Current Research in Food Science 4 (2021) 336–344

Contents lists available at ScienceDirect

Current Research in Food Science

journal homepage: www.editorialmanager.com/crfs/

Assessing the antioxidant and metabolic effect of an alpha-lipoic acid and acetyl-L-carnitine nutraceutical

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ARTICLE INFO

Keywords:
Alpha-lipoic acid
Acetyl-L-carnitine
Nutraceutical
Antioxidant
Pilot study

ABSTRACT

Personalized nutrition (PN) is seen as a potentially effective and affordable strategy for the prevention of non-communicable diseases (NCDs). In this study we aimed to evaluate the antioxidant and metabolic effect of a dietary supplement based on alpha-lipoic acid (ALA) and acetyl-L-carnitine (ALC) in order to include this product in a novel PN service. The antioxidant properties of the commercial nutraceutical were investigated at physiological conditions (through *in vitro* digestion) and at mitochondrial conditions. The metabolic activity was assessed in a human pilot study using a Gas Chromatography-Mass Spectrometry (GC-MS) methodology in dried urine samples. The nutraceutical exerted an elevated antiradical activity and reducing capacity, especially at mitochondrial conditions, after *in vitro* digestion. This increase in mitochondrial activity was also evidenced *in vivo* by a significant increase in the urinary phosphate concentration ($p = 0.004$). As pro-oxidant effect was reached with the concentration of 4 capsules, 2 capsules at the same time could be a reasonable dose. No adverse effects were recorded *in vivo* with this dose. Thus, although its metabolic effect was not so conclusive, ALA + ALC combination might be beneficial as a dietary supplement for the prevention of the oxidative stress and an interesting dietary supplement to consider in large scale studies.

1. Introduction

Non-communicable diseases (NCDs) kill 41 million people each year, accounting for 71% of all deaths globally (WHO, 2018). Most of these deaths are largely preventable, as diet and other lifestyle behaviours are considered shared and important risk factors (Celis-Morales et al., 2015; WHO, 2018). However current strategies targeting modifiable lifestyle factors are of limited efficacy, as the global burden of NCDs continues to rise (Ordovas et al., 2018; WHO, 2018). This only highlights the need to develop novel, effective and affordable strategies for the prevention and control of these diseases. Personalized nutrition (PN) is seen as the way forward to tackle this challenge (Celis-morales et al., 2017; Ordovas et al., 2018).

There is no agreed definition of PN, although experts coincide in that the goal of PN is to advance human health and wellbeing by tailoring nutrition recommendations and interventions to individuals or groups of individuals with similar traits (Ordovas et al., 2018; Bush et al., 2020). In this sense, personalized dietary interventions are designed according to key characteristics of the individual like gender, weight, age, level of physical activity, possible nutritional deficiencies or disorders, among others (Celis-morales et al., 2017). Conversely, not only regular foods and lifestyle habits can play a determinant role in PN, since some dietary supplements have the potential of improving human metabolism and wellbeing (Zmora et al., 2019).

Stance4Health (Smart Technologies for personAlized Nutrition and Consumer Engagement) is a project funded by European Union's Horizon 2020 research and innovation programme, aiming at evaluate the benefits of a novel smart PN service in a large clinical study. Stance4Health will also test the hypotheses that a PN including the use of nutraceuticals may allow for a targeted control of the general dietary advice.

AlcaLip® is a commercial nutritional supplement of alpha-lipoic acid (ALA) and acetyl-L-carnitine (ALC). These bioactive compounds have a high impact in the regulation of oxidative stress and in the improvement of the mitochondrial function, organelle where they exert their main action (Gorą;ca et al., 2011). An adequate redox state will have special relevance for health. A persistently altered redox balance is typically

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https://doi.org/10.1016/j.crfs.2021.05.002
Received 8 March 2021; Received in revised form 28 April 2021; Accepted 17 May 2021
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observed in many NCDs, together with others features as inflammation and dysregulated authophagy, thus being considered as key targets to tackle NCDs (Liguori et al., 2018; Peña-Oyarzun et al., 2018; Nediani and Giovannelli, 2020).

On the other hand, ALA and ALC also play an important role in the human metabolism. ALA is a cofactor for several enzymes including pyruvate dehydrogenase complex and α-ketoglutarate dehydrogenase complex, two mitochondrial enzymes involved in glucose metabolism and energy production (Tibullo et al., 2017). ALC also plays some roles in glucose metabolism, although its primary physiological role is associated with the lipid metabolism (Pooyandjoo et al., 2016; Bene et al., 2018). ALC facilitates the transport of long-chain fatty acids from the cytosol into the mitochondria, where their degradation takes place via β-oxidation (Bene et al., 2018).

In this way, the combination of improving the organism's redox and metabolic state, due to its effects on mitochondrial function and redox state, makes AlcaLip® an interesting dietary supplement to evaluate. Previous studies have highlighted that ALA and ALC combination could exert a synergistic effect. For example, the combined treatment of ALA and ALC offered protection in a rat model of Parkinson's, slowing down the progression of the disease (Zaitone et al., 2012). Zhang et al. (2010) also observed that this kind of combination potently inhibited the rotenone-induced mitochondrial dysfunction and oxidative damage in a chronic Parkinson's disease model with human neuroblastoma cells. However, few studies have been carried out in humans using this combination, and some of them have not even obtained significant results (Soczynska et al., 2008; Coit et al., 2013).

With regard to security, the safety of both ALC and ALA has been previously evaluated in several studies (Gorą;ca et al., 2011; Ussher et al., 2013; Serban et al., 2016; Song et al., 2017). In fact, ALA is used as a drug for the treatment of diabetic polyneuropathy. However, some concerns have been raised in the recent years regarding ALC supplementation, as some studies have linked high levels of ALC to cardiovascular diseases (Koeth et al., 2013; Chen et al., 2019).

Taking all this information into account, the aim of the present study was to investigate the *in vitro* antioxidant properties of a commercial supplement of ALA + ALC (AlcaLip®) under conditions as similar as possible to the physiological ones, and, once established, test its safety and metabolic effect *in vivo* in a short human intervention pilot study using a urine medical test. The results obtained will allow us to know more accurately the effects of this supplement in order to use it in a large-scale clinical trial.

2. Materials and methods

2.1. Chemicals and reagents

AlcaLip and the medical urine test (MetaCliniq®) were kindly provided by RM FARMAMÉDICAL (Spain). The AlcaLip capsule is a gastric resistant one and its composition is shown in Table 1. Ascorbic acid (AA) L type and resveratrol (RESV) trans type pure compounds were from Sigma-Aldrich (Germany). All chemical reagents and the mitochondrial enzyme used for the assays and *in vitro* digestion were from Sigma-Aldrich (Germany), Alpha Aesar (United Kingdom) and R&D Systems (USA).

2.2. In vitro antioxidant capacity analysis

2.2.1. Extraction of samples for antioxidant capacity analysis

The total antioxidant capacity of AlcaLip was compared to RESV 10 mM and AA 10 mM (concentrations in the final volumes after dissolution at physiological conditions, after *in vitro* digestion or after mitochondrial activation), which are well known to possess high antioxidant capacity. The determination was carried out following different protocols (Fig. 1) to investigate how different conditions can affect the antioxidant capacity of the dietary supplement. Thus, a standard condition of solubilization, an *in vitro* digestion and the conditions found within the mitochondria, were examined as follows:

Table 1
Composition of a capsule of the commercial nutraceutical Alcalip®.

Nutritional information	1 capsule (750 mg)
ALC	400 mg
ALA	100 mg
Vitamin B1	0.183 mg
Vitamin B12	0.416 μg

- Standard conditions: as AlcaLip capsule is a gastric resistant one it was, the first step was the solubilization of the capsule in distilled water at pH 7.2 (intestinal pH) under stirring for 30 min. The solubilization had to be carried out at intestinal pH as it is at that pH that the components of the product get separated (capsule dissolved).
- *In vitro* digestion: was performed through the global antioxidant response (GAR) method as described by Pastoriza et al. (2011). All the pH, time and temperature conditions are reported in Fig. 1. After digestion of the capsule, two fractions were obtained: a soluble fraction (potentially absorbable by the intestine) and an insoluble fraction (non-absorbable by the intestine). The soluble fraction was submitted to the analyses.
- Mitochondrial conditions: the mitochondrial enzyme dihydrolipoamide dehydrogenase (5U/mg protein) was added to the soluble fraction deriving from the *in vitro* digestion described above. Dihydrolipoamide dehydrogenase acts in the human mitochondria reducing the alpha lipoic acid to dihydroxylipoic acid, the main species responsible for the antioxidant capacity of the acid.

2.2.2. Antioxidant capacity analysis

The antioxidant capacity of the different samples was determined by two different methods ($TEAC_{FRAP}$ and $TEAC_{OH}$). All the results were corrected considering their respective blanks (enzymes and chemicals). The obtained results are expressed as mmol Trolox equivalents per g of sample (referring to g of capsule, not g of inside content). The colorimetric assays were carried out using a FLUO Star Omega microplate reader (BMG Labtech, Germany) with temperature control (37 °C).

2.2.2.1. $TEAC_{FRAP}$ assay. The ferric reducing antioxidant power was established as proposed by Benzie and Strain (1996). The assay was performed using 20 μl of sample and 280 μl of FRAP reagent solution. Trolox was used as a standard reference ranging from 0.01 mg/mL to 1 mg/mL. The absorbance values were those obtained after 30 min at 595 nm.

2.2.2.2. $TEAC_{OH}$ assay. The method allows the evaluation of the antioxidant capacity of a foodstuff against hydroxyl radicals, and was carried out as described by Pérez-Burillo et al. (2018). Hydroxyl radicals were generated by the Fenton reaction using H2O2 and ferric ions, and detected by using indigo carmine as indicator. The assay was carried out using 90 μl of sample, 190 μl of $TEAC_{OH}$ daily solution and 20 μl of H2O2. Trolox stock solutions ranging from 0.01 to 10 mg/mL were used to perform the calibration curves. The absorbance was read at 610 nm after 60 min.

2.3. Human intervention

2.3.1. Study subjects and features of the intervention

We conducted a small non-controlled pilot human intervention between October 2019 and December 2019. Eleven healthy volunteers aged 21–46 years were recruited from the Centro de Investigación Biomédica de la Universidad de Granada, Spain. One of them finally rejected to participate in the study, so 10 volunteers were included to start the intervention. Among the inclusion criteria it was stabilised that the

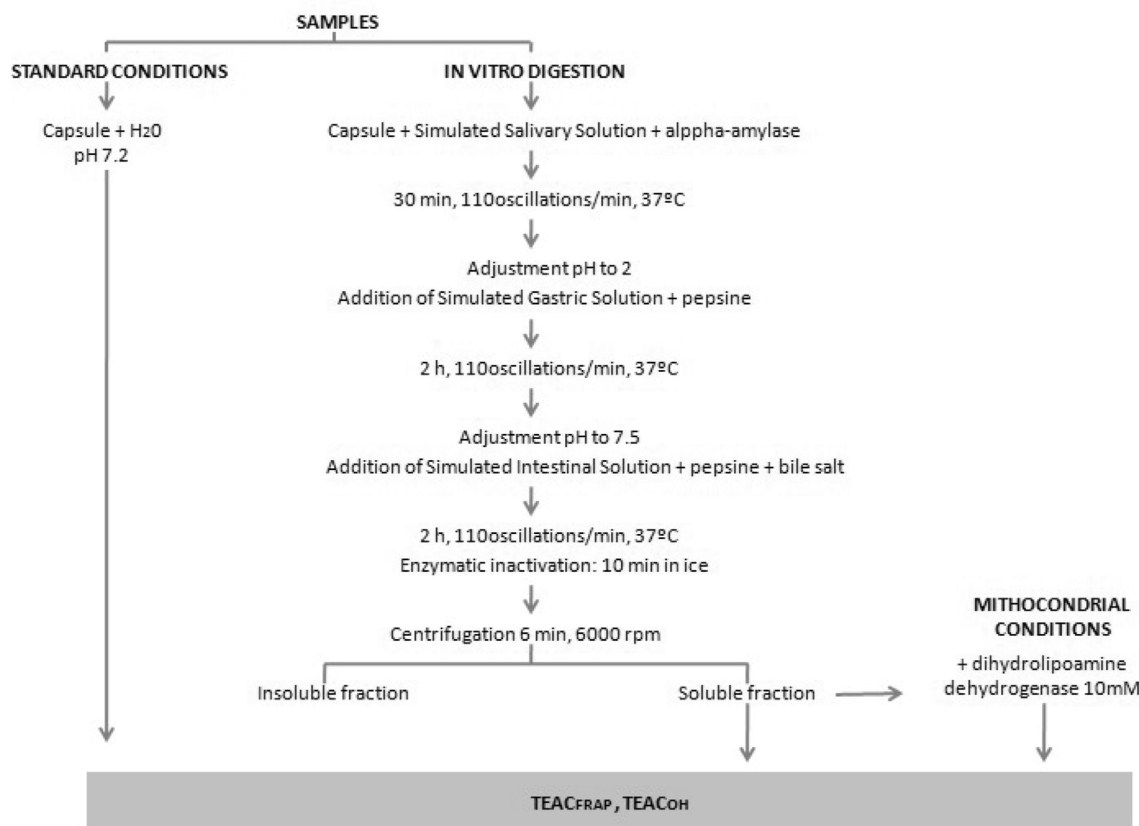

Fig. 1. Schematic description of the extraction processes to evaluate the antioxidant activity *in vitro*.

participant had to be healthy, 20–65 aged and with a BMI 20–28 kg/m^2. Medically prescribed diet or specific dietary regimens were the exclusion criteria for the study.

Before starting the study all the participants signed an informed consent. This pilot study also has the approval of the ethics committee of the Universidad de Granada No. 1080/CEIH/2020, and followed the ethical guidelines of the Declaration of Helsinki (World Medical Association, 2008).

The effect of the commercial dietary supplement was assessed using a diagnostic tool called MetaCliniq. This diagnostic tool is based on an *in vitro* test in urine and is able of detecting more than a hundred metabolic alterations (Supplementary Materials, Table S1). A total of 36 metabolites, including creatinine, were measured for our study (Supplementary Materials, Table S2). It is important to underline that the metabolites included in the medical test are considered key metabolic products as they participate in numerous metabolic pathways and processes in the body (Bouatra et al., 2013). For example, citric acid, pyruvic acid or carboxycitric acid are products of the Krebs Cycle (Chen y col.,2019). This important metabolic pathway takes part in the mitochondria, the main place of action of ALA and ALC (Gorą;ca et al., 2011).

Taking into account the results from the *in vitro* digestion, participants were instructed to take 2 pills before each main meal (breakfast, lunch, dinner), 3 times a day (total 6 pills) during 30 days of treatment, and to record any kind of symptom associated with their consumption. At the end of the study, participants were also asked for any kind of problem with the intervention and pills intake. The entire participant (n = 10) correctly completed the intervention.

2.3.2. Sample collection and GC-MS measurement

The urine test was applied before, after 30 days of treatment, and 14 days after finishing the intervention. Volunteers collected their second morning urine in a sterile urine storage container and bring it to the laboratory at the Centro de Investigación Biomédica. There, a Whatman 903 filter paper strip was inserted into the container filled with the urine. This way the filter paper strip was soaked with the urine by an adsorption process. Then, the samples were dried at room temperature for 3 h, taking care not to contaminate the paper strip. Once dried, the filter paper strip was placed in a protective, airtight bag, and was sent to the RM FARMAMÉDICAL's laboratory for analysis. There the samples were pre-treated with urease, followed by deproteinization with alcohol and derivatization by silylation, as described previously (Matsumoto & Kuhara, 1996; Kuhara, 2007). The samples were also eluted and pre-analytically processed for GC-MS analysis using Kuhara's protocol (Matsumoto & Kuhara, 1996; Kuhara, 2007). The equipment used for analysis was gas chromatograph Agilent 7890 A coupled to a mass spectrometer Agilent 5975C. Once the metabolites were separated and correctly identified by GC-MS (see a chromatogram example in Supplementary Materials, Fig. S1), the results were analyzed and interpreted using The Human Metabolome Database (Bouatra et al., 2013).

2.3.3. Quality control

RM FARMAMÉDICAL's laboratory routinely conducts internal quality controls and participates in external quality assessments organized by the European Research Network for Evaluation and Improvement of Screening, Diagnosis and Treatment of Inherited Disorders of Metabolism (ERNDIM).

2.4. Statistical analysis

Statistical analysis of the results was carried out with SPSS software (version 23, SPSS, Chicago, IL, USA). P values < 0.05 were regarded as statistically significant. To investigate the antioxidant properties of the commercial supplement, all measurements were performed at least in triplicate and expressed as means ± standard deviations. Statistical

differences were evaluated by using analysis of unpaired t-Students test and one-way ANOVA, followed by the Bonferroni post-hoc test.

Regarding to the pilot study, the results were first standardized by the urinary creatinine excretion and expressed as a metabolite/creatinine ratio. Creatinine urinary concentration in the spot urine samples was quantified for each participant by GC-MS and then used to standardize the results by dividing each metabolite concentration by the creatinine ones. Then, a descriptive analysis of the different metabolites was carried out. Since missing data were obtained for 22 metabolites, we could not properly carry out the statistical analysis for them, so we finally included in our study 14 metabolites (see Supplementary Materials, Table S2). After testing the normality of the variables, we transformed data by log10. To analyze the evolution of the variables throughout the study, parametric tests for continuous variables (Student's T test for paired samples) were used. We studied 3 different associations: changes from baseline (preT) to the end of the treatment (30daysT), from the end of the treatment to 14 days after finishing the intervention (postT) and from baseline to 14 days after finishing the intervention.

3. Results

3.1. Antioxidant capacity

3.1.1. Antioxidant capacity after standard solubilization

Figs. 2 and 3 show the results obtained after solubilization at physiological pH of the AlcaLip content. The antioxidant capacity increased in a dose-dependent manner. In general, reductive activity averaged 3.5 times higher than antiradical activity. In addition, AlcaLip showed a lower scavenging and reducing capacity ($p < 0.05$) compared to 10 mM AA and 10 mM RESV. These antioxidant species were chosen as standard since AA is widely consumed around the world, while RESV is the main bioactive compound of other nutraceuticals similar to AlcaLip (like Revidox®). Finally, AlcaLip showed no signs of pro-oxidant activity, either against hydroxyl radical or through its reducing activity, at the doses assayed in the present study.

3.1.2. Antioxidant capacity after the in vitro gastrointestinal digestion

The results obtained after the *in vitro* digestion of AlcaLip, AA and RESV are shown in Figs. 4 and 5. It can be observed that the activity of AA decreased compared to that exerted after a simple solubilization, while that of RESV slightly increased in both assays. The antioxidant capacity of AlcaLip increased to a large extent ($p < 0.05$) in comparison to the results obtained after simple solubilization, again showing an increase in a dose-dependent manner. Similar to standard solubilization, the values obtained by the $TEAC_{OH}$ assay were lower compared to the $TEAC_{FRAP}$ one.

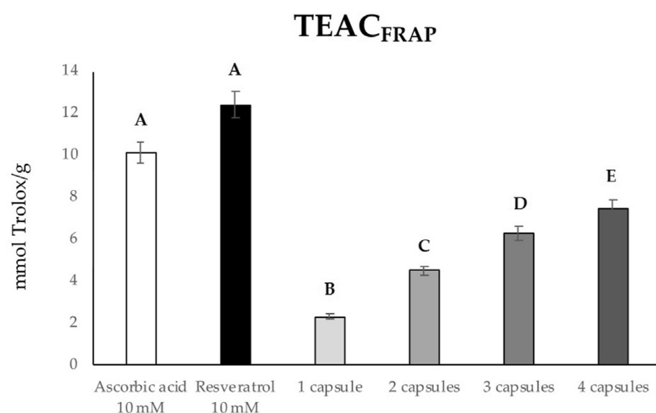

Fig. 2. *In vitro* antioxidant capacity of AA, RESV and different dosage of AlcaLip (1–4 capsules) at standard conditions by $TEAC_{FRAP}$ assay. The obtained results are expressed as mmol Trolox equivalents per g of sample (g of capsule). All measurement were performed in triplicate. Data is expressed as means ± standard deviation. Different letters denote significant differences among values at $p < 0.05$ significance level.

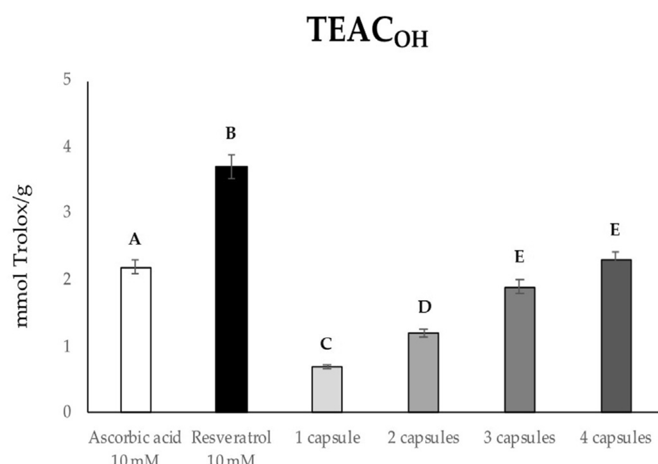

Fig. 3. *In vitro* antioxidant capacity of AA, RESV and different dosage of AlcaLip (1–4 capsules) at standard conditions by $TEAC_{OH}$ assay. The obtained results are expressed as mmol Trolox equivalents per g of sample (g of capsule). All measurement were performed in triplicate. Data is expressed as means ± standard deviation. Different letters denote significant differences among values at $p < 0.05$ significance level.

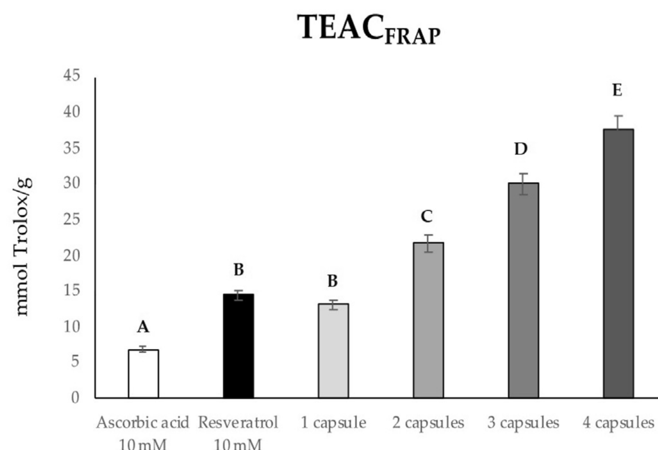

Fig. 4. *In vitro* antioxidant capacity of AA, RESV and different dosage of AlcaLip (1–4 capsules) after *in vitro* digestion by $TEAC_{FRAP}$ assay. The obtained results are expressed as mmol Trolox equivalents per g of sample (g of capsule). All measurement were performed in triplicate. Data is expressed as means ± standard deviation. Different letters denote significant differences among values at $p < 0.05$ significance level.

3.1.3. Antioxidant capacity under mitochondrial conditions

After *in vitro* digestion and subsequent metabolic activation under mitochondrial conditions, AA and RESV maintained almost the same activity against hydroxyl radicals (1.4 and 4.6 mmol Trolox/g, respectively) and reducing capacity (6.9 and 13.2 mmol Trolox/g, respectively). On the contrary, the antioxidant capacity of AlcaLip increased∽ 10-fold (Figs. 6 and 7). Such a high increase in the antioxidant capacity had a side effect: a pro-oxidant effect was reached with the concentration of 4 capsules. In the case of the $TEAC_{OH}$ assay, the activity of AlcaLip increased from the dose of 1 capsule up to 3 capsules (from 27.6 to 49.2 mmol Trolox/g, respectively), but decreased with 4 capsules (24.3 mmol Trolox/g; Fig. 7). The same trend was observed for reducing activity (Fig. 6), but with an approximately 8-fold higher reductive capacity than

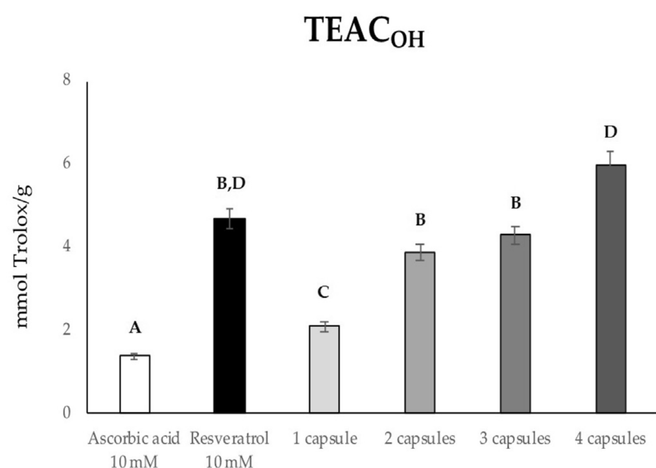

Fig. 5. *In vitro* antioxidant capacity of AA, RESV and different dosage of AlcaLip (1–4 capsules) after *in vitro* digestion by $TEAC_{OH}$ assay. The obtained results are expressed as mmol Trolox equivalents per g of sample (g of capsule). All measurement were performed in triplicate. Data is expressed as means ± standard deviation. Different letters denote significant differences among values at $p < 0.05$ significance level.

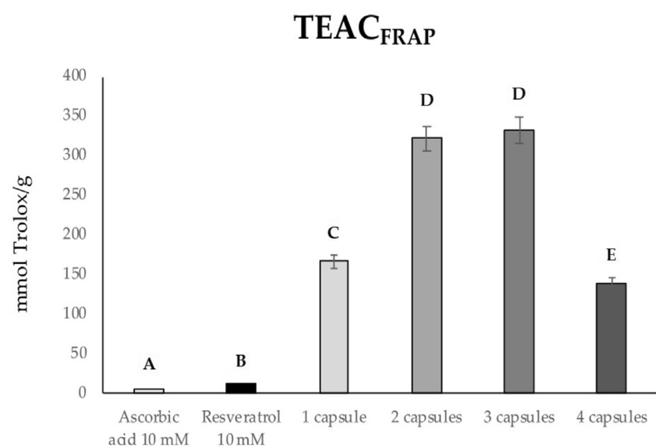

Fig. 6. *In vitro* antioxidant capacity of AA, RESV and different dosage of AlcaLip (1–4 capsules) at mitochondrial conditions by $TEAC_{FRAP}$ assay. The obtained results are expressed as mmol Trolox equivalents per g of sample (g of capsule). All measurement were performed in triplicate. Data is expressed as means ± standard deviation. Different letters denote significant differences among values at $p < 0.05$ significance level.

hydroxyl scavenging capacity assessed with the $TEAC_{OH}$ assay.

Comparing AlcaLip with the reference compounds, the doses of 1, 2 and 3 capsules of AlcaLip were respectively 20, 30 and 35 times more effective in stabilizing hydroxyl radicals than AA, and approximately 6, 9 and 10 times more effective than RESV (Fig. 7). In the case of the reducing capacity, AlcaLip was 24 (1 capsule), 47 (2 capsules) and 48 (3 capsules) times more reducing than AA, and approximately 13 (1 capsule), 24 (2 capsules) and 25 times (3 capsules) more reducing than RESV (Fig. 6).

3.2. Human intervention

Our study included a total of 5 women and 5 men aged between 21 and 45 years (mean 30.4 ± 8.2 SD) as it is shown in Table S3. None of the participants reported any side effects from the treatment. On the other hand, the urine test also ruled out the presence of any type of in born alteration in the amino acids, carbohydrates and fatty acids metabolism,

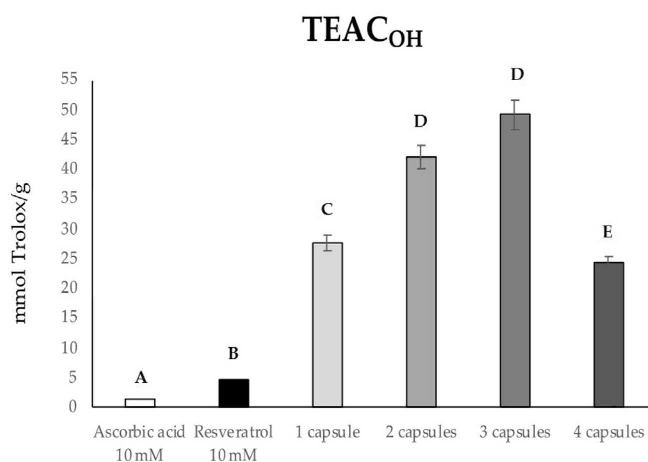

Fig. 7. *In vitro* antioxidant capacity of AA, RESV and different dosage of AlcaLip (1–4 capsules) at mitochondrial conditions by $TEAC_{OH}$ assay. The obtained results are expressed as mmol Trolox equivalents per g of sample (g of capsule). All measurement were performed in triplicate. Data is expressed as means ± standard deviation. Different letters denote significant differences among values at $p < 0.05$ significance level.

among others. Table 2 shows the standardized metabolite/creatinine ratio of urinary metabolites (presented as mean ± standard deviation and transformed data by log10 to normalize the results) in the 3 different times of study (mean chromatographic area of metabolites and creatinine shown in Table S4), and graphically represented in the Fig. 8 and Supplementary Materials (Figs. S2 and S3).

A paired sample T-test was used to compare the concentrations of the different metabolites. We observed a statistically significant increase in the concentration of phosphate with the 30 days of treatment ($p = 0.004$). This increase was not maintained after the cessation of treatment, but significantly reduced its concentration ($p = 0.046$) to its initial values, as no significant difference was observed between pre-T and post-T phosphate concentration ($p = 0.837$).

On the other hand, the vanillylmandelic acid (VMA) increased its concentration with the consumption of AlcaLip, although this increase was not statistically significant ($p = 0.237$). However, the decrease in its concentration in the comparison 30daysT-postT was significant ($p = 0.034$). The VMA concentration also returned to the initial one ($p = 0.975$).

With regard to the rest of the metabolites, we observed that the means of their concentrations were the same at the different times of the study; no significant changes were observed in their values after the intake of the nutraceutical AlcaLip (Table 2 and Supplementary Figs. S2 and S3).

4. Discussion

4.1. Antioxidant capacity

The commercial dietary supplement AlcaLip showed a high antioxidant capacity against hydroxyl radicals and a potent reducing activity. The greatest reductive activity of AlcaLip at physiological pH could be explained taking into account that hydroxyl radicals are strong oxidizing species, so it is more difficult to avoid their activity than to cause the reduction of the reaction medium.

The *in vitro* method established by Pastoriza et al. (2011) allows the evaluation of total antioxidant capacity of foods and derivatives, including both soluble and insoluble fractions. In this study we decided to analyze only the soluble fraction, which is considered potentially absorbable, so that the bioactive species could cross the intestinal barrier and reach the systemic circulation. Under this point of view, the evaluation of the antioxidant capacity is essential since all dietary supplements must follow a digestion process, where the conditions of pH,

Table 2
Standardized concentrations (calculated area in the chromatogram divided into creatinine concentration in the samples) of the urinary metabolites presented as mean ± standard deviation in the 3 different moments of study (preT, 30daysT and postT). Data shown were previously transformed by log10. n = 10 per each group. p values < 0.05 were regarded as statistically significant.

Metabolite	PreT[a]	30daysT[b]	PostT[c]	p preT-30daysT	p 30daysT-postT	p preT-postT
	Mean (±SD)					
4-HBA[d]	−1.619 (0.327)	−1.665 (0.319)	−1.803 (1.028)	0.828	0.767	0.699
4-HPA[e]	−1.663 (0.272)	−1.493 (0.534)	−1.539 (0.517)	0.454	0.831	0.510
5 HIA[f]	−2.280 (0.335)	−2.033 (0.521)	−2.360 (0.777)	0.202	0.491	0.833
HPHPA[g]	−1.246 (0.587)	−1.287 (0.772)	−1.349 (0.318)	0.857	0.864	0.614
Arabinose	−0.704 (0.187)	−0.464 (0.397)	−0.552 (0.286)	0.340	0.658	0.328
Citric Acid	−0.464 (0.300)	0.044 (0.744)	−0.256 (0.520)	0.176	0,.70	0.525
Phosphate	−0.880 (0.341)	0.184 (0.149)	−0.976 (0.905)	0.004	0.046	0.837
Gluconate	−0.983 (0.169)	−0.682 (0.562)	−0.791 (0.302)	0.213	0.606	0.235
Hippuric Acid	−0.118 (0.393)	−0.170 (0.685)	−0.455 (0.600)	0.821	0.169	0.369
Lactic Acid	−1.556 (0.113)	−1.182 (0.876)	−1.376 (0.428)	0.360	0.802	0.390
Mannitol	−0.381 (0.369)	−0.645 (0.570)	−0.569 (0.556)	0.451	0.444	0.525
MioInositol	−1.393 (0.286)	−1.356 (0.737)	−1.336 (0.527)	0.669	0.943	0.797
Pseudouridine	−0.740 (0.219)	−0.559 (0.564)	−0.715 (0.178)	0.579	0.582	0.828
VMA[h]	−2.324 (0.321)	−1.941 (0.470)	−2.317 (0.313)	0.237	0.034	0.975

[a] Before treatment.
[b] End of the 30 days treatment.
[c] 14 days after finishing the intervention.
[d] 4-Hydroxybenzoic Acid.
[e] 4-Hydroxyphenylacetic Acid.
[f] Isoniazid.
[g] 3-(3-Hydroxyphenyl)-3-hydroxypropanoic acid.
[h] Vanillylmandelic acid.

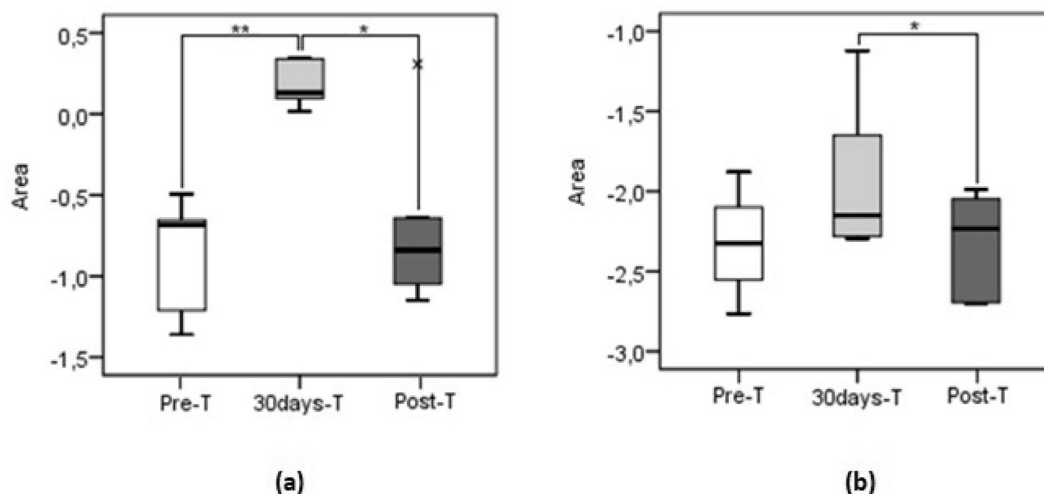

Fig. 8. Variation of standardized phosphate levels (a) and VMA (b) urinary concentration at the 3 different times of study (X axis). The Y axis corresponds with the metabolite/creatinine ratio of the chromatographic peaks and transformed by log10. n = 10 per each group. *Significant differences between groups $p < 0.05$, ** $p < 0.01$ Outliers correspond with the " × " symbol.

temperature, ionic strength or enzymatic activity can alter the antioxidant capacity of the sample. Noteworthy, AlcaLip showed an increase of the antioxidant capacity in contrast to other antioxidant compounds (AA and RESV), which decreased their antioxidant activity due to digestion conditions.

The antioxidant capacity of AlcaLip was also determined in the conditions where it could possibly exert its full antioxidant potential: after *in vitro* digestion and subsequent metabolic activation under mitochondrial conditions. The boost of the antioxidant capacity in a dose-dependent manner, stopped at the dose of three capsules. Then, a pro-oxidant effect was reached with the concentration of 4 capsules, both in the case of the $TEAC_{FRAP}$ and $TEAC_{OH}$ assays. The pro-oxidant activity observed at an excessive concentration of the supplement is an effect previously described for all antioxidant compounds, natural or synthetic, known at the present moment (Lambert and Elias, 2010). Therefore, 2 capsules at the same time could be a reasonable AlcaLip dose, reaching a high antioxidant activity but with enough therapeutic range to avoid toxic (pro-oxidant) effects, which could be closer if 3 capsules were taken at the same time.

AlcaLip was particularly antioxidant within the cellular mitochondrion, due to an enzymatic activation process. The main components of AlcaLip, ALA and ALC are known to penetrate the mitochondrion, which is the main cellular organelle generating free radicals. In particular, mitochondrial ALA is known to be metabolized to dihydrolipoic acid, a compound that exerts a very high antioxidant activity (Packer et al., 1995; Moini et al., 2002; Castañeda-Arriaga and Alvarez-Idaboy, 2014). This could be the reason behind the higher antioxidant capacity of AlcaLip under mitochondrial conditions, compared to physiological pH and *in vitro* digestion conditions. The ability of AlcaLip to behave *in situ* as an antioxidant would give a great advantage in decreasing the levels of

free radicals, especially in conditions where they are abundantly generated (i.e. sport practice and different pathologies). Moreover, natural antioxidants of the diet could have an additive effect with those provided by AlcaLip, then the pro-oxidant effects could be even closer.

4.2. Human intervention

The *in vitro* urine test, MetaCliniq, is a medical test based on the analysis by GC-MS of some important metabolites present in urine deposited on a filter paper. There are many studies that have demonstrated the validity of collecting biological fluids, either blood or urine, on filter paper for analysis (Ito et al., 2000; Hao et al., 2015; Hampe et al., 2017). In fact, nowadays such technique is used worldwide in the detection of well-known diseases, such as Phenylketonuria, in public health programmes.

Urine is a biological matrix that contains hundreds of metabolic end products at various concentrations (Khodadadi and Pourfarzam, 2020). Its use presents a number of important advantages over the use of blood samples. Firstly, urine can be collected in large quantities, easily, and in a non-invasive way, which makes it less annoying for the patient (Jóźwik and Kałużna-Czaplińska, 2016). Conversely the use of dry urine samples has the added advantage of inactivating the ongoing metabolism of the sample (Hernández Redondo et al., 2012; Hampe et al., 2017).

On the other hand, GC-MS has shown to have a high sensitivity and specificity, making it one of the preferred techniques for conducting urinary metabolomic investigations (Jóźwik and Kałużna-Czaplińska, 2016; Khodadadi and Pourfarzam, 2020). Moreover GC-MS has a long history of achievements in the description of complex biological systems (Khodadadi and Pourfarzam, 2020). Related to urine samples, it has especially been used in mass-screening tests for early diagnosis and treatment of the inborn errors of metabolism (Hao et al., 2015; Hampe et al., 2017).

AlcaLip supplementation seems to be safe and exerted some metabolic changes. In our study, the GC-MS analysis also enabled us to rule out the presence of different alterations in the metabolism (Supplementary Materials, Table S1). Moreover, we observed some significant changes in the phosphate and VMA urine concentration.

Phosphate is considered an essential component of life as it is involved in numerous biological functions, highlighting its important role in energy metabolism, and being excreted in sweat, faeces and urine as the result of this metabolism (Bouatra et al., 2013). We observed a significant increase in urine phosphate concentration after 30 days of AlcaLip treatment, which could be related with a significant rise in the activity of the citric acid cycle, an important metabolic pathway that takes place in the mitochondrion. As stated before, the mitochondrion is the main place of action of AlcaLip's bioactive compounds, ALA and ACL (Gorą;ca et al., 2011). In fact, ALA is considered an essential cofactor for mitochondrial bioenergetics processes, and together with ALC, contribute to ATP synthesis in different ways (Pekala et al., 2011). Phosphate, among other important metabolites like citric acid, pyruvic acid or carboxycitric acid, are key products of the citric acid cycle (Bouatra et al., 2013; Chen y col., 2019). However, the rise in the urinary concentration of phosphate was not followed by a significant increase in the urinary levels of the other measured metabolites involved in the citric acid cycle, such as citrate. We hypothesize that in future large-scale studies these changes would be appreciated.

Conversely VMA is one of the most important catecholamine metabolites (Bouatra et al., 2013). In our study, no volunteer had altered levels of this metabolite, either before taking AlcaLip, or after taking AlcaLip, although we observed a small significant decrease in urinary concentrations of VMA in the 30daysT-postT comparison. We must bear in mind that the urinary excretion of VMA may also be affected by physical exercise or stress factors (Bouatra et al., 2013).

Our pilot study did not record any adverse effects, which was expected given the dose applied and the wide use that ALA and ALC supplements have had in clinical trials. However, in 2013 a controversial article was published describing that oral ingestion of L-carnitine in both, animals and humans, resulted in generation of trimethylamine by the gut microbiota, which is absorbed and then metabolized to trimethylamine-N-oxide (TMAO), a pro-atherogenic compound (Koeth et al., 2013). Such observations differ from a multitude of studies demonstrating beneficial effects of ALC on metabolic and cardiovascular health (Di Nicolantonio et al., 2013; Ussher et al., 2013; Serban et al., 2016; Song et al., 2017; Asadi et al., 2020). In fact, a systematic review and meta-analysis of 13 controlled trials including 3629 patients concluded that, compared to placebo, L-carnitine caused a 27% reduction in all-cause mortality, a 65% reduction in ventricular arrhythmias, and 40% reduction in angina symptoms in patients experiencing an acute myocardial infarction (Di Nicolantonio et al., 2013). More recently, Song et al. (2017) observed that there were no significant differences in all-cause mortality but that there was a considerable improvement in clinical symptoms and cardiac functions in chronic heart failure. In addition, these researchers reported that the supplement had a good tolerance. This last point is important considering that a possible explanation for these discrepant findings by Koeth et al. (2013), may be due to extremely large doses of ALC supplementation (Ussher et al., 2013). Several meta-analyses have evaluated different randomized controlled trials (RCTs) using doses up to 6 g per day, without describing any adverse effects. In fact, Shang et al. recommend an optimal dose of 3 g/day in acute myocardial infarction patients (Shang et al., 2014). This dosage is approximately 25–35 times lower than that reported by Koeth et al. (2013) and Ussher et al. (2013). Furthermore, several human studies could not confirm that elevated fasting blood TMAO levels predict increased risk of cardiovascular disease (Mueller et al., 2015; Kaysen et al., 2016; Samulak et al., 2019).

In our study we used a dose of 800 mg three times a day, 2.4 g in total per day. So, the dose used should be safe. On the other hand, an adequate redox state has been associated with cardiovascular health (Liguori et al., 2018; Noce et al., 2019; Zmora et al., 2019). Future interventional large-scale studies will allow us to assess more accurately this relationship.

4.3. Strengths and limitations

For a better interpretation of our findings, several limitations should be mentioned. One of the main drawbacks has to do with the potential bacterial contamination associated with the use of urine samples, although this factor was taken into account in the study protocol. It is also important to mention that urine samples were only processed in accordance with a sample acceptance protocol: urine samples were accepted only when they were received on the filter paper strips suitable for that use and when there was no fungal contamination or foreign material. Conversely, we must bear in mind that the pilot study was conducted with a convenience sample (volunteers aiming at testing the nutraceutical) and that there was no placebo group.

Our study also presents some valuable strengths. We evaluated the activity of the ALA and ALC nutraceutical in conditions as similar as possible to the physiological ones, and in its main place of action: the mitochondria. On the other hand, the urine test used was based on a highly sensitive and specific technique: GC-MS. Furthermore, the validity of urine collection on filter paper has long been evaluated in previous studies.

5. Conclusions

In summary, the nutraceutical AlcaLip showed a high antioxidant capacity against hydroxyl radicals and a strong reducing activity. Moreover, this activity increased after the digestive process, unlike other antioxidant compounds present in our diet, being especially antioxidant within the cellular mitochondria due to a process of enzymatic activation. To observe this effect, a maximum of 2 capsules at the same time would be enough, dosage that also was found to be safe in our *in vivo* study. Conversely the *in vitro* urine test used was easy to use, non-invasive

and allowed us to monitor the changes at metabolic level that occurred throughout the intervention study. Although only phosphate and VMA urinary concentration showed to be significantly altered, it is expected that future large-scale clinical studies will provide us a more accurate knowledge of the effects of ALA and ALC on human beings.

Funding

This work was supported by the research project Stance4Health funded by the European Union's Horizon 2020 research and innovation programme, grant agreement Nº. 816303 and by the Plan propio de Investigación y Transferencia of the University of Granada under the program "Intensificación de la Investigación, modalidad B". This study was also supported by a Spanish predoctoral fellowship F.P.U. Nº 18/06440 from the Spanish Ministry of Science, Innovation and Universities.

CRediT authorship contribution statement

Alicia Lopez-Maldonado: Formal analysis, Investigation, writing—original draft preparation, Visualization, All authors have read and agreed to the published version of the manuscript. **Silvia Pastoriza:** Conceptualization, Methodology, Formal analysis, Investigation, writing—review and editing, Supervision. **José Ángel Rufián-Henares:** Conceptualization, Methodology, writing—review and editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments:

This paper will form part of the doctoral thesis by Alicia Lopez-Maldonado, conducted within the context of the "Clinical Medicine and Public Health Programme" at the University of Granada.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2021.05.002.

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