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RESEARCH ARTICLE

Comparative effect of resveratrol, carnosic acid and hernandulcin on target enzymes and biochemical markers linked to carbohydrate and lipid metabolism in mice

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ABSTRACT

Resveratrol (RV), carnosic acid (CA) and hernandulcin (HE; a non-caloric sweetener) are envisioned as promising nutraceuticals to design new functional foods for improving lipid and carbohydrate metabolism. This study aimed to investigate the *in vitro* inhibitory effect of these molecules on specific enzyme targets and their capacity to improve distinctive markers associated to carbohydrate and lipid metabolism in murine model. The enzymes explored were alpha-amylase, alpha-glucosidase, and pancreatic lipase whereas ICR male mice were used for *in vivo* testing. Saturation curves (10-200 μM mL^{-1}) and Lineweaver-Burk regressions suggested that RV, CA and HE exerts non-competitive inhibition on pancreatic lipase, alpha-glucosidase and alpha-amylase but, CA produced a strong competitive activity on alpha-amylase. RV was more effective to inhibit alpha-glucosidase (IC_{50}, 22.1 μM) whereas CA was the most effective to inhibit both alpha-amylase (IC_{50}, 11.7 μM) and pancreatic lipase (IC_{50}, 31.5 μM). The effects of the oral administration of RV (300 mg/kg) HE (100 mg/kg) and CA (100 mg/kg) as well as the simultaneous administration of the three compounds at the same concentration was also explored in normoglycemic and diabetic mice. In addition, the prolonged administration of these substances combined with hypercaloric/atherogenic diet for 30 days was performed. Our results revealed a clear modulatory activity in both postprandial glucose and triglyceride levels as well an improvement in biochemical markers of mice treated with hypercaloric/atherogenic diet. The administration of HE produced a notable change ($p < 0.01$) in postprandial glucose assimilation at 60 min post-treatment in diabetic mice, whereas the other two compounds exerted a stronger depletion of glucose levels from 30 to 120 min post-treatment. A similar trend was recorded by RV and CA in postprandial triglyceride content, however, the latter compound was more effective ($p < 0.05$) at lower doses than RV. The simultaneous administration of the three compounds produced a significant improvement ($p < 0.01$) in biochemical parameters associated to carbohydrate (insulin and glucose) and lipid metabolism (total cholesterol, LDL-c, HDL-c, triglycerides, leptin, and adiponectin). Outstandingly, the mixture of the three compounds was more effective ($p < 0.01$) than the administration of sole compounds to ameliorate the side effects of the hypercaloric/atherogenic diet. Finally, the body weight of treated mice significantly decreased (from 5 to 20%; $p < 0.05$) in comparison with mice only fed with hypercaloric/atherogenic diet. Our results suggest that mixtures of RV, HE and CA may work better than their sole administration in mice and part of their biological activity could be associated with their inhibitory properties on the enzyme targets evaluated in this investigation.

Keywords: Carnosic acid; Hernandulcin; Resveratrol; Hypoglycemic; Hypolipidemic; Murine model; Target enzymes

INTRODUCTION

Bioactive molecules from plant sources represent an alternative to treat pandemic disorders such as obesity, type 2 diabetes mellitus and cancer (Kato, 2019). As a consequence of the co-evolution between plants and humans, the metabolism of latter organisms has adapted to vegetable consumption and fortunately, these

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substances may produce substantial improvements in human physiology (Taroncher et al. 2021). Now, these natural compounds are tagged as nutraceuticals because of the beneficial effects that exert in human health (Taroncher et al. 2021; Shrestha et al. 2021). The molecules considered as nutraceuticals need to be extensively tested to prove their biological activity, nevertheless, one clear limiting factor is that regarded to their low-dependent accumulation in natural sources (Taroncher et al., 2021; Shrestha et al., 2021). This is the particular case of resveratrol (RV), carnosic acid CA) and hernandulcin (HE) which are found in low concentrations in the natural sources in which they are biosynthesized (Villa-Ruano and Pacheco-Hernández, 2020). RV (3,5,4'-trihydroxy-trans-stilbene) is a natural stilbene found in several berries (i.e. grapes and blackberries) which possesses a wide spectrum of nutraceutical activities mainly related to *in vivo* antioxidant capacity (Zhang et al., 2021). In addition, this phenolic compound exerts antiinflammatory, anti-platelet aggregation, anti-atherogenic, oestrogen-like, immunomodulatory and chemopreventive activities (Zhang et al., 2021). On the other hand, CA is highly accumulated in sage and rosemary and visualized as a promising nutraceutical because of its inhibitory properties on pancreatic lipase (antiobesity), antiproliferative, anxiolytic and anti-metabolic syndrome properties (Birtić et al., 2015). Such beneficial effects are apparently produced in a dose-dependent manner (Birtić et al., 2015). Currently, rosemary-standardized extracts containing CA have been recognized with the "Generally as Safe" status in the United States granted by the Food and Drug Administration (Villa-Ruano and Pacheco-Hernández, 2020). Due to this fact, several patents claiming the use of this diterpene as a food additive have been emerged. Conversely, HE is sweetener naturally biosynthesized by *Phyla scaberrima*, a medicinal plant also known as the "Mexican stevia" (Villa-Ruano and Pacheco-Hernández, 2020). HE is basically a sesquiterpene derived from bisabolol that shows high stereospecificity and currently, there is not enough information on their biological activity (Villa-Ruano and Pacheco-Hernández, 2020). Nevertheless, some insights on their antifungal and weak antibacterial properties have recently been reported (Villa-Ruano et al., 2020). Considering that this natural sweetener is a sesquiterpene with several potentialities, more investigation is required to elucidate its possible application for therapeutic aims. Recent efforts of our research group were focused in the controlled production of RV, HE and CA through biotechnological platforms which may exert possible benefits under moderate consumption (Villa-Ruano et al. 2020; 2021; 2023). Specifically, few is known on the biochemical effect of these compounds at the kinetic level for enzymes involved in fat and lipid metabolism. In the same context, the synergistic *in vivo* action of RV, CA and HE for improving biochemical parameters associated with the same metabolism, need to be addressed. A is widely known, obesity and related comorbidities such as type 2 diabetes mellitus (DM2), hypertension, dyslipidemia and diverse types of cancer have been dramatically increased around the world (Yamada et al. 2023). This trend is linked to a highly diabetogenic environment in which hypercaloric diets are implicit (Yamada et al. 2023). The observed trends strongly suggest that the prevalence of DM2 in third world countries will continue to rise in the next couple of years if preventive measures are not applied (Zhang et al. 2023). Then, the gradual use of nutraceuticals in the form of supplements or food additives may help to ameliorate the side effects of these diseases. Due to the latter arguments, this work was focused in the evaluation of RV, CA and HE in specific enzyme targets and biochemical markers of murine model in order to compare their modulatory activity on lipid and carbohydrate metabolism.

MATERIALS AND METHODS

Chemicals, reagents, and enzymes

Ethanol (HPLC grade) used to emulsify authentic standards of RV, CA and RV was from J.T. Baker (Radnor, PA, USA). Streptzotocin, alpha-glucosidase (AG), alpha-amylase (AA), and pancreatic lipase (PL), triolein, p-Nitrophenyl-α-D-glucopyranoside and 4-nitrophenyl-4,6-ethylidene-a-D-maltoheptaoside, cholesterol, colic acid, sucrose and the kits for determining insulin, leptin and adiponectin were purchased from Sigma-Aldrich Co. (St. Louis MO, USA). Glucose solution for oral glucose tolerance tests were of the label GlucoX (Pharmalab, Mexico). Kits for determining plasma glucose, total cholesterol, LDL-c, HDL-c and triglycerides were from Spinreact® (Sta. Coloma, Spain). Pork lard was from Pragana (S.A de C.V. México). Heparin tubes were from BD Vacutainer® (Becton, Dickinson, Mexico). Corn oil was from Mazola (S.A. de CV Mexico).

Sources of RV, HE, and CA

RV was constantly obtained by fermentation process using the engineered yeast strain pESC-Pa4CL1- PcPKS5 previously reported by Villa-Ruano et al. (2020). HE was produced and purified through the cell suspension system of *Phyla scaberrima* fed with (+)-epi-α-bisabolol as a biosynthetic precursor (Villa-Ruano et al., 2021). CA was produced and isolated from batch cultures using cell suspensions of *Lepechinia meyenii* treated with miltiradiene/abietatriene as biosynthetic precursors (Villa-Ruano et al., 2023).

Assays on target enzymes

The enzymatic reactions were performed with AG, AA, and PL. The effect of RV, HE and CA was explored at

kinetic level through dose-response curves of 10-200 μM. Blocking properties on AG, AA, and PL were determined using 1U of each enzyme and two distinct concentrations of RV, HE and CA (50 and 100 μg mL^{-1}) as potential inhibitors. Lineweaver-Burk regressions were performed using GraphPad Prims 7.0 in order to determine the type of inhibition exerted by each molecule and the values of K_m and V_{max} were calculated using the same linear regression in each enzyme (Villa-Ruano et al. 2013; 2017). *p*-Nitrophenyl-α-D-glucopyranoside and 4-nitrophenyl-4,6-ethylidene-a-D-maltoheptaoside were used as substrates for AG and AA, respectively. The hydrolytic activity of both enzymes was recorded at 405 nm as previously described (Villa-Ruano et al., 2017). The inhibitory examination on PL was determined by the hydrolysis of fatty acids from triolein at 340 nm (Villa-Ruano et al., 2013). The IC_{50} of each molecule was calculated by linear regression considering the specific activity of each enzyme under our experimental conditions. All assays were replicated twenty-five times (n=25) for each enzyme

Animals

In vivo assays were done with ICR male mice maintained at 25°C and 60% relative humidity in normal photoperiod (12 h light, 12h dark) feed with water *ad libitum*. Experiments were done under approval of the bioethical committee of the Interdisciplinary Group of Natural Resources (Code P1577) on the basis of animal welfare described in the Mexican norm NOM-062-ZOO-1999. ICR male mic fed with standard laboratory LabDiet® of 10 weeks old weighting approximately 30 g were used for further experiments.

Evaluation of RV, HE, and CA on postprandial glucose levels

All formulations were administered by oral gavage to normoglycemic and streptozotozin diabetic mice showing glucose levels higher than 200 mg/dL (Rivera et al., 2020). Streptozin diabetic mice were prepared in accordance with Rivera et al. (2020). Oral glucose tolerance tests (OGTT) were done with a soluble glucose solution Glucox ^{MR} (1.5 g/kg). Three experimental groups of 10 mice (n=10) were separately fed with soluble glucose solution mixed with RV (300 mg/kg), HE (100 mg/kg), and CA (100 mg/kg). These compounds were dissolved in 10% ethanol before mixed with glucose solution. A fourth experimental group was fed with same glucose solution combined with a mixture of the three compounds at the same concentration. These concentrations were chosen on the basis of previous assays performed in murine model (Xiang et al., 2013; Wang et al., 2020). The final volume of the formulations administered to the mice was 100 μL. The control groups comprised normoglycemic and diabetic mice subjected to standard OGTT. Glucose levels were measured from blood extracted from the tail vein at 0, 15, 30, 60 and 120 min and conserved in heparin tubes for immediate analysis using the Spinreact® kit as reported by Rivera et al. (2020).

Evaluation of RV, HE, and CA on postprandial triglyceride accumulation

As for anti-hyperglycemic tests, anti-hypertriglyceridemic tests were performed with four groups of ten mice (n=40) administered by oral gavage with corn oil (2 mL/kg) mixed with RV (300 mg/kg body weight) HE (100 mg/kg body weight), CA (100 mg/kg body weight) and a mixture of the three compounds at the same concentration. Control groups were only administered with corn oil (2 mL/kg) and water (2 mL/kg). Blood samples were extracted from the tail vein at 0, 1.5, 3, 4.5, and 6 h and stored in heparin tubes for immediate analysis. Blood plasma was obtained by centrifugation at 3500 g and then subjected to triglyceride quantification using the Spinreact® kit in accordance with Villa-Ruano et al. (2018).

Oral administration of RV, HE and CA in mice simultaneously treated with hypercaloric/atherogenic diet

Four groups of 10 male mice (n=40) were orally administered one time a day for 30 days with a hypercaloric/atherogenic diet (2 mL/kg body weight). The formulation of the atherogenic diet consisted in 1% cholesterol, 0.5% colic acid, 5% pork lard and 10% sucrose. The diet of the three groups was enriched with RV (300 mg/kg body weight) HE (100 mg/kg body weight) and CA (100 mg/kg body weight). A fourth experimental group was treated with the same hypercaloric/atherogenic diet containing a mixture of RV (300 mg/kg body weight) HE (100 mg/kg body weight) and CA (100 mg/kg body weight). Two control groups of ten mice (n=20) were treated with hypercaloric/atherogenic diet and with a standard laboratory diet for mice (LabDiet®; 2 g/kg body weight), respectively. Triglycerides and cholesterol (total, LDL and HDL) were measured at 10, 20 and 30 days using the SPINREACT® kit. The levels of glucose, insulin, leptin and adiponectin were measured at 5, 15 and 28 days using commercial Spinreact® and ELISA Kits from Sigma-Aldrich Co. (St. Louis MO, USA) according to manufacturer instructions. Body weight was measured every seven days during 28 days.

Statistical analysis

Analysis of Variance and Dunnett´s test ($p < 0.05/<0.01$) were conducted to determine statistically significant differences among the biochemical parameters of the animal groups assayed. Data were processed using GraphPad Prism 7.02 software.

RESULTS AND DISCUSSION

Inhibitory activity on enzymes linked to carbohydrate and lipid metabolism

The kinetic parameters for AG, AA and PL are shown in Table 1. Optimal specific activity (100% activity) for AG, AA, and PL was 0.034, 0.063, 0.085 mM min^{-1}, respectively. The values of K_m and V_{max} of each precursor in the assayed enzymes showed clear divergences which derived from their affinity to specific substrates. Interestingly, RV produced non-competitive inhibition in all enzymes (Fig. 1; Table 2). Previous studies revealed that RV exerts more potent inhibitory activity on AA than on PL (Carpéné et al., 2019). This stilbene causes a fast depletion in glucose transport of mature adipocytes by blocking the effect of insulin-like lipogenic agents (Carpéné et al., 2019). Similarly, RV and related derivatives such as viniferin enantiomers exert an inhibition in AA (Mattio et al., 2019). Inhibition of AA and PL by current commercial drugs such as acarbose and orlistat reduces the bioavailability of oligosaccharides and triglycerides avoiding both hyperglycemia and hyperlipidemia during postprandial situation (Zhang et al., 2021). However, undesirable side effects such as flatulence, abdominal pain, and diarrhoea are often reported during the administration of these inhibitors (Mattio et al., 2019). The results of kinetic analysis suggest that RV could elude fat accumulation by blocking the activity of enzymes linked to carbohydrate and lipid metabolism in gut lumen. According to our kinetic studies, CA exerted a non-competitive inhibition on PL and AG but, competitive inhibition on AA (Fig. 1; Table 2). These data partially match with those reported by Ninomiya et al. (2004) and Sheng et al. (2018) regarding the effect of these molecules on same enzymes. To the best of our knowledge, the non-competitive effect of HE had not been reported before. Our results suggest that this non-caloric sweetener is able to block AA, AG, and PL (Fig. 1) but, the estimated IC_{50} found for the three enzymes indicates that high amounts of HE are required to decrease their specific activity (Table 3). According to IC_{50}, RV was more effective to inhibit AG whereas CA was the most effective to inhibit both AA and PL.

Table 1: Kinetic parameters for pancreatic lipase, alpha-glucosidase and alpha-amylase under standard conditions

Enzyme	Vmax (100% specific activity)	Km
Pancreatic lipase	0.085 mM min^{-1}	0.31 Mm
alpha-Glucosidase	0.034 mM min^{-1}	0.12 Mm
alpha-Amylase	0.063 mM min^{-1}	0.23 mM

Effect of RV, CA, and HE in mice subjected to oral glucose tolerance test

The administration of HE (100 mg/kg body weight) did not produce substantial changes in normoglycemic but, a significant hypoglycemic effect ($p < 0.01$) was observed at 60 min in diabetic mice (Fig. 2A-B). However, no statistically significant differences were observed in other points of the OGTT in both experimental groups. Previous studies state that alpha-bisabolol (HE's biosynthetic precursor) produced an inhibitory activity on AG and AG (Yin et al., 2014; Capetti et al., 2020). According to our results, HE may exert non-

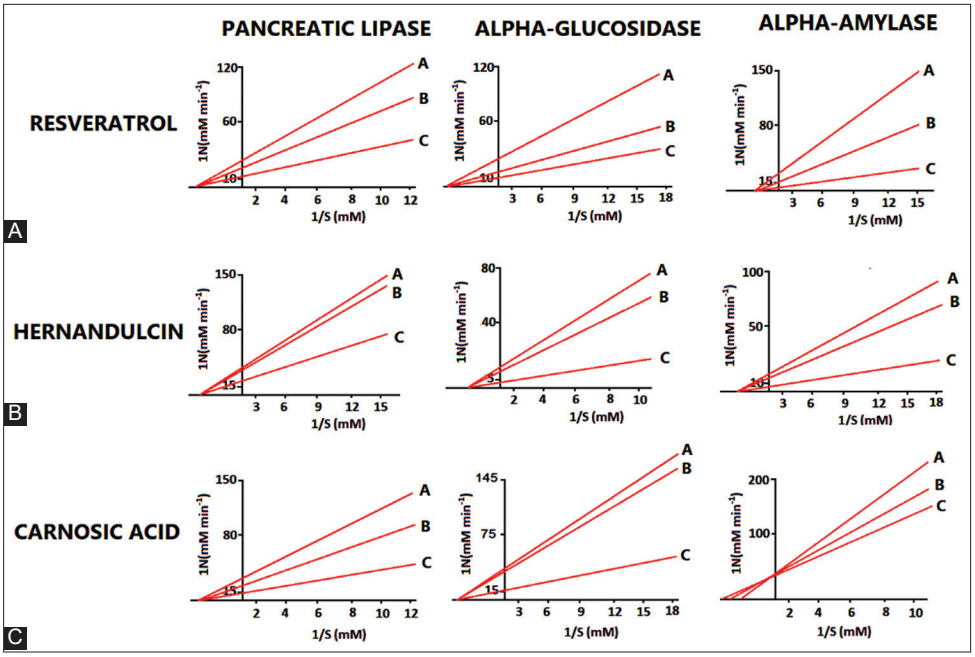

Fig 1. Lineweaver-Burk plots for resveratrol, hernandulcin and carnosic acid on alpha-glucosidase, alpha-amylase and pancreatic lipase. The effect of 100 (A) and 50 μg mL^{-1} (B) on standard kinetics (C) is shown.

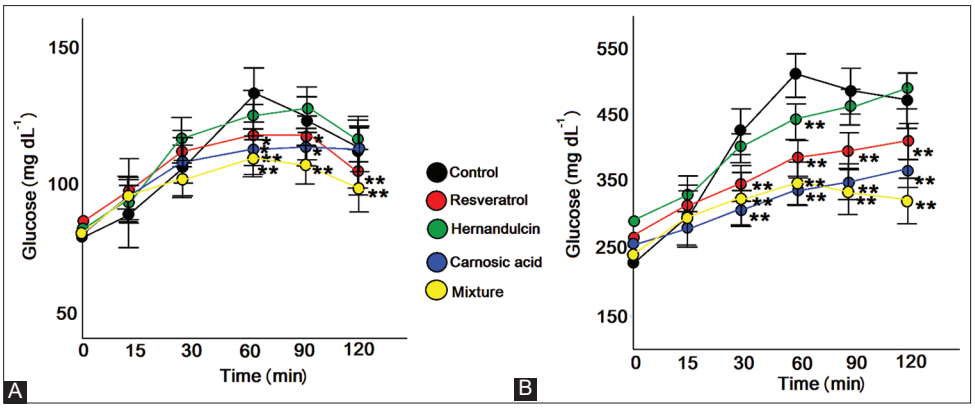

Fig 2. Oral glucose tolerance test performed with the simultaneous administration of resveratrol (300 mg/kg BW) carnosic acid (100 mg/kg) and hernandulcin (100 mg/kg) and a mixture of the three natural products at the same concentration in nomoglycemic (A) and (B) diabetic mice. The kinetics are presented as means ± SD of ten replicates (n=10) and statistically significant differences were estimated at $p < 0.05$ (*) and $p < 0.01$ (**) by ANOVA-Dunnett´s tests. These differences were determined between treated mice versus untreated mice.

Table 2: Effect of resveratrol, hernandulcin and carnosic acid on the kinetic parameters of pancreatic lipase, alpha-glucosidase and alpha-amylase under *in vitro* conditions

Compound	Pancreatc lipase (Vmax/Km)	alpha-Glucosidase (Vmax/Km)	alpha-Amylase (Vmax/Km)
Resveratrol	0.037 mM min^{-1}/0.030 mM	0.014 mM min^{-1}/0.12 mM	0.043 mM min^{-1}/0.24 mM
Hernandulcina	0.053 mM min^{-1}/0.031 mM	0.019 mM min^{-1}/0.13 mM	0.050 mM min^{-1}/0.22 mM
Ácido carnósico	0.024 mM min^{-1}/0.031 mM	0.021 mM min^{-1}/0.12 mM	0.062 mM min^{-1}/0.31 mM

Table 3: IC_{50} of resveratrol, hernandulcin and carnosic acid on pancreatic lipase, alpha-glucosidase and alpha-amylase.

Compound	Pancreatic lipase (V_{max}/K_m)	alpha-Glucosidase (V_{max}/K_m)	alpha-Amylase (V_{max}/K_m)
Resveratrol	55.2 µM	22.1 µM	38.6 µM
Hernandulcina	83.4 µM	105.6 µM	67.9 µM
Ácido carnósico	31.5 µM	73.8 µM	11.7 µM

competitive inhibition in both enzymes. This biochemical evidence may suggest that HE could probably interact with hydrolytic enzymes of gut lumen, since the metabolism of bisabolol and its derivatives are slowly metabolized under *in vivo* conditions (Capetti et al., 2020). To the best of our knowledge, this work is he first one reporting the interaction of a natural non-caloric sweetener with both alpha-amylase and alpha-glucosidase and its beneficial effect as regulator of glucose metabolism under postprandial conditions.

RV (300 mg/kg body weight) caused a marked depletion in both normoglycemic and diabetic mice ($p < 0.01$), however, CA exerted a more potent effect at lower concentrations than RV (100 mg/kg body weight). Statically significant differences were found between mice treated with RV and CA at 60-90 min in normoglycemic mice whereas marked differences were observed in diabetic mice at 30-90 min (Fig. 2A-B). These results suggest that low concentrations of CA are more effective than high concentrations of RV to regulate postprandial glucose levels. Several clinical trials sustain that RV enhance the therapeutic effects of metformin hydrochloride which is a common hypoglycemic drug as well as improves insulin sensitivity and cardioprotection (Nanjan and Betz, 2014). On the other hand, recent studies revealed that CA administration causes an efficient depletion of postprandial blood glucose in mice (Wang et al., 2019). Interestingly, the simultaneous administration of the three metabolites produced a more evident hypoglycemic effect ($p < 0.05$) than the sole administration of CA being noticeable in diabetic mice (Fig. 2A-B). This evidence strongly suggest that these compounds may be synergistically working to exert hypoglycemic activity in mice. Then, more combinations of these metabolites should be further explored.

Anti-hypertriglyceridemic activity of RV, CA, and HE

The anti-hypertriglyceridemic activity of the three compounds was palpable after 1.5 hours but it was kept until 4.5 h post-treatment ($p < 0.05$). According to our results, the most effective treatment was that based in the mixture of the three metabolites (Fig. 3). Nevertheless, the hypolipidemic effect of the mixture was maintained until 4.5 h but, it had no statistically significant differences with that of CA administration (Fig. 3). This result suggested that the administration of pure CA may be more effective than that of the other compounds and the mixture. Interestingly, the effect of these components was negligible after 6 h post-treatment. The hypolipidemic activity of prolonged

RV administration has extensively been documented in murine model (Miura et al. 2003; Cho et al., 2008). However, few information on the regulatory activity of RV on postprandial absorption of triglycerides is currently available. This activity could probably be related to the inhibitory action of RV on pancreatic lipase which has previously been described at kinetic level (Carpéné et al., 2019). Interestingly, HE produced moderate hypolipidemic activity at dose of 100 mg kg in ICR mice. As for RV, the *in vivo* effect of HE may be related to its inhibitory activity on pancreatic lipase (Fig. 1). According to this evidence, more investigation is required to determine HE pharmacokinetics of and its possible transformation during digestion in the gut lumen. CA produced a similar effect than that of the mixture of three compounds. The effect of CA as regulator of postprandial triglyceride content in murine model has been verified (Nonomiya et al., 2004).

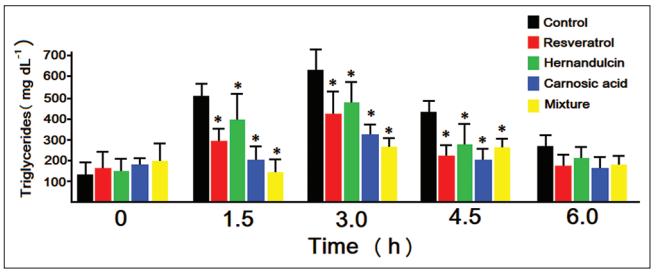

Fig 3. Anti-hypertriglyceridemic test performed with the simultaneous administration of resveratrol (300 mg/kg) carnosic acid (100 mg/kg), hernandulcin (100 mg/kg) and a mixture of the three natural products at the same concentration in ICR male mice. The kinetics are presented as means ± SD of ten replicates (n=10) and statistically significant differences were estimated at $p < 0.05$ (*) by ANOVA-Dunnett´s tests. These differences were determined between treated mice versus untreated mice (control).

In addition, the mixture of the three compounds showed no statistically significant differences in comparison with the sole administration of CA (Fig. 3). This fact strongly suggest that the administration of CA may be more efficient and less expensive than that of the mixture.

Effect of RV, CA, and HE in mice treated with hypercaloric/atherogenic diet

As expected, the oral administration of the hypercaloric/atherogenic diet increased the levels of total cholesterol, LDL-c and triglycerides during 30 days treatment (Fig. 4). Conversely, RV and CA caused a significant depletion ($p < 0.01$) of total cholesterol and LDL-c whereas the levels of HDL-c improved with the administration of both compounds as well as with the mixture (Fig. 4A-C). Nevertheless, the best improvement on lipid profile was obtained with the simultaneous administration of the three compounds (Fig. 4). CA caused a more stable hypocholesterolemic effect than RV since levels of total cholesterol at the end of the experimental period (30 days) were slightly less significant ($p < 0.05$) at day 30 than those found by CA administration. This trend was undoubtedly observed when the levels of cholesterol were contrasted with those of mice fed with hypercaloric/atherogenic diet. In all observed cases, the sole administration of RV, CA, and the mixture of the three compounds never caused a comparable depletion of total cholesterol as that found in healthy mice. In the same context, the levels of LDL-c were not equivalent to those found in healthy mice only treated with standard laboratory diet (Fig. 4A-C). These results strongly suggested that these substances may help to ameliorate hypercholesterolemia but they cannot revert it to normal levels, at least not under our experimental conditions.

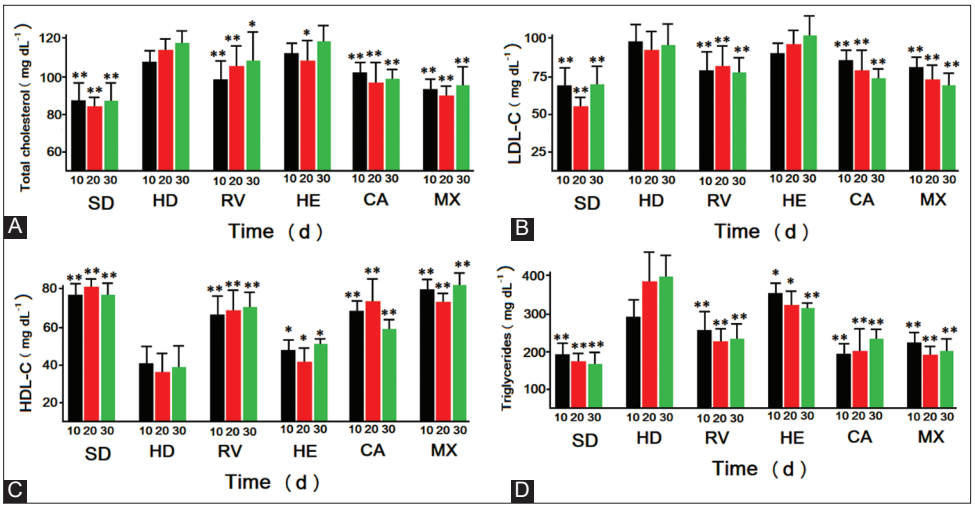

Fig 4. Effect of the oral administration of resveratrol (RV), hernandulcin (HE), and carnosic acid (CA) and the mixture of the three compounds (MX) in the levels of total cholesterol (A), LDL-c (B), HDL-c (C) and triglycerides (D) of ICR male mice during 30 days. The kinetics are presented as means ± SD of ten replicates (n=10) and statistically significant differences were estimated at $p < 0.05$ (*) and $p < 0.01$ (**) by ANOVA-Dunnett´s tests. Control groups were mice administered with standard laboratory diet (SD) and with a hypercaloric/atherogenic diet (HD). The differences were determined between treated mice and mice only feed with HD.

Unexpectedly, the administration of the mixture improved the amount of HDL-c in treated mice when contrasted with healthy mice ($p < 0.05$). Despite the hypolipidemic activity of RV under prolonged administration seems to be controversial, many studies suggest that daily doses over 100 mg/kg could reduce cholesterol levels (Akbari et al., 2020). On the other hand, a recent study states that CA induces a significant change in the levels of cholesterol in lambs (Bialek, et al., 2021). This evidence endorses the results of the present investigation which propose that this diterpene exerts a more potent hypocholesterolemic activity than RV. The administration of HE did not produce significant changes in total cholesterol or LDL-c levels during 30 days of treatment but, it induced an accumulation of HDL-c ($p < 0.05$) in comparison with mice fed with hypercaloric/atherogenic diet (Fig. 4A-C). In the same context, this sesquiterpene produced a weaker hypotriglyceridemic effect than RV and CA ($p < 0.05$) (Fig. 4D). This result partially matches with the inhibitory activity of HE on pancreatic lipase under *in vitro* conditions and suggests that frequent consumption of the non-caloric sweetener may collaterally help in regulating triglyceride metabolism. To the best of our knowledge, our work is the first evidence suggesting that HE induces favorable changes in lipid metabolism. Nevertheless, further experiments are required to elucidate the pharmacokinetics of this sesquiterpene in murine model. As for cholesterol levels, similar trends were observed by the action of RV and CA in the accumulation of triglycerides. Nevertheless, CA exerted a more potent effect than RV whereas the mixture of the three compounds produced a similar potency than sole CA (Fig. 4).

The levels of glucose increased by the administration of hypercaloric/atherogenic diet whereas mice fed with standard laboratory diet did not show significant fluctuation in their levels (Fig. 5). HE only produced a statistically significant depletion ($p < 0.05$) of glucose at day 5 but, no clear tendency was observed in subsequent days (Fig. 5A). RV, CA and the mixture of the three compounds exerted a substantial attenuation of glucose levels during the treatments, however, the hypoglycemic effect of sole CA was comparable to that of the mixture. The levels of insulin in healthy ICR male treated with standard diet were around 13 mU L^{-1} whereas those fed with hypercaloric/atherogenic diet were almost four folds higher (~40 mU L^{-1}) (Fig. 5B). Hyperinsulinemia was significantly ameliorated ($p < 0.01$) by the administration of CA and RV (20-28 mU L^{-1}) and moderately reduced by HE. Similarly, the levels of leptin were high (19 ng mL^{-1}) in the mice treated with hypercaloric/atherogenic diet but these were reduced by the simultaneous administration of the three compounds (single or combined) from day 5 to day 28 post-treatment (Fig. 5C). A contrary trend was detected in the levels of adiponectin which were low (16-6 μg mL^{-1}) in mice treated with hypercaloric/atherogenic diet but, a significant depletion of this hormone was produced by the administration of single or combined molecules (Fig. 5D). Several studies suggest that insulin resistance can be produced by diets rich in carbohydrates and lipids (Rivera et al., 2020; Fahed et al., 2022). As is known, insulin resistance is enhanced by high levels of leptin (Fahed et al., 2022), however, the addition of natural antioxidants to daily diet improves the regulation of these hormones involved in carbohydrate and lipid metabolism until normal levels (Jiang et al., 2021). Adiponectin is considered another

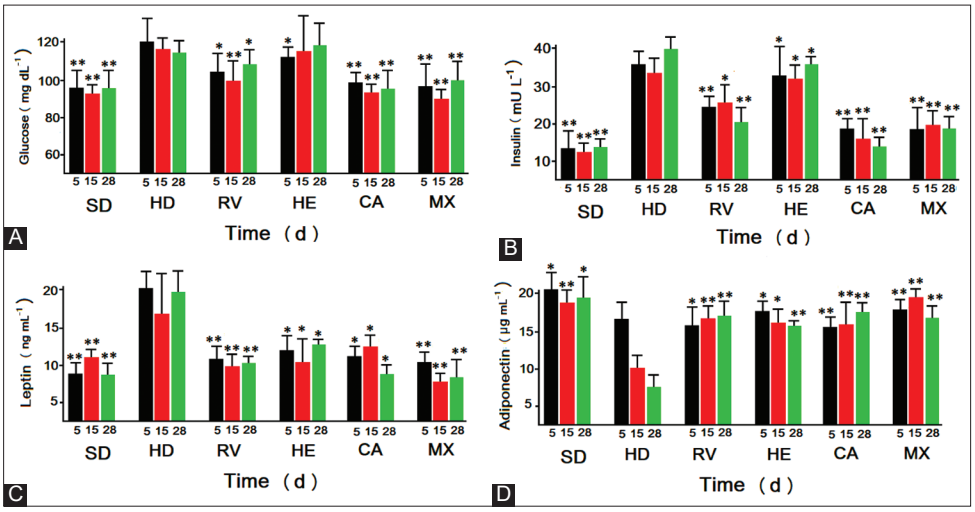

Fig 5. Effect of the oral administration of resveratrol (RV), hernandulcin (HE), carnosic acid (CA) and the mixture of the three compounds (MX) in the levels of glucose (A), insulin (B), leptin (C) and adiponectin (D) of ICR male mice during 30 days. The kinetics are presented as means ± SD of ten replicates (n=10) and statistically significant differences were estimated at $p < 0.05$ (*) and $p < 0.01$ (**) by ANOVA-Dunnett´s tests. Control groups were mice administered with standard laboratory diet (SD) and with a hyercaloric/atherogenic diet (HD). The differences were determined between treated mice and mice only feed with HD.

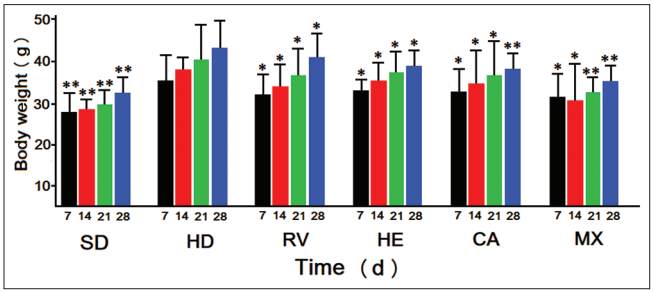

Fig 6. Body weight of ICR mice feed with resveratrol (RV), hernandulcin (HE), carnosic acid (CA) and the mixture of the three compounds (MX) during 30 days. The kinetics are presented as means ± SD of ten replicates (n=10) and statistically significant differences were estimated at $p < 0.05$ (*) and $p < 0.01$ (**) by ANOVA-Dunnett´s tests. Control groups were mice administered with standard laboratory diet (SD) and with a hyercaloric/atherogenic diet (HD). The differences were determined between treated mice and mice only fed with HD.

key biochemical parameter because of its involvement in both carbohydrate and lipid metabolism (Nguyen, 2020). It is also related with weight loss, caloric restriction and improves insulin sensitivity (Nguyen, 2020). Adiponectin signaling pathway is visualized as a relevant target for the development of new and efficient drugs to treat DM2 and other comorbidities of obesity (Nguyen, 2020). The evidences earned in the present investigation, strongly suggest that the concurrent administration of RV, HE and CA could ameliorate some diseases associated to metabolic syndrome.

Effect of RV, CA and HE on body weight of mice

The average weight of healthy ICR male mice was around 29.4 g and the administration of hypercaloric/atherogenic diet resulted in an evident gain of weight gain from day 7 (33 g) to day 28 (46.7 g) (Fig. 6). Noticeable differences were determined in mice treated with standard diet versus those treated with hypercaloric/atherogenic diet ($p < 0.01$). Mice fed with the latter diet gained approximately 30% body weight at the end of the experimental period (day 28). Interestingly, animals treated with RV, HE and CA also gained weight but, the levels were significantly lower ($p < 0.05$) than those of mice only treated with hypercaloric/atherogenic diet (Fig. 6). Interestingly, the body weight of mice treated with HE was lower (39.5 g) than that of mice treated with RV (41.2 g). Nevertheless, mice feed with CA and the mixture of the three compounds weighed 35.3 and 33.8 g, respectively. High statistically significant differences were observed at day 28 and days 21-28 in mice treated with RV ($p < 0.01$) and the mixture of the three compounds ($p < 0.01$) (Fig. 6). Nevertheless, the administration of the mixture was the best alternative to avoid the triggering of body weight in ICR male mice treated with hypercaloric/atherogenic diet. According to previous studies, RV produced a significant reduction in body weight in healthy and ovariectomized rats. As is known, ovariectomy causes body weight gaining due to the lack of estrogen production. Interestingly, RV substantially decreased body weight in this murine model (Sharma et al., 2017). Despite good results have bee obtained in standardized trials with diverse murine models, other investigations sustain that the heterogeneity of trials performed in humans cannot cross-validate the properties of RV as a redactor of body weight (Hillsley et al., 2022). This fact envisions that the beneficial effect of RV could be influenced by several environmental and genetic factors which need to be considered. The beneficial effects of CA to ameliorate disorders associated to metabolic syndrome have been reported (Zhao et al., 2015). However, few studies endorse the potential application of CA to regulate body weight (Park and Sung, 2015). According to our results, CA had better weight-losing properties than RV and HE. This fact may suggest that CA plays a preponderant role in the results observed by the simultaneous administration of the three compounds.

CONCLUSION

The oral administration of RV, CA, and HE produced substantial improvements in diverse parameters linked to carbohydrate and lipid metabolism in mice which can be probably related with is ability to interact with specific target enzymes such as alpha amylase, alpha-glucosidase and pancreatic lipase. Our results suggest that standardized mixtures of the three compounds may work better than their sole administration in mice. Nevertheless, CA produced a better improvement in postprandial glucose levels in normoglycemic and streptozotocin diabetic mice as well as prevented the triggering of cholesterol, triglycerides and glucose in mice feed with prolonged hypercaloric/atherogenic diet. Our data demonstrate that RV, CA and HE may act as a regulators of body weight and key hormones involved in lipid and carbohydrate metabolism. Further studies on the potential hepatotoxicity and pharmacokinetics are required, especially for HE which has been less studied than other nutraceuticals.

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Authors' contributions

N.V.-R., J.M.C.-F., G.L.-C. and G. M. H.-V. designed and performed the experiments with assayed enzymes and analyzed the respective data. N.V.-R., Y.P-H. and S.A. R.-G, designed and performed the assays with murine model and analyzed the respective data. G.L.-C., J.M.C.-F. and G. M. H.-V. contributed with funds and materials to perform the investigation. N.V.-R., Y.P-H., G.L.-C, and S.A. R.-G

wrote the first draft of the article. All authors read and approved the final version of this document.

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