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Eruca sativa Mill. seed extract promotes anti-obesity and hypoglycemic effects in mice fed with a high-fat diet

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Obesity is currently considered a major source of morbidity, with dramatic complications on health status and life expectancy. Several studies demonstrated the positive effects of *Brassicaceae* vegetables on obesity and related diseases, partially attributing these beneficial properties to glucosinolates and their derivatives isothiocyanates. Recently, isothiocyanates have been described as a hydrogen sulfide (H₂S)-releasing moiety, suggesting that H₂S may be at least in part responsible for the beneficial effects of *Brassicaceae*. In this work, the metabolic effects of an extract obtained from *Eruca sativa* Mill. seeds (E.S., *Brassicaceae*), containing high levels of glucoerucin, were evaluated in an experimental model of obesity. Male balb/c mice were fed for 10 weeks with standard (Std) diet or high fat (HF) diet supplemented with E.S. E.S. significantly contained the body weight gain in this obesity model, improving also glucose homeostasis. Interestingly, lower values of white adipose tissue mass and a significant reduction of adipocytes size were also observed. Moreover, E.S. enhanced the adipocytes metabolism, improving the citrate synthase activity and reduced triglyceride levels in mice fed with HF diet. Taken together, these results suggest that E.S. is endowed with an interesting translational and nutraceutical value in the prevention of metabolic disorders, suggesting that H₂S could be a key player.

KEYWORDS

Brassicaceae, *Eruca sativa* Mill., glucosinolates, hydrogen sulfide, obesity, rocket

1 | INTRODUCTION

Obesity is a main health issue among children and adults worldwide. According to the World Health Organization (WHO), 39% of the global population is overweight and 13% is affected by obesity (N.R.F. C. (NCD-RisC), 2016; Kopelman, 2000; Kopelman, 2007). Obesity is currently considered a major source of morbidity, with dramatic complications also on health status and life expectancy (Cheung, Cunningham, Narayan, & Kramer, 2016; Younossi et al., 2016). Several studies have demonstrated a strong correlation between obesity and increased risk of cardiovascular disorders onset, especially heart failure, hypertension and coronary heart disease. Moreover, an increased incidence of type II diabetes in obese people has been observed (Garg, Maurer, Reed, & Selagamsetty, 2014; Kachur, Lavie, de Schutter, Milani, & Ventura, 2017; Michalakos, Mintzioti, Kaprara, Tarlatzis, & Goulis, 2013).

Obesity is a condition characterized by an excessive body weight for a given height; in particular in humans, it has been defined by the National Institutes of Health (the NIH) as a body mass index (BMI) greater than 30. However, obesity is a more complex condition typically accompanied by systemic oxidative stress and low-grade inflammation (Hotamisligil, Shargill, & Spiegelman, 1993). Moreover, health risks are related not only with the total amount of fat, but also with the sites of fat deposition and the type of the adipose tissue. Indeed, two different types of adipose tissue have been described: the white adipose tissue (WAT), localized at subcutaneous and visceral level, accumulates large amounts of triglycerides during periods of energy excess and mobilizes them under conditions of energy deficiency (Tchernof & Després, 2013), and the brown adipose tissue (BAT) highly vascularized and mitochondria-rich. BAT represents a small percentage of body fat and its main function is the thermogenesis. Growing evidence suggests that the activation of BAT may be responsible for beneficial effects on obesity, insulin resistance and hyperlipidemia (Harms & Seale, 2013).

Eruca sativa Mill. (E.S.) is an edible vegetable from *Brassicaceae* botanic family, commonly known as rocket salad. Very recently, Martelli and colleagues described the vasoactive and antihypertensive properties of erucin, the characteristic isothiocyanate derived from E.S., and these health promoting effects have been mainly attributed to the hydrogen sulfide (H₂S)-releasing properties of erucin (Martelli, Citi, Testai, Brogi, & Calderone, 2020a). Moreover, glucoerucin, the glucosinolate typical of E.S., exhibits H₂S-mediated anti-nociceptive effects in diabetic rats (Lucarini et al., 2019). In addition, E.S. extracts show inhibition of platelet activation, aggregation and release of inflammatory mediators, through the inhibition of the pro-inflammatory transcription factor NF- κ B (Fuentes, Alarcón, Fuentes, Carrasco, & Palomo, 2014).

In this work, we investigate the metabolic effects of an extract obtained from E.S. seed, containing high levels of glucoerucin (precursor of erucin) in mice fed with a HF diet.

2 | MATERIALS AND METHODS

2.1 | Plant material

Seeds of E.S. var. NEMAT were collected at CREA-CI (Lazzeri et al., 2013; Lazzeri, Errani, Leoni, & Venturi, 2004) and extract was obtained following procedures as reported in supplementary materials. According to literature (Franco et al., 2016) the amount of GSLs were estimated through HPLC-UV analysis and using purified sinigrin as an internal standard.

2.2 | In vivo chronic treatment

All the procedures were performed according to European (EEC Directive 2010/63) and Italian (D.L. March 4, 2014 n. 26) legislation (protocol number 12/2019-PR, 10/01/2019). Animals were housed in cages with food and water ad libitum, and they were exposed to 12 hr:12 hr light:dark cycles.

12-Week-old Balb/c male mice (20–25 g, Envigo, Italy) were randomly assigned into four groups (10 animals per group) and were treated for 10 weeks. One group was fed with pulverized standard diet (Std group, Envigo, Italy; Table 1); another one was fed with pulverized high-fat (HF) diet (HF group, 45% Kcal derived from fat, SAFE, France; Table 1); the third one was fed with Std diet enriched with the E.S. seed extract 0.75% w/w (Std + E.S. group); the last group was fed with HF diet enriched with the E.S. seed extract 0.75% w/w (HF + E.S. group). This percentage of E.S. extract (0.75% w/w) ensured a daily intake of 15 μ mol of glucosinolates for each mouse (corresponding to 577 μ mol kg⁻¹), and this dosage has been selected on the basis of previous papers of others (Nagata et al., 2017).

Mice were weighed twice a week and food intake was daily monitored over a period of 10 weeks. At the end of the treatment, mice were sacrificed with an overdose of an aqueous solution of urethane (20% w/v, Sigma-Aldrich, USA), and BMI was measured. Then, the blood was collected and the WAT and the liver were explanted, weighed and stored at -80°C for functional and histological analysis.

2.3 | Measurement of cardiometabolic parameters

Before anesthesia, fasting blood sugar levels were measured by collecting blood from the tail tip of each animal. Glucose concentration was determined using Glucocard™ blood glucose meter (Menarini, Italy). Then, mice were anaesthetized with an intraperitoneal injection of urethane 20% w/v and intracardiac blood was collected in tubes containing the anticoagulant EDTA (BD Vacutainer, BD Diagnostics Preanalytical Systems, Canada) to measure glycated haemoglobin (HbA1c) and lipids (total cholesterol, LDL and triglycerides) levels with Cobas b 101 instrument (Roche Diagnostics, Switzerland).

TABLE 1 Composition of Std and HF diets

	Proteins (%)	Fats (%)	Carbohydrates (%)	Micro-nutrients (%)	Energy from Proteins (%)	Energy from Fats (%)	Energy from Carbohydrates (%)	Energy (kcal g ⁻¹)
Std	14.3	4.0	48.0	33.7	20.0	13.0	67.0	2.9
HF	20.3	24.0	43.3	12.4	17.3	45.9	36.8	4.7

2.4 | Evaluation of citrate synthase activity in WAT

WAT was finely cut and homogenized with an Ultra-Turrax homogenizer (IKA-Werke GmbH & Co., Germany) in an ice-cold isolation buffer (composition: sucrose 250 mM, Tris 5 mM, EGTA 1 mM, Triton X-100 0.02%, pH 7.4); three homogenization cycles were performed on ice. The suspension was centrifuged at 12000×g for 15 min at 4°C (Speed Master 14 R centrifuge; Euroclone, Italy) and the supernatant was used for the assay. Protein concentration was measured spectrophotometrically by Bradford's protein assay (Sigma-Aldrich, USA). Then, samples were diluted in Tris-buffer 100 mM (pH 8.2) containing 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB, 100 μM) and acetyl-coenzyme A (100 μM). The assay was performed in 96-well plates and the reaction was initiated by the addition of oxalacetate (500 μM). Absorption was measured spectrophotometrically at 30°C and 412 nm every 30 s for 15 min using a microplate reader (EnSpire; PerkinElmer, USA). All reagents were purchased from Sigma-Aldrich. Citrate synthase (CS) activity was determined by using a calibration curve obtained with known concentrations of the enzyme.

2.5 | Histological evaluation of adipocytes size

Six WAT samples per group were thawed in 4% paraformaldehyde. After routine processing for paraffin embedding, sections (4 μM) were stained with Hematoxylin and Eosin (H&E) for morphological examination. Then, they were analyzed by visual examination to identify any diet-associated changes. Representative images were acquired by the Nis-elements Br software associated to a Ni-E microscope (Nikon Instruments SPA, Japan).

2.6 | Statistical analysis

All data were expressed as mean ± standard error (SEM). One-way ANOVA followed by Bonferroni's post hoc test has been selected for analyzing differences among experimental groups. $p < .05$ was considered representative of significant differences (software: GraphPad Prism 6.0).

3 | RESULTS

3.1 | Characterization of the extracts

The total glucosinolate content of E.S. defatted seed meal was 101 μmol g⁻¹. 35 g of brown powder were obtained enriched with

23 ± 2 μmol g⁻¹ glucoraphanin and 498 ± 2 μmol g⁻¹ glucoerucin, with an overall yield of extraction of 45% referring to glucosinolate content in defatted meal. No traces of residual E.S. seed oil was detected in final extract after standard automated continuous extraction by using a E-816 ECE (Economic Continuous Extraction) extraction unit (BÜCHI Labortechnik AG, Switzerland), and hexane as solvent.

3.2 | Effects of *Eruca sativa* Mill. seed extract on HF diet-induced body weight gain

The baseline weight of mice was 26.2 ± 0.5 g. During the 10 weeks of treatment, mice fed with standard diet (Std group) showed a physiological increase in the body weight (13.2 ± 2.2% at the last day of treatment). Interestingly, mice daily fed with a standard diet plus E.S. seed extract (Std + E.S. group) showed body weight increase significantly lowered (9.0 ± 1.1% on the last day of treatment). Mice fed with the high-fat diet (HF group) showed a body weight increase significantly higher if compared with Std group (27.7 ± 2.1% on the last day of treatment). Conversely, daily oral treatment with E.S. seed extract (HF + E.S. group) significantly contained HF-induced body weight gain in mice fed with high-fat diet, since the percentage value of body weight increase on the last day of treatment was 20.7 ± 1.4% in this experimental group (Figure 1).

Accordingly, daily consumption of E.S. contained also HF diet-induced BMI increase (Figure 2). Indeed, as expected, HF fed mice showed a significantly higher BMI value, if compared with STD diet; conversely, the supplementation with E.S. contributed to contain this parameter, that it was superimposable to STD diet.

3.3 | Effects of *Eruca sativa* Mill. Seed extract on the WAT

According to the composition of the HF diet, the weight of WAT of mice belonging to HF group was significantly higher than that of mice fed with Std diet (Figure 3a). Interestingly, daily treatment with E.S. seed extract for 10 weeks clearly, albeit not significantly, reduced adipose tissue weight of animal fed with HF diet (Figure 3a). Daily consumption of HF diet for 10 weeks deeply modified the metabolic activity of adipose tissue. Indeed, CS activity, which is an index of tissue metabolic condition, was significantly lower in HF group compared to Std group (0.480 ± 0.03 nmol min⁻¹ mg⁻¹ proteins vs 0.996 ± 0.21 nmol min⁻¹ mg⁻¹ proteins). In contrast, CS activity was almost completely recovered (0.919 ± 0.11 nmol min⁻¹ mg⁻¹ proteins) in HF + E.S. group (Figure 3b). No significant changes have been observed between Std and Std + E.S. groups.

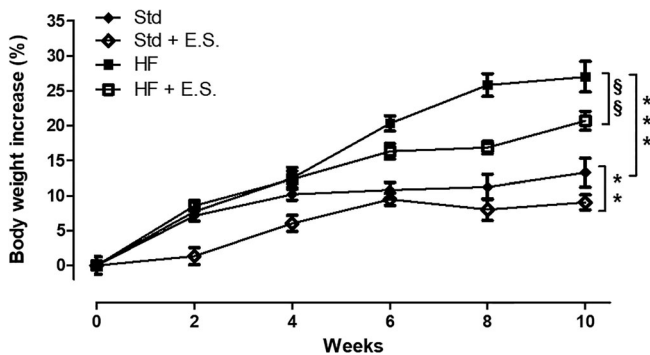


FIGURE 1 Effects of *Eruca sativa* Mill. (E.S.) seed extract on high-fat diet-induced body weight increase. Graph shows the weight increase (%) of mice fed with standard diet (Std), standard diet plus *Eruca sativa* Mill. (Std + E.S.), high-fat diet (HF) or high-fat diet plus *Eruca sativa* Mill. (HF + E.S.) for 10 weeks. Data are expressed as mean \pm SEM. “*” indicates significant difference vs Std group (** $p < .01$; *** $p < .001$) while “\$” indicates significant difference vs HF group (§§ $p < .01$). Number of animals for each group is $n = 10$

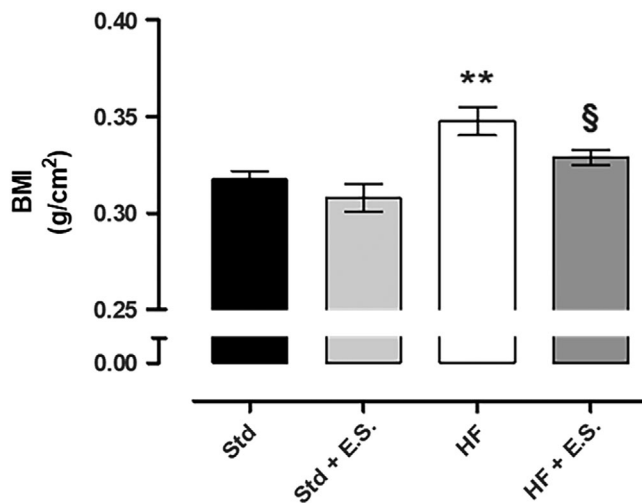


FIGURE 2 Effects of *Eruca sativa* Mill. on BMI. Histograms show the effects of *Eruca sativa* Mill. (E.S.) on the body mass index (BMI) of mice fed with standard diet (Std), standard diet plus *Eruca sativa* Mill. (Std + E.S.), high-fat diet (HF) and high-fat diet plus *Eruca sativa* Mill. (HF + E.S.) for 10 weeks. Data are expressed as mean \pm SEM. “*” indicates significant difference vs Std group (** $p < .01$) while “\$” indicates significant difference vs HF group (§ $p < .05$). Number of animals for each group is $n = 10$

Furthermore, the histological analysis of adipose tissue showed a significant increase in size of adipocytes of HF group mice, compared to the Std group; interestingly, the dietary intake of E.S. significantly reduced the adipocyte size of animals fed with a HF diet (Figure 4a–d).

3.4 | Effects of *Eruca sativa* Mill. seed extract on lipid profile

HF-diet fed mice showed higher levels of total cholesterol, LDL and triglycerides if compared with Std group. The supplementation of the

standard diet with E.S. seed extract (Std + E.S. group) did not affect total cholesterol and LDL values, while a reduction in the levels of triglycerides was observed. Conversely, in the HF group the supplementation with E.S. seed extract contributed to reduce all the parameters, particularly the triglyceride levels (Table 2).

3.5 | Hypoglycemic effects of *Eruca sativa* Mill. seed extract

After 10 weeks of treatment, both fasting blood glucose and HbA1c levels were measured. No significant differences have been observed in blood glucose levels between Std group and HF group, suggesting that such HF diet did not induce evident hyperglycemic conditions during the 10 weeks of treatment. Nevertheless, an increasing trend in levels of HbA1c was measured in the HF group compared to the Std group, indicating that HF may have actually acted as a diabetogenic factor. Notably, dietary intake of E.S. strikingly and significantly reduced both blood glucose and HbA1c levels in mice fed with a HF diet (Figures 5a,b).

4 | DISCUSSION

Obesity is a condition that impairs quality of life and is a main risk factor for several chronic diseases, including type II diabetes and cardiovascular diseases (Williams, Mesidor, Winters, Dubbert, & Wyatt, 2015; N.R.F.C. (NCD-RisC), 2016; González-Muniesa et al., 2017); therefore, the identification of effective nutraceuticals is a goal in prevention and treatment of obesity. In this study, the anti-obesity and hypoglycemic effects of dietary supplementation with E.S. seed extract on HF diet-induced obesity has been evaluated in mice. HF diet is considered a reliable experimental model of obesity; indeed, it promotes body weight gain, hyperlipidemia and an alteration of glucose homeostasis in rodents (Pinheiro-Castro, Silva, Novaes, & Ong, 2019).

E.S. belongs to the *Brassicaceae* family. It contains numerous edible vegetables commonly used as food, including broccoli, cabbage, cauliflower, raphanus and brussels sprouts. To date, several preclinical and clinical evidence shows the cardiovascular beneficial effects of these edible plants (Raiola et al., 2017).

The main finding of this work is the capability of E.S. seed extract to reduce the body weight gain in an experimental model of obesity, improving also glucose homeostasis. Importantly, the reduction of body weight gain was not due to an anorexigen effect, since both HF and HF + E.S. diets, although associated with a lower food intake compared to Std group, did not significantly affect daily caloric intake of mice (data not shown).

Interestingly, beside body weight containment, lower values of BMI and WAT mass were observed; such a beneficial effect was confirmed from histo-morphological evaluation, in which a significant reduction of adipocytes size has been highlighted. Indeed, in mice fed with a HF diet WAT expands by storing triglycerides in the central lipid droplet; therefore, the reduction of triglyceride levels, observed

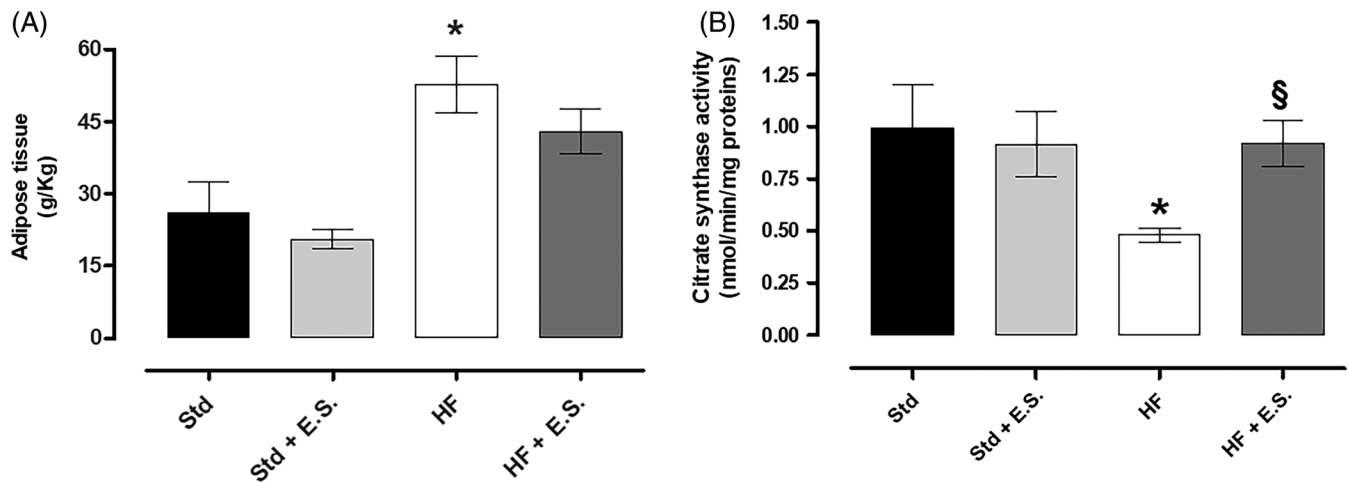


FIGURE 3 Effects of *Eruca sativa* Mill. seed extract on adipose tissue. Graphs represent the weight of WAT (a) and the activity of CS (b) in the adipose tissue of mice fed with Std diet, standard diet plus *Eruca sativa* Mill. (Std + E.S.), HF or HF diet plus *Eruca sativa* Mill. (HF + E.S.) for 10 weeks. Data are expressed as mean \pm SEM. “*” indicates significant difference vs Std group ($*p < .05$) while “§” indicates significant difference vs HF group ($§p < .05$). Number of animals for each group is $n = 10$

from us, strengths this paradigm. Moreover, a well-recognized early marker of alteration of adipose tissue metabolism is the CS, a mitochondrial enzyme that acts as a rate-limiting enzyme in the first step of the Krebs cycle. It is largely used for evaluating mitochondrial oxidative capacity and its activity results almost half following an HF-feeding. Noteworthy, a compromised activity of CS is evident even before that ultra-structural alterations or mitochondrial dysfunctions appear (Cummins et al., 2014). In these experimental conditions, the supplementation with E.S. seed extract contributed to enhance CS activity in WAT, demonstrating an improvement of the metabolism of adipocytes.

These results are in accordance with the literature published on other *Brassicaceae* vegetables; indeed, many preclinical and clinical reports demonstrated their antioxidant activity (Bahadoran et al., 2011), anti-obesity and hypo-lipidemic effects in rats fed with HF diet (Lee, Kim, & Lee, 2018). For instance, 4-week supplementation with *Brassica rapa* L. (var. *perviridis*) juice contributed to reduce cholesterol levels in middle-aged men, through an improvement of cholesterol metabolism (Aiso, Inoue, Seiyama, & Kuwano, 2014). Another study reported the hypo-lipidemic effects of broccoli; in particular, 400 g of broccoli, containing high amount of glucoraphanin, improved fatty acid oxidation and tricarboxylic acid cycle activity within mitochondria, probably due to Nrf2-mediated modulation of cellular redox status (Armah et al., 2013; Armah et al., 2015). Recently, Lee et al. reported that supplementation with a *Brassica juncea* leaf extract exerted positive effects on lipid profile and on body fat in rats fed with HF diet. Indeed, a reduction of body fat accumulation and an improvement of lipid profile, by modulating lipogenesis and cholesterol metabolism, have been described (Lee et al., 2018). Similar results have been obtained with a supplementation of red cabbage (*Brassica oleracea* L. var. *capitata*) in rats fed with a HF diet. Huang and colleagues highlighted its hypocholesterolemic effects without showing significant impact on body weight; moreover, reduction of

pro-inflammatory cytokines and increase of ACAT1 expression, which is involved in the fatty acid oxidation, have been found (Huang et al., 2016).

Recently, it has been also suggested that many *Brassicaceae* plants exert promising hypoglycemic effects. For example, *B. oleracea* L. leaves produced, in streptozocin-induced diabetic rats, marked reduction in both blood glucose and HbA1c levels, and a marked increase in insulinemia (Shah, Shaker, & Gousuddin, 2016). In addition, Akbari and colleagues have recently described the hypoglycemic properties of *Brassica napus* L. on diabetic rats (Akbari et al., 2016). Finally, the consumption of *Brassicaceae* vegetables has been associated with significant decrease of serum insulin concentration in type II diabetes patients (Bahadoran et al., 2012; Yokozawa, Kim, Cho, Yamabi, & Choi, 2003; Yun et al., 2018).

In our experimental conditions, although there was not a hyperglycemic condition, higher HbA1c levels have been measured in blood of mice fed with a HF diet, suggesting an early alteration of glucose homeostasis, which is prodromal to type II diabetes (Qaseem et al., 2018). Interestingly, E.S. markedly reduced both blood glucose and HbA1c levels in mice fed with a HF diet, indicating that E.S. extract represents a promising nutraceutical approach to improve the glucose metabolism. In the light of these results, it is possible to hypothesize that E.S. extract can positively influence the metabolism of glucose, although further evaluations are still needed.

Notable, key bioactive components in *Brassicaceae* vegetables are glucosinolates and their derivatives isothiocyanates, which are produced by the myrosinase enzyme (Palliyaguru et al., 2018). Very recently, the well-known glucosinolate glucoraphanin, similarly to crude *Brassica* extracts, ameliorated inflammation and reduced insulin resistance associated with obesity. Nrf2 has been recognized as a main player in these beneficial effects, since the activation of Nrf2 promotes the stimulation of both AMPK and uncoupling protein UCP1, which are involved in the energy consumption (Xu, Nagata, &

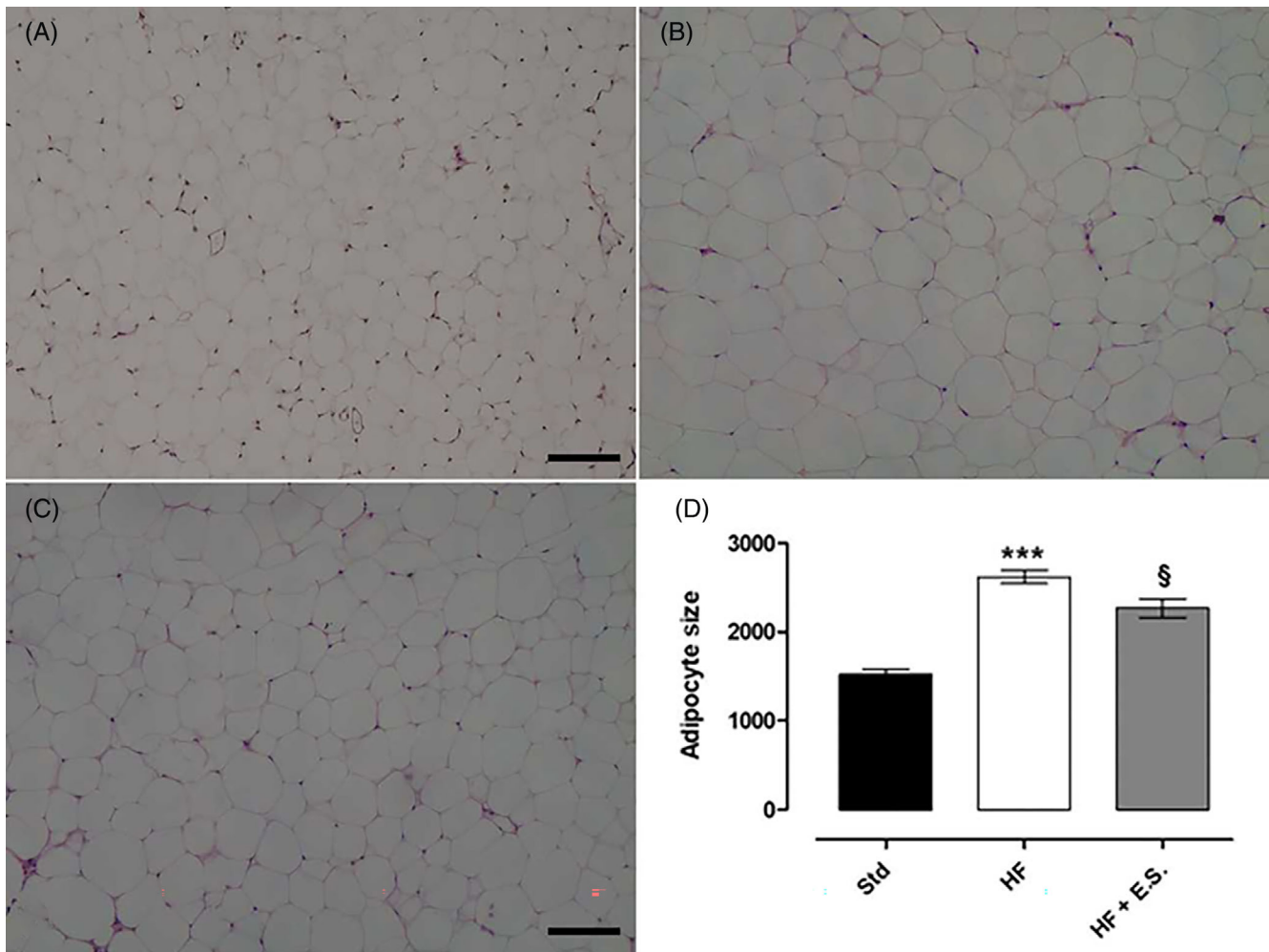


FIGURE 4 Histological evaluation of adipocyte size. Photomicrographs of hematoxylin and eosin (H&E) stained adipose tissue sections from: (a) mice fed with a Std diet; (b) mice fed with a HF diet; (c) mice fed with a HF diet plus *Eruca sativa* Mill. (HF + E.S.) for 10 weeks. Scale bar is equal to 10 μm . Histograms (d) represent the mean size of adipocytes from each experimental group. Data are expressed as mean \pm SEM. “***” indicates significant difference vs Std group (** $p < 0.001$) while “§” indicates significant difference vs HF group (§ $p < 0.05$). Number of animals for each group is $n = 5$ [Colour figure can be viewed at wileyonlinelibrary.com]

	Std	Std + E.S.	HF	HF + E.S.
Total cholesterol (mg dl^{-1})	148.0 \pm 12.0	177.0 \pm 17.1	247.2 \pm 15.1**	221.8 \pm 7.4*
LDL (mg dl^{-1})	80.3 \pm 9.5	75.6 \pm 7.4	114.8 \pm 7.2**	104.0 \pm 6.6*
Triglycerides (mg dl^{-1})	119.5 \pm 8.4	84.8 \pm 10.4*	150 \pm 20.5	103.2 \pm 3.8§

Note: Table shows the total cholesterol, LDL and triglycerides levels measured in mice fed with standard diet (Std group), standard diet plus *Eruca sativa* Mill. seed extract (Std + E.S.), high-fat diet (HF group) and HF diet plus *Eruca sativa* Mill. seed extract (HF + E.S. group). The values are expressed as mean \pm SEM. “**” indicates significant difference vs Std group (** $p < .01$) while “§” indicates significant difference vs HF group (§ $p < .05$).

TABLE 2 Measurement of cardiometabolic parameters

Ota, 2018). Interestingly, natural isothiocyanates have been described as H_2S -releasing moieties and H_2S has been proposed to be the likely player of many biological and pharmacological effects of isothiocyanates and of *Brassicaceae* plants/extracts (Citi et al., 2014; Martelli et al., 2020a). As above reported, H_2S -releasing properties of erucin have been recently demonstrated (Martelli et al., 2020b).

H_2S has recently gained significant attention as biological mediator implicated in several pathological conditions, including obesity. Indeed, it is produced in adipose tissue and is involved in adipogenesis, metabolism and adipokine production (Bełtowski & Jamroz-Wiśniewska, 2016). Reduced levels of H_2S -synthesizing enzymes have been reported in fat tissues of obese mice, as well as in db/db animals (Katsouda, Szabo, & Papapetropoulos, 2018).

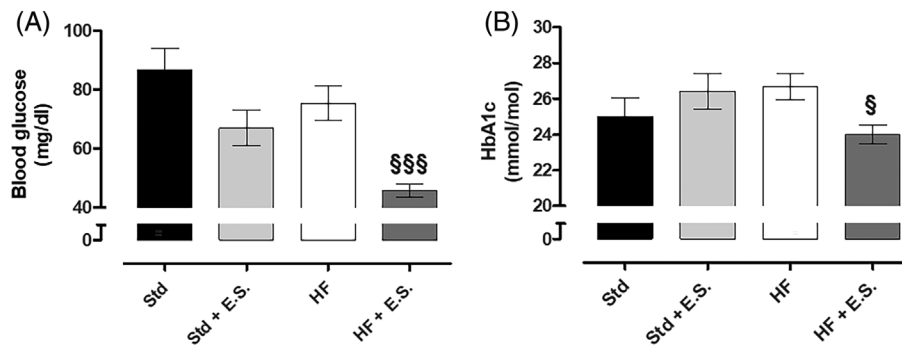


FIGURE 5 Evaluation of the effects of *Eruca sativa* Mill. seed extract on glycaemic parameters. Histograms represent the blood glucose (a) and the HbA1c levels (b) measured in mice fed with Std diet, standard diet plus *Eruca sativa* Mill. (Std + E.S.), HF or high-fat diet plus *Eruca sativa* Mill. seed extract 0.75% p/p (HF + E.S.) for 10 weeks. Data are expressed as mean \pm SEM. “\$” indicates significant difference vs HF group ($^{**}p < .01$; $^{***}p < .001$). Number of animals for each group is $n = 10$

At this regard, down-regulation of endogenous H₂S production and alteration of glucose metabolism were also observed in HF diet-treated mice, suggesting that H₂S deficiency promoted a reduction in levels of the myokine recently described irisin and subsequent metabolic imbalance. Interestingly, treatment with exogenous sources of H₂S led to beneficial effects on muscle glucose homeostasis via the irisin pathway (Parsanathan & Jain, 2018).

Therefore, it can be hypothesized that H₂S, released from sulfur secondary metabolites (namely glucoerucin and erucin) may be a relevant player in the metabolic beneficial effects of E.S. seed extract observed in this experimental work. Future studies will be specifically focused on the exploration of the mechanisms of action, with particular attention on the potential role of H₂S.

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CONFLICT OF INTEREST

The authors declare no competing financial interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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