (Day 31 - Day 0)**:

   - **Wilcoxon signed-rank test**: p = 0.1289 (non-significant).
   - This indicates no significant difference between baseline and post-treatment concentration levels for the placebo.

2. **Test Water (Day 96 - Day 36)**:

   - **Paired T-test**: p = 0.0200 (significant).
   - This suggests a significant improvement or change in NAD+ concentration after consuming Analemma Water compared to the baseline.

For Comparison of Placebo (Day 31 - Day 0) and Test Water (Day 96 - Day 36), we used Wilcoxon Rank-Sum Test.

Comparison Between Treatments:

- **Wilcoxon Rank-Sum Test** (non-parametric test for two independent samples):
  - p = 0.0071 (significant).
  - This indicates a statistically significant difference between the baseline-corrected concentrations of the test water (Analemma Water) and placebo. The test water showed a greater effect than the placebo.

Safety Results:

There were no SAEs reported during the study. Overall, 02 AEs were reported during the study. Out of which, no AE was reported during the study periods and all the 02 AEs were reported during post-study safety assessments. Out of 02 AEs 01 was mild and 01 was moderate in intensity. Both the AEs were resolved completely.

Overall Conclusion:

- Analemma Water showed a significant improvement in NAD+ concentration from baseline to post-treatment (p = 0.0200), whereas the placebo showed no significant change (p = 0.1289). This suggests that Analemma Water may have a positive effect on NAD+ levels.

- The significant difference between Analemma Water and placebo (p = 0.0071) further strengthens the evidence that Analemma Water has a favorable impact compared to

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placebo drinking water.

This study provides preliminary evidence that Analemma Water may positively affect NAD+ levels.

There were no SAEs observed during the study and no unresolved AEs with either the test product or the placebo. The water treated with test product was well tolerated during the study and was found safe for consumption**.**