

QUALITY ASPECTS TO CONSIDER IN FLAXSEED AS A VALID OMEGA-3 DIETARY SUPPLEMENT: A RANDOMIZED CONTROLLED TRIAL

Vanessa M García-Hernández¹, Marta Beltrá-García-Calvo¹, Joaquín Sánchez-Soriano², María A Iborra-Campos¹, Marina Cano-Lamadrid³, Ángel A Carbonell-Barrachina³, Enrique Roche^{1,4,5}, Elena García-García*¹

Address(es): Dra Elena García-García

¹ Department of Applied Biology-Nutrition and Institute of Bioengineering, Miguel Hernández University, Elche, Alicante (Spain).

² U.I. Center of Operations Research (CIO), Miguel Hernández University, Elche, Alicante (Spain).

³ Group of Food Quality and Safety, Agro-Food Technology Department, University Miguel Hernández, Orihuela, Alicante (Spain).

⁴ Institute for Health and Biomedical Research (ISABIAL), Alicante, Spain.

⁵ CIBER Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Instituto de Salud Carlos III (ISCIII), 28029-Madrid (Spain).

*Corresponding author: egarcia@umh.es

<https://doi.org/10.55251/jmbfs.5932>

ARTICLE INFO

Received 4. 3. 2022
Revised 6. 10. 2022
Accepted 28. 10. 2022
Published 1. 12. 2022

Regular article



ABSTRACT

Dietary supplements, based on essential polyunsaturated fatty acids, help in normalizing the circulating lipids in overweight people. These fatty acids are abundant in fish oils and certain vegetable seed oils. In this context, the fatty acid profile of 9 commercial flaxseed trademarks was analyzed. Regarding fatty acid composition, α -linolenic acid represented more than 60% of the total fatty acids. In addition, sample 3 displayed the highest oxidative stability; thus, it was selected to be used in a pilot nutritional intervention study. The objective was to assess the hypolipidemic effects of flaxseed together with an isocaloric fish-rich diet. Two groups of women aged 25-70 years presenting high levels of circulating cholesterol ($> 200 \text{ mg dL}^{-1}$) and triglycerides ($> 150 \text{ mg dL}^{-1}$) participated. Group F consumed flaxseed, while group D did not. At the end of intervention, participants from group F presented significantly reduced total cholesterol and lower systolic and diastolic pressure as compared to group D. Triacylglycerols decreased in both groups with respect to initial values, suggesting a role of diet. The flaxseed trademark is an influencing variable in its fatty acid composition and flaxseed supplementation combined with a fish-rich diet can help to reduce blood lipid parameters.

Keywords: Cardiovascular risk, fish-rich diet, flaxseed oil, flaxseed supplementation, hyperlipidemia, α -linolenic acid

INTRODUCTION

Flaxseed is an increasingly popular diet supplement due to the presence of 4 major components (fiber, lignans, protein and α -linolenic acid) with important nutritional interest (Ganorkar & Jain, 2013). Flaxseed presents a high percentage of fiber, both soluble:insoluble in a proportion between 20-40:80-60, respectively (Ganorkar & Jain, 2013). The second component is lignans, a type of phytoestrogen. Flaxseed contains one of the highest contents in plants (13 mg g^{-1} of whole seed) (Madhusudhan *et al.*, 2000). Protein could be considered as the third component due to its abundance in the seed (20-30%) with a biological value of 77.5% and a high digestibility of almost 90% (Ganorkar & Jain, 2013). Finally, flaxseed contains an outstanding amount of the essential fatty acid, α -linolenic (ALA, 18:3n-3) and other omega-3 fatty acids. This is a unique feature among the plant sources used for human nutrition. ALA and the other fatty acids (FAs) are present in the form of triacylglycerols (TG) stored in discrete oil bodies, which are distributed heterogeneously in all parts of the seed, including testa or hull together with endosperm. The oil component represents 35-45% of the seed weight, with ALA being approximately half of the total fatty acid content (Ganorkar & Jain, 2013).

On the other hand, the leading causes of death in the European Union are cardiovascular diseases, becoming a major health problem in modern societies. Prevention strategies are necessary to decrease the prevalence, high morbidity and mortality, the impact on the patient's quality of life and the associated cost for the public health systems. In this way, the accumulated evidences indicate that the consumption of omega-3 FAs, within a balanced diet, can reduce circulating cholesterol levels (Wang & Fu, 2017). Foods with a high content of omega-3 FAs are fish and seeds. The international recommendations for ALA intake are around 2 g per day or 0.5-1% of the total energy, which can be easily achieved by consuming 10 g daily of flaxseeds (Rodríguez-Leyva *et al.*, 2010). A meta-analysis shows that flaxseed administration in the diet seems to help in normalizing the lipid profile by reducing the contents of total cholesterol and the cholesterol bound to low density lipoproteins (LDL) (Pan *et al.*, 2009). The cholesterol lowering effects were more apparent in postmenopausal women and in individuals with high circulating cholesterol levels (Pan *et al.*, 2009). However, no effects

were observed using flaxseed or its derivatives on high density lipoproteins (HDL)-cholesterol or circulating TG levels (Ganorkar & Jain, 2013).

Altogether, these observations suggest that the use of flaxseed in foods could be an interesting option to supplement diets with polyunsaturated fatty acid (PUFAs). In this way, flaxseed could be used as a condiment or supplement in ready-to-eat cooked foods, salads and dairy products. The role of specific diets supplemented with flaxseed to lower circulating lipid levels has not been explored because the majority of the studies have been designed to maintain body weight regardless of the diet of the participants (Pan *et al.*, 2009). Nevertheless, omega-3 FAs display a high susceptibility to oxidation and when the oxidized forms are consumed, they produce an opposite effect to the expected hypocholesterolemic action (García-Hernández *et al.*, 2013).

Therefore, the aim of the present study was to analyze the PUFA composition of 9 trademarks of flaxseed as well as their stability to forced oxidation. After this first stage, the product with the best FA profile and resistance to oxidation will be selected to assess the effect of its consumption in an isocaloric fish-rich diet in a population of Spanish women with high circulating cholesterol and TG levels.

MATERIAL AND METHODS

To carry out this study, 9 commercial trademarks of flaxseed were purchased from Pharmacy Offices, Health Shops and Mall Stores distributed throughout the city of Elche (Alicante, Spain). Flaxseed trademarks are characterized by different presentations: (i) samples 1 to 5 are raw golden (golden flaxseeds are mostly cultivated in Dakota-USA, contain less fat and calories but a higher percentage of protein than brown flaxseeds); (ii) sample 6 is raw mix of brown (brown flaxseeds are mostly cultivated in Canada, contain more fat and calories than golden flaxseeds) and golden; (iii) sample 7 is a grinded mixture of brown and golden seeds; (iv) sample 8 comes from a milled mixture of brown and golden seeds; and, (v) number 9 comes from milled golden seeds. The oil extraction yield of these same trademarks was analyzed in a previous report (García-Hernández *et al.*, 2017/18).

Fatty acid analysis

Flaxseed oil was obtained from ground using the Soxhlet method and petroleum ether as described previously (García-Hernández et al., 2017/18). The fatty acid composition of the obtained oils was immediately analyzed by gas chromatography-mass spectrometry (GC-MS) after methylation of the fatty acids into their corresponding methyl esters (FAMES) with some modifications. Basically, 30 mg of hydrolyzed flaxseed were transferred into a test tube with 20 mg of C17:0 n-hexane solutions (1 mL) as internal standard. Then, 100 µL of dichloride methane and 1 mL of 0.5N NaOH in methanol were added. The tubes were heated in a water bath at 90°C for 10 min, and 1 mL of BF₃ in methanol was added and the mixture was left at room temperature (25°C) for 30 min to prevent intrasomerization of conjugated linoleic acid isomers (Calín-Sánchez et al., 2011). Finally, 1 mL of distilled water and 600 µL of hexane were added. Then, FAMES were extracted by vigorous shaking for about 1 min, following centrifugation (3000 rpm, 5 min). Aliquots were dried with sodium sulfate anhydrous, and the top layer was transferred into a vial flushed with N₂ and stored at -20°C until GC analysis.

The identification of the FAMES was done in a SHIMADZU GC-17A (Shimadzu Corporation, Kyoto, Japan), coupled with a SHIMADZU mass spectrometer detector GC-MS QP-5050A. The GC-MS system was equipped with a TRACASIL Meta.X5 column, 95% dimethyl-polsiloxane and 5% diphenyl-polysiloxane (Teknokroma S. Co. Ltd., Barcelona, Spain) 30 m × 0.25 mm i.d. × 0.25 µm film thickness. Analyses were carried out using He as carrier gas at a flow rate of 0.9 mL min⁻¹ in a split ratio of 1:10 and a program: (a) initial temperature 80°C hold for 2 min, (b) rate of 8°C min⁻¹ from 80 to 160°C, (c) rate of 4°C min⁻¹ from 160 to 240°C and hold for 30 min. Injector and detector were held at 250 and 300°C, respectively. 2 µL of the extracts were injected (Andreu-Coll et al., 2019). FAME peaks identification was performed by comparing the retention times of the FAME SUPELCO MIX-37 standards. Analyses were run in triplicate.

FAME quantification was done using a HEWLETT-PACKARD 6890 gas chromatograph equipped with a flame ionization detector (FID) and a DB-23 capillary column (30 m length × 0.25 µm film × 0.25 mm internal diameter) (J&W Scientific, Agilent Technologies, Santa Clara, CA, USA). The flow rate of the carrier gas (He) was 1.2 mL min⁻¹ and 35 mL min⁻¹ at the make-up point. The injector and detector temperatures were 200 and 220°C, respectively. The injection volume was 0.5 µL (splitless). The temperature program was as following: initial temperature 70°C, temperature from 70 to 190°C at 10°C min⁻¹ for 20 min, from 190 to 240°C at 5°C min⁻¹ and at 240°C held isothermally for 3 min. Identification of the FAME peaks was performed by comparing the retention times of the FAME standards (Calín-Sánchez et al., 2011). AGILENT TECHNOLOGIES software (G2072AA Rev.A.05.02 Chemstation) was used for integration of peaks.

Oxidative stability of oils under accelerated conditions (Rancimat)

The Rancimat method is included in different national and international standards (AOCS, 2017). The accelerated oxidation process occurs by heating the reaction vessel while continuously passing air through the sample. This test was run in triplicate using a METROHM Rancimat equipment, model 743 (Metrohm, Switzerland) with 2.5 g of flaxseed oil. The sample placed in the reaction vessel was heated and air was continuously circulating at a rate of 20 L h⁻¹ and 120°C. Fatty acids were decomposed and the volatile organic acids formed were transported by the air stream into a measuring vessel containing distilled water where the electrical conductivity was measured continuously. The test was stopped when conductivity increased sharply.

Statistics for flaxseed composition analysis

Statistical analysis was carried out using STATGRAPHICS Centurion XV software (StatPoint Technologies, Inc Warrington, VA, USA). Significance was established at $p < 0.05$. Data were reported as mean ± SD (standard deviation). Inter-group comparisons were performed by one-way analysis of variance, as well as HSD Tukey's multiple range test to determine which means were significantly different from the others.

Study of flaxseed consumption

Participants

The recruitment process began in November 2018. The intervention was carried out in March 2019 and lasted 30 days, with a subsequent monitoring of participants, ending in April-May 2019. Participant flow diagram is indicated in Figure 1. Data from 133 women were processed by an initial test consisting of 3 brief questions to be able to include them in the study: (i) age > 24, (ii) presence of high circulating cholesterol and TG levels, and (iii) participant is not currently taking any medication to control these levels. Individuals that met the inclusion criteria for the study were eligible (n= 133), and were all women aged between 25 and 75 years, with dyslipemia, no indication of drug treatment, blood cholesterol levels above 200 mg dL⁻¹. Exclusion criteria were pregnancy and lactation, participation in another diet-supplement program, and cholesterol and TG levels

over 300 mg dL⁻¹. According to these criteria, a total of 69 women were excluded. The location where the women were recruited for the study was a pharmacy in the city center of Elche (Alicante, Spain); Elche is an industrial city located in the southeast of Spain, with a population of 230,000 inhabitants. Selected volunteers that agreed to participate in the study signed a written consent. The study was in accordance with Helsinki Declaration for research on human beings and approved by the Ethics Committee of University Miguel Hernández (Reference number: IB-EGG-01-13). Volunteers were monitored using a survey on dietary intake and provided by a follow-up form for possible incidents and a non-compliance form if they wished to abandon the study. Weight and blood pressure were assessed on-site at the pharmacy office under standard conditions (García-Hernández et al., 2013). Total cholesterol, TG and glucose levels were measured in capillary blood with a Rapid Control Gauge on a REFLOTROTRON[®] PLUS desktop analyzer with serial number 5072278 (Roche, Barcelona, Spain). Fasting blood samples were obtained by capillary puncture between 9:00 and 11:00 am, at the beginning (day 1) and end (day 30) of the study.

Trial design

The assignment of women to the different groups was carried out following the classification criteria established by the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. (2021). Women were randomly double-blindly assigned to one of the two parallel groups, in a 1:1 ratio by the research team. One group (n=32) took 15 g of flaxseed daily, mixed in 125 mL of skimmed yoghurt in the context of an isocaloric fish-rich diet (flaxseed group or F group). A second group (n=32) was recruited as a comparison control group and consumed daily 125 mL of skimmed yoghurt in the context of the same diet (diet group or D group). Therefore, the design was a parallel group, randomized controlled trial, double-blind (participants and contact scientists were unaware who was taking the flaxseed or not) conducted at the Miguel Hernández University (Elche, Spain).

Estimation of sample size

Since the main interest was detecting sensitive variations in each of the variables measured of the lipid profile within each group after 1 month following flaxseed intake and diet, it was considered a big (Cohen) effect size (d=1), with a 2-sided 5% significance level and a power of 80%. Using these variables, the minimum sample size to use was 16 women per group. On the other hand, to detect sensitive variations in each of the secondary variables between the two groups, considering again a big (Cohen) effect size (f=0.5), with a 5% significance level and a power of 80%, the number obtained was 17 women per group. The number of volunteers at the end of the study fitted with these theoretically calculated values: n=30 for F group and n=18 for D group.

Intervention

During a 1-month intervention period, all participants attended in-person training sessions, receiving the flaxseed package (only F group), instruction manuals that included a personal meal plan indicating the brand of skimmed yoghurt to purchase, recipes and information regarding physical activity. Body weight (kg) and blood pressure (mmHg) were measured under standard conditions. The energy intake of the diet designed was similar in the two groups (ranging 1900-2000 kcal per day), used on a previous study (García-Hernández et al., 2013). The daily energy expenditure was calculated according to Harris-Benedict equation considering basal metabolism values plus an estimation for moderate daily physical activity (30-35% of basal metabolism).

Concluding guidelines

The only rule considered to prematurely conclude the study was in the case that there was a drastic decrease in sample size with respect to the necessary number of participants in order to obtain statistically significant results (see below). In addition, particular cases were excluded from the study (n=2 in group F and n=14 in group D). The reasons for leaving the study in group F (2 people for personal reasons) and in group D (2 people for pregnancy, 5 for protocol violation and 7 for personal reasons). Therefore, at the end of the intervention period the distribution of participants was as follows: n=30 for group F and n=18 for group D (Figure 1).

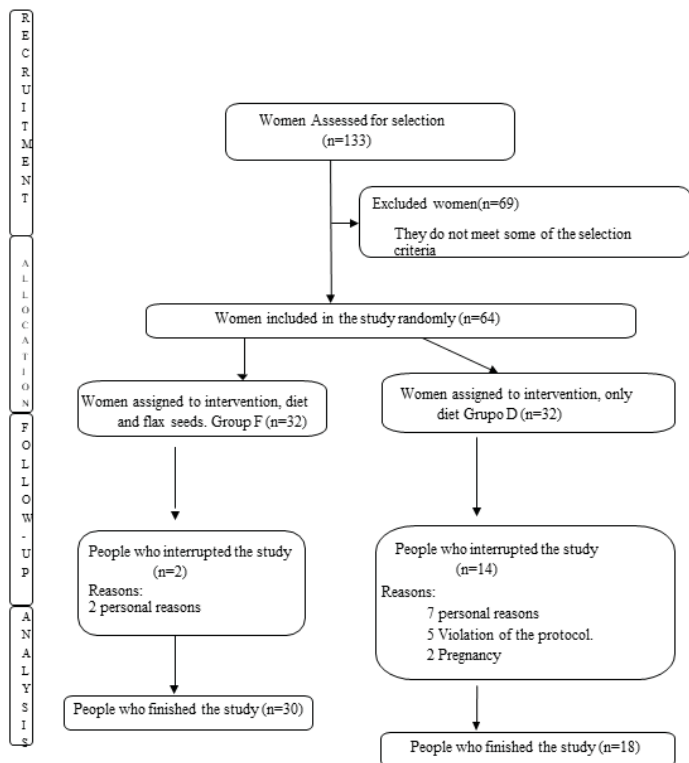


Figure 1 Participant's flow diagram

Statistics for data analysis from intervention

Statistical analysis was carried out using STATGRAPHICS Centurion XV software mentioned before; likewise, significance was defined at p< 0.05. Data are reported as mean ± standard error of the mean (SEM). The homogeneity between the initial conditions of glucose, cholesterol, weight, systolic pressure and diastolic pressure of both groups were performed using the following parametric and non-

parametric tests: t-test, W-test and K-S-test, the first for the difference of means, the second for the difference of medians and the last one for the difference of distributions. Intra-group statistical comparisons were performed using the following hypothesis tests, both parametric and non-parametric: t-test, sign test and signed rank test for paired samples.

RESULTS AND DISCUSSION

Composition of fatty acids from flaxseed oils

Flaxseeds have a woody structure and for this reason samples must be crushed previously, to allow a greater area of contact between the solid material and the solvent. The resulting small particles allow direct contact solvent-oil. This explains why sample 9 gave the highest extraction performance, because the milling allowed smaller particle sizes that facilitated oil solvent contact.

The chromatographic analysis of the oils from seed samples allowed identifying 10 FAMES:

- Saturated fatty acids (SFAs) (n=6): myristic (C14:0), pentadecanoic (C15:0), palmitic (C16:0), stearic (C18:0), arachidic (C20:0) and behenic (C22:0) acids.
- Monounsaturated fatty acids (MUFAs) (n=2): palmitoleic (C16:1) and oleic (C18:1) acids.
- Polyunsaturated fatty acids (PUFAs) (n=2): linoleic (C18:2) and α-linolenic (C18:3) acids.

The order from highest to lowest amount of each FA, determined by GC-MS/FID, present in flaxseeds is (Table 1): α-linolenic (61.83 %) > palmitic (9.94 %) ≈ oleic (9.75 %) ≈ linoleic (9.67 %) > stearic (7.58 %) > arachidic (0.49 %) ≈ behenic (0.41 %) ≈ palmitoleic (0.14 %) ≈ myristic (0.11 %) ≈ pentadecanoic (0.07 %)

Significant differences (p<0.05) for all FAMES, except palmitic acid, were found. Regarding compounds with high nutritional interest, the content of linoleic acid in the different samples can be statistically ranked in 2 separate groups: {1, 3-9} ≤ {2}. The analysis for oleic acid resulted in 3 groups: {2} ≥ {3, 5} > {1, 4, 6-9}. Similar analysis for linolenic acid resulted in 4 groups: {2} ≥ {5, 6, 9} > {3, 4, 7, 8} > {1}. The average content of oil obtained from raw flaxseeds was 39.59 g per 100 g (García-Hernández et al., 2017/18). This content was slightly higher but close to that 36.6 g per 100 g previously reported (Khan, 2010).

Table 1 Fatty acid percentage values of the average of three measurements made in 9 flaxseed oil samples

FAME	Sample									Average
	1	2	3	4	5	6	7	8	9	
	(g kg ⁻¹)									
Myristic (C14:0)	0.14 ^a	0.12 ^a	0.11 ^a	0.08 ^b	0.08 ^b	0.12 ^a	0.12 ^a	0.14 ^a	0.09 ^{ab}	0.11
Pentadecanoic (C15:0)	0.08 ^{ab}	0.07 ^b	0.06 ^b	0.05 ^b	0.05 ^b	0.06 ^b	0.07 ^{ab}	0.09 ^a	0.06 ^b	0.07
Palmitoleic (C16:1)	0.18 ^a	0.12 ^b	0.11 ^b	0.10 ^b	0.11 ^b	0.20 ^a	0.15 ^{ab}	0.16 ^{ab}	0.09 ^b	0.14
Palmitic (C16:0)	10.9 ^a	9.11 ^a	9.95 ^a	8.84 ^a	8.87 ^a	10.2 ^a	11.0 ^a	11.7 ^a	8.84 ^a	9.94
Linoleic (C18:2)	6.92 ^d	15.2 ^a	8.73 ^c	8.77 ^c	10.7 ^b	9.16 ^{bc}	8.93 ^c	8.44 ^c	10.2 ^b	9.7
Linolenic (C18:3)	66.2 ^a	29.3 ^b	64.2 ^a	68.5 ^a	65.8 ^a	64.7 ^a	64.7 ^a	66.0 ^a	67.0 ^a	61.8
Oleic (C18:1)	4.84 ^c	35.1 ^a	8.95 ^b	6.48 ^c	7.28 ^{bc}	6.51 ^c	6.65 ^c	5.86 ^c	6.05 ^c	9.8
Stearic (C18:0)	9.47 ^a	9.93 ^a	7.13 ^b	6.45 ^b	6.47 ^b	8.29 ^{ab}	7.41 ^b	6.23 ^b	6.85 ^b	7.58
Arachidic (C20:0)	0.66 ^{ab}	0.52 ^{ab}	0.34 ^b	0.35 ^b	0.29 ^b	0.47 ^b	0.48 ^b	0.86 ^a	0.46 ^b	0.49
Behenic (C22:0)	0.61 ^a	0.48 ^{ab}	0.39 ^b	0.36 ^b	0.35 ^b	0.32 ^b	0.42 ^b	0.47 ^b	0.32 ^b	0.41

[†]Values (mean of 3 replications) followed by the same letter, within the same row, were not significantly different (p<0.05), Tukey's least significant difference test.

Oxidative stability

A high degree of unsaturation of the fatty acids makes the oils highly susceptible to oxidation. This is a key point, because in the nutritional intervention, it was planned to incorporate flaxseed into a yoghurt matrix, increasing thereby its susceptibility to oxidation (Jhala & Hall, 2010). As a result of oxidation, PUFAs and vitamins lose their biological properties and can even form toxic products under extreme conditions (Shim et al., 2015). The oxidative stability ranged between 9 and 140 min in samples 9 and 3, respectively (Table 2). Sample 3 was the most stable sample to oxidation and thus, it was the selected for the nutritional study. It is also important to highlight the high stability of sample 2, which presents high contents of the essential linoleic and linolenic acids, as well as oleic acid.

Nutritional intervention

Outcomes

The first objective of the nutrition study was to assess the effect of consuming 15 g of flaxseed (sample 3) mixed in yoghurt during breakfast and combined with a fish-enriched diet on blood total cholesterol and TG levels daily during 1 month. The second objective was to assess the effect of the diet supplemented with 15 g

of flaxseed on weight (kg), systolic (mmHg) and diastolic pressure (mmHg) after one month. Results indicated a significant decrease in total cholesterol and blood pressure in the F group. TG levels decreased very similarly in both groups.

Table 2 Oxidative stability performed in duplicate of the different flaxseed samples

Sample	Oxidative stability (min)
1	62.4 ± 3.0 ^d
2	119.1 ± 0.3 ^b
3	140.0 ± 0.4 ^a
4	42.0 ± 0.6 ^e
5	67.8 ± 6.6 ^d
6	19.2 ± 9.0 ^f
7	16.2 ± 6.0 ^{fg}
8	91.8 ± 2.4 ^c
9	9.0 ± 1.2 ^g

[†]Values (mean of 3 replications) followed by the same letter, within the same column, were not significantly different (p<0.05), Tukey's least significant difference test.

Study

Groups F and D presented at the beginning of the study circulating total cholesterol above the healthy limits ($> 200 \text{ mg dL}^{-1}$) (Table 3). The initial conditions of both groups were not significantly different except for glucose ($p < 0.05$), which was slightly higher in participants from group D. Therefore, it can be considered that the starting point of both groups was homogeneous.

Results from the intragroup analysis after one month of intervention indicated that group F presented significant differences in total cholesterol, with an approximately 10.7% decrease (Table 3). Group D also reduced their total cholesterol significantly, although in a lesser degree (4.2%). In both cases, the final values still remained over the healthy limit (above 200 mg dL^{-1}) because both groups presented a pronounced hypercholesterolemia, possibly requiring a longer intervention period to normalize their circulating cholesterol levels. In any case, the results indicated that the diet exerted a hypocholesterolemic effect, being more effective when combined with flaxseed. As for TG circulating levels, both groups experienced a similar reduction (25.2% and 25.4% in groups F and D, respectively). According to previous results, this reduction could mainly be due to the effect exerted by diet alone (García-Hernández et al., 2013). Interestingly, the TG levels reached at the end of the study, in both groups, were within the healthy range ($< 150 \text{ mg/dL}$) or near it. Altogether, these results suggested that the daily intake of 15 g of flaxseed in the context of a fish-enriched diet reduces circulating total cholesterol and TG levels. Interestingly, an increase in the glucose levels was

observed in group F (Table 3), but differences were not statistically significant and values were maintained into the healthy range. This observation coincided with our previous report that indicated increased glycaemia in diets supplemented with ω -3 fatty acids (García-Hernández et al., 2013).

The values obtained were also analysed over time, comparing the initial and final values of the parameters into the same group. This was analysed using the general regression model. As a result, the only parameters that were significantly changed over time were circulating cholesterol and TG, as observed in the intragroup analysis. Thus, the intervention with flaxseed (F group) had a more significant impact than diet alone (D group).

Regarding blood pressure, it must be mentioned that the rates recommended for an adequate control of hypertension are 140-90 mmHg for systolic and 90-60 mmHg for diastolic. A significant reduction of both pressures was detected in the F group, while no significant changes were observed in the D group (Table 3). Regarding body weight, significant changes were only observed in group D; although this change was modest and could mainly be due to the design of an isocaloric diet, because the main objective was to normalize circulating lipid parameters and to correct bad diet habits. Interestingly, several participants continued the consumption of flaxseed together with a hypocaloric diet for an additional 30 days, resulting in an additional 1-2 kg weight loss. Unfortunately, this period of time was not included in the intervention and thereby out of the scope of the study.

Table 3 Changes in certain parameters of volunteers at the beginning (day 1) and at the end (day 30) of the study. Results are expressed as means \pm SEM

Parameter	Group F (day 1)	Group F (day 30)	Group D (day 1)	Group D (day 30)
Glucose (mg/dL)	85.8 \pm 3.5	88.9 \pm 2.5	99.7 \pm 4.6	101.3 \pm 3.6
(mmol/L)	4.8 \pm 0.2	4.9 \pm 0.1	5.6 \pm 0.3	5.6 \pm 0.2
Cholesterol (mg/dL)	241.5 \pm 4.9	215.9 \pm 6.1*	236.8 \pm 4.2	227.2 \pm 6.1
(mmol/L)	6.3 \pm 0.1	5.6 \pm 0.2*	6.2 \pm 0.1	5.9 \pm 0.2
Triacylglycerols (mg/dL)	205.7 \pm 12.2	153.6 \pm 11.0*	92.6 \pm 17.1	144.4 \pm 11.1*
(mmol/L)	2.4 \pm 0.1	1.8 \pm 0.1*	2.2 \pm 0.2	1.6 \pm 0.1*
Weight (kg)	77.1 \pm 0.7	76.9 \pm 0.7	75.3 \pm 1.1	74.8 \pm 1.2*
Systolic pressure (mmHg)	124.6 \pm 0.1	119.7 \pm 0.5*	124.7 \pm 0.9	124.1 \pm 0.8
Diastolic pressure (mmHg)	75.9 \pm 0.7	73.6 \pm 0.8*	74.7 \pm 0.8	74.6 \pm 0.2

*Statistically significant difference compared to day 1, ($p < 0.05$).

DISCUSSION

Recently, there has been a growing interest about the beneficial effects of flaxseed consumption on cardiovascular disease (Parikh et al., 2019). The beneficial effects are mostly due to flax lipids. Flax oil is the richest plant source of the essential FAs (18-24% of linoleic and 36-50% of linolenic acids), including as well significant amounts of the non-essential MUFA (16-24% of oleic acid), which seems to play a key role in prevention of cardiovascular pathology (Martínez-González et al., 2015). However, in the present study the FAs profile depended on the trademark of flaxseed studied. The analysis showed that in all the analyzed samples, the predominant PUFAs were α -linolenic and linoleic. Interestingly, the concentration of oleic acid was the highest one in the sample number 2 (32.6%), while it was much lower in the rest of samples (around 5%) (Table 1). In any case, this value of oleic acid is well below of the concentration found in olive oil (58-83%). All flaxseed oils analyzed, including sample 2, display a very low percentage of SFAs (Table 1). In addition, sample 3 was the most resistant to forced oxidation and had the second highest oleic acid content and an equivalent content of linolenic acid as compared to the other brands. These were the reasons why the raw golden flax seed (sample 3) was considered as the most interesting from a nutritional point of view and was selected for the nutritional intervention.

Trial limitations

The limitations that the nutritional intervention presents stem from the REFLOTRO[®] PLUS apparatus used to determine circulating parameters that could differ from the results obtained by blood analysis performed in certified laboratories. In this respect, during the intervention several participants underwent blood extractions prescribed under routinely medical controls. Patients voluntarily provided the analytical values and these were compared with the determinations obtained from the REFLOTRO[®] PLUS. In all available cases, no significant differences were observed.

External validity and applicability of the trial findings

The female participants in this study were frequent clients of one pharmacy located in Elche, a south-eastern Spanish city. Thus, it is quite reasonable to think that results and findings are applicable to women in urban environments, taking into

account that lifestyles and eating habits are similar in most Spanish cities and by extension in cities of Mediterranean countries.

Discussion of results from the intervention

This study assessed the effects of consuming 15 g of flaxseed (sample 3) with skimmed yoghurt during breakfast in the context of a fish-enriched diet. As a result, beneficial effects were observed regarding circulating lipid parameters, with a 10.7% reduction in total cholesterol in group F, effectively doubling the results observed in group D. This finding suggests that flaxseed may enhance the hypocholesterolemic effect exerted by the diet. Regarding circulating TG levels, they were significantly reduced in both groups (~25%), possibly due to the fish-enriched diet, as previously observed with a similar experimental design (García-Hernández et al., 2013). Reductions in cholesterol levels were corroborated by previous reports, where 6-11% decreases in normocholesterolemic volunteers were observed (Rodríguez-Leyva et al., 2010), and 5-17% in hypercholesterolemic subjects after consuming flaxseed for 4-24 weeks (Soni et al., 2016). No effect regarding TG levels were observed in any of the studies commented.

Although the results obtained in the present study agree with data published by other researchers (Pan et al., 2009), some particularities arise and should be considered to explain the beneficial effects observed and associated with the flaxseed consumption. These include: (i) the moment of the day when flaxseed is consumed, (ii) the way that flaxseed is presented in diet, (iii) the amount of flaxseed consumed, and (iv) the time duration for flaxseed consumption.

The moment of the day chosen for the flaxseed intake in the current study was during breakfast, a moment of the day that follows 8h of sleep, the longest resting period of the day. This is a particular moment when the absorption of nutrients should be favored and consequently the beneficial effects of flaxseed could be optimized better, i.e. by reducing the amount of flaxseed administered.

The way flaxseed is introduced in the diet could be a key point as well. From a nutritional point of view, flaxseed can be administered as whole grain or milled. Regarding milled administration, one could expect that this procedure may favor the exposure of the PUFA acids to the ambient oxygen, and being consequently oxidized, as it was observed in sample 9 when studied in the Rancimat method (Table 2). In this context, it has been recently demonstrated that the intake of oxidized PUFAs does not have the capacity of lowering high circulating total cholesterol levels (García-Hernández et al., 2013). In addition, whole grain

(sample 3) can be easily digested in the human intestine, allowing the access to the nutrients present in the seed.

The daily dose administered is another instrumental aspect of the present study. It has been reported that doses as small as 20 g per day of grinded flaxseed for 60 days reduced total cholesterol by 17% in hypercholesterolemic adults (Mandasescu et al., 2005). In the present study, hypocholesterolemic effects for a lower dose and during half the time using raw golden flaxseed have been reported. It seems that these effects are only evident in people with hyperlipemia, because flaxseed consumption (40 g per day) had no effect on the lipid profile after a one-year period of intake in healthy women (Dodin et al., 2008).

Another key point of the study concerns which flaxseed component is the one responsible for the lowering effects on cholesterol levels. Scientific evidence indicates that PUFAs (present in diet and flaxseed) and lignans (Pan et al., 2009) are the likely candidates. On the other hand, it was observed that flaxseed combined with a balanced isocaloric fish-rich diet was also able to reduce TG levels, a point that has not been confirmed in previous studies only consuming flaxseed with an unbalanced diet (Pan et al., 2009).

Another interesting finding in the present study was the lowering of the systolic and diastolic blood pressure in the flaxseed-consuming group (F). There is scientific evidence that the ω -3 in flaxseed can stimulate endothelial production of NO. This molecule relaxes the smooth muscles, allowing the dilatation of blood vessels, which in turn reduces blood pressure and endothelial activation (Poudyal et al., 2013). Blood pressure lowering is beneficial, as the literature confirms that a systolic blood pressure value of 20 mmHg above normal levels doubles the risk of death due to cardiovascular disease. Changes in body mass index (data not shown) were not significant, as expected from an isocaloric diet.

Acknowledgments: The study was carried out in the Pharmacy of M. Asunción Iborra Campos. Authors want to thank Pepa Garry for providing the flaxseed samples used in this study and Tomás Sáez-Rodes (LTS Análisis Clínicos) for its help in blood analysis. CIBEROBN is an initiative of Instituto de Salud Carlos III, Spain.

REFERENCES

- Andreu-Coll, L., Cano-Lamadrid, M., Sendra, E., Carbonell-Barrachina, A., Legua, P., & Hernández, F. (2019). Fatty acid profile of fruits (pulp and peel) and cladodes (young and old) of prickly pear [*Opuntia ficus-indica* (L.) Mill.] from six Spanish cultivars. *Journal of Food Composition and Analysis*, 84, 103294. <https://doi.org/10.1016/j.jfca.2019.103294>.
- AOCS (American Oil Chemists' Society). (2017). Official Method Cd 12b-92. Oil Stability Index. *Illinois, USA*. <https://www.aocs.org/attain-lab-services/methods/methods/search-results?method=111524>.
- Calín-Sánchez, A., Martínez, J. J., Vázquez-Araujo, L., Burló, F., Melgarejo, P., & Carbonell-Barrachina, A. A. (2011). Volatile composition and sensory quality of Spanish pomegranates (*Punica granatum* L.). *Journal of the Science of Food and Agriculture*, 91, 586-592. <https://doi.org/10.1002/jsfa.4230>.
- Dodin, B., Cunnane, B. C., Mâsse, B., Lemay, A., Jacques, H., Asselin, G., Tremblay-Mercier, J., Marc, I., Lamarche, B., Légaré, F., & Forest, J. C. (2008). Flaxseed on cardiovascular disease markers in healthy menopausal women: a randomized, double-blind, placebo-controlled trial. *Nutrition*, 24, 23-30. <https://doi.org/10.1016/j.nut.2007.09.003>.
- Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. (2001). Executive Summary of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). *JAMA*, 285, 2486-2497. <https://doi.org/10.1001/jama.285.19.2486>.
- Ganorkar, P. M., & Jain, R.K. (2013). Flaxseed, a nutritional punch. *International Food Research Journal*, 20, 519-525.
- García-Hernández, V. M., Gallar, M., Sánchez-Soriano, J., Micol, V., Roche, E., & García-García, E. (2013). Effect of omega-3 dietary supplements with different oxidation levels in the lipid profile of women: a randomized controlled trial. *International Journal of Food Science and Nutrition*, 64, 993-1000. <https://doi.org/10.3109/09637486.2013.812619>.
- García-Hernández, V. M., Hojjati, M., Carbonell-Barrachina, A. A., Sánchez-Soriano, J., Roche, E., & García-García, E. (2017/18). Comparison of SOXHLET and ultrasound methods for oil extraction from spanish flaxseeds. *Journal of Microbiology, Biotechnology and Food Science*, 7, 332-336. <https://doi.org/10.15414/jmbfs.2017/18.7.3.332-336>.
- Jhala, A. J., & Hall, L. M. (2010). Flax (*Linum usitatissimum*): current uses and future applications. *Australian Journal of Basic and Applied Sciences*, 4, 4304-4312.
- Khan, M. L., Sharif, M., Sarwar, M., Sameea, M., & Ameen, M. (2010). Chemical composition of different varieties of linseed. *Pakistan Veterinary Journal*, 30, 79-82.
- Mandasescu, S., Mocanu, V., Dăscălița, A. M., Haliga, R., Nestian, I., Stitt, P. A., & Luca, V. (2005). Flaxseed supplementation in hyperlipidemic patients. *Revista Medico-Chirurgicală a Societății de Medici și Naturaliști din Iași*, 109, 502-506.
- Madhusudhan, B., Wiesenborn, D., Schwartz, J., Tostenson, K., & Gillespie, J. (2000). A dry mechanical method for concentrating the lignin secoisolariciresinol diglucoside in flaxseed. *Food Science and Technology*, 33, 268-275. <https://doi.org/10.1006/fstl.2000.0652>. <https://doi.org/10.1006/fstl.2000.0652>.
- Martínez-González, M. A., Salas-Salvadó, J., Estruch, R., Corella, D., Fitó, M., Ros, E., & PREDIMED Investigators. (2015). Benefits of the mediterranean diet: Insights from the PREDIMED study. *Progress in Cardiovascular Diseases*, 58, 50-60. <https://doi.org/10.1016/j.pcad.2015.04.003>.
- Pan, A., Yu, D., Demark-Wahnefried, W., Franco, O. H., & Lin, X. (2009). Meta-analysis of the effects of flaxseed interventions on blood lipids. *American Journal of Clinical Nutrition*, 90, 288-297. <https://doi.org/10.3945/ajcn.2009.27469>.
- Parikh, M., Maddaford, T. G., Alejandro-Austria, J., Aliani, M., Netticadan, T., & Pierce, G. N. (2019). Dietary flaxseed as a strategy for improving human health. *Nutrients*, 11, 1171. <https://doi.org/10.3390/nu11051171>.
- Poudyal, H., Kumar, S. A., Iver, A., Waanders, J., Ward, L. C., & Brown, L. (2013). Responses to oleic and α -linolenic acids in high-carbohydrate, high-fat diet-induced metabolic syndrome in rats. *Journal of Nutritional Biochemistry*, 24, 1381-1392. <https://doi.org/10.1016/j.nutbio.2012.11.006>.
- Rodríguez-Leyva, D., Bassett C. M. C., McCullough, R., & Pierce, G. N. (2010). The cardiovascular effects of flaxseed and its omega-3 fatty acid, alpha-linolenic acid. *Canadian Journal of Cardiology*, 26, 489-496. [https://doi.org/10.1016/S0828-282X\(10\)70455-4](https://doi.org/10.1016/S0828-282X(10)70455-4).
- Shim, Y. Y., Gui, B., Arison, P. G., Wang, Y., & Reanye, M. J. T. (2015). Flaxseed (*Linum usitatissimum* L) bioactive compounds and peptide nomenclature: A review. *Trends in Food Science and Technology*, 38, 5-20. <https://doi.org/10.1016/j.tifs.2014.03.011>.
- Soni, R. P., Mittukatoch, A. K., & Verma, P. (2016). Flaxseed-composition and its health benefits. *Research in Environment and Life Sciences*, 9, 310-316.
- Wang, D.D., & Fu, F.B. (2017). Dietary fat and risk of cardiovascular disease. Recent controversies and advances. *Annual Review of Nutrition*, 37, 423-446. <https://doi.org/10.1146/annurev-nutr-071816-064614>. <https://doi.org/10.1146/annurev-nutr-071816-064614>.