Poplar Propolis Ethanolic Extract Reduces Body Weight Gain and Glucose Metabolism Disruption in High-Fat Diet-Fed Mice

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Nicolas Cardinault[3]*, Franck Tourniaire[1,2]*, Julien Astier[1], Charlène Couturier[1], Estelle Perrin[1], Julie Dalifard[1], Eva Seipelt[1], Lourdes Mounien[1], Claire Letullier[3], Lauriane Bonnet[1], Esma Karkeni[1], Naïma Delbah[4], Stéphane Georgé[4], Jean-François Landrier[1,2]

[1] Aix-Marseille Université, C2VN, INRA, INSERM, 13000, Marseille, France

[2] CriBioM, Criblage Biologique Marseille, Faculté de médecine de la Timone, Marseille, France.

[3] Pollenergie, La Grabère, 47450 Saint Hilaire de Lusignan, France.

[4] CTCPA, 449 Avenue Clément Ader, 84911 Avignon.

* These authors contributed equally to this work.

Running title: Propolis improves obesity and associated disorders

Corresponding author and person to whom reprint requests should be addressed:

Jean-François Landrier, C2VN ; UMR 1260 INRA/1263 INSERM/Université d'Aix-Marseille, 27 Bd Jean Moulin, 13385 Marseille cedex 05, France. Phone: +33 4 91 29 42 75; E-mail: jean-francois.landrier@univ-amu.fr

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Abstract

Scope: Current evidence supports the beneficial effect of polyphenols on the management of obesity and associated comorbidities. This is the case for propolis, a polyphenol-rich substance produced by bees. The aim of the present study was to evaluate the effect of a poplar propolis ethanolic extract (PPEE) on obesity and glucose homeostasis, and to unveil its putative molecular mechanisms of action.

Methods and results: Male high-fat (HF) diet-fed mice were administered PPEE for 12 weeks. PPEE supplementation reduced the HF-mediated adiposity index, adipocyte hypertrophy and body weight gain. It also improved HOMA-IR and fasting glucose levels. Gene expression profiling of adipose tissue (AT) showed an induction of mRNA related to lipid catabolism and mitochondrial biogenesis and inhibition of mRNA coding for inflammatory markers. Interestingly, several Nrf2-target genes were induced in AT following administration of PPEE. The ability of PPEE to induce the expression of Nrf2-target genes was studied in adipocytes. PPEE was found to transactivate the Nrf2 response element and the Nrf2 DNA-binding, suggesting that part of the effect of PPEE could be mediated by Nrf2.

Conclusion: PPEE supplementation may represent an interesting preventive strategy to tackle the onset of obesity and associated metabolic disorders.

Introduction

Obesity is a major metabolic risk factor for type 2 diabetes mellitus (T2DM) and a public health problem throughout the world. In T2DM, insulin signaling is progressively lost, leading to an increase in pancreatic insulin production and pancreas exhaustion at the latest stages of the disease [1]. T2DM represents 90-95% of diabetes mellitus cases worldwide and s a multifactorial disease in which obesity plays a central role [2]. Indeed, obesity generates low-grade inflammation, which is related to the activation of inflammatory pathways (JNK and NF-$_K$B) in adipose tissue (AT) [3] and increased production of cytokines such as interleukin-6 (IL-6), interleukin-1beta (IL-1β), tumor necrosis factor alpha (TNF-α), and chemokines [4] (e.g. monocyte chemotactic protein 1 (MCP-1)) and other mediators of the immune response [3], including microRNA [5-7]. When AT lipid storage capacities are exhausted, ectopic fat depots develop in other tissues, including insulin sensitive tissues (e.g. liver, skeletal muscle). Such ectopic fat accumulation, together with inflammation, interferes with insulin signaling and is central in the development and persistence of insulin resistance[8], observed in T2DM. So far, drug-based therapeutic approaches to tackle obesity and associated disorders remain limited and have either deleterious side effects or poor efficiency. Therefore, alternative "natural" treatments are urgently needed.

Propolis is a complex natural resinous substance from buds and exudates of selected plant sources, collected by bees, mixed with wax, and transformed in the presence of bee enzymes [9]. Propolis has been used as a folk medicine in many countries since ancient times and has been reported to possess various pharmacological effects such as immunomodulatory, antitumoral, antimicrobial, antiviral, antifungal, anti-inflammatory and antioxidant properties [10]. These

effects are mostly attributed to the nature and amount of phenolic compounds contained in propolis. The chemical composition of propolis is highly dependent upon its plant source(s) and varies with the site of collection [9].

Interestingly, several clinical trials have been performed to test the effect of propolis supplementation in T2DM patients [11]. These recently reviewed and meta-analyzed studies suggest that propolis supplementation decreases fasting blood glucose and Hb1Ac levels [11]and indicate that propolis is able to delay or slow down the progression of the disease, and acts by decreasing oxidative stress and inflammatory processes associated with T2DM.

Preclinical studies have been performed in animal models of obesity and/or diabetes, to investigate the phenotypical impact of propolis supplementation in the diet [12-17]. The molecular mechanisms underlying the observed effects in terms of obesity management and improvement of glucose homeostasis, however, still remain unclear. Thus, the aim of the present study was to evaluate the effect of a standardized poplar propolis ethanolic extract (PPEE) on obesity glucose homeostasis and the physiology of the adipose tissue in high-fat diet-fed mice. The putative molecular mechanisms involved in these effects was also investigated.

Materials and methods

Propolis source and extraction

Poplar propolis was collected in the southern regions of France using dietary propolis traps according to special specifications to minimize contamination. It was stored at -20 °C prior to extraction. The latter was performed according to a previously published method [18]. Briefly, the propolis sample was cut into small pieces and soaked into 75% ethanol for 8 days. Following filtration, the filtrate was concentrated under reduced pressure using a rotary evaporator, to afford a poplar propolis ethanolic extract (PPEE). The dry residue was ground to a fine powder by adding food excipients and kindly provided by Pollenergie (Agen, France). The PPEE powder used in this study fully complies with European regulations on food supplements and those more specific of propolis.

Total polyphenol content -The total polyphenol content was measured according to the method described by Popova *et al*.. The results were expressed in mg/100 g of propolis powder as pinocembrin/galangin equivalent for poplar propolis. Methanolic solutions of pinocembrin/galangin (2/1) were used for calibration purposes [19]. The phenolic profile was determined by HPLC as previously reported. [20]. Briefly, a sample of 1 g of PPEE powder was dissolved in 30 mL of ethanol (70%) in a 50 mL Falcon tube then heated for 2h at 70 °C. Following centrifugation, the alcoholic phase was recovered and the extraction was repeated twice. The three extracts were combined and adjusted to 100 mL with 70% ethanol in a volumetric flask. PP solution was finally diluted 1 to 50 times in methanol / water (1:1). Separation was achieved by HPLC using a Symmetry (250 x 4.6 mm, C_{18}) column (Waters, Saint

Quentin en Yvelines, France). A linear gradient of acetonitrile and 0.2% aqueous formic acid (v/v) was used as the mobile phase with a flow rate of 1.2 mL/min and DAD data were acquired in the 200–450 nm range. Polyphenolic compounds were identified by comparing their retention time and UV characteristics to authentic standards. The total polyphenol content and the detailed composition of polyphenols present in PPEE are reported on Table 1.

Animal Experiments - Six-week-old male C57BL/6J mice were obtained from Janvier Labs (Le Genest Saint Isle, France) and fed *ad libitum* with control food (chow diet A04, Safe-diets, France) and with full access to drinking water for a 1-week acclimatization period. The animals were maintained at 22 °C under a 12-h light/dark cycle and a 20 % relative humidity level. The mice (3 or 4 per cage) were randomly assigned (10 per group) into one of three experimental groups depending on their diet, i.e.standard (chow diet A04 from Safe diets), high fat (HF: 45% energy from lipids, Test Diet ref. 58V8) or HF-supplemented with PPEE (20 mg/mouse/day, corresponding to 4.5 mg of total polyphenols/mouse/day). The pellets were replace every 3 days. The weight gain was measured once a week and dietary intake was recorded every two weeks. After 12 weeks, the mice were fasted overnight and blood was collected by cardiac puncture using a general anesthetic (sevoflurane, Baxter, France). The plasma was isolated by centrifugation at 3000 rpm for 15 min at 4 °C and was stored at -80 °C until further analysis. Animals were euthanized by cervical dislocation under general anesthesia, and their tissues (liver and various white AT deposits) were collected, weighted and stored at -80 °C until further analysis This protocol was approved by the local Ethics committee and the French Minister of Research and Education (n° 01549.04).

Dosage information – The PPEE has been mixed in the food and delivered to mice at the dosage of 20 mg / mouse / day, which corresponding to 4.5 mg of total polyphenols / mouse / day, for 12 weeks. This dosage was chosen to be relevant in human, as it correspond to a dose of 12 mg of total polyphenol / kg of body weight, taking into account the conversion factor for dose translation between mice and human [21].

Oral glucose and insulin tolerance tests - Glucose and insulin tolerance tests were performed two weeks before animals were euthanized. For the glucose tolerance test, the mice were fasted for16 h and then gavaged with glucose (2 g/kg). For the insulin tolerance test, the mice were fasted for 5 h and then injected *i.p*. with insulin (0.5 U/kg). Blood samples were taken from the tail tips at specific time intervals to measure glucose levels (Accu-Check glucometer, Roche).

Biochemical analyses – The concentration of glucose in plasma was evaluated using glucose RTU (bioMerieux, Craponne, France). Triglycerides and free fatty acids (FFA) were measured using a colorimetric test (RANDOX, Crumlin, Co. Antrim, United Kingdom). Insulin was measured using an enzyme-linked immuno-sorbent assay ELISA (ALPCO Diagnostics, New Hampshire, United States). Leptin and adiponectin were quantified by ELISA (R&D Systems quantikine ELISA). β-hydroxybutyrate concentration was measured using a colorimetric test according to the manufacturer's procedure (BEN srl, Milano, Italy). The HOMA-IR index was calculated according to the following formula: fasting insulin (microU/L) x fasting glucose (nmol/L)/22.5.

Adipose tissue histology - Epididymal AT samples were fixed in 10% buffered formalin, embedded in paraffin and sliced to prepare 5 µm tissue sections that were stained with hematoxylin and eosin (H&E) as previously reported [22]. The images were captured by a light microscope (EZAD, Leica, Germany). The adipocyte area (μm^2) were determined using the Image J software.

Cell culture - 3T3-L1 preadipocytes (ATCC, Manassas, VA) were seeded in 3.5-cm diameter dishes at a density of 15×10^4 cells/well. Cells were grown in DMEM supplemented with 10% FBS at 37 °C in a 5% CO_2 humidified atmosphere as previously reported [23]. To induce differentiation, 2-day post-confluent 3T3-L1 preadipocytes (day 0) were stimulated for 48 h with 0.5 mM isobutylmethylxanthine, 0.25 µM dexamethasone, and 1 µg/mL insulin in DMEM supplemented with 10 % FBS. The cells were maintained in DMEM supplemented with 10% FBS and 1 µg/mL insulin. To examine the effect of PPEE, 3T3-L1 adipocytes received either PPEE (3 - 30 µg/mL) or ethanol only (0.1%). All treatments were performed on day eight. The data obtained are the mean of three independent experiments, each performed in triplicate.

RNA interference - The 3T3-L1 differentiated cells were seeded in 6-well plates and transfected with either targeted siRNA (against Nrf2) or a non-targeting siRNA according to the manufacturer's instructions (Dharmacon, Inc. Lafayette, CO) and as previously reported [24, 25]. Briefly, the cells were transfected overnight using a mixture of 100 nM siRNA and 2 µL of DharmaFECT reagent per well. The media were subsequently replaced with fresh media with or without PPEE and the plates were re-incubated for 24 h.

Reporter assays - The reporter plasmid containing the gene for firefly luciferase under the control of the wild-type or mutated antioxidant response element (ARE) [26] was transfected into 3T3-L1 preadipocyte cells, together with the pGL4 plasmid (1 µg) coding for the renilla luciferase. The transfection was performed using Lipofectamine LTX (0.9 µL) (Thermo, Saint-Aubin, France) and the Plus Reagent (1 µL) as previously reported [27]. After overnight incubation the medium was replaced by DMEM supplemented with 10% FBS and PPEE (30 µg/mL) . After 24 h, the cells were lyzed and assayed for luciferase activity using a Dual-Glo luciferase assay system (Promega, Madison, WI) withfirefly luciferase activity normalized to renilla luciferase. The transfection experiments were performed in triplicate and repeated three times independently.

Nfr2 transAM assay - 3T3-L1 cells were incubated for 6 h with or without PPEE (30 µg/mL). Nuclei were prepared as recommended and used in the TransAM Nrf2 assay according to the manufacturer's protocol (Active Motif; La Hulpe, Belgium)

RNA extraction and real-time PCR - Total RNA was extracted from the liver, kidney and epididymal AT or from cells using TRIzol reagent (Thermo, Courtaboeuf, France). Total RNA (1 µg) was used to synthetize cDNAs using random primers and Moloney murine leukemia virus reverse transcriptase (Thermo, Courtaboeuf, France). Real-time quantitative PCR analyses were performed using the Mx3005P Real-Time PCR System (Stratagene, La Jolla, USA) as previously described [28]. For each condition, the expression was quantified in duplicate, and 18S rRNA was used as the endogenous control in the comparative cycle threshold (CT) method [29].

Statistical analysis - All data are expressed as the mean ± SEM. Significant differences between the control and treated groups were determined using ANOVA, followed by the Tukey-Kramer post hoc test using the Statview software (SAS Institute, Cary, NC). $P < 0.05$ was considered statistically significant.

Results

Poplar propolis ethanolic extract supplementation protects against diet-induced obesity

The results showed that PPEE supplementation (20 mg/mouse/day, equivalent to 4.5 mg of total polyphenols/mouse/day) significantly limited weight gain ($p<0.0001$) caused by a HF diet (Fig 1A and 1B). The energy intake was higher in the HF diet fed group compared to the control group, but no significant difference *(*$p<0.002$) was observed in the PPEE supplemented group compared to the HF diet fed group (Fig 1C). The limitation of body weight gain in PPEE supplemented mice was associated with a reduction of absolute and relative fat mass in epididymal, retroperitoneal or inguinal fat pads (Fig 1D and 1E, $p<0.05$). Consequently, the adiposity index (sum of epididymal, inguinal and retroperitoneal AT mass relative to total body mass) in PPEE supplemented mice was reduced, compared to HF diet fed mice (Fig 1F, $p<0.001$). Interestingly no significant difference of adiposity index was observed between PPEE supplemented mice and control fed mice after 12 weeks of supplementation (Fig 1F). Triglycerides, free fatty acids and alanine transaminase (ALAT) were neither affected by the HF diet nor the EEPP supplementation ($p>0.05$). Adiponectin was not affected by PPEE supplementation compared to the HF diet fed group, but was significantly reduced compared to the control group ($p<0.05$). The levels of leptin and β-hydroxybutyrate were increased in the HF diet fed group compared to the control group and were reduced in the PPEE supplemented group ($p<0.05$) (Table 2).

Poplar propolis ethanolic extract supplementation improves glucose homeostasis

The effect of PPEE supplementation on glucose homeostasis was evaluated using the oral glucose tolerance test (OGTT) and the insulin tolerance test (ITT). PPEE supplementation improved (p<0.001), glucose tolerance in OGTT (Fig 2A) as highlighted by area under the curve (AUC) of the glycemic response (Fig 2B) which was reduced compared to what is observed in the group of HF fed animals. Additionally, PPEE supplementation reduced blood glucose levels, compared to HF fed mice (Fig 2C and 2D). In fasted conditions, insulinemia and glycaemia that were induced by the HF diet, were normalized in PPEE supplemented mice and were not significantly different from control mice (Fig 2E, p<0.05). Similarly, the HOMA-IR index was induced (p<0.005) by the HF diet and corrected by PPEE supplementation (Fig 2F,

Poplar propolis ethanolic extract supplementation changes adipocyte cellularity and modulates gene expression in adipose tissue

To evaluate the effect of PPEE supplementation on the phenotype of AT, epididymal AT was used for histological analysis. A HF diet promoted adipocyte hypertrophy with a significant increase in the adipocyte area compared to the control group. Interestingly, this effect was significantly prevented (p<0.05) by PPEE supplementation (Fig 3A). Furthermore, the effect of PPEE on inflammation in AT was evaluated by measuring the expression of different markers of inflammation including cytokines (Tnfa) and chemokines (Chemokine C-C motif ligand 5 (Ccl5) and Ccl2). As expected, the HF diet induced a strong increase in the mRNA level of these inflammatory markers compared to the control group., PPEE supplementation, on the other hand, caused a significant decrease (p<0.05) of those levels (Fig 3B).

To understand the modification of cellularity and fat mass increase, the expression of genes encoding for transcription factors involved in energy metabolism, fatty acid oxidation and

mitochondrial biogenesis were studied. PPEE supplementation stimulated ($p<0.05$) the expression of genes promoting fatty acid oxidation, such as the peroxisome proliferator activated receptor a (Ppara), the carnitine palmitoyl-transferase 1a (Cpt1a), the medium- and long-chain acyl-CoA dehydrogenases (Mcad and Lcad), and adipose triglyceride lipase (Atgl), compared to HF diet fed mice (Fig 3C). PPEE supplementation also induced ($p<0.05$) genes related to white AT browning, including genes encoding for the uncoupling protein 1 (Ucp1), the transcription factors peroxisome proliferator-activated receptor gamma coactivator 1-alpha (Ppargc1a), PR domain containing 16 (Prdm16) and cell death-inducing DNA fragmentation factor α-like effector A (Cidea) (Fig 3D) as well as the mitochondrial DNA relative content (Fig 3E).

Poplar propolis ethanolic extract supplementation induces the Nrf2 pathway in adipose tissue and adipocytes

PPEE contains several polyphenols considered as ligands of the natural nuclear factor (erythroid-derived 2)-like 2 (Nrf2) [30], and Nrf2 gonists are known to induce a similar metabolic phenotype [31] the activation of this signaling pathway was investigated. PPEE supplementation induced the gene expression of the glutamate-cysteine ligase catalytic subunit (Gclc), glutamate-cysteine ligase modifier subunit (Gclm), NADPH: quinone oxidoreductase 1 (Nqo1) and heme oxygenase 1 (Hmox1) in the adipose tissue of mice ($p<0.05$) (Fig 4A). No effect on Nrf2 gene expression was observed. PPEE (30 µg/mL) significantly ($p<0.05$) induced the expression of Nrf2, Gclc, Gclm and Nqo1 in 3T3-L1 cells (Fig 4B). The ability of PPEE to transactivate a Nrf2 response element (ARE) was confirmed using transient transfection. PPEE increased luciferase activity in 3T3-L1 cells transfected with the wildtype ARE (ARE wt), whereas no induction was observed with the mutated ARE (ARE mut) (Fig 4C, $p<0.05$). Furthermore, the increased ability

of Nrf2 to bind to its DNA binding domain in the presence of PPEE was confirmed in 3T3-L1 cells (TransAM Nrf2 assay; Fig 4D, $p<0.05$). The involvement of Nrf2 in the PPEE-mediated induction of Nrf2, Gclc, Gclm and Nqo1 was confirmed by RNA interference. This PPEE-mediated induction was blunted or abolished in3T3-L1 cells transfected with siRNA against Nrf2 compared to control siRNA (Fig. 4E, 4F, 4G and 4H, $p<0.05$).

Discussion

In the present study, we reported a beneficial role of PPEE supplementation on weight gain limitation and glucose homeostasis improvement in mice fed a HF diet. In addition, we provided evidences of a direct effect of poplar propolis supplementation on AT biology, strongly suspected to be related to Nfr2 signaling activation in adipocytes.

We observed that PPEE supplementation reduced weight gain in mice fed a HF diet. This seems to be mainly related to the limitation of fat mass accumulation as highlighted by a reduced adiposity index. It is noteworthy that these morphological changes were not caused by a reduced energy intake. Interestingly, both subcutaneous (inguinal) and visceral (epididymal and retroperitoneal) adipose pads masses were reduced by PPEE supplementation. The plasma levels of leptin, which is known to be correlated to fat mass, s [32] also decreased. Similar results have been previously observed with Brazilian propolis supplementation, which reduced adiposity and limited the development of perirenal and mesenteric fat depots in high fat-fed rats It was postulated that the anti-obesity effect of propolis correlated to PPARγ inhibition in AT, and reduced triglycerides absorption (after a fat loading test [12].. In mice subjected to a high fat diet, Brazilian propolis also limited body weight gain and visceral AT mass [14], or both epididymal and subcutaneous fat masses. No modification of energy expenditure was observed under propolis supplementation, but an increase of fecal lipids was observed, suggesting that Brazilian propolis reduced intestinal fat absorption [13] The molecular mechanism(s) underlying the effect of propolis on weight gain limitation remains elusive.

We studied the cellularity of adipocytes and found that PPEE supplementation could normalize the mean adipocyte area compared to control mice. To explain this effect, we quantified gene

expression linked to lipid metabolism. We observed that PPEE supplementation induced the expression of genes coding for proteins involved in fatty acid oxidative metabolism (Pparα, Cpt1, Mcad, Lcad). This suggests that PPEE drove a global fatty acid oxidative program. Previous studies have reported the induction of PPARα, a master regulator of fatty acid oxidation, in the liver of rats supplemented with Brazilian propolis [12]. We also observed a strong induction of genes linked to mitochondria biogenesis and function, such as Ucp1, Ppargc1α, Prdm16 and Cidea, and an increase in DNA mitochondrial content with PPEE supplementation. The stimulation of mitochondrial biogenesis and fatty acid oxidative capacity in white adipocytes, when linked to increased energy expenditure in such cells through increased energy uncoupling, *via* Ucp1 expression and/or waste (e.g., futile cycles), is a potential novel target for the control of obesity and related comorbidities [33], and could explain the PPEE-induced anti-obesity effect. We, and others, have already proposed a similar mechanism to explain the anti-obesity effect of other molecules,(e.g. *trans*-retinoic acid) [34].

In this study, we also observed a beneficial effect of PPEE on glucose homeostasis. Indeed, PPEE supplementation in our diet induced obesity (DIO) mice model improved fasted glycemia and insulinemia and the HOMA-IR index. An the glucose tolerance and sensitivity to insulin were also improved, but were probably overestimated since doses were not adjusted to total mass [35]. Such observations agree with a recent meta-analysis reporting that propolis administrated to T2DM patients decreased fasting blood glucose and Hb1Ac levels[11], and with several preclinical studies on the effect of propolis on glucose homeostasis. These studies showed that propolis partly restored glucose tolerance and insulin sensitivity in ob/ob mice [15]. The anti-diabetic effect of propolis has also been described in streptozotocin /high fat diet fed

rodents [16, 17, 36], where it was found to stop systemic and pancreatic β-cells inflammation and oxidative stress [17, 37].

AT plays a major role in the insulin resistance genesis in the context of obesity, notably through the establishment of a low-grade inflammatory status [3]. The effect of propolis on glucose homeostasis could be due, at least in part, to the impact of PPEE on the inflammation present in AT. When investigating the expression of genes linked to inflammation, we observed that a HF fed diet induced pro-inflammatory cytokines. This was reversed by PPEE supplementation, suggesting that PPEE reduced the inflammatory process in AT. It is presently unclear if such effect is a cell-autonomous effect in adipocytes or if it is related to the reduction of fat mass accumulation, which is associated with an indirect anti-inflammatory effect. This will require further investigations.

To further identify the molecular mechanism of action of PPEE we focused on Nrf2. Several compounds found in propolis have been demonstrated to induce the Nrf2 signaling pathway [30], including CAPE [38]. We observed that a large set of Nrf2-target genes were induced in white AT following PPEE supplementation, suggesting that it mediated a strong antioxidant effect in white AT. In addition, we demonstrated a cell-autonomous effect of PPEE supplementation in adipocytes in our in vitro studies. We stimulated the induction of the canonical Nrf2 target genes and demonstrated the specific involvement of Nrf2 using RNA interference. We demonstrated that PPEE transactivated a wild type ARE but not a mutated ARE, and that PPEE induced Nrf2 DNA-binding. This demonstrated that PPEE was able to activate the Nrf2 signaling pathway in adipocytes.

Nrf2 belongs to the Cap'n collar type transcription factor family (for review [39-41]). In the inactive state, Nrf2 is bound to the protein Keap 1, itself associated with the cytoskeleton, which

prevents Nrf2 accumulation in the nucleus. The dissociation of Nrf2 and Keap 1 enables nuclear translocation of Nrf2, which binds to ARE after heterodimerisation with the transcription factor Maf. This type of response element is present in many genes encoding antioxidant enzymes: Nqo1, Gclc, Gclm, Hmox-1 and phase II enzymes. In general, molecules capable of inducing Nrf2 will induce the antioxidant system and detoxification system for xenobiotics and other potentially deleterious molecules. The role of Nrf2 in the control of AT metabolism and adipogenesis has also been extensively studied but remains far from clear [42, 43]. Mice lacking Nrf2 have been found to display either a lean phenotype [44, 45] or no protection against obesity [46, 47]. Nevertheless, if the role of Nrf2 invalidation remains complex, the effect of Nrf2 agonists are clearer and more consensual. Treatment of mice with CDDO-IM fed a HF diet prevented body weight gain possibly through a Nrf2-dependant modulation of hepatic lipid metabolism [48]. Similarly, oltipraz, a well-known Nrf2 inducer [49], as well as glycyrrhizin [50]., blunted the HF-mediated obesity and insulin resistance in mice. Recently, glucoraphanin administration was also found to prevent obesity, insulin resistance and NAFLD in HF fed mice via the Nrf2 pathway [31]. The mechanisms by which Nrf2 activators restore energy homeostasis in DIO mice models remains elusive. Such beneficial effect of Nrf2 inducers could be related to an enhancement of mitochondrial biogenesis and function as reported in several cells types including cardiomyocytes [51] and hippocampus neurons [52]. In addition, Nrf2 activation is well-known to reduce oxidative stress and inflammation, which interfere with energy homeostasis and insulin sensitivity [53, 54]. Nrf2 activation could also improve metabolic disorders induced by a HF diet. Finally, the effect of Nrf2 activation could be related to the induction of lipid catabolism by PPEE in AT, since Nrf2 signaling has already been associated to an induction of lipid oxidation in the liver [55]. All these metabolic pathways are strongly

interconnected and involved in mediating the Nrf2 effects elicited by PPEE. Further work is required to elucidate their relative contribution.

In conclusion, our results indicate that EEPP protects against HF-mediated obesity by limiting weight gain and regulating glucose homeostasis This is likely to be achieved via interfering with the mitochondrial biogenesis,uncoupling activity, enhancing of lipid oxidation, and limitating oxidative stress and inflammation

Author contributions

Study design (NC, FT, JFL), data collection (FT, CC, JA, EP, JD, ES, LM, CL, LB, EK, ND), data interpretation (NC, FT, LM, SG, JFL), manuscript preparation (NC, JFL) and funds collection (NC JFL). All the authors have read and approved the final version of this manuscript.

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Conflict of interest statement

NC and CL are employed by Pollenergie. Other authors have nothing to disclose.

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Figure legends

Fig 1. Poplar propolis ethanolic extract limits weight gain associated with diet-induced obesity. A. Body weight evolution was quantified for each mouse (*n* =10 per group). **B**. Before sacrifice, animal weight was recorded. **C**. Energy intake was quantified by measuring food intake every two weeks for a period of 12 weeks. **D**. Absolute organ weights. **E**. Relative organ weights (absolute organ weight / body weight). **F.** Adiposity index was calculated by dividing the sum of adipose tissues weight by the body weight of animal. Values are presented as means ± SEM. Bars not sharing the same letter were significantly different in Tukey-Kramer post hoc test $p < 0,05$.

Fig 2. Poplar propolis ethanolic extract improves glucose homeostasis. A. Glucose levels during the oral glucose tolerance test (OGTT; n=10 mice per group). **B**. Area under the curve (AUC) for the glycemic response during OGTT. **C**. Glycemia evolution during the insulin tolerance test (ITT; n=10 mice per group). **D**. Area under the curve for the glycemic response during ITT. **E**. Fasted glycemia and insulinemia of mice (n=10 mice per group). **F**. HOMA-IR mean values. Values are presented as means ± SEM. Bars not sharing the same letter were significantly different in Tukey-Kramer post hoc test $p < 0,05$.

Fig 3. Poplar propolis ethanolic extract effect on adipocyte cellularity, gene expression and DNA mitochondrial content in adipose tissue. A. Representative histological images of epididymal fat pads H&E stained, taken at 10X magnification. Adipocyte area determined using Image J software. **B**. Relative expression of epididymal adipose tissue genes related to

inflammatory markers measured through qPCR and expressed relative to 18S ribosomal RNA. **C**. Relative expression of epididymal adipose tissue genes related to lipid metabolism measured through qPCR and expressed relative to 18S ribosomal RNA. **D**. Relative expression of epididymal adipose tissue genes related to mitochondrial metabolism measured through qPCR and expressed relative to 18S ribosomal RNA. **E**. Mitochondrial DNA quantification in epididymal adipose tissue. Mitochondrial DNA to nuclear DNA ratio was determined by qPCR. N=10 mice per each group, values are presented as means ± SEM. Bars not sharing the same letter were significantly different in Tukey-Kramer post hoc test $p < 0,05$.

Fig 4. Poplar propolis ethanolic extract induces Nrf2 pathway in adipose tissue and adipocytes. A. Relative expression of epididymal adipose tissue Nrf2-target genes coding for antioxidant enzymes measured through qPCR and expressed relative to 18S ribosomal RNA (n=10 mice per group). **B**. 3T3-L1 adipocytes were incubated with growing concentrations of PPEE for 24 h. Expression of Nrf2-target genes coding for antioxidant enzymes measured through qPCR and expressed relative to 18S ribosomal RNA. **C**. 3T3-L1 adipocytes were transiently transfected with plasmid containing wild-type or mutated antioxidant response element (ARE) and cells were incubated for 24 h with 30 µg/ml of PPEE. Luminescence was monitored and standardized. **D**. 3T3-L1 adipocytes were incubated with PPEE 30µg/ml) for 24 h. DNA binding activity was monitored using transAM Nrf2 assay. **E to H**. 3T3-L1 adipocytes were transiently transfected with non-targeted (NT) siRNA or siRNA directed against Nrf2 for 24h. cells were incubated for an additional 24h, and gene expression was quantified by qPCR and expressed relative to 18S ribosomal RNA. Values are presented as means ± SEM of 3

independent cultures per treatment condition. Bars not sharing the same letter were significantly different in Tukey-Kramer post hoc test $p < 0,05$.