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In vivo and in vitroevaluation of the Acrocomia aculeatapulp oil - lipid profile and oxidative stress

Ana Cristina Jacobowski[1]*, Ana Laura Colín-González[2], Ariana Lizbeth Paz-Loyola[2], Abel Santamaría[2], Maria LígiaRodrigues Macedo[1]

[1]Protein Purification Laboratory and their Biological Functions - Center for Biological and Health Sciences / Federal University of Mato Grosso do Sul, Campo Grande, MS, Brazil;

anacristinaj@gmail.com, bioplant@terra.com.br

[2]Laboratory of Excitatory Amino Acids- National Institute of Neurology and Neurosurgery; Mexico City, Mexico

laura_coling@yahoo.com.mx, aracne_hola32@hotmail.com, absada@yahoo.com, anacristinaj@gmail.com

ABSTRACT

Acrocomia aculeata is a plant species with high levels of antioxidant compounds, rich in carotenoids with high bioavailability and monounsaturated fatty acids. This study was designed to investigate the biological effect of Acrocomia aculeata pulp oil (AaPO) on body weight, epididymal adipose tissue mass, liver weight and blood analytes of high-fat-diet-induced obeseC57BL/6j mice.In addition, the antioxidant properties of AaPO were evaluated against the iron sulfate ($FeSO_4$)-induced oxidativedamage to rat brain synaptosomes. Obese mice receiving oral gavage with AaPO showed a significant decrease in body weight, adipose tissue weight,plasma triacylglycerol and aspartate aminotransferase. Histological analysis showed that AaPO attenuated high-fat-diet-induced liver injury decreasing hepatic steatosis and adipocyte hyperplasia. In addition, the in vitro assays showed that AaPOprevented the $FeSO_4$-induced oxidative damage in synaptosomal fractions. Results from this study suggest that AaPO can reduce body weight, plasma triacylglycerol, adipose tissue and oxidative stress.

Keywords: Acrocomia aculeata; Hypercaloric diet; Monounsaturated fatty acid; Oleic acid; Obesity; Oxidative stress; Brain synaptosomes.

Academic Discipline and sub-disciplines: Biochemistry; Nutrition.

SUBJECT CLASSI FICATION: Metabolism; Essential fat acids; Antioxidants.

TYPE (METHOD/APPROACH): Biological assays; Experimental dates; Literary analysis.

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1.INTRODUCTION

Acrocomia aculeata (Jacq.)Lodd.ex Mart., popularly known as Bocaiuva or Macaúba, is a perennial palm tree, widely distributed in the tropical and subtropical Americas. In Brazil is found in the Cerrado e Pantanal biomes, reaching Central America and the Mexican territory [1]. Acrocomia aculeata pulp oil (AaPO)has an intense orange color and it is characterized by the presence of fatty acids: 67% mono unsaturated fatty acid (MUFA), 25% saturated fatty acid (SFA) and 8% of poly unsaturated fatty acid (PUFA). Oleic acid (cis C18:1 ω9) is the predominant fatty acid in AaPO (60%) [2]. In addition, AaPO has a high content of carotenoids (300.01 ± 2.29 µg/g; [3], of which 80% are β-carotene[4].

Considering the absence of data related to the biological activity of this oil, the aim of this study was to investigate the biological its effectsthrough the following experiments: for in vitro investigations, we usedrat brain synaptosomal fractions to examine the influence of AaPOiron sulfate ($FeSO_4$)-induced lipid peroxidation and formation of reactive oxygen species (ROS); for in vivo investigations, we used high-fat-diet-induced obese mice to examine the influence of AaPO on body mass decrease, liver fat accumulation, epididymal adipose tissue weight, blood lipid level and blood hepatic enzymes.

2. MATERIAL AND METHODS

2.1 Assay to obtain AaPO

AaPO was obtained from fruits of A. aculeata collected in the urban area of Campo Grande, MS, Brazil (20º27'54.39''S 54º38'43.732''W) in December 2013.The species has been identified by the botany department of UFMS. The pulp oil content was obtained by direct extraction in Soxhlet apparatus (50 ºC) using petroleum ether (Merck, Rio de Janeiro, Brazil) [5].

2.2 Assays to estimate the antioxidants effects of AaPO in rat brain synaptosomal fractions

2.2.1 Achievement of synaptosomal fractions

Rats were handled according to the Guidelines for the Use of Animals in Neuroscience Research from the Society of Neuroscience, the local Ethical Committees and in compliance of the arrive guidelines. Six adult male Rattus norvegicus Wistar strain rats (250-300 g) were obtained from the Vivarium at the Universidad Nacional Autónoma de México. The isolation of synaptosomal P2 fractions (SF) from rat brains was carried out according to the method described by LoPachin[6] with modifications. The weighted tissues were gently homogenized in 10 volumes (g/mL) of sucrose (0.32 M) and centrifuged for 10 min at 1,073 x g (4 ºC). The supernatants were re-centrifuged for 15 min at 17,000 x g (4 ºC) and the recovered pellets (P2 fractions) were resuspended in 40 mL of HEPES-buffer (NaCl 0.1 M, NaH_2PO_4 0.001 M, $NaHCO_3$ 0.005 M, $CaCl_2$ 0.001 M, glucose 0.006 M and HEPES 0.01 M pH 7.4), constituting the SF.

2.2.2 Lipid peroxidation assay

The oxidative damage to lipids (lipid peroxidation) was quantified in SF as the formation of TBARS, according to a previous report [7]. Aliquots (200 µL) obtained from the homogenate synaptosomes were added to each of five tubes according to the following protocol: 1) only SF (control); 2) SF + ethanol 10% (AaPO diluent); 3) SF + AaPO 0.1%, incubated for 30 minutes; 4) SF + AaPO 0.1%, incubated for 30 minutes + $FeSO_4$ incubated for 30 minutes; 5) SF + $FeSO_4$, incubated for 30 minutes. The formation of TBARS (estimated as an index of malondialdehyde (MDA) production) was calculated by interpolation of values in a constructed standard curve of tetramethoxypropane. Data were calculated as nmoles of MDA per mg of protein and expressed as the percent of lipid peroxidation vs. control.

2.2.3 Measurement of reactive oxygen species formation

The formation of ROS was estimated according to a previous report [8]. Aliquots (200 µL) obtained from the SF were diluted in 9 volumes 40 mMTris plus HEPES buffer and added to each of five tubes according to the following protocol: 1) only SF (control); 2) SF + ethanol 10% (AaPO diluent); 3) SF + AaPO 0.1%, incubated for 30 minutes; 4) SF + AaPO 0.1%, incubated for 30 minutes + $FeSO_4$ incubated for 30 minutes; 5) SF +d $FeSO_4$, incubated for 30 minutes. Results were expressed as µmoles of dichlorofluorescein (DCF/g wet tissue).

2.3 Anti-obesity effect of AaPO on the diet-induced-obesity mice model

2.3.1 Preparation of hyperlipidic diet

The hyperlipidic diet (HD) was based in cafeteria diet from Estadella [9] and consisted of normal diet (ND, commercial rat chow Nuvilab CR-1®, Sogorb Inc., São Paulo – SP, Brazil), roasted peanuts, milk chocolate, and sweet biscuit in the proportion of 3:2:2:1, respectively. The nutritional composition of these diets is shown in Table 1.

Table 1 - Nutritional composition of experimental diets.

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Composition	Normal Diet (ND)	Hyperlipidic Diet (HD)
Lipids (%)	3.8	20.0
Proteins (%)	21.0	20.0
Carbohydrates (%)	36.9	48.0
Calorie (kcal/100 g diet)	265.8	452

2.3.2 Animals and experimental design

Twenty-four male C57BL/6j mice (3 weeks old, weighing 8-9 g) were obtained from Laboratório Central Animal (UFMS) and housed (six per cage) in a temperature and humidity controlled room with a 12 h artificial light-dark cycle. The protocol used for this research was employed according to the guidelines of the Brazilian College for Animal Experimentation and received approval from the Ethical Committee for Animals Research of Federal University of Mato Grosso do Sul (UFMS), Campo Grande, MS, Brazil (License number 353/2011).

- Obesity period: The mice were randomly divided in the control group (n = 12, receiving normal diet), and the obese group (n = 12, receiving hyperlipidic diet to create diet-induced obesity mice). The obesity period was 16 weeks.

- Supplementation period: After the obesity period, the groups received daily oral gavage as follows: Control group was divided in two subgroups: CGW (n = 6, receiving normal diet + 2 µL/g water) and CGO (n = 6, receiving normal diet + 2 µL/g AaPO). In turn, the obese group was divided in two subgroups: OGW (n = 6, receiving hyperlipidic diet + 2 µL/g water) and OGO (n = 6, receiving hyperlipidic diet + 2 µL/g AaPO). The gavage period was eight weeks.

All animals were allowed free access to normal diet or hyperlipidic diet and water. Food intake was monitored daily while the body weight (BW) was measured once a week throughout the study. Obesity was diagnosed in the end of obesity period by Lee index, which was calculated dividing the cube root of body weight in grams by naso-anal length in centimeter and multiplying by ten [10].

2.3.3 Oral glucose tolerance test (OGTT)

Glucose solution (1 g/Kg body weight; 20% glucose monohydrate, Merck, São Paulo, Brazil) was orally given to the mice. Blood samples were taken from the tail of mice before glucose load at time zero and at 15, 30, 60 and 120 min thereafter. Blood glucose concentrations were determined using an automated apparatus for determining blood glucose (Accu-Chek glucometer Roche Diagnostic, Brazil).

2.3.4 Intraperitoneal insulin tolerance test (IpITT)

Blood glucose concentrations were determined using an automated apparatus for determining blood glucose (Accu-Chek glucometer Roche Diagnostic, Brazil) on a blood sample obtained using tail vein (zero time). Insulin solution (Insunorm® Reg, Cellofarm, Rio de Janeiro, Brazil) was then administered intraperitoneally (1 U/Kg body weight). Glucose levels were then assessed in the course of 15, 30, 60 and 120 minutes after insulin injection.

2.3.5 Biochemical evaluation of serum

At the end of experimental test, the blood samples were collected in tubes without anticoagulant and centrifuged at 3,000 x g at 4 ºC for 10 min to isolate the serum for analysis. The epididymal adipose tissue and liver were excised immediately, rinsed with PBS (phosphate-buffered saline) and then weighted. The samples were stored in 10 % phosphate-buffered formaldehyde for histological evaluation. Fasting glucose, total cholesterol, high density lipoprotein and triacylglycerol, were determined using enzymatic colorimetric kits (Labtest, MG, Brazil). Aspartate aminotransferase and alanine aminotransferase were determined using kinetic kits (Analisa, MG, Brazil).

2.3.6 Histological evaluation of adipocytes and liver

Hydrated 5.0 µm sections of paraformaldehyde-fixed, paraffin-embedded liver and epididymal specimens were stained with hematoxylin-eosin to be histologically evaluated in original magnification of 200x.

3. RESULTS and DISCUSSIONS

3.1 Antioxidants effects of AaPO

AaPO did not cause any damage to synaptosomes (compared to control; Figure 1). In contrast, AaPO attenuated the effect of $FeSO_4$1 mM(22 %), when incubated at 0.1% when compared to $FeSO_4$1 mMalone. The quantification of TBARS showed that AaPO 0.1% has a partially protective effect against lipid peroxidation induced by $FeSO_4$.

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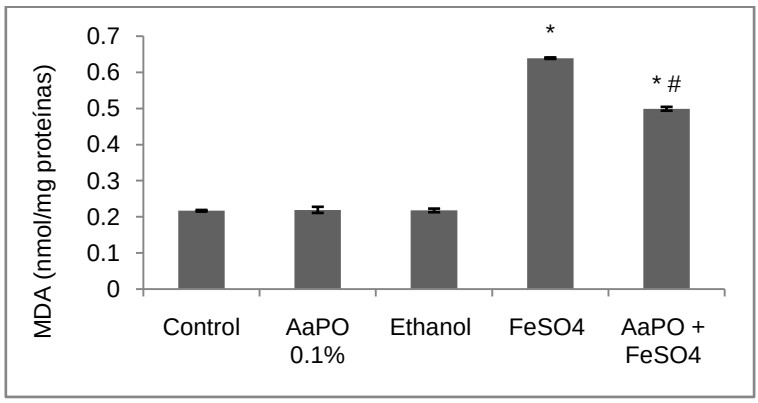


Figure 1 - Effects of AaPO 0.1% on the formation of thiobarbituric acid reactive substances (TBARS) in synaptosomes from rat brain. Data are expressed as mean ± SD (n = 6). One-way analysis of variance was performed (ANOVA) followed by post hoc Tukey's test to determine statistical differences between groups. * P < 0.001 compared control; # P < 0.001 compared $FeSO_4$.

AaPO not only prevented the formation of ROS induced by $FeSO_4$ (51% following treatment with $FeSO_4$ 1 mM; P < 0.001), but also led to values of ROS levels below baseline (43% lower than control, P< 0.001) (Figure 2). These results demonstrate the protective effect of AaPO to avoid the formation of oxidative stress in brain synaptosomes when exposed to oxidative insults like $FeSO_4$ 1 mM.It is known that the micronutrient β carotene has important antioxidant actions and influence chelating oxygen in oxidative reactions, protecting cell membranes from the toxic action of oxidizing agents [11]. Thus, it is possible to suggest that the presence of this micronutrient in AaPO may contribute to our results, since it is vital in eliminating free radicals and preventing the degeneration of oxidative stress scenario.

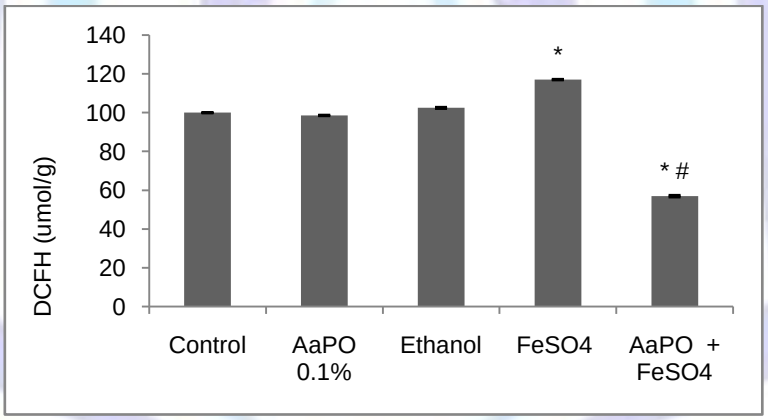


Figure 2 -Effects of AaPO on the formation of reactive oxygen species (ROS) in synaptosomes from rat brain. Data are expressed as mean ± SD (n = 6). One-way analysis of variance was performed (ANOVA) followed by post hoc Tukey's test to determine statistical differences between groups. * P < 0.001 compared control; # P < 0.001 compared $FeSO_4$.

3.2 Anti-obesity effect of AaPO on the diet-induced-obesity mice model

Diets rich in oleic acid have a beneficial effect on the regulation of lipid metabolism and the homeostasis of body weight [12].

The groups had the same food intake (g/day) (fed with normal diet vs the fed with hyperlipidic diet), indicating normofagia between them. Our results differ from other reports, such as the one of Nascimento[13], in which the group fed a high fat diet had a lower food intake than the group fed a standard diet, presumably due to the higher caloric intake of the fat diet.

At the end of the obesity period, intake of HD resulted in an important increase in body weight of the obese group, visibly larger than control group (P < 0.0001), confirmed by Lee index (Table 2).At the end of the supplementation period, the final body weight was significantly lower (P < 0.001) in control group oil (CGO) and obesity group oil (OGO) (Table 2) than that of control group water (CGW) and obesity group water (OGW). The supplementation of AaPO significantly suppressed the body weight gain. Considering that the CGW had a normal physiological increase in their weight during this period, weight loss demonstrated by CGO was readily significant (lost 2.5 ± 0.3% of their initial weight). Among obese groups, there was also significant weight gain (P < 0.001) in the supplementation period: OGO increased 9.05 ± 1.0% and OGW increased 24.35 ± 2.1%, compared to its initial weight. It is likely that the AGS fat diet acted directly on the hypothalamus, inducing inflammation and causing stress in the endoplasmic reticulum, leading to hypothalamic

dysfunction. This local effect is responsible for the loss in the fine control of food intake, satiety and energy expenditure, resulting in increased adiposity and metabolic disarray [14].

Table 2 - Initial and final body weight (BW), weight gain and Lee index in the Obesity and Supplementation periods and relative liver and epididymal adipose tissue (EAT) in the Supplementation period in C57BL/6j mice.

	Parameters	Control Group	Obesity Group		
Obesity Period	Initial BW (g)	9 ± 0.10	8.5 ± 0.50$^{£}$		
	Final BW (g)	32.06 ± 0.87	38.6 ± 0.56$^{£}$		
	Weight gain (g)	23.1 ± 0.67	30.10 ± 0.50$^{£}$		
	Lee Index	3.96 ± 0.05	4.22 ± 0.04$^{£}$		
		CGW	CGO	OGW	OGO
Supplementation Period	Initial BW (g)	32.63 ± 0.64	31.09 ± 0.30	38.87 ± 0.55***	38.4 ± 0.46***
	Final BW (g)	34.96 ± 0.39	31.01 ± 0.37*	48.2 ± 0.51*	41.9 ± 1.05$^{*\#}$
	Weight gain (g)	2.1 ± 0.64	-0.8 ± 0.4*	9.04 ± 0.49*	3.08 ± 1.21$^{\#}$
	Lee Index	3.63 ± 0.03	3.49 ± 0.04*	4.04 ± 0.04*	3.86 ± 0.06$^{*\#}$
	Relative liver weight (%)	3.19 ± 0.18	3.57 ± 0.31	3.86 ± 0.17**	3.49 ± 0.22
	Relative EAT weight (%)	2.80 ± 0.48	0.94 ± 0.39*	5.66 ± 0.51*	4.49 ± 0.31$^{*\#\#}$

Values shown are mean ± SD (n = 6); Statistical significance (P) was estimated using a two-tailed Student t test for the Obesity period. Analysis of variance (ANOVA) followed by post hoc Tukey's test were used to determine the statistically significance among the groups; $^{£}$ P < 0.001compared Control Group; * P < 0.001compared CGW; ** P < 0.01compared CGW; *** P < 0.05 compared CGW $^{\#}$ P < 0.001compared OGW; $^{\#\#}$ P < 0.01compared OGW. Lee Index = cube root of body weight (g) by naso-anal length (cm) and multiplying thousand; CGW, normal diet + oral daily gavage with water; CGO, normal diet + oral daily gavage with AaPO; OGW, hyperlipidic diet + oral daily gavage with water, OGO, hyperlipidic diet + oral daily gavage with AaPO. AaPO, Acrocomia aculeata pulp oil.Control Group Water, CGW; Control Group Oil, CGO; Obesity Group Water, OGW; Obesity Group Oil, OGO; epididymal adipose tissue, EAT.

As shown in Table 2, the relative liver weight in OGW was significantly higher (P < 0.01) than in CGW. The supplemented groups had lower relative weight of epididymal adipose tissue (EAT) compared to unsupplemented groups. CGO presented 66.43 ± 5.4% less EAT weight than CGW, and OGO 20.7 ± 1.4% less than OGW. Among obese groups there were significant difference (P < 0.01), suggesting that AaPO supplementation in OGO decreased the concentration of epididymal fat. MUFA have been shown to possess anti-inflammatory effects, acting directly on the hypothalamus [15] affecting the balance between decreasing satiety and energy expenditure, therefore suggesting that this was the mechanism leading to weight reduction in the supplemented groups with AaPO. In humans, MUFA intake is associated with low abdominal fat [16], confirming our results since the epididymal fat was noticeably reduced in mice supplemented with AaPO.Table 3 shows the effect of AaPO supplementation on the serum parameters including glucose (GLU), total cholesterol (TC), high density lipoprotein (HDL), triacylglycerol (TAG), aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

Table 3 - Effect of AaPO on the serum biochemical parameters in C57BL/6j mice fed with normal diet and hyperlipidic diet supplemented with AaPO or water (8 weeks).

Parameter	CGW	CGO	OGW	OGO
GLU (mg/dL)	131.4 ± 6.35	150.0 ± 16.67	494.4 ± 33.5*	453 ± 50.14*
TC (mg/dL)	90.6 ± 1.67	84.6 ± 6.19	199.6 ± 2.89*	176.5 ± 58.72*
HDL (mg/dL)	86.8 ± 3.85	84.6 ± 3.36	132.6 ± 8.17*	142.2 ± 7.79*
TAG (mg/dL)	126.6 ± 6.39	29.2 ± 5.07*	174.4 ± 9.82*	90 ± 8.31*
AST (U/L)	239.2 ± 7	229.2 ± 6	339.0 ± 4.1*	345.0 ± 6.9*
ALT (U/L)	45.1 ± 2.5	39.3 ± 2.0$^{\$}$	69.9 ± 3.5*	55.9 ± 2.0$^{*\#}$

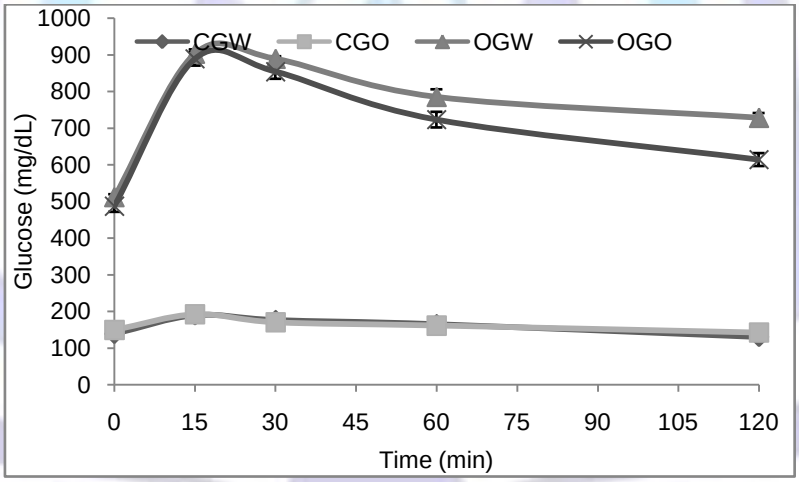
Data are expressed as means ± standard deviation (n = 6). Analysis of variance (ANOVA) followed by post hoc Tukey's test were used to determine the statistically significance among the groups; * P < 0.01compared CGW; $^{\#}$ P < 0.01compared OGW; $^{\$}$ P < 0.05compared CGW. AaPO, Acrocomia aculeata pulp oil; CGW, normal diet + oral daily gavage with water; CGO, normal diet + oral daily gavage with AaPO; OGW, hyperlipidic diet + oral daily gavage with water, OGO, hyperlipidic diet + oral daily gavage with AaPO; GLU, glucose; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; TAG, triacylglycerol; AST, aspartate aminotransferase; ALT, alanine aminotransferase. AaPO,

Acrocomia aculeata pulp oil.Control Group Water, CGW; Control Group Oil, CGO; Obesity Group Water, OGW; Obesity Group Oil, OGO.

The fasting blood glucose in the obesity groups was significantly higher than those in control groups.Our results demonstrated that AaPO was not effective in modifying the levels of TC and HDL-C altered by obesity in OGW and OGO groups. Binkoski et al. [17] demonstrated that PUFA in the diet has a major effect as hypocholesterolemic compared with a diet rich in MUFA. Covas et al. [18] and Urpi-Sarda et al. [19] found that the hypocholesterolemic effect of extra virgin olive oil is due to the polyphenols present in olive oil, not the oleic acid.

Our results also show that TAG is high in OGW, according to the above dyslipidemia and obesity in this group. However, AaPO was able to reduce the levels of triacylglycerols both in CGO as in OGO. These data confirm the results of the work of Kris-Etherton et al. [20] who showed a reduction in TAG levels in patients that replaced SFA by a carbohydrate MUFA enriched diet. Roche et al. [21] suggested that a diet rich in MUFA alters the metabolism of TAG lowering blood levels. This mechanism was clarified by Zheng et al. [22], who showed that the intake of a diet rich in MUFA actives the catabolism of TAG-rich lipoproteins in a process involving apolipoprotein E (Apo-E) and III-C (apo C-III). Thus, decreasing circulating TAG levels considerably lowers their blood concentration. As our results showed very significant decreases of the TAG levels in both groups supplemented with AaPO, it can be hypothesized that oleic acid is catabolized via synthesis of these TAG enriched lipoproteins, then reducing the concentration of triacylglycerol in serum-supplemented groups.

To quantify glucose tolerance, we calculated the OGTT area under curve (AUC) for all groups (Figure 3 C). The average AUC value was higher for OGW and OGO, indicating the deterioration of glucose tolerance. In the IpITT AUC, insulin sensitivity was markedly worsened in OGW and OGO than in CGW and CGO, indicating intolerance to insulin. The obesity mechanisms of the OGO and OGW groups could be related with the development of diabetes mellitus II, which is confirmed by the presence of insulin resistance and glucose intolerance. Daily supplementation with 2 uL/g body weight AaPO for eight weeks was not enough to reverse this situation, not improving impaired insulin. Similar to our results, Gillam et al. (2009) [23] found no effect of fish oil 10% (rich in ω-3) added to the diet in obese Zucker rats for 9 weeks compared to insulin resistance, glucose and lipid metabolism. Rather, Pocai et al. (2006) [24] found that obesity and insulin resistance induced in mice receiving a supersaturated diet, may be reversed by the use of MUFA (dose response), presumably through the restoration of the hypothalamic sensitivity by inhibition of fatty acid oxidation, feeding behavior and normalizing glucose homeostasis. Hence, our hypothesis for future studies is that higher doses of supplementation with AaPO are likely to reverse this insulin resistance profile, leading to glucose homeostasis.



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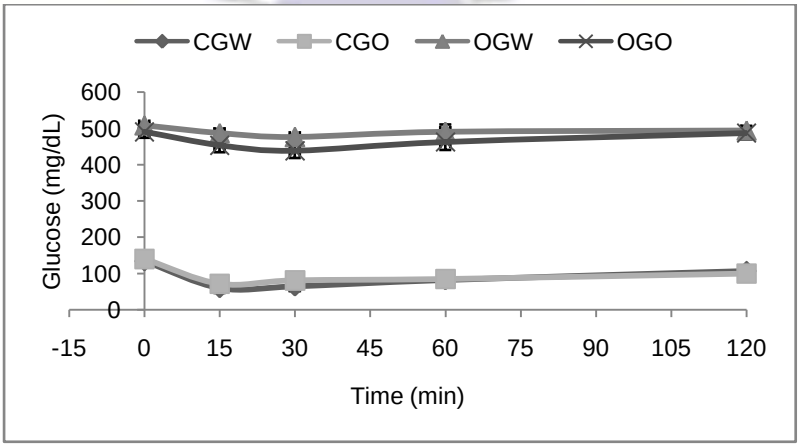

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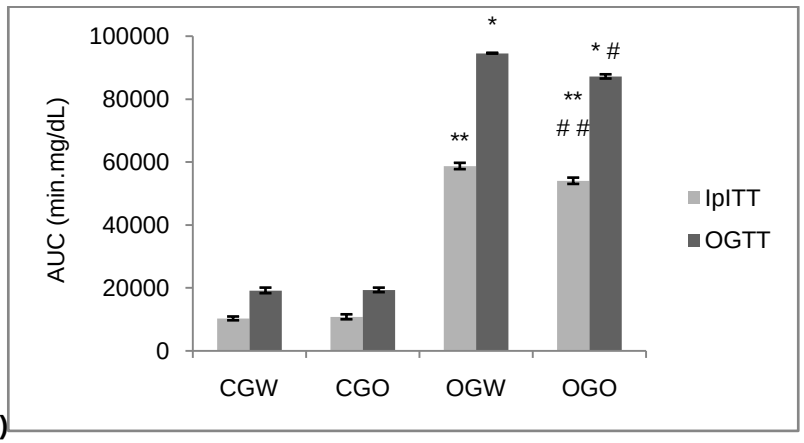

(C)

Figure 3 - Effects of AaPO on blood glucose and insulin tolerance. (**A**) OGTT; (**B**) IpITT; (**C**) OGTT and IpITT AUC, calculated according to OGTT and IpITT respectively. Data are expressed as mean ± SD (n = 6). One-way analysis of variance was performed (ANOVA) followed by post hoc Tukey's test to determine statistical differences between groups. For OGTT, * $P < 0.001$ compared CGW; $^{\#}$ $P < 0.01$ compared OGW; for IpITT, ** $P < 0.001$ compared CGW; $^{\#\#}$ $P < 0.01$ compared OGW.Control Group Water, CGW; Control Group Oil, CGO; Obesity Group Water, OGW; Obesity Group Oil, OGO.

Obesity is a risk factor for developing non-alcoholic fatty liver disease (NAFLD) with a spectrum of liver injury (steatosis, hepatitis, cirrhosis and cancer) (Chiang et al., 2011 [25]). ALT resulting from obese groups demonstrates that obesity-induced DH was efficient in causing liver damage manifested in increased serum ALT and vacuoles in hepatocytes. This change was significantly decreased after supplementation with AaPO (OGO), suggesting that AaPO ameliorated NAFLD, probably due to the presence of antioxidants in the AaPO reducing liver fat. This observation was confirmed by the decrease in relative liver weight in OGO.

3.3 Morphological changes

The size of adipocytes was larger in obese groups than in control groups, but OGO showed reduced adipocyte hypertrophy. These observations suggested that AaPO supplementation suppressed fat accumulation in adipose tissues. The liver sections from mice of OGW and OGO groups exhibited visible intracellular vacuolization with microvesicular fat deposits, although the hepatic steatosis was significantly alleviated by the treatment with AaPO.

Significant differences were observed for hepatic cells and adipocyte size in the supplemented groups compared with OGW. These results suggest that AaPO has antiobesity effects by influencing visceral fat mass and adipocyte size rather than influencing the body mass increase. Our histological examination suggested that adipocyte hypertrophy induced by HD is reversed after treated with AaPO for 8 weeks.

4. CONCLUSIONS

This study constitutes a key report establishing a protective role of AaPO in different toxic paradigms, thus emphasizing its potential for the design of therapeutic strategies for different human disorders.

Acknowledgments

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Author' biography with Photo

Ana Cristina Jacobowski is pharmaceutical biochemistry formed by UniversidadeFedral do Paraná in 1989, a master's degree in Biotechnology (UniversidadeCatólica Dom Bosco) and doctorate in Health (Universidade Federal de Mato Grosso do Sul). Currently a post-doc stage in Biochemistry and Molecular Biology.works with animal models of scientific research and plant peptides with antibiotic potential.