Investigating the Effects of Monarda Didyma L. Extract Supplementation on Biological Aging of Susceptible Population: Protocol of a Randomized, Placebo- Controlled, Double-blind Parallel Clinical Trial

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Study protocol

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Abstract

Background

Manipulations to decelerate biological aging and extend health span are major challenges for health given the social and healthcare costs of aging population. Excessive oxidative stress and inflammation are primary drivers in biological aging and age-related diseases. Didymin, a natural bioactive flavonoid and the major polyphenol of *Monarda didyma* L., has showed a crucial anti-inflammatory role. This clinical study aims to investigate the potential benefits of a 12-weeks supplementation of *Monarda didyma* L. extract on reducing / delaying the biological aging of a susceptible population of aging workers.

Methods

This is a randomized, placebo-controlled, double-blind, parallel design and monocentric clinical trial. Study population will comprise 80 participants, aged 45–65 years, randomly allocated to both Intervention group (supplementation of *Monarda didyma* L. extract) and placebo control group (placebo supplementation) for a 12-weeks period of treatment. All study participants, who will be enrolled at the Occupational Medicine Unit – Azienda Ospedale Università of Padova providing a written informed consent, will undergo clinical examination, an interview with structured questionnaires (demographic data, lifestyle, information quality of life and sleeping patterns) and collection of fasting blood for evaluation of indicators of biological aging as primary outcomes (DNA methylation age, telomere length, mitochondrial DNA copy number), as well as secondary outcomes including basic biochemistry and inflammation markers (emocrome, glycemia, insulin, total cholesterol, low and high density lipoproteins, triglycerides, creatinine, telomerase expression, C-reactive protein, interleukin6, aspartate transaminase, alanine transaminase, gamma-glutamyl transferase) and salivary sample for cortisol analysis. Clinical examination and sample collection, to assess primary and secondary outcomes, will be performed at enrollment and at the follow up after of this treatment. Tracking of additional parameters (heart rate, sleep, mobility) will be performed along the study period by using a wearable MiBand 7 watch and a daily diary to determine compliance and record effects on stress, well-being and health status.

Discussion

The success of this research could represent a turning point in the healthy aging research, leading to rejuvenation by means of a sustainable and safe intervention, accessible to everybody in order to extend health span.

Trial registration:

ClinicalTrials.gov PRS (NCT05399966), registration date: May 17, 2022.

1. Background

Manipulations to slow down biological aging and extend health span are major challenges for health, nutrition and quality of life, given the social and healthcare costs of aging population. Although aging is an individual, natural, inexorable and biologically complex process, people do not age at the same rate (1). Excessive oxidative stress exposures and altered inflammation responses are related to biological aging and involved in the pathogenesis of age-related diseases (2).

Our body has a biological aging clock in epigenetic signature and telomeres. A powerful marker of non-mitotic cellular aging in humans is the epigenetic age, also defined as DNA methylation age (DNAmAge). DNAmAge is assessed from methylation at a species-specific subset of cytosine–guanine dyads (CpG), and it is strongly correlated with chronological age. Development of epigenetic predictors has addressed to an “chronological epigenetic clock” theory of aging, according to which the difference between DNAmAge and age is defined as “age acceleration” (AgeAcc), which is indicative of altered biological functions and elevated risk for morbidity and mortality (3, 4). Furthermore, Leukocyte telomere length (LTL) is an estimator of mitotic cellular aging. Telomeres act as a mitotic clock that, by reducing itself at each cell division, leads to cellular senescence (replicative senescence) or cell death (4). Aging also appears to affect mitochondria. Mitochondria, which have diminished protective histones and DNA repair capacity compared with nDNA, are highly prone to be altered, and compensate by replicating their mtDNA molecules and increasing their cellular mtDNA copy number (mtDNAcn) (5, 6). Even if molecular/epigenetic alterations and mtDNA dysfunction are suspected driving forces in cellular aging, these pathways have rarely been investigated together.

Our previous studies have demonstrated that 60 days of intensive relaxing training reverses the biological aging, together with improvement of clinical blood parameters (i.e., stress hormones, inflammatory markers) and endothelial function, in patients after myocardial infarction, and even more in healthy subjects. These studies suggest that youthful genetic and epigenetic information can be recovered, and indicators of biological aging examined may represent an accurate tool to measure the effectiveness of lifestyle-based interventions in the prevention of age-related diseases (3, 4). These outcomes also provide a strong indication that significant changes in biological aging markers, such as DNAmAge and LTL, can be discerned within a relatively short timeframe.

Didymin (DID) is a natural compound and the major polyphenol of Monarda didyma L., a herb of the mint family (Lamiaceae). DID is an orally bioactive flavonoid glycoside commonly found in several citrus fruits, such as oranges, lemons, grapefruits, and mandarins (7). In vitro studies demonstrated anti-oxidative and anti-inflammatory activities (7–9), antidiabetic and cardiovascular-protective properties (7, 8, 10), and neuroprotective effects (7, 11) as well as the anti-tumor efficacy and therapeutic effects of DID (8, 12, 13). In vivo studies showed also anticancer proprieties of DID in mice (12), and revealed its anxiolytic-like effect (14), and its role in mitigating hepatic fibrosis in rats (15). Furthermore, a

randomized, placebo-controlled, double-blind, crossover clinical trial showed that consumption of DID-rich orange juice decreases inflammatory biomarkers (16) and plasma concentration of endothelial biomarkers (17) in overweight and obese subjects, decreasing cardiovascular risk. Therefore, since inflammation and the consequent oxidative stress are distinctive signs of biological aging and since DID has an anti-inflammatory role, a possible role in slowing down aging is suggested.

This research aims to explore the potential benefits of supplementation of the natural ingredient, the *Monarda didyma* L. extract, to reduce / delay the biological aging of a susceptible population by a randomized, placebo-controlled, double-blind, parallel design, monocentric clinical trial. Biological aging will be detected by DNA methylation age, telomere length and mitochondrial DNA copy number for each participant at the beginning and at the end of this treatment period in order to catch a potential deceleration of biological aging.

2. METHODS

2.1 GENERAL AIM OF THE STUDY

The general aim of the study is to investigate the effect of a 12-week (84 days) supplementation of *Monarda didyma* L. extract on reducing / delaying the biological aging of a susceptible population of health aging workers.

2.2 SPECIFIC AIMS OF THE STUDY

The specific aims of the study are as follows:

- To comprehensively evaluate primary outcomes, including DNAmAge, LTL, and mtDNAcn, as well as secondary outcomes such as basic biochemistry and inflammation markers, among participants receiving *Monarda Didyma* L. extract supplementation.
- To compare the effects of *Monarda Didyma* L. extract supplementation with a placebo control group to determine its potential impact on primary and secondary outcomes.
- To investigate whether *Monarda Didyma* L. extract supplementation influences additional parameters such as heart rate, sleep patterns, mobility, stress levels, and general health status, as monitored using wearable Miband7 watches, questionnaires and daily diaries.

2.3 STUDY TYPE

Randomized, placebo-controlled, double-blind, parallel design, monocentric clinical trial.

2.4 STUDY POPULATION

Study population will comprise a total of 80 participants, aged 45–65 years.

A group of N = 40 subjects will be randomly allocated to Intervention group (with supplementation of *Monarda didyma* L. extract), while a group of N = 40 subjects will be randomly allocated to placebo-only control group (with placebo supplementation). Non-active controls will facilitate understanding of appropriateness of the intervention under consideration.

Inclusion criteria:

- Subject is able and willing to follow the study protocol procedures to sign the Informed Consent Form prior to screening evaluations
- Age: 45–65

Exclusion criteria:

Relevant history or presence of any medical disorder potentially interfering with this study (heavy depression, diabetes, active cancer, severe liver disease, heavy cardiovascular disease (e.g., stroke, heart attack)); Chronic intake of medication/dietary supplements with impact on stress levels (psychologically and physiological); Consumption of any dietary/nutritional supplements or functional foods; Smokers; Gastrointestinal diseases/conditions (colitisulcerosa, Crohn'sdisease, pepticulcres, celiacdisease); Drug-, alcohol- and medication abuses; Known HIV-infection ; Known acute or chronic hepatitis B and C infection; Known allergies against the mint family (labiate), especially lemon balm (cedronella; melissa, citronella); Pregnancy, breast feeding or intention to become pregnant during the study; Participation in another clinical study within the last 4 weeks and concurrent participation in another clinical study; Blood donation within 4 weeks prior to study start (screening) or during study; Anticipating any planned changes in lifestyle for the duration of the study; Subjects considered inappropriate for the study by investigators, including subjects who are unable or unwilling to show compliance with the protocol.

2.5 STUDY DESIGN

All study participants will be employed by the University of Padua and will include in the research during their routine health assessments conducted at the Occupational Medicine Unit - Azienda Ospedale Università di Padova (AOUP). Eligible subjects, who satisfy the inclusion criteria and do not present any exclusion criteria, will receive the *Information form for study participation*, the *Informed Consent Form* (ICF) and the *Personal Data Processing Form to be signed (the Italian version is reported in Supplementary material)*. The informed consent form (ICF) will be written in accordance with established criteria of the Institutional Review Board (IRB) and the appropriate federal regulations to describe the study plan, procedures, and risks.

All participants will be informed of the purpose of the study by the personal staff during the recruitment at the Occupational Medicine Unit – Azienda Ospedale Università di Padova (AOUP). After collection, informed consent form will be kept by members of the coordinating Centre and steering committee, at the Department of Cardiac, Thoracic, Vascular Sciences and Public Health - University of Padua (Italy).

Enrolment

Enrolment and assignment of the study population will place at the Occupational Medicine Unit – Azienda Ospedale Università di Padova (AOUP). ICFs will be collected by the personal staff during the recruitment and then stored by members of the coordinating centre and steering committee.

Eligible subjects for study participation will be randomized in two different groups at day of enrolment (T0):

- Intervention group with nutritional supplementation of *Monarda didyma* L. extract

Monarda didyma L. extract; 100 mg/day. 1 capsules/day; daily (1 capsule with the lunch) and plenty of water.

- and placebo-only control group with placebo supplementation

Maltodextrin; 100 mg/day. 1capsules/day; daily (1 capsule with the lunch) and plenty of water.

The *Monarda didyma* L. extract and placebo will be provided in capsules (Hydroxypropylmethylcellulose) with the same shape and appearance to allow double blind performance. Capsules will be provided in plastic flasks (pill box) with 60 capsules per flask. Each flask will be consecutively numbered for each participant according to the randomization schedule. Each participant will be assigned an order number and receive the capsules in the corresponding flask.

The coordinating centre and steering committee will generate the two comparison groups using simple randomization, with an equal allocation ratio, by referring to a table of random numbers. Subjects with the same age, gender, education, to avoid possible confounding variables, will be called for enrolment in groups of 10. Blocking ensures that the numbers of participants to be assigned to each of the comparison groups will be balanced within paired blocks of five in one group and five in the other for every 10 entered participants. The block size may be randomly varied to reduce the likelihood of foreknowledge of intervention assignment. The allocation sequence will be concealed from the personal staff enrolling and assessing participants in sequentially numbered, opaque, sealed and stapled envelopes. Aluminium foil inside the envelope will be used to render the envelope impermeable to intense light. To prevent subversion of the allocation sequence, the name and date of birth of the participant will be written on the envelope. Corresponding envelopes will be opened only after the enrolled participants completed all baseline assessments and it will be time to allocate the intervention.

Screening

All study participants, who will provide a written informed consent, will undergo clinical examination, an interview with structured questionnaires administered by trained interviewers, and collection of fasting blood [for laboratory tests including biological age analyses (primary outcomes), basic biochemistry and inflammation markers (secondary outcomes)] and salivary sample for cortisol analysis. Clinical examination and sample collection, to assess all primary and secondary outcomes, will be performed at

enrollment (T0) and at the follow up (T1) after 12 weeks (84 days) of treatment with dietary supplementation (*Monarda didyma* L. extract or placebo capsules).

An overview of clinical examinations, questionnaires administration, samples collection and analyses is reported in the Gantt chart (Fig. 1).

Tracking of physical parameters (e.g., heart rate (HR), sleep, mobility) will be carried out during the study period (from baseline (T0) to Follow up (T1)) by using Miband 7 watch wearable devices.

The participants will also compile a diary throughout the time course of the study to determine compliance and to further monitor participant well-being, health status, stress levels and treatment effects.

To further implement data collection and management, an ad hoc data collection form (database) will be created as excel file at the enrolment (T0).

Except for the coordinating center and steering committee, other personal staff and investigators will be kept blind to intervention assignment of the participants. The trial adhered to established procedures to maintain separation between personal staff that collect biological samples, clinical parameters and information by questionnaires during medical examination at enrolment (T0) and follow up (T1), and personal staff that take outcome measurements.

Criteria for discontinuing or modifying allocated interventions for a given trial participant

- Adverse events* onset
- Tolerability evaluation.

*Assessment of an adverse event (AE) involves: subjective reports from the subject, observation by members of the research team and clinically significant abnormal laboratory results.

The following information will be collected for all AEs: Date/time of onset; description of the AE; severity; relation to study product; action taken with the study product; outcome; date of resolution.

All potential AE will be recorded and signalized on VigiErbe ("http://www.vigierbe.it"). VigiErbe is a web site through which suspected adverse reactions that occurs after taking products of natural origin and herbal products can be signalized to the Istituto Superiore di Sanità, Ministero della Salute- Italy.

Furthermore, all potential AE will be also recorded and signalized to Mibelle AG Biochemistry (Buchs, Switzerland), as company producing both capsules of *Monarda didyma* L. extract and placebo.

2.6 OUTCOMES

PRIMARY OUTCOMES

- DNA methylation age (DNAmAge) (years) and estimation of Age acceleration (AgeAcc) as difference between DNAmAge and chronological age (years).
- Leukocyte telomere length (LTL) as ratio (T/S) of number telomere repeat sequences (T) to single-copy gene (S).
- Mitochondrial DNA copy number (mtDNAcn) as ratio (MT/S) of mitochondrial DNA copy number (MT) and the singular copy number of a gene (S).

Data on primary outcomes are of paramount relevance to determine the biological aging of each participant. To confirm their reliability and reproducibility, the analyses of biological aging indicators (primary outcomes) will be performed in triplicate or at least 20% of the samples will be analysed in different days, in order to verify that the coefficient of variation is similar to the reproducibility originally reported for each method.

SECONDARY OUTCOMES

Telomerase expression; Blood pressure; Salivary cortisol; Emocrome; Inflammation markers (C reactive protein – CRP (mg/l); interleukin6 -IL6 (ng/l)); Questionnaires about of quality life, stress levels, anxiety, sleeping patterns, general well-being; Tracking of physical parameters using Miband 7 watch (heart rate (HR), sleep, mobility); Total cholesterol (mg/dl); low density lipoprotein - LDL (mg/dl); high density lipoprotein - HDL (mg/dl) ;Triglycerides (mg/dl); Glycemia (mg/dl); Insulin (mcU/ml); Homeostasis model assessment (HOMA) index; Aspartate transaminase - AST (IU/l); Alanine transaminase – ALT (IU/l); Gamma-glutamyltransferase - GGT (U/l); Creatinine (mg/dl); Waist circumference (cm); Weight (kg); Total cholesterol/HDL; LDL/HDL.

Most data on secondary outcomes are of clinical relevance to determine the physiological condition such as inflammation status, cardiovascular, renal and hepatic status. Data obtained from questionnaires and Miband 7 watch are essential to determine lifestyle, stress levels, anxiety, sleeping patterns and well-being of each participant. To confirm their reliability and reproducibility, all questionnaires and laboratory tests (secondary outcomes) will be administered or performed, and finally evaluated as described in the protocol for each method.

ADDITIONAL PARAMETER

Evaluation of diary protocol to determine compliance and further effects on stress levels, well-being and health status.

2.6.1 OUTCOMES MEASUREMENTS

Clinical examination

Anthropometric measurements (e.g., height, weight (kg), waist circumference (cm), blood pressure, heart rate variability) will be collected for each study participant.

Questionnaires

Each eligible subject will be characterized by a data collection form properly structured to collect information on demographic variables, lifestyle, and lifetime history of smoking, coffee and other liquid consumption, diet habits, marital status, education in years, institutionalization, occupations, environmental and occupational exposure to genotoxic factors, body mass index (BMI), parental age at birth, including all factors associated with biomarkers of aging.

Furthermore, for each eligible subject:

- - Quality of life questionnaire will be assessed using the World Health Organization's Quality of Life Assessment, BREF version (WHOQOL-BREF).
- - Self-reported sleep quality and efficiency will be measured with the Pittsburgh Sleep Quality Index (PSQI).
- - Global assessment test will be administered.

Wearables Monitoring through MiBand 7 watch

HR monitoring, Sleep tracking, Daily step count, physical activity and Energy Expenditure data during the study period, will be recorded through MiBand 7 wearable devices (Xiaomi) provided by DCTV - University of Padova, on loan for use. The MiBand 7 watch will be worn on the left wrist approximately 2.5 cm distal to the styloid process and measurements will be taken as outlined in the manufacturer instructions. The participants will be asked to avoid removing the device during the study period if not strictly necessary, and only for short periods. The devices will be worn during the sleeping periods and activity monitoring will be manually activated during physical activities.

The devices supposedly won't need to be removed in order to charge their batteries during the week. The devices will provide continuous saturation, respiratory rate, heart rate monitoring, energy expenditure and sleep profile throughout the study period in order to evaluate metabolic demands and exercise pattern. Calibration will be performed by comparing the data obtained with laboratory tests and with questionnaires administered including data from, life style, physical activity, life and sleep quality.

Sample collection

At enrollment (T0) and at the follow up (T1), whole blood samples from the antecubital vein will be collected for each study participant.

Blood samples collected at T0, as part of routine worker surveillance at the Unit of Occupational Medicine Unit – AOUP, will be sent to the Laboratory Medicine Unit –AOUP for routine analysis of blood parameters, as well as an additional aliquot of blood on which to perform non-routine analyses of CRP, IL-6, AST, ALT and insulin, and morning saliva samples collected in Salivette device (SARSTEDT AG & Co, Nümbrecht, Germany) for salivary cortisol levels, according to Laboratory Medicine Unit instructions (AOUP). A part of the blood samples, with the addition of 1 Blood RNA PaxGene Tube, will be used by the

Laboratory of Environmental Mutagenesis (Department of Cardiac, Thoracic, Vascular and Public Health Sciences - DCTV, University of Padova) to analyze the genetic and epigenetic indicators of biological aging.

Blood samples and morning saliva samples collected at the follow up (T1) will be sent to the Laboratory Medicine Unit – AOUP, and the Laboratory of Environmental Mutagenesis – DCTV- University of Padova, to repeat all analyses performed at T0 as non-routinely analyses.

Samples collected will be coded with the ID numbers of the corresponding study participant.

Data recording and sampling operations will be carried out on the "Request and registration of the sample" form, which must be attached to the samples and completed in all its parts by the study participant and who carrying out the collection.

Basic biochemistry, inflammatory markers and salivary cortisol

Data of basic biochemistry will include emocrome, total cholesterol (mg/dl), LDL (mg/dl), HDL (mg/dl), Triglycerides (mg/dl), Glycemia (mg/dl), Insulin (mcU/ml), HOMA index, total cholesterol/HDL, LDL/HDL. Analysis of inflammatory markers (CRP and IL6), AST (IU/l), ALT (IU/l), GGT (U/l), creatinine (mg/dl) and salivary cortisol will be also performed. All analyses will be performed according to Laboratory Medicine Unit instructions – AOUP.

DNA/RNA extractions

Nuclear/mitochondrial DNA will be extracted from whole blood by using QIAmpDNAmini kit (QIAGEN) on QIAcube System (QIAGEN, Milano, Italy) for automated DNA purification, following the manufacturer's instructions as previously described (3). DNA samples will be quantified and checked for quality using QIAexpert Quantification System (QIAGEN, Milano, Italy).

DNAmAge

DNAmAge will be determined by analysis the methylation levels from five selected markers ELOVL2, C1orf122, KLF14, TRIM59 and FHL2) in genomic DNA using bisulfite conversion and Pyrosequencing® methodology on PyroMark Q48 Autoprep (QIAGEN, Milano, Italy), as previously described (4, 18, 19). The resulting Pyrograms® generated by the instrument will be automatically analyzed using Pyromark Q48 Autoprep Software (QIAGEN, Milano, Italy). The level of methylation will be expressed as a percentage of methylated cytosines at the 5 CpG sites considered and will be used for estimation of biological aging (years) as previously reported (4, 18, 19).

AgeAcc

AgeAcc will be estimated as the difference between the detected DNAmAge of blood leucocytes and the chronological age of the study participants (years) (18, 19).

LTL and telomerase expression

LTL will be measured by the real-time quantitative PCR method as previously described (3, 20, 21). This method measures the relative TL in genomic DNA by determining the ratio of telomere repeat copy number (T) to single-copy gene (S) in experimental samples relative to the T/S ratio of a reference pooled sample.

The expression of hTERT will be analyzed by real-time PCR using TaqMan Gene Expression assay (Life Sciences Solutions, Thermo Fisher Scientific, Monza, Italy) as previously described (3).

mtDNAcn

mtDNAcn will be determined in the same DNA of LTL testing by means of the real-time quantitative PCR method as previously specified (5). This assay appraises mtDNAcn in experimental samples by establishing the relation between the mitochondrial (MT) DNA copy number and the singular copy number of a gene (S) relative to the MT/S ratio of a reference assembled DNA sample (5).

Storage of biological material

Biological specimens will be stored for a period of 20 years at the Genomic and Environmental Mutagenesis Laboratory – DCTV - University of Padova, anonymously and not accessible to outsiders. A new favorable opinion and the approval of the Ethics Committee must be required for any other future studies on these samples by Italian and foreign researchers.

2.7 STATISTICAL ANALYSIS

Sample size

The sample size per group was calculated for a two-time longitudinal study, on the base of data obtained from our previous study (3), by using the StastDirects software. In particular, since the telomere analysis represents the limiting analysis, considering a difference in LTL of -0.20 (SD 0.29), 22 subjects are required to achieve 80% power of detecting the association with an alfa error ≤ 5%. Therefore, although the number would be 22, we include 40 subjects for both the intervention and control groups to take into account any abandonment of participation in the study (dropout).

Statistical Analysis

Data of the study will be evaluated exploratory. All data obtained and documented in this study will be listed and summarized with descriptive statistics or frequency tables as appropriate.

All statistical tests will be performed two-sided with StastDirects software and STATA command. The efficacy endpoints will be tested using an analysis of variance (ANOVA) and pairwise test for each versus group to placebo, between the product group (active product or placebo) and variable of interest

(all primary and secondary outcomes). Each comparison will be considered an independent question of interest, and each comparison will be interpreted with $p \leq 0.05$ will being considered significant.

Intermediate analyses will be performed on the data collected at T0. The analyses and interim results will be the responsibility of the steering committee, whose members will have exclusive access.

Additional analyses will be performed if possible, including analyses on subgroups (e.g. male / female subjects only, younger vs. older subjects) or adjusted analyses for age, sex, and/or other variables, using the same statistical methods.

Definition of analysis population relating to protocol non-adherence

Data will be analyzed by the Per-Protocol (PP) method - consisting of all subjects who completed all scheduled visits, had no protocol deviations that (in the judgment of the principal investigator) would have invalidated their efficacy data, and were between 80 and 120% compliant with the specified product dosing. If a subject is only slightly outside the allowable compliance limits, the principal investigator can include that subject in the PP population. If the overall compliance is not available for one of the testing phases of the study (e.g. if the subject has forgotten to return the unused product), the PI can make a determination of compliance based on the familiarity with the subject or on the subject's compliance during the other testing phase(s) of the study.

2.8 PLANNED TIMELINE

As reported in the Gantt chart-proposed time plan (Fig. 1), the study will last a total of 12 months. The study will start in January 2023 and will be completed by January 2024.

2.9 CHARACTERISTICS OF *MONARDA DIDYMA* L.

Monarda didyma L. is a source for different dietary flavonoids, namely didymin, linarin, sakuranetin and isosakuranetin. They are also described to be present in various citrus fruits like sweet orange, blood orange and mandarin and polyphenol-rich orange juice with high didymin content. Furthermore, the plant material contains other polyphenols like rosmarinic acid and chlorogenic acid, components widely distributed in edible plants.

Scientific name: *Monarda didyma* L.

Common commercial name: Scarlet beebalm, Oswego tea, golden balm, gold melissa, melissa d'oro, fiore del bergamotto and menta selvatica.

Taxonomically related to lemon balm.

Plant Family: Lamiaceae.

Part used: Aerial parts.

Geographical origin: Switzerland.

Growing conditions and harvesting period: From organic (Bio Suisse), wild harvest agriculture located in the Swiss mountains at 800–1400 meter of altitude. Areal parts are harvested before flowering time in July-September.

2.10 INFORMATION ON THE PLANT EXTRACT

Plant raw material manufacturing process

Upon harvest, plant material is collected, air dried (2–3 days), sieved and packed by a Swiss company specialized in the cultivation, drying and sales of aromatic and medicinal herbs.

End-product manufacturing process

End-product manufacturing process involves a water extraction (maceration) step followed by filtration, evaporation with the addition of maltodextrin as a carrier, heat treatment and spray drying.

Solvent: Purified water

Plant extract ratio: 2,0–4,0 : 1

The manufacturing process is carried out in compliance with the quality standard ISO 22000:2018. All raw materials as well as packaging and food contact materials are food grade. A HACCP system is in place to guarantee food safety and prevent contamination and non-conformances.

3. DISCUSSION

3.1 Brief summary and potential implications

This clinical study aims to investigate the potential benefits of a 12-week (84 days) supplementation of *Monarda didyma* L. extract on reducing / delaying the biological aging of a susceptible population of healthy aging workers. This is a randomized, placebo-controlled, double-blind, parallel design, monocentric clinical trial. Study population will comprise a total of 80 participants, aged 45–65 years, randomly allocated to both Intervention group (with supplementation of *Monarda didyma* L. extract) and placebo control group (with placebo supplementation) that will facilitate understanding of relevance of the intervention under consideration. All study participants will be aging workers of the same ethnicity, employed by the University of Padua (Italy), to minimize potential confounding variables that could arise from population heterogeneity, which could impact the validity and interpretability of our results. By selecting participants from a relatively homogenous group, we aim to control for potential factors that could introduce variability in the outcomes. While our current study focuses on a specific demographic, future research endeavors could indeed explore the effects of *Monarda didyma* L. extract supplementation in a more heterogeneous population. This approach could provide insights into how the intervention interacts with various genetic backgrounds, lifestyles, and environmental factors.

Our previous studies suggests that youthful genetic and epigenetic information can be recovered, and indicators of biological aging examined may represent an accurate tool to measure the effectiveness of lifestyle-based interventions in the prevention of age-related diseases (3, 4). Therefore, we chose to analyze biological aging indicators as primary outcomes (DNAmAge and AgeAcc, LTL and mtDNAcn), in combination with secondary outcomes that are of clinical relevance to determine the physiological condition such as inflammation status, cardiovascular, renal and hepatic status, as well as lifestyle information and psychological aspects such as stress levels, anxiety, sleeping patterns and well-being of each participant.

The broad goal is to create a green nutraceutical product that offers potential benefits for both human health and environment, contributing to the implementation of anti-aging strategies and / or healthy aging for wellbeing and to environmental sustainability that currently represent two of the most relevant topics in science with important social and economic implications.

Results of the present research could lead to the identification of a “natural treatment” to reverse aging. This opens new scenarios in which the ability to reprogram genetic and epigenetic information will allow the control of cellular physiology, dreaming of a future with one more weapon to counteract aging. The success of this research could represent a turning point in the healthy aging research, leading to rejuvenation by means of a sustainable and safe intervention, accessible to everybody in order to extend health span.

3.2 Practical or operational issues involved in performing the study

Strategies for achieving adequate participant enrolment and to improve adherence to intervention protocols

To achieve the adequate participant enrolment and to improve adherence to intervention protocols, we have developed the following strategies: a dedicated personal staff for study information and purpose; a Miband 7 watch on loan for use by which to acquire the daily data of the participants useful for the study purpose; release of the reports obtained from all biological and biochemical analyses performed during the study; release of the reports obtained from all psychological tests performed during the study. Each subject will be informed verbally and by means of specific written documents about the purposes and procedures of the study (Information form for study participation) and on the processing of personal data (Personal Data Processing Form). The subject will be able to consult reliable people and he/she will have an appropriate amount of time to identify critical issues, to ask for further information, etc. Adherence we will provide to each participant by a diary for daily supplementation intake registration together with the record of effects on stress levels and well-being, and all residual study products (both capsules of *Monarda didyma* L. and placebo) will be gathered and counted at the end of the intervention.

Finally, he/she will be asked to sign the ICF, in the eventuality to accept the study participation. Study participation is voluntary, free and aware and the subject’s consent can be withdrawn at any time for any

reason or even without any reason.

Circumstances under which unblinding is permissible

The coordinating center and steering committee have defined the voluntary withdrawal from the study and the withdrawal from the study after adverse event onset or poor tolerability as circumstances under which unblinding is permissible, stopping the study participation and revealing the participant's allocated intervention during the trial.

Safety data of the product

Monarda didyma L. is listed on several European positive plant lists safe for human consumption and for use in dietary supplements, including Italy. The intended product is not produced by any technology and does not contain any ingredients regulated by the new Regulation (EU) 2015/2283 or the old Regulation (EC) No 258/97 on novel foods and novel food ingredients. Therefore, based on our current regulatory knowledge and experience, the product should be broadly accepted as a conventional (non-novel) food in the EU. In Italy, the extract of *Monarda didyma* L. is included in Annex 1 to the Ministerial Decree of 10 August 2018 concerning the regulation of the use of herbal substances and preparations in food supplements as updated with the Decree of 9 January 2018 and most recently with the Decree of 26 July 2019, in which it is indicated among botanicals that can be used in food supplements (22).

Furthermore, the manufacturing process of the product is carried out by Mibelle AG Biochemistry in compliance with the quality standard ISO 22000:2018. All raw materials as well as packaging and food contact materials are food grade. A HACCP system is in place to guarantee food safety and prevent contamination and non-conformances. The product is free of the following food allergens (celery, dairy products, egg, fish, crustacean shellfish, mollusc, thee nuts, peanuts, mustard, wheat, soybeans, sesame, lupin, sulphur dioxite/sulfit), and free of gluten (Reg. (EU) No 282/2014).

In the present study we propose a daily dosage of *Monarda didyma* L. extract equal to 100 mg/day (1 capsule with the lunch) containing ca. 4–10 mg of DID, which is a significant lower dosage (2-6-fold lower) than that used in the clinical trial published by Rangel et al. (16, 17), during which no side effects have been reported demonstrating the safety of daily DID consumption.

For all of the above-mentioned reasons no sever or permanent consequences should result from trial participation, even after the adverse effect onset. However, all potential adverse events will be reported and recorded on VigiErbe (www.vigierbe.it) and also to Mibelle AG Biochemistry and the study documentation also includes clinical trial insurance.

Efficacy data of the product

DID is a natural extract and the major polyphenol of *Monarda didyma* L. DID is an orally bioactive citrus flavonoid glycoside normally found in several citrus fruits, such as oranges, lemons, grapefruits, and

mandarins (7). Recent studies provided newer insights into this compound, which could regulate multiple biological activities of many important signaling molecules in health and disease (7).

In vitro studies demonstrated the anti-oxidative and anti-inflammatory activities of DID and prevented endothelial dysfunction and death in HUVECs and THP1 monocytic cells at concentration equal to 20 μM (7–9), as well as anti-oxidative and neuroprotective effects in SH-SY5Y cells at concentrations of 0.5, 1, 5, 10, 20 μM (7, 23). Furthermore, the antidiabetic properties of DID have been revealed in HepG2 cells at concentration equal to 10–20 μM, showing potent inhibitory activity against the key enzymes involved in diabetes mellitus (7, 8, 24). Moreover, DID showed that the anti-tumor efficacy and therapeutic effects of DID prevent the growth of cancer cells such as breast (MDA-MB-221 cells) and lung cancer (A549 e H460 cells) at concentration of 10–20 μM (7, 8, 12).

In vivo studies confirmed the anticancer properties of DID in mice treated with 6 mg/kg/day of DID (12), and revealed its anxiolytic-like effect through the interaction with the GABAA receptors in mice treated with DID's concentration of 1, 10, 20, 40 mg/kg/day (14), and its role in mitigating Hepatic Fibrosis in rats with a daily dosage of 0.5 mg/kg (15).

A randomized, placebo-controlled, double-blind, crossover clinical trial has been performed to evaluate the effects of a DID-rich orange juice-based beverage on endothelial function markers and plasma inflammatory biomarkers in overweight and obese adults (BIONAOS STUDY) (16, 17). The authors found that a consumption of two daily doses of a DID-rich orange juice (250ml each) containing 24mg of DID for 12 weeks improves condition of cardiovascular risk biomarkers, decreasing plasma concentration of resistin and soluble forms of intercellular adhesion molecule (sICAM-1) (17), as well as of pro-inflammatory biomarkers, IL- 6 and IL- 8 (16). According to authors DID-rich orange juices have been well tolerated by study volunteers and no side effects have been reported (16, 17).

In vitro, in vivo and human studies do not show any adverse effects related to the extract of *Monarda didyma* L. (7–9, 12, 14–17, 23, 24).

Furthermore, since the anti-inflammatory effect seems to be one of the most relevant aspects of DID, and since inflammation and the consequent oxidative stress are distinctive signs of biological aging, we hypothesize that DID, contained in the extract of *Monarda didyma* L., may have a potential role in reducing / delaying biological aging, and this is the purpose of our study.

Trial status

The local Ethics Committee - University of Padova approved the study protocol (5518/AO/22; Study Code: EMODISU) on December 6, 2022. The recruitment of the study population will carry out between January 2023 and March 2023.

The study was also registered in the database for Protocol Registration and Results System ClinicalTrials.gov PRS (NCT05399966, registration date: May 17, 2022. ID: BIOAGELAB202201; title: Effects of *Monarda didyma* L. extract supplementation on biological aging (EMODISU)).

All items from the World Health Organization Trial Registration Data Set are reported in the Supplementary Material.

Abbreviations

AE: adverse event; AgeAcc: age acceleration; ALT: alanine transaminase; AST: Aspartate transaminase; CRP: C reactive protein; DID: didymina; DNAmAge: DNA methylation age; GGT: Gamma-glutamyltransferase; HDL: high density lipoprotein; HOMA index: homeostasis model assessment; HR: heart rate; IL6: interleukin6; LDL: low density lipoprotein; mtDNAcn: mitochondrial DNA copy number; PSQI: Pittsburgh Sleep Quality Index; TL: telomere length; WHOQOL-BREF: World Health Organization's Quality of Life Assessment, BREF version.

Declarations

Ethics approval and consent to participate

The local Ethics Committee - University of Padova approved the study protocol (5518/AO/22; Study Code: EMODISU) on December 6, 2022.

If necessary, the protocol modifications decided by the coordinating centre and steering committee team will be submitted for approval by the Ethics Committee as amendment to the original protocol. The approved protocol modifications then will be communicated to Mibelle AG Biochemistry, trial participants, other investigators and trial registries, as relevant parties.

Consent for publication

Not applicable

Availability of data and materials

Data collected from each participant will be reported in an ad hoc Database created as an excel file at the recruitment (T0). The computerized data will be anonymized; subject identifiers will be limited to ID numbers. Prior to analysis, the investigators will review all data, all data elements will be evaluated for reasonableness and consistency, and any suspicious or impossible values will be referred back to source documents for verification or correction.

Data management and monitoring will be executed by members of the coordinating centre and steering committee, who will have an exclusive access.

As per contract, neither Party (PI and Mibelle AG Biochemistry) will, either during the project period or for five years after the end of the project period, disclose to any third Party, nor use for any purpose except carrying out the project, any of the other Party's confidential information.

Competing interests

The authors declare that they have no competing interests.

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Mibelle AG Biochemistry (Buchs, Switzerland) supports the dietary supplementation production and supplying (capsules of *Monarda didyma* L. extract and placebo), costs of enrolment and sample collection, costs of biological aging analyses as primary outcomes and secondary outcomes, and publication costs.

University of Padua supports a PhD Fellow.

Authors' contributions

The coordinating center and steering committee, that conceived the clinical trial and wrote the protocol, are represented by SP, who is the Chief Investigator, MC and LC.The team will carry out the data management and monitoring. JB from Mibelle Group Biochemistry provided all information about *Monarda didyma* L. extract.

This protocol study has been written according to the SPIRIT reporting guidelines (25).

All authors read and approved the final manuscript

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Figures

Description		Task	Staff	Timeline (12 Months)											
				1	2	3	4	5	6	7	8	9	10	11	12
Kick off activities and Ethical Committee approval	1	Write research protocol, request and **approval ethics committee**	Principal Investigator and two research assistants	x	x	x									
	2	Starting meeting, staff training, data collection form		x	x	x									
	3	Interim Report		x	x	x	x	x	x	x	x	x	x	x	x
Assessment for eligibility	1	N=80 subjects eligible for study participation		x	x	x									
Enrolment	1	N=40 subjects randomly allocated to intervention group (with supplementation of Monarda Didyma extract)	Medical staff and other members of the research team				x	x							
		N=40 subjects randomly allocated to placebo-only control group (with placebo supplementation)					x	x							
Intervention and follow up	1	Dietary supplementation of Monarda Didyma extract (1 capsule/day)	Participants				x	x	x	x	x				
		Dietary supplementation of Placebo (1 capsule/day)					x	x	x	x	x				
	2	Compiling treatment diary					x	x	x	x	x				
	3	Follow up (T1)							x	x	x				
Samples collection and Clinical assessment	1	Clinical data collection, and questionnaires for all subjects enrolled in the study, including both groups, at enrollment (T0) and follow up (T1)	Nursing and medical staff, and researchers				x	x	x	x	x				
	2	Collection of whole blood and saliva samples from all subjects enrolled in the study, including both groups, at enrollment (T0) and follow up (T1)	Nursing and medical staff, and researchers				x	x	x	x	x				
Analysis of biological aging indicators	1	Analyses of primary and secondary outcomes at enrollment (T0)	Researchers				x	x	x	x	x	x	x		
	2	Analyses of primary and secondary outcomes at follow up (T1)	Researchers						x	x	x	x	x		
Statistical analyses	1	Data base creation and statistical analysis between primary and secondary outcomes, clinical data, and information collected by lifestyle questionnaire for all participants at enrollment (T0)	Researchers				x	x	x	x	x	x	x		
	2	Data base management and statistical analysis between primary and secondary outcomes, clinical data, and information collected by lifestyle questionnaire for all participants at follow up (T1)	Researchers				x	x	x	x	x	x	x		
	3	Evaluation of changes (Δ=T1-T0) in primary outcomes	Researchers									x	x		
	4	Evaluation of changes (Δ=T1-T0) in clinical data and secondary outcomes	Researchers									x	x		
	5	Identification of dietary supplemental' effect in slowing down biological aging acting on primary outcomes	Researchers									x	x		
	6	Identification of dietary supplemental' effect on secondary outcomes										x	x		
Results evaluation and dissemination	1	Final Report	All researchers										x	x	x
	2	Manuscript preparation	All researchers										x	x	x
	3	Manuscript submitted to peer-review journal	All researchers												x
	4	Participation to national and international meetings	All researchers												x
	5	Conference organisation	All researchers												x

Figure 1

Gantt chart-Proposed time plan. The Gantt chart shows a description of the activities that will be carried out during the clinical study, including the Ethical Committee approval, assessment for eligibility, enrolment, intervention and follow up, samples collection and clinical assessment, analysis of biological aging indicators, statistical analyses, results evaluation and dissemination. For each of them, the relative tasks, the staff who will conduct the various activities and the timeline have been reported.