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Effects of Long-Term Dietary Administration of Kale (*Brassica oleracea* L. var. *acephala* DC) Leaves on the Antioxidant Status and Blood Biochemical Markers in Rats

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Kale (*Brassica oleracea* L. var. *acephala* DC) is a leafy green vegetable which belongs to the Brassicaceae family, one of the most commonly cultivated and consumed edible plants. The aim of this study was to investigate the potential adverse effects and antioxidant properties of freeze-dried kale leaves in 90-day dietary experiment on Wistar rats. The kale leaf powder was added to a diet at levels: 10, 30, and 60 g/kg feed. The standard blood biochemical and hematological markers, antioxidant enzyme activities, level of lipid peroxidation, reduced glutathione content, and damage to DNA in the liver were measured. Antioxidant potential in serum was measured and expressed as Trolox equivalents antioxidant capacity (TEAC). The kale leaf phytochemicals, *i.e.*, glucosinolates, phenolic acids and flavonols, were quantified. Major glucosinolates were glucoiberin (164.6 mg/100 g d.w.) and glucobrassicin (130.6 mg/100 g d.w.), whereas kaempferol (159.1 mg/100 g d.w.), quercetin (119.6 mg/100 g d.w.), and sinapic acid (73.8 mg/100 g d.w.) dominated among phenolics. The lowest dose of kale leaves augmented the activity of catalase by 34% in males and by 44% in females. The highest applied dose increased activities of glutathione reductase by 31%, superoxide dismutase by 27%, and glutathione *S*-transferase by 24% in males only. Kale administration did not affect the activities of glutathione peroxidase and paraoxonase 1, glutathione concentration, lipid peroxidation level, and the level of DNA damage in the liver and the whole blood leukocytes. TEAC increased upon all doses of kale leaves by 30–90% in males and by 40–90% in females. The diet with kale did not affect blood biochemical and hematological markers. Results confirm no adverse effects of dried kale leaves used in model rat's diet up to 60 g/kg for 90 days. The changes in hepatic antioxidant enzymes and plasma TEAC suggest beneficial effect of kale leaves on the antioxidant status in rats. Interestingly, these changes were more pronounced in male *versus* female animals.

INTRODUCTION

Nutrient- and non-nutrient phytochemical diet components of diverse chemical structures and ways of action contribute to health preservation, *e.g.* antioxidants are capable of preventing and/or attenuating inflammation-dependent pathological conditions (cardiovascular and neurodegenerative diseases, cancer, and aging impairments) [Pizzino *et al.*, 2017; Zhang *et al.*, 2015] and glucosinolates are particularly active in the prevention of breast cancer [Soundararajan & Kim, 2018]. The contribution of edible phytochemicals to human wellbeing resulted in the development of the concept of functional foods, which are dietary products with the additional function apart from their basic nutritional value and which exert a beneficial effect on human health [Aronson, 2017]. Kale (*Brassica oleracea* L. var *acephala* DC),

which is examined in the current study, belongs to the group of *Brassica* vegetables. These edible plants exert chemopreventive activity, resulting primarily from the content of glucosinolates (GLS), including aliphatic (*e.g.*, glucoiberin, glucoraphanin) and indolic (*e.g.*, glucobrassicin) GLS found in various proportions [Šamec *et al.,* 2019]. The absorption, bioavailability, and biological activity of these organic sulfur derivatives depend on the initial stage of metabolism, namely hydrolysis with myrosinase (β-thioglucosidase) [Yagishita *et al.*, 2019]. As a result of this breakdown, a variety of products appear, among which isothiocyanates (sulforaphane and phenethyl isothiocyanate) and indoles (indole--3-carbinol and 3,3'-diindolylmethane) have been studied for their chemopreventive potential [Soundararajan & Kim, 2018]. Various products of GLS hydrolysis have been found to affect diverse pathways: they induce drug-metabolizing

enzymes [James *et al.*, 2012]; induce apoptosis by the p53, bax, and bcl-2 signaling pathways [Soundararajan & Kim, 2018]; and reduce transcription and increase degradation of an estrogen receptor or arrest the breast cancer cell cycle [Cheng *et al.*, 2019].

Next to GLS, phenolics of diverse structures are components of kale, where flavonoids including kaempferol and its derivatives are dominant [Schmidt *et al.*, 2010]. The majority of reports have demonstrated that dietary polyphenols protect the body against the effects of reactive oxygen species (ROS) and exert anticarcinogenic, anti-inflammatory, hepatoprotective, atheroprotective, and neuroprotective effect, as well as maintain gut microbiota homeostasis [Qu *et al.*, 2018]. However, there are data presenting adverse, pro-oxidant and toxic effects of high doses/concentrations of phenolics used in diverse experimental models, *e.g.* green tea polyphenols have been implicated in inducing hepatotoxicity, carcinogenesis and nephrotoxicity in animals. Moreover, some flavonoids (*e.g.* quercetin or kaempferol) could be converted into genotoxic derivatives [Qu *et al.*, 2018]. Sakr *et al.* [2013] described a genotoxic effect caused by grapefruit juice polyphenols in rats. Also isolated flavonoids, such as silymarin, myricetin, quercetin, kaempferol, rutin, and kaempferol 3-rutinoside, appeared genotoxic to human lymphocytes and sperm [Anderson *et al.*, 1997]. Hence, before the development of functional foods or supplements, safe levels of intake should be established.

The number of *in vivo* biological studies of kale is very limited. Its antioxidant and anticarcinogenic activities have been proved in *in vitro* experiments [Šamec *et al.,* 2019]. Beneficial effects of kale on gastric ulcer and inflammatory bowel disease have been demonstrated in rodents which might be associated with its antimicrobial activity against *Helicobacter pylori*. Moreover, kale suppressed cognitive decline in a mouse model of accelerated senescence. It has been found in intervention study that kale consumption reduced the risk of coronary disease in men. Drinking kale leaf juice for 24 months resulted in bone mass stabilization in postmenopausal women. Inhibition of acetylcholinesterase might suggest that kale seeds can be used in therapy of neurodegenerative diseases [Šamec *et al.,* 2019].

Health-beneficial properties of kale have been widely investigated, but its potential adverse effects have not been studied because it is considered a safe food. No data referring to the long-term ingestion of kale preparations in a natural food matrix were found in the available literature. Therefore, the aim of this study was to assess the antioxidant and potential adverse effects of 90-day dietary administration of freeze-dried kale leaves to rats.

MATERIALS AND METHODS

Chemicals

The normal melting point agarose for comet assay was Prona Plus Agarose (Madrid, Spain). Triton X-100 was obtained from PS PARK Scientific Ltd. (Northampton, Great Britain). Sodium acetate was from Chempur (Piekary Śląskie, Poland), whereas glucotropaeolin was purchased at Roth (Karlsruhe, Germany). Other chemicals were from Sigma Aldrich Chemie GmbH (Steinheim, Germany) and Polish Chemical Reagents Company (Gliwice, Poland). Reagents for the determinations of blood biochemical markers were purchased from Biosystems (Barcelona, Spain).

Materials

Leaves of kale (*Brassica oleracea* L. var. *acephala* DC) of Reflex F1 variety were obtained from the Organic Horticultural Farm in Pamiątkowo near Poznań, Poland. Freeze-dried kale leaves were prepared and analyzed at the Institute of Food Technology of Plant Origin, Poznań University of Life Sciences, Poznań, Poland. The raw material was washed, shredded into pieces, and deep frozen at −50°C. The freeze-drying process was performed at Celico S.A. company (Poznań, Poland) in a tunnel continuous freeze dryer with a separate freezing chamber and ice condensers. The following freeze-drying parameters were applied: pressure of 0.03 mbar, drying temperature of 5°C (shelf temperature), forced drying (conduction drying) temperature of 30°C, freezing chamber temperature of −50°C, and total drying time of 30 h. Freeze-dried kale was packed in plastic bags (OPA/PE), vacuum-sealed (A-300, Multivac, Wolfertschwenden, Germany), and stored at 6°C until use. The freeze-dried kale powder was added to the certified laboratory feed (Labofeed H, ISO 22 000, Animal Fodder Producer Morawski, Kcynia, Poland), thoroughly mixed with the feed to produce pellets with freeze-dried kale leaf levels: 10, 30, and 60 g/kg feed.

Analysis of kale leaf phytochemicals

In order to determine the phenolic compound composition, 2 g of freeze-dried kale leaves were extracted with 70% (*w/v*) methanol (50 mL) at room temperature by sequentially homogenization (Ultra Turrax T-25 homogenizer, IKA-WERKER, Staufen, Germany), shaking for 15 min (Water Bath Shaker 357, Elpan, S.C., Lubawa, Poland), and centrifugation for 30 min at 4,000×*g* (MPW-351R centrifuge, MPW, Warsaw, Poland). The procedure was performed twice. The supernatants were combined and vacuum-evaporated at 40°C (Büchi R-205, BÜCHI Labortechnik, Flawil, Switzerland). The volume of the aqueous residue was adjusted to 25 mL in water [Vallejo *et al.*, 2002]. The phenolic analysis was performed by an Agilent Technologies 1200 rapid resolution liquid chromatography (RRLC) system equipped with a 1260 diode-array detector and a Zorbax SB-C18 (4.6×150 mm, 5 μm) column (Agilent Technologies, Santa Clara, CA, USA). Briefly, the mobile phase consisted of 6% (*v/v*) acetic acid in 2 M sodium acetate (solvent A) and 100% acetonitrile (solvent B), which were used in the following gradient program: 0–15% B for 15 min, 15–30% B for 10 min, 30–50% B for 5 min and 50–100% B for 5 min. The flow rate of the mobile phase was 1 mL/min and the total run time was 40 min [Tsao & Yang, 2003]. The phenolic compounds were detected based on the maximum wavelength of UV-Vis absorption, *i.e.*, hydroxycinnamic acid derivatives at 320 nm and flavanols at 360 nm. Chlorogenic acid, quercetin, and kaempferol were used as external standards. The calibration curve of each standard was used for its quantification. The results were expressed in mg/100 g dry weight (d.w).

Prior to the analysis of glucosinolates, the kale leaf powder was supplemented with glucotropaeolin as an internal standard, extracted with methanol, and the procedure followed the steps described in the official method ISO 9167–1:1992. Glucosinolate separation was carried out with the same LC Agilent Technologies equipment and chromatographic column which were mentioned above to determine phenolic compounds. The total running time of separation was 30 min and the mobile phase was used in the gradient system which was changed as follows: 100% A (water)/0% B (acetonitrile in water, 20:80, *v/v*) for 1 min, then to 100% B over 22 min and isocratically 100% B over 5 min, after which the column was equilibrated at 100% A for 2 min. Absorbance was read out at 229 nm. The peaks were identified by comparison with data obtained from the literature [Verkerk *et al.,* 2001]. The quantity of GLS was determined based on the internal standard (glucotropaeolin) and expressed as mg/100 g d.w.

Trolox equivalent antioxidant capacity (TEAC) of kale leaves was evaluated based on 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid radical cation (ABTS$^{\bullet+}$) scavenging activity of extract, which was determined according to Re *et al.* [1999] method. The ABTS$^{\bullet+}$ was produced by reacting 7 mM ABTS water solution with 2 mM potassium persulphate. The 70% (*w/v*) methanol extract of leaves prepared as described above (50 μL) was added to 5 mL of the ABTS$^{\bullet+}$ solution, which was then incubated at 30°C for 6 min, and afterward absorption was measured at 734 nm. The results were expressed as TEAC of kale leaves based on the calibration curve plotted for Trolox and shown as μmol Trolox/100 g d.w.

In order to determine the ascorbic acid content, the freeze-dried powdered leaves (2 g) were homogenized with metaphosphoric acid (10 g/L) using an Ultra Turrax T-25 homogenizer (IKA-WERKER), then the suspension was shaken (Water Bath Shaker 357, Elpan, S.C.) and centrifuged at 4,000×*g* (MPW-351R centrifuge). The procedure was performed twice. The supernatants were combined and an aliquot of 1 mL was transferred to a volumetric flask. Then, 1 mL of a dithiothreitol solution (50 g/L) was added and the mixture was adjusted to 10 mL with metaphosphoric acid. This solution was analyzed using an LC Agilent Technologies 1200 RRLC system, equipped with a Zorbax SB-C18 column (4.6×150 mm, 5 μm; Agilent Technologies). To elute the ascorbic acid, a mobile phase with a flow rate of 0.7 mL/min in a gradient system of methanol (solvent A) and 5 mM KH_2PO_4 at pH 2.6 (solvent B) was used according to the following program: linear increment from 5% A to 22% over 6 min, then a return to the initial conditions over 9 min. The eluate was detected using a DAD set to 245 nm [Kurilich *et al.*, 1999]. The ascorbic acid was identified by comparing its retention time with that of standard.

Animals

Wistar rats used in the current study were bred at the Breeding Facility of the Department of Toxicology, Poznań University of Medical Sciences, Poznań, Poland. Male (184±47 g) and female (135±22 g) rats were housed in polycarbonate cages (30×20×25 cm; 4≤rats/cage) containing hardwood chip bedding and kept in a 12-h light/dark cycle at an average temperature and humidity of 21°C and 50%, respectively and in the controlled circulation of air. Animals had unlimited access to food and drinking water.

Experimental design

Thirty-two male and thirty-two female rats were divided randomly, each sex, into 4 groups, 8 animals each: controls and rats given pellets containing kale leaves (10, 30, 60 g/kg feed). The experiment lasted 90 days. The animals were monitored throughout the experiment for changes in their health status. Their body weight was recorded once a week and feed intake was measured daily. The daily dose levels (g/kg b.w.) were calculated using the nominal content of preparation tested in the diet, the mean daily feed consumption, and the body weight for a week. The calculated mean daily intake of kale leaves was 0.38±0.04, 1.10±0.16, and 2.19±0.23 g/kg b.w., respectively.

On the 91st day of the experiment, rats were anesthetized with ketamine and xylazine (100/7.5 mg/kg b.w., i.p.), and the blood was withdrawn from the heart to obtain samples for comet assay and aliquots for plasma and serum separation. The livers were excised, a needle was inserted to the portal vein and ice-cold 1.15% KCl was injected until the liver had blanched. The portions of the liver were dissected and stored at –80°C for the determination of biochemical markers. Comet assay in the liver was performed in a portion of the tissue shortly after collection.

The animal experiment followed the animals welfare regulation according to EU Directive 201/63/EU, and was approved by the Local Ethics Committee for Animal Experimentation in Poznań, Poland (protocol No 28/2012).

Blood biochemical markers

Biochemical markers assayed in serum included alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, total protein, total cholesterol, chloride, inorganic phosphorus, glucose, creatinine, blood urea, potassium, sodium, and calcium. The markers were determined using a chemistry analyzer XL 300 (Erba Diagnostics, Mannheim, Germany).

Hematological markers were determined using an automated hematology analyzer Cell-Dyn 3700 (Abbott Laboratories, Chicago, IL, USA) and included: red blood cells count, white blood cells count, and differential leukocyte count (neutrophils, lymphocytes, monocytes, eosinophils, and basophils), hemoglobin concentration, and platelet count.

Parameters of antioxidant status

In the liver, cytosol superoxide dismutase (SOD) activity was measured on the basis of the inhibition of spontaneous epinephrine oxidation, catalase (CAT) assay was based on monitoring the rate of hydrogen peroxide reduction, glutathione peroxidase (GPx) activity was determined with hydrogen peroxide as a substrate, the rate of NADPH depletion at 340 nm was a measure of the enzyme activity, whereas glutathione reductase (GR) activity was analyzed by measuring NADPH oxidation with oxidized glutathione as a substrate.

In the liver homogenate, the measurement of thiobarbituric acid reactive substances (TBARS) was used to assay lipid peroxidation, and Ellman's reagent was applied to determine

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reduced glutathione (GSH) content. The above-mentioned methods were described in detail in our previous article by Jodynis-Liebert *et al.* [2009].

Glutathione-*S*-transferase (GST) assay in the liver cytosol was based on the reaction of the reduced glutathione with 1-chloro-2,4-dinitrobenzene (CDNB) [Krajka-Kuźniak *et al.*, 2011]. Protein concentrations in the liver cytosol and homogenate were determined by the routine method with phenolic reagent using bovine serum albumin as the standard. Paraoxonase-1 (PON-1) in rat serum was analyzed with phenylacetate as a substrate, and the rate of generated phenol was measured at 270 nm [Kumru *et al.*, 2004]. Antioxidant potential was measured in the rat plasma using the ABTS assay and expressed as TEAC [Re *et al.*, 1999].

Statistical analysis

The mean values and standard deviations (SD) were calculated separately for males and females. One-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for multiple comparisons was used (GraphPad InStat software, San Diego, CA, USA), and $p<0.05$ was considered the limit of significance.

RESULTS AND DISCUSSION

Kale has been recently gaining interest as a crop of a great nutritional value and health-promoting properties resulting from its components, mainly glucosinolates, phenolics, and vitamins. Kale is represented by numerous botanical varieties which differ in chemical composition depending on the cultivar, growing region, agricultural practices employed, and geo-climatic conditions [Sarıkamış *et al.*, 2008; Satheesh & Workneh Fanta, 2020].

Results of the quantification of glucosinolates in the freeze-dried kale leaves are presented in Table 1. Total GLS content in kale leaves was 487.1 mg/100 g d.w. Aliphatic GLS accounted for 60.8%, and indolic GSL for 39.1% of total GLS, which is consistent with literature data [Cieślik *et al.*, 2007; Korus *et al.*, 2014]. Glucoiberin, glucoraphanin, and synigrin were found as major aliphatic glucosinolates of fresh kale juice, while glucobrassicin prevailed in the group of indolic GLS [Korus *et al.*, 2014; Sasaki *et al.*, 2012; Velasco *et al.*, 2007]. Our data corroborate these findings (Table 1): the major GLS turned out to be glucoiberin (164.6 mg/100 g d.w.). The kale leaves were also rich in glucoraphanin (73.8 mg/100 g d.w.) and glucobrassicin (130.6 mg/100g d.w.), the precursor molecules of the biosynthesis of sulforaphane and indole-3-carbinol, respectively, which are known for their anticarcinogenic and antioxidant potential [Cheng *et al.*, 2019; Fuentes *et al.*, 2015; Satheesh & Workneh Fanta, 2020; Soundararajan & Kim, 2018]. The effect of cultivation condition on kale GLS composition was presented in the report of Sarıkamış *et al.* [2008], which showed that the content of glucobrassicin, the major indolic GLS, in kale grown in Turkey depended on the year of cultivation (range from 2094.56 to 2184.05 mg/100 g d.w.), and that aliphatic glucoraphanin and glucoiberin were present in minor quantities, *i.e.* 32.42 to 37.45 mg/100 g d.w. and 8.87 to 120.6 mg/100 g d.w., respectively. The authors have suggested that temporarily high temperatures during the cultivation period boosted up the indole GLS synthesis.

TABLE 1. The content of glucosinolates in freeze-dried kale leaves.

Glucosinolates		Content (mg/100 g d.w.)
Aliphatic	Glucoiberin	164.6±2.2
	Progoitrin	17.9±0.6
	Sinigrin	29.1±0.1
	Glucoraphanin	73.8±1.2
	Gluconapin	10.9±0.0
Indolic	4-Hydroxyglucobrassicin	31.2±0.8
	Glucobrassicin	130.6±2. 9
	4-Methoxyglucobrassicin	12.4±0.6
	Neoglucobrassicin	16.6±0.1
Sum of glucosinolates		487.1± 8.5

Results are presented as mean±standard deviation ($n=6$); d.w. – dry weight.

Phenolic compounds of kale leaves are represented by various phenolic acids and flavonols, including quercetin and kaempferol, that have been extensively examined for their strong biological activities and health effects [Qu *et al.*, 2018; Satheesh & Workneh Fanta, 2020]. The phenolic profile of the freeze-dried kale leaves used in our study is shown in Table 2. Contents of kaempferol and quercetin were 159.1 and 119.6 mg/100 g d.w., respectively. Satheesh & Workneh Fanta [2020] presented a review of data where kaempferol content ranged from 537 to 343 mg/100 g d.w. and that of quercetin from 272 mg/100 g d.w. to 319 mg/100 g d.w. in kale leaves, which was higher than the results obtained in our study. However, our values and these cited data show that kaempferol was the major flavonoid of kale leaves. In turn, Schmidt *et al.* [2010] identified a total of 71 flavonoid glycosides of kale by high-performance liquid chromatography/mass spectrometry and among them kaempferol glycosides (32 compounds) were found to prevail.

The content of ascorbic acid in freeze-dried kale leaves equaled 319.3±6.1 mg/100 g d.w. Literature data demonstrate that vitamin C content in kale has been quantified in the range of 62–969 mg/100 g and that it is higher than in other salad vegetables [Satheesh & Workneh Fanta, 2020]. The recommended daily allowance (RDA) value is 90–120 mg/day [Aly *et al.*, 2010].

The TEAC of kale leaves was 9241±5.2 μmol Trolox/100 g d.w. This value was higher than that quantified by other authors for fresh kale leaves, namely: 1175–3620 μmol of Trolox/100 g d.w. [Šamec *et al.*, 2018].

It is known that in some circumstances, certain phenolics can paradoxically behave as pro-oxidants [Qu *et al.*, 2018]. Hence, we examined the effects of freeze-dried kale leaves on parameters of the antioxidant status in rats. The impact exerted by kale leaves administration on the activity of antioxidant enzymes is presented in Table 3. The activity of SOD was increased by 27% only upon the intake of the highest dose of kale leaves in males, whereas in other groups of animals,

TABLE 2. The content of phenolic compounds in freeze-dried kale leaves.

Phenolic compound	Content (mg/100 g d.w.)
Caffeoyloquinic acid	49.3±2.2
Ferulic acid	21.4±0.6
Caffeic acid	22.7±0.2
Sinapic acid	73.8±0.9
Other hydroxycinnamic acids	5.9±0.03
Quercetin	119.6±1.3
Kaempferol	159.1±2.1
Sum of phenolic compounds	451.8±7.3

Results are presented as mean±standard deviation (n=6); d.w. – dry weight.

no significant differences were found in comparison to controls. The activity of CAT was raised in males and females receiving the lowest dose of kale leaves, by 34% and 44%, respectively. The activity of GPx was changed in none of the experimental groups. There was no response of GR to the intake of kale leaves except for the 31% increase in the highest-dose males. A 24% increase in the GST activity was found in males receiving the highest dose, whereas no changes were observed in female rats. Our results indicate that changes in the antioxidant enzymes activities occurred in single groups of animals treated with kale leaves. The only consistency was observed in the response of SOD, GR, and GST, whose activities were elevated in males administered the highest dose.

The increase in the activity of enzymes involved in GSH metabolism, GR and GST, is in contrast with literature data and our previous observations. It has been found that multiple

flavonoids can inhibit the activity of isolated GR [Güller et al., 2021]. A decrease in the GR activity was observed in rat hepatocytes cultured with delphinidin, (−)epicatechin, kaempferol, quercetin, luteolin, naringenin, and apigenin [Galvez et al., 1995]. Breinholt and co-workers reported on the GR activity diminishment in the liver of rats treated with flavonoids (quercetin and genistein) [Breinholt et al., 1999]. A decrease in the GR activity was also noticed in our previous similarly designed experiment, where Wistar rats were administered feed containing freeze-dried chokeberry juice rich in anthocyanins and proanthocyanidins for 90 days [Jodynis--Liebert et al., 2009]. GST is a phase II enzyme that detoxifies xenobiotics and potential carcinogens by conjugation with glutathione and, as such, modulates the cellular response to the exposure to electrophiles and oxidants [Krajka-Kuźniak et al., 2011]. Boušová & Skálová [2012] reported on the inhibition of the purified enzyme by multiple flavonoids. Similarly, a decrease in GST activity has been found in rats fed a diet enriched in quercetin and (+)-catechin [Wiegand et al., 2009].

The impact of glucosinolates on antioxidant enzymes in animals was studied by Vang et al. [1995], who treated rats for one week with broccoli cultivated at various fertilizer concentrations. Their experiment proved that even a short period of cruciferous vegetable administration upregulated SOD, GR, and GPx in the liver, kidney, and colon. Hence, it could be suggested that the inducing effect of glucosinolates present in kale on antioxidant enzymes prevails over inhibiting the action of kale phenolic compounds.

In our study, kale administration to rats caused a modest increase in the GST activity only in the highest-dose male group (Table 3). However, in the experiment reported by Krajka-Kuźniak et al. [2011], after 30 days of cabbage juice

TABLE 3. Antioxidant enzyme activities in the liver and serum of rats fed a diet with freeze-dried kale leaves for 90 days.

Parameter	Sex	Content of kale leaves in feed (g/kg)			
		0	10	30	60
Superoxide dismutase (SOD) (U/mg protein)	M	26.0±1.9	29.2±5.2	21.9±3.4	33.0±2.4* [↑27%]
	F	25.4±4.1	30.6±4.6	27.3±4.2	30.1±2.4
Catalase (CAT) (U/mg protein)	M	4.72±0.76	6.33±1.13* [↑34%]	5.53±0.91	6.01±0.75
	F	3.20±0.42	4.61±0.43* [↑44%]	3.20±0.38	3.36±0.51
Glutathione peroxidase (GPx) (nmol NADPH / (min×mg protein))	M	627±59	630±86	595±72	771±100
	F	920±67	1048±117	854±89	965±71
Glutathione reductase (GR) (nmol NADPH/ (min×mg protein))	M	68.9±8.3	69.7±7.1	62.1±7.7	89.9±6.7* [↑31%]
	F	54.6±8.5	63.1±7.7	55.5±5.8	66.1±6.8
Glutathione S-transferase (GST) (nmol CDNB/(min×mg protein))	M	589±65	610±53	584±58	729±57* [↑24%]
	F	486±27	504±57	457±32	517±31
Paraoxonase 1 (PON-1) in serum (U/mL)	M	55.7±5.5	55.5±6.7	55.4±4.8	59.1±5.8
	F	66.8±2.1	66.2±3.8	61.7±6.2	63.1±8.7

Results are presented as mean±standard deviation of 8 values corresponding to 8 animals, in each gender group, each value being the mean of quadruplicate assays; NADPH – nicotinamide adenine dinucleotide phosphate equivalent; CDNB-1-chloro-2,4-dinitrobenzene equivalent; M – males; F – females; *p<0.05 for statistical difference $vs.$ control.

TABLE 4. Oxidative stress parameters in the liver and whole blood leukocytes of rats fed a diet with freeze-dried kale leaves for 90 days.

Parameter	Sex	Content of kale leaves in feed (g/kg)			
		0	10	30	60
TBARS (nmol MDA/mg protein)	M	17.6±1.8	14.6±4.6	15.8±4.5	19.2±2.8
	F	16.4±2.3	20.7±5.2	15.2±1.8	14.6±0.8
Reduced glutathione (μmol/g liver)	M	5.22±0.81	5.13±0.88	4.98±0.82	5.17±0.96
	F	3.72±0.55	3.40±0.89	2.81±0.55* [↓24%]	3.56±0.60
DNA damage in hepatocytes (arbitrary points)	M	73±5	71±10	71±6	74±7
	F	68±8	69±7	69±8	70±10
DNA damage in the whole blood leukocytes (arbitrary points)	M	70±9	72±8	69±7	68±7
	F	70±10	72±11	73±7	70±10

Results are presented as mean±standard deviation of 8 values corresponding to 8 animals, in each gender group, each value being the mean of quadruplicate assays; TBARS – thiobarbituric acid reactive substances; MDA – malondialdehyde equivalent; M – males; F – females; *$p<0.05$ for statistical difference *vs.* control.

administration to rats, a distinct rise in the enzyme activity was found, which corroborates the capability of glucosinolates and their derivatives to induce the enzyme. Accordingly, after a 21-day administration of sprout extract of Tuscan black cabbage to Sprague-Dawley rats, a significant induction of GST was only noticed when enzymatic decomposition of GLS to isothiocyanates occurred [Melega *et al.*, 2013]. Thus, the differences in the impact of various *Brassica* products on the GST activity might result from the technology of juice/extract preparation, conservation of myrosinase function, and different composition of phytochemicals. Moreover, another reason for these discrepancies may be attributable to various doses applied and experiment duration.

PON-1 is a calcium-dependent enzyme synthesized in the liver and released into plasma, where it associates with high density lipoproteins (HDL). It protects low density lipoproteins (LDL) and HDL molecules against oxidation through breaking up ester bonds in phospholipid peroxides and thus is considered an anti-atherogenic factor. PON-1 strongly depends on the exogenous conditions: (dietary) antioxidants strengthen, whereas toxic xenobiotics diminish its protective activity [Lou-Bonafonte *et al.*, 2015]. In our experiment, the administration of kale leaves to male and female rats did not affect PON-1 activity (Table 3).

Effects of the kale leaves intake on oxidative stress parameters in rats are presented in Table 4. Freeze-dried kale leaves did not affect the TBARS level, an index of lipid peroxidation. The concentration of reduced glutathione was diminished by 24% in females receiving kale leaves in the dose of 30 g/kg feed. In other experimental groups, no changes were observed in comparison to results from related controls.

We included the comet assay in the set of parameters tested because there are some reports referring to the DNA-damaging properties of phenolic compounds. The comet assay detects strand breaks resulting from oxidative DNA damage and is a common method of genotoxicity testing. Lesions appear at sites of oxidized purines and pyrimidines. Background radiation and basal levels of ROS in aerobic organisms contribute to endogenous, steady-state DNA damage [Møller

et al., 2004]. In our experiment, no significant changes were found in the extent of DNA damage, both in blood leukocytes and in the liver, in rats consuming kale-enriched feed (Table 4). However, the report of Sakr *et al.* [2013] describes an increase in the genotoxicity range measured by the comet assay in blood lymphocytes of rats treated for five weeks with a grapefruit juice which is a rich source of flavonoids and other phenolics. Isolated flavonoids, including silymarin, myricetin, quercetin, kaempferol, rutin, and kaempferol 3-rutinoside, incubated with human lymphocytes, and sperm cells appeared genotoxic. Nevertheless, in combination with the food mutagens, these compounds showed protective effects since DNA damage was reduced in the lymphocytes [Anderson *et al.*, 1997]. On the other hand, Møller *et al.* [2004] showed that in a 3-week controlled parallel intervention study with volunteers consuming blackcurrant juice or anthocyanin-enriched drink, there were no differences between controls and treated participants in DNA damage markers. The authors concluded that even ingestion of large amounts of dietary antioxidants did not decrease the steady-state levels of oxidative DNA damage and suggested that protective effect might be only observed in subjects exposed to the oxidative stress. Similarly, no effect on the steady-state DNA damage was found when human lymphocytes were incubated with the Brussels sprout extract containing glucosinolates, whereas this extract provided protection against DNA strand breaks in cells exposed to hydrogen peroxide [Zhu & Loft, 2001].

The effect of freeze-dried kale leaves on the antioxidant potential measured in rat plasma as TEAC is presented in Figure 1. TEAC values in rats consuming feed with freeze-dried kale leaves revealed a statistically significant increase compared to the control group value. However, the dose-response relationship was different in both sexes. Whereas in males, the highest TEAC was found in the group fed with 60 g of kale/kg of feed, in females, inversely, the highest TEAC was observed in the lowest dose group. In males, statistically significant differences were observed between 10 g/kg and 30 g/kg dose-group *vs.* 60 g/kg dose-group. In females, such differences were found between all dose-groups ($p<0.05$).
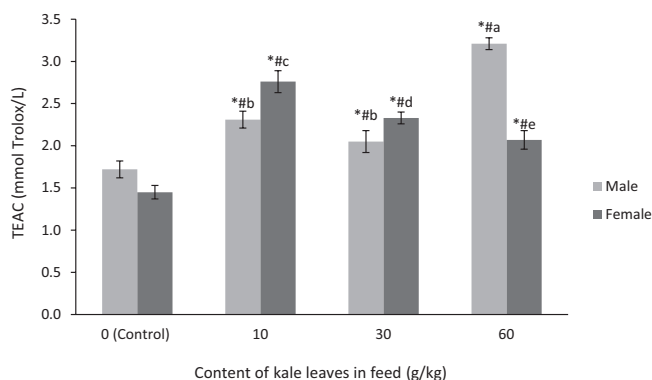
FIGURE 1. Trolox equivalent antioxidant capacity (TEAC) of plasma of rats fed kale leaves. Each column represents mean±standard deviation of 8 values corresponding to 8 animals, each value being the mean of quadruplicate assays. *$p<0.05$ for statistical difference *vs.* control; #$p<0.05$ for male *vs.* female group; different letters over the bar mean statistically significant differences ($p<0.05$) between dose-groups within each sex.

It has been shown that female rats have a higher basal level of antioxidant capacity in tissues than males [Katalinic *et al.*, 2005]. This difference is due to various factors, for example, higher activities of some antioxidant enzymes, and greater levels of endogenous antioxidants in females or the protective effect of estrogen [Massafra *et al.*, 2000; Tiidus *et al.*, 1999; Yamamoto *et al.*, 2002]. Our findings might suggest that this difference is enhanced by the ingestion of natural antioxidants present in kale leaves; however, only in a limited range of doses because the response to the highest dose was much weaker. The observed increase in TEAC values correlates with the results of the antioxidant status analysis in humans consuming *Brassica rapa* L. [Gul *et al.*, 2013]. A relatively high level of TEAC was probably due to the specificity of the preparation production: a low temperature applied for drying kale leaves prevented the loss of antioxidants, including vitamin C and phenolics [Korus & Lisiewska, 2011].

There were no statistically significant differences in the final body weight and mean feed intake between controls and rats administered freeze-dried kale leaves (data not shown).

In contrast, some differences were found in the hematological and biochemical markers between control and treated rats (Tables S1 and S2 in Supplementary Materials). The observed changes were not comprehensive, occurred in single groups of animals or in one sex only, and were not dose-related. The majority of these changes were not extensive.

The highest daily dose of kale leaves tested on animals, 2.16 g/kg b.w., which was safe, corresponds approximately to a daily freeze-dried kale portion of 153.3 g for an individual of 70 kg b.w. This amount is much higher than a likely intake of kale leaves in an everyday human diet or in dietary supplements.

The presented results reflect the response of animals to chemically diverse active ingredients present in the feed. It must be herein underlined that the antioxidant phytochemicals of foods are poorly absorbed in the intestine, rapidly metabolized by the intestinal flora, and excreted. Their absorption may be affected by fiber content, accelerating

the intestinal passage, as well as modulation of specific transport mechanisms. Bioavailability, stability, and metabolism of these phytochemicals might be some of the key factors regulating their influence on the antioxidant status [Pizzino *et al.*, 2017; Zhang *et al.*, 2015]. The observed effects might also be affected by *in vivo* interactions of active substances with the nutritional matrix. Thus, the antioxidant effects found in plasma and/or tissues might depend mostly on the stimulating impact of these compounds on the endogenous systems of antioxidant defense [Phan *et al.*, 2018]. Further studies, including the impact of active phytochemicals on other physiological functions and disease prevention in animals and humans, are necessary to elucidate the involved mechanisms.

CONCLUSIONS

Results of the current study confirm the safety of a diet enriched in freeze-dried kale leaves applied for 90 days to rat males and females. No toxicologically relevant changes were found in hematological and biochemical markers. The tested material did not enhance the hepatic level of lipid peroxidation and the basic range of DNA damage in the liver and leukocytes. Antioxidant defense system of rats treated with kale leaves was fortified as evidenced by the increase in hepatic activities of catalase, superoxide dismutase, glutathione reductase, and glutathione *S*-transferase as well as by the enhanced antioxidant potential in plasma.

SUPPLEMENTARY MATERIALS

Supplementary data related to this article can be found at http://journal.pan.olsztyn.pl/Effects-of-Long-Term-Dietary-Administration-of-Kale-Brassica-oleracea-L-var-acephala,152434,0,2.html. Table S1: Biochemical parameters in blood of rats fed diet with kale leaves for 90 days. Table S2: Hematology parameters in rats fed diet with kale leaves for 90 days.

RESEARCH FUNDING

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

INSTITUTIONAL REVIEW BOARD STATEMENT

The animal experiment followed the animal welfare regulation according to EU Directive 201/63/EU, and was approved by the Local Ethics Committee for Animal Experimentation (protocol No 28/2012).

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