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Treatment with *Parkinsonia aculeata* combats insulin resistance-induced oxidative stress through the increase in PPARγ/CuZn-SOD axis expression in diet-induced obesity mice

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Abstract *Parkinsonia aculeata* L. (Caesalpiniaceae) is a traditional ethnomedicine and has been used for the empiric treatment of hyperglycemia, without scientific background. Mechanistic analyses at molecular level from the antioxidant mechanism observed by *P. aculeata* are required. Herein the effects of the treatment by hydroethanolic extract partitioned with ethyl acetate of *P. aculeata* aerial parts (HEPa/EtOAc) in mice fed a high-fat diet that share many obesity phenotypes with humans were evaluated. The animals were treated orally with HEPa/EtOAc (125 and 250 mg/kg/day) and pioglitazone (5 mg/kg/day), for 16 days. After the treatment, HEPa/EtOAc reduced fasting serum glucose and insulin levels, as well as homeostasis model assessment for insulin resistance. In addition, an improvement in glucose intolerance was also observed. Indeed, a reduction in the circulating levels of TNF-α and IL-6 was also observed. Furthermore, at molecular level, it was demonstrated that the HEPa/EtOAc treatment was able to improve these physiological parameters, through the activation of peroxisome proliferator-activated receptor γ (PPARγ) *per si*, as well as the enhancement of antioxidant mechanism by an increase in PPARγ/Cu^{2+}, Zn^{2+}-superoxide dismutase (CuZn-SOD) axis expression in liver and adipose tissue. In sum, *P. aculeata* is effective to improve insulin resistance in a mouse model of obesity and this effect seems to involve the antioxidant and anti-inflammatory mechanisms through the increase in PPARγ/CuZn-SOD axis expression.

Keywords *Parkinsonia aculeata* · Fabaceae · Oxidative stress · Antioxidant · Obesity · Insulin resistance · PPARγ · CuZn-SOD

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Introduction

Obesity has been described as the biggest global health issue. Recently, the World Health Organization (WHO) statistics reports that up to 35 % of adults aged over 20 years are currently considered overweight (BMI >25 kg/m^2) and 11 % are obese (BMI >30 kg/m^2), meaning that almost 2.5 billion people are affected [1, 2]. In this scenario, the human, social, and economic consequences related to obesity are devastating. These diseases being responsible for dramatically increasing the risk for serious life-threatening diseases include insulin resistance (IR), dyslipidemia, type 2 diabetes mellitus (T2D), heart disease, and cancer [2].

It is well established that one of the main features of IR and dyslipidemia state is the deleterious capacity of these diseases to promote oxidative stress. This disturbance is the consequence of a reduction in the antioxidant systems, including superoxide dismutase (SOD), glutathione peroxidase (GSH), catalase, total antioxidant capacity, and/or an increase in the production of free radicals and reactive oxygen species (ROS) [3, 4]. Among some potential mechanisms against the IR-induced oxidative stress, we

can emphasize the role of peroxisome proliferator-activated receptor γ (PPARγ); this plays a critical role in glucose and lipid homeostasis [5] and is involved in the regulation of IR. In this way, PPARγ is associated with the expression of Cu^{2+}, Zn^{2+}-superoxide dismutase (CuZn-SOD) [6–8], which scavenges the ROS.

Parkinsonia aculeata L. (Caesalpiniaceae) is small spiny deciduous tree, native from tropical America. It is traditionally described to treat fever, malaria, rheumatism [9], and hyperglycemia [10, 11]. The use of natural antioxidants has been associated with reduced risks of diabetes, cancer, cardiovascular disease, and other illnesses associated with aging [12–14]. These natural phytochemicals act as free radical scavengers, reducing agents, quenchers of singlet oxygen molecule, and activators for antioxidative enzyme, so striking the oxidative damage [12–14]. Several studies have demonstrated, using different types of extract, the antioxidant effects of *P. aculeata* in different in vitro assays [13, 14]. Deeply in this issue, we investigated, through molecular methods, the effects of *P. aculeata* on PPARγ by itself, as well as on PPARγ/CuZn-SOD axis in diet-induced obesity mice.

Materials and methods

Plant material

Aerial parts of *P. aculeata* were collected from the Xingó region (Sergipe, Brazil) (the geographical coordinates given by GPS—latitude: −37.8396; longitude: −9.619; altitude: 121 m), during February 2012. The plant was identified by Professor H. P. Bautista (INCRA-BA) and a voucher specimen was deposited (n° 500) in the Xingó Herbarium (Canindé do São Francisco, Sergipe, Northeast region, Brazil).

Preparation of plant extract

Dehydrated and powdered *P. aculeata* aerial parts were macerated with EtOH:H_2O (1:1; v/v) at the ratio of 100 mL of ethanol to 10 g of dried plant. The suspension was submitted to mechanic agitation for two 24-h cycles at 23 °C, and subsequently at the end of each cycle the material was filtered and submitted to a Soxhlet apparatus. After complete removal of ethanol, the resulting extract was partitioned with ethyl acetate P.A. at a ratio of (1:1; v/v). The biphasic suspension was subjected to separation by decanting funnel and the polar phase was collected. Finally, the material was lyophilized and stored at −20 °C until use. The final yield of the hydroethanolic extract partitioned with ethyl acetate of *P. aculeata* aerial parts (HEPa/EtOAc) was 5.2/50 g of the dried powdered aerial parts. Based on three previous studies by our group [10, 11, 15], for pharmacological assays, a fresh dilution of lyophilized HEPa/EtOAc extract in vehicle (distilled water) was prepared on the day of treatment and administered by gavage at the doses of 125 and 250 mg/kg b.w. in a fixed volume of 0.2 mL.

Animals

Male C57BL/6 J mice (7 weeks old; obtained from the State University of Campinas Central Breeding Center) were housed under a 12/12 h light/dark cycle in a controlled environment (room temperature: 22 ± 3 °C, humidity: 55 ± 5 %) and also were randomly allocated into two dietary regimens of either the standard rodent chow diet Nuvilab CR-1 (Nuvital, Colombo, Paraná, Brazil) (8 % fat, 26 % protein, 54 % carbohydrate, as a percentage of total kcal) and water ad libitum or high-fat diet (HFD) (55 % fat, 16 % protein, 29 % carbohydrate, as a percentage of total kcal) and water ad libitum, for a period of 12 weeks. The composition details and preparation of HFD were the same as described previously [16, 17]. Food intake was determined by measuring the difference between the weights of the high-fat or chow diet given and their weights at the end of the 24-h period. All animal protocols of the experiment were approved by the animal care and use committee at the State University of Campinas and are in accordance with the guidelines for the care and use of laboratory animals.

Evaluation of the subchronic oral treatment

After 12 weeks under the two different diet regimens, the mice were then randomly divided into five groups, consisting of six animals each. The control group (CTL) and the diet-induced obesity group (DIO) received the vehicle, once daily, orally for 16 days. The other groups of obese mice received HEPa/EtOAc by gavage, once daily, at the doses of 125 mg/kg (PA125) or 250 mg/kg (PA250) for 16 days. Additionally, another group of obese mice was treated with pioglitazone (PIO), once daily, at the dose of 5 mg/kg, orally for 16 days, and was adopted as a positive treatment group. Pioglitazone hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO) and for oral administration it was suspended in a vehicle (distilled water). All the groups of mice except the CTL group were on a HFD throughout the period of treatment. The weight of epididymal fat pads was determined to provide a measure of adiposity. The fat pads were dissected from each animal according to defined anatomical landmarks.

Biochemical assays

At the end of the treatments, mice were fasted overnight, and the blood samples were withdrawn from the retrobulbar intraorbital capillary plexus. The serum was separated by centrifugation (2500×*g*, 5 min). Glucose values were measured from the tail venous blood with a glucose monitor (glucometer; Bayer Diagnostics, New York, NY). Readouts of IL-6, TNF-α, and fasting serum insulin level were determined by ELISA (Millipore, Bedford, MA). The homeostasis model of assessment (HOMA-IR) score, as a surrogate measurement of insulin resistance, was calculated as (fasting insulin (μU/mL) × fasting glucose (mmol/L)/22.5), to determine the degree of insulin resistance [18, 19].

Intraperitoneal glucose tolerance test (ipGTT)

An ipGTT was performed subsequently after a 6-h fasting. After collection of an unchallenged sample (time 0), a bolus of 1.0 g/kg body weight of glucose was administered into the peritoneal cavity, and the blood samples were collected from the tail vein at different time points up to 120 min to determine blood glucose levels, as previously described [20, 21].

Tissue extraction and immunoblotting

After an overnight fasting, the mice were anesthetized (anesthesia was ensured by the loss of pedal and corneal reflexes). The liver, gastrocnemius muscle, and epididymal adipose tissue were removed, minced coarsely, and homogenized immediately in extraction buffer, as previously described [20–22]. In direct immunoblot experiments, protein extracts were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted, as previously described [20–22], with anti-PPARγ and anti-Cu–Zn superoxide dismutase-1 (SOD-1). The homogeneity of gel loading was evaluated by blotting the membranes with antibody against β-actin.

Materials

Routine reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless specified elsewhere. Reagents for SDS-PAGE and immunoblotting were from Bio-Rad (Richmond, CA, USA). All antibodies were from Santa Cruz Technology (Santa Cruz, CA).

Statistical analysis

Data are displayed as mean ± standard error of the mean (SEM) of at least two independent experiments. The results of blots are presented as direct comparisons of bands or spots in autoradiographs and quantified by optical densitometry (UN SCAN IT gel®, Silk Scientific Inc., Orem, UT, USA). Multiple comparisons were tested by one-way ANOVA, followed by Tukey's post hoc test, with the significance level set at $P < 0.05$ using SPSS software (SPSS for Windows, version 16.0, Chicago, IL, USA).

Results

Effects of *P. aculeata* administration on physiological and metabolic parameters

Figure 1 shows the comparative data of animals fed on standard rodent diet (CTL), animals fed on high-fat diet nontreated (DIO), animals fed on high-fat diet and treated with HEPa/EtOAc at 125 mg/kg (PA125) or 250 mg/kg (PA250), and animals fed on high-fat diet and treated with pioglitazone (PIO) that was used as a positive control for insulin resistance treatment. Concerning body weight and epididymal fat weight, as expected, all animals fed on high-fat diet showed a significant increase when compared to the animals of CTL group; however, no significant differences were found among all groups fed on high-fat diet (Fig. 1a, b). In accordance, the results of food consumption showed that there is no significant difference among all groups subjected to high-fat feeding (Fig. 1c). By assessing fasting glycemic and insulin levels, we observed increased levels in DIO group compared to CTL group, and that both HEPa/EtOAc doses resulted in reductions very similar to the results observed in PIO group (Fig. 2a, b). We next calculated the HOMA index, and the results showed consistent insulin resistance in DIO animals compared to the CTL group, and both PA125 and PA250 groups displayed improvement in this index (Fig. 2c). And finally, after an ip glucose challenge during the GTT, DIO group was less efficient in clearing the glucose than CTL animals, and both HEPa/EtOAc doses were able to improve glucose tolerance, although not as effective as pioglitazone treatment (Fig. 2d). Taken together, these results point out that HEPa/EtOAc treatment may be an important alternative therapy for insulin resistance.

HEPA/EtOAc treatment reduces the circulating levels of pro-inflammatory cytokines (IL-6 and TNF-α)

Next, we decided to evaluate the effects of HEPa/EtOAc over inflammatory markers on blood by analyzing IL-6 and TNF-α. In this regard, as expected, high-fat diet resulted in higher levels of these two cytokines when compared to the CTL group (Fig. 3a, b). On the other hand, the results also

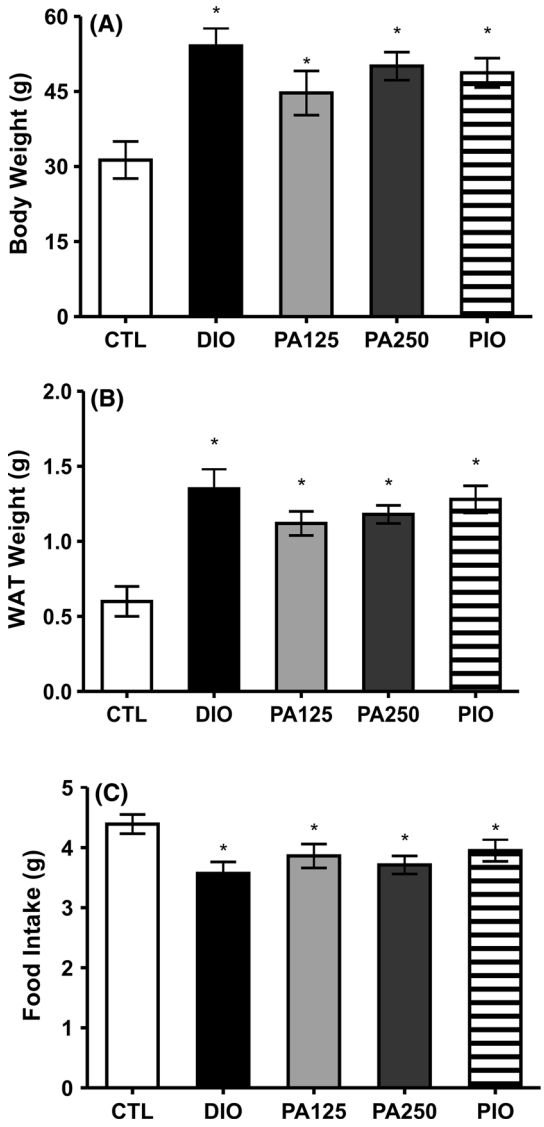

Fig. 1 Body weight (**a**), white adipose tissue (WAT) weights (**b**), and average daily food intake (**c**) in control mice, obese mice, and obese mice submitted to the administration of HEPa/EtOAc or Pioglitazone for 16 days. CTL and DIO groups received vehicle only. All groups except CTL group received the high-fat diet throughout the study. Data represent the mean ± SEM ($n = 6$). One-way ANOVA with Tukey's post hoc test. *$P < 0.05$ versus control

showed that both *P. aculeata* treatments, i.e., PA125 and PA250, were able to induce a significant attenuation in IL-6 and TNF-α levels in a similar way to that observed in PIO group (Fig. 3a, b).

P. aculeata treatment attenuates oxidative stress in high-fat diet-fed animals

After observing the positive effects of HEPa/EtOAc on the physiological parameters of insulin resistance, we decided to investigate its effects on proteins that attenuate the IR-induced oxidative stress, such as PPARγ and SOD. Regarding PPARγ, as expected, the results showed that DIO animals had a decrease in its expression in liver, adipose tissue, and muscle, when compared to CTL group (Fig. 4a–c). Conversely, the treatment of HEPa/EtOAc was able to improve PPARγ in liver and adipose tissue, but not in muscle when compared to the expression of DIO group (Fig. 4a–c). When analyzing SOD expression behavior, we observed a reduction in all studied tissues from DIO animals compared to CTL animals, and the *P. aculeata* treatment resulted in a significant increase in the SOD protein expression only in liver and adipose tissue (Fig. 4a, c). It is important to mention that the results of PA250 in both proteins were more impressive than those observed in the PA125 group, and we can also observe that they were similar to the results observed in the positive control group, i.e., PIO group, which highlighted a dose–response effect (Fig. 4a, c).

Discussion

Data presented by the World Health Organization (WHO) show that approximately 65–80 % of the population from developing countries relies on medicinal plants as a primary source of treatment [23]. Medicinal herbs contain diverse bioactive compounds and can have multiple actions against the deleterious effects of the obesity process [24, 25]. One of these actions that deserve special attentions is the improvement of antioxidant mechanism that is considered an important strategy in the pharmacological treatment for both obesity and T2D. Due to its relevance and widespread use, it is necessary to conduct further studies related to mechanistic analyses of these medicinal plants. In this regard, the present study demonstrates that *P. aculeata* is effective in improving insulin resistance in a mouse model of obesity and this effect seems to involve the antioxidant mechanisms through the increase in PPARγ/CuZn-SOD axis expression. In addition to these important effects, we can assume that *P. aculeata* is toxicologically safe, since a previous study has demonstrated that the oral administration of a maximum dose of 5 g/kg of HEPa/EtOAc did not result in any lethality or observable behavioral changes [10].

Certainly, to fully understand the mechanism of new antidiabetic agents, it is important to study the crucial molecules that could be therapeutic targets for the treatment of IR. Peroxisome proliferator-activated receptor gamma (PPARγ) is a transcription factor that belongs to a subfamily of nuclear hormone receptors; it is responsible for adipocyte differentiation, glucose uptake, and regulation of fatty acid metabolism [26, 27]. In the main insulin target tissues, PPARγ increases the insulin sensitivity,

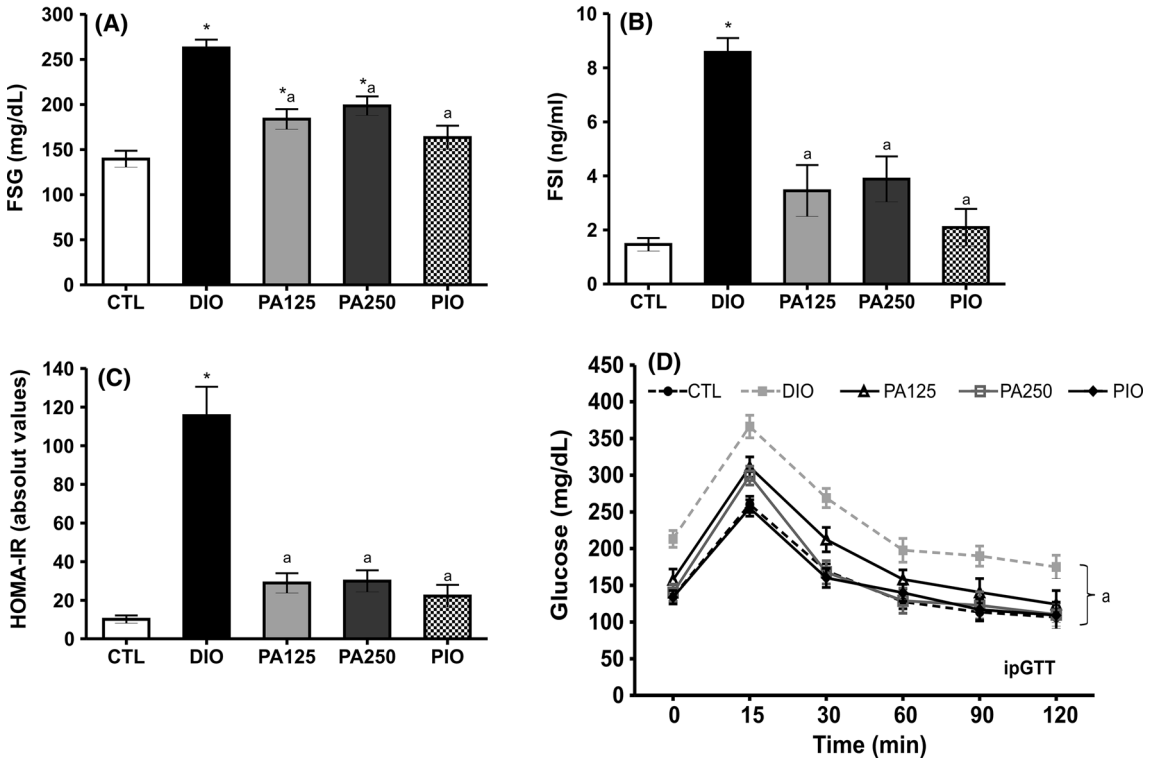

Fig. 2 Physiological and metabolic parameters in mice fed a high-fat diet (HFD) and treated for 16 days with daily oral doses of 125 and 250 mg/kg of HEPa/EtOAc. CTL and DIO groups received vehicle only. All groups except CTL group received the high-fat diet throughout the study. Fasting serum glucose (FSG) (**a**), fasting serum insulin (FSI) (**b**), HOMA-IR (**c**), and intraperitoneal glucose tolerance test (ipGTT) (**d**). The values represent the mean ± SEM ($n = 6$). One-way ANOVA with Tukey's post hoc test. $^{*}P < 0.05$ versus control; $^{a}P < 0.05$ versus DIO

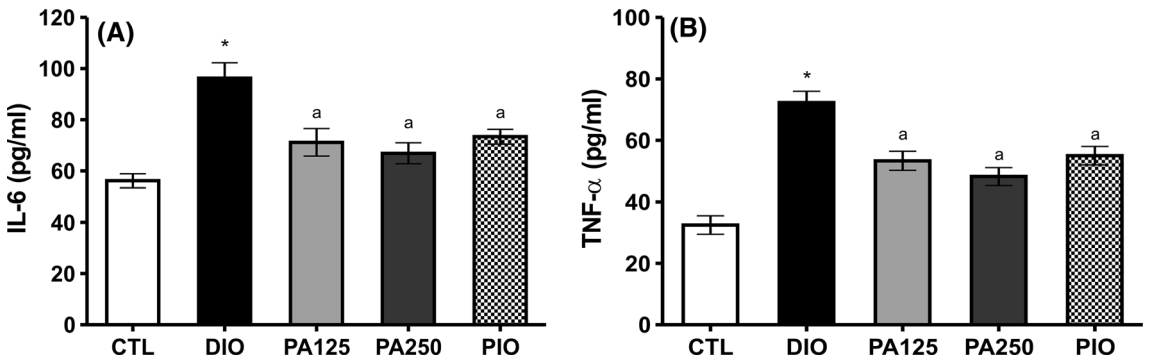

Fig. 3 Effects of treatments on circulating levels of IL-6 and TNF-α. Determination of serum IL-6 (**a**) and serum TNF-α (**b**) by ELISA. *Data* are presented as mean ± SEM of six mice per group. One-way ANOVA with Tukey's post hoc test. $^{*}P < 0.05$ versus control; $^{a}P < 0.05$ versus DIO

leading to the enhancement of IR background [26, 27]. Thus, PPARγ agonists, such as pioglitazone and rosiglitazone, have been widely used as insulin sensitizers in the diabetes treatment [28]. In the current studies, plant-derived compound extracts have been described to improve insulin sensitivity and promote adipocyte differentiation by activating PPARγ [29–31]. Herein, we demonstrated for the first time that HEPa/EtOAc treatment increased the expression of PPARγ in liver and with more intensity in adipose tissue. Since PPARγ is a key factor in adipogenesis and its physiological response to its agonists seems to derive mainly from its activation in adipose tissue [26, 27], we can justify this greater response to HEPa/EtOAc treatment in adipose tissue. In addition, it also deserves highlight that the dosage of 250 mg/kg from HEPa/EtOAc treatment presented similar results as pioglitazone treatment, thus reinforcing the surprisingly significant effect of HEPa/EtOAc extract.

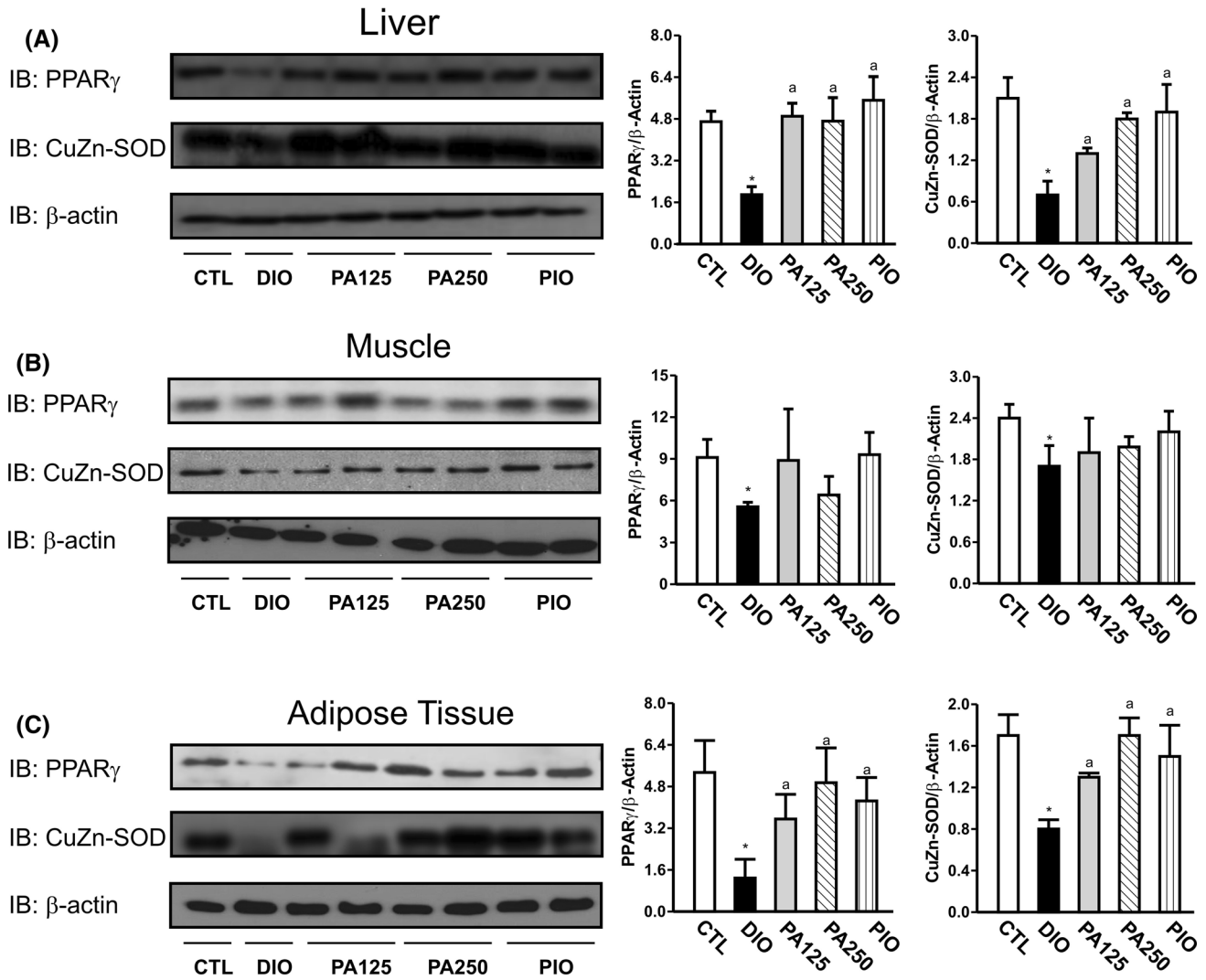

Fig. 4 Effects of HEPa/EtOAc administration on PPARγ and CuZn-SOD expression in high-fat diet-fed mice. Representative blottings show PPARγ and CuZn-SOD expression in liver (**a**), muscle (**b**), and adipose tissue (**c**) of all studied groups. Western blots were quantified after standardization with β-actin. Data were representative of two independent experiments. The values represent the mean ± SEM ($n = 6$). $^{*}P < 0.05$ versus control; $^{a}P < 0.05$ versus DIO. *IB* immunoblot

Taken together, from this effect observed by HEPa/EtOAc treatment on PPARγ activation, it is possible to create a linkage between the physiological effects observed in this study. In this way, it is justified the reduction in fasting blood glucose and insulin levels, as well as the improvement in glucose tolerance observed in carrying out the ipGTT test after the HEPa/EtOAc treatment. Indeed, the HOMA-IR, which is considered as surrogate measurement that provides an acceptable and reliable approximation of regular measurements of insulin resistance, has been commonly used in diabetes research for decades, and an increase of HOMA-IR is associated with worsening of IR [18, 19, 32]. Our experiment demonstrated that the HEPa/EtOAc treatment in obese mice significantly decreased the levels of HOMA-IR similar to the pioglitazone-treated rats, suggesting a relevant effect on the IR associated with the aforementioned results.

It is stated that persistent hyperglycemia results in increased production of ROS [33]. On the other hand, nonenzymatic and antioxidant enzymatic ROS scavengers, such as SOD and GSH, attenuate the ROS concentration. Following this reasoning, several studies demonstrated that chronic obesity depletes the activity of antioxidant enzymes over time [34, 35], contributing to the vicious cycle of IR. Emphasizing the SOD enzyme, it constitutes the main enzymatic mechanism for superoxide degradation, i.e., catalyzes the conversion of superoxide to H_2O_2. Featuring the SOD, it has been identified in three isoforms: the cytosolic, copper/zinc-containing SOD (Cu/Zn-SOD); the mitochondrial manganese SOD (MnSOD); and the

extracellular SOD (ecSOD) [36]. Due to its abundance and significance [36], we decided to study the Cu/Zn-SOD. In this regard, our experiments demonstrated that the expressions of Cu/Zn-SOD were lower in liver, muscle, and adipose tissue from obese mice than in control animals. After the treatment with HEPa/EtOAc, the Cu/Zn-SOD expression levels in liver and adipose tissue are increased in comparison with untreated obese mice, and also being then similar to that seen in the group treated with pioglitazone. In the same direction, other tools used in the prevention and combating of IR, such as exercise and metformin, also show the increase in SOD expression as one of the mechanisms of action [37–39]. Therefore, it was confirmed that HEPa/EtOAc treatment presents in the same line of action of the most important therapeutic tools in the fight against IR.

As mentioned above, SOD is one of the agents that combat the increased flux of ROS, thereby preventing the deleterious effects of obesity and IR. Although several studies have presented that the expression of SOD is also associated with PPARγ activation [6, 40, 41], few studies have addressed the probable association between the phenomena of oxidative stress and the PPARγ/SOD axis expression, as well as its therapeutic target. One study that deserves attention was performed by Matsumoto and colleagues (2007), which demonstrated the strong association between the PPARγ activation and increase in Cu/Zn-SOD expression after the treatment with pioglitazone in diabetic rats [41]. Then, as mentioned, the antioxidant effect related to PPARγ activation in obese and T2D mice is probably due to the altered expression of certain enzymes that participate in the production or elimination of ROS, such as SOD. Expanding this knowledge, we found that HEPa/EtOAc treatment at the same level of pioglitazone treatment selectively restored Cu/Zn-SOD in liver and adipose tissue of obese mice via an increase in PPARγ. In this regard, it emphasizes the importance of PPARγ/CuZn-SOD axis as a therapeutic target of *Parkinsonia aculeata*. Furthermore, it seems to be important to clarify and further develop the study of this axis that emerges as a possible target to combat the deleterious effects of IR.

It is well known that obesity state leads to an increased production of several inflammatory cytokines, which play a critical role in obesity-related inflammation and metabolic pathologies. TNF-α and IL-6 are potent pro-inflammatory cytokines that have deleterious effects on glucose transport, lipid metabolism, and insulin action [42, 43]. In this regard, several studies have reported that in obese individuals and animal models, the levels of TNF-α and IL-6 are persistently elevated [44–46]. In this paper, HEPa/EtOAc changed the expression of an antioxidant axis (PPARγ/CuZn-SOD). Whereas, PPAR-γ expression can control secreted factors such as hormones and inflammatory cytokines, therefore reducing inflammation and oxidative stress [27]; we observed that the HEPa/EtOAc treatment reduced the circulating levels of TNF-α and IL-6 from obese mice in the same manner as the pioglitazone treatment. For that reason, *P. aculeata* could be effective in modulating the inflammation.

Phytomedicine intervention for the prevention of metabolic disease is presently a major topic of interest in

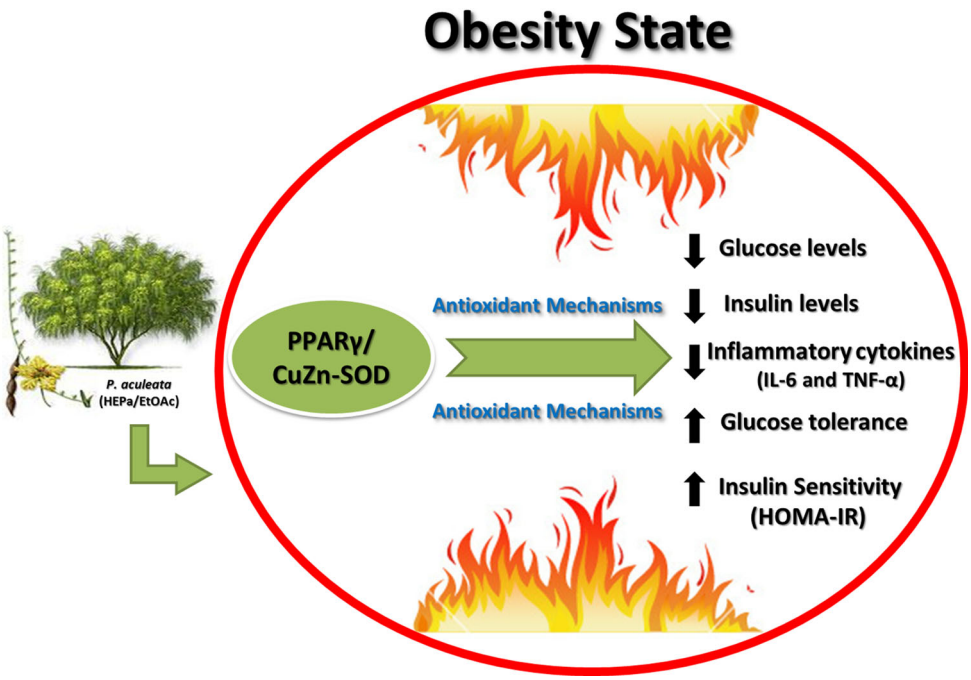

Fig. 5 HEPa/EtOAc effects in DIO mice. HEPa/EtOAc treatment was able to improve the physiological parameters from obese mice, through the activation of PPARγ *per si*, as well as the enhancement of antioxidant mechanism by an increase in PPARγ/CuZn-SOD axis expression in liver and adipose tissue

scientific community [24, 25, 31]. In this way, it has been demonstrated that compounds from plant sources can modulate nuclear receptors such as PPARs [29–31, 47]. Additionally, several studies have suggested that the large fraction of flavonoids could be responsible for PPARγ activation and consequently the cellular effects observed [47, 48]. Accordingly, the *P. aculeata* extract stands out because it has shown the presence of orientin, isoorientin, vitexin, and isovitexin (all glycosylated flavonoids) [49–51]. Furthermore, other studies through in vitro assays, such as DPPH, CUPRAC, reducing power assay, deoxyribose degradation (site and nonsite specific), ferric reducing antioxidant potential (FRAP), ferric thiocyanate (FTC), thiobarbituric acid (TBA), and molybdate ion reduction, have demonstrated the antioxidant effects from different extracts of *P. aculeata* [13, 14]. Thus, it is clear for us that these scientific findings endorse our results presented in this paper.

Evidently, well-designed analytics and standardized studies are critically important to ensure reproducible therapeutic effects of herbal medicines. Like this, mechanistic analyses at molecular level from phytomedicines are required. Thus, in sum, our study demonstrated at molecular level that the HEPa/EtOAc treatment was able to improve the physiological parameters from obese mice, through the activation of PPARγ *per si*, as well as the enhancement of antioxidant and anti-inflammatory mechanisms by an increase in PPARγ/CuZn-SOD axis expression in liver and adipose tissue (Fig. 5). Altogether, evidence gained by molecular insights into the action of HEPa/EtOAc suggests its therapeutic application against the deleterious effects of the obesity state.

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Compliance with ethical standards

Conflict of interest No potential conflicts of interest relevant to this article were reported.

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