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

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1 **Poplar propolis ethanolic extract reduces body weight gain and glucose metabolism**  
2 **disruption in high-fat diet-fed mice.**

3

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7

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13

14 Running title: Propolis improves obesity and associated disorders

15

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20

21 Keywords : adipose tissue, insulin resistance, NRF2, obesity, propolis.

22

23 **Abstract**

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

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

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

40

41

42

43 **Introduction**

44

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

61

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

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

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

75  
76 Preclinical studies have been performed in animal models of obesity and/or diabetes, to  
77 investigate the phenotypical impact of propolis supplementation in the diet [12-17]. The  
78 molecular mechanisms underlying the observed effects in terms of obesity management and  
79 improvement of glucose homeostasis, however, still remain unclear. Thus, the aim of the present  
80 study was to evaluate the effect of a standardized poplar propolis ethanolic extract (PPEE) on  
81 obesity glucose homeostasis and the physiology of the adipose tissue in high-fat diet-fed mice.  
82 The putative molecular mechanisms involved in these effects was also investigated.

83

84 **Materials and methods**

85

86 **Propolis source and extraction**

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

96

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

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

112

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

129

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

135

136 **Oral glucose and insulin tolerance tests** - Glucose and insulin tolerance tests were performed  
137 two weeks before animals were euthanized. For the glucose tolerance test, the mice were fasted  
138 for 16 h and then gavaged with glucose (2 g/kg). For the insulin tolerance test, the mice were  
139 fasted for 5 h and then injected *i.p.* with insulin (0.5 U/kg). Blood samples were taken from the  
140 tail tips at specific time intervals to measure glucose levels (Accu-Check glucometer, Roche).

141

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

151



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

157  
158 **Cell culture** - 3T3-L1 preadipocytes (ATCC, Manassas, VA) were seeded in 3.5-cm diameter  
159 dishes at a density of  $15 \times 10^4$  cells/well. Cells were grown in DMEM supplemented with 10%  
160 FBS at 37 °C in a 5% CO<sub>2</sub> humidified atmosphere as previously reported [23]. To induce  
161 differentiation, 2-day post-confluent 3T3-L1 preadipocytes (day 0) were stimulated for 48 h with  
162 0.5 mM isobutylmethylxanthine, 0.25  $\mu\text{M}$  dexamethasone, and 1  $\mu\text{g}/\text{mL}$  insulin in DMEM  
163 supplemented with 10 % FBS. The cells were maintained in DMEM supplemented with 10%  
164 FBS and 1  $\mu\text{g}/\text{mL}$  insulin. To examine the effect of PPEE, 3T3-L1 adipocytes received either  
165 PPEE (3 - 30  $\mu\text{g}/\text{mL}$ ) or ethanol only (0.1%). All treatments were performed on day eight. The  
166 data obtained are the mean of three independent experiments, each performed in triplicate.

167  
168 **RNA interference** - The 3T3-L1 differentiated cells were seeded in 6-well plates and transfected  
169 with either targeted siRNA (against Nrf2) or a non-targeting siRNA according to the  
170 manufacturer's instructions (Dharmacon, Inc. Lafayette, CO) and as previously reported [24, 25].  
171 Briefly, the cells were transfected overnight using a mixture of 100 nM siRNA and 2  $\mu\text{L}$  of  
172 DharmaFECT reagent per well. The media were subsequently replaced with fresh media with or  
173 without PPEE and the plates were re-incubated for 24 h.

174

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

185

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

189

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

197

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

202

203 **Results**

204

205 *Poplar propolis ethanolic extract supplementation protects against diet-induced obesity*

206 The results showed that PPEE supplementation (20 mg/mouse/day, equivalent to 4.5 mg of total  
207 polyphenols/mouse/day) significantly limited weight gain ( $p<0.0001$ ) caused by a HF diet (Fig  
208 1A and 1B). The energy intake was higher in the HF diet fed group compared to the control  
209 group, but no significant difference ( $p<0.002$ ) was observed in the PPEE supplemented group  
210 compared to the HF diet fed group (Fig 1C). The limitation of body weight gain in PPEE  
211 supplemented mice was associated with a reduction of absolute and relative fat mass in  
212 epididymal, retroperitoneal or inguinal fat pads (Fig 1D and 1E,  $p<0.05$ ). Consequently, the  
213 adiposity index (sum of epididymal, inguinal and retroperitoneal AT mass relative to total body  
214 mass) in PPEE supplemented mice was reduced, compared to HF diet fed mice (Fig 1F,  
215  $p<0.001$ ). Interestingly no significant difference of adiposity index was observed between PPEE  
216 supplemented mice and control fed mice after 12 weeks of supplementation (Fig 1F).  
217 Triglycerides, free fatty acids and alanine transaminase (ALAT) were neither affected by the HF  
218 diet nor the EEPP supplementation ( $p>0.05$ ). Adiponectin was not affected by PPEE  
219 supplementation compared to the HF diet fed group, but was significantly reduced compared to  
220 the control group ( $p<0.05$ ). The levels of leptin and  $\beta$ -hydroxybutyrate were increased in the HF  
221 diet fed group compared to the control group and were reduced in the PPEE supplemented group  
222 ( $p<0.05$ ) (Table 2).

223

224 *Poplar propolis ethanolic extract supplementation improves glucose homeostasis*

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

234

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

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

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

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

257

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

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

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

276

277

278 **Discussion**

279

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

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

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



301 expression linked to lipid metabolism. We observed that PPEE supplementation induced the  
302 expression of genes coding for proteins involved in fatty acid oxidative metabolism (Ppara,  
303 Cpt1, Mcad, Lcad). This suggests that PPEE drove a global fatty acid oxidative program.  
304 Previous studies have reported the induction of PPAR $\alpha$ , a master regulator of fatty acid  
305 oxidation, in the liver of rats supplemented with Brazilian propolis [12]. We also observed a  
306 strong induction of genes linked to mitochondria biogenesis and function, such as Ucp1,  
307 Ppargc1 $\alpha$ , Prdm16 and Cidea, and an increase in DNA mitochondrial content with PPEE  
308 supplementation. The stimulation of mitochondrial biogenesis and fatty acid oxidative capacity  
309 in white adipocytes, when linked to increased energy expenditure in such cells through increased  
310 energy uncoupling, *via* Ucp1 expression and/or waste (e.g., futile cycles), is a potential novel  
311 target for the control of obesity and related comorbidities [33], and could explain the PPEE-  
312 induced anti-obesity effect. We, and others, have already proposed a similar mechanism to  
313 explain the anti-obesity effect of other molecules,(e.g. *trans*-retinoic acid) [34].

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

323 rodents [16, 17, 36], where it was found to stop systemic and pancreatic  $\beta$ -cells inflammation  
324 and oxidative stress [17, 37].

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

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

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

346 prevents Nrf2 accumulation in the nucleus. The dissociation of Nrf2 and Keap 1 enables nuclear  
347 translocation of Nrf2, which binds to ARE after heterodimerisation with the transcription factor  
348 Maf. This type of response element is present in many genes encoding antioxidant enzymes:  
349 Nqo1, Gclc, Gclm, Hmox-1 and phase II enzymes. In general, molecules capable of inducing  
350 Nrf2 will induce the antioxidant system and detoxification system for xenobiotics and other  
351 potentially deleterious molecules. The role of Nrf2 in the control of AT metabolism and  
352 adipogenesis has also been extensively studied but remains far from clear [42, 43]. Mice lacking  
353 Nrf2 have been found to display either a lean phenotype [44, 45] or no protection against obesity  
354 [46, 47]. Nevertheless, if the role of Nrf2 invalidation remains complex, the effect of Nrf2  
355 agonists are clearer and more consensual. Treatment of mice with CDDO-IM fed a HF diet  
356 prevented body weight gain possibly through a Nrf2-dependant modulation of hepatic lipid  
357 metabolism [48]. Similarly, oltipraz, a well-known Nrf2 inducer [49], as well as glycyrrhizin  
358 [50]., blunted the HF-mediated obesity and insulin resistance in mice. Recently, glucoraphanin  
359 administration was also found to prevent obesity, insulin resistance and NAFLD in HF fed mice  
360 via the Nrf2 pathway [31]. The mechanisms by which Nrf2 activators restore energy homeostasis  
361 in DIO mice models remains elusive. Such beneficial effect of Nrf2 inducers could be related to  
362 an enhancement of mitochondrial biogenesis and function as reported in several cells types  
363 including cardiomyocytes [51] and hippocampus neurons [52]. In addition, Nrf2 activation is  
364 well-known to reduce oxidative stress and inflammation, which interfere with energy  
365 homeostasis and insulin sensitivity [53, 54]. Nrf2 activation could also improve metabolic  
366 disorders induced by a HF diet. Finally, the effect of Nrf2 activation could be related to the  
367 induction of lipid catabolism by PPEE in AT, since Nrf2 signaling has already been associated to  
368 an induction of lipid oxidation in the liver [55]. All these metabolic pathways are strongly

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

371

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

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379 **Author contributions**

380 Study design (NC, FT, JFL), data collection (FT, CC, JA, EP, JD, ES, LM, CL, LB, EK, ND), data  
381 interpretation (NC, FT, LM, SG, JFL), manuscript preparation (NC, JFL) and funds collection (NC JFL).

382 All the authors have read and approved the final version of this manuscript.

383

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389 **Conflict of interest statement**

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

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393 **References**

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548 **Figure legends**

549 **Fig 1. Poplar propolis ethanolic extract limits weight gain associated with diet-induced**  
550 **obesity.** **A.** Body weight evolution was quantified for each mouse ( $n = 10$  per group). **B.** Before  
551 sacrifice, animal weight was recorded. **C.** Energy intake was quantified by measuring food intake  
552 every two weeks for a period of 12 weeks. **D.** Absolute organ weights. **E.** Relative organ weights  
553 (absolute organ weight / body weight). **F.** Adiposity index was calculated by dividing the sum of  
554 adipose tissues weight by the body weight of animal. Values are presented as means  $\pm$  SEM.  
555 Bars not sharing the same letter were significantly different in Tukey-Kramer post hoc test  $p <$   
556  $0,05$ .

557  
558 **Fig 2. Poplar propolis ethanolic extract improves glucose homeostasis.** **A.** Glucose levels  
559 during the oral glucose tolerance test (OGTT;  $n = 10$  mice per group). **B.** Area under the curve  
560 (AUC) for the glycemetic response during OGTT. **C.** Glycemia evolution during the insulin  
561 tolerance test (ITT;  $n = 10$  mice per group). **D.** Area under the curve for the glycemetic response  
562 during ITT. **E.** Fasted glycemia and insulinemia of mice ( $n = 10$  mice per group). **F.** HOMA-IR  
563 mean values. Values are presented as means  $\pm$  SEM. Bars not sharing the same letter were  
564 significantly different in Tukey-Kramer post hoc test  $p < 0,05$ .

565  
566 **Fig 3. Poplar propolis ethanolic extract effect on adipocyte cellularity, gene expression and**  
567 **DNA mitochondrial content in adipose tissue.** **A.** Representative histological images of  
568 epididymal fat pads H&E stained, taken at 10X magnification. Adipocyte area determined using  
569 Image J software. **B.** Relative expression of epididymal adipose tissue genes related to

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

578

579 **Fig 4. Poplar propolis ethanolic extract induces Nrf2 pathway in adipose tissue and**  
580 **adipocytes.** **A.** Relative expression of epididymal adipose tissue Nrf2-target genes coding for  
581 antioxidant enzymes measured through qPCR and expressed relative to 18S ribosomal RNA  
582 (n=10 mice per group). **B.** 3T3-L1 adipocytes were incubated with growing concentrations of  
583 PPEE for 24 h. Expression of Nrf2-target genes coding for antioxidant enzymes measured  
584 through qPCR and expressed relative to 18S ribosomal RNA. **C.** 3T3-L1 adipocytes were  
585 transiently transfected with plasmid containing wild-type or mutated antioxidant response  
586 element (ARE) and cells were incubated for 24 h with 30  $\mu$ g/ml of PPEE. Luminescence was  
587 monitored and standardized. **D.** 3T3-L1 adipocytes were incubated with PPEE (30 $\mu$ g/ml) for 24  
588 h. DNA binding activity was monitored using transAM Nrf2 assay. **E to H.** 3T3-L1 adipocytes  
589 were transiently transfected with non-targeted (NT) siRNA or siRNA directed against Nrf2 for  
590 24h. cells were incubated for an additional 24h, and gene expression was quantified by qPCR  
591 and expressed relative to 18S ribosomal RNA. Values are presented as means  $\pm$  SEM of 3

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

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