To Chew or Not to Chew?: Assessing the Differences Between Chewing and Swallowing Arugula Extract Capsules on Plasma Nitrate Levels, Blood Pressure, and Heart Rate

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Synopsis
(optional)

Title	To Chew or Not to Chew?: Assessing the Differences Between Chewing and Swallowing Arugula Extract Capsules on Plasma Nitrate Levels, Blood Pressure, and Heart Rate
Study Duration	3/1/2023 – 6/31/2024
Study location(s)	HHPR Research Labs
Objectives	Aim 1: Determine the efficacy of chewing an arugula extract capsule compared to swallowing. Aim 2: Examine levels of nitrate, nitrite and downstream markers of NO breakdown when swallowing or chewing arugula extract capsules.
Number of Subjects	12
Main Inclusion/Exclusion Criteria	The goal is to recruit 12 apparently healthy males currently not taking any medications and with no underlying medical conditions.

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1.0 BACKGROUND AND RATIONALE

Current literature has demonstrated positive effects of dietary nitrates (NO_3) on the regulation of the endothelium's dilatory tone, cell growth, and vascular homeostasis which are largely influenced by the presence or production of nitric oxide (NO)(1, 2). NO is synthesized from the amino acid l-arginine by the enzyme endothelial nitric oxide synthase (eNOS) (3, 4). However, in patients with cardiometabolic diseases (CMD's) like chronic kidney disease (CKD), eNOS activity could very well be blunted by various factors (e.g., inflammation, cytokines, eNOS inhibitors) stemming from CKD (1, 5). As noted by Ma et al. (6), when conditions of hypoxia and ischemia are present, eNOS activity is down regulated. Thereby limiting the presence and production of NO in the endothelium due to halted synthesis of l-arginine to NO (6). Though NO production may be limited from diminished eNOS activity, the need for NO is still of utmost importance. NO can still be provided to the endothelium through non-dependent enzymatic processes such as the conversion of dietary nitrate (NO^3) to NO (**Figure 1**) (7, 8). Dietary NO^3's are degraded by salivary NO^3-reducing bacteria (e.g., *Veillonella* and *Rothia* species) into nitrites (NO^2). Thereafter NO^2's have been ingested, they meet with gastric acid to form nitrous oxide (HNO^2) and are further degraded into NO (7, 8). The necessary production of NO can now be met and is provided through the consumption of dietary NO^3's.

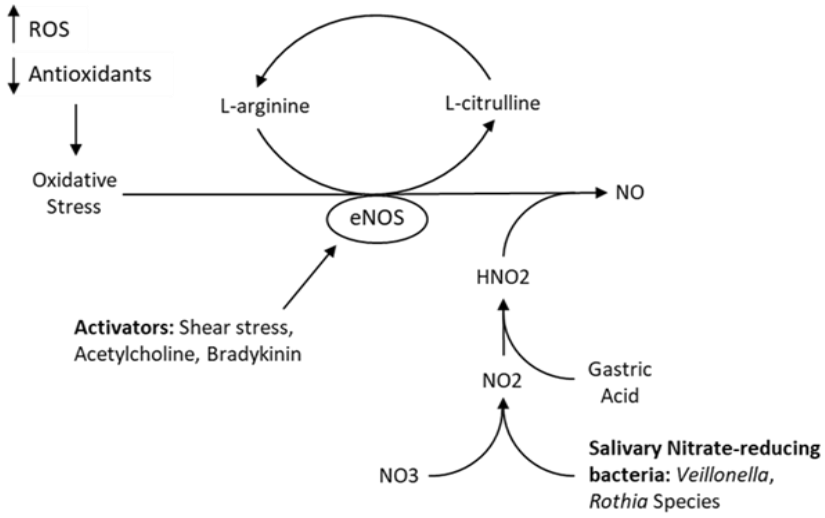


Figure 1. NO production from L-arginine, L-citrulline, and non-dependent enzymatic processes.

Nitrate-rich vegetables (e.g., beetroot, spinach) have commonly been studied for their effects on cardiovascular health benefits (9–11). Current literature suggests that diets high in nitrate (NO^3) content (e.g., Mediterranean diet, dietary approach to stop hypertension [DASH]) tend to see improved cardiovascular health by lowering blood pressure and preventing declines in endothelial function (e.g., flow-mediated dilation [FMD], pulse wave velocity [PWV], shear rate [SR]) seen after an ischemic stimulus when measuring brachial artery FMD (9, 12–15). While these studies have shown promising results toward cardiovascular health, there appears to be one cruciferous vegetable that has gone unnoticed. Eruca Vesicaria, otherwise known as Arugula, has been reported to have the highest known nitrate contents of all cruciferous vegetables and lacks adequate research on its effectiveness of mitigating deteriorations in vascular health (16–18).

Previous literature has shown the benefits of beetroot, as well as spinach, on endothelium function due to their high levels of nitrates (9). In a recently published meta-analysis, Jackson et al. (11) found that oral consumption of inorganic nitrate and nitrite was significantly associated

with improved vascular and endothelial function. Likewise, Bahrami et al. (10) conducted a meta-regression of randomized controlled trials on beetroot supplementation and found supplementing with beetroot significantly decreased arterial stiffness (e.g., PWV) and increased endothelial function (e.g., FMD). Not only that, Bahrami et al. (10) determined beetroot supplementation as a potentially safe method for improving endothelial function. Thus, if beetroot supplementation has shown positive efficacy and results towards the improvement of endothelial function and inflammation, then perhaps Arugula, which has a substantially higher nitrate content [Arugula: >5000mg/kg fresh weight (FW); Beetroot: 200-1000mg/kg FW], will provide parallel or better results (19, 20).

From that perspective, it may seem likely that arugula extract in capsules would produce comparable results. However, if capsules are swallowed and there are no NO_3-reducing bacteria that will aid in the degradation process prior to reaching gastric acid, then it may not yield the same results had an individual chewed the capsule. This would allow the extract to meet NO_3-reducing bacteria in saliva and undergo the breakdown process prior to reaching the stomach lining. In turn, this may provide more NO to the endothelium compared to swallowing the capsule. Thus, the purpose of this pilot study is to 1) identify if arugula extract in capsules will increase plasma nitrate/nitrite levels, 2) assess the differences seen between swallowing and chewing the capsule prior to ingestion, and 3) examine the effects on downstream markers indicative of NO production (e.g., 3-Nitrotyrisine). While future studies in our laboratory will examine the endothelial response using ultra-sound measurements (e.g., FMD, SR, PWV) from the consumption of arugula extract. The implication of these findings may identify newer realms that have yet to be explored in arugula extract supplementation and could potentially be relayed into numerous avenues such as clinical, athletic, and even general populations.

2.0 STUDY OBJECTIVES

Aim 1: Determine the efficacy of chewing an arugula extract capsule compared to swallowing in healthy individuals.

Aim 2: Examine levels of nitrate, nitrite and downstream markers of NO breakdown when swallowing or chewing arugula extract capsules.

3.0 SUBJECT SELECTION & RECRUITMENT

3.1 INCLUSION CRITERIA

- Males between the ages of 18 and 50
- Non-smoker and do not use tobacco of any kind
- No previously diagnosed or known cardiometabolic diseases
- Considered apparently healthy according to guidelines established by the Amercian College of Sports Medicine

3.2 EXCLUSION CRITERIA

- Females
- Previously or currently diagnosed as having a cardiometablic or chronic disease

- Diagnosed as having hypertension (SBP>120mmHg; DBP>80mmHg)
- Currently taking any prescription that may affect blood pressure, heart rate, or mental state
- Currently taking any prescription stimulants or anti-depressants

4.0 RESEARCH DESIGN & METHODS

The goal is to recruit 12 male participants who are considered apparently healthy. Potential participants will be recruited by phone, mail, and physician referral. Eligible individuals will be provided both verbal and written information regarding the research study and will have the opportunity to ask questions pertaining to the research study. Participants will sign and return the informed consent document and will undergo further health screening before admittance into the study.

This randomized, single-blind, cross-over designed study will begin by having an initial orientation where the study details and scheduling will be discussed with the participants, along with the appropriate informed consent documents being explained thoroughly and signed by both parties. The initial orientation will include and be done in the following order: 1) assessing appropriate inclusion criteria for study, 2) explaining study details with proper authorization from both the researcher and participant, 3) showing participant the lab rooms and where the study will be conducted, and 4) a whole body dual-energy x-ray absorptiometry (DEXA) scan for body composition classifications. Following the orientation, participants will be instructed to maintain the same eating and exercise habits throughout the duration of the study.

On the subsequent visit, participants will arrive at the laboratory at 07:00 hours, having fasted for 8 hours and refrained from exercise for 24 hours. Participants will be randomized and counterbalanced for all three conditions (e.g., swallow, chew, nothing). At the beginning of each condition, participants will have a venous catheter inserted into the most prominent vein in the antecubital space of the lower arm. Participants will then be asked to either do nothing, swallow the arugula extract capsule (2 capsules), or chew the arugula extract capsule (2 capsules) until the contents are dispersed into their mouth (at which point the chewing will continue for 15 total seconds) and then swallowed. Blood sampling will occur at 4 time points for each condition (e.g., prior to ingestion, 1-hour, 2-hours, and 3-hours proceeding ingestion). **See Study Protocol in Table 1.** A wash-out period of approximately 72-hours will be implemented in between each session. Along with blood sampling, blood pressure will be measured at each time point and heart rate will be monitored using a standardized heart rate monitor (Polar Electro, Kempele, Finland).

Arugula extract:
The arugula extract will be obtained from Rucola Health. Rucola Health will send each canister needed with 60 capsules and each capsule is packaged by the manufacturer with 500mg. The primary investigators (Dr. Forsse and Tomas Chapman-Lopez) will remove two capsules each visit and give the capsules to the research paritipant with detailed instructions on how to consume the capsule [based on the assigned condition (visits 2, 3, & 4)].

Sample size determination:
Based on an a-priori power analysis with an effect size of 0.80, an alpha level of 0.05, and power (1-B) set at 0.80 we determined the sample size needed was a minimum of 8 participants (G*Power 3.1.9.7, Universität Kiel, Germany). However, we expect a participant attrition rate of roughly 20-25%. Therefore, to ensure adequate statistical power is met, we plan to recruit 12 total participants.

Sample Collection:
Blood samples will be obtained on day one before beginning the randomized conditions. A wash out period of 72-hours will be required in-between condition changes. Serum samples (1 tube per time point) will be allowed to clot for 30 minutes at room temperature, whereas plasma EDTA (1 tubes per time point) samples will be stored on ice for 30 minutes, followed by centrifugation at 3330 RPM for 10 minutes. All blood samples will be aliquoted into 2 mL micro test-tubes and stored in an ultra-low temperature (ULT) freezer at -80°C until all data is collected and ready to be analyzed.

5.0 STUDY ACTIVITIES

Health History Review:
Participants will review personal health and physical activity history and current health status with a PI/co-PI. This process will be used to identify any characteristic or condition that may preclude them from participating in the study.

Body Stature and Composition:
We will measure the participant's height by stadiometer and weight by digital scale. We will measure waist girth using a cloth tape. Waist girth will be measured at the level of the umbilicus with the participant standing with feet shoulder-width apart, weight distributed equally, and after a normal exhalation. Participants will undergo a single body composition assessment using dual x-ray absorptiometry (DXA). The participant lies still on a padded table while an x-ray emission arm moves above their body. DXA utilizes low-level x-ray beams at two different energy levels to assess tissue densities. When the energy absorption of soft tissue (soft tissue includes muscles, tendons, ligaments, fascia, nerves, fibrous tissues, fat, blood, and blood vessels) is accounted for, we can determine bone density. Differences between the dual x-ray energy absorbances may also be used to differentiate between fat and lean soft tissues. Participants will be exposed to low-dose radiation. The amount of radiation is many times less than what you would get during many routine medical exams, such as a chest x-ray.

Blood Sampling:
Prior to each experimental condition (2-4), the following standardization protocols will be adhered to for all research participants. After two (2) days on a standardized diet and a 6- to 8-hr fast pariticipants will arrive at the lab to complete whichever randomized condition they have for that day. Participants will have a venous catheter inserted into the most prominent vein site in the antecubital space of the lower arm. A blood sample, equal to about 1.08 tbsp (16) mL, will be taken after insertion of venous catheter and then sampled every hour for a total of 3 hours (e.g., baseline, 1-hour, 2-hours, 3-hours post-ingestion) using 10mL red top serum collection tubes and 6mL plasma EDTA tubes, through a standardized vacutainer apparatus. The Teflon catheter will

be maintained patent by introducing 0.5 mL of 10 USP/mL sodium-heparin flush after each sample is taken. We will obtain a total of four (4) smaple time points for each experimental condition. One (1) 10mL red top tube and one (1) 6mL plasma tube will be taken at each sampling time point. Each condition (visits 2-4) will have a total blood draw volume of 64mL, while the initial visit will be a total blood draw volume of 16mL.

Biochemical Analysis:
Whole blood samples will be sent to a CLIA-certified lab for complete blood count, and lipid profiles as the data is collected. Biomarkers, 3-nitrotyrosine and nitrates/nitrites will be assessed via commercially- available single analyte ELISAs and colorimetric assays. Biochemical analysis will be conducted upon completion of human participant data collection.

Visit 1	Visit 2	Visit 3	Visit 4
Conditions Will be randomized	A	B	C
Assessment Informed Consent Protocols	Swallow Arugula Extract Capsules 1000mg	Chew Arugula Extract Capsules 1000mg	Nothing
(1) Blood Draw	(4) Blood Draws (e.g., baseline, 1-HR, 2-HR, 3-HR post-consumption)	(4) Blood Draws (e.g., baseline, 1-HR, 2-HR, 3-HR post-consumption)	(4) Blood Draws (e.g., baseline, 1-HR, 2-HR, 3-HR post-consumption)
Blood Pressure DEXA	(4) Blood Pressure Heart Rate Monitor	(4) Blood Pressure Heart Rate Monitor	(4) Blood Pressure Heart Rate Monitor
A minimum of 72-hours is required between all conditions that will serve as a wash out period. The expected amount of time to complete the study is 3 weeks.			

Table 1. Study Protocol

6.0 RISKS & BENEFITS

1. The primary risk associated with dual-energy x-ray absorptiometry (DXA) is with radiation exposure. The radiation dose for one DXA scan to measure body composition is 1.5 millirem. This is similar to the amount of natural background radiation you would receive in one month while living in Waco, TX, or less than what you would be exposed to during an airplane flight from New York City to Los Angeles (2 to 5 millirem). The maximal permissible x-ray dose for non-occupational exposure is 500 millirem per year.
2. Blood sampling may impose minor bruising, swelling, and itching of the affected area. As with any similar procedure that disrupts the skin barrier, there is an increased risk of

infection.

3. Ultrasound assessment may be uncomfortable due to the blood pressure cuff being inflated for 5 minutes. We will adhere to the standard FMD protocols that are widely accepted in the research and medical community.

Methods to limit risks:

1. Our DXA equipment is checked by Baylor University's Risk Management and Safety regularly to ensure radiation exposure remains at very low levels. In order to keep your exposure to a minimum, we will calibrate our DXA equipment before participants arrive for their DXA assessment. Participants will undergo only one DXA scan.

2. Universal precautions will be observed for each blood sample and the following procedures will be used to help minimize any potential risks to the participant and investigator: the technician will use surgical non-latex gloves; the antecubital area will be cleansed with an alcohol pad prior to puncturing the skin; all blood draw equipment and instruments will be sterile. All punctured sites will be properly dressed with antiseptic and bandage following each sample collection. Dr. Forsse will oversee all phlebotomy procedures throughout the experimental blood sampling.

3. The tolerance for the blood flow occlusion procedure improves with practice. We will review all procedures, including cuff placement and inflation pressure, with participants prior to obtaining ultrasound measurements of their brachial artery.

Compensation:

Individuals who choose to participate in the research study will not be compensated for their participation.

7.0 STATISTICAL ANALYSIS

Based on an a-priori power analysis with an effect size of 0.80, an alpha level of 0.05, and power (1-B) set at 0.80 we determined the sample size needed was a minimum of 8 participants (G*Power 3.1.9.7, Universität Kiel, Germany). However, we expect a participant attrition rate of roughly 20-25%. Therefore, to ensure adequate statistical power is met, we plan to recruit 12 total participants. All statistical analyses will be done using IBM SPSS 29.0 (IBM, Armonk, NY, USA). A repeated measures 2x3 ANOVA will be employed to assess the differences between each of the three treatment conditions. If there are any significant differences seen between treatments, Tukey's post-hoc test will be used to compare the means of the treatments. Level of significance will be set at $\alpha = 0.05$.

8.0 DATA SECURITY & PRIVACY/CONFIDENTIALITY

All data collection forms for a particular participant will be kept in a single folder during the assessment. The folder will be in the possession of the investigators that are managing the assessments. Investigators involved with data collection will not discuss a participant's information with any other person without the express consent of the participant and in the

presence of the participant. After the assessment, each file folder with participant data will be kept in a locked filing cabinet in Dr. Forsse's office (MMG 110.09) until an individualized report has been completed. Participant reports will be sent to the individual in a sealed envelope via U.S. mail. After the report has been sent, participant data will be scanned and stored in electronic format on a jump drive stored in Dr. Forsse's office (MMG 110.09).

9.0 DATA & SAFETY MONITORING

Data will be obtained by hard copy and on lab computers. Data collected from lab computers will be downloaded to a jump drive after completing all assessments in a particular session and then deleted from the computer's hard drive. Data recorded on data collection forms, included in this protocol package, will be maintained in separate files for each participant. The data will be used only to provide the individual with their health report. We will ask the participants, as part of the consent process, to maintain their files indefinitely in electronic format to verify database fidelity. We will destroy all hard copy files of participant data immediately after scanning forms into electronic format and generating individual reports. Hard copy files will be destroyed by a paper shredder.

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APPENDICES

- DATA COLLECTION FORMS
- RECRUITMENT FLYER