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Dietary supplementation with polyphenol-rich *Salicornia ramosissima* extracts: Assessing safety, efficacy, and impact on cardiovascular health biomarkers in healthy volunteers

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ABSTRACT

The importance of diet in preventing non-communicable diseases is well established, with polyphenol consumption suggested to impact cardiovascular disease development. *Salicornia ramosissima synthesizes* high amounts of phytochemicals under environmental stress. In a randomized controlled clinical trial on 90 healthy volunteers, we evaluated the safety of supplementation with 1 g of polyphenol-rich *S. ramosissima* extracts from salt marshes and hydroponic sources over three months. No differences in adverse effects were observed between extract-treated and placebo subjects. Salicornia extract from marshes (SM) increased glomerular filtration rate and reduced LDL cholesterol. SM treatment also modulated plasma markers related to cardiovascular disease: MERTK, Gal-9, ADM, TF, PRSS27, HAOX1, IL-18, PAPPA, TNFRSF1A, TIE2 and FGF-21 proteins were down-regulated while SRC levels were upregulated. Therefore, under the studied conditions of use, Salicornia extracts consumption is safe and SM induced biochemical and proteomic changes related to cardiovascular health.

1. Introduction

A healthy diet has been shown to have a protective effect against non-communicable diseases, such as heart disease, cancer, stroke or chronic respiratory disease, which are associated with very high mortality and morbidity worldwide. Therefore, dietary habits are a major modifiable risk factor for their prevention and progression, as well as for preserving good general health, stronger immunity and increased longevity (Cena & Calder, 2020; Locke, Schneiderhan, & Zick, 2018). In fact, the impact of diet on non-communicable diseases in 2017 was estimated to cause 11 million deaths and 255 million disability-adjusted life-years (DALYs) (Collaborators, 2019).

Several dietary patterns have been associated with health benefits, but the Mediterranean diet is the best studied one (Dominguez, Di Bella,

Abbreviations: AEs, Adverse Events; BMI, Body mass index; CVD, Cardiovascular disease; CRF, Case Report Form; DALYs, Disability-adjusted life-years; GAE, Gallic Acid Equivalent; IPAQ, International Physical Activity Questionnaire; MeDRA, Medical Dictionary for Regulatory Activities; MEDAS, Mediterranean diet adherence questionnaire; MFA, Multiple Factor Analysis; PCA, Principal Component Analysis; SM, Salicornia Marshes; SH, Salicornia hydroponia.

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Veronese, & Barbagallo, 2021). The PREDIMED trial has already shown that the Mediterranean diet was able to reduce the incidence of stroke, myocardial infarction, or cardiovascular death in a high risk population (Estruch et al., 2018). The high consumption of plant-based foods as a source of dietary polyphenols is largely responsible for the beneficial effects of this diet, due to its antioxidant and anti-inflammatory properties and its ability to modulate mechanisms associated with many diseases (Yammine et al., 2021).

Different clinical trials reported a beneficial effect of polyphenolic supplementation on lowering blood pressure (Moreno-Luna et al., 2012; Tjelle et al., 2015), which is one of the main stroke risk factors (Johnson et al., 2015). Dietary supplementation with polyphenols have been also shown to influence the levels of circulating biomarkers associated with cardiovascular damage such as C reactive protein (Espinosa-Moncada et al., 2018), TNF-α (Grabez et al., 2022), or IL-6 (Al-Aubaidy et al., 2021; Grabez et al., 2022), but their exact mechanisms of action and metabolic pathways have not been fully elucidated.

Salicornia ramosissima, commonly known as glasswort, is a seasonal halophyte plant of salt marsh ecosystems that, in response to saline stress and UV radiation, synthesizes high amounts of polyphenols (Limongelli, Crupi, Clodoveo, Corbo, & Muraglia, 2022). Salicornia species grow and have a long history of consumption in traditional food in many different regions, particularly in Mediterranean countries as a fresh vegetable or as a seasoning (Lopes, Roque, Cavaleiro, & Ramos, 2021). Salicornia species have been attributed many health benefits. Pre-clinical studies with different species of Salicornia have demonstrated *in vivo* their cardiovascular protective effects, such as anti-inflammatory (Noh et al., 2022), anti-obesity (Chrigui et al., 2023; On et al., 2023); as well as anti-dyslipidemic activities (Chrigui et al., 2023; Park, Ko, Choi, & Chung, 2006). Moreover, Salicornia extracts have shown to play a role against hyperglycemia and diabetes prevention in mice (Park et al., 2006) and regulate vascular remodeling (Won et al., 2017). *S. herbacea* showed anti-osteoporotic effects *in vitro* (Karadeniz, Kim, Ahn, Kwon, & Kong, 2014). *S. europaea* extracts were shown to protect against hypertension and induced vasorelaxation, which was attributed to the presence of *trans*-ferulic acid (Panth, Park, Kim, Kim, & Oak, 2016). The beneficial effects of many individual polyphenols identified in *S. ramosissima* support the use of this species for the treatment of ischemia (Nájar, Romero-Bernal, Del Rio, & Montaner, 2023). Recently, we showed that supplementation with *S. ramosissima* extracts prevented hypoxia-induced death in Drosophila. Moreover, we demonstrated that treatment with a Salicornia ethanolic extract for 4 weeks reduced brain infarct volume and lowered the plasma levels of oxidative markers after experimental brain ischemia in mice (Garcia-Rodriguez et al., 2022).

Our previous work suggested that *S. ramosissima* could be a promising option for the prevention of neurovascular disease but the safety and efficacy in controlling of cardiovascular risk factors need to be addressed in human studies. A previous study reported that a single high-dose oral administration of *S. herbacea* did not exert any toxic effects in mice and was determined to be effective in reducing lipid contents in the blood (Lee, 2016). To our knowledge there are only two human studies assessing Salicornia extracts. However, these studies investigated either a different Salicornia species (*S. europaea*) (Lee, Shin, Kim, Kweon, & Kim, 2020) or the use of *S. ramosissima* as a skin cream application (Giordano et al., 2022). None of these studies assessed blood haematological or biochemical changes, indicating the need for further research. In the present work, we have evaluated for the first time the safety of oral administration of *S. ramosissima* extracts and investigated whether dietary supplementation of healthy volunteers with the polyphenol-rich extracts is associated with changes in circulating levels of protein biomarkers linked to the development of cardiovascular disease (CVD).

2. Methods

2.1. Study design

A randomised, triple-blind, parallel-group, placebo-controlled pilot trial was conducted to assess the safety and efficacy of administration of a food supplement containing halophyte plant extracts compared to placebo in healthy volunteers. The study consisted of a three month treatment with two scheduled clinic visits: at baseline (visit 1) and at 3 months (end-of-treatment, visit 2). The work has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The study was registered at clinicaltrials.gov (NCT06076122) and was approved by the Andalusian Ethics Committee (ID HALOFITAS) on February 18th, 2022. Written informed consent was signed by the volunteers, and participants were able to withdraw from the study at any time without giving a reason.

Two hydroalcoholic extracts from different *S. ramosissima* sources were assessed: *S. ramosissima* grown and harvested at marshes (Salicornia Marshes (SM) extract; Isla Cristina, Huelva, Spain [https://marshfoods.weebly.com/]) and *S. ramosissima* grown hydroponically (Salicornia hydroponia (SH) extract; Faro, Portugal, [https://riafresh.com/]).The use of extracts from two suppliers is based on the understanding that marshland plants, exposed to more stress, contain higher polyphenol levels. This distinction between wild and hydroponically cultivated plants offers an opportunity to study the dose–response effect, hypothesising that more diverse polyphenols yield greater clinical benefits Salicornia from marshes and from hydroponia were harvested and dried during summer of 2021. Then, the extraction process took place during May 2022. The aerial part of the plants was left to dry and hydroalcoholic extracts were manufactured by EVESA (Cadiz, Spain [https://evesa.com/]). The encapsulation as well as the blinding process, were carried out by BIO-DIS laboratories (Seville, Spain [https://www.bio-dis.com/]). The study comprised three arms according to the treatment administered (ratio 1:1:1): (a) SM extract; (b) SH extract and (c) placebo.

2.2. Participant selection, randomization, and blinding

From September 2022 to November 2022, a total of 90 healthy volunteers were enlisted through advertisements distributed via the institutional networks at Institute of Biomedicine of Seville (IBiS) and the University Hospitals. The inclusion criteria were healthy people ≥18 years old that agree to participate in the study and have read and signed the informed consent form. People with the following criteria were excluded: previous neurovascular disease; other chronic diseases for which the subject takes medication on a regular basis; hyperthyroidism; volunteers taking vitamins or polyphenol-containing nutritional supplements in the 30 days prior to the screening visit; known allergies or intolerance to halophyte plants; pregnant or breastfeeding women; and frequent consumption of halophyte plants.

Participants were randomly assigned to one of the study arms and instructed to take two capsules daily for the following three months. The daily dose of polyphenols found to be well tolerated and protective against experimental ischemia in mice (4.63 mg of Gallic Acid Equivalent (GAE)/kg/day) (Garcia-Rodriguez et al., 2022) would be equivalent to approximately 26 mg in humans (https://drughunter.com/resource/practical-pk-calculators). Therefore, 1 g of extracts with total polyphenol content of 24 mg GAE/g and 17.2 mg GAE/g for SM and SH, respectively, or placebo were administered. The nutritional composition of each extract is shown in Supplementary Table 1. Indistinguishable treatment bottles were prepared with unique coding for each participant. The coding list was securely held by the Pharmacy Department of the Virgen Macarena University Hospital, ensuring the triple-blind protocol was maintained.

2.3. Safety evaluation

The safety and tolerability of the supplement were quantified in terms of the incidence of Adverse Events (AEs). All AEs were recorded on Case Report Form (CRF) and coded according to corresponding Medical Dictionary for Regulatory Activities (MedDRA) coded system organ classes. In addition, classification according to causality (unrelated events or events related or possibly related to treatment) and severity (mild or serious) were madre by study neurologists. Only those that could be related to treatment were analysed (Supplementary Fig. 1). The frequency of AEs and the percentage of AEs causing subject withdrawal from the study were determined.

2.4. Nutritional and physical activity data assessment

At baseline (visit 1), clinical data regarding medical history and other conditions were recorded. Participants' body mass index (BMI) and blood pressure were measured at visit 1 and 2. Toxic habits (alcohol and tobacco consumption), allergies and intolerances were also collected.

Dietary habits were assessed at baseline using two validated questionnaires: the 14-item Mediterranean diet adherence questionnaire (MEDAS) and a 137-item food frequency questionnaire (PREDIMED study) from which the daily intake of food groups, energy, macronutrients and micronutrients was calculated using the residual model. In addition, physical activity was assessed using the short version of the 7-item International Physical Activity Questionnaire (IPAQ).

2.5. Blood sampling and analytical evaluation

Blood samples were collected at the beginning and the end of the study after overnight fasting (8:00 a.m.-11:00 a.m.) in an ethylenediaminetetraacetic acid (EDTA) anticoagulant tubes (BD Vacutainer K2EDTA spray-coated tubes) for plasma cell count using the Sysmex XN-2000 analyzer (Sysmex, Kobe, Japan), and without anticoagulant (BD Vacutainer PST), for serum analysis of biochemical parameters using the Hitachi Cobas c 702 modular analyzer (Roche Diagnostics, Rotkreuz, Switzerland).

For proteomic analysis, blood samples were collected using EDTA containing tubes, which were centrifuged at 1,500*xg* for 10 min. Plasma was then aliquoted and stored at −80 °C until analysis.

2.6. Proteomic analysis

Plasma was analysed using the Olink Cardiovascular II target 96 panel (Cobiomic Bioscience, S.L) to perform Proximity Extension Assay (PEA) measurement of the levels of 92 proteins associated with biological functions linked to cardiovascular diseases.

2.7. HPLC-ESI-QTOF-MS analysis

For analytical characterisation, all chemicals were of analytical reagent grade and used as received. LC-MS grade acetonitrile and formic acid for mobile phases were purchased from Riedel-de-Haën (Honeywell, NC, USA). For solutions, ultrapure water was obtained with a Milli-Q system Millipore (Bedford, MA, USA) and absolute ethanol was purchased from VWR chemicals (Radnor, PA, USA). The analytical standards Luteolin-7-glucoside, Quercetin 3-b-d-glucoside were acquired from Sigma-Aldrich (Steinheim, Germany). Isorhamnetin-3-O-β-D-Glucoside and 3,5-dicaffeoylquinic acid were acquired from Cymit Química S.L. (Barcelona, Spain), MedChemExpress (Monmouth Junction, NJ, USA), respectively.

A chemical standard mix (Luteolin-7-glucoside, Quercetin 3-b-d-glucoside, Isorhamnetin-3-O-β-D-Glucoside and 3,5-Dicaffeoylquinic acid) at 100 mg/L was prepared for quantification purposes. The mixture of these 4 chemical standards was diluted at different concentrations (0.1, 0.5, 1, 5, 10, 25 and 50 mg/L) for the analysis. These standard dilutions and the bioactive extracts, which were prepared at 5 mg/mL, were analysed by high performance liquid chromatography (Agilent 1290 HPLC, Agilent Technologies, Palo Alto, CA, USA) coupled to mass spectrometry with a quadrupole time-of-flight analyser (Agilent 6545 QTOF Ultra High Definition, Agilent Technologies, Palo Alto, CA, USA). Chromatographic analysis was performed in a reversed phase with a C18 ACQUITY UPLC BEH column (1.7 μm, 2.1 mm, 150 mm, 130 Å, Waters Corporation). The mobile phases were (A) acidified water with 0.1 % of formic acid (v/v), and (B) acetonitrile, with gradient for optimal separation was: 0.00 min [A:B 100/0], 5 min [A:B 90/10], 18 min [A:B 15/85], 24 min [A:B 0/100], 25.50 min [A:B 0/100], 26.50 min [A:B 95/5] and 32.50 min [A:B 95/5]. A mobile phase flow rate of 0.4 mL/min and an injection volume of 5 μL was used. MS acquisition was performed in electrospray negative ionisation (ESI) mode in a mass range between 50–1200 *m/z*. Other parameters were as follows: gas flow rate 10 L/min; gas temperature 200 °C; nebuliser 20 psig, enveloping gas temperature 350 °C, enveloping gas flow rate 12 l/min, VCap 4000 V, nozzle voltage 500 V. Finally, the acquired data were processed through MZmine 2.53 and Sirius v5.8.6. The compounds were annotated by comparison of the MS/MS spectra with those from analytical standards or published in the literature and databases, such as SciFinder®, CEU Mass Mediator, Human Metabolome DataBase (HMDB) and Kyoto Encyclopedia of Genes and Genomes (KEGG). Calibration curves were obtained for the analytical standards analysed. The limits of detection and quantification were then calculated based on Signal-to-Noise ratios of 3 and 10, respectively, in accordance with IUPAC guidelines (Supplementary Table 2). The main phenolic compounds in the bioactive extract were quantified using their commercial standards or tentatively semi-quantified by structural analogy to the standard corresponding to their chemical family.

2.8. Statistical analysis

Sample size was calculated using free sample size calculating software G*Power version 3.1.9.7 (Franz, Universitat Kiel, Germany). An a priori power analysis for repeated-measures Analysis of Variance (ANOVA) for within-between interaction, with an expected effect size f of 0.20, alpha of 0.05, and statistical power of 90 %, indicated that the required sample size was 84 subjects among the three treatment groups. SPSS v.29 and R V.4.3.1. were used for statistical analysis.

The safety analysis was performed on an intention-to-treat basis (subjects who have initiated treatment). Descriptive statistics were employed, using tables and graphs representing the absolute and relative values of qualitative variables, as well as measures of position and variability of quantitative variables. The Shapiro–Wilk normality test was used to determine the distribution of the variables. Variables showed a non-normally distribution, therefore, the Kruskal Wallis test was used to compare medians between treatment types. Chi-square test was used for testing relationships on qualitative variables by type of treatment. Statistical significance was established for p-value < 0.05.

Explorative dimensional reduction methods were performed for proteomic analysis. Multiple Factor Analysis was performed on clinical variables groups and Principal Component Analysis on Nutritional Data subset and Olink NPX protein expression data. factoMiner package (Lê, Josse & Husson, 2008) was used. Results were represented on heatmaps with hierarchical clustering using complete link.

Differential expression analysis was performed using limma package. The repeated measure design was incorporated into the fit in a random effect manner estimating correlation structure from the data (Ritchie et al., 2015). Linear models fitted each protein expression by extract, visit and an interaction of both factors. Proteins are ranked and listed according effect contrast absolute t-statistic value, which show decreasing evidence of association. Estimates of log2 fold change expression for extracts at visit 2 are also obtained. Due to the exploratory nature of this study and concern for false negatives no adjustment for

multiplicity has been considered on reported results. As a sensitivity analysis, we performed the same ranking analysis on 2000 bootstrap replicates and present main quantiles of resulting ranking distribution reordered by 2.5th percentile of ranking distribution.

Interpretations of evidence of differential expression (p-values) should acknowledge the 96 probes setting (as no multiplicity adjustment has been reported. A q-value according to Benjamini & Hochberg (BH) method has been computed to ease the interpretation in the context of false discovery control.

To adjust for potential clinical and nutritional cofounders of expression in a feasible manner we performed two alternative fits incorporating the first two dimensional reduction scores of the nutritional questionnaire, and overall clinical data as covariates.

The present study is compliant with the journal's data availability standards, and any data not provided in the article may be shared by request of other qualified investigators.

3. Results

3.1. Analytical characterisation of S. ramosissima SM and SH extracts by HPLC-ESI-QTOF

SM and SH extracts were tentatively characterised by HPLC-ESI-QTOF-MS. This characterization was carried out based on retention times, *m/z*, main fragments, and predictions from different software and other studies previously published in the literature. All analytical information is provided in Supplementary Table 3. A total of 45 compounds were annotated in both samples, where 41 and 31 compounds were detected in SM and in SH, respectively. Among the most relevant compounds were chlorogenic acid derivatives. Most of these compounds have been previously reported in Salicornia species (Chung et al., 2005; Hwang et al., 2010) although two new derivative isomers, peaks 5 and 12, have been detected (only in SM) for the first time. Moreover, several fatty acids have been also annotated in both extracts. In Table 1, the bioactive compounds in each extract were quantified using their commercial standards. Chlorogenic acid levels were comparable in both extracts. We found that the concentrations of tungtungmadic acid, quercetin, and isorhamnetin glucosides were higher in the SM extract compared to the SH extract. Caffeoyl dihydrocaffeoyl quinic acid derivatives were found exclusively in the SM extract, while luteolin glucoside was present only in the SH extract.

Table 1
Quantification of phenolic compounds in *S. ramosissima* SM and SH extracts by HPLC-ESI-QTOF-MS.

Peak	PROPOSED COMPOUND	Quantification (μg/mg extract)	
		SM	SH
3,5-dicaffeoylquinic acid			
4	Chlorogenic acid	1.44 ± 0.03	1.60 ± 0.07
5	Caffeoyl-dihydrocaffeoyl quinic acid derivative	0.560 ± 0.008	ND
9	Tungtungmadic acid (caffeoyl-dihydrocaffeoyl quinic acid)	1.53 ± 0.03	0.92 ± 0.03
10	Tungtungmadic acid (caffeoyl-dihydrocaffeoyl quinic acid)	2.43 ± 0.03	1.01 ± 0.03
11	Isochlorogenic acid isomer	0.678 ± 0.008	1.14 ± 0.03
12	Caffeoyl-dihydrocaffeoyl quinic acid derivative	1.34 ± 0.03	ND
13	Isochlorogenic acid A (3,5-dicaffeoylquinic acid)	0.9 ± 0.1	1.77 ± 0.03
15	Tungtungmadic acid (caffeoyl-dihydrocaffeoyl quinic acid)	2.31 ± 0.07	0.92 ± 0.03
16	Isochlorogenic acid isomer	1.1 ± 0.0	1.55 ± 0.07
Luteolin 7-O-β-D-glucoside			
6	Luteolin 7-O-β-D-glucoside	ND	0.36 ± 0.05
Quercetin 3-β-D-glucoside			
8	Quercetin 3-β-D-glucoside	1.29 ± 0.03	0.5 ± 0.0
Isorhamnetin-3-O-β-D-Glucoside			
14	Isorhamnetin-3-O-β-D-Glucoside	1.54 ± 0.06	0.08 ± 0.01
17	Isorhamnetin acetylglucoside	0.92 ± 0.06	ND
32	Genkwanin	0.56 ± 0.04	0.61 ± 0.01

ND: compound non detected

3.2. Enrolment, baseline characteristics and participant reported outcomes

A total of 131 subjects were invited to participate, of whom 90 (63.8 %) were finally included and randomised. Among the 41 subjects who were excluded, 24 decline to participate, and 17 were not eligible. Participants were assigned to SM treatment (n = 30), SH treatment (n = 30) or placebo group (n = 30) and 84 of them finished the study. The causes of withdrawal from the study were: treatment intolerance (1 subject, SM treatment); protocol noncompliance (3 subjects, SH treatment); and loss of follow-up (2 subjects, placebo group) (Fig. 1).

The subjects recruited had a median age of 40 years, ranging from 21 to 80 years, and were predominantly female (64.4 %). At baseline, there were no significant differences between the three groups with respect to the distribution of individuals based on sex, the presence of other minor conditions, alcohol or tobacco consumption, allergies or intolerances (Supplementary Table 4) as well as BMI, heart rate or blood pressure (Table 2). Among the three groups we only found differences in age between the SM extract and SH extract treatment groups (median age 32 years vs 42.5 years, respectively; p = 0.022).

Clinical variables including BMI, systolic and diastolic blood pressure and heart rate were also assessed at the end of the study. No significant differences were observed between treatment groups (Table 2).

During the three-months intervention, no serious AEs were reported. No significant differences were found in the incidence of mild AEs or side effects among the three study arms (26.67 % in SM group, 33.33 % in SH group and 13.33 % in placebo group, p = 0.186). Most of the reported AEs or side effects were mild gastrointestinal disorders, like gastroesophageal reflux or urinary disturbances such as polyuria or pollakiuria (Table 3).

3.3. Analytical evaluation

The hemogram and biochemical profile were analysed at the beginning and the end of the study. There were no pathological alterations in the hemogram at any of the visits for the placebo or treatment groups (data not shown). Biochemical profiles are shown in Table 4. At baseline, significant differences in albumin (4.8, 4.7 and 4.6 g/dL, p = 0.017), total protein (7.45, 7.15 and 7.1 g/dL, p = 0.008), LDL-cholesterol (101.5, 117 and 113, p = 0.018) and glycosylated haemoglobin (5.2, 5.3 and 5.2 %, p = 0.038) were observed (Table 4). Therefore, we carried out pairwise analysis to identify differences between specific groups. For median values of albumin (4.8 vs 4.7 g/dl, p = 0.027) and LDL cholesterol (101.5 vs 117 mg/dl, p = 0.018) significant differences were found between SM and SH treatments. For total proteins (7.45 vs 7.1 g/dl, p = 0.009) differences were observed between SM treatment and placebo. No significant differences were found for glycosylated haemoglobin after pairwise analysis.

When analysing biochemical data after 3 months of treatment, these differences continued for LDL-cholesterol (94, 122 and 119.5 mg/dL, p = 0.014) and glycosylated haemoglobin (5.2, 5.4, 5.2 %, p = 0.036) and

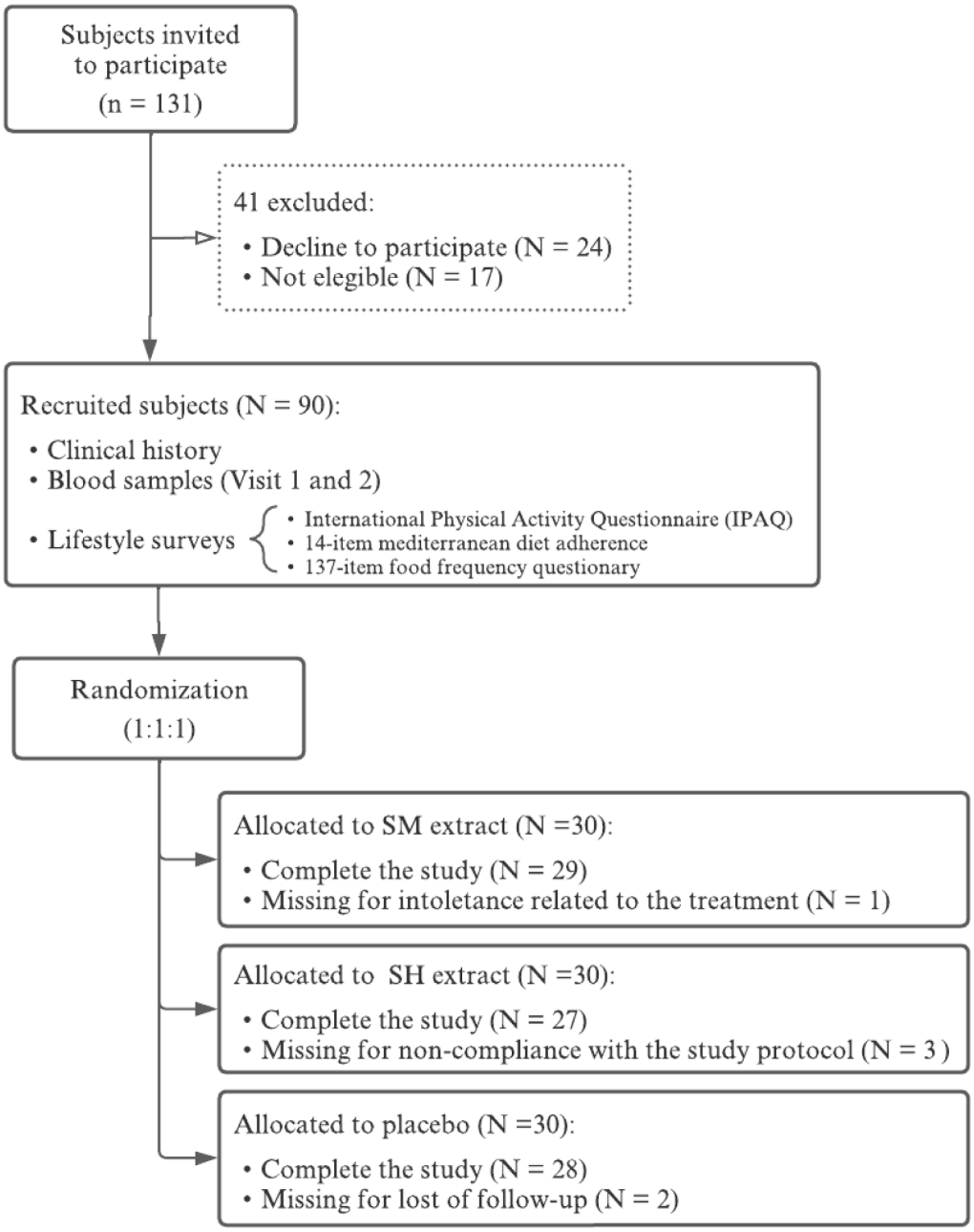


Fig. 1. Study flowchart.

significant differences appeared for glomerular filtration rate (110, 100, 96.5 ml/min, p = 0.013) and sodium (139, 140 and 141 mEq/L, p = 0.001). Pairwise analysis revealed that SM treatment increased glomerular filtration rate (110 vs 96.5 ml/min, p = 0.02) and reduced sodium (139 vs 141 mEq/L, p = 0.0005) and LDL cholesterol (94 vs 119.5 mg/dl, p = 0.024) levels compared to placebo.

Additionally, we performed intra-group analyses and observed that treatment with both extracts (SM and SH) for 3 months increased glomerular filtration rate and decreased homocysteine. In addition, SM extract increased potassium levels and reduced gamma glutamyl transferase (GGT) (Supplementary Fig. 2).

3.4. Nutritional and physical activity data assessment

The 14-item Mediterranean diet adherence and 7-item IPAQ questionnaires revealed that most study participants (83.3 %) had good adherence to the Mediterranean diet, with a mean score of 9.74, and moderate (36.6 %) or high (37.7 %) level of physical activity. Participants were also asked about their consumption of foods (cereals and grains, pulse, fruit, vegetables, dairy products, meat, fish, sweets, industrial bakery and olive oil) and frequency of servings during the twelve months prior to their recruitment into this study through the 137-item food frequency questionnaire. Baseline characteristics showed no significant differences concerning Mediterranean diet adherence, physical activity level or the total consumption of food categories between the three treatment groups (Table 5).

Estimated total energy and nutrient intake were calculated by the frequency of consumption and portion size of each food item. The daily intake of nutrients by subject in each treatment group is summarised in Supplementary Table 5. There were no significant differences in the median total energy consumed in subjects receiving the SM extract, SH extract or placebo, which were 2244.9, 2366.1 and 2141.45 Kcal/day, respectively. All treatment groups were also comparable with respect to their median for all other parameters except for vitamin B3-niacin

Table 2
Clinical assessment at baseline (visit 1) and after three months of treatment (visit 2) by groups. Median and interquartile range (IQR) are shown. Data were analysed using Kruskal Wallis test.

	Treatment			p-value
	SM extract Median (IQR)	SH extract) Median (IQR)	Placebo Median (IQR)	
Visit 1				
Body mass index (kg/m^2)	22.9 (21.4–25.03)	23.7 (21.45–25.78)	24.4 (22.75–27.03)	0.090
Systolic blood pressure (mm Hg)	112 (102.75–119.5)	113.5 (102.5–126)	107 (102.5–124.25)	0.491
Diastolic blood pressure (mm Hg)	74 (70.5–81)	80 (72.75–88.25)	75 (66.75–82.25)	0.166
Heart rate (bpm)	73 (65.75–83)	71 (59.75–77)	72 (64.75–77.25)	0.404
Visit 2				
Body mass index (kg/m^2)	23.1 (21.7–25.35)	24.1 (21.5–26)	24.35 (22.28–27.58)	0.322
Systolic blood pressure (mm Hg)	112 (98.5–130)	114 (107–129)	111 (101.25–120)	0.418
Diastolic blood pressure (mm Hg)	74 (69.5–84.5)	78 (73–90)	73 (68–76)	0.064
Heart rate (bpm)	74 (66.5–86.5)	73 (65–82)	74.5 (67–80)	0.601

Table 3
Related adverse and side effects by treatment group. Data were analysed using the chi-square test.

	Treatment			p-value
	SM extract n (%)	SH extract n (%)	Placebo n (%)	
Global	8 (26.67)	10 (33.33)	4 (13.33)	0.186
Gastrointestinal disorders	4 (50)	6 (60)	2 (50)	0.896
Gastroesophageal reflux	2 (25)	4 (40)	1 (25)	0.754
Constipation	1 (12.5)	0 (0)	0 (0)	0.400
Upset stomach	2 (25)	2 (20)	1 (25)	0.962
Nervous system disorders	0 (0)	2 (20)	1 (25)	0.36
Headache	0 (0)	2 (20)	1 (25)	0.36
Renal and urinary disorders	4 (50)	1 (10)	1 (25)	0.165
Coluria	1 (12.5)	0 (0)	0 (0)	0.400
Pollakiuria	2 (25)	0 (0)	0 (0)	0.146
Polyuria	1 (12.5)	1 (10)	1 (25)	0.756
Reproductive system and breast disorders	0 (0)	2 (20)	0 (0)	0.267
Hypermenorrhea	0 (0)	1 (10)	0 (0)	0.533
Decreased dysmenorrhea	0 (0)	1 (10)	0 (0)	0.533
Decreased breast tenderness	0 (0)	1 (10)	0 (0)	0.533
Vascular disorders	0 (0)	1 (10)	0 (0)	0.533
Mild hypertension	0 (0)	1 (10)	0 (0)	0.533

equivalents (40.88, 48.23 and 40.27 mg/day for SM extract, SH extract and placebo, respectively; p value = 0.025) and arachidonic acid n-6 (0.14, 0.17 and 0.13 g/day respectively; p value = 0.014).

3.5. Proteomic analysis of cardiovascular disease markers

In order to delineate the proteomic changes induced by supplementation with polyphenol-rich Salicornia extracts during 3 months, the levels of 92 proteins related to cardiovascular diseases were examined in plasma samples at baseline and the end of the study. Amongst the 92 proteins analysed, 1 protein had levels below the lower limit of detection and was discarded from further analysis.

To explore multivariate relationships, we performed dimension reduction exploration for nutritional and clinical variables First, nutritional data (67 variables) were subjected to Principal Component Analysis (PCA) to reduce the multivariate dataset. Of the resulting ten principal components, the first and second dimensions were retained, which explained 49.5 % of dataset variance (34.9 % and 14.6 % respectively) (Fig. 2A, upper panel). Therefore, the resulting dimension 1 and 2 include the variables that best represent data variability (Fig. 2A, bottom panel).

Secondly, Multiple Factor Analysis (MFA) analysis was used to identify which clinical and nutritional data modulated the effect of supplementation with Salicornia extracts on proteins related to vascular health. Visualisation of the variables along the first and second dimensions explained 11.2 and 9.9 % of the variance respectively, a total of 21.1 % (Fig. 2B upper panel) and were the components retained for the following analysis (Fig. 2B, bottom panel). These variables included parameters related to BMI (weight, height and BMI itself), age, systolic and diastolic blood pressure, heart rate, adherence to the Mediterranean diet and the percentage of protein and carbohydrates consumption.

To study the differential expression after three months of supplementation with SM and SH extract, all proteins were adjusted by visit and treatment using linear regression. In addition, clinical and nutritional covariates were introduced via MFA/PCA dimensions covariates adjustment (the first two dimensions were included in the analysis). Contrasts were performed on protein expression after 3 months and intra-patient correlation was estimated from the sample and applied in the linear modelling. Significant changes in protein expression were evaluated after adjustment by treatment group, nutritional or clinical principal components. Adjustment by treatment group and visit showed that treatment with SM extract for 3 months significantly altered 7 cardiovascular disease-related proteins (6 downregulated and 1 upregulated) compared to placebo. When nutritional adjustment was performed the number of significant changes increased to 11 proteins (10 downregulated and 1 upregulated) in the SM group and 1 protein in SH group versus placebo. However, after clinical adjustment was performed, only 4 proteins remained altered (3 downregulated and 1 upregulated) after SM treatment (Fig. 3A). A volcano plot was used to represent significance for serum markers after SM treatment in each adjustment (Fig. 3B).

To quantify order stability, we ranked all proteins according to evidence of association over 2000 bootstrap samples, obtaining a different ranking in each iteration. The proteins that after resampling have a lower classification interval, or in other words, their worst classification in the ranking has been higher, would point to expressions less prone to worsen on data perturbation, so its location is more stable. In the case of treatment with SM extract, MERTK was the protein that ranked better in the three adjustments. In addition to MERTK, there were seven proteins that were common to the three analyses and included SRC, TF, ADM, HAOX-1, PRSS27, GAL-9 and TNFRSF11A (Fig. 3C).

Since treatment with SM extract was associated with most of the changes in protein expression, we conducted a comparative analysis of the proteins significantly modulated by SM treatment and a functional enrichment analysis. Significant changes in 3 proteins were common to the three adjustments (MERTK, TF and SRC) while there were 4 proteins showing significant changes after adjustment by both visit and treatment and nutritional principal component adjustment, 3 proteins common to visit and treatment group and clinical adjustment and 1 protein showing changes after clinical and nutritional adjustments. In addition, significant changes in 3 proteins were exclusive to nutritional adjustment (Fig. 3D). Finally, enrichment analysis to study whether the changes identified in protein expression regulate specific pathways was performed using the STRING platform. Results showed that proteins altered after SM treatment were involved in biological processes associated to endothelial cells apoptosis and angiogenesis, the regulation of blood pressure and body fluid levels, blood coagulation, the response to stress and the regulation of cellular signaling through MAPK and PKB

Table 4
Biochemical profile at baseline (visit 1) and after three months of treatment (visit 2) by treatment group. Data were analysed using Kruskal Wallis test.

	Treatment			p-value
	SM extract Median (IQR)	**SH extract** Median (IQR)	**Placebo** Median (IQR)	
Visit 1				
Glucose (mg/dl)	77 (71.75–86)	81 (75.75–87.75)	83 (78–89)	0.050
Albumin (g/dl)	4.8 (4.65–5)	4.7 (4.5–4.8)	4.6 (4.48–4.8)	**0.017***
Total protein (g/dl)	7.45 (7.1–7.63)	7.15 (7–7.5)	7.1 (6.8–7.48)	**0.008***
Urea (mg/dl)	29 (25–36.25)	31 (26–37)	31 (26.5–39)	0.727
Creatinine (mg/dl)	0.77 (0.69–0.94)	0.79 (0.74–0.95)	0.82 (0.7–0.91)	0.773
Glomerular filtration rate (ml/min)	103 (89.75–118.5)	96.5 (89–105.5)	100.5 (89–111.25)	0.104
Cholesterol (mg/dl)	182.5 (160.75–209.75)	199 (177–241)	192 (170.75–208.25)	0.256
HDL Cholesterol (mg/dl)	70.5 (59.75–81.25)	62 (52.75–80.25)	61.5 (52.75–75)	0.080
LDL Cholesterol (mg/dl)	101.5 (86.75–115.5)	117 (99.75–146.5)	113 (97–135.25)	**0.018***
Triglycerides (mg/dl)	56.5 (50–79.5)	69 (52.75–84)	62 (47.5–101.5)	0.759
Gamma glutamyltransferase (GGT) (U/L)	15.5 (12.75–20.25)	16.5 (12–26.25)	17 (11.5–23)	0.796
Aspartate aminotransferase (AST) (U/L)	19 (15.25–21.25)	19 (15–21)	19 (15.75–23.25)	0.527
Alanine aminotransferase (ALT) (U/L)	12 (10–16.75)	13.5 (11.75–17)	16.5 (11.75–25)	0.103
Sodium (mEq/L)	139.5 (139–141)	140 (138–141)	140 (139–141)	0.928
Potassium (mEq/L)	4.25 (4.08–4.5)	4.3 (4.2–4.53)	4.5 (4.2–4.73)	0.057
C-reactive protein (CRP) (mg/L)	0.6 (0.3–1.15)	0.6 (0.4–1.55)	0.7 (0.48–1.53)	0.852
Homocysteine (mg/L)	12.45 (10.8–14.63)	12.75 (10.7–15.48)	12.15 (10.98–15.3)	0.903
Glycosylated hemoglobin (HbA1c) (%)	5.2 (5.1–5.3)	5.3 (5.2–5.5)	5.2 (5.08–5.43)	**0.038***
Thyroid-stimulating hormone (TSH) (U/ml)	1.48 (1.03–2.08)	1.58 (1.24–2.17)	1.47 (1.18–2.17)	0.670
Visit 2				
Glucose (mg/dl)	87 (81.5–90)	86 (81–92)	86.5 (81.5–93.25)	0.843
Albumin (g/dl)	4.6 (4.5–4.8)	4.6 (4.5–4.7)	4.6 (4.43–4.7)	0.660
Total protein (g/dl)	7.2 (7–7.45)	7.2 (6.9–7.5)	7.1 (6.7–7.48)	0.673
Urea (mg/dl)	27 (25.25–34.75)	31 (24–39)	32 (27.25–36.75)	0.466
Creatinine (mg/dl)	0.72 (0.65–0.89)	0.76 (0.7–0.88)	0.8 (0.7–0.96)	0.281
Glomerular filtration rate (ml/min)	110 (97.5–120)	100 (94–109)	96.5 (88.75–108.25)	**0.013***
Cholesterol (mg/dl)	178 (152.5–208.5)	197 (176–228)	199 (184.25–221.75)	0.081
HDL Cholesterol (mg/dl)	69 (54.5–80.5)	59 (53–79)	66 (54–72)	0.492
LDL Cholesterol (mg/dl)	94 (77–119)	122 (97–138)	119.5 (101–143.25)	**0.014***
Triglycerides (mg/dl)	62 (46.5–85.5)	74 (51–108)	63 (54–96.25)	0.685
Gamma glutamyltransferase (GGT) (U/L)	13 (9.5–18.5)	16 (13–34)	17 (11–24)	0.151
Aspartate aminotransferase (AST) (U/L)	18 (16–21.75)	18.5 (15.75–22)	19 (16–23)	0.882
Alanine aminotransferase (ALT) (U/L)	14 (11–19)	14 (12–22)	15.5 (11.25–26.25)	0.556
Sodium (mEq/L)	139 (138–141)	140 (139–142)	141 (141–143)	**0.001***
Potassium (mEq/L)	4.4 (4.2–4.6)	4.5 (4.2–4.7)	4.4 (4.3–4.5)	0.801
C-reactive protein (CRP) (mg/L)	1 (0.4–2.75)	1 (0.6–2.8)	0.95 (0.6–1.48)	0.946
Homocysteine (mg/L)	10.9 (9.55–13.5)	11.9 (10.4–13.2)	12.95 (10.85–15.95)	0.104
Glycosylated hemoglobin (HbA1c) (%)	5.2 (5–5.3)	5.4 (5.2–5.6)	5.2 (5–5.4)	**0.036***
Thyroid-stimulating hormone (TSH) (U/ml)	1.43 (1.08–2.65)	1.65 (1.28–2.23)	1.79 (1.11–2.45)	0.774

pathways (Fig. 3E).

4. Discussion

This randomized controlled clinical trial study shows for the first time that supplementation with polyphenol-rich ethanolic extracts of *S. ramosissima* in healthy volunteers for three months is safe, with no reported serious side effects. In addition, we showed potential effects in modifying some biochemical parameters, including an increase in glomerular filtration rate and a reduction of homocysteine and potassium blood levels, as well as biomarkers of cardiovascular damage. Only one study investigated Salicornia oral tolerability, which focused on evaluating the effect of a desalted ethanolic extract of *S. europaea L.* on cognitive performance. Although no differences in the primary outcome were observed in that study, the frequency of adverse events between treatment and placebo groups did not differ after 12 weeks of treatment with 600 mg/day (Lee, Shin, Kim, Kweon, & Kim, 2020). In our study, a higher dose during 3 months did not result in any significant side effects or pathological alterations. However, the polyphenols quantification or phenolic composition of *S. europaea L.* extract was not reported, which restricts our ability to make comparisons.

The mean MEDAS score for all the participants recruited (9.74) was higher than that reported for the general adult Spanish population in previous studies (Leon-Munoz et al., 2012; Martinez-Gonzalez et al., 2012; Quarta et al., 2021; Vidal-Peracho et al., 2017). This may be related to the fact that our study excluded people with cardiovascular risk factors that were present or even an inclusion criterion in other studies. According to the results of MEDAS, a high percentage of subjects have adequate adherence to the Mediterranean diet (defined as a score of ≥ 9). This is consistent with previous studies that have related a good adherence to the Mediterranean diet to a higher level of education, the absence of smoking habits, and a higher level of physical activity (Hu et al., 2013; Schroder et al., 2011), which are predominant in our study subjects.

In our study, the main side effects reported were related to gastrointestinal alterations (especially when the capsules were consumed on an empty stomach) and urinary alterations (mainly reported by patients as polyuria), but these were not significantly different from controls, and need to be monitored in future trials. A previous study using a different species of Salicornia, *S. europaea L.,* showed similar results and only mild adverse events such as gastroesophageal reflux and dyspepsia were reported (Lee, Shin, Kim, Kweon, & Kim, 2020). Urinary alterations, which were mainly reported in the SM treatment group, could be partially explained by the effects of the hydroxycinnamic acids present in the plant. Several compounds such as chlorogenic acid (Angappan, Devanesan & Thilagar, 2018) or caffeic acid (Veeren et al., 2021) have shown to play a role in the regulation of diuresis and can also be found in other foods such as artichoke (Ben Salem et al., 2015). In addition, the

Table 5
Physical activity level, adherence to the Mediterranean diet and daily food groups intake by participant during the 12 months prior to their inclusion in the study assessed by the 7-item IPAQ questionnaires, 14-item Mediterranean diet adherence, and 137-item food frequency questionnaire (PREDIMED), respectively. Data were analysed using ([1]) chi-square or ([2]) Kruskal Wallis test.

	SM extract n (%)	SH extract n (%)	Placebo n (%)	p value
Physical activity level[1]				
Low	9 (30)	9 (30)	5 (16.67)	0.138
Moderate	12 (40)	13 (43.33)	8 (26.67)	
High	9 (30)	8 (26.67)	17 (56.67)	
Adherence to Mediterranean diet[1]				
Low Adherence	7 (23.33)	3 (10)	5 (16.67)	0.383
Good Adherence	23 (76.67)	27 (90)	25 (83.33)	
	Median (IQR)	Median (IQR)	Median (IQR)	
Daily food intake[2]				
Sum of vegetables (g/day)	291.06 (178.92–397.21)	285.18 (210.56–393.12)	206.24 (154.51–372.29)	0.242
Sum of fruits (g/day)	273 (149.74–361.59)	268.22 (181.3–473.25)	264.35 (111.93–463.84)	0.836
Sum of pulses (g/day)	25.2 (16.8–35.61)	24.84 (20.46–29.22)	20.46 (16.08–32.5)	0.526
Sum of cereals (g/day)	164.07 (126.12–241.73)	133.01 (110.71–201.8)	142.74 (91.79–190.47)	0.247
Sum of whole grains (g/day)	33.9 (5.03–75)	10.5 (0–75)	11.51 (0–52.35)	0.134
Sum of dairy products (g/day)	266.55 (142.48–343.33)	281.61 (113.38–449.46)	233.14 (95.07–363.62)	0.973
Sum of meat and meat products (g/day)	124.55 (104.59–172.47)	161.61 (128.68–226.1)	140.65 (79.34–173.36)	0.071
Total olive oil (g/day)	25 (12.8–30.34)	25 (10–25)	25 (10–32.8)	0.646
Sum of fish (g/day)	107.51 (80.92–129.39)	101.07 (76.72–159.97)	80.89 (53.86–134.32)	0.248
Sum of biscuits, cakes and sweets (g/day)	21.74 (12.33–37.39)	14.74 (8.04–34.34)	17.42 (9.77–35.05)	0.669
Sum of industrial bakery products (g/day)	9.38 (4.69–19.91)	10.05 (3.35–16.75)	10.2 (6.2–19.4)	0.882

increase in glomerular filtration rate after treatment with Salicornia extracts could be related to the urinary disturbances reported by the subjects, especially polyuria. Many plants with diuretic activity associated with their polyphenol content (Masood et al., 2023; Nirumand et al., 2018; Paltinean et al., 2017; Schlickmann et al., 2018) would support this association. The loss of water leads to a passive loss of sodium by osmosis, which could explain the decrease in sodium levels in the blood in the SM group. Furthermore, the increase in blood potassium in the SM group is consistent with this finding.

Salicornia, growing in saltwater areas, has a high mineral content (Barreira et al., 2017) and each dose of active treatment in our study contained 0.58 and 0.63 g of salt for SH and SM, respectively. Therefore, one of our main concerns was its potential interference with blood pressure values. In our pilot trial, blood pressure values were not altered in the participants after 3 months of treatment. This finding is supported by preclinical data showing that in animals predisposed to hypertension *S. europaea* extract administration was associated with significantly lower mean arterial pressure and did not induce vascular dysfunction (Panth, Park, Kim, Kim, & Oak, 2016). This is partly explained by the potassium content of the plant, which has been identified as a deficient nutrient, and whose increase in the diet has a potential benefit in reducing the risk of hypertension (Stone, Martyn, & Weaver, 2016). Interestingly, potassium levels rose in the SM treatment group without reaching hyperkalemia. Hypertension is the main risk factor for cardiovascular diseases and diuretics are often used as first-line treatment for hypertension due to their vascular action (Arumugham & Shahin, 2024). On the other hand, the protective effect of potassium on blood pressure is widely known (Gallen et al., 1998; Guo et al., 2017) and may have a cumulative benefit on blood pressure.

An important analytical finding was the reduction in homocysteine levels in SM and SH treatment groups. Homocysteine has deleterious effects on vascular health, as it is linked to endothelial dysfunction and extracellular matrix proliferation that may cause vessel damage (Pinzon, Wijaya, & Veronica, 2023). Previous studies on dietary supplements in stroke prevention have shown that only folic acid supplementation with or without vitamin B12 is effective for this purpose (An et al., 2022). Interestingly, homocysteine increases the risk associated with hyperlipidemia, including LDL (Daly et al., 2009). The causal relationship between low LDL levels and reduced risk of cardiovascular disease is well known (Cziraky, Watson, & Talbert, 2008). After three months of treatment, no change was observed in the SH group but LDL levels were significantly reduced in the SM extract treatment group compared to placebo. In this sense, the presence of higher levels and wider variety of caffeoylquinic acid derivatives in SM extract could explain this effect given that these compounds have been attributed with anti-hyperlipidemic effects (Zhang et al., 2013; Huang, Liang, Zhong, He & Wang, 2015). Phenolic acids could be also behind this phenomenon, due to their potential lipid-lowering effect (Sun et al., 2021). In fact, *S. arabica* extract has been recently reported to have anti-dyslipidemic effects in mice receiving a high fat diet (Chrigui et al., 2023). Salicornia has also been associated with the control of obesity through its anti-adipogenic properties, producing a weight reduction in a rat model of high fat diet-induced obesity (Rahman et al., 2018). However, we found no changes in BMI in our study, probably due to the administration of the treatment to subjects within a healthy weight range.

In the SM extract treatment group, we documented a decrease in the enzyme GGT. Elevated serum GGT levels are associated with an increased risk of hypertension, type 2 diabetes and cardiovascular disease, and are an independent marker of oxidative stress, antioxidant insufficiency and inflammation. Furthermore, oxidative stress and inflammation are key components in the progression of atherosclerosis, which, like atherosclerotic plaque instability, has also been directly linked to elevated GGT. In this sense, epidemiological studies reported an inverse relationship between dietary polyphenol intake and blood GGT levels (Lee, Steffen, & Jacobs, 2004; Taguchi, Kishimoto, Kondo, Tohyama, & Goda, 2018).

Treatment with SM significantly reduced plasma levels of Galectin-9 (Gal-9), Tyrosine-protein kinase Mer (MERTK), Interleukin-18 (IL18) and Angiopoietin-1 receptor (Tie-2). High levels of these biomarkers have been related to diabetes development (Fischer, Perstrup, Berntsen, Eskildsen, & Pedersen, 2005; Hennings et al., 2016; Kurose et al., 2013; Su et al., 2024; Thorand et al., 2005). In addition, the reduction in Gal-9 and Tie-2 levels was associated with increased GFR (Hennings et al., 2016; Kurose et al., 2013), which is in line with our results. In this sense, polyphenols have been shown to modulate TIE2 expression. Wen et al documented that resveratrol treatment in a rat model of type 1 diabetes

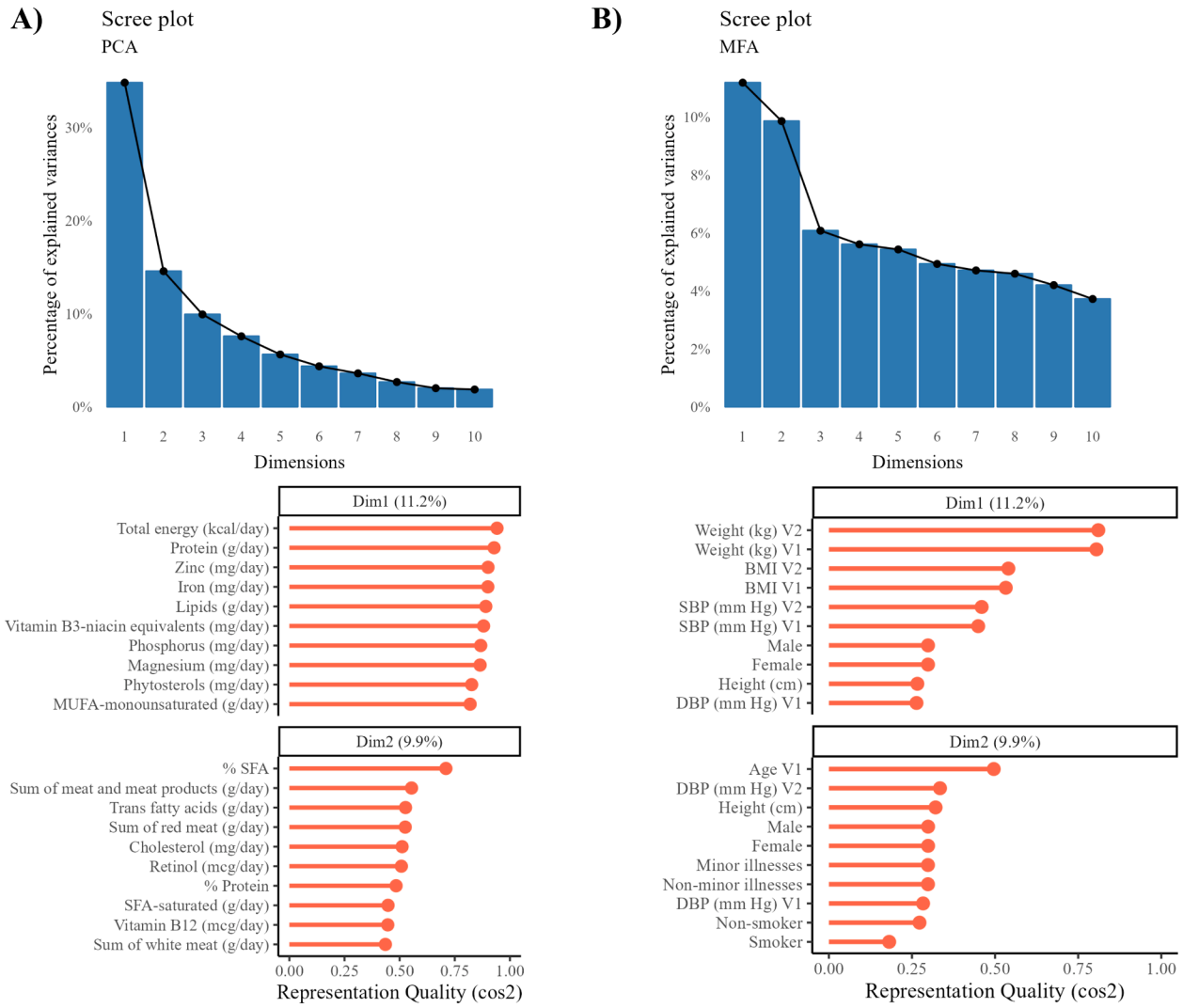


Fig. 2. Explorative dimensional reduction analysis. Percentage of variance explained by each dimension in Multiple Factor Analysis (A) and Principal Component Analysis (B) and the variables represented on Dimension 1 and 2.

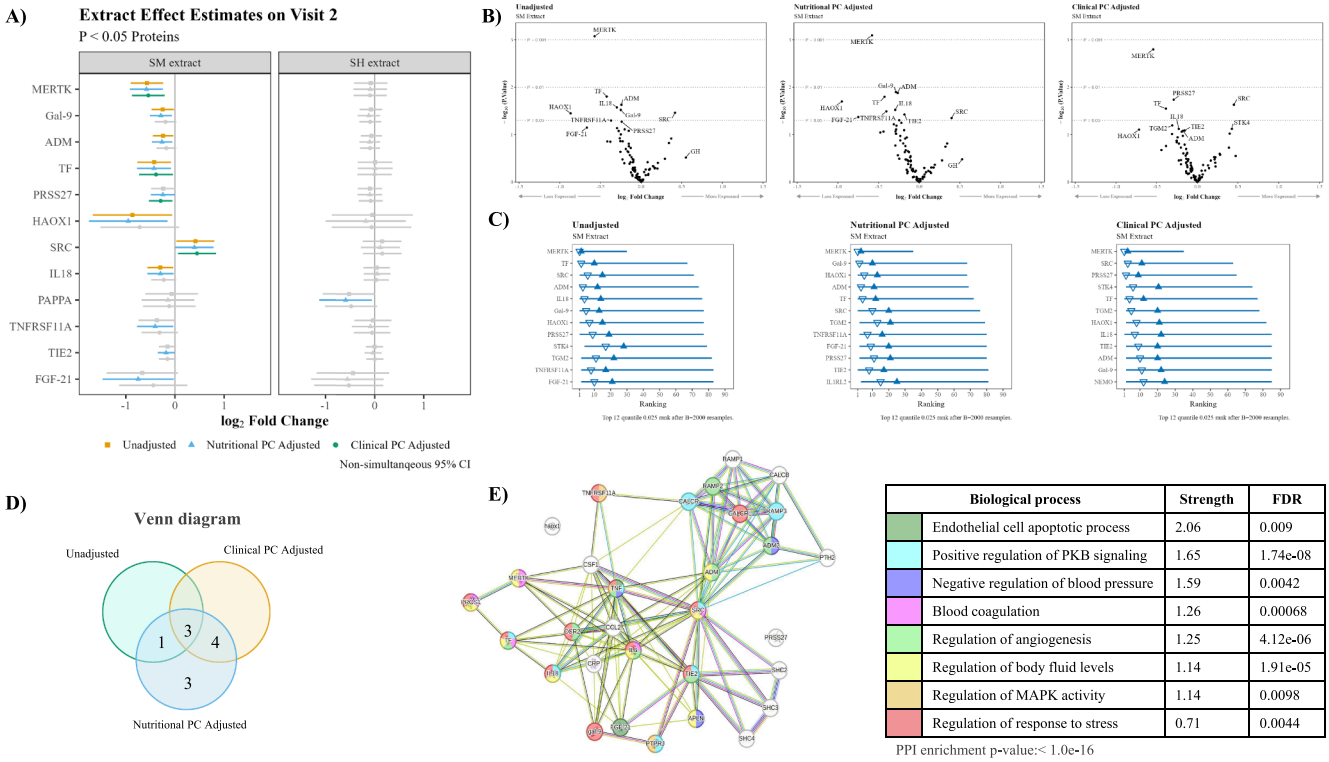
reduced TIE-2 levels in the renal cortex (Wen et al., 2013).

Studies have also established a positive association between hypertension and elevated blood levels of Adrenomedullin (ADM) (Wong, Cheung, & Cheung, 2012) and Tissue Factor (TF) (Felmeden et al., 2003), which were reduced after treatment with SM extract. Preclinical work showed that a polyphenol-rich extract was able to reduce ADM levels in rats with chronic heart failure (Cheng et al., 2020). Moreover, different grape extracts with high polyphenol content showed to inhibit TF synthesis *in vitro* (Carrieri et al., 2013). Interestingly, TF plays a role in blood coagulation and elevated levels increase the risk of thrombotic events (Mackman, 2004). *Rheum rhaponticum and R. rhabarbarum* plant extracts reported a significant reduction of TF-induced coagulation (Liudvytska et al., 2023). Circulating levels of FGF-21 are associated with a higher risk of developing atherosclerosis (Chow et al., 2013). A controlled clinical trial in healthy volunteers reported a significant reduction in serum FGF-21 values after consuming of 5 g of fresh grapes per kg of body weight for 21 days (Notarnicola et al., 2022).

The only upregulated marker after treatment with SM extracts was proto-oncogene tyrosine-protein kinase (SRC), which has an important role in the migration of endothelial and tubulogenic cells required in angiogenesis. Despite some polyphenols showing to deactivate SRC during proinflammatory stimuli (Li et al., 2022; Socodato et al., 2015), 3-Hydroxytyrosol, a phenolic alcohol found in olive oil and widely described in the literature for its cardioprotective properties has shown to promote angiogenesis *in vitro* through the activation of SRC (Abate et al., 2020). In fact, the promotion of angiogenesis through this route and subsequent increased cerebral blood flow can be effective in mitigating ischemic lesions (Kanazawa et al., 2019).

The vascular and metabolic beneficial effects of Salicornia are possibly the best known. Regarding cellular processes involved in vascular disorders, it was shown that a Salicornia extract could suppress platelet-derived growth factor (PDGF)-induced migration of vascular smooth muscle cells by down-regulating p38 MAPK and ERK1/2 phosphorylation, leading to reduced neointimal hyperplasia during vascular remodeling (Won et al., 2017). Moreover, numerous preclinical studies have attributed anti-dyslipidemic, anti-inflammatory, anti-hyperglycemic, and other effects related with cardiovascular health to different species of Salicornia (Nájar, Romero-Bernal, Del Rio, & Montaner, 2023). Accordingly, preclinical studies in animal models of ischemia in our laboratory pointed out neurovascular benefits, achieving reductions in infarct volumes in those animals that had been previously treated with an ethanolic extract of *S. ramosissima* (Garcia-Rodriguez et al., 2022). Thus, these extracts might be a useful dietary supplement to avoid abnormal vascular events that could precede stroke, such as vascular dysfunction and remodeling, and clinical studies evaluating their safety and efficacy in humans were needed.

Interestingly, the only protein altered by treatment with SH extract is not modified by the SM treatment. Pregnancy associated plasma protein-



Biological process	Strength	FDR
Endothelial cell apoptotic process	2.06	0.009
Positive regulation of PKB signaling	1.65	1.74e-08
Negative regulation of blood pressure	1.59	0.0042
Blood coagulation	1.26	0.00068
Regulation of angiogenesis	1.25	4.12e-06
Regulation of body fluid levels	1.14	1.91e-05
Regulation of MAPK activity	1.14	0.0098
Regulation of response to stress	0.71	0.0044

PPI enrichment p-value:< 1.0e-16

Fig. 3. Effect of *S.ramosissima* supplementation on cardiovascular health biomarkers in plasma. Differential expression and significant effects of SM and SH treatments adjusted by clinical PC, nutritional PC and by visit and treatment (A). Volcano plots showing the results of differential expression analyses by clinical PC, nutritional PC and by visit and treatment (B). Sensitivity analysis plots show the ranking of proteins differentially expressed according to the absolute value of t-statistic contrast after 2000 bootstrap iterations. Non-filled triangles represent apparent results from our data, filled triangles and blue lines represent the median and the interval between the quantile 0.025 and the quantile 0.975 of resample rankings (C).Venn diagram illustrating the proteins altered in each of the different adjustments (D). Enrichment analysis of proteins altered after SM treatment for 3 months in healthy subjects and functional classification of proteins by STRING platform (E).

A (PAPPA) is a proteolytic enzyme that increases Insulin-like growth factor-1 (IGF-1) availability. IGF signaling contributes to atherosclerotic plaque progression, inflammation and lipid uptake, and its reduction was associated with increased lifespan and healthspan. Therefore, the inhibition of PAPPA has been suggested as a therapeutic approach to indirectly decrease IGF signaling and promote healthy longevity (Conover & Oxvig, 2017).

We identified several plasma markers of cardiovascular damage that are differentially expressed after three months of treatment with Salicornia extracts. The most significant changes were observed in the group supplemented with SM extract, which is in agreement with the greater effect on biochemical parameters observed in the SM extract group. SM showed a higher amount of total phenolic content and the presence of several compounds that are exclusive to this extract. Among them, caffeoylquinic acid derivatives present in SM been reported to modulate several pathways including MAPK (Hwang et al., 2009) and may inhibit platelet activation by neutralising reactive oxygen species, blocking cyclooxygenase enzymes, and reducing P-selectin expression (Park, 2015). In addition, isorhamnetin glucoside, which is present in the SM extract to a much greater extent, has also been reported to play a role in atherosclerotic responses in vascular smooth muscle cells and modulate MAPK signaling (Won et al., 2017). The presence of betavulgaroside II, IV, and VIII in SM extracts is notable, as these compounds are absent in SH extracts. Only one in vivo study has been found on betavulgaroside I, II, III, and IV, which reported their hypoglycemic activity in an oral glucose tolerance test in rats (Yoshikawa et al., 1996). However, we did not find antihyperglycemic effects of SM extract in our cohort. Due to the wide variety of compounds present in the extract and the potential synergistic effects among them, it is difficult to ensure that the clinical improvements are solely due to these polyphenols. It would be necessary to design new studies using these compounds individually.

Among the limitations of the study is the length of treatment. Three months of treatment does not allow us to obtain data on potential long-term side effects, and it may also be too short to get certain efficacy data. In addition, despite randomization, the groups were not age balanced which could limit some of the findings. Nevertheless, this limitation has been minimized with intra-group variability studies.

5. Conclusions

We can conclude that dietary supplementation with 1 g of two different *S. ramosissima* extracts for 3 months in healthy volunteers is safe. A total of 41 and 31 compounds were detected in SM and in SH, respectively. Major compounds were chlorogenic acid derivatives and triterpenoids. Treatment with SM resulted in a higher glomerular filtration rate, lower LDL cholesterol, and modulation of cardiovascular disease-related plasma markers. Specifically, MERTK, Gal-9, ADM, TF, PRSS27, HAOX1, IL-18, PAPPA, TNFRSF1A, TIE2, and FGF-21 proteins were downregulated, while SRC levels were upregulated. Compounds of greater abundance or exclusive to SM that could explain this effect include caffeoylquinic acid derivatives, betavulgarosides and isorhamnetin glucoside. Treatment with SM for three months has modified some analytical and proteomic markers related to improved vascular health. However, further studies are needed to prove their individual or synergic effect and to analyse SM safety in vascular disease conditions and to investigate its efficacy in larger samples and for a longer period.

Ethics statement

The work has been carried out in accordance with the Code of Ethics

of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The study was registered at clinicaltrials.gov (NTC06076122) and was approved by the Andalusian Ethics Committee (ID HALOFITAS) on February 18, 2022. Written informed consent was signed by the volunteers, and participants were able to withdraw from the study at any time without giving a reason.

CRediT authorship contribution statement

Ana M. Nájar: Writing – original draft, Investigation, Formal analysis, Data curation. **Soledad Pérez-Sánchez:** Writing – original draft, Resources, Methodology, Formal analysis, Conceptualization. **Carmen del Río:** Investigation, Data curation, Writing – original draft. **Carmen Domínguez:** Investigation, Data curation. **Cristina López Azcárate:** Investigation, Data curation. **Reyes de Torres:** Investigation. **Marcel Lamana-Vallverdú:** Formal analysis. **Marina Romero-Bernal:** Investigation. **Ángela González-Díaz:** Investigation. **María de la Luz Cádiz-Gurrea:** Investigation. **Francisco Javier Leyva-Jiménez:** Investigation. **Álvaro Fernández-Ochoa:** Investigation. **Antonio León:** Investigation. **Joan Montaner:** Writing – original draft, Resources, Methodology, Funding acquisition.

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Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2024.106539.

Data availability

Data will be made available on request.

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