A novel nutraceutical formulation increases telomere length and activates telomerase activity in middle-aged rats

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Abstract. Telomeres are major contributors to cell fate and aging through their involvement in cell cycle arrest and senescence. The accelerated attrition of telomeres is associated with aging-related diseases, and agents able to maintain telomere length (TL) through telomerase activation may serve as potential treatment strategies. The aim of the present study was to assess the potency of a novel telomerase activator on TL and telomerase activity *in vivo*. The administration of a nutraceutical formulation containing *Centella asiatica* extract, vitamin C, zinc and vitamin D3 in 18-month-old rats for a period of 3 months reduced the telomere shortening rate at the lower supplement dose and increased mean the TL at the higher dose, compared to pre-treatment levels. TL was determined using the Q-FISH method in peripheral blood mononuclear cells collected from the tail vein of the rats and cultured with RPMI-1640 medium. In both cases, TLs were significantly longer compared to the untreated controls (P≤0.001). In addition, telomerase activity was increased in the peripheral blood mononuclear cells of both treatment groups. On the whole, the present study demonstrates that the nutraceutical formulation can maintain or even increase TL and telomerase activity in middle-aged rats, indicating a potential role of this formula in the prevention and treatment of aging-related diseases.

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Introduction

Telomeres have been characterized as molecular clocks, playing a pivotal role in cell division arrest. These chromatin structures, composed of tandem DNA sequence repeats coated by the sheltering protein complex, are formed at chromosomal termini as protective caps against DNA degradation and recombination to ensure genomic stability and integrity (1). Each cell division leads to shorter telomeres due to the inability of DNA polymerase to fully replicate the end part of the lagging strand of DNA, described as the end replication problem. Telomere length (TL) loss can be compensated by telomerase, which functions as a reverse transcriptase, adding telomeric repeats at the end of chromosomes. The telomerase complex consists of a catalytic unit denominated as telomerase reverse transcriptase (TERT) and the telomerase RNA component (2). This ribonucleoprotein polymerase is abundantly expressed in highly proliferating cells, such as stem cells; however, its expression in somatic cells is reduced or absent (3). In cells with a low telomerase activity, telomeres continue to shorten, and when they reach a critically short length, cells undergo apoptosis or cell senescence. Beyond this critical length, excessive telomere shortening can lead to chromosomal fusions and genomic instability that increase the risk of cancer tumorigenesis through genomic alterations. Ultimately, cells need to reactivate telomerase to become immortal during tumor progression (4). The replicative past and potential of cells can be assessed through TL and telomerase activity estimation methods, focusing on short telomere load and the rate of telomere shortening that reflects cellular aging (5).

Telomere attrition and cellular senescence are identified as major contributors to the physiological process of aging (6). Furthermore, increased aging and age-related diseases have been shown to be associated with the disruption of telomere homeostasis, which has been mainly attributed to increased levels of oxidative stress and inflammation (7). To date, cumulative oxidative stress and inflammation have been strongly

associated with age-related TL shortening (8). Research on the associations of telomere shortening with age has revealed that the inhibition of telomere attrition via telomere gene therapy in mice results in delayed physical aging (9). By contrast, it has been suggested that a short leukocyte TL is an independent risk factor that accounts for functional decline in elderly European populations (10). Furthermore, critically short telomeres have been shown to be associated with higher mortality rates and shorter lifespans (11).

The human lifespan has been considerably extended, leading to an increase in the elderly populations globally, stressing the need to develop 'healthy aging' strategies that will prevent and treat aging-related diseases (12). Research focusing on identifying effective natural or synthetic compounds with anti-aging properties has intensified in recent years (13). Specifically, there is increasing evidence to indicate that bioactive compounds, including nutrients and vitamins present in foods and supplements with antioxidant and anti-inflammatory properties can ameliorate age-related phenotypes, including telomere shortening (14,15). Of note, telomerase activity can be positively modified by natural compounds, such as *Astragalus membranaceus* plant extracts, cycloastragenol (CAG), curcumin C3 and vitamins, among others, which enable telomerase activation (13). Moreover, in a previous study, the authors demonstrated that a formulation containing *Centella asiatica* (*C. asiatica*) extract markedly enhanced telomerase activity in human peripheral blood mononuclear cells (PBMCs) *in vitro* (16). Notably, the demonstrated increase in telomerase activity was the highest reported *in vitro*, to date, at least to the best of our knowledge (16). Another study also demonstrated that the administration of the formulation containing *C. asiatica* extract, vitamin C, zinc and vitamin D3 for a period of 3 months restored TERT expression and increased telomerase activity in the brains of middle-aged rats. Furthermore, a structural reversibility effect was observed in the brains of middle-aged rats treated with the formulation, close to the differentiation of the grey matter from a young control group (17). Supporting evidence was provided from a complementary behavioral study of the same treated rats, where supplementation with the formulation improved the locomotor activity and decreased stress significantly in a dose-dependent manner in aged rats (12).

The present study aimed to examine the effects of the nutraceutical formulation containing *C. asiatica* extract, vitamin C, zinc and vitamin D3 on telomerase activity and TL using a murine model. The obtained results shed light onto the potential association of supplement administration with the abating of the aging process.

Materials and methods

Animal model. All animals were obtained from the University of Medicine and Pharmacy of Craiova Animal House, Craiova Romania, authorization no. 76/20.04.2016. The Ethical Committee of the University of Medicine and Pharmacy of Craiova, Craiova, Romania, approved the animal study (protocol no. 102/23.09.2019). All the procedures were according to the European directives for animal experiments (E.U. Directive 2010/63/E.U.as amended by Regulation E.U.2019/1010). A total of 24 Sprague-Dawley (CD-SD) male rats divided into four groups were used in the present study. Of the total 24 animals, 18 rats were 18 months old [body weight (bw) range, 500-580 g]. The middle-aged animals were randomly assigned to three groups as follows: Old group 1, old group 2 and old control group, with 6 rats in each group. The remaining 6 rats were 3 months old, and were denominated as the young control group. Male rats were selected over female animals to reduce the variability of responses due to potentially asynchronous estrous cycles (18). All animals were acclimatized to their new housing conditions for 2 weeks before the commencement of the study. The rats were kept in cages containing 2 or 3 animals during the study, under standard conditions with a constant temperature (22±1°C) and humidity (50±10%) and a 12-h dark/light cycle, receiving free access to standard animal feed and tap water. The study animals received the Reverse™ (Natural Doctor S.A.) supplement treatments presented in Table I for 3 months and were evaluated daily for signs of morbidity and mortality. At the end of the experiment (3 months), the animals received 5% sevoflurane anesthesia via nasal administration and were monitored until they were fully anesthetized. The rats were then sacrificed by exsanguination from the abdominal aorta for the analysis. As stated in current regulatory testing guidelines, when animals are evidently in pain, exhibiting signs of severe and enduring distress, or characterized as moribund, they were humanely euthanized rather than allowing them to survive to the end of the scheduled study period. However, in the present study, no animal death was observed (19,20). The animals were examined daily for any walking impairments that prevented access to food or water, excessive weight loss and emaciation, a lack of physical or mental alertness, difficulty breathing, and an inability to stand upright for long periods of time.

Treatment dose selection and administration. Reverse™ (Natural Doctor S.A.), notified as a food supplement (Notification no. 6704/21 1 2020) at the Greek National Organization for Medicines was administered at a dose of 1 or 2 capsules/kg bw/day. The supplement contains 9 mg *C. asiatica* extract (consisting of a >90% high purity single chemical entity, as assessed using high-performance liquid chromatography and gas chromatography) (21), vitamin C (200 mg as magnesium ascorbate), zinc (5 mg as zinc citrate) and vitamin D3 (50 μg as cholecalciferol) per capsule.

The doses administered to the rats were extrapolated from the human doses using the correction factor (fc) ratio for the species used and the safety factor value for humans. The fc is estimated as the ratio of the mean bw (kg) of the species used, to the species' body surface area (m^2), according to the Food and Drug Administration guidelines being 6 for rats and 37 for humans, respectively. The fc ratio for drug dose conversion from rats to humans corresponds to a value of 6.2. The safety factor (fs) value for converting rat doses to humans is 1043. The reference bw for humans is 60 kg (22), which means the dose for humans (dH) is calculated to 1/60 capsules per kg bw for a single dose of 1 capsule and 2/60 capsules per kg bw for a single dose of 2 capsules. Based on the information provided above, the dose in rats (dR) was calculated as follows: dR=dH x fcratio x fs (Equation 1).

Based on Equation 1, the old group 1 receiving the equivalent of the 1 capsule/day human dose, was administered

Table I. Treatments administered to the animal groups for 3 months daily and sampling time points (pre-and post-treatment).

Animal groups	Old group 1 (n=6)	Old group 2 (n=6)	Old control group (n=6)	Young control group (n=6)
Treatments	1 capsule per kg/body weight of Reverse™ supplement	2 capsules per kg/body weight of Reverse™ supplement	1.5 ml corn oil	1.5 ml corn oil
Age (months) (pre-treatment)	18	18	18	3
Age (months) (post-treatment)	21	21	21	6

dR=1/60 capsules x 6.2x10=1.03 capsule per kg/bw. The Old Group 2, receiving the equivalent of the 2 capsules/day human dose, was issued with dR=2/60 capsule x 6.2x10=2.06 capsule per kg/bw.

Prior to treatment administration, the content of the capsules was suspended in corn oil, widely used as an inert suspension agent for non-water-soluble drugs in animal experiments (23), as a stock suspension with 1 capsule/1 ml corn oil concentration or 2 capsules/1 ml corn oil. Each rat received the equivalent of 1 capsule per kg/bw or 2 capsules per kg/bw, once per day at the same time for 3 months (Table I). The respective equivalent dose calculated according to the bw of each animal was diluted with corn oil until the final volume of 1.5 ml and administered by gavage.

The dose administered was within clinical recommendations relative to bw and similar to other animal studies evaluating the effects of vitamin C (24,25), vitamin D3 (25-hydroxyvitamin D) (26,27) and zinc (28). Specifically, as regards vitamin D3, the administered dose per capsule was below the upper limit and was safely administered without medical supervision. Concerning the safety levels of the ingested dose for vitamin D, the most common concern is the risk of hypercalcemia, which may be evoked when the serum 25-hydroxyvitamin D levels exceed 700 ng/ml, which is >7-fold higher than the levels of sufficiency (29). Cholecalciferol was selected over ergocalciferol since ergocalciferol is less stable and less potent than cholecalciferol, characterized as the only vitamin D form suitable for supplementation (21). For *C. asiatica*, there is no established clinical recommendation, at least to the best of our knowledge. The dose administered in the present study was markedly lower than the levels reported in the literature (30).

Sample collection and preparation for quantitative-fluorescent in situ hybridization (Q-FISH). Peripheral blood from the tail vein (2 ml) was collected from each rat at the start of the experiment (pre-treatment) and after 3 months (post-treatment). The collected heparinized blood cultured in 50-ml falcon tubes with RPMI-1640 culture medium supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin, phytohemagglutinin 100 μg/ml and streptomycin was stimulated for 72 h in a CO_2 incubator with phytohemagglutinin. The cells were incubated at 37°C with 10 μg/ml colcemid for 2 h to obtain metaphases, followed by KCl hypotonic shock, and then were harvested and fixed in methanol/acetic acid (3:1). All reagents were obtained from MilliporeSigma.

Quantitative-fluorescent in situ hybridization (Q-FISH). Several drops of a fixative solution containing cells from each culture were applied to three slides. Slides with the fixative solution containing cells were thereupon dried and incubated on a hot plate (55°C) overnight prior to hybridization. Fluorescence staining for telomeric DNA was performed using the peptide nucleic acid (PNA) fluorescent probe Cy3-(C3TA2)3 obtained from Panagene Inc. Each slide was hybridized with 20 μl of 60% formamide (MilliporeSigma) and 0.3 mg/ml Cy3 (C3TA2)3 PNA probe (HLB Panagene) diluted in 20 mM Tris pH 7.4 (MilliporeSigma) and covered using a coverslip (76x26 mm). Cell DNA was denatured by heat treatment for 10 min at 85°C. Following hybridization for 2 h at room temperature, the slides were washed at 55°C with a washing solution containing 0.05% Tween-20 (MilliporeSigma) (2x10 min). The chromosome preparations were counterstained with 4',6-diamino-2-phenylindole (DAPI) (Thermo Fisher Scientific, Inc.) (0.5 μl/ml SSC) at room temperature for 20 min and then washed with SSC solution (MilliporeSigma) (2x2 min). The slides were air-dried and covered using mounting media and a coverslip before proceeding with image acquisition.

Image analysis. The analysis of FISH Images from metaphase cells was performed using a Leica TCS Sp8 inverted laser scanning spectral confocal microscope (Leica Microsystems GmbH). For each slide, a total of 20-30 optical sections were captured. Metaphase spread images were captured using a 63X objective and a charge-coupled device camera at a 1024x1024 pixel resolution and 8-bit depth with a step of 250 nm. A 405-nm laser was used for the excitation and detection of DAPI for nuclear staining. In comparison, a 568-nm laser was utilized for the excitation and detection of Alexa Fluor 647 for telomere staining. Exposure and gain settings remained unaltered between captures to avoid differences between the replicates. For each slide, >10 different scanned images were obtained. The maximum projections and deconvolution of the images were performed using Leica Q-FISH software (Leica Application Suite-Advanced Fluorescence version 3.1.3 for Leica TCS SP8; Leica Microsystems, Inc.). The quantification of telomere fluorescence intensity was performed twice by two different researchers using ImageJ software 1.8.0 (National Institutes of Health). A total of two calibration steps were undertaken to ensure the accuracy of telomere fluorescence intensity quantification. First, images of fluorescent beads (orange beads, size 0.2 μm, Thermo Fisher Scientific, Inc.) were obtained before the samples. Fluorescence intensities of the beads (obtained using ImageJ software) were used to

normalize and adjust the lamp intensity and alignment prior to sample analysis. Second, for the determination of the telomere fluorescence intensity the L5178Y-S cells (cat. no. 93050408; European Collection of Authenticated Cell Cultures) were included as a calibration standard in each experiment. The mean value of L5178Y-S cell telomere fluorescence intensities was used to normalize the respective values of samples between slides. Telomere fluorescence values were converted into kb according to the telomere fluorescence intensity of L5178Y-S cells that have an established TL of ~7 kb (31).

Telomerase activity assay. Blood samples for telomerase activity assay were collected from each animal at the beginning of the experiment (pre-treatment) and after 3 months (post-treatment). PBMCs were isolated from the blood samples using Ficoll-Hypaque (MilliporeSigma) gradient centrifugation at 277 x g for 5 min at room temperature in 15-ml falcon tubes. The extracted PBMCs were cultured in DMEM (F0455, Biochrom AG) supplemented with 10% FBS (10500-064, Invitrogen; Thermo Fisher Scientific, Inc.) with 4 mM glutamine (XCT1715, Biosera) and antibiotics (gentamycin; 15710-049, Gibco; Thermo Fisher Scientific, Inc.; and 100 U/ml penicillin/streptomycin; LMA4118, Biosera). Telomerase activity was quantified using a TeloTAGGG telomerase PCRELISA TRAP kit (MilliporeSigma), based on the telomeric repeat amplification protocol, as previously described (16). All measures for each condition were performed in triplicate.

Statistical analysis. The obtained data on TL were implemented into the specialized spreadsheet (BIOTEL 2.4) (32) to produce TL statistics, including percentiles, medians and telomere distribution. The TL data were further statistically analyzed using the IBM SPSS Statistics 24.0 package (IBM Corp.), while data for telomerase activity were processed using GraphPad Prism version 5.0 for Windows (GraphPad Software, Inc., www.graphpad.com). TL data are presented in two forms as follows: i) As the mean with 95% confidence intervals (CIs); and ii) as the median and interquartile range (IQR). Parametric (independent samples t-test) and non-parametric tests (Mann-Whitney test) for independent samples were applied to compare the means of two independent groups or, additionally/alternatively, the distribution of TL data. A similar approach using one-way ANOVA (parametric) and Kruskal-Wallis (non-parametric) for independent samples was applied to compare means for more than two separate groups or, additionally/alternatively, the distribution of TL data. These analyses (ANOVA and Kruskal-Wallis) was followed by post hoc analyses using the Bonferroni adjusted t-test, SNK, Dunnett's for ANOVA, and Dunn's test for Kruskal-Wallis.

Differences in paired design (pre-and post-treatment measures) were examined using a t-test for paired samples (parametric) or a Wilcoxon ranked sum test (non-parametric). Boxplots and scatterplots were applied for the graphical representation of the data. A value of P<0.05 was set as a significant statistical hypothesis.

Results

Effects of the nutraceutical formulation on the TL of the rats. Initially, the present study evaluated TL variations during the

3-month study period in the animals that did not receive treatment. These animals demonstrated a significant reduction in the mean and median TL during this period (Table II). Specifically, the pre-treatment mean TL of the young control group of 19.468 bp (19.146-19.789) was significantly longer compared to the TL mean value [14.535 bp (14.329-14.741)] at the study termination. Similarly, the pre-treatment mean TL values of the old control were decreased during the 3-month period (Table II).

Of note, a mean TL reduction was not observed in the rats that received the treatment. Specifically, no significant differences were detected between the mean TL values at pre-treatment compared to post-treatment in the old group 1 [t (449)=1.45, P=0.148] (Table II). In the old group 2, the mean TL values at 13.600 bp (12.755-14.446) were statistically significantly higher at post-treatment compared to the baseline TL values of 10.469 bp (10.203-10.734), while the distribution was not significantly altered (z=-1.179, P=0.238) (Table II). The mean telomere length levels at baseline and at 3 months post-treatment are illustrated in Fig. 1 for the control groups and the groups that received treatments and indicative images from Q-FISH analysis referring to old group 2 pre-treatment and to old group 2 post-treatment are also presented.

One-way ANOVA and the Kruskal-Wallis test were applied to examine the differences between the baseline TL of the old group 1, old group 2 and the old control group. No statistically significant differences were determined for the baseline mean TL among the old control groups. Similarly, no significant differences were detected in TL distributions [median of the overall old control group, 10.068; median old group 1, 10.221; and old group 2, 10.292; $\chi^2(2)=0.463$, P=0.793] (Table II).

On the other hand, significant differences in the mean and median TLs among these groups were identified post-treatment (Table II). The old control group displayed lower mean TL values (8.420, 8.127-8.713 bp) compared to the intervention groups [old group 1, 10.369 bp (10.010-10.729 bp); old group 2, 13.600 bp (12.755-14.446 bp); F (2.1581)=104.93, P<0.001]. The median TL values post-treatment exhibited similar differences [$\chi^2(2)=136.40$, P<0.001]. The TL distribution per animal at the two set time points (pre-and post-treatment) is presented in Fig. 2. The results of the Kolmogorov-Smirnov test for normality are presented in Table SI.

Independent statistical analysis using the adjusted t-test (LSD, Bonferroni or Dunnett's) likewise did not detect any differences in the TL pre-treatment values between the old controls and the old treatment groups (old group 1 and old group 2). On the other hand, significant differences in TL values were detected between the old controls and the rats in old group 1, and old group 1 vs. the rats in old group 2. A detailed post hoc analysis of pairwise differences is presented in Tables SII and SIII.

Effects of treatment with the nutraceutical formulation on the TL reduction rate. To evaluate the effects of the nutraceutical formulation treatment on the rate of TL change, the mean and median difference in TL per month (TL pre- and TL post-treatment)/month was calculated and represented for each group (Table SIV). A decrease in the median difference in TL per month was detected in all the study groups. In younger rats (untreated young control), the magnitude of the TL reduction rate of TL values was shown to be relatively high, whereas the

Table II. Descriptive statistics of TL and comparisons between pre-treatment (baseline).

Statistics	Young control		Old control		Old group 1		Old group 2	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Min	8.061	6.542	2.805	1.952	2.671	4.075	2.543	2.905
95% LB	19.146	14.329	10.294	8.127	10.240	10.010	10.203	12.755
Mean	19.468	14.535	10.569	8.420	10.472	10.369	10.469	13.600
95% UB	19.789	14.741	10.843	8.713	10.703	10.729	10.734	14.446
1st	16.613	12.613	7.697	5.593	8.737	7.407	8.088	7.117
Median	19.297	14.439	10.068	7.103	10.221	9.142	10.292	9.064
3rd	22.458	16.366	13.220	10.682	12.009	12.637	12.502	19.385
Max	31.379	22.438	22.178	21.616	17.833	22.792	19.305	34.881
P-value[a]	$t(587)=82.42$, P<0.001		$t(671)=76.35$, P<0.001		$t(449)=1.45$, P=0.148		$t(449)=-10.08$, P<0.001	
P-value[b]	$z=-21.00$, P<0,001		$z=-22.46$, P<0.001		$z=-3.14$, P=0.002		$z=-1.179$, P=0.238	
P-value[c]	Pre: $F(2, 1581)=0.19$, P=0.825 Post: $F(2, 1581)=104.93$, P<0.001							
P-value[d]	Pre: $\chi^2(2)=0.463$, P=0.793 Post: $\chi^2(2)=136.40$, P<0.001							

[a]Paired samples t-test for pre-post comparisons for each group; [b]Wilcoxon signed rank sum test for pre-post comparisons for each group; [c]one way ANOVA comparing TL measures between groups of rats at pre-intervention (pre) and post-intervention (post) (young controls were not included in the analysis); [d]non-parametric Kruskal-Wallis test comparing TL measures between groups of rats at pre-intervention (pre) and post-intervention (post) (young controls were not included in the analysis).
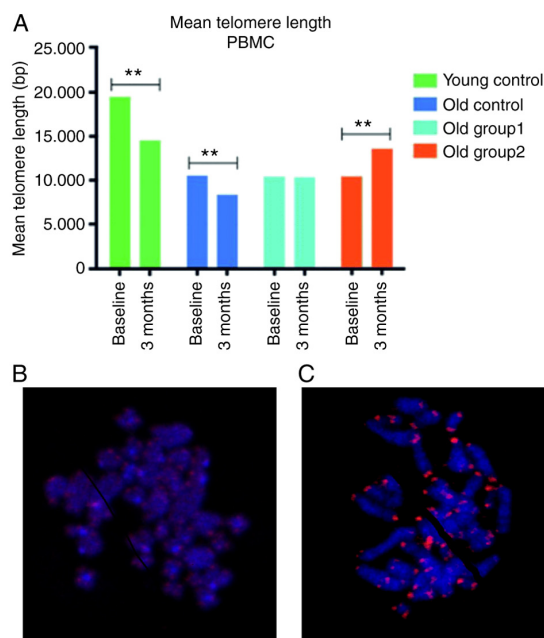

Figure 1. (A) PBMC mean telomere length values (bp), at baseline and post-treatment (3 months). Horizontal bars with two asterisks (**) indicate significant comparisons at P<0.01 in paired comparisons. (B) An indicative image from Q-FISH analysis is presented from the old group 2 pre-treatment. (C) An indicative image from Q-FISH analysis is presented from the old group 2 post-treatment. PBMC, peripheral blood mononuclear cell.

reduction rate of TL values for the old untreated control group was decreased to ~50% as compared to the reduction rate of the young controls. Treated with supplements, rats showed a complex pattern in TL changes. The median TL reduction rate of the old group 1 and old group 2 was <50% compared to the reduction rate of the old control group (Fig. 3). Nevertheless, the mean TL reduction rate was low for old group 1, whereas the TL of old group 2 exhibited a considerable increase with a mean of -1044 bases/month (Fig. 3).

Effects of aging on rat TL. Furthermore, the effects of aging on TL were evaluated in the old and young rat groups. The median TL and quartile range values at 3 months (young control pre-), 6 months (young control post), 18 months (old control pre) and 21 months (old control post) are presented (Fig. 4). A TL decrement is apparent, and TL vs. time dependence can be characterized by a linear regression line TL=-1743 * time + 19.700 bp with an R^2=0.932.

Telomerase activity. The baseline telomerase activity of the PBMCs collected from all rat groups measured as fluorescence at 450 nm OD is presented in Fig. 5. The mean telomerase activity of the PBMCs was 0.355±0.033 for the young control group, while the old control group exhibited a mean activity of 0.163±0.03. Old group 1 and old group 2 exhibited similar baseline telomerase activities with a mean of 0.168±0.03 and 0.163±0.03, respectively. One-way ANOVA revealed a statistically significant difference between the young group and all old groups [(F(3, 12)=37,94, P<0.001)]. Similar results were obtained with the post-hoc Dunnett's test (t=8.784, P<0.001).

The telomerase activity of the PBMCs form the control and treated groups at 3 months post-treatment is presented in
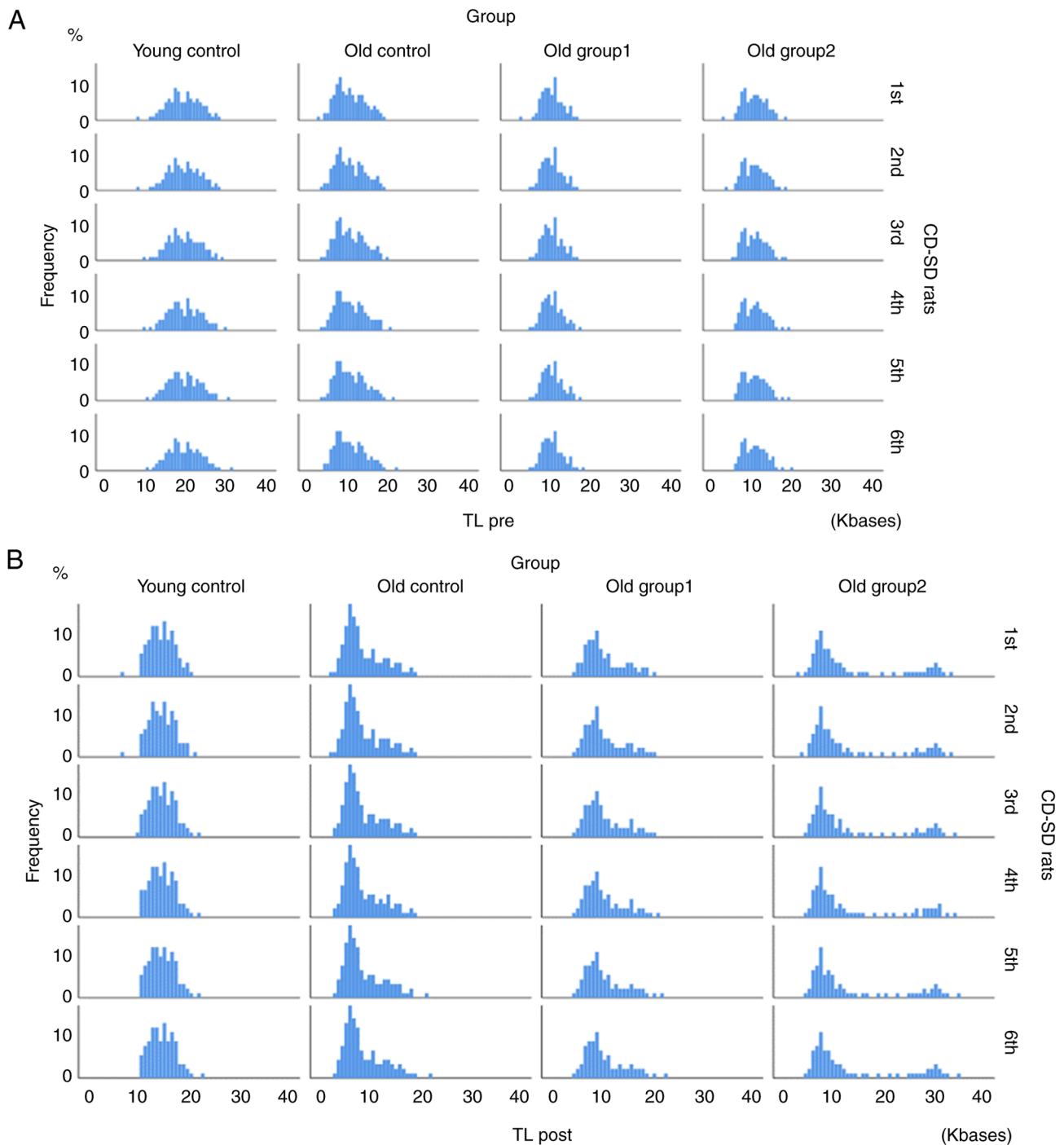
Figure 2. Histograms for the TL distribution per treatment group and per animal of Sprague-Dawley rats at (A) baseline (start of the experiment) and (B) 3 months following the onset of the experiments. TL, telomere length.

Fig. 6. One-way ANOVA revealed a statistically significant difference between the young control group (0.343±0.03), old control group (0.118±0.02), treated old group1 (0.258±0.017) and treated old group2 (0.298±0.033) with an $F_{(3, 12)}=52.17$ and a P-value of <0.001. In continuation, the analysis of telomerase activity values with the Dunnett's test revealed a significant difference between the old and young controls (t=11.82, P<0,05), and between old group 1 and the young controls (t=4.464, P<0.05). Notably, no marked differences in telomerase activity were detected between old group 2 and the young controls (t=2.364, P>0.05). Statistically significant differences at the P<0.001 level were found for old group 1 and old group 2 vs. the old control CD-SD rats (P>0.001).

A two-way ANOVA was applied to compare the telomerase activity of PBMCs expressed at OD450 nm fluorescence between four groups (two controls and two treated groups) at two time points (baseline and following 3 months of treatment). The analysis revealed that treatment x time interaction presented a significant effect [$F_{(3, 12)}=16.72$, P=0.007]. The administration of the *C. asiatica*-containing supplements induced significant effects on PBMC telomerase activity with $F_{(3, 12)}=69.95$, P<0.001, and $F_{(1, 12)}=5.46$, P=0.005,
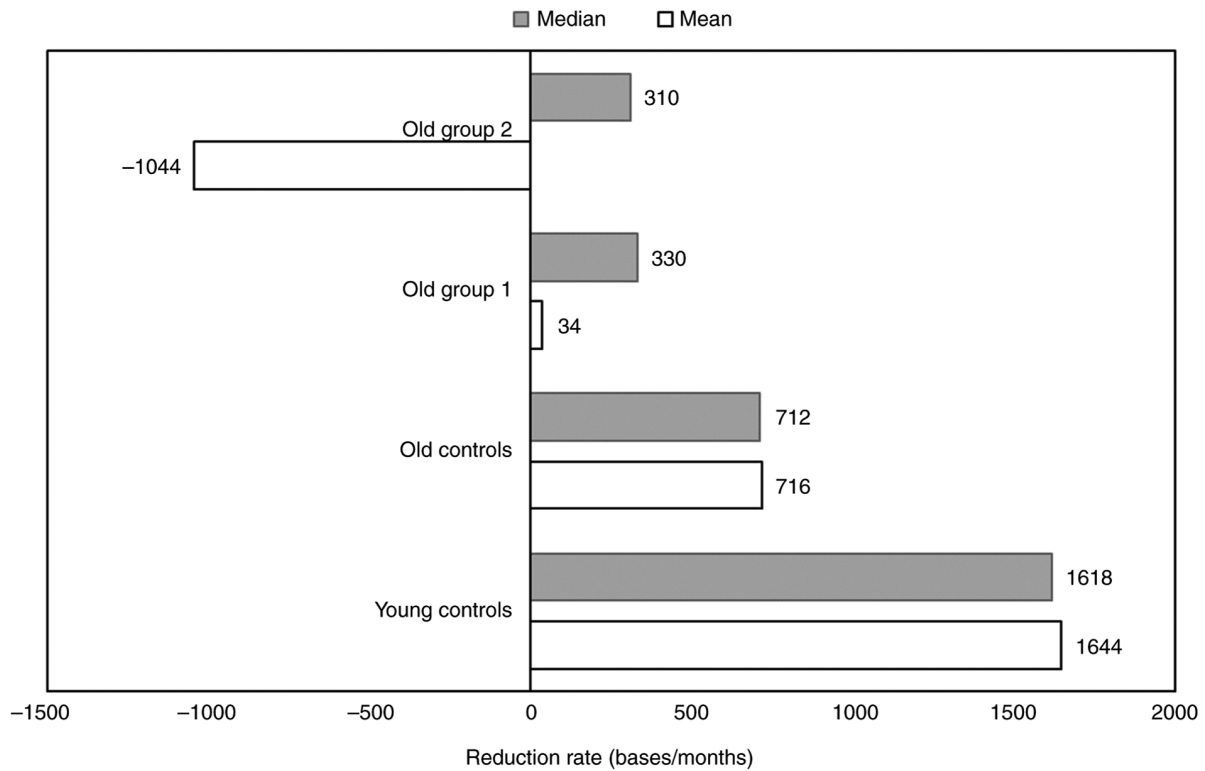
Figure 3. Mean and median reduction rate of TL (bases/month) during the 3-month experimental period, for each group separately (treated: old group 2 and old group 1; untreated: old controls, young controls). Negative values of mean reduction rate of TL of old group 2 (-1,044 bases/month) indicate telomere elongation, mean reduction rate of TL of old group 1 (34 bases/month), lower than both control groups (old controls, 716 bases/month; and young controls, 1,644 bases/month). TL, telomere length.
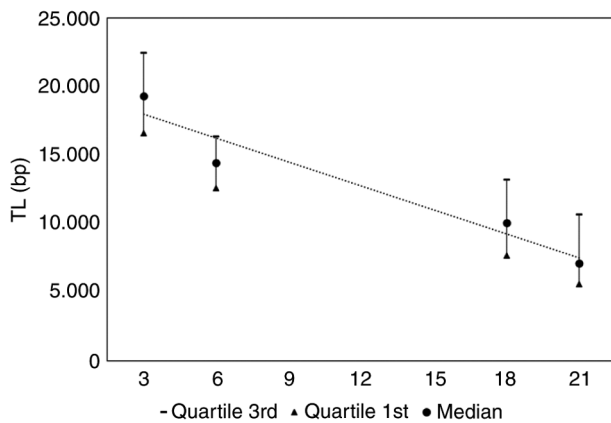

Figure 4. The TL scatterplot with error bars of the median TL (bp) (dots) and inter-quartile range (bars) at different time points (months) in the control groups representing the effects of aging on TL (young controls, 3 to 6 months; old controls, 18 to 21 months). TL, telomere length.
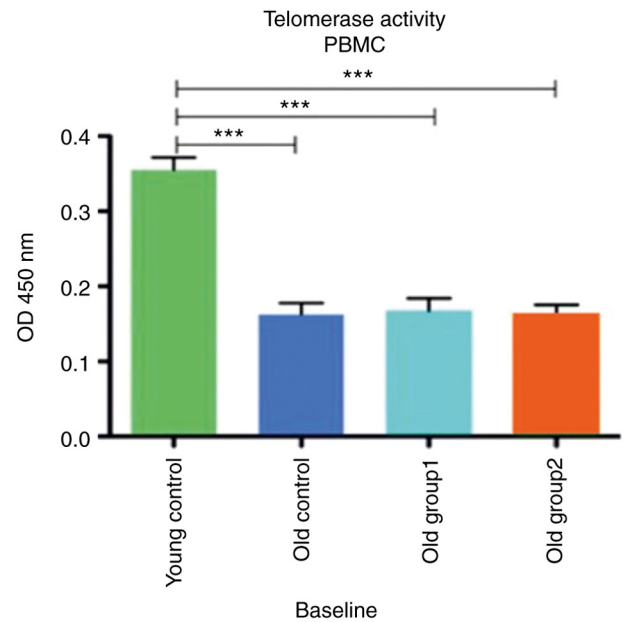

Figure 5. Mean PBMC telomerase activity at baseline expressed as OD450 fluorescence arbitrary units. Vertical bars represent the standard deviation of measurements. The three asterisks (***) resulting from Dunnett's post hoc tests indicate significant differences in comparisons between the young control vs. the other groups (P<0.001). PBMC, peripheral blood mononuclear cell.

respectively (Fig. 7). Furthermore, the Bonferroni post hoc test revealed differences in telomerase activity in old group 1 CD-SD rats (t=3.657, P<0.05) and old group 2 CD-SD rats (t=5.485, P<0.001) when comparing the baseline and the levels at 3 months after treatment.

Discussion

Telomere shortening is strongly associated with DNA damage, oxidative stress and inflammation (1), without omitting various other factors, including genetic and epigenetic mediators, which affect telomere length regulation (33).
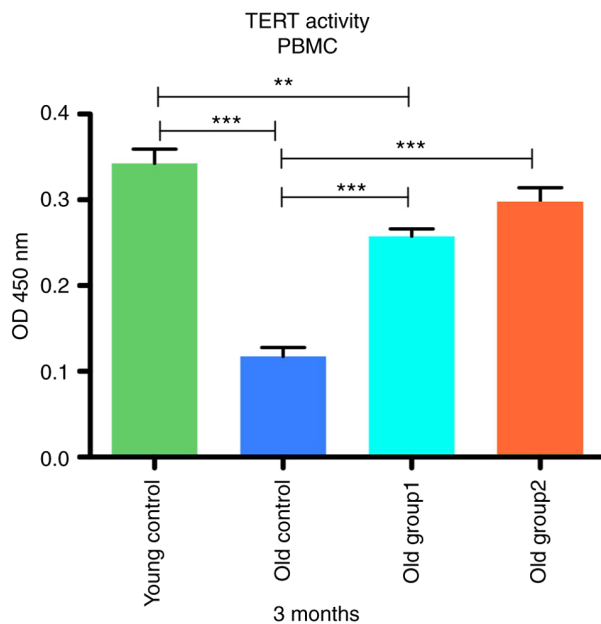
Figure 6. Mean PBMC telomerase activity after 3 months of exposure expressed as OD450 fluorescence presented as colored bars. Vertical thin bars represent the standard deviation of measurements. The horizontal bars indicate the significance of the two groups' comparisons using post hoc tests. The two (**) or three (***) asterisks refer to P<0.01 and P<0.001 significant differences, respectively between groups. PBMC, peripheral blood mononuclear cell.
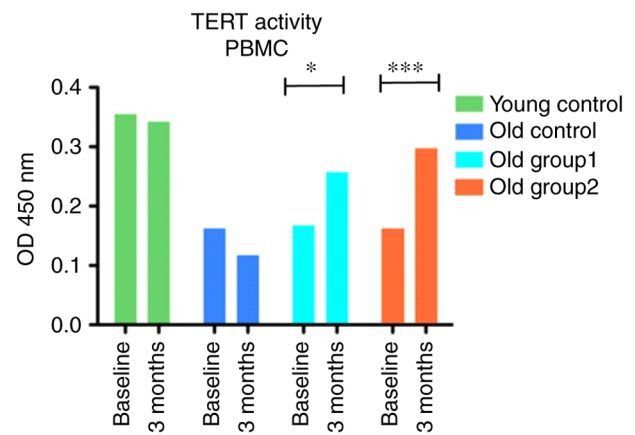

Figure 7. Mean PBMC telomerase activity expressed as OD450 fluorescence at baseline and post-treatment (3 months). Horizontal thin bars indicate significant differences within groups between baseline and post-treatment. Three (***) and one (*) asterisk indicate significant comparisons at the P<0.001 and P<0.05 levels, respectively. PBMC, peripheral blood mononuclear cell.

Natural molecules with demonstrated antioxidant and anti-inflammatory capacities are being increasingly recognized as potent agents which can be used to restore telomere attrition for the improvement of aging-related diseases (34-36).

The effects of natural compounds on TL regulation have been ascribed to the central role of oxidative stress and inflammation to telomere shortening and telomerase, suggesting that nutrients with anti-inflammatory and antioxidant capacity can reduce cell sensitivity to telomere loss (37). Th authors have previously demonstrated that the nutraceutical formulation containing *C. asiatica* extract, vitamin C (as magnesium ascorbate), zinc (as zinc citrate) and vitamin D3 (as cholecalciferol) has a beneficial effect on aging-associated telomerase decline, behavior and brain morphology change *in vivo* (17). The present study examined the potency of the formulation in middle-aged rats and revealed that it results in telomere shortening attenuation and the activation of telomerase of PBMCs in a dose-dependent manner. Specifically, the treatment maintained the rat PBMC TL at the lower dose, whereas the higher dose increased the median TLs, compared to the pre-treatment levels. In terms of translating these findings in humans, it is supported that two capsules of the nutraceutical formulation per day were more potent in activating telomerase and increasing the telomere length of PBMCs than 1 capsule. Clinical trials on the efficacy of the nutraceutical formulation to telomere shortening and other aging biomarkers are required to further evaluate these effects.

Data from epidemiological studies and clinical trials have demonstrated that the increased intake of micronutrients, such as vitamins D, C, E and A, and dietary fiber in the context of a balanced diet, e.g., the Mediterranean diet, is associated with longer telomeres, as reviewed by Galiè *et al* (38). In addition, the consumption of nuts and seeds, a rich source of anti-inflammatory polyunsaturated fatty acids, has been showon to be associated with longer telomeres, and improved metabolic and blood lipid profiles, possibly mediated by an antioxidant effect (39).

The formulation tested herein consisted of nutrients that have been previously reported to improve aging-related and/or oxidative stress markers. *C. asiatica* is a herb utilized in traditional Chinese medicine for the treatment of various pathologies (40). The extract of this herb contains some pentacyclic triterpenoids, mainly asiaticoside, madecasosside, asiatic acid, madecassic acid and other components, including centellose and centelloside (41). Notably, the extracts of *C. asiatica* have been shown to exert potent antioxidant and anti-inflammatory effects. Specifically, the oral administration of *C. asiatica* has been shown to attenuate inflammatory markers and enhance the antioxidative function of diabetic rats (42). Moreover, carbon tetrachloride (CCl4)-induced liver fibrosis has been shown to be ameliorated by oral treatment with *C. asiatica*. Specifically, *C. asiatica* was shown to decrease CCl4-induced inflammation, oxidative stress and hepatocyte apoptosis, and to modify Bcl-2/Bax signaling in the livers of rats (43).

Furthermore, vitamin D is a fat-soluble micronutrient that participates in critical cellular functions beyond the absorption of calcium and its deficiency has been related to numerous inflammatory and aging-associated pathologies, including hypertension, cognitive decline and cardiometabolic complications (44). One of its extraskeletal effects is the regulation of the pace of aging as a regulator of mitochondrial function, oxidative stress and senescence (45). The results of an epidemiological study suggest that vitamin D can reduce the production of inflammatory mediators, such as C-reactive protein and interleukin-6, known to mitigate telomere shortening (46). Moreover, it was previously demonstrated that supplementation with vitamin D for 12 months improved cognitive function by reducing oxidative stress in older adults with mild cognitive impairment. This was associated with an increased TL (47). A separate, recent epidemiological study suggested that genetic variations in the gene encoding

vitamin D binding protein (GC-rs2282679) were associated with a low TL, suggesting that the vitamin D status may affect TL early in life (48).

A cross-sectional study performed on a cohort of 2,483 males demonstrated that 25-hydroxyvitamin D (25(OH)D) and 1,25-dihydroxy vitamin D plasma levels were not associated with a low TL (49). On the other hand, higher plasma 25(OH)D levels appear to be associated with longer telomeres in women, and this association appears to be modulated by calcium intake (50). There is additional epidemiological evidence linking high serum levels of vitamin D with a longer TL. As previously demonstrated, the treatment of patients undergoing hemodialysis with vitamin D resulted in a longer peripheral PBMC TL compared to that of untreated patients (51). Additionally, epidemiological studies have positively associated vitamin C intake with a longer TL, and the vitamin's antioxidative properties have further been associated with abated telomere shortening of peripheral blood cells (52-54). There is also *in vitro* evidence to support the potency of zinc to decrease the aging of mesenchymal stem cells via an increase in TL, telomerase expression and activity, with an additional decrease in the percentage of senescent cells and the epigenetic modification of the hTERT gene promoter (55).

The present study further demonstrated that the administration of the nutraceutical formulation containing *C. asiatica* extract, vitamin C, zinc and vitamin D3 increased the telomerase activity of PBMCs in a dose-dependent manner in a rat model. Specifically, the telomerase activity of medium-aged rats was reversed to the levels of the young controls at the higher dose. These findings are in line with those of previous studies demonstrating the potency of formulation and its naturally occurring constituents on telomerase activation (17). Moreover, the authors also recently demonstrated that *C. asiatica* extract increased telomerase activity in human PBMCs (16). The potency of the *C. asiatica*-containing formulation to activate telomerase was significantly more pronounced compared to other multi-nutrient formulations (16). Furthermore, vitamin C treatment has been found to be capable of mesenchymal stem cell sheet formation and tissue regeneration by inducing telomerase activity *in vitro* (56). In addition, vitamin C has been shown to activate telomerase in periodontal ligament stem cells, resulting in their enhanced expression of extracellular matrix components and stem cell markers (57). Moreover, the treatment of human pluripotent stem cell-derived cardiomyocytes with vitamin C has been found to result in the upregulation of human telomerase RNA (57). Likewise, supplementation with vitamin D has been demonstrated to significantly enhance the PBMC telomerase activity in a cohort of overweight African Americans (58). In a separate cohort of obese African Americans, vitamin D supplementation was associated with reduced epigenetic aging (59). Notwithstanding, it has been highlighted that the combination of nutrients and natural compounds exerts more significant effects than single compounds (13,16,60). Thus, it can be hypothesized that the beneficial effects of treatment on TL and telomerase may be due to the synergy of the constituents of the supplement. However, further studies are required to validate this.

The present study has several limitations which should be mentioned. First, the relatively low number of animals in the control and treatment groups can be mitigated by increasing animal numbers in future studies. In addition, the present study did not assess the putative mechanisms of action. In future studies, this point can be addressed by examining antioxidant, anti-inflammatory and various epigenetic markers. Finally, there is a general limitation in extrapolating results obtained in animal models to clinical efficacy that can be attributed to internal and external validity (61). In the present study, strict measures were taken to ensure internal validity and control bias. However, the issue of external validity, including the incapacity of animal models to emulate the complexity of human conditions and species-related differences, remain. One approach would be to perform the study with a larger number of animals exposed to some level of stress, e.g., sleep interruption, to better mimic the everyday pressures facing the majority of the human population. Furthermore, there are several differences between rats, as well as mice and humans regarding telomeres, telomerase and lifespan. Small animals such as mice and rats are characterized by longer telomeres and a higher telomerase expression in various tissues compared to humans, albeit with a lower lifespan (62). Still, they are the gold standard in aging research, with numerous reports on the impact of telomerase activation and underlying anti-aging mechanisms (63). Collectively, it was observed that the administration of the nutraceutical supplement resulted in telomerase activation in both PBMCs and the brains of rats in accordance with the amelioration of brain structure and function and the increase in TL. However, additional studies are required to define the association of these effects, given that rats have long telomeres, and other non-canonical pathways of TERT may be involved (64). Finally, epidemiological studies would also provide a better overview of the putative role of multi-nutrient supplements on TL and telomerase activity in humans.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

AT, DT, DAS, DN and AOD conceptualized the study. AT, DT, AOD, DN and DC were involved in the supervision of

the study. AMB, AOD, DC, ER, ES, EV and PF performed the experiments, sample treatment and analyses. ER, DN, ES, DT, AMB, EV, PF, AA, AOD, AT and DAS were involved in the writing and editing of the manuscript. AA performed the statistical analyses. AT and AA confirm the authenticity of the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All animals were obtained from the University of Medicine and Pharmacy of Craiova Animal House, Craiova Romania, authorization no. 76/20.04.2016. The Ethical Committee of the University of Medicine and Pharmacy of Craiova, Craiova, Romania, approved the animal study (protocol no. 102/23.09.2019). All the procedures were according to the European directives for animal experiments (E.U. Directive 2010/63/E.U.as amended by Regulation E.U.2019/1010).

Patient consent for publication

Not applicable.

Competing interests

DAS is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article. DT is a scientific advisor for Natural Doctor S.A. The other authors declare that they have no competing interests.

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