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Consumption of *Ocimum sanctum* L. and *Citrus paradisi* infusion modulate lipid metabolism and insulin resistance in obese rats

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ABSTRACT

High saturated fat and fructose diet leads to metabolic disorders through dysregulation of genes involved in lipid metabolism. Consumption of plant infusions reduces these obesity alterations, but the precise mechanism remains unclear. In this study, we investigated the effect and the possible mechanism of *Ocimum sanctum* L. (OS) and *Citrus paradisi* (CP) infusions in diet-induced obese rats. CP and OS infusions suppressed hepatic tissue fat accumulation, and significantly down-regulated the mRNA levels of two hepatic lipogenesis genes: sterol regulatory element binding protein 1c (SREBP1c) and fatty acid synthase (FAS) compared with obese control. Treatment with these infusions up-regulated the hepatic expression of mRNA related with mitochondrial fatty acid uptake: peroxisome proliferator activated receptor alpha (PPARα) and the expression of carnitine palmitoyl-transferase 1a (CPT1a). Both infusions improved insulin resistance, OS showing the major effect. Consumption of these infusions reduces the damage caused by free radicals, protecting hepatic lipid and proteins. Additionally, plant infusions increase activity of hepatic enzymes: glutathione S-transferase (GST), glutathione peroxidase (GPX), and catalase (CAT). Our results suggest that the effects of CP and OS infusions on lipid metabolism are related to the down-regulation of genes involved in lipogenesis, particularly for OS, and with the increase in lipid β-oxidation, especially for CP infusion. In conclusion, the consumption of these plant infusions is a feasible adjuvant therapy for metabolic changes induced by obesity.

Keywords: *Hibiscus sabdariffa*; *Ocimum sanctum* L.; *Citrus paradisi*; Obesity, lipid metabolism, insulin resistance.

Abbreviation list: *Hibiscus sabdariffa* (HS),*Citrus paradisi* (CP), *Ocimum sanctum* L. (OS) High-saturated-fat and fructose diet (HFFD), Triglycerides (TG), Free fatty acids (FFA), hepatic insulin resistance (HIR).

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1 **1. Introduction**

2 Obesity is the most prevalent metabolic disease in the western world which is
3 related to high consumption of calories from high saturated fat food and caloric
4 beverages, especially those sweetened with fructose.[1] Excess of dietary
5 carbohydrates are transformed in triglycerides (TG) in the liver and subsequently
6 stored in white adipose tissue. A flaw in free fatty acid (FFA) oxidation or TG
7 exportation leads to an increase in the lipid stored in hepatocytes in form of TG
8 condition that eventually produce steatosis.[2]

9 Previous studies demonstrated that the development of steatosis is associated with
10 an increase of lipogenesis, mediated by an increase in the activity of the
11 transcription factor: sterol regulatory element–binding protein-1c (SREBP-1c).[3]
12 SREBP-1c regulates genes required for lipogenesis such as fatty acid synthase
13 (FAS).[4] Another factor associated to steatosis is the decrease oxidation of the lipids
14 into the mitochondria. β-oxidation is regulated mainly by the transcription factor:
15 peroxisome proliferator activated receptor alpha (PPARα). PPARα regulates the
16 expression of genes that participate in FFA oxidation (included CPT1a).[5] Down-
17 regulation of PPARα and CPT1a could be related to a development state of
18 steatosis.

19 Several studies have documented a strong relationship between steatosis and
20 hepatic insulin resistance (HIR), with an overproduction of glucose despite the
21 presence of high levels of circulating insulin.[6] Furthermore, evidence *in vitro*
22 indicates that steatosis and HIR is accompanied by numerous adverse effects on
23 mitochondrial function, including the uncoupling of oxidative phosphorylation,[7] thus
24 with generation of reactive oxygen species (ROS), including superoxide.[8]

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25 Additionally, excess of lipids also increase HIR through decreased endogenous
26 antioxidant defenses such as hepatic glutathione (GSH) levels and catalase (CAT)
27 activity.[9,10]
28 Several studies on obesity treatment and its complications have focused on the
29 potential that plant infusions exert on lipid metabolism. Previous reports showed that
30 *Hibiscus sabdariffa* (HS) aqueous extract decreases body weight gain and liver
31 steatosis in diet-induced obese mice; this latter through down-regulated SREBP-1c.
32 [11] Treatments with *Ocimum sanctum* L. (OS) leaf extract showed a decreased
33 hyperlipidemia and improved oxidative state in liver and heart in high-cholesterol diet
34 fed rats.[12] Similarly, treatment with *Citrus paradisi* (CP) significantly decreases total
35 cholesterol and low-density lipoprotein.[13] However to our knowledge, the
36 mechanism of the effects on lipid metabolism of these plants has been not reported.
37 In the present study, we investigated the effect of OS and CP infusions on lipid
38 metabolism and insulin resistance, using obese rats induced with high-saturated fat
39 and fructose diet (HFFD).
40 **2. Materials and methods**
41 **2.1 Preparation of plant infusions**
42 We used three different plant materials: *Citrus paradisi* (CP), *O. sanctum* L. (OS),
43 and *Hibiscus sabdariffa* (HS); this latter was used for comparative purposes.
44 CP and HS were obtained at the locality Apatzingan (Michoacán, Mexico) and Santo
45 Domingo (Guerrero, Mexico) respectively in properly managed fields. OS was
46 collected from a greenhouse (Querétaro, Mexico). A representative sample from the
47 production of the 2011 season was collected. CP (previously sliced in transverse

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48 sections) and OS were dried in a convection oven at 50 ºC for 24 h. HS flowers were
49 sun dried at ambient temperature according to the instructions from the producers.
50 Dried materials were ground (1.13 mm), afterwards stored in the darkness for later
51 analysis. Infusions (1 %) were prepared by adding 200 mL of freshly boiled water to
52 2 g of dried ground sample, then let stand for 10 minutes and strained using a
53 commercial paper filter.

54 **2.2 Animals and experimental design**

55 Male Sprague-Dawley rats (Rismart SA de CV, Mexico City, Mexico), eight weeks of
56 age, were housed in a light and temperature-controlled room (12-12 h light-dark
57 cycle; 24 ± 1 ºC). Experiments on animals were performed in accordance with the
58 Animal Care and Use protocol of the University of Queretaro, as recommended by
59 NIH (Publications No. 80-23).

60 Rats were randomly divided into groups of seven animals. Healthy control group
61 was fed with a base diet (proteins 22 %, lipids 5 % [1 % saturated fat], and
62 carbohydrates 45 %). Obese control, HS, OS, and CP groups were fed with a high
63 fructose and high saturated fat diet (HFFD) (proteins 14 %, lipids 40 % [38 %
64 saturated fat], and carbohydrates 51 % [17 % fructose]) for nine months. Starting the
65 sixth month, instead of water, infusion treatments (1 %) were administered to HS,
66 OS, and CP groups. Measurement of body weight was performed weekly; food and
67 infusion intake were performed daily.

68 **2.3 Measurements and blood sampling**

69 After nine months, rats were sacrificed. Liver and adipose tissue were removed,
70 weighed, and immediately frozen in liquid nitrogen, stored at 80 °C until analysis. A

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71 fraction of omental adipose tissue and liver from each sample was immersed in 10
72 % formalin solution.

73 Blood was withdrawn via cardiac puncture; serum was separated and immediately
74 frozen at 80 °C until analysis. Glucose, serum triglyceride (TG), total cholesterol
75 (TC), and high-density lipoprotein cholesterol (HDL-c) concentrations were
76 measured using commercial assay kits (Randox Laboratories Ltd.). Serum insulin
77 levels were measured using a rat insulin ELISA kit (Millipore, USA). Afterwards, the
78 insulin resistance index was calculated with the Homeostasis Model Assessment
79 (HOMA) using the values from insulin and glucose under fasting conditions:

80 $$\text{HOMA} = \text{fasting insulin } (\mu\text{U mL}^{-1}) \times [\text{fasting glucose } (\text{mmol L}^{-1})\ 22.5^{-1}]$$

81 **2.4 Measurement of triglyceride concentration in liver and adipose tissue**

82 Lipids from omental adipose tissue and liver were extracted in a process previously
83 described.[14] The extract was washed with 0.2 volumes of saline solution (NaCl
84 0.9%) and centrifuged at 4,000 x *g* for 10 min. The lipid phase was then recovered,
85 and triglyceride content was determined using an enzymatic method (Randox
86 Laboratories Ltd.).

87 **2.5 Histology and cell-size measurement**

88 Paraffin embedded fraction of liver and omental adipose tissue from each sample
89 was used for steatosis and adipocyte size evaluation. Samples were observed and
90 photographed (100X for adipose tissue and liver, 400X only for liver) in hematoxylin-
91 eosin–stained tissue sections (5-μm). Adipocyte mean volumes were determined by
92 computerized image analysis (KS400 software, Carl Zeiss, Oberkochen, Germany).

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93 Cells were examined by selecting five sections for each fat sample; systematic
94 random sampling was used to select ten fields for each section. Cell volume was
95 calculated as follows, volume = $4(3\pi r^3)^{-1}$ (where r is radius); results were expressed
96 in picoliters.[15]

97 **2.6 mRNA isolation and cDNA synthesis**

98 Total RNA from liver tissue was obtained using a Total RNA Isolation System
99 (Promega, Madison, MI). Its concentration and purity were measured with a
100 NanoDrop ND-1000 instrument (Thermo Scientific, Wilmington, DE, USA), and the
101 integrity was verified after electrophoresis in a 1 % agarose gel stained with SYBR®
102 Gold Nucleic Acid Gel Stain (Invitrogen) under UV-light. cDNA synthesis was
103 performed using 2 µg of total RNA and 1 µL of oligo dT (15 bases, 0.25 µM) plus 8
104 µL of M-MLV 5X reaction buffer, 1 µL 10 mM dNTP mix (Invitrogen), 1 µL (200 U) of
105 M-MLV RT (Promega, Madison, WI), and RNAse-free water, reaching a final volume
106 of 20 µl and samples were incubated at 37 °C for one h, then stopped by heating to
107 55 °C for 15 min.

108 **2.7 Real-time PCR**

109 The cDNA samples were diluted 1:10 and used as templates for PCR in a
110 LightCycler1 (Roche Diagnostics, Indianapolis, IN, USA). qPCR amplification was
111 performed with the lightcycler Fast Start DNA master SYBR Green I from *Roche*
112 using 3 µL of the diluted cDNA, 0.5 µM of the corresponding oligonucleotides (Table
113 1). PCR conditions were as follows: 95 °C 1', 40 cycles: 95 °C 10'' 68 °C10'' and 72
114 °C 12''. PCR product identity was confirmed by sequencing in an ABI prism 310
115 from Applied Biosystems, big dye v 3.1. The results were evaluated with the $2^{-\Delta\Delta CT}$

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116 method[16] using the geometric mean of SOD and CYCA as reference genes. mRNA
117 expression in treated animals is reported as fold difference to obese control.

118 **2.8 Hepatic markers of oxidative stress**

119 Liver was homogenized in 50 mM phosphate buffer (pH 7), containing 0.5 mM EDTA
120 and 0.5 % Triton, using a tissue homogenizer (Kinematica, Switzerland) and
121 centrifuged at 8,000 *g* for 15 min at 4 ºC. Homogenized liver was then centrifuged at
122 8,000 *g* for 15 min at 4 ºC. The cytosolic fractions were stored at -70 ºC until
123 analysis. Catalase (CAT), Glutathione peroxidase (GPx), and Glutathione S-
124 transferase (GST) activity was assayed.[17-19] Results were expressed as µmol min^{-1}
125 mg $protein^{-1}$ respectively. Extinction coefficient of 6.22 mM^{-1} cm^{-1} was used to
126 calculate GPx. Protein concentration in the cytosolic fractions was determined by the
127 bicinchoninic acid protein assay (Pierce Inc., Rockford, IL), using bovine serum
128 albumin as a standard. Lipid peroxidation was determined by the thiobarbituric acid
129 reaction (TBARS) with malondialdehyde (MDA), a product formed due to the
130 peroxidation of lipids[20] and expressed as nmol mg $protein^{-1}$. Oxidative protein
131 damage was measured by protein carbonyl levels (C=O) and determined as
132 previously described.[21] Carbonyl content was calculated from the maximum
133 absorbance using a molar absorption coefficient of 22,000 M^{-1} cm^{-1}. Protein content
134 was determined by using bovine serum albumin as the standard.[22]

135 **2.9 Statistical analysis**

136 Data were expressed as mean values ± standard error (SE). Statistical significance
137 was determined by one-way variance analysis (ANOVA) ($P < 0.05$) followed by the
138 Tukey's test multiple comparisons. Kruskal-Wallis one way analysis of variance on

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139 ranks and multiple comparison procedures by Dunn's Method was performed for
140 enzymatic activity. All statistical analyses were performed using JMP 5.0.1 software.

141 **3. Results**

142 **3.1 Plant infusions (HS, OS, and CP) and its effect on body weight, lipid**
143 **accumulation in liver and omental adipose tissue.**

144 After nine months following their feeding pattern, obese control weighed around 25
145 % more than animals fed with normal diet (data not shown). The same trend was
146 observed in the liver and adipose tissue weight (Table 2). The amount of adipose
147 tissue increased more than 3 times for the obese control, and its content of TG was
148 almost 3 times higher compared to healthy control (Table 2). The adipocyte volume
149 augmented around 3.5 times respected to that of rats fed with a normal diet (Figure
150 1).

151 HFFD feeding in obese control led to significant increase in liver weight (around 40
152 %). In addition, hematoxylin-eosin-staining of liver section showed that HFFD
153 induced severe steatosis (85-95 %) characterized by the presence of large and
154 medium lipid droplets, as well as altered hepatocytes and sinusoids (Figure 2).
155 These results were in agreement with liver triglycerides content, which was 2.7 fold
156 higher compared to the healthy control (Table 2).

157 Animals fed with HFFD and treated with HS infusion decreased body weight (10 %),
158 whereas the other two groups treated with CP and OS did not change their weight
159 (data not shown) compared to obese control animals. No significant statistical
160 difference of food (range of food intake was 20-23 g $rat^{-1}day^{-1}$) and infusion intake
161 [Obese control (water intake): 32.1 ± 2.4 mL $rat^{-1}day^{-1}$; HS treated group: 28.9 ± 2.7

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162 mL $rat^{-1}day^{-1}$; OS treated group: 33.6 ± 2.4 mL $rat^{-1}day^{-1}$; CP treated group: 32.4 ±
163 2.2 mL $rat^{-1}day^{-1}$] were observed between treated groups and obese control.
164 In agreement with the effect of infusions on body weight, only HS was able to
165 decrease the weight of omental adipose tissue (29 %), while the other two infusion
166 treatments did not lead to any difference compared with the obese control group
167 (Table 2).
168 In contrast, rats under HFFD, and treated with plant infusions exhibited decreased
169 adipocyte volume (Figure 1). Group treated with HS infusion showed adipose cell
170 reduction (43 %), and their adipocytes showed minor volume (1.8 fold) compared to
171 adipocytes from obese control rats. OS and CP treated groups also exhibited a
172 reduction in adipocyte size and volume, but showed intermediate values between
173 the obese control and the HS treated group (Figure 1). These results are in
174 agreement with levels of adipose tissue TG, which were reduced in 46 %, 16 % and
175 38 % for HS, CP and OS respectively.
176 Treatment with infusions (HS and OS) decreased the weight of liver up to 21 %;
177 however, these values were not statistically significant (Table 2). Nonetheless, rats
178 treated with HS had a liver with preserved architecture and lower steatosis (30-40
179 %). OS showed intermediate values (50-60 %) between HS and obese control. CP
180 treated group showed a less reduction of steatosis (20-30 %) compared with the
181 other treated group (Figure 2).
182 The positive effect of plant infusions on liver steatosis was associated with a
183 reduction of hepatic TG content, which was decreased considerably compared with
184 their obese counterparts, up to 59 % and 57 % for HS and OS respectively. CP
185 decreased only 31 % (Table 2).

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186 **3.2 Plant infusions (HS, OS, and CP) regulate mRNA hepatic gene levels**
187 **involved in lipid metabolism**

188 We studied the expression of two lipogenic genes (SREBP-1c and FAS), which were
189 selected according to their metabolic functions involved in the biosynthesis of fatty
190 acids. There was an increase in SREBP-1c and FAS gene expression in HFFD-fed
191 animals, nearly 10 times compared with that of the healthy control group (Figure 3).
192 These results are in agreement with the increment of steatosis and hepatic TG
193 content. Treatment with the three infusions reduced the expression of these two
194 genes, being HS the most effective followed by OS and finally CP (Figure 3).

195 Two genes related with fatty acid β-oxidation (PPARα and CPT1a) were also
196 measured. Animals fed with HFFD exhibited a relative expression of the PPARα
197 gene five times lower than the healthy control (Figure 4). Similarly, the expression of
198 the CPT1a gene was reduced 13 times in liver (Figure 4). The best recovery of
199 PPARα expression was observed in rats treated with HS (4 fold) followed by animals
200 treated with OS (3 fold) infusions. Although, CP infusion treatment showed only a
201 slight increase in the expression of PPARα, this infusion consumption recovered the
202 expression of CPT1a by a factor of six in liver (Figure 4).

203 **3.3 Effect of Infusion treatments (HS, OS, and CP) on hyperlipidemia and**
204 **hyperglycemia.**

205 Obese control presented hyperlipidemia, hyperglycemia, and insulin resistance at
206 the end of the experimental period. We observed elevated quantities of TG in serum
207 from HFFD fed rats, 1.6 fold compared to healthy control (Table 3). There was a
208 hypolipidemic effect in rats receiving infusions in contrast to the obese control. We
209 observed a reduction in serum TG: 34 % for HS, 48 % for CP, and 27 % for OS

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210 infusions (Table 3). Serum cholesterol concentration was not altered with HFFD;
211 however, HFFD decreased HDL-c levels in obese rats. All plant infusion treatments
212 increased HDL-c concentration, being similar to the healthy group (Table 3).
213 HFFD-fed animals presented values of circulating glucose, which are considered
214 pre-diabetic (Table 3). Additionally, obese control displayed a 1.7-fold increase in
215 insulin over the non-obese control group. Therefore, the HOMA value increased
216 almost 4 times (Table 3). Plant infusion consumption improved diabetic parameters
217 in obese treated rats. A significant decrease in circulating levels of glucose and
218 insulin was observed for the three treated groups; consequently, HOMA values also
219 declined. Results for HS and OS were close to those of healthy control animals
220 (Table 3).

221 **3.4 Effect of Infusion treatments (HS, OS, and CP) improves hepatic oxidative**
222 **markers.**

223 As expected, HFFD feeding decreased activity in CAT, GPx, and GST enzymes,
224 increased hepatic lipid peroxidation (expressed as TBARS), and hepatic proteins
225 oxidation (expressed as C=O) in comparison with healthy rats (Table 4).
226 HS and OS infusion treatment on obese rats reestablished the enzymatic antioxidant
227 system to normal levels (Table 4). These two infusions displayed enzymatic activity
228 (CAT, GPx, and GST) similar to the non-obese animals and clearly different from the
229 obese group, except for GST in rats treated with HS. CP infusion produced slight but
230 significant increments in GPx activity compared with that of the obese control rats
231 (Table 4). In addition, we demonstrated that the consumption of infusions reduced
232 the levels of MDA, a marker of lipid peroxidation (approximately 84 %), particularly
233 those treated with HS and OS infusion (Table 4). Treatment with infusions

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234 diminished hepatic protein oxidation compared with obese control group (up to 36
235 %).

236 **4. Discussion**

237 Elevated consumption of caloric diets resulted in metabolic alterations, leading to
238 physiological changes in organs such as adipose tissues and liver. In this study, we
239 examined the effects of CP and OS infusion on HFFD fed obese rats. We included
240 HS infusion as an infusion control, due to its reported anti-obesity potential and
241 elucidated mechanism of action.[23,24]

242 Results showed that treatment with these plant infusions improved alterations
243 related with obesity. Most of the reported health benefits of these plant infusions are
244 associated with their bioactive compounds such as polyphenols. Among the
245 compounds reported for these plant materials are: cyanidin-3-sambubioside,
246 protocatecuic, coumaric and chlorogenic acid, for HS, naringin and narirutin for CP,
247 and kaempferon and rosmarinic acid for OS. [25-27] Rats treated with infusions
248 consumed some of these polyphenols, for example: HS treated group 4.6 μg rat^{-1}
249 day^{-1} of coumaric acid, OS 4.1 μg rat^{-1} day^{-1} of rosmarinic acid, and CP 2.4 μg rat^{-1}
250 day^{-1} of naringin (Data obtained by HPLC analysis, Anexo 1). In a previous report,
251 these compounds have demonstrated anti-obesity, antioxidant, and anti-
252 inflammatory effects.[28]

253 We analyzed the omental adipose tissue since previous reports have demonstrated
254 that this tissue differs metabolically from other fat cells. For example, omental fat cell
255 enlargement in obesity increases the production of cytokines more than other fat
256 tissues. These cytokines are associated with the development of insulin

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257 resistance.[29] In our study, consumption of infusions (OS and CP) decreased
258 adipocyte volume and TG in adipose tissue.
259 Plant infusions also reduce hyperlipidemia and lowered lipid accumulation in liver,
260 thus the presence and progression of steatosis. In order to demonstrate whether this
261 effect is related to the modulation of lipid metabolism by infusions, we evaluated the
262 expression of genes involved in synthesis and oxidation of FFA in the liver. We
263 observed a down-regulation of SREBP-1c and FAS because of the consumption of
264 HS infusion. In agreement with our results, previous studies reported that an HS
265 aqueous extract exerted a hypolipidemic effect, reduced fat liver accumulation, and
266 decreased the lipid content of hepatocyte through down regulated of SREBP-1c.[30]
267 Similarly to HS, we have shown that treatment with OS and CP infusion decreased
268 the expression of both genes compared with the obese control, being more
269 important for group treated with OS. This down-regulation suggests that the
270 attenuation observed in liver steatosis by treated groups is associated with minor
271 fatty acid synthesis.
272 Reduction of hepatic FFA levels induced by infusions could be related with an
273 increased mitochondrial fatty acid uptake and oxidation through the up-regulation of
274 PPARα and CPT1a expression.[31] Previous studies report that HS extract showed an
275 enhancement of lipid metabolism through up regulation of the PPARα expression in
276 type 2 diabetic rat model.[24] Similarly to HS, we demonstrated that OS and CP
277 infusions induced the relative expression of PPARα compared with obese control.
278 On the other hand, rats treated with CP infusions showed a main up regulation of
279 CPT1a (1.5 fold) compared to OS. This could be because the transcriptional

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280 regulation of CPT1a involves several PPARα-independent pathways such as
281 peroximal proliferator activated receptor gamma cofactor 1 (PGC1).[32]
282 Previous studies report that up-regulation of CPT1a reduces hepatic steatosis and
283 insulin resistance in genetically obese db/db mice. [33] CPT1a controlled the entry of
284 long chain fatty acids through the mitochondrial outer membrane by binding them to
285 carnitine in order to carry out β-oxidation.[33] Therefore, our results suggest that
286 proportional decreased of hepatic steatosis observed in CP group, could be
287 associated to mitochondrial fatty acid uptake. However, this mechanism was lower
288 compared with FFA synthesis.
289 Treatment with CP infusions also improved the concentration on serum TG. This
290 effect could be related to the decreased absorption of dietary lipids. We found that
291 consumption of CP infusion increased the TG in feces of HFFD fed rats (1.4-fold that
292 obese control) (data not published). In agreement with our results, other authors
293 reported that hypolipidemic action in plant materials is probably due to the ability of
294 bioactive compounds to modify absorption and intracellular transport of lipids.[25,27]
295 Steatosis seems to be one of the first steps in the development of insulin resistance,
296 and then of diabetes type 2. Therefore, we evaluated the hypoglycemic effect of the
297 infusions. All treatment with plant infusions significantly decreased circulating levels
298 of glucose and insulin; consequently, the HOMA values declined more than 50%,
299 OS showed quantities similar to healthy control. These data are in agreement with
300 several studies performed with ethanolic, methanolic, and non-infusion aqueous
301 extracts of different parts of these plant materials.[23,34-35] One possible mechanism
302 for the hypoglycemic action of infusions may be attributed to an increased utilization
303 of glucose by the liver for glycogen synthesis. As well as a reduction of

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304 gluconeogenesis, decreasing sugar delivery into the blood stream.[36] However, there
305 is insufficient research providing evidence for these effects exerted by such
306 infusions (OS and CP).
307 Improvement of insulin resistance was accompanied by a decrease in steatosis and
308 hepatic oxidative stress markers. *In vitro* evidence indicates that elevated lipid
309 levels induce the synthesis of ROS and impair endogenous antioxidant defenses.[8]
310 High levels of ROS and depleted endogenous antioxidant system lead to the
311 activation of multiple serine/threonine kinases signaling cascades. These activated
312 kinases can act on a number of potential targets in the insulin signaling pathway,
313 including the insulin receptor and inducing insulin resistance.[37] This latter effect was
314 observed in HFFD through a decreased activity in CAT, GPX, and GST enzymes,
315 and an increased hepatic lipid and protein oxidation.
316 However, rats treated with plant infusions restored hepatic endogenous antioxidant
317 system, mainly OS. These results indicate that consumption of infusions could either
318 increase the biosynthesis of antioxidant enzymes or reduce their degradation. The
319 improvement in activity of antioxidant enzymes by infusions can be related to some
320 compounds of plant infusions quantified in this work (Anexo 1), such as rutin and
321 coumaric acid. It has been reported that the treatment with these compounds
322 enhanced the levels of GPx and GST in the hepatic tissue of rats with HFD-induced
323 obesity.[28]
324 We also demonstrated that treatment with infusions reduced MDA and protein
325 oxidation due to its antioxidant activity. In agreement with our results, several
326 studies report that different extracts of these plants exhibit antioxidant activity. For
327 instance, treatment with aqueous HS extract reduces lipid peroxidation attributed to

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328 the improvement in the cellular antioxidant system in high fat diet-induced obese
329 mice.[38] Furthermore, OS leaf extract suppressed the high levels of serum lipid profile
330 and hepatic lipids through the reduction in TBARS and raised the low activities of
331 GPx and CAT in rats fed with high-cholesterol (HC) diet.[12] Therefore, OS and CP
332 infusions are an interesting target to improve the metabolic alterations studied in this
333 work.

334 **5. Conclusion**

335 Consumption of OS and CP infusions decreased liver fat accumulation through the
336 down-regulation of gene involved in lipogenesis (SREBP-1c and FAS) and lipid
337 oxidation (PPARα and CPT1a), being better for OS. These regulations are
338 associated with an improvement in insulin resistance and hepatic oxidative stress,
339 thus suggesting that plant infusion (OS and CP) consumption is a viable alternative
340 treatment for these metabolic alterations in obesity induced by obesogenic diets.

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446 **Table 1.** Primer pairs used in the real-time quantitative PCR reactions.

Gene	NCBI ID	Forward primer	Reverse primer
SREBP-1c[39]	AF286470	GCCCACAATGCCATTGAGA	CAGGTCCTTGAGCTCCACAATC
FAS	NM_017332	GGACATGGTCACAGACGATGAC	GTCGAACTTGGACAGATCCTTCA
PPARα	NM_013196	TGGAGTCCACGCATGTGAAG	CGCCAGCTTTAGCCGAATAG
CPT1	NM_031559.2	CCCATATCCAGGCAGCGAGA	AGCCAGACCTTGAAGTACCG
SOD	NM_017051.2	TGGACAACCTGAGCCCTAA	GACCCAAAGTCACGCTTGATA
CYCA[40]	XM_345810	AGCACTGGGGAGAAAGGATT	AGCCACTCAGTCTTGGCAGT

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449 **Table 2.** Relative organ weight and lipid concentration of obese rats treated with infusions. Values are expressed as
450 mean ± SE (n = 7).

	Healthy Control	Obese Control	HS	CP	OS
Organ Weight					
Liver (g)	9.37 ± 0.6[c]	15.24 ± 0.50[a]	12.08 ± 1.1[b]	14.40 ± 0.60[a]	12.63 ± 0.90[b]
Abdominal Adipose Tissue (Omental) (g)	4.44 ± 0.3[b]	17.72 ± 1.0[a]	12.44±3.2[a]	16.59±1.0[a]	17.82±1,0[a]
Lipids in Organs					
Hepatic Triglycerides (mg g^{-1})	26.8 ± 4.10[b]	72.7 ± 3.90[a]	29.6 ± 3.10[b]	50.2 ± 1.10[c]	31.4 ± 5.30[b]
Omental Adipose Tissue Triglycerides (mg g^{-1})	82.7 ± 5.10[d]	239.7 ± 6.30[a]	129.6 ± 16.90[cd]	201.5 ± 13.40[b]	147.2 ± 5.00[c]

Values in a row followed by different letters are significantly different ($p < 0.05$) by Tukey's test. *Hibiscus sabdariffa* (HS), *Citrus paradisi* (CP), *Ocimum sanctum* L. (OS).

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455 **Table 3.** Serum metabolites and Homeostasis Model Assessment (HOMA) of obese rats treated with infusions. Values
456 are expressed as mean ± SE (n = 7).

	Healthy control	Obese control	HS	CP	OS
Total Cholesterol (mg dL^{-1})	94.3 ± 4.6^{a}	104.0 ± 2.2^{a}	102.3 ± 3.8^{a}	109.0 ± 2.9^{a}	101.2 ± 2.4^{a}
High Density Lipoprotein (mg dL^{-1})	52.30 ± 3.0^{a}	35.34 ± 2.4^{b}	51.1 ± 0.2^{a}	48.3 ± 2.4^{a}	49.0 ± 5.9^{a}
Triglycerides (mg dL^{-1})	95.0 ± 7.8^{bc}	159.6 ± 9.4^{a}	105.2 ± 9.4^{bc}	82.7 ± 9.5^{c}	117.0 ± 9.6^{b}
Glucose (mg dL^{-1})	94.8 ± 4.0^{b}	122.4 ± 1.0^{a}	93.9 ± 5.5^{b}	102.1 ± 2.4^{ab}	87.8 ± 5.8^{b}
Insulin (µU mL^{-1})	29.9 ± 0.5^{b}	51.9 ± 2.8^{a}	31.3 ± 0.8^{b}	31.3 ± 2.5^{b}	32.4 ± 2.2^{b}
HOMA (arbitrary units)	4.8 ± 0.6^{d}	18.7 ± 0.6^{a}	6.9 ± 0.3^{c}	9.1 ± 0.5^{b}	6.5 ± 0.5^{cd}

Values in a row followed by different letters are significantly different ($p < 0.05$) by Tukey's test. *Hibiscus sabdariffa* (HS), *Citrus paradisi* (CP), *Ocimum sanctum* L. (OS).

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459 **Table 4.** Hepatic markers of oxidative stress: Thiobarbituric acid reactive substance (TBARS), carbonyl content of protein
460 (C=O), catalase (CAT), glutathione peroxidase and glutathione (GPx), and glutathione S-transferase (GST) activities in
461 liver fraction of obese rats treated with infusions. Values are expressed as mean ± SE (n = 7).

	Healthy control	Obese control	HS	CP	OS
TBARS (nmol mg protein^{-1})	3.7 ± 0.1[b]	21.0 ± 2.8[a]	3.3 ± 0.4[b]	5.3 ± 1.1[b]	3.6 ± 0.3[b]
C=O (nmol carbonyl mg Protein^{-1})	18.2 ± 0.5[d]	34.3 ± 0.3[a]	21.5 ± 0.7[c]	26.5 ± 1.3[c]	23.3 ± 0.7[c]
CAT (μmol min^{-1} mg^{-1} protein)	6.1 ± 0.1[a]	4.0 ± 0.2[b]	6.0 ± 0.4[a]	4.5 ± 0.4[b]	7.7 ± 0.9[a]
GPx (μmol min^{-1} mg^{-1} protein)	12.2 ± 0.2[a]	10.4 ± 0.1[b]	12.5 ± 0.1[a]	11.8 ± 0.1[a]	12.5 ± 0.1[a]
GST (μmol min^{-1} mg^{-1} protein)	686.3 ± 31.0[a]	471.0 ± 27.6[b]	588.1 ± 32.9[b]	491.4 ± 35.9[b]	695.7 ± 40.0[a]

Values in a two first row followed by different letters are significantly different with ($P \leq 0.05$) by Tukey's test.
Values in enzymatic activity row followed by different letters are significantly different with ($P \leq 0.05$) by .Dunn's Method
Hibiscus sabdariffa (HS), *Citrus paradisi* (CP), *Ocimum sanctum* L. (OS).

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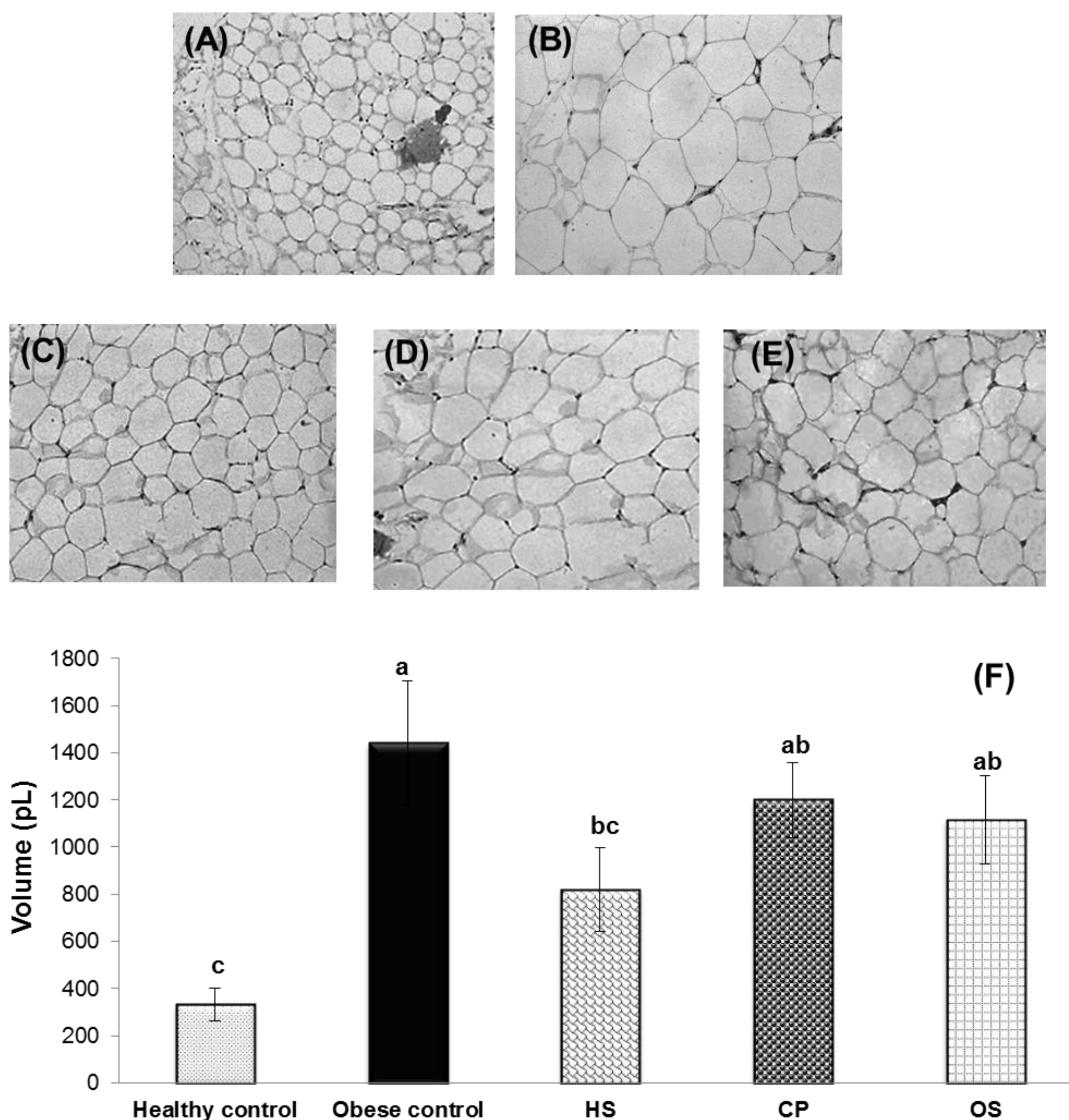

463

464 **Figure 1.** Hematoxylin-eosin–stained adipose tissue sections (100x) of obese rats
465 treated with infusions: (A) healthy control, (B) obese control, (C) *Hibiscus sabdariffa*
466 (HS), (D) *Citrus paradisi* (CP), and (E) *Ocimum sanctum* L. (OS) treated group. (F)
467 Adipocytes volume expressed in picoliters (pL). Values are expressed as mean ± SE
468 (n= 7). [a,b,c] Different letters are significant different (P≤0.05) by Tukey's test.

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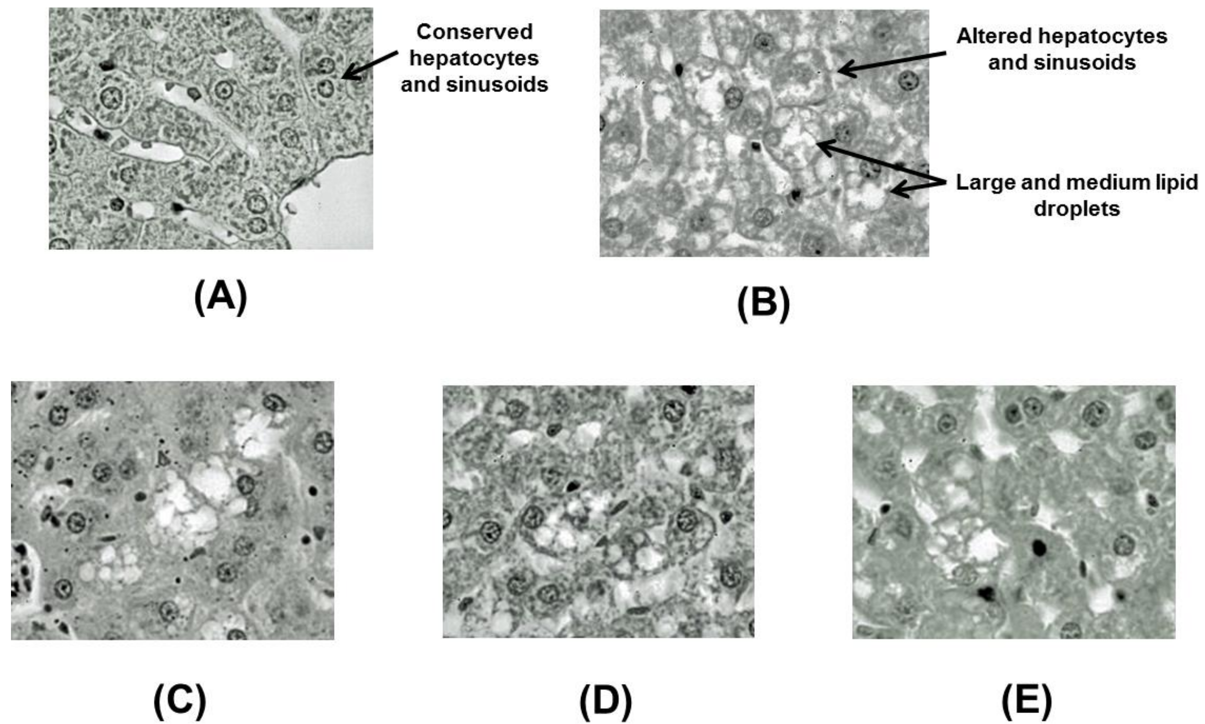

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472 **Figure 2**. Representative photographs of Hematoxylin-eosin–stained hepatic tissue
473 sections (400X): (A) healthy control, (B) obese control, (C) *Hibiscus sabdariffa* (HS),
474 (D) *Citrus paradisi* (CP), and (E) *Ocimum sanctum* L. (OS) treated group.

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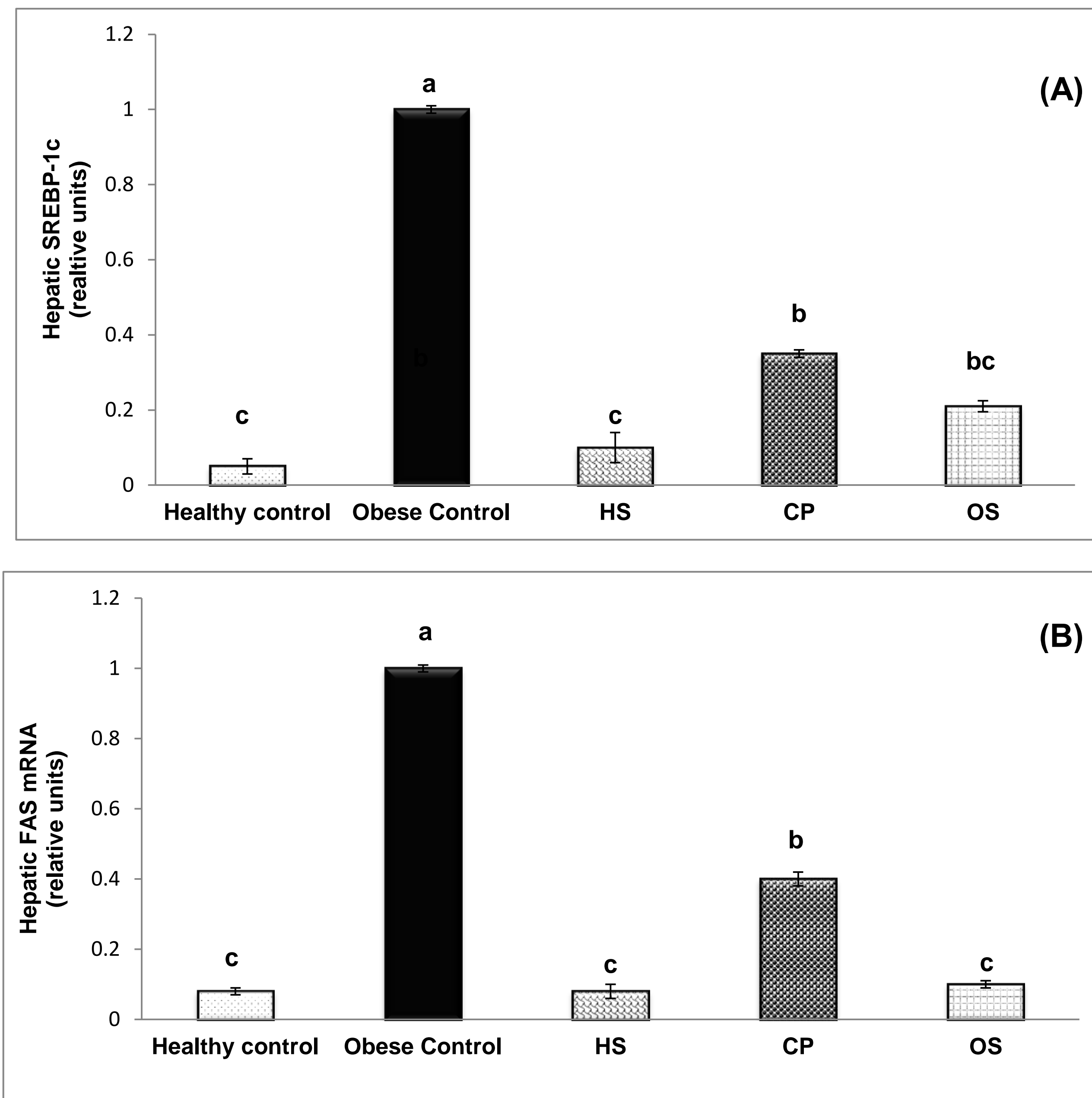

479

480 **Figure 3.** Quantitation of mRNA of hepatic genes involved in fatty acids synthesis of
481 obese rats treated with infusions. (A) SREBP1 and (B) FAS. The geometric mean of
482 SOD and CYCA was used as a reference gene. HS= *Hibiscus sabdariffa*, CP=
483 *Citrus paradisi*, OS= *Ocimum sanctum*. Values are the means ± SE (n = 7). The
484 values for rats treated with infusions were expressed relative to the value for obese
485 rats. [a,b,c] Different letters are significant different (P≤0.05) by Tukey's test.

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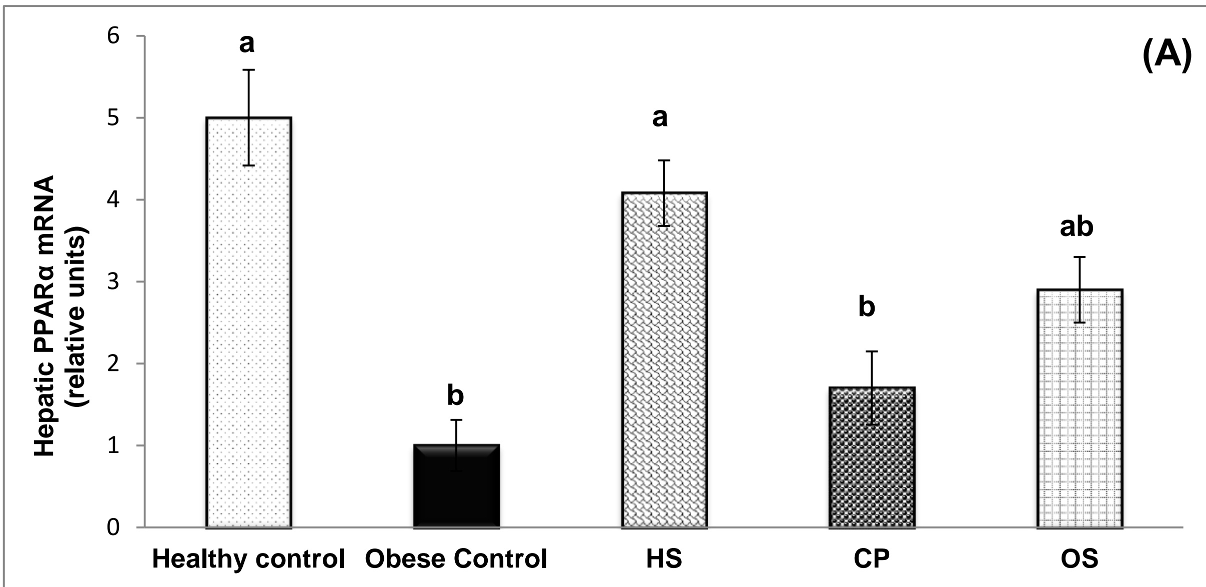

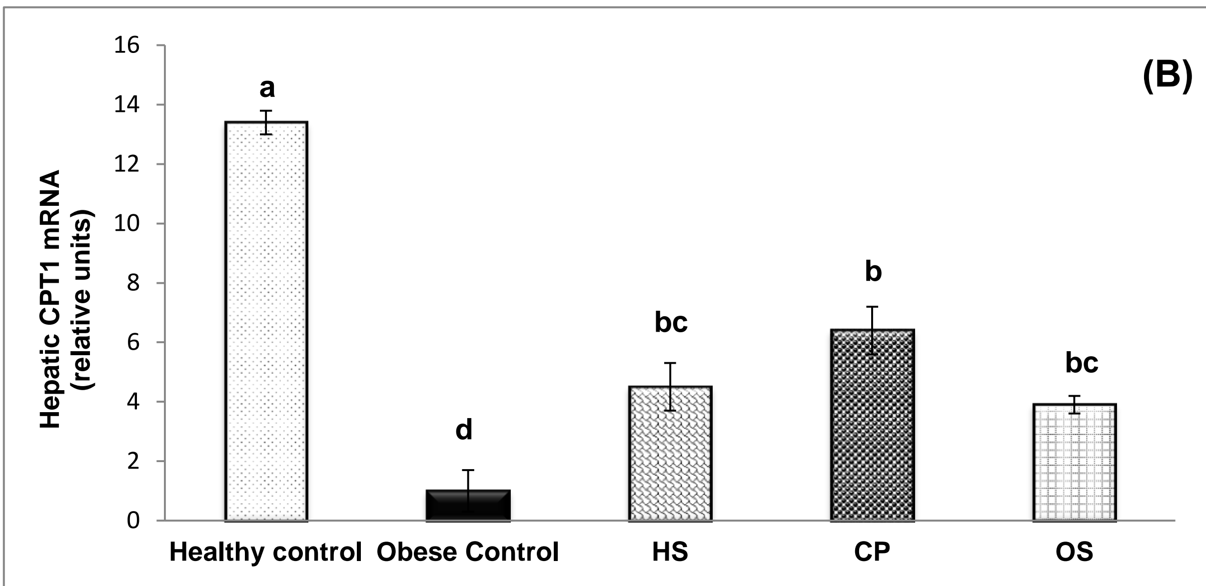

486

487 **Figure 4.** Quantitation of mRNA of (A) hepatic PPARα and (B) hepatic CPT1a. The
488 geometric mean of SOD and CYCA was used as a reference. HS= *Hibiscus*
489 *sabdariffa*, CP= *Citrus paradisi*, OS= *Ocimum sanctum*. Values are the means ± SE
490 (n = 7). The values for rats treated with infusions were expressed relative to the
491 value for obese rats. [a,b,c,d] Different letters are significant different (P≤0.05) by
492 Turkey's test.

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