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Antioxidant and anti-inflammatory effects of cauliflower leaf powder-enriched diet against LPS induced toxicity in rabbits

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Brassica phytochemicals exert a broad spectrum of health-promoting activities. The aim of this study was to investigate the possible beneficial effects of a cauliflower leaf powder (CLP)-enriched diet to prevent inflammation and oxidative stress resulting from injection of lipopolysaccharide (LPS) into rabbits. Animals (24 rabbits) were randomly divided into two groups and fed with a standard diet (SD) or a standard diet supplemented with a 100 g kg^{-1} diet of CLP. After 60 days, six rabbits of both groups received a LPS injection (100 μg per kg body weight). Serum samples collected after 90 min of LPS injection were assessed for their content of both inflammatory biomarkers such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and matrix-metalloproteinases (MMP-2 and MMP-9) and oxidative stress biomarkers such as thiobarbituric acid reactive substances (TBARS), glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT). LPS increased the levels of TNF-α, IL-6, and TBARS as well as MMP-2 and MMP-9 activities, whereas it decreased the GSH levels and SOD and CAT activities. In conclusion, preventive supplementation with CLP can protect rabbits from the inflammation and oxidative stress induced by LPS.

Introduction

The endotoxin lipopolysaccharide (LPS), one of the major structural components of the cell wall of Gram-negative bacteria, is an inducer of inflammatory response[1] and septic shock[2] and is classically used to stimulate the immune response in animals. The response to the toxic effects of LPS is highly variable among different animal species,[3] as it depends on the increase of circulating tumor necrosis factor alpha (TNF-α) induced by LPS. Mice and rabbits are equally sensitive to the effects of higher levels of circulating TNF-α, but mice are more resistant than rabbits to the induction of TNF-α by LPS.[4] Injection of LPS into animals promotes inflammation and oxidative stress,[2] and the release of cytokines such as TNF-α, interleukins (IL)-1 and IL-6, and reactive oxygen species (ROS), leads to fever[5] and causes multiple organ injury.[6] The administration of dietary supplements with antioxidant/anti-inflammatory action to animals treated with LPS could reduce its toxic effects. The most important and useful compounds with anti-inflammatory and antioxidant properties are the phytochemicals produced by plants to protect themselves from environmental stress and pathogens. These compounds offer various biological benefits to human and animal health. The *Brassicaceae* (*Cruciferae*) family is composed of 350 genera and about 3500 species. *Brassica* vegetables (for example, broccoli, cabbage, kale, mustard greens, Brussels sprouts, cauliflower, *etc.*) represent a rich source of health-promoting phytochemicals; their beneficial effects have been attributed to the anticancer properties of glucosinolates (GLSs) and their isothiocyanate (ITC) derivatives.[7] More generally, the contribution of *Brassica* vegetables to health improvement has been associated with their antioxidant and anti-inflammatory properties due to the presence of carotenoids, phenolic compounds and vitamins.[8,9] Some authors have reported on the anti-inflammatory properties of glucosinolates and their derived isothiocyanates in cultured cells and *in vivo* models.[10–13] However, some glucosinolates and their degradation products (isothiocyanates, thiocyanates, oxazolidinethiones and nitriles) originating from enzymatic cleavage by myrosinase have been linked to toxic and/or anti-nutritional effects. In this respect, it is worth mentioning their ability to inhibit iodine uptake and the biosynthesis of the thyroid hormones T3 and T4, leading to hypothyroidism and subsequent enlargement of the thyroid gland (goitre).[14] Glucosinolates are also very unpalatable, causing

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animals to reduce their feed intake, which causes less growth.[15] The oil meal of *Brassica* origin is a good source of protein for animal feed but the GLS content limits its efficient utilization. For this purpose, several processing techniques were used to remove or lower the GLS content in order to minimize their negative effects on animals.[15] However, as the recommended dietary content of GLSs for rabbits is only 7.9 mmol kg^{-1} at most,[16] we have tried to apply to the rabbits under study a preventive dietary treatment based on food supplemented with cauliflower leaf powder (*Brassica oleracea* var. *botrytis*) prior to injection of LPS. As a result, anti-inflammatory treatment with cauliflower leaf powder has proved to be effective.

Results and discussion

Phytochemicals and their antioxidant activity in cauliflower leaf powder

Phenolic compounds have received much attention due to their potential health benefits as antioxidants and protective agents against cancer and several other diseases, and therefore they have been largely studied in *Brassica* vegetables.[9,17] In *Brassica* crops, phytochemical contents and their antioxidant activity significantly vary among the types, cultivars and their different parts. The total phenolic content (TPC) of cauliflower leaf powder, as determined by the Folin–Ciocalteu method, was reported in terms of gallic acid equivalents per g of dry weight (mg GAE per g DW) (Table 1). The observed TPC content of 9.62 ± 0.78 mg GAE per g DW was similar to that reported by other authors.[18,19]

Flavonoids, due to their hydroxyl groups, show antioxidant activity and have a radical scavenging effect in plants, as well as providing health beneficial effects.[20] The total flavonoid content of leaves powder, as determined by the aluminum chloride method, was reported in terms of quercetin equivalents per g of dry weight (mg QE per g DW) (Table 1). The extract showed a total flavonoid content of 4.44 ± 0.22 mg QE per g DW, representing about 46% of the total phenolic compound content. A similar finding was reported in another study.[18]

Vitamin C is another health-promoting nutrient in *Brassica* crops that protects against cell death and ROS and acts as a lipid peroxidation chain-breaking agent. Cauliflower leaf powder showed a vitamin C content of 3.72 ± 0.37 mg per g of dry weight. The value observed in this study was lower than that found in the fresh product.[21] The loss of ascorbic acid can probably be ascribed to water leaching and its thermal degradation during the drying process.[22] The DPPH radicals are commonly used to evaluate the free radical scavenging activities of compounds. In this context, lower IC_{50} values reflected higher antioxidant activity. Methanolic leaves extract showed a moderate radical scavenging activity, with an IC_{50} value of 48.74 ± 2.09 μg mL^{-1} (Table 1), compared to ascorbic acid with an IC_{50} of 5 μg mL^{-1}, used as a control.

Glucosinolate content of cauliflower leaf powder

Ten glucosinolates were identified in the leaves of cauliflower (Table 2). Sinigrin was a predominant glucosinolate (5.34 ± 0.44 μmol g^{-1}), followed by glucoiberin (4.21 ± 0.39 μmol g^{-1}) and glucobrassicin (2.49 ± 0.11 μmol g^{-1}). These three major GLSs represented approximately 82% of the total GLSs. The other minor GLSs were progoitrin, glucobrassicanapin, gluconapin, neoglucobrassicin, gluconasturtiin, 4-metoxyglucobrassicin and 4-OH-glucobrassicin. The total glucosinolate content (14.59 μmol g^{-1}) was similar to that described by other studies.[23,24] Leaves extract showed a similar glucosinolated pattern to that reported by Cabello-Hurtado *et al.*,[23] except for the level of glucobrassicin, 17% instead of 7–12%, and for slight variations among the level of minor glucosinolates. These differences could be due to environmental factors as

Table 2 Glucosinolate (GLS) composition of cauliflower leaf powder

	μmol per g DW	%
Sinigrin	5.34 ± 0.44	36.60
Glucoiberin	4.21 ± 0.39	28.86
Glucobrassicin	2.49 ± 0.11	17.07
Progoitrin	0.62 ± 0.05	4.25
Glucobrassicanapin	0.51 ± 0.02	3.50
Gluconapin	0.42 ± 0.03	2.88
Neoglucobrassicin	0.39 ± 0.01	2.67
Gluconasturtiin	0.28 ± 0.01	1.92
4-Metoxyglucobrassicin	0.25 ± 0.02	1.71
4-OH-Glucobrassicin	0.08 ± 0.01	0.55
Total glucosinolates	14.59	

For GLS quantification, two independent extractions were performed, and HPLC analyses for each extract were performed in duplicate. Results were expressed as micromoles of GLS per gram of dry weight. Values are reported as the mean ± S.E.M. of two replicates each analysed in duplicate.

Table 1 Antioxidant compounds and their antioxidant activity in cauliflower leaf powder

Total phenolic content (mg GAE per g DW)	Total flavonoids (mg QE per g DW)	L-Ascorbic acid (mg AA per g DW)	DPPH radical scavenging activity (IC_{50} μg mL^{-1})
9.62 ± 0.78	4.44 ± 0.22	3.72 ± 0.37	48.74 ± 2.09

Total phenolic content was determined using Folin–Ciocalteu reagent and expressed as mg of gallic acid equivalents (GAE) per g of dry weight. Total flavonoid content was determined by the aluminum chloride method and expressed as mg of quercetin equivalents (QE) per g of dry weight. L-ascorbic acid was determined by HPLC and expressed as mg L-ascorbic acid (mg AA) per g of dry weight. DPPH: 2,2-diphenyl-1-picrylhydrazyl. The radical scavenging activity was expressed in terms of IC_{50}, where IC_{50} denotes the concentration (μg mL^{-1}) of the sample required to scavenge 50% of the DPPH free radicals. Values are reported as the mean ± S.E.M. of two replicates each analysed in triplicate.

well as origin, plant age, climate conditions and, in general, abiotic stress.[25]

Body weight

No animal died during the study. During the first week of experiments, in the group of rabbits fed with the supplement of cauliflower leaf powder, a slight reduction of feed intake and growth rate was observed, probably due to the unpalatable nature of the glucosinolates present in the diet. However, the mean body weight (3.45 ± 0.24 kg) of the rabbits at the end of the study was not significantly different among the animal groups.

Effect of diet on anti-inflammatory factors

Rectal temperature. In both the groups treated with LPS (LPS-SD and LPS-CLP), 30 minutes after injection there was an increase in temperature compared to the baseline and untreated groups (SD and CLP, respectively) (Fig. 1), whereas no increase was observed in the untreated group (CLP); this clearly demonstrates that fever represents a response to LPS injection. The maximum peak temperature was reached 180 minutes after injection (ΔT = 2.21 ± 0.22 and 1.11 ± 0.06 for LPS-SD and LPS-CLP, respectively); these data are in line with those observed in other studies conducted in adult male rabbits.[1,5] The temperature gradually decreased after 180 min and reached the basal level after 24 and 48 h in LPS-CLP and LPS-SD groups, respectively. In the LPS-CLP group the increase of temperature observed at all times was significantly less than that of the LPS-SD group ($P < 0.05$), which suggests the protective biological effect of the supplementation with cauliflower leaves (100 g kg^{-1} diet).

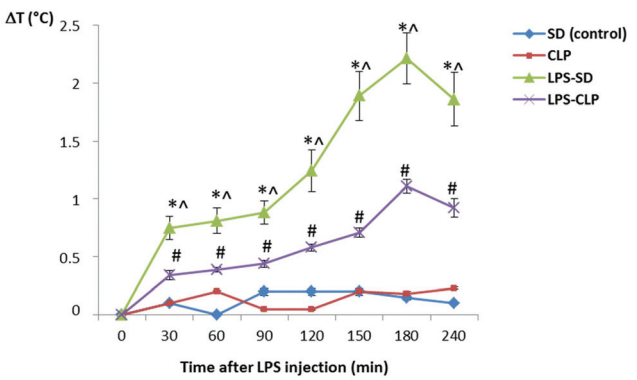

Fig. 1 Effect of dietary supplementation on the rectal temperature. ΔT: difference between the measured temperatures at each time-point and the baseline. Data were expressed as the means ± S.E.M. (n = 6). *Significant difference ($P < 0.05$) as compared to the control (SD group). ^Significant difference ($P < 0.05$) as compared to the LPS-CLP group. #Significant difference ($P < 0.05$) as compared to the CLP group (Tukey's test).

TNF-α and IL-6 serum levels. Table 3 shows the effect of the diet supplemented with cauliflower leaf powder on the rabbit serum levels of the pro-inflammatory cytokines TNF-α and IL-6 after 90 min of LPS injection at a dose of 100 μg kg^{-1}. Results showed that, compared to the control group SD (60.18 ± 3.24 pg mL^{-1}, 21.43 ± 1.86 pg mL^{-1}), the levels of TNF-α and IL-6 were significantly ($P < 0.05$) increased in the LPS-treated groups (189.55 ± 8.97 pg mL^{-1}, 57.35 ± 4.32 pg mL^{-1} for LPS-SD and 131.22 ± 4.56 pg mL^{-1}, 31.28 ± 3.74 pg mL^{-1} for LPS-CLP, respectively), whereas no difference was observed in the LPS-untreated group (CLP). Data also demonstrated that supplementation of *Brassicaceae* in rabbit injected with LPS lowered ($P < 0.05$) the LPS-induced levels of TNF-α and IL-6 (−45% and −72%, respectively), exerting anti-inflammatory activity and mitigating the toxic effect of LPS, since the toxicity of LPS appears to be correlated with the levels of circulating TNF-α.[4] These results are in agreement with those of other authors who suggest anti-inflammatory properties of *Brassica*-derived phytochemicals.[11,12,26] The anti-inflammatory effects of supplementation may be due to the presence in cauliflower leaves of phenolic compounds such as flavonoids and hydroxycinnamic acids that inhibit the expression of inflammatory cytokines through the inhibition of transcription factors such as the nuclear factor kappa B (NF-κB),[27,28] a key player in inflammatory processes that is induced by several factors including LPS.

Gelatinase serum levels. Gelatinases-A (MMP-2) and -B (MMP-9) belong to the matrix metallo-proteinases (MMPs), a heterogeneous family of zinc- and calcium-dependent matrix-degrading endopeptidases; their activity is under tight control of their endogenous tissue inhibitors, namely (TIMPs).[29] Matrix metallo-proteinases are implicated in a variety of physiological (embryonic development, cell differentiation, proliferation, adhesion and regeneration of tissues) and pathological

Table 3 Effect of the diet on TNF-α and IL-6 serum levels at 90 min after LPS injection

	Groups			
	SD (control)	CLP	LPS-SD	LPS-CLP
TNF-α (pg mL^{-1})	60.18 ± 3.24	62.04 ± 2.13	189.55 ± 8.97*^	131.22 ± 4.56$^{\#}$
IL-6 (pg mL^{-1})	21.43 ± 1.86	20.94 ± 2.01	57.35 ± 4.32*^	31.28 ± 3.74$^{\#}$

Serum Tumor Necrosis Factor-alpha (TNF-α) and interleukin-6 (IL-6) levels were quantified by enzyme-linked immunosorbent assay (ELISA) using specific anti-rabbit TNF-α and IL-6 antibodies (rabbit TNF-α and IL-6 DuoSet, R&D Systems, Abingdon, UK) following the manufacturer's recommendations. Data were expressed as the means ± S.E.M. (n = 6). *Significant difference as compared to the control group (SD or CLP groups). ^Significant difference of LPS-SD from the LPS-CLP group. #Significant difference ($P < 0.05$) as compared to the CLP group (Tukey's test).

processes such as neurodegenerative and vascular disorders, inflammation, arthritis, atherosclerosis, or cancer.[30] It is largely known that different cell types can produce MMPs in response to LPS and that MMP production in the course of inflammation is upregulated. LPS induces the transcription of gelatinases, directly through the activation of NF-κB[31,32] or indirectly promoting the synthesis and release of large amounts of mediators involved in the inflammatory, in particular, cytokines (*e.g.* TNF-α, IL-1β and, IL-6).[33] The effect of diet on the inflammatory status of rabbits after LPS injection was assessed by zymographic analyses of the serum gelatinases MMP-9 and MMP-2. These MMPs were chosen as markers of the inflammatory status of the rabbits, and their levels were evaluated by monodimensional zymography (1-DZ) under non-reducing conditions. Zymograms of serum samples from the four rabbit groups showed, for all, a similar proteolytic pattern (Fig. 2), characterized by three major clear bands corresponding to proteolytic activities with an M_r of 98 (pro-MMP-9), 82 (MMP-9), and 68 kDa (MMP-2), respectively. The gelatinolytic activities of MMP-2 and MMP-9 in the serum of all animal groups are presented in Fig. 3. For all the enzymes, the LPS-treated rabbit groups (LPS-SD and LPS-CLP) showed a higher serum activity than the untreated groups (SD and CLP, respectively) ($P < 0.05$); they were the following for the pro-enzymatic form of MMP-9 (pro-MMP-9): 1.8 and 1.3 fold, respectively; for the active form of MMP-9: 1.6 and 1.3 fold, respectively, and finally for MMP-2: 2.0 and 1.4 fold, respectively, whereas no statistical differences were found among the untreated group (CLP) and the control. Results clearly show that supplementation of the diet with cauliflower leaves significantly decreases the level of LPS-induced activity to about

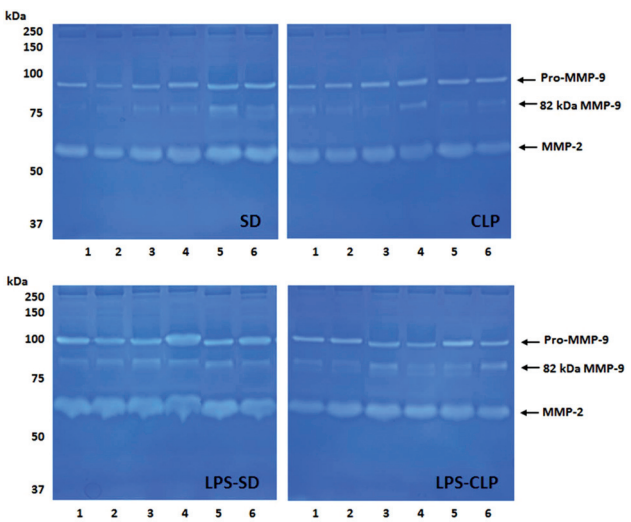

Fig. 2 Monodimensional zymography (1-DZ) of serum gelatinases. Zymography was performed on 8% polyacrylamide gel copolymerized with 0.1% (w/v) gelatin. For each sample, aliquots of sera containing 30 μg of proteins were loaded. Gels: SD (control, Standard Diet); CLP (supplemented with Cauliflower Leaf Powder); LPS-SD (LPS-treated Standard Diet); LPS-CLP (LPS-treated and supplemented with Cauliflower Leaf Powder).

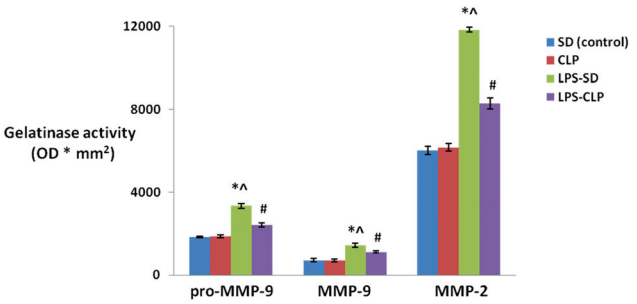

Fig. 3 Gelatinase activity. Gelatinolytic activity of the bands was quantified by computerized densitometric analysis using the Image Master 1D program (Pharmacia Biotech, Uppsala, Sweden). Gelatinase activity was expressed as optical density (OD) × mm^2, representing the scanning area under the curves which takes into account both the brightness and width of the substrate lysis zone. Data were expressed as the means ± S.E.M. (n = 6). *Significant difference ($P < 0.05$) as compared to the control (SD group). ^Significant difference ($P < 0.05$) as compared to the LPS-CLP group. #Significant difference ($P < 0.05$) as compared to the CLP group (Tukey's test).

two-thirds (62% for pro-MMP-9, 55% for MMP-9 and 61% for MMP-2) with respect to LPS-treated rabbits fed with a standard diet (LPS-SD). Probably, this effect may be ascribed to the different phytochemical compounds present in cauliflower leaves, for example, phenolic, glucosinolates and their isothiocyanate derivatives. Actually, several studies indicate that natural compounds of plant origin have inhibitory effects towards MMP activities. Examples of natural MMP inhibitors are: catechins derived from green tea,[34,35] curcumin,[36] caffeic acid,[37] tyrosol/hydroxytyrosol and resveratrol.[38] The inhibitory effects of *Brassica* isothiocyanates (sulforaphane and 7-methylsulphinylheptyl isothiocyanate) derived from broccoli and watercress on MMP-9 activity were reported by Rose *et al.*[39]

Effect of diet on TBARS and GSH levels and SOD and CAT serum activities

We evaluated the oxidative stress status in the serum of LPS-treated rabbits by measuring the levels of TBARS and GSH and the activities of SOD and CAT enzymes. LPS significantly ($P < 0.05$) increased serum TBARS levels by 150 and 54% and lowered the GSH levels by 28 and 17% in LPS-SD and LPS-CLP groups, respectively, compared with the untreated groups SD and CLP (Table 4), while these were significantly lowered (41%) for TBARS and increased (34%) for GSH, respectively, in LPS-CLP compared with the LPS-SD group. Finally, in the CLP group, the GSH level was 1.3 fold higher than that for the SD group, whereas no statistical difference was found for the TBARS level. In LPS treated rabbits fed with the standard diet (LPS-SD), LPS decreased significantly the serum SOD and CAT activities (39 and 63%, respectively) compared with the control group, while in the LPS-CLP group, supplementation with cauliflower leaves prevented the decrease of both SOD and CAT activities, and maintained their values at levels similar to those of the untreated group. One of the toxic effects of LPS is the generation of a high level of reactive oxygen species (ROS)

Table 4 Effect of the diet on TBARS and GSH levels and on SOD and CAT activities at 90 min after LPS injection

	Groups			
	SD (control)	CLP	LPS-SD	LPS-CLP
TBARS	4.15 ± 0.11	3.96 ± 0.22	10.35 ± 0.39*^	6.09 ± 0.14$^{\#}$
GSH	6.47 ± 0.23	8.53 ± 0.15*	4.65 ± 0.18*^	7.04 ± 0.52$^{\#}$
SOD	44.28 ± 4.26	42.78 ± 3.07	26.97 ± 2.94*^	46.22 ± 3.32
CAT	68.12 ± 3.27	69.28 ± 3.96	25.28 ± 0.15*^	71.36 ± 4.04

TBARS (thiobarbituric acid reactive substances) was expressed as malondialdehyde equivalents (nmol mL^{-1}). The GSH (glutathione) level was expressed in mg mL^{-1}. SOD (superoxide dismutase) activity was expressed as U mL^{-1}, where one unit (U) of SOD activity is defined as the amount of the enzyme causing 50% inhibition of the auto-oxidation of pyrogallol. CAT (catalase) activity was expressed as U mL^{-1}, where one unit (U) of CAT activity corresponds to μmol H_2O_2 decomposed per min. Data were expressed as the means ± S.E.M. (n = 6). *Significant difference as compared to the control group (SD or CLP groups). ^Significant difference of LPS-SD from the LPS-CLP group. $^{\#}$Significant difference ($P < 0.05$) as compared to the CLP group (Tukey's test).

in cells, leading to a deleterious condition termed oxidative stress.[40] Oxidative stress plays an important role in the pathogenesis of several diseases such as cancer, neurodegenerative, cardiovascular and cerebrovascular disorders. This condition is generated when the production of ROS overcomes the cellular antioxidant defenses represented by antioxidative enzymes (superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase) and non-enzymatic antioxidants (vitamins C and E and glutathione), resulting in the damage of macromolecules such as nucleic acids, proteins and lipids. TBARS are considered standard markers for lipid peroxidation induced oxidative stress. TBARS assay values are reported in terms of MDA equivalents. Malondialdehyde represents one of the products of the oxidative deterioration of polyunsaturated fatty acids in biomembranes, induced by the action of reactive oxygen species and free radicals. MDA is one of the most important indicators of the lipid peroxidation in biological systems and it is used as an indicator of cell membrane injury.[41] GSH is one of the major endogenous antioxidants produced in all cell compartments; the GSH molecule is also involved in the maintenance of protein structure and function, regulation of protein synthesis and degradation, and maintenance of immune function and detoxification. As an antioxidant, it participates in the neutralization of free radicals, as well as in the maintenance of exogenous antioxidants such as vitamins C and E in their active forms. Superoxide dismutase, together with catalase and peroxidase, is one of the most effective intracellular enzymatic antioxidants; it catalyzes the conversion of superoxide radical into hydrogen peroxide and molecular oxygen, while peroxidase and catalase convert hydrogen peroxide into water and into oxygen and water. In our study, in rabbits fed with a standard diet, LPS leads to a significant increase in MDA equivalent levels and simultaneously to a significant GSH depletion, as well as to a decrease of CAT and SOD serum activities, indicating the presence of the serum oxidative stress status. Our data suggest that dietary supplementation with cauliflower leaf powder protects against oxidative stress, reducing the levels of lipid peroxidation and preventing the reduction of the activities of the antioxidant enzymes CAT and SOD. These protective effects could be ascribed to their antioxidant capacity, due to their content of vitamins and phenolic compounds.[9,17] Among them, vitamin C and flavonoids are the most important. These latter represent about 46% of the total phenolic content. Furthermore, the beneficial antioxidant effects of supplementation with cauliflower leaves may also be due to the induction of antioxidant and phase 1/2 genes by GSLs and their hydrolysis products.[42] In fact, while vitamins C, E and phenolic compounds are direct antioxidants as they neutralize free radicals, GSLs and their derivatives act as indirect antioxidants as they do not neutralize free radicals directly, but rather by modulating the activity of xenobiotic metabolizing (phases I and II) enzymes, which trigger long lasting antioxidant activity. Thus, one of the major effects of isothiocyanates, namely to protect against oxidative stress, is the activation of phase 2 detoxifying enzymes (quinone reductase, glutathione transferase and glutathione reductase) and increase the glutathione level.[25] These findings agree with other studies in which *Brassica* phytochemicals showed antioxidant effects and decreased oxidative damage.[43,44]

Experimental

Chemicals

All the reagents used were of the highest grade and were purchased from Sigma-Aldrich (St Louis, MO, USA), Carlo Erba (Milan, Italy), Bio-Rad Laboratories (Segrate, Italy) and GE Healthcare (Uppsala, Sweden).

Plant material

Leaves of cauliflower (*Brassica oleracea* var. *botrytis*) were harvested from a private crop grown in Southern Italy (Metaponto – Basilicata, Italy). The plant material was air-dried in an oven at 37 °C for 5 days and pulverized. Then the leaf powder was kept under vacuum in a plastic bag and stored at room temperature in the dark.

Determination of antioxidant compounds and their antioxidant activity in cauliflower leaf powder

Ten grams of the leaf powder were extracted in duplicate for 12 h at 30 °C in a conical flask at 150 rpm with 50 mL of

methanol/water (70/30, v/v). The extract was recovered after centrifugation (18 000*g* for 4 min at 20 °C), filtered through filter paper and stored at 4 °C.

Total phenolic content

The total phenolic content (TPC) of the leaf powder extracts was determined spectrophotometrically using Folin–Ciocalteu reagent[45] with some modifications. 2.5 mL of Folin–Ciocalteu reagent (diluted 10 times with water) was added to 0.5 mL of leaf extract and, after 3 minutes, 2 mL of 7.5% sodium carbonate was added. Then, after 2 h of incubation at room temperature (in the dark) the absorbance was measured at 765 nm using an Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). The total phenolic content was determined as gallic acid equivalents (GAE) and expressed in terms of mg GAE per gram of dry weight (mg GAE per g DW). Values are reported as the mean ± S.E.M. of two independent experiments performed in triplicate (n = 6).

Total flavonoid content

Total flavonoid content (TFC) was determined by the aluminum chloride method using quercetin as a reference compound.[46] Briefly, 0.3 mL of extract was mixed with 0.9 mL of methanol, 0.06 mL of 10% aluminum chloride, 0.06 mL of 1 M potassium acetate and 1.7 mL of distilled water. The reaction mixture was incubated at room temperature and after 30 min the absorbance was measured at 415 nm. Total flavonoid content was determined as quercetin equivalents (QE) and expressed in terms of mg QE per gram of dry weight (mg QE per g DW). Values are reported as the mean ± S.E.M. of two independent experiments performed in triplicate (n = 6).

Vitamin C (L-ascorbic acid) content

The vitamin C analysis was performed with HPLC according to the methods described by Bhandari *et al.*[47] Cauliflower leaf powder (0.5 g) was extracted with 5% metaphosphoric acid solution. Then, after centrifugation and filtration (a 0.2 μm filter), the aliquot was analyzed using a liquid chromatograph equipped with a Varian ProStar model 210 pump, a Rheodyne injector with a 20 μL loop, and a Varian ProStar model 325 UV-Vis detector at 254 nm wavelength and using Galaxie™ chromatography software (Varian, Inc., Walnut Creek, CA, USA). The samples were injected into a HPLC system using a Hypersil GOLD C18 column (250 × 4.6 mm, 5 μm) connected with a Hypersil GOLD guard column (10 × 4.0 mm, 5 μm) (Thermo Fischer Scientific, Milan, Italy). Separation was performed using an isocratic mobile phase composed of 0.1% formic acid in water at 1 mL min^{-1}. The content of L-ascorbic acid was calculated on the basis of the calibration curve of L-ascorbic acid (0.05–1.00 mg mL^{-1}), and the results were expressed as mg of L-ascorbic acid per gram of dry weight (mg AA per g DW). Values are reported as the mean ± S.E.M. of two independent experiments performed in triplicate (n = 6).

DPPH radical scavenging activity

The antioxidant capacity of the leaf extract was measured on the basis of free radical scavenging activities of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$).[48] Briefly, 0.5 mL of the different concentrations of the extract were mixed with 0.5 mL of 0.24 mM freshly prepared DPPH$^{\bullet}$ in methanol solution. After 30 min of incubation in the dark at room temperature, the absorbance was measured at 517 nm. The radical scavenging activity of the samples was expressed in terms of IC_{50}, where IC_{50} denotes the concentration (μg mL^{-1}) of the sample required to scavenge 50% of the DPPH free radicals. Ascorbic acid was used as a positive control. Values are reported as the mean ± S.E.M. of two independent experiments performed in triplicate (n = 6).

Glucosinolate analysis

Glucosinolates were extracted with hot methanol from a dried sample followed by enzymatic desulfation.[11] Briefly, 500 mg of the dried sample was added to 0.3 mL of 2 μM benzylglucosinolate (internal standard), extracted with 4 mL of 100% methanol at 70 °C and heated for 20 min on a heating block. The extracting procedure was repeated twice and the supernatants were combined. After centrifugation (10 000*g* for 8 min), 1 mL of supernatant was added to a Sephadex A25 column and desulfated (for 16 h) using 0.5 mL of aryl sulfatase (20 U mL^{-1}; Sigma, St Louis, MO). Desulfoglucosinolates were eluted with 2 mL of deionized water, filtered through a 0.2 μm filter, and separated with a Waters Alliance 2695 HPLC system (Waters, Milford, MA) equipped with a UV detector set at 229 nm and a Spherisorb ODS2 column (150 × 4.6 mm, 3 μm, Waters). Elution was performed with a linear gradient mobile phase from 100% A (0.5% trifluoroacetic acid in water) to 30% B (acetonitrile) in 30 min at 1 mL min^{-1}. Desulfoglucosinolates were identified and quantified by comparison of the HPLC retention time with that of a glucosinolate standard (Sigma) after on-column sulfatase treatment as described above. Total and individual glucosinolates were reported as micromoles per gram of dry weight (μmol per g DW). For GLS quantification, two independent experiments were performed; HPLC analyses for each extract were performed in duplicate.

Animals

Twenty-four male rabbits (New Zealand White, 25 days of age), with approximately equal mean body weight (2.02 ± 0.23 kg), were acclimatized for ten days under laboratory conditions and were randomly divided into two groups of 12 animals each and housed individually in cages (at 22 ± 2 °C, 65 ± % relative humidity) under a 16 h light/8 h dark cycle. One group was fed with a standard diet based on commercial rabbit pellets, and the second group was fed with a diet supplemented with 100 g kg^{-1} diet of cauliflower leaf powder (Table 5). The experimental diet was prepared by adding the cauliflower leaf powder to the commercial diet before the pelleting process. The animals were fed *ad libitum* and had free access to food and water until the end of the trial. Rabbits were weighed

Table 5 Ingredients and chemical composition of diet

	Standard diet	Diet supplemented with cauliflower leaf powder
Ingredients (g kg^{-1} diet)		
Soybean meal	230	160
Alfalfa hay	220	140
Wheat bran	210	130
Alfalfa meal dehydrated, 17% CP	100	86.8
Beet pulp	100	70
Barley	77	60
Wheat	20	10
Calcium carbonate	20	20
Soybean oil	10	10
Sodium chloride	4	4
Dicalcium phosphate	1.8	2
Vitamin and mineral premix[a]	2.5	2.5
Methionine (99%)	2.3	2.3
Lysine (78.5%)	1.4	1.4
Choline (75.0%)	1	1
Cauliflower leaf powder	0	100
Chemical composition[b] (g kg^{-1})		
Dry matter (DM)	909.0 ± 1.0	915.7 ± 1.4
Crude protein	165.8 ± 0.6	168.2 ± 0.8
Crude fiber	171.4 ± 1.1	173.6 ± 1.0
Ether extract	26.6 ± 0.3	33.4 ± 0.5
Crude ash	61.8 ± 0.3	62.6 ± 0.1
Neutral detergent fiber	366.3 ± 1.1	375.3 ± 1.1
Acid detergent fiber	207.1 ± 1.7	210.1 ± 1.2

[a] Supplied per kg of feed: vitamin A 2000 I.U., vitamin D_3 320 I.U., vitamin E 4.0 mg, vitamin B_2 0.52 mg, vitamin B_6 0.40 mg, vitamin B_{12} 0.006 mg, vitamin K 0.32 mg, vitamin H 0.020 mg, vitamin PP 3.2 mg, folic acid 0.10 mg, D-pantotenic acid 2.4 mg, copper 5.6 mg, manganese 4.0 mg, iron 12.0 mg, zinc 16.0 mg, iodine 0.060 mg, selenium 0.040 mg. [b] $n = 3$; mean ± S.E.M.

weekly throughout the 60 days duration of the study. The experiments were conducted in accordance with the European ethical guidelines for the care and use of laboratory animals. The protocol was assessed and approved by the Ethical Institutional Committee on Animal Experimentation as per protocol number 6 (ISPAAM).

Experimental protocol for the LPS treatment

Lyophilized LPS from *E. coli* (0127:B8, Sigma-Aldrich) was dissolved (100 μg kg^{-1} of weight) in 1.0 mL of saline solution (0.9% NaCl) under sterile conditions. At 95 days of age, for both groups, six rabbits received an intra-peritoneal injection of LPS, whereas the remaining six rabbits received 1.0 mL of saline solution. The experimental groups were classified as follows: a standard diet (the control, SD group); supplemented with cauliflower leaf powder (CLP group); LPS-treated standard diet (LPS-SD group); and supplemented with cauliflower leaf powder and LPS-treated (LPS-CLP group).

Health status was assessed by clinical examination of the animals before injection. Blood samples were collected from the auricular vein at 90 min after LPS injection; then serum was extracted from the blood samples by centrifugation (3000*g* at 4° C for 5 min) and stored at −70 °C until analysis.

Assays of inflammatory markers

Rectal temperature measurements. The inflammatory response was determined by measuring rectal temperatures before (time 0, baseline) and 30, 60, 90, 120, 150 and 180 min after the LPS treatment. Rectal temperature was recorded with a digital thermometer. Data were expressed as the difference between the measured temperatures at each time point and the baseline.

TNF-α and IL-6 determination. Serum Tumor Necrosis Factor-alpha (TNF-α) and interleukin-6 (IL-6) levels were quantified by enzyme-linked immunosorbent assay (ELISA) using specific anti-rabbit TNF-α and IL-6 antibodies (rabbit TNF-α and IL-6 DuoSet, R&D Systems, Abingdon, UK) following the manufacturer's recommendations.

Zymographic analysis of serum gelatinases. Serum gelatinolytic activity was assessed by 1D gelatin gel zymography according to the established procedures.[49] 1D zymography (1-DZ) was carried out as follows. Aliquots of sera containing 30 μg of proteins were supplemented with 15 μL of non-reducing electrophoresis loading buffer (4% SDS, 12% glycerol, 0.01% bromophenol blue, 50 mmol L^{-1} Tris-HCl at pH 6.8) and analyzed in 8% polyacrylamide gel copolymerized with 0.1% (w/v) gelatin. The stacking gel contained 4% polyacrylamide. Electrophoresis was carried out on a Mini-PROTEAN Tetra Cell system (Bio-Rad) at 4 °C for 90 min at 150 V. Precision Plus and Protein Standards Dual Color (Bio-Rad) were used as molecular weight markers. After electrophoresis, gels were washed (2 × 40 min) with 2.5% (w/v) Triton X-100, 20 mmol L^{-1} Tris-HCl, pH 7.5 (washing buffer) in order to remove SDS, and then incubated for 14 h at 37 °C in developing buffer: 1% (w/v) Triton X-100, 20 mmol L^{-1} Tris-HCl pH 7.5, 10 mmol L^{-1} $CaCl_2$. For the development of enzyme activity, the gels were stained with Coomassie Brilliant Blue R-250, destained with methanol/acetic acid/H_2O and scanned using an ImageMaster DTS (Pharmacia Biotech) scanner. Gelatinase activity was detected as a white band on a blue background and was quantified by computerized image analysis through bidimensional scanning densitometry using the Image Master 1D program (Pharmacia Biotech, Uppsala, Sweden). Gelatinase activity was expressed as optical density (OD) × mm^2, representing the scanning area under the curves which takes into account both the brightness and width of the substrate lysis zone.

Assays for oxidative stress

Determination of serum lipid peroxidation by thiobarbituric acid reactive substances (TBARS). TBARS are naturally present in the biological matrix and include lipid hydroperoxides and aldehydes, which increase in concentration as a response to oxidative stress. TBARS assay values are usually reported in terms of malonaldehyde (MDA) equivalents, one of the products of the oxidative deterioration of polyunsaturated fatty acids in biomembranes, induced by the action of reactive oxygen species and free radicals. The method is based on the reaction of MDA and TBA, in which MDA in combination with TBA forms a chromogen compound detected spectrophoto-

metrically at 532 nm.[50] MDA (malondialdehyde tetrabutylammonium salt, 96% pure, Sigma-Aldrich) was used as the standard; results were expressed as nmol mL^{-1} of malondialdehyde equivalents.

Determination of serum GSH levels. The serum levels of acid-soluble thiols, mainly (glutathione) GSH, were determined as described by Ellman.[51] After protein precipitation with trichloroacetic acid, the collected supernatant was treated with Ellman's reagent [5,5′-dithio-bis(2-nitrobenzoic acid)]. The absorbance was read at 412 nm and the concentration was expressed as mg mL^{-1}.

Determination of serum SOD activity. The enzymatic activity of superoxide dismutase (SOD) was assessed according to the method of Marklund.[52] SOD activity was expressed as U mL^{-1}, where one unit of SOD activity is defined as the amount of the enzyme causing 50% inhibition of the auto-oxidation of pyrogallol.

Determination of serum CAT activity. The catalase (CAT) activity was assayed by H_2O_2 consumption following the protocol described by Aebi.[53] The decrease in absorbance was recorded at 240 nm. The CAT activity was expressed as U mL^{-1}, where one unit of CAT corresponds to μmol H_2O_2 decomposed per min.

Data analysis

Data were reported as the mean ± standard error of the mean (S.E.M.). Statistical analysis was performed by one-way ANOVA, and multiple comparisons were made by Tukey's test. *P*-Values less than 0.05 ($P < 0.05$) were considered significant.

Conclusions

In recent years, there has been an increasing interest in edible vegetables, especially those rich in phytochemicals (secondary metabolites), owing to their dietary antioxidant and anti-inflammatory action. It is indeed well known that dietary factors influence the metabolism of cells and modulate inflammation as well as oxidative stress.[54] In the case of the plant used in the present study, it has however been ascertained that *Brassica* phytochemicals could play a dual role, either beneficial or deleterious. This double-faced character is substantiated by the ever-increasing evidence of their nutritional value and health-promoting activities such as anti-cancer, antioxidant and anti-inflammatory, while the negative effects of *Brassica* on human and animal health may be due to their goitrogenic (thiocyanates, isothiocyanates, and oxazolidine-2-thiones) or anti-nutritional (phytates) activities. Our results show that preventive dietary supplementation with *Brassica* plants can attenuate in a rabbit model the LPS-induced oxidative stress and inflammation status. In conclusion, our findings clearly demonstrate the beneficial effect of a cauliflower leaf powder enriched-diet (100 g kg^{-1} diet), which is able to prevent the expression of inflammatory cytokines and the activity of pro-inflammatory enzymes, modulating antioxidant enzyme activities as well as suppressing lipid peroxidation, resulting from the action of injected LPS.

Conflicts of interest

The authors declare no competing financial interest.

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