

***Phaseolus vulgaris* L. extract: alpha-amylase inhibition against metabolic syndrome in mice**

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Supplementary materials

Material and methods

Rota Rod test

The apparatus consisted of a base platform and a rotating rod with a diameter of 3 cm and a nonslippery surface. The rod was placed at a height of 15 cm from the base. The rod, 30 cm in length, was divided into 5 equal sections by 6 disks. Thus, up to five mice were tested simultaneously on the apparatus, with a rod-rotating speed of 16 revolutions per minute. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 s. The performance time was measured on week 19 before (0 min) and 15, 30, and 45 min after the beginning of the test.

Von Frey test

Mechanical allodynia was measured with the dynamic plantar aesthesiometer (Von Frey instrument) (Ugo Basile, Comerio, Italy) on week 19 as described by Baptista-de-Souza et al. (2014). The mice were placed in individual Plexiglas cubicles (8.5 × 3.4 × 3.4 cm) on a wire mesh platform. After approximately 1 h accommodation period, during which exploratory and grooming activity ended, the mechanical paw withdrawal threshold was measured as the hind paw withdrawal responded to von Frey hair stimulation. The mechanical stimulus was delivered to the plantar surface of the hind paw of the mouse from below the floor of the test chamber by an automated testing device. A steel rod (2 mm) was pushed with electronic ascending force (0 – 5 g in 35 s). When the animal withdrew its hind paw, the mechanical stimulus was automatically withdrawn, and the force recorded to the nearest 0.1 g. Nociceptive response for mechanical sensitivity was expressed as paw withdrawal threshold in grams. The test was repeated 5 times for each animal and the final values were obtained by averaging results of the 5 tests.

Hargreaves' plantar test

Paw withdrawal latency in response to radiant heat (infrared) was assessed using the plantar test apparatus (Ugo Basile, Comerio, Italy). Each mouse was placed under a transparent Plexiglas box (7.0 × 12.5 × 17.0 cm) on a 0.6-cm-thick glass plate and allowed to acclimatize for 1–2 h before recording. The radiant heat source consisted of an infrared bulb (Osram halogen-bellaphot bulb; 8 V, 50 W) that was positioned 0.5 cm under the glass plate directly beneath the hind paw. Paw withdrawal latency after switching on the infrared radiant heat stimulus was measured automatically. The infrared light intensity was chosen to give baseline latencies of about 10 s in control mice. A cut-off of 20 s was used to prevent tissue damage. Each hind paw was tested two to three times, alternating between paws with an interval of at least 1 min between tests, and averaged for each animal. The interval between two trials on the same paw was of at least 5 min. Nociceptive response for thermal sensitivity was expressed as thermal paw withdrawal latency in seconds.

Elevate plus maze

The apparatus consisted of 2 open arms (40 × 5 cm) and 2 arms enclosed with 15 cm high dark walls, all of which were elevated 55 cm above the floor (Ugo Basile, Varese, Italy). Arms of the same type were located opposite from each other. A mouse was placed in the central square of the maze (5 × 5 cm) so that it faced one of the enclosed arms. Time latency before the animals enter into the closed arms and the time spent into the closed arms was measured. The test was repeated after 24 h. Data acquisition and analysis were performed automatically using ANY-maze software.

Liver and kidney histopathology

Liver sections (5 μ m) were stained with hematoxylin and eosin (H&E). The quantitative scoring of H&E-stain based liver tissue sections was conducted according to the previous methods with slight modifications, regarding steatosis (Brunt, 2001; Kleiner et al., 2005). Briefly, each section was examined by a specialist who was blinded to the sample information and hepatic steatosis and inflammation scores were evaluated. The degree of steatosis was graded '0' to '10' based on the average percent of fat-accumulated hepatocyte per field at 20 \times magnification under H&E staining (grading 0, <5% of fat accumulation and grading 10, >75% of fat accumulation).

Fixed kidneys were embedded in paraffin and were sectioned (5 μ m). To evaluate the renal structural changes, we performed PAS (Periodic Acid Schiff) staining on these sections as described previously (Koya et al., 2000). From each mouse, 70 glomeruli for sections for 3 consecutive section were supplied for morphometrical analysis. The extent of the mesangial matrix (defined as mesangial area) was determined by assessing the PAS-positive and nuclei-free area in the mesangium using a computer-assisted color image analyzer at 20X magnification. Other sections were also used for H&E staining by which the glomerular area (μ m²) was evaluated. Results were expressed as a percentage average compare to the control animals and represent the mean of three consecutive sections. The kidney sections were imaged with a microscope at 4X magnification.

Lipid peroxidation (thiobarbituric acid-reactive substances assay)

The TBARS determination was carried out in mice liver homogenate obtained from liver tissue homogenized in PBS at the final concentration of 10% w/v. Then were added FeCl₃ (20 μ M, Sigma-Aldrich, St. Louis, MO, USA) and ascorbic acid (100 μ M, Sigma-Aldrich) to obtain the Fenton reaction. At the end of incubation, the mixture was added to 4 mL reaction mixture consisting of 36 mM thiobarbituric acid (Sigma-Aldrich) solubilized in 10% CH₃COOH, 0.2% SDS, and pH was adjusted to 4.0 with NaOH. The mixture was heated for 60 min at 100°C and the reaction was stopped by placing the vials in an ice bath for 10 min. After centrifugation (at 1600 g at 4°C for 10 min) the absorbance of the supernatant was measured at 532 nm (PerkinElmer spectrometer) at 550 nm (PerkinElmer spectrometer) and TBARS were quantified in μ mol/mg of total proteins using 1,1,3,3-tetramethoxypropane as the standard.

Carbonylated proteins

Samples were separated on a 4–12% sodium dodecyl sulfate (SDS)-polyacrylamide gel by electrophoresis and transferred onto nitrocellulose membranes (Biorad, Italy). Membranes were blocked with 5% non fat dry milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBST) and then probed overnight with primary antibody specific versus DNPH (Sigma-Aldrich, Italy) 1 : 5000 in PBST/5% non fat dry milk. After washing with PBST, the membranes were incubated for 1 hour in PBST containing the appropriate horseradish peroxidase-conjugated secondary antibody (1 : 5000; Cell Signalling, USA) and again washed. ECL (Pierce, USA) was used to visualize the peroxidase-coated bands. Densitometric analysis was performed using the "ImageJ" software (NIH Bethesda, Meryland, USA). For each experiment the density of all bands showed in a lane was reported as mean. GAPDH (1 : 2000 mouse antiserum, Cell signaling, USA) normalization was performed for all samples.

Western blot analysis

The suspension was sonicated on ice using three 10-second bursts at high intensity with a 10-second cooling period between each burst. After centrifugation (13000 \times g for 15 minutes at 4°C) aliquots containing 20 μ g total protein underwent to western blot analysis using a mouse anti-NADH dehydrogenase antiserum (1 : 2000; Abcam, Cambridge, UK), a mouse anti-catalase antiserum (1 : 1000; Novus Biologicals, CO, USA) and a mouse anti-glutathione reductase antiserum (1 : 500; Santa Cruz Biotechnology, CA, USA). Densitometric analysis was performed using the "ImageJ" analysis software (NIH Bethesda, Meryland, USA), and results were normalized to GAPDH immunoreactivity (1 : 2000 mouse antiserum, SantaCruz Biotechnology) as internal control.

Supplementary Table S1. Hematic metabolic parameters

	Total cholesterol (mg/dL)	Triglyceride (mg/dL)	Glucose (mg/dL)
group	week 11	week 11	week 11
normal diet + vehicle	101.0 ± 1.0	71.3 ± 11.3	103.5 ± 7.9
HFD + vehicle	130.5 ± 3.4**	103.9 ± 4.4**	144.1 ± 8.8**

Effects of normal and high fat diet (HFD) on total cholesterol, tryglicerides glucose blood levels, prior to the beginning of compounds administration. Each value represents the mean of ± s.e.m. of at least 12 mice per group. **P< 0.01 *vs* normal diet + vehicle.

Supplementary Table S2. GTT and ITT AUC

group	GTT AUC	ITT AUC
normal diet + vehicle	4194 ± 35.0	5079 ± 42.8
HFD + vehicle	6960 ± 46.8**	7609 ± 37.2**
HFD + <i>P. vulgaris</i> 500 mg/kg	4843 ± 31.5^^	5605 ± 40.1^^
HFD + metformin 100 mg/kg	4473 ± 40.9^^	5439 ± 33.6^^
HFD + atorvastatin 10 mg/kg	4778 ± 48.0^^	7914 ± 50.3

Effects of normal diet and HFD on GTT and ITT on week 19. Each value represents the mean of ± s.e.m. of at least 12 mice per group. **P< 0.01 *vs* normal diet + vehicle; ^^P<0.01 *vs* HFD + vehicle.

Supplementary Table S3. HOMA-IR

group	HOMA-IR
normal diet + vehicle	1.02
HFD + vehicle	15.56
HFD + <i>P. vulgaris</i> 500 mg/kg	2.35
HFD + metformin 100 mg/kg	2.79
HFD + atorvastatin 10 mg/kg	11.49

Effects of normal diet and HFD on insulin resistance index. Each value represents the mean of ± s.e.m. of at least 12 mice per group.

Supplementary Table S4. Plantar test

group	Withdrawal latency (s)
normal diet + vehicle	13.4 ± 1.0
HFD + vehicle	15.7 ± 1.3

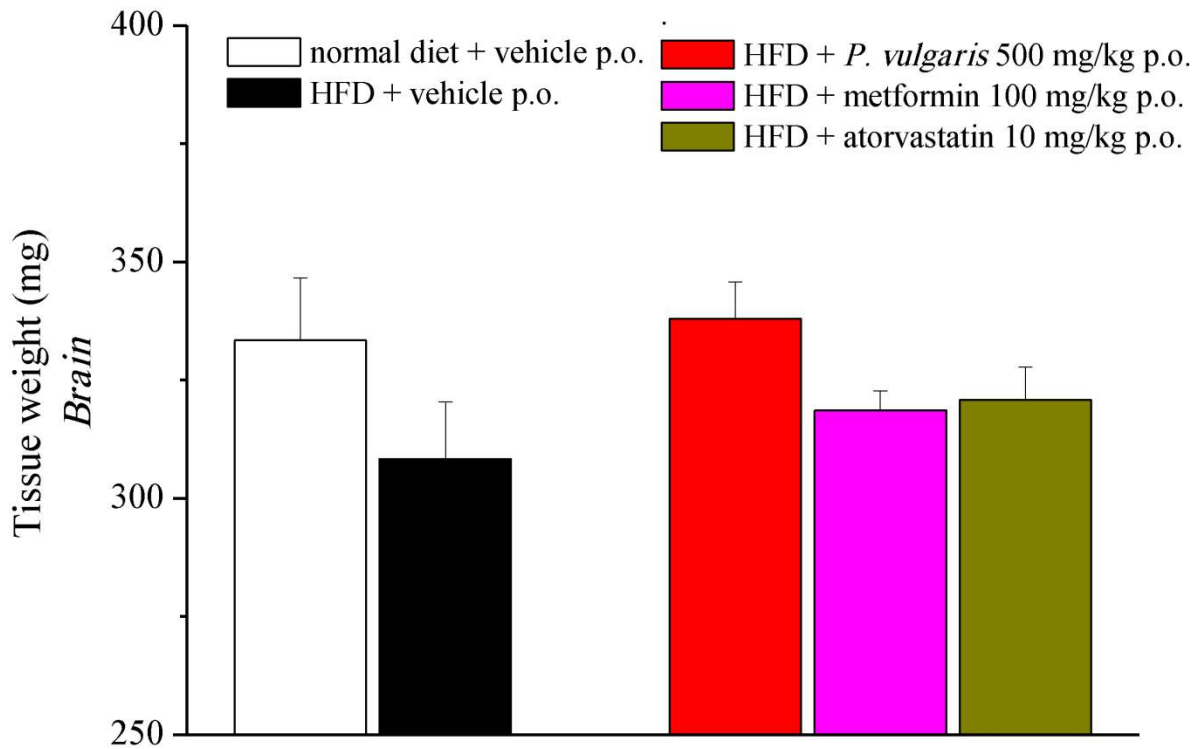
Effects of normal diet and HFD on pain threshold (week 19). Plantar test was performed to assess the response to a thermal stimulus. Each value represents the mean of ± s.e.m. of at least 12 mice per group.

Supplementary Table S5. Elevated plus maze

group	Latency time (s)	Time spent into the closed arms (s)	Latency time (s) at 24h
normal diet + vehicle	21.9 ± 6.7	232.3 ± 3.4	10.6 ± 3.4
HFD + vehicle	22.0 ± 8.2	257.1 ± 6.2	9.7 ± 2.1

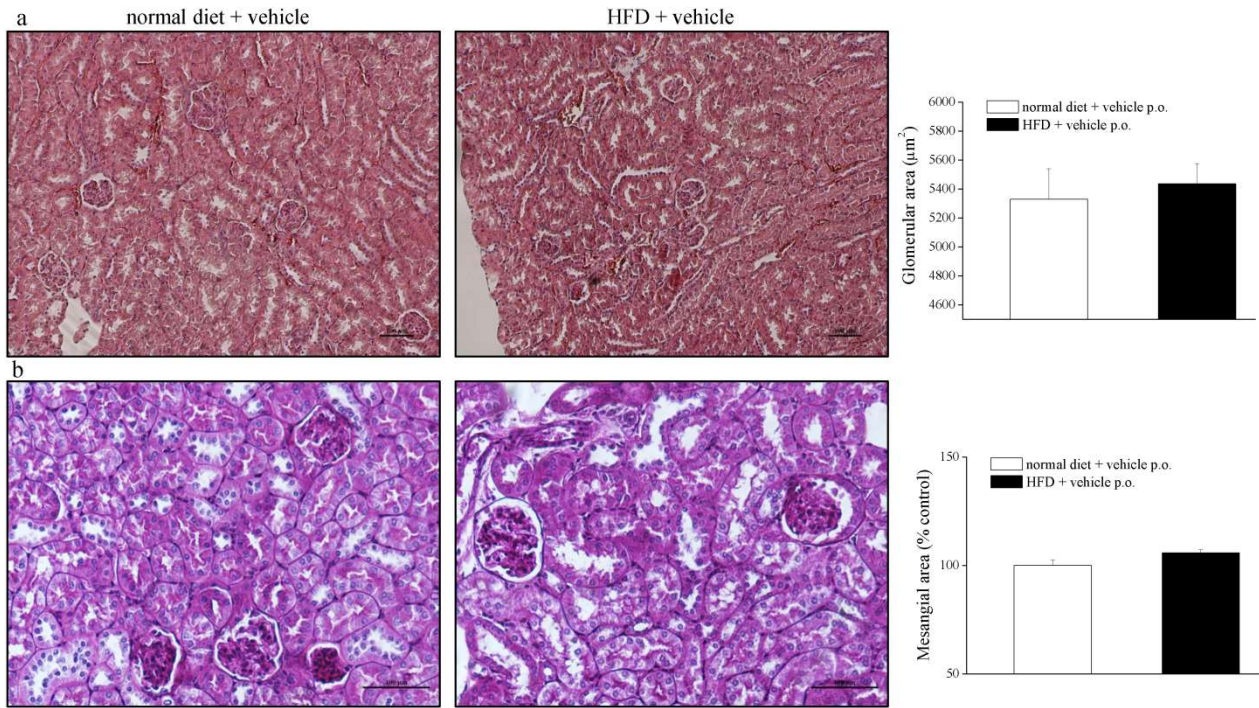
Effects of normal diet and HFD on anxious behaviour (week 19). The elevated plus maze test was performed. Each value represents the mean of ± s.e.m. of at least 12 mice per group.

Supplementary figure S1



Supplementary Figure 1. Brain weight. On week 19, after in vivo examinations brain was collected and weighted. Each value represents the mean of ± s.e.m. of 12 mice per group.

Supplementary figure S2



Supplementary Figure 2. Kidney histological evaluation in normal diet fed animals in comparison with HFD. a) Representative images of cortical kidney sections stained with hematoxyllin-eosin, 4X magnificated; graph: glomerular area in HFD group is expressed as percentage compared to control tissues (set as 100%). b) Representative images of cortical kidney sections stained with PAS stain, 20X magnificated; graph: mesangial area in HFD group is expressed as percentage compared to control tissues (set as 100%). Each value represents the mean of \pm s.e.m. of 12 mice per group.