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ORIGINAL ARTICLE

Investigation of the Effects of *Chlorella vulgaris* Supplementation on the Modulation of Oxidative Stress in Apparently Healthy Smokers

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SUMMARY

Background: **Smoking is among the established yet modifiable risk factors for cancers, cardiovascular diseases, and pulmonary disorders. Oxidative stress has been proposed as a key mechanism mediating the deleterious consequences of smoking. The present study evaluated the effect of supplementation with *Chlorella vulgaris*, a nutrient and bioactive green microalgae with proven antioxidant capacity, on the burden of oxidative stress in Iranian smokers.**

Methods: **Thirty-eight smokers (mean age: 37.11 ± 1.69 years; females: 18.4%) were administered *C. vulgaris* extract (3600 mg/day) for a period of 6 weeks. Fasted serum samples collected at baseline and after the completion of study were analyzed for the concentrations of vitamin C, vitamin E, glutathione, and malonedialdehyde (MDA) as well as activities of superoxide dismutase, glutathione peroxidase, and catalase. Total antioxidant capacity of serum was also determined by the ability of serum to inhibit the formation of ferryl myoglobin radical species.**

Results: **Six-week supplementation with *C. vulgaris* extract in smokers was associated with marked elevation of all assessed serum antioxidant measures ($p < 0.001$) and significant reduction of MDA levels ($p = 0.002$). After gender segregation, a similar pattern of changes was observed for both male and female subjects apart from lack of significant change in serum vitamin E status in females. Although the magnitude of change in serum vitamin E was significantly greater in males compared to females ($p = 0.014$), there was no significant change in the magnitude of changes for other assessed parameters between the genders.**

Conclusions: **Supplementation with *C. vulgaris* extract significantly improves antioxidant status and attenuates lipid peroxidation in chronic cigarette smokers. Hence, *C. vulgaris* might prevent the disease burden and mortality rate associated with smoking.**

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KEY WORDS

smokers, *Chlorella vulgaris*, cigarette, algae, antioxidant, oxidative stress

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INTRODUCTION

Free radicals are highly reactive molecules/atoms which, due to their unpaired electrons, could impair several vital biomolecules such as lipids, proteins, carbohydrates, and DNA [1].

Any imbalance between the production of these radicals

and the natural antioxidant defense systems of the body would lead to a situation called oxidative stress. Heretofore, oxidative stress has been suggested to be implicated in the pathophysiology of around one hundred disorders. Smoking is one of the most well-known contributors of oxidative stress and yet a main risk factor for noncommunicable and chronic diseases. The prevalence of smoking among US adults has been reported to be 20.6% [2]. Based on the findings of a recent study among Tehran residents, some 20.6% of males and 2.9% of females are smokers [3].

Chlorella vulgaris is a green unicellular microalga of fresh water, which has long been used as a popular foodstuff in the Far East countries. *C. vulgaris* is a natural all-in-one supplement and a rich source of proteins and amino acids, vitamins, minerals, dietary fiber, and unsaturated fatty acids [4,5]. Owing to its extraordinary high protein content (~50%), *C. vulgaris* has gained increasing popularity and demand to be used as a superfood and biomass. In addition to its use as a food source, *C. vulgaris* has also diverse medicinal properties. Previous investigations have unveiled hepatoprotective, immunomodulatory, anti-hypertensive, anti-atherogenic, anti-diabetic, anti-hyperlipidemic, anti-inflammatory, anti-hyperglycemic, antioxidant, anti-tumor, anti-bacterial and anti-viral effects [6-8]. Such beneficial effects of *C. vulgaris* are directly related to its diverse content of bioactive micronutrients. It has also been demonstrated that *C. vulgaris* is a powerful detoxification aid for cadmium, dioxin and many other types of heavy metals, toxins, and pesticides [9,10]. Finally, this microalga has been affirmed as "generally recognized as safe" (GRAS) by FDA and its consumption has not been reported to be associated with any adverse event in previous clinical trials.

Given the high rates of smoking-induced disorders such as cardiovascular disease, lung cancer and chronic obstructive pulmonary disease, and smoking-associated mortality (~5 million deaths annually) [11-13], there is an apparent need for medications that could counterbalance the biochemical impairments that are induced by smoking. There has been considerable evidence on the reduction of antioxidant status in smoker vs. non-smoker subjects [14-17]. One way to mitigate the deleterious effects of smoking is to compensate the oxidative damage via consumption of natural antioxidant supplements. Alongside the aforementioned effects, *C. vulgaris* has promising antioxidant activity [17-19]. However, most of the findings on the antioxidant effects of *Chlorella* pertain to animal studies and clinical evidence, especially in cigarette smokers, is minimal. Therefore, the present study aimed to evaluate the effectiveness of supplementation with *C. vulgaris* on serum antioxidant measures of cigarette smokers.

MATERIALS AND METHODS

This study was performed as a prospective open-label clinical trial. Recruited subjects were apparently healthy smokers (defined as smoking ≥ 20 cigarettes per day) aged 17 - 62 years from the personnel of Loghman-Hakim Hospital (Tehran, Iran). Patients who had any chronic disease or history of hypersensitivity to herbal preparations were excluded from the study. A complete explanation about the intervention and its effects was given to all recruited subjects. Then, *C. vulgaris* extract was administered at a dose of 3600 mg/day (1800 mg b.i.d.) for 6 weeks. The study protocol was approved by the Ethics Committee of the Baqiyatallah University of Medical Sciences and written informed consent was obtained from participants.

C. vulgaris extract used in the study was in the form of 300 mg tablets which are commercially available under trade name ALGOMED® (Bioprodukte Prof. Steinberg Produktions- und Vertriebs GmbH & Co. KG, Klötze, Germany). The tablets contained 98% *C. vulgaris* powder, 1% separating agent (silicic acid), and 1% plant-based magnesium stearate). The tablets were ~9 mm in diameter and ~300 mg in weight. The ingredients of tablets are summarized in Table 1 (based on the manufacturer's information).

Fasted serum samples were collected at baseline as well as at the end of trial. Baseline samples were frozen at -80°C and analyzed in parallel with the post-trial samples. Biochemical factors that were measured in serum samples included vitamin C, vitamin E, Malonedialdehyde (MDA) and glutathione (GSH) concentrations, superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) activities, and total antioxidant status (TAS).

GSH measurement was based on the method of Beutler, Duron, and Kelly [20]. In this method, the reaction between GSH and 5,5`-dithiobis-2-nitrobenzoic acid (DTNB) produces a reduced chromogen. The yellowish product has an absorbance at 405 nm which could be measured spectrophotometrically.

The activity of SOD was determined using the method described by Ewing and Janero with slight modifications [21]. The method is based on the ability of superoxide radicals - generated by the reaction between NADH and phenazine methosulfate (PMS) - to reduce nitro blue tetrazolium (NBT) to formazan at acidic PH. The production of formazan could be detected spectrophotometrically at 560 nm. SOD is able to react with produced superoxides radicals, thereby preventing NBT conversion to the chromogen product. The change in absorbance over a period of 10 min was monitored at 560 nm with a microplate reader using a kinetic mode. One unit of enzyme activity was defined as the amount of enzyme that gave 50% inhibition of NBT reduction in one minute.

Serum catalase activity was measured using a spectrophotometric method described by Aebi [22]. The reaction is based on the disappearance rate of H_2O_2 at

Table 1. Some ingredients of *Chlorella vulgaris* tablets and their respective amounts.

	Ingredient	Quantity
Ingredient	Fat (g/100g)	8.65
	Protein (g/100g)	52.0
	Carbohydrates (g/100g)	13.6
	Ash (g/100g)	6.56
	Water (g/100g)	3.63
	Dietary fiber (g/100g)	15.6
	Energy (Kcal/100g)	340
Fatty acids	Saturated fatty acid (g/100g)	2.16
	Monounsaturated fatty acid (g/100g)	1.69
	Poly unsaturated fatty acid (g/100g)	3.34
	Trans fatty acid (g/100g)	0.06
ω-3 fatty acids	Linoleic acid (g/100g)	1.282
	α-Linolenic acid (g/100g)	1.964
ω-6 fatty acids	Octodecatetaenoic acid (g/100g)	0.003
	Eicosadienoic acid (g/100g)	0.011
	Arachidonc acid (g/100g)	0.009
	Docosatetraenoic acid (g/100g)	0.020
Vitamins	β-Carotene (mg/100g)	180.8
	Vitamin B1 (mg/100g)	1.5
	Vitamin B2 (mg/100g)	4.8
	Vitamin B3 (mg/100g)	23.8
	Vitamin B5 (mg/100g)	1.3
	Vitamin B6 (mg/100g	1.7
	Vitamin B12 (µg/100g)	125.9
	Vitamin C (mg/100g)	15.6
	Folic acid (µg/100g)	26.9
	Biotin (µg/100g)	191.6
	Para-amino-benzoic acid (mg/100g)	0.6
Minerals	Phosphorus (mg/100g)	959
	Potassium (mg/kg)	21450
	Magnesium (mg/kg)	4425
	Calcium (mg/kg)	2710
	Iron (mg/kg)	680
	Copper (mg/kg)	19.0
	Zinc (mg/kg)	54.5
	Manganese (mg/kg)	39.5
	Iodine (mg/kg)	12.9
	Chromium (mg/kg)	0.575
Miscellaneous	Lutein (mg/100g)	84.3
	Lycopin (mg/100g)	0.307
	Zeaxanthin (mg/100g)	0.679
	Chlorophyll (g/kg)	15.21

Administered *C. vulgais* tablets were from Bioprodukte Prof. Steinberg (Produktions- und Vertriebs GmbH & Co KG, Klötze, Germany).

Table 2. Summary of demographic characteristics of the study population.

		Total	Male	Female	p-value
n		38	31	7	-
Age (yrs)		37.11 ± 1.69	37.45 ± 10.73	35.57 ± 9.43	> 0.05
Duration of smoking (yrs)		7.89 ± 1.65	9.03 ± 10.69	2.86 ± 5.18	> 0.05
Physical activity (minutes/day)		10.00 ± 2.16	8.87 ± 11.67	15.00 ± 19.36	> 0.05
Fruit/vegetable consumption (unit/day)*	0	25 (65.8)	21 (67.4)	4 (57.1)	> 0.05
	1	8 (21.1)	6 (19.4)	2 (28.6)	
	2	5 (13.2)	4 (12.9)	1 (14.3)	

***Each unit was equivalent to 200g or an apple.**

Table 3. Effect of *C. vulgaris* on serum oxidative stress biomarkers in the study population.

Parameter	Pre-trial	Post-trial	p-value
Vit E (µg/dL)	0.84 ± 0.02	1.42 ± 0.06	< 0.001
Vit C (mg/dL)	0.82 ± 0.02	1.29 ± 0.05	< 0.001
GPx (U/mL)	4.66 ± 0.05	6.92 ± 0.07	< 0.001
SOD (U/mL)	2.34 ± 0.05	3.37 ± 0.07	< 0.001
CAT (U/mL)	42.89 ± 0.52	53.45 ± 0.51	< 0.001
GSH (µg/mL)	22.82 ± 0.18	32.31 ± 0.39	< 0.001
TAC (mmol/L)	1.39 ± 0.03	1.70 ± 0.03	< 0.001
MDA (nmol/mL)	9.76 ± 0.15	7.73 ± 0.11	0.002

Values are expressed as mean ± SEM. Vit E - vitamin E, VitC - vitamin C, GPx - glutathione peroxidase, SOD - superoxide dismutase, CAT – catalase, GSH – glutathione, TAC - total antioxidant capacity, MDA - malonedialdehyde.

240 nm in a medium containing 50 mM KPi-buffer (pH 7.0), 0.5 mM EDTA and serum.

GPx activity was evaluated using a coupled enzymatic assay [23]. In this assay, glutathione is first oxidized by H_2O_2 in the presence of GPx. In the second step, oxidized glutathione is reduced by glutathione reductase (GR) in the presence of NADPH. The disappearance rate of NADPH (conversion of NADPH to NADP) was measured as absorbance at 340 nm for 30 s. The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 0.33 mM NADPH, 0.11 M NaN3, 0.12 mM glutathione (GSH), 5 units of GR and serum sample. GPx activity was expressed as micromoles of NADPH oxidized per minute per mg protein.

MDA was measured using the TBARS method [24]. Serum samples were mixed with HCl 0.1N containing 1% phosphoric acid, 10 mM BHT (dissolved in ethanol) and 0.6% TBA. The mixture was then heated at 90°C for an hour cool and then allowed to cool. The pink colored complex was then extracted into *n*-butanol phase, vortexed and centrifuged at 5000 g for 10 min. The absorbance of supernatant was read at 532 nm. MDA levels are expressed as nanomoles of thiobarbituric acid reactive substances formed per milliliter of plasma. Total antioxidant status (TAS) was measured based on a colorimetric reaction [25]. In this assay, 2,2'-azino-di-[3-ethylbenzethiazoline sulphonate] (ATBS) serves as the reconstituted chromogen and is incubated with sample, a peroxidase (metmyoglobin) and H_2O_2 to produce a blue-green colored radical cation. The optical density of the radical cation is measured at 600 nm. The initial (A1) and final (A2; after 3 minutes) absorbances of the assay mixture were read prior and 3 minutes after addition of H_2O_2 to the assay mixture. TAS of each sample was then calculated using the following formula and expressed as mmol/L:

TAS = Concentration of Standard × (ΔA Blank - ΔA Sample)/(ΔA Blank - ΔA Standard).

Serum vitamin E was measured using an HPLC method and vitamin C using a colorimetric assay, with modifycations of the previously described methods [26].

Table 4. Effect of *C. vulgaris* on serum oxidative stress biomarkers in male and female subgroups.

	Males			Females		
	Pre-trial	Post-trial	p-value	Pre-trial	Post-trial	p-value
Vit E (µg/dL)	0.83 ± 0.02	1.48 ± 0.06	< 0.001	0.85 ± 0.03	1.16 ± 0.16	> 0.05
Vit C (mg/dL)	0.81 ± 0.02	1.29 ± 0.05	< 0.001	0.89 ± 0.03	1.29 ± 0.16	0.032
GPx (U/mL)	4.68 ± 0.05	6.95 ± 0.08	< 0.001	4.56 ± 0.09	6.79 ± 0.15	< 0.001
SOD (U/mL)	2.34 ± 0.05	3.36 ± 0.08	< 0.001	2.36 ± 0.13	3.39 ± 0.18	0.006
CAT (U/mL)	42.63 ± 0.62	53.47 ± 0.62	< 0.001	44.06 ± 0.60	53.34 ± 0.53	< 0.001
GSH (µg/mL)	22.89 ± 0.20	32.30 ± 0.46	< 0.001	22.53 ± 0.40	32.37 ± 0.73	< 0.001
TAC (mmol/L)	1.38 ± 0.04	1.71 ± 0.04	< 0.001	1.40 ± 0.05	1.68 ± 0.06	0.005
MDA (nmol/mL)	9.67 ± 0.13	7.70 ± 0.12	< 0.001	10.16 ± 0.56	7.90 ± 0.35	0.007

Values are expressed as mean ± SEM. Vit E - vitamin E, VitC - vitamin C, GPx - glutathione peroxidase, SOD - superoxide dismutase, CAT - catalase, GSH - glutathione, TAC - total antioxidant capacity, MDA - malonedialdehyde.

Table 5. Comparison of magnitude of changes in serum oxidative stress biomarkers between males and females.

Parameter	Male	Female	p-value
Vit E (µg/dL)	0.65 ± 0.05	0.31 ± 0.16	0.014
Vit C (mg/dL)	0.48 ± 0.06	0.40 ± 0.14	> 0.05
GPx (U/mL)	2.27 ± 0.08	2.23 ± 0.14	> 0.05
SOD (U/mL)	1.02 ± 0.10	1.03 ± 0.25	> 0.05
CAT (U/mL)	10.84 ± 0.96	9.29 ± 0.99	> 0.05
GSH (µg/mL)	9.41 ± 0.45	9.84 ± 0.69	> 0.05
TAC (mmol/L)	0.32 ± 0.04	0.28 ± 0.06	> 0.05
MDA (nmol/mL)	-1.98 ± 0.12	-2.26 ± 0.56	> 0.05

Values are expressed as mean ± SEM. Vit E - vitamin E, VitC - vitamin C, GPx - glutathione peroxidase, SOD - superoxide dismutase, CAT - catalase, GSH - glutathione, TAC - total antioxidant capacity, MDA - malonedialdehyde.

RESULTS

Out of the 40 individuals who initially entered the trial, 2 were dropped due to gastrointestinal side effects while 38 (male/female: 31/7) completed the trial and were included in the final analyses. There was no significant difference between males and females regarding age, duration of smoking habit, physical activity level and daily consumption of fruits and vegetables. Demographic characteristics of the study population are summarized in Table 2.

Effect of *C. vulgaris* supplementation on oxidative stress biomarkers

In the overall study population, supplementation with *C. vulgaris* was associated with significant elevations in serum levels of GSH, SOD, GPx, CAT, vitamin E, vitamin C, and TAS ($p < 0.001$) while a decrease in MDA ($p = 0.002$) (Table 3).

In males, all assessed antioxidant measures were significantly increased by the end of trial ($p < 0.001$). There was a similar trend for females ($p = 0.032$ for vitamin C, $p = 0.006$ for SOD; $p = 0.005$ for TAS, and $p < 0.001$ for GPx, CAT, and GSH) except for serum Vit E concentrations which remained unchanged compared to baseline ($p > 0.05$). Serum MDA levels decreased by the end of trial in both males ($p < 0.001$) and females ($p = 0.007$) subgroups (Table 4). As for the magnitude of changes in antioxidant measures and MDA, no significant difference was observed between males and females ($p > 0.05$). The only exception was the amount of Vit E elevation, which was significantly greater in males compared to females ($p = 0.014$) (Table 5).

Bivariate analyses

Bivariate correlations between serum antioxidant indices and MDA levels were evaluated at baseline as well as at the end of trial.

Table 6. Summary of clinical trials investigating the pharmacological effects of *C. vulgaris*.

Trial	Design	Study population	Intervention	Dose	Duration	Outcome
Panahi et al. [42]	Randomized open-label clinical trial	Dyslipidemic patients	*C. vulgaris* + atorvastatin (n =26) or atorvastatin (n = 37)	600 mg/day	8 weeks	No benefit for *C. vulgaris* as an adjunct to atorvastatin for the treatment of dyslipidemia
Panahi et al. [43]	Randomized open-label clinical trial	Patients with non-alcoholic fatty liver disease	*C. vulgaris* + low-dose metformin + vitamin E (n = 33) or high-dose metformin + vitamin E (n = 43)	1200 mg/day	12 weeks	Favorable effects on serum levels of transaminases and triglycerides as well as insulin sensitivity.
Panahi et al. [44]	Randomized open-label clinical trial	Patients with asthma or chronic obstructive pulmonary disease (COPD)	*C. vulgaris* + standard anti-asthma/anti-COPD treatment (n =28) or standard anti-asthma/anti-COPD treatment (n =29)	2700 (n =26)	8 weeks	Amelioration of serum antioxidant status but no clinical efficacy on respiratory function of patients with asthma or COPD
Lee et al. [41]	Randomized double-blind placebo-controlled trial	Smokers	*C. vulgaris* (n = 28) or placebo (n = 24)	6300 mg/day	6 weeks	Conservation of plasma antioxidant nutrient status and improvement in erythrocyte antioxidant enzyme activities
Shimada et al. [45]	Randomized double-blind placebo-controlled trial	Subjects with high-normal blood pressure and borderline hypertension	GABA-rich *Chlorella* (n = 38) or placebo (n = 39)	20 tablets of GABA-rich *Chlorella*/day equivalent to 20 mg GABA/day	12 weeks	Significant reduction of high-normal blood pressure and borderline hypertension
Nakamura et al. [46]	Open-label clinical trial	Subjects with mild hypertension	GABA-rich *Chlorella* (n = 10)	30 tablets of GABA-rich *Chlorella* (200 mg/ tablet)/ day	8 weeks	Reduction in systolic but not diastolic blood pressure + reductions in plasma adrenaline, noradrenaline, and dopamine
Sansawa et al. [47]	Open-label clinical trial	Patients with mild hyper-cholesterolemia	*Chlorella* (n = 11); 9 patients served as controls	6000 mg/day	12 weeks	Reduction of serum total cholesterol; LDL-cholesterol and atherogenic index

There were significant correlations between baseline serum SOD and Vit C (p = 0.035), GSH and TAS (p = 0.049), and borderline significant correlations between Vit E and CAT (p = 0.067), GPx and TAS (p = 0.060), and GPx and SOD (p = 0.062). In males, there were significant correlations between SOD and Vit C (p = 0.006) and borderline significant correlations between SOD and MDA (p = 0.055), Vit E and MDA (p = 0.051), GPx and TAS (p = 0.057), GPx and SOD (p = 0.063), and GPx and MDA (p = 0.057). In females, baseline GPx and GSH were significantly correlated with Vit E (p = 0.015) and Vit C (p = 0.013), respectively. With respect to the post-trial values, there was no significant correlation among the evaluated parameters,

neither in the overall population nor in the male subgroup. In females, Vit E levels were found to be correlated with CAT ($p = 0.029$) and MDA ($p = 0.067$).When the association between changes in the evaluated parameters was assessed, no significant correlation was observed, neither in the overall population nor in each individual gender ($p > 0.05$).

DISCUSSION

The purpose of the current study was to determine the antioxidant potential of *C. vulgaris* in smokers. The results clearly supported the relevance of *C. vulgaris* in the enhancement of serum antioxidant status and reducing lipid peroxidation.

C. vulgaris contains a broad spectrum of micro- and macronutrients including essential antioxidant vitamins, trace elements, ω-3 and ω-6 fatty acids and miscellaneous antioxidants such as lutein, zeaxanthin, chlorophyll, and lycopene [4,5; Table 1]. Due to this rich composition, *C. vulgaris* is supposed to function as a powerful antioxidant supplement. Analysis of serum oxidative stress biomarkers indicated a raised level of MDA along with a depleted status of SOD, GPx, vitamins E and C, CAT, and GSH compared to values previously reported for healthy individuals [27-30].

Our findings in the present study confirmed this notion as *Chlorella* boosted serum levels of all seven antioxidant measures that were measured in the serum of smokers. In addition, *C. vulgaris* supplementation reduced serum levels of MDA, which is a well-known lipid peroxidation product and widely used biomarker of oxidative stress. These beneficial effects of *C. vulgaris* in smoker subjects are of special importance as these subjects are subjected to a heightened burden of oxidative stress [14-17] and are therefore more susceptible to developing subsequent vascular and respiratory disorders. This elevated level of oxidative stress in smokers could be attributed to two factors. First, cigarette smoke contains around 3500 chemicals of which the majority is toxic, carcinogenic, and mutagenic [31]. Therefore, cigarette smoke is a rich source of free radicals and may directly induce oxidative stress. On the other hand, there has been epidemiologic evidence indicating that smokers consume fewer amounts of phytonutrient-rich foods such as fruits and vegetables in their daily diet [32,33]. Our findings corroborate those of previous studies. In a recent investigation on the antioxidant capacities of 12 strains of microalga, *C. vulgaris* showed promising activity in FRAP and DPPH-HPLC assays and was among the most active tested species [18].

Shibata and colleagues reported the reduction of serum peroxides in streptozocin-induced diabetes following 11-week dietary feeding with *Chlorella* powder [34]. In another study on mice fed with an atherogenic diet, addition of 5% *Chlorella* was associated with significant reductions in thiobarbituric acid reactive substances and superoxide anion generation as well as enhancement of hepatic SOD and CAT activities [19]. In a survey by Blas-Valdivia and colleagues, *C. vulgaris* administration was shown to protect against $HgCl_2$-induced oxidative stress and cellular damage in kidney by decreasing lipid peroxidation and reactive oxygen species and increasing glutathione content [35]. Yun et al. showed that 4-week supplementation with *C. vulgaris* exerts protective antioxidant effects against lead-induced oxidative stress, manifested by increased SOD, GPx and glutathione reductase activities, elevated glutathione content, and decreased MDA levels [36]. *C. vulgaris* extract has also been shown to protect against carbon tetrachloride-induced acute hepatic injury in mice. Inhibition of lipid peroxidation together with boosting hepatic glutathione content and activities of SOD, GPx, and glutathione-S-transferase enzymes have been reported as key mechanisms for this hepatoprotection [37]. There has also been further evidence confirming the antioxidant potential of *C. vulgaris* in different oxidative systems [7,34,38-40].

Heretofore, *C. vulgaris* has been the subject of a number of trials (Table 6). The robust trial by Lee et al. is the study most related to the present work. In the referred study, the effect of 6-week supplementation with *C. vulgaris* (6.3 g/day) was investigated on antioxidant status of Korean male smokers. Based on their results, the authors demonstrated that *C. vulgaris* increased plasma Vit C (by 44.4%), α-tocopherol (by 15.7%), and erythrocyte catalase and superoxide dismutase activities [41]. The results of the present study further confirmed these findings and provided additional evidence with respect to the antioxidant efficacy of a much lower *C. vulgaris* dose i.d. 1800 mg/day. In addition, the percentages of Vit C and Vit E elevations in the present (57.3% and 69.0% for Vit C and Vit E, respectively) study were much higher than those reported by Lee et al. (44.4% and 15.7%).

Overall, *C. vulgaris* supplementation was found to be safe and there was no report of serious adverse events following *Chlorella* consumption. This is consistent with the findings of our previous clinical investigations, in which there was no negative effect of *C. vulgaris* extract on circulating biomarkers of hepatic function (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and bilirubin), renal function (creatinine and blood urea nitrogen) as well as albumin, uric acid, and glucose levels [42-44].

In summary, the findings of the present trial favored the antioxidant impact of 6-week supplementation with *C. vulgaris* extract in chronic cigarette smokers. The most important limitation of the current study lies in its lack of blindness, placebo-controlling, and dietary intake assessment together with the relatively small number of recruited subjects. Hence, a large-scale double blind placebo-controlled trial with full nutrition intake assessment would have eliminated possible bias and reflect the effectiveness of *C. vulgaris* in a more precise manner. Another limitation is the lack of precise monitoring

of daily smoking. Such information allows the evaluation of the association between the degree of smoking and severity of alterations in serum oxidative stress biomarkers. In addition, future prospective investigations are warranted to clarify whether these antioxidant effects of *C. vulgaris* in smokers are translated into a lower frequency of cardiovascular and pulmonary outcomes and decreased mortality rates. Finally, given the close association between oxidative stress and inflammation, it would be interesting to explore if the observed antioxidant properties of *C. vulgaris* are associated with a significantly decreased burden of inflammation and alterations in the circulating levels of inflammatory biomarkers.

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Declaration of Interest:
The authors have no conflict of interest to declare.

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