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Consumption of orange fermented beverage reduces cardiovascular risk factors in healthy mice

Blanca Escudero-López [a], Genoveva Berná [a,b], Ángeles Ortega [a,b], Griselda Herrero-Martín [a], Isabel Cerrillo [a,c], Franz Martín [a,b], María-Soledad Fernández-Pachón [a,c,*]

[a] Area of Nutrition and Food Sciences, Department of Molecular Biology and Biochemistry Engineering, Pablo de Olavide University, Carretera de Utrera Km 1, E-41013 Seville, Spain
[b] Spanish Biomedical Research Centre in Diabetes and Associated Metabolic Disorders (CIBERDEM), Spain
[c] Associated Researcher at Autónoma de Chile University, Av. Pedro de Valdivia 641, Santiago de Chile, Chile

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ABSTRACT

The consumption of fruits prevents the risk of cardiovascular diseases. Alcoholic fermentation has been carried out in fruits resulting in products which provide high concentration of bioactive compounds and variable alcohol content. The aim of this study was to assess the potential beneficial effect of an orange beverage obtained by alcoholic fermentation and pasteurization of orange juice on cardiovascular risk biomarkers. For this purpose, four mice groups (n = 8) ingested orange beverage (equivalent volume to 250 mL/day in human), orange juice, alcoholic solution (at the proportional amount of orange beverage) or water during 12 weeks. The equivalent amount to double serving of orange beverage (500 mL/day) was administered to mice in a subsequent intervention, and a control group was also evaluated. Orange beverage consumption increased levels of glutathione and uric acid, improved lipid profile, decreased oxidized LDL and maintained levels of IL-6 and C-reactive protein. Synergistic effects between the bioactive compounds and the alcohol content of orange beverage may occur. The intake of double serving also increased antioxidant enzyme activities, bilirubin content and plasma antioxidant capacity. These results suggest that orange beverage may produce greater protection against cardiovascular risk factors than orange juice in healthy mice.

1. Introduction

Epidemiologic and clinical studies have shown that the intake of fruits and vegetables reduces the risk of cardiovascular diseases. This beneficial effect has been attributed to the presence of bioactive compounds (Liu, 2013). A large number of physiological effects have been attributed to these compounds such as antioxidant and anti-inflammatory activity, lipid profile and blood glucose regulation, improvement of endothelial function, antithrombotic effect or vasodilator action (Tresserra-Rimbau et al., 2014). Orange juice is among the most consumed fruit juices worldwide and it is known to be a rich source of bioactive compounds such as flavonoid, carotenoid, ascorbic acid and melatonin (Sae-Teaw et al., 2013; Tounsi et al., 2011). Numerous studies have shown that orange juice consumption reduces the prevalence of cardiovascular risk factors such as oxidative or inflammatory stress, dyslipidemia, hyperglycemia, endothelial dysfunction, hypertension or obesity (Aptekmann and Cesar, 2013; Buscemi et al., 2012; O'Neil et al., 2012). In recent years, fermentation processes have been carried out in fruit juices (Pérez-Gregorio et al., 2011), resulting in products which provide a higher concentration of bioactive compounds than the respective substrate. Our group has previously described the influence of controlled alcoholic fermentation on the orange juice composition showing that flavanones, carotenoids and melatonin content significantly increased during the process, and the antioxidant capacity was enhanced (Escudero-López et al., 2013; Fernández-Pachón et al., 2014). Thermal treatment in this fermented orange juice has been carried out and the final composition of the beverage will be discussed in the present study. On the other hand, this fermented product of orange presents a moderate alcohol content. Moderate alcohol consumption reduces the risk of cardiovascular diseases (Arranz et al., 2012). Potentially, bioactive compounds and

Abbreviations: PAC, plasma antioxidant capacity; ORAC, oxygen radical absorbance capacity; FRAP, ferric reducing antioxidant power; TEAC, trolox equivalent antioxidant capacity; GSH, reduced glutathione; GSSG, oxidized glutathione; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase; TC, total cholesterol; LDL-ox, oxidized low-density lipoprotein; TBARS, thiobarbituric acid reactive species; MDA, malondialdehyde; CRP, C-reactive protein.

* Corresponding author. Area of Nutrition and Food Sciences, Department of Molecular Biology and Biochemistry Engineering, Pablo de Olavide University, Carretera de Utrera Km 1, E-41013, Seville, Spain. Tel.: +34 954977613; fax: +34 954349813.
E-mail address: msferpac@upo.es (M.-S. Fernández-Pachón).

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moderate alcohol content of this novel fermented orange juice would exert synergistic benefits in the organism, similar to beer or wine (Chiva-Blanch et al., 2012), and increase the health effects already tested with orange juice.

Thus, the aim of the present study was to assess the potential beneficial effect of a novel fermented orange beverage on cardiovascular risk markers in mice evaluating the possible synergistic effects of its bioactive compounds and alcohol content, and the influence of the volume ingested. Therefore, the antioxidant status (plasma antioxidant capacity, endogenous antioxidants content and antioxidant enzyme activities), the lipid profile, the lipid peroxidation (thiobarbituric acid reactive species (TBARS) level and oxidized low-density lipoprotein (LDL-ox) content) and the inflammatory status (IL-6 and C-reactive protein (CRP) levels) have been evaluated after repeated consumption of the fermented orange beverage in healthy mice.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals were purchased from Sigma-Aldrich Quimica (Alcobendas, Spain).

2.2. Orange beverage preparation

The company Grupo Hespérides Biotech S.L. carried out the controlled alcoholic fermentation of commercial orange juice made from *Citrus sinensis* L. var. *Navel late* (Huelva, Spain). The fermentation process was carried out in a 5 L pvc tank at 20 °C for 10 days in repose. The yeast strain *Saccharomycetaceae* var. *Pichia kluyveri* was selected from the natural microbiota present in the orange fruit and used for the inoculation of the fermentation because this yeast strain ferments only reducing sugars, resulting in a final product with low alcohol content and sweet taste. The thermal pasteurization was achieved at 85 °C for 30 s in a semi-tubular pasteurizer 25 L/h (Mipaser Prototype, Murcia, Spain). After treatment, the orange beverage was cooled to 10 °C in an ice-water-bath and stored at –20 °C until its consumption. Quality parameters, bioactive compounds content and antioxidant activity of orange juice and the fermented-pasteurized product (orange beverage) were analyzed as described by Escudero-López et al. (2013), Organisation Internationale de la Vigne et du Vin (OIV) (2014) and Collado-González et al. (2014).

2.3. Animals and experimental design

Eight week-old male OF1 mice were obtained from Charles River Laboratories (Barcelona, Spain) and all methods were performed in accordance with the Legislation for the protection of animals used for scientific purposes (EU Directive 2010/63/EU). All mice were housed and maintained under the same laboratory conditions of temperature (22 °C) and lighting (12 h light–dark cycle) and were given free access to standard nonpurified diet (Scientific Animal Food and Engineering, Spain) and tap water. The mice were acclimated to the laboratory conditions for 1 week before the experiments. The review boards of animal ethics at our University approved this study. We followed the requirements regarding the protection of animals used for experimental and other scientific purposes.

To assess the potential beneficial effects of orange beverage on cardiovascular risk and the possible synergistic effects of its bioactive compounds and alcohol content, a total of thirty-two mice were randomly divided into four groups (n = 8 per group, 1 mice/cage). Each group received different drinks solutions administered *ad libitum* during 12 weeks: (1) Control group (CTRL): water; (2) OJ group: orange juice diluted 1:10 in tap water (this dilution factor was calculated based on a proportional intake of 250 mL/day of orange juice – one serving – in humans which had shown beneficial effects (Johnston et al., 2003); (3) OB group: orange beverage diluted 1:10 in tap water; (4) AS group: aqueous alcohol solution diluted 1:1000 from a 96% ethanol stock (this dilution factor provides the equivalent amount of alcohol of a proportional intake of 250 mL of orange beverage/day in humans).

To investigate whether the effects obtained are dependent on the dose of orange beverage, the equivalent amount to double serving (equal to 500 mL/day in humans) was administered subsequently to 18 mice during 12 weeks (OB-2 group). A control group (n = 18) was also evaluated (CTRL-2).

Bottles were replaced every 2 days to avoid oxidization and precipitate formation, and the liquid volume consumption was also measured (difference between initial and final volumes) every 2 days in both interventions. Body weight of mice was recorded weekly.

2.4. Sample collection

Mice were fasted overnight prior to being sacrificed by cervical dislocation. Blood samples were collected by intracardiac puncture and plasma was separated by centrifugation at 3000 *g* (10 min, 4 °C). Plasma samples were stored at –80 °C for subsequent analysis. Liver was carefully dissected, weighed and immersed in liquid N_2 before storage at –80 °C.

2.5. Plasma antioxidant capacity (PAC)

2.5.1. Oxygen radical absorbance capacity (ORAC) assay

Plasma samples were diluted (1:2000) in phosphate buffer (75 mM, pH 7.4). ORAC assay was performed according to Ou et al. (2001).

2.5.2. Ferric reducing antioxidant power (FRAP) assay

Plasma samples were diluted (1:10) in distilled water. The ferric reducing ability was estimated according to Delgado-Andrade et al. (2005).

2.5.3. Trolox equivalent antioxidant capacity (TEAC) assay

Plasma samples were diluted (1:10) in water/methanol (1:1). TEAC assay was performed following the procedure described by Delgado-Andrade et al. (2005).

2.6. Endogenous antioxidants content

Albumin, bilirubin and uric acid contents were measured in plasma samples using the manufacture's protocols established by Roche Diagnostics. Contents of total glutathione, oxidized (GSSG) and reduced (GSH) were determined in liver samples using a commercial kit (Enzo Life Sciences, Plymouth Meeting, USA). Liver was homogenized using a Thomas-Teflon homogenizer in a solution of 5% metaphosphoric acid 1:20 (w/v), and kept on ice. Homogenates were centrifuged at 15700 *g* (10 min, 4 °C), and the supernatants were used for the analysis.

2.7. Antioxidant enzyme activities

Activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) were evaluated in liver samples according to Cohen and Somerson (1969), McCord and Fridovich (1969), Carmagnol et al. (1983) and Cribb et al. (1989), respectively. Samples were homogenized in a 1:4 (w:v) buffered solution (100 mM Tris-HCl with 0.1 mM EDTA, 0.1% triton X-100) using a Miccra D-1 homogenizer (Miccra, Germany). The homogenates were centrifuged at 20800 *g* (30 min, 4 °C). The resulting supernatants were collected and stored at –80 °C until analysis. Total protein content was assessed by standard Bradford's procedure (Bradford, 1976).

2.8. Lipid profile

Commercial available kits were used to measure the lipid profile in plasma samples: total cholesterol (TC) (Invitrogen Life Technologies, Oregon, USA), HDL (Roche Diagnostics Systems Inc., New Jersey, USA) and TAG (Thermo Scientific, Middletown, USA). LDL was obtained using the Friedewald's formula (Friedewald et al., 1972): LDL = TC – HDL – (TAG/5).

2.9. Lipid peroxidation

The lipid peroxidation was evaluated by the thiobarbituric acid reactive species level and oxidized low-density lipoprotein content. TBARS was measured in liver homogenates according to Buege and Aust (1978), and the results were expressed as malondialdehyde (MDA) concentration. LDL-ox was measured in plasma samples using a commercial kit (Cusabio Biotech, Wuhan, China).

2.10. Inflammation status

Plasmatic levels of IL-6 and C-reactive protein were measured with ELISA kits purchased from Ray Biotech Inc. (Georgia, USA) and USCN Life Science Inc. (Wuhan, China), respectively.

All markers were recorded on a SynergyTM HT-multimode microplate reader (Biotek Instruments, Winooski, USA) except albumin, bilirubin and uric acid which were recorded on a COBAS Integra 400 Plus biochemistry analyzer (Roche Diagnostics, Indianapolis).

2.11. Statistical analysis

All analyses were in triplicate. The values are given as mean ± SEM. Differences between the CTRL, OB, OJ and AS groups were tested by one-way analysis of variance (ANOVA) followed by Tukey's test. Student's *t* test was applied to establish differences between CTRL-2 and OB-2 groups. A probability value of $p < 0.05$ was adopted as the criteria for significant differences. These analyses were carried out by SPSS 15.0 Software (SPSS Inc., Chicago, USA).

3. Results and discussion

The current study is the first intervention involving an orange juice derivative with low content of alcohol (0.87% v/v) obtained

Table 1
Quality parameters, bioactive compounds content and antioxidant activity of orange juice and orange beverage.

Composition	Orange juice	Orange beverage
pH	3.48 ± 0.20	3.45 ± 0.20
TA (g citric acid/L)	8.48 ± 0.02	8.83 ± 0.02
Total sugars (g/L)	78.2 ± 5.64	52.3 ± 4.23
Reducing sugars (g/L)	48.5 ± 3.63	23.9 ± 2.54
Non-reducing sugars (g/L)	29.7 ± 2.01	29.5 ± 2.33
Amino acids (mg/L)	8194 ± 250	9265 ± 214
TSS (°Brix)	11.0 ± 0.50	10.0 ± 0.50
% Pulp	12.0 ± 2.00	8.00 ± 0.50
Alcohol (% v/v)	0.00	0.85 ± 0.01
Ascorbic acid (mg/L)	423 ± 1.8	203 ± 0.60
Total phenolic (mg/L)	713 ± 73.9	585 ± 52.3
Total flavanones (mg/L)	762 ± 50	647 ± 6.9
Total carotenoid (mg/L)	10.5 ± 0.43	7.07 ± 0.18
Provitamin A (RAEs/L)	144 ± 5.32	90.1 ± 1.36
ORAC (μmol/L)	6361 ± 261	6353 ± 307
TEAC (mmol/L)	6.17 ± 0.40	5.45 ± 0.58

Values are expressed as mean (n = 3) ± SD.
TA, titratable acidity; TSS, Total soluble solids; RAEs, Retinol Activity Equivalents; ORAC, oxygen radical absorbance capacity; TEAC, trolox equivalent antioxidant capacity.

by a controlled alcoholic fermentation process and subsequent pasteurization. The bioactive compounds profile of this novel beverage was evaluated (Table 1). The orange beverage showed lower bioactive compounds content than the orange juice used for the fermentation process caused by the thermal treatment. However, its high content of total flavanones (647 mg/L), total carotenoids (7.07 mg/L), provitamin A (90.1 RAEs/L) and ascorbic acid (203 mg/L) is notable, comparable to other commercial orange juices (Stinco et al., 2012; Vallejo et al., 2010). Moreover the antioxidant capacity (ORAC and TEAC values) was preserved in spite of the partial loss of these compounds (Table 1).

Mice appear to perfectly tolerate the replacement of drinking water for diluted orange juice (1:10), orange beverage (1:10) or alcohol (1:100). The average of liquid volume intake every 2 days was significantly higher in the experimental groups OJ (13.9 ± 0.2 mL), OB (14.9 ± 0.3 mL) and AS (10.8 ± 0.1 mL) in relation to CTRL (10.0 ± 0.2 mL). However, the difference of the liquid volume ingested did not influence the final weight of mice. Mice from all groups showed similar body weight at the end of the experiment (CTRL: 45.6 g ± 4.4 g; OB: 47.9 ± 5.9 g; OJ: 45.1 ± 3.3 g; AS: 49.1 ± 6.8 g). In addition no significant differences in total weight gain were presented among them. On the other hand, the dilution factor of the orange beverage as double-serving (1:5) did not influence on the average volume of liquid ingested (OB-2: 14.2 ± 2.6 mL). Furthermore, the consumption of a double dose of the orange beverage did not influence the total weight gain of mice and all showed similar body weight at the end of the experiment (CTRL-2: 50.6 ± 5.4 g; OB-2: 51.4 ± 4.5 g).

3.1. *Effect of orange beverage intake on antioxidant status*

Fig. 1 shows PAC values (ORAC, FRAP and TEAC) in the four mice groups (CTRL, OJ, OB and AS groups). Results revealed that the intake of orange beverage did not lead to significant changes in PAC in relation to CTRL. In others studies with rats or humans, the authors did not observe changes in PAC using similar volume of antioxidant-rich beverages (Arendt et al., 2005; da Silva et al., 2013). In the present study, fasting blood samples were collected about 12–14 h after the consumption of the orange beverage, and the possible antioxidant potential of flavanones may not have been maintained after this time. In previous study, flavanone metabolites of orange beverage, such as naringenin-7-*O*-glucuronide and hesperetin-7-*O*-glucuronide, reached maximal concentration in rat plasma at 6 h after ingestion of a single dose (2 mL), and the levels returned to basal values after 10 h (Escudero-López et al., 2014). No differences were observed in PAC among the four mice groups except for ORAC which significantly increased when alcoholic solution was administered (AS group) compared to CTRL. This may be due to the interactions between the alcohol content of the plasma sample and the fluorescent compound used in the assay reaction (fluorescein), as previously observed Villaño et al. (2005).

Fig. 2 shows endogenous antioxidants content (albumin, bilirubin, uric acid and glutathione (total, GSSG and GSH)) in the four mice groups (CTRL, OJ, OB and AS groups). Albumin and bilirubin concentrations were not affected in any group after the intervention period (Fig. 2A). Uric acid, GSH and total glutathione levels were significantly increased after orange beverage consumption compared to CTRL (Fig. 2A and B). Other authors showed similar results in humans ingesting moderate volume of alcoholic and/or flavonoid-rich beverages (Matthaiou et al., 2014; Modun et al., 2008; Panza et al., 2008; Rajdl et al., 2007). Both OJ and AS groups were not different in relation to CTRL. Thus, a synergistic effect between the bioactive compounds and the alcohol contained in orange beverage may occur in order to enhance these endogenous antioxidants. This could be an advantage of orange beverage with respect to the orange juice. GSH plays an important role in providing protection against oxidative damage. Thus, an increase in the concentration of glutathione (total and GSH) might be due to a lower degree of oxidative stress in the organism caused by an improvement in the antioxidant defense by the dietetic antioxidants. For uric acid content

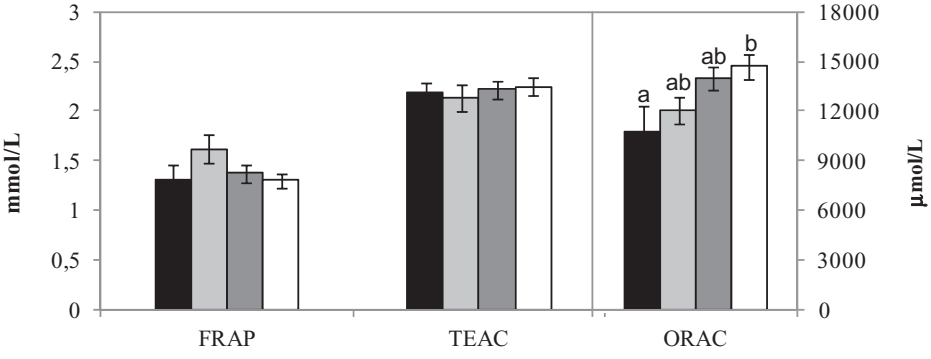

Fig. 1. Plasma antioxidant capacity in control (CTRL: water), OJ (orange juice 1:10), OB (orange beverage 1:10) and AS (aqueous alcoholic solution 1:1000) groups of mice. Dilution factors 1:10 and 1:1000 are based on a proportional intake of 250 mL/day – one serving – in humans and of alcohol in 250 mL orange beverage/day, respectively. FRAP and TEAC values (mmol/L) are on the left axis and ORAC values (μmol/L) are on the right. Black bars: CTRL; light gray bars: OJ group; dark gray bars: OB group; white bars: AS group. Data are expressed as mean ±SEM. Values with different letters indicate significant difference by one-way analysis of variance followed by Tukey's test ($p < 0.05$).

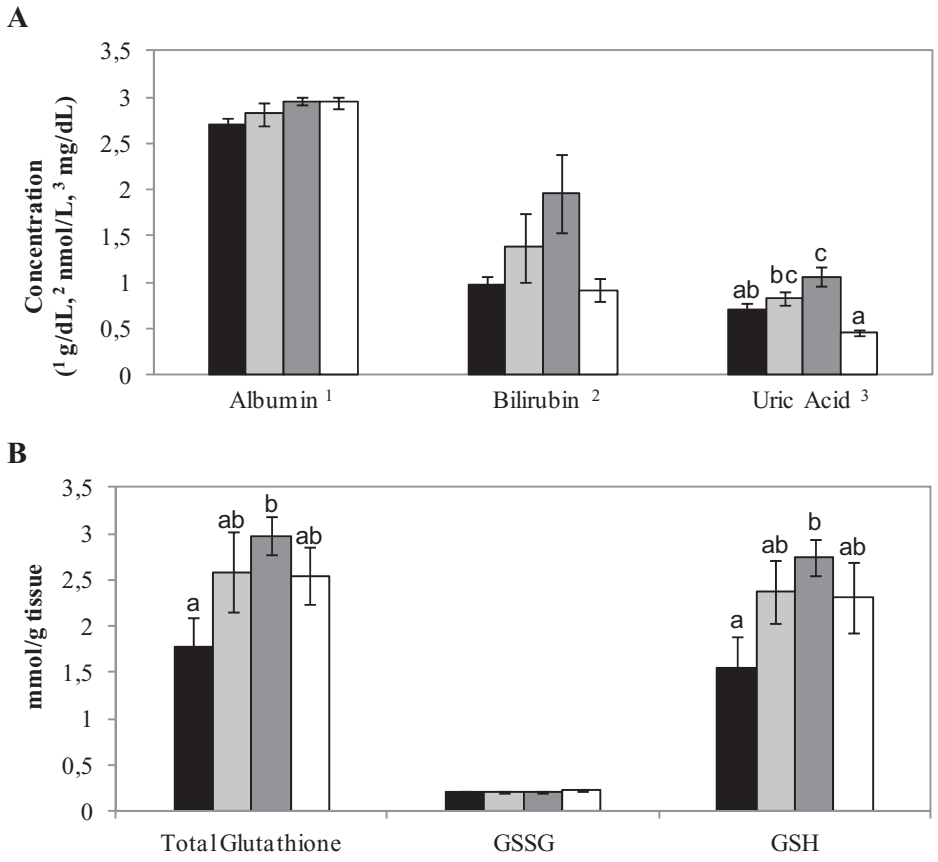

Fig. 2. Endogenous antioxidants content in control (CTRL: water), OJ (orange juice 1:10), OB (orange beverage 1:10) and AS (aqueous alcoholic solution 1:1000) groups of mice. Dilution factors 1:10 and 1:1000 are based on a proportional intake of 250 mL/day – one serving – in humans and of alcohol in 250 mL orange beverage/day, respectively. (A) Albumin (g/dL), bilirubin (mmol/L) and uric acid (mg/dL) values. (B) Total, oxidized (GSSG) and reduced (GSH) glutathione values (mmol/g). Black bars: CTRL; light gray bars: OJ group; dark gray bars: OB group; white bars: AS group. Values with different letters indicate significant difference by one-way analysis of variance followed by Tukey's test ($p < 0.05$).

the additional significant differences observed between OB and AS groups and OJ *vs* AS groups may also indicate a main contribution of the bioactive compounds in this case, and a minor effect exerted by alcohol content (Fig. 2A). Flavonoids consumption may increase plasma uric acid, although the underlying mechanisms are not elucidated (Lotito and Frei, 2006). Alcohol induces the lactate metabolism which reduces the renal clearance of uric acid (Burch and Kurke, 1968). On the other hand, fructose contained in both orange beverage and orange juice could also intervene in the uric acid enhancement. Fructose metabolism leads to a transient decrease in hepatic ATP and inorganic phosphate which are important inhibitors of 5′-nucleotidase and AMP deaminase, respectively, and thus increase degradation of AMP to uric acid (Heuckenkamp and Zöllner, 1971).

Fig. 3 shows the antioxidant enzyme activities (CAT, SOD, GR and GPx) in the four mice groups (CTRL, OJ, OB and AS groups). The antioxidant enzyme activities were not affected significantly by orange beverage intake compared to CTRL. These results are consistent with

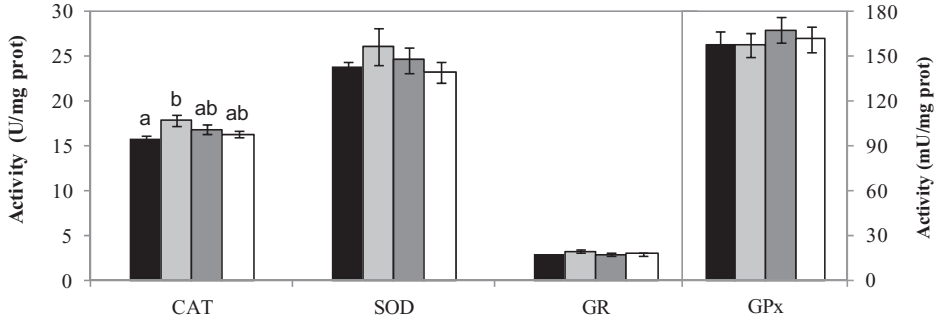

Fig. 3. Antioxidant enzyme activities in control (CTRL: water), OJ (orange juice 1:10), OB (orange beverage 1:10) and AS (aqueous alcoholic solution 1:1000) groups of mice. Dilution factors 1:10 and 1:1000 are based on a proportional intake of 250 mL/day – one serving – in humans and of alcohol in 250 mL orange beverage/day, respectively. Catalase (CAT), superoxide dismutase (SOD) and glutation reductase (GR) values (U/mg prot) are on the left axis and glutathione peroxidase (GPx) values (mU/mg prot) are on the right. Black bars: CTRL; light gray bars: OJ group; dark gray bars: OB group; white bars: AS group. Values with different letters indicate significant difference by one-way analysis of variance followed by Tukey's test ($p < 0.05$).

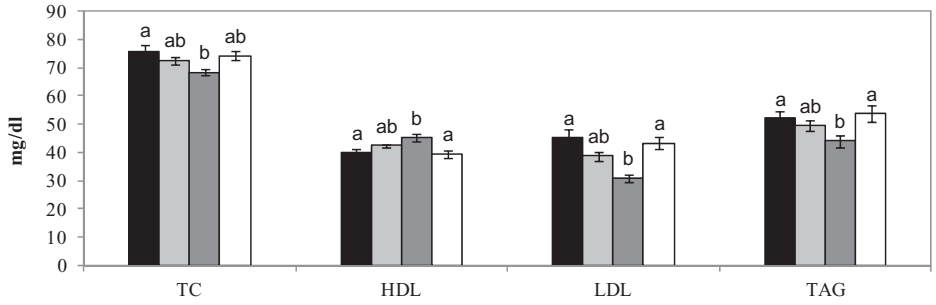

Fig. 4. Lipid profile in control (CTRL: water), OJ (orange juice 1:10), OB (orange beverage 1:10) and AS (aqueous alcoholic solution 1:1000) groups of mice. Dilution factors 1:10 and 1:1000 are based on a proportional intake of 250 mL/day – one serving – in humans and of alcohol in 250 mL orange beverage/day, respectively. Total cholesterol (TC), HDL, LDL and TAG values (mg/dL). Black bars: CTRL; light gray bars: OJ group; dark gray bars: OB group; white bars: AS group. Values with different letters indicate significant difference by one-way analysis of variance followed by Tukey's test ($p < 0.05$).

others reported in human studies showing no significant effect on antioxidant enzymes after beer or red wine consumption (Van der Gaag et al., 2000). The only significant difference obtained among the four mice groups was in CAT activity for OJ group *vs* CTRL. Since levels of endogenous antioxidants did not increase in OJ group, CAT increase could be a defense mechanism against natural oxidative stress.

3.2. Effect of orange beverage intake on lipid profile

Fig. 4 shows the levels of TC, HDL, LDL and TAG in the four mice groups (CTRL, OJ, OB and AS groups). Values of TC, LDL and TAG were significantly lower in OB group than CTRL. Furthermore, HDL level was significantly increased in OB group compared to CTRL. In contrast, OJ and AS groups did not present significant changes in their lipid profile compared to CTRL. Thus, a synergistic effect between the bioactive compounds and the alcohol content of orange beverage may also occur. This would suggest other additional healthy effect of orange beverage compared to orange juice. Moreover, the OB group was significantly different with respect to AS group for HDL, LDL and TAG levels which could reveal a greater effect of the bioactive compounds in comparison to the alcohol to improve the lipid profile. Flavonoids and their colonic and human metabolites were proposed as blood cholesterol-lowering agents and enhancers of HDL in different *in vivo* studies (Cesar et al., 2010; Zanotti et al., 2015). It was hypothesized that these compounds may mediate this effect via reduction of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity, a key enzyme in cholesterol synthesis, and increasing the expression of LDL receptors in the liver (Lee et al., 1999). These compounds were also shown to reduce the net secretion of apolipoprotein B, which in turn may help in inhibiting cholesterol ester synthesis (Borradaile et al., 1999). On the other hand, plasma cholesteryl ester transfer protein inhibition could be a possible mechanism for the elevation of plasma HDL concentration (Lam et al., 2008). In addition to flavonoids, moderate consumption of alcohol has been shown to increase levels of HDL in different interventional studies (Brien et al., 2011). Humans with high cardiovascular risk showed HDL increase after red wine and gin interventions, but their levels remained constant with dealcoholized red wine (Chiva-Blanch et al., 2013).

3.3. Effect of orange beverage intake on lipid peroxidation

Fig. 5 shows the lipid peroxidation markers (TBARS and LDL-ox) in the four mice groups (CTRL, OJ, OB and AS groups). The TBARS concentrations were not significantly different among the four mice groups. In contrast, LDL-ox levels were significantly lower in OB group compared to CTRL. A significant difference between OJ group and CTRL was also obtained, and similar LDL-ox levels were observed in mice given orange beverage compared to those given orange juice. This implies that a potential synergistic effect between the bioactive compounds and alcohol content of the orange beverage was not evident and the bioactive compounds could be responsible for this beneficial effect, which has been previously attributed to flavonoids (Tirkey et al., 2005) or carotenoids (Tapiero et al., 2004). These antioxidant compounds could be incorporated into LDL, react with singlet molecular oxygen and peroxyl radicals generated in the process of lipid peroxidation and be oxidized. Other authors also obtained decreased levels of lipid peroxidation markers after consumption of orange juice or other beverages rich in these bioactive compounds in mice or humans (Foroudi et al., 2014; Potter et al., 2011; Saric et al., 2009). Single alcohol intake would exert some effect (no differences: OB *vs* AS groups), although lower (no differences: AS group *vs* CTRL). Moderate alcohol consumption has been proposed as protector against lipid peroxidation via decrease of LDL-ox (Xia et al., 1998).

3.4. Effect of orange beverage intake on inflammatory markers

Fig. 6 shows IL-6 and CRP levels in the four mice groups (CTRL, OJ, OB and AS groups). Orange beverage consumption did not alter CRP concentrations compared to CTRL. Rajdl et al. (2007) also did

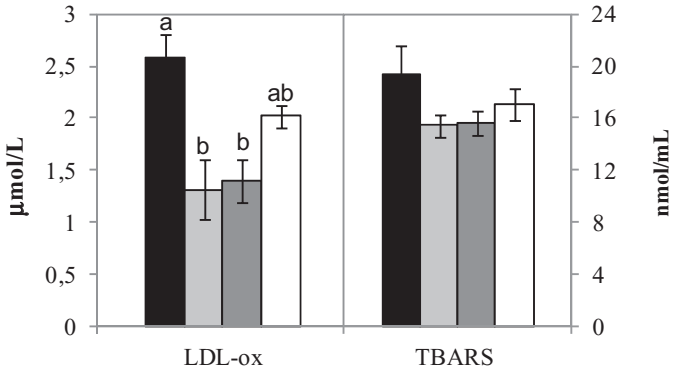

Fig. 5. Lipid peroxidation in control (CTRL: water), OJ (orange juice 1:10), OB (orange beverage 1:10) and AS (aqueous alcoholic solution 1:1000) groups of mice. Dilution factors 1:10 and 1:1000 are based on a proportional intake of 250 mL/day – one serving – in humans and of alcohol in 250 mL orange beverage/day, respectively. Oxidized low-density lipoproteins cholesterol (LDL-ox) values (µmol/L) are on the left axis and thiobarbituric acid reactive species (TBARS) values (nmol/mL) are on the right. Black bars: CTRL; light gray bars: OJ group; dark gray bars: OB group; white bars: AS group. Values with different letters indicate significant difference by one-way analysis of variance followed by Tukey's test ($p < 0.05$).

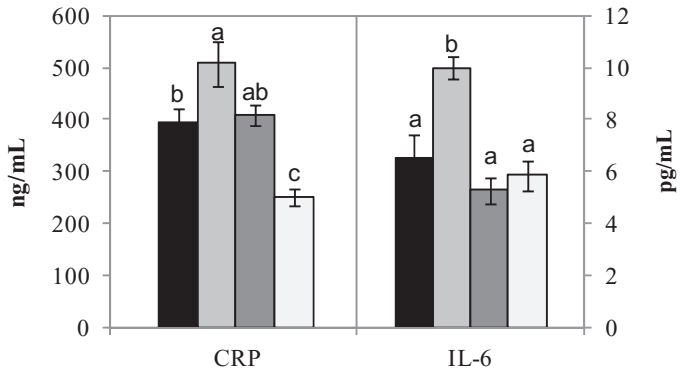

Fig. 6. Inflammatory markers in control (CTRL: water), OJ (orange juice 1:10), OB (orange beverage 1:10) and AS (aqueous alcoholic solution 1:1000) groups of mice. Dilution factors 1:10 and 1:1000 are based on a proportional intake of 250 mL/day –one serving – in humans and of alcohol in 250 mL orange beverage/day, respectively. C-reactive protein (CRP) values (ng/mL) are on the left axis and IL-6 values (pg/mL) on the right. Black bars: CTRL; light gray bars: OJ group; dark gray bars: OB group; white bars: AS group. Values with different letters indicate significant difference by one-way analysis of variance followed by Tukey's test ($p < 0.05$).

not obtain variation in CRP of humans after consumption of white wine. However, orange juice and alcoholic solution induced a significant increase and decrease in relation to CTRL, respectively. In this case, the alcohol content could be the only one responsible for this positive effect. Several studies found that light to moderate alcohol consumption is associated with lower CRP levels (Imhof et al., 2001). The increment of CRP in OJ group and the significant difference between OB and AS groups would reveal that other components, such as sugars, contained in the orange juice and the orange beverage, could induce inflammation reaction. The orange juice used contains 78.2 mg/L of total sugars (glucose, fructose, saccharose). This amount decreases to 52.3 mg/L (saccharose) in the orange beverage as a result of the fermentation process (Table 1). It is possible that sugar intake increases the intracellular concentration of glucose, followed by increased reactive oxygen species production that induces inflammatory response (Esposito et al., 2002). Dalgard et al. (2009) found increased levels of CRP after supplementation with a sugar-containing beverage. Moreover, in the OB group the moderate alcohol content would counteract the effect of residual saccharose. IL-6 concentration was unaffected after consumption of orange beverage compared to CTRL, whereas mice given orange juice also had elevated IL-6 value. The IL-6 level of AS group was similar to OB group and CTRL. Similarly, we propose that the alcohol contained in orange beverage could counteract the inflammation process induced by sugars. Therefore, intake of orange beverage did not alter the inflammation state mainly due to its moderate alcohol content.

3.5. Evaluation of the dose–response relationship of orange beverage on the cardiovascular risk markers

Table 2 shows the percentages of change calculated in the different markers evaluated between values obtained in mice which ingested orange beverage (OB (equal to 250 mL/day in humans) and OB-2 (equal to 500 mL/day) groups) with respect to their corresponding control (CTRL and CTRL-2, respectively). Markers values were not significantly different among CTRL and CTRL-2 (data not shown). The double-serving intake had greater effects on the antioxidant status than those observed previously. The significant increase of all antioxidant enzymes induced by double serving of orange beverage, not obtained with one-serving consumption, is remarkable. Moreover, a significant increase in PAC evaluated with FRAP assay was obtained. Other authors observed an increase in PAC of rat after orange juice consumption using a similar daily dose (equals 600 mL for humans) (Gorinstein et al., 2004). With regard to the endogenous antioxidants, the effect was similar with both volume intakes but bilirubin content also increased after double-serving consumption. Diverse biological pathways have been proposed whereby the flavonoid-rich fruit increases bilirubin level such as induction of hemeoxygenase through modulation of P-450-dependent metabolic activities, elimination of reactive oxygen species that may reduce the rate of decay in heme oxygenase protein expression or decreasing cellular Na+ and Ca+ contents that help to reduce edema formation (Loprinzi and Mahoney, 2015). So, two servings of orange beverage are required to induce significant and higher changes in markers of antioxidant status in mice. The higher consumption of bioactive compounds and alcohol derivative of double serving compared to single serving would explain this increased effect. The effects of double-serving on the lipid profile and lipid peroxidation were equal. On the other hand, the double serving intake showed variable effects on the markers of inflammation state (increased CRP and decreased IL-6). The increase of CRP content could be due to a higher intake of sugars previously discussed. Increased consumption of alcohol from double serving may decrease IL-6 concentration. Alcohol consumption has been shown to increase, decrease or not affect IL-6 concentrations, depending on alcohol consumption level. Studies focusing on a low content consumption report a decrease in IL-6 concentration (Marques-Vidal et al., 2012). More detailed studies are necessary to fully establish the effect of the orange beverage on the inflammation status evaluating others markers.

4. Conclusion

Orange beverage may protect against cardiovascular risk factors in healthy mice by (1) enhancing in a dose dependent manner antioxidant status via increase of antioxidant enzyme activities and/or endogenous antioxidants, (2) improving lipid profile and lipid peroxidation, and (3) preventing inflammation state. The orange beverage consumption could produce greater beneficial effects than

Table 2
Effect of the volume of orange beverage ingested on cardiovascular risk biomarkers.

Biomarkers	One serving[a]	Double serving[b]
ORAC	↑12	↑18
FRAP	↑4	**↑30**
TEAC	↑1	0
Albumin	↑10	↑3
Bilirubin	↑102	**↑83**
Uric acid	**↑49**	**↑46**
Total glutathione	**↑68**	**↑47**
Reduced glutathione	**↑76**	**↑53**
Oxidized glutathione	↑4	↑4
Catalase	↑7	**↑41**
Superoxide dismutase	↑3	**↑42**
Glutathione peroxidase	↑6	**↑53**
Glutathione reductase	↑2	**↑38**
Total cholesterol	**↓10**	**↓13**
LDL	**↓32**	**↓60**
HDL	**↑14**	**↑13**
TAG	**↓15**	**↓12**
Thiobarbituric acid reactive substance	↓19	↓6
Oxidized low-density lipoprotein	**↓46**	**↓59**
C-Reactive protein	↑3	**↑13**
IL-6	↓19	**↓14**

Values represent the percentages of change in biomarkers between the values obtained in mice groups ingesting a volume equal to [a] 250 or [b] 500 mL/day in humans of orange beverage: OB (n = 8) and OB-2 (n = 18), respectively, and their corresponding control (CTRL (n = 8) and CTRL-2 (n = 18)).
Values in bold indicate significant changes from the control ($p < 0.05$).
↑, Increase; ↓, Decrease; ORAC, oxygen radical absorbance capacity; FRAP, ferric reducing antioxidant power; TEAC, trolox equivalent antioxidant capacity.

orange juice due to possible synergistic effects between the bioactive compounds and the moderate alcohol content. In relation to the beverage volume, the intake of one daily serving (250 mL) would be enough to decrease the cardiovascular risk, although the intake of double serving (500 mL) may be more beneficial to enhance the antioxidant status of the body. Further studies are required to evaluate the potential beneficial effects of the novel orange beverage in the treatment of cardiovascular disease.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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