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Article

Gender differences in biochemical markers and oxidative stress of rats after 28 days oral exposure to a mixture used for weight loss containing *p*-synephrine, ephedrine, salicin, and caffeine

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The association of *p*-synephrine, ephedrine, salicin, and caffeine in dietary supplements and weight loss products is very common worldwide, even though ephedrine has been prohibited in many countries. The aim of this study was to evaluate a 28-day oral exposure toxicity profile of *p*-synephrine, ephedrine, salicin, and caffeine mixture (10:4:6:80 w/w respectively) in male and female Wistar rats. Body weight and signs of toxicity, morbidity, and mortality were observed daily. After 28 days, animals were euthanized and blood collected for hematological, biochemical, and oxidative stress evaluation. No clinical signs of toxicity, significant weight loss or deaths occurred, nor were there any significant alterations in hematological parameters. Biochemical and oxidative stress biomarkers showed lipid peroxidation, and hepatic and renal damage ($p < 0.05$; ANOVA/Bonferroni) in male rats (100 and 150 mg/kg) and a reduction ($p < 0.05$; ANOVA/Bonferroni) in glutathione (GSH) levels in all male groups. Female groups displayed no indications of oxidative stress or biochemical alterations. The different toxicity profile displayed by male and female rats suggests a hormonal influence on mixture effects. Results demonstrated that the tested mixture can alter oxidative status and promote renal and hepatic damages.

Uniterms: *p*-Synephrine/toxicity profile. Ephedrine/toxicity profile. Salicin/toxicity profile. Caffeine/toxicity profile. Dietary supplements/evaluation. Weight loss products/evaluation.

A associação de *p*-sinefrina, efedrina, salicina, e cafeína em suplementos alimentares e produtos para perda de peso é muito utilizada em todo o mundo, embora a efedrina tenha sido proibida em muitos países. O objetivo deste estudo foi avaliar o perfil de toxicidade à exposição oral de 28 dias à associação de *p*-sinefrina, efedrina, salicina e cafeína (na proporção de 10:4:6:80 m/m respectivamente) em ratos Wistar machos e fêmeas. Diariamente, os animais foram observados quanto ao peso corporal, sinais de toxicidade, morbidade e mortalidade. Após 28 dias, os animais foram sacrificados e o sangue coletado para avaliações hematológicas, bioquímicas e de estresse oxidativo. Não se observaram sinais clínicos de toxicidade, tampouco perda significativa de peso, mortes, ou quaisquer alterações significativas nos parâmetros hematológicos. Biomarcadores do estresse oxidativo e bioquímicos mostraram peroxidação lipídica, danos renais e hepáticos ($p < 0,05$; ANOVA/Bonferroni) em ratos machos (100 e 150 mg/kg) e a redução ($p < 0,05$; ANOVA/Bonferroni) nos níveis de glutationa reduzida (GSH) em todos os grupos de machos tratados. Nas fêmeas, não houve indícios de estresse oxidativo, nem alterações bioquímicas. O diferente perfil de toxicidade entre os gêneros sugere influência hormonal nos efeitos de mistura administrada. A associação testada pode alterar o estado oxidativo e promover danos renais e hepáticos.

Unitermos: *p-Sinefrina/perfil de toxicidade. Efedrina/perfil de toxicidade. Salicina/perfil de toxicidade. Cafeína/perfil de toxicidade. Suplementos alimentares/avaliação. Produtos para perda de peso/avaliação.*

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INTRODUCTION

Overweight and obesity are highly prevalent worldwide. Many consumers are seeking strategies for reducing weight to healthier levels, including the use of dietary supplements and weight loss products. These products typically stimulate or enhance weight loss through mechanisms such as reducing hunger or increasing resting metabolism (Dwyer *et al.*, 2005). There are a large number of products being marketed for weight loss, most usually containing a mixture of ingredients including *p*-synephrine (*Citrus aurantium* L.), ephedrine (Ma Huang, *Ephedra sinica*, *Ephedra* sp.), salicin (*Salix* sp.) and caffeine (*Paullinia cupana, Cola nitida, Cola acuminata*, *Camellia sinensis*), as well as vitamins, minerals, and other herbs (Arbo *et al.*, 2008, 2009a, 2009b).

Ephedrine is an adrenergic agonist for both α and β receptors affecting both the cardiovascular and central nervous system (Schaneberg *et al.*, 2003). The use of *p*-synephrine, a biogenic amine found in *Citrus aurantium*, became very popular after the banning of ephedrine-containing dietary supplements by the Food and Drug Administration (FDA) due to the occurrence of significant adverse effects, mainly related to the cardiovascular system (Bouchard *et al.*, 2005). It quickly replaced ephedrine in supplements called "ephedra-free" due to their structural similarity (Fugh-Berman, Myers, 2004; Jordan *et al.*, 2004; Grollman, 2005).

However, like ephedrine, *p*-synephrine is an α and β adrenergic agonist and both are structurally and pharmacologically related to amphetamines (Firenzuoli *et al.*, 2005) and other adrenergic amines such as epinephrine and norepinephrine (Rossato *et al.*, 2011a). For this reason, it is believed that it may also cause adverse effects, especially on the cardiovascular system (Bouchard *et al.*, 2005). On the other hand, Rossato *et al.* (2011b) observed that only *m*-synephrine (positional isomeric form of synephrine) induced a potentially toxic effect, while *p*-synephrine showed negligible cardiotoxicity in an *in vitro* model. Arbo *et al.* (2008, 2009a, 2009b) demonstrated that extracts of both *C. aurantium* and isolated *p*-synephrine showed low toxicity in mice, even at high doses.

These facts therefore lead us to believe that reports of toxicity from this amine could be related to its association with other substances, specifically caffeine, salicin and even ephedrine in many products (Schmitt *et al.*, 2012). Although ephedrine has been banned or restricted in several countries, it can be found in some formulations due to adulterations to improve product performance (Schmitt *et al.*, 2012). Caffeine, a stimulant with α-adrenergic-like properties, acts on cardiovascular system (Kalman *et al.*, 2002) and may enhance the effects of sympathomimetics such as ephedrine (Astrup *et al.*, 1992; Haller *et al.*, 2004) and probably synephrine (Schmitt *et al.*, 2012) because their structural similarities (Pellati and Benvenuti, 2007).

Salicin is a phenolic glycoside extracted from *Salix* sp. (white willow bark), which displays analgesic properties (Zaugg *et al.*, 1997), but there is no scientific support for its presence in weight loss formulations. It is believed to enhance the effects of other substances in a synergistic pathway, extending or increasing the activity of thermogenic ingredients through prolonging ephedrine action time (Schmitt *et al.*, 2012). Acetylsalicylic acid, which belongs to the same salicylate family as salicin, showed increases in the thermogenesis effects of both ephedrine and caffeine in obese women (Horton, Geissler, 1991).

The association of *p*-synephrine with ephedrine, salicin, and caffeine is found in several marketed products, and it is known that the association of similarly acting substances can increase and/or strengthen their pharmacological actions. Acute toxicity of this association was seen by Schmitt *et al.* (2012). However, these products are usually consumed over long periods and it is necessary to evaluate the effects after repeated doses. The aim of this study was to evaluate 28 days oral exposure to a mixture of *p*-synephrine, ephedrine, salicin and caffeine (10:4:6:80 w/w respectively) in male and female rats and their actions on oxidative stress biomarkers. The relative proportions of the substances in the mixture were based on several weight loss products available on the market. However, this does not linearly correspond to products used by humans because proportions and dose indications vary greatly from product to product.

MATERIAL AND METHODS

Chemicals and reagents

Chemicals and reagents were obtained thus: (±)-*p*-synephrine (99%) from MP Biomedicals (California, USA); ephedrine (99.5%) from Aldrich (St. Louis, USA); salicin (99%) from FlukaBioChemika (Switzerland); caffeine (99%) from Merck (Darmstadt, Germany);5,5'-dithio-*bis*(2-nitrobenzoic acid) from Sigma (St. Louis, MO, USA);and HPLC grade acetonitrile, methanol and n-butanol from the Tedia Company (Fairfield, USA). Water was purified using a Milli-Q system (Millipore, Bedford, USA). All other chemicals were of analytical grade.

Animals

Experiments were performed after approval from the University Ethics Committee (deliberation number 2007982) and were carried out in accordance with current guidelines for the care of laboratory animals (Goldim, Raymundo, 1997; Olfert *et al.*, 1998). Male and female Wistar rats, weighing 306.3 ± 24.5 g and 196.9 ± 17.6 g respectively, were obtained from Central Animal House of the Institute of Basic Health Sciences, Federal University of Rio Grande do Sul. Animals were housed in 47 × 34 × 18 cm polyethylene cages under standard temperature conditions (22 ± 2 °C), controlled humidity, and a 12 h light/dark cycle. All experimental procedures were performed during the light phase of the cycle.

Repeated dose 28-day oral toxicity study

The experimental protocol was based on OECD (Organization for Economic Co-operation and Development) guideline 407 (Repeated Dose 28-day Oral Toxicity Study in Rodents). The experiment was conducted distributing the rodents in groups of 8 male and 8 female rats each. Animals were treated by oral gavage for 28 consecutive days with water (control) or 50, 75, 100, and 150 mg/kg of an aqueous mixture of *p*-synephrine, ephedrine, salicin and caffeine (proportion of 10:4:6:80 w/w respectively) administered at a constant volume of 10 mL/kg. The substance proportions in the mixture were defined considering several weight loss products available in the market.

Body weight was measured every day and animals were observed daily for signs of toxicity, morbidity, and mortality. At the end of the experiment, animals were euthanized with ketamine/xylazine (50 mg/kg and 10 mg/kg respectively) and necropsied. Selected organs (heart, liver, lung, brain, spleen, kidneys, and adrenal glands) were collected and observed for macroscopic alterations.

Biochemical and hematological analysis

After euthanasia, blood was collected from caudal vein using heparin or EDTA as anticoagulant for biochemical and hematological evaluations. In whole blood with EDTA, hemoglobin and hematocrit were performed according to ICHS (1978) and ICHS (1982) respectively. Part of the blood was centrifuged and heparinized plasma used to evaluated alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatinine using commercial kits (Labtest Diagnóstica).

Oxidative stress biomarker evaluations

Part of the blood collected with anticoagulant was centrifuged at 1500g for 10 minutes and immediately separated into plasma and erythrocytes. EDTA plasma was used to evaluate lipid peroxidation through an HPLC method with visible detection at 532 nm (Grotto *et al.*, 2007). This method quantifies malondialdehyde (MDA) levels after alkaline hydrolysis and extraction with n-butanol. Erythrocytes were used to determinate reduced glutathione (GSH), which was measured as non-protein thiols, according to Ellman (1959) modified. Aliquots (0.3 mL) of erythrocytes were added to a phosphate buffer 0.3 M (0.85 mL), pH 7.4 and the reaction was read in spectrophotometer at 412 nm after addition of 10 mM 5,5'-dithio*bis*(2-nitrobenzoic) acid (DTNB) (0.05 mL). The results were expressed as μmol/mL erythrocytes.

The whole blood collected with heparin was used to analyze antioxidant enzyme glutathione peroxidase (GPx) activity using glutathione reductase and NADPH. The method is based on the oxidation of NADPH, which is indicated by a decrease in absorbance at 340 nm (Paglia, Valentine, 1967).

Statistical analysis

Data were expressed as mean ± SEM (standard error of mean). Organ weights were determined and expressed as relative weight and were analyzed by one-way analysis of variance (ANOVA). Body weight gain was evaluated by repeated measures ANOVA. Relative body weight gain was obtained considering initial weight measurement as 100% and all gain was compared to the initial measurement. Differences between groups were evaluated by one-way ANOVA, followed by post hoc Bonferroni, when appropriate. The significance level was set at 5% ($p < 0.05$).

RESULTS AND DISCUSSION

Daily oral exposure (28 days) of *p*-synephrine associated to ephedrine, salicin and caffeine in male and female rats did not show signs of toxicity; there were also no deaths of either male or female rats during the 28-day experimental period. This suggests that the tested doses (50, 75, 100, and 150 mg/kg) presented low toxicity. In previous studies with mice, higher doses (300, 350, and 400 mg/kg) of the mixture, showed clear signs of toxicity including death, especially in males (Schmitt *et al.*, 2012).

Body weight did not significantly change during the 28-day toxicity test. However, it was possible to observe that

body weight gain was higher in females than males (Figure 1). Necropsy showed no changes in relative organ weights and no macroscopic alterations in either male or female rats.

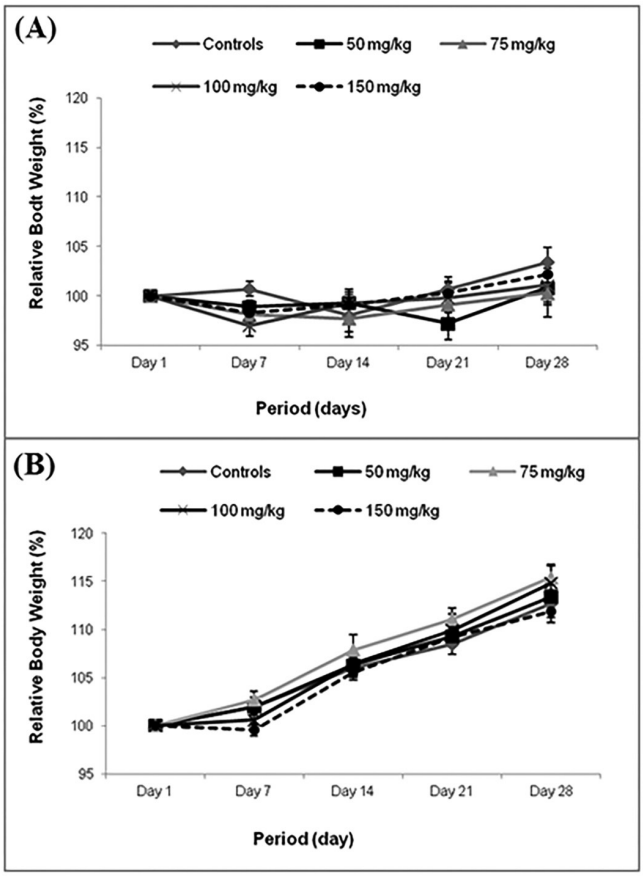

FIGURE 1 - Relative body weight gain in Wistar rats male (A) and female (B) after oral treatment for 28 consecutive days with *p*-synephrine associated to ephedrine, salicin and caffeine (10:4:6:80 w/w). Each point represent the mean ± standard error of the mean (n=8).

Animals did not lose weight in contrast to the purported aims of the products. Interestingly, the results revealed a different profile between genders. Females showed more body weight gain, demonstrating them as less susceptible to the effects of the mixture. This fact could be related to hormonal differences between males and females; further studies focusing on hormones could elucidate this point.

Hematological and biochemical analysis results are presented in Table I. No significantly alterations were observed in male or female rat hematological parameters. Biochemical analysis revealed increases ($p < 0.05$) in AST, ALT, and creatinine levels in male rats treated with 100 and 150 mg/kg of the mixture. However, there was a significant decrease ($p < 0.05$) in AST for female groups treated with 100 and 150 mg/kg of the mixture.

Males displayed an upward trend in both AST and ALT enzyme levels as dose increased. According to adopted reference values, AST is normal whereas ALT is increased and may indicate some liver damage. In the same way, creatinine levels in all male treated groups showed a progressive increase, which was significantly higher in the 100 and 150 mg/kg treated groups compared to controls ($p < 0.05$). It was also outside the normal range (0.2-0.6 mg/dL) for male Wistar rats (Giknis, Clifford, 2008) indicating possible renal damage. Although there is undeniable evidence of the nephrotoxic effects of the mixture, the mechanism responsible is unclear. It may be caused by a similar mechanism to amphetamines, whose vasoconstrictor properties predispose the renal medulla to ischemia and hypoxia. In addition, there could be direct toxic effects from the drugs and/or their metabolites, as well as enhanced oxidative stress in the kidney (Carvalho *et al.*, 2012).

The liver is a target for amphetamines and their analogues (as in the case of synephrine and ephedrine) since they are biotransformed primarily in this organ, as is caffeine. During the biotransformation process (e.g. 3,4-Methylenedioxy-methamphetamine -MDMA,

TABLE I - Hematological and plasmatic biochemical parameters evaluated in male and female Wistar rats after 28 days of treatment with a mixture of *p*-synephrine, ephedrine, salicin and caffeine (10:4:6:80 w/w)

Parameters	MALE					FEMALE				
	Control	50 mg/kg	75 mg/kg	100 mg/kg	150 mg/kg	Control	50 mg/kg	75 mg/kg	100 mg/kg	150 mg/kg
Hematocrit (%)	41.9 ± 1.1	43.4 ± 1.7	44.7 ± 1.3	43.5 ± 1.3	44.4 ± 1.0	40.7 ± 0.7	40.3 ± 0.7	41.5 ± 0.4	41 ± 0.8	43.1 ± 0.9
Hemoglobin (g/dL)	17.0 ± 0.4	17.3 ± 0.9	16.8 ± 1.1	16.7 ± 0.8	17.2 ± 0.5	14.1 ± 0.3	13.8 ± 0.3	14.7 ± 0.2	14.2 ± 0.9	14.1 ± 0.4
AST (U/mL)	66.8 ± 5.2	77.6 ± 8.3	99.4 ± 6	124.3 ± 17.3*	147 ± 19.6*	135.5 ± 17	114.1 ± 7.7	94.4 ± 3.8	87.1 ± 6.6*	86.6 ± 6.7*
ALT (U/mL)	35.7 ± 3.7	34.1 ± 2.8	43.7 ± 3.5	62 ± 5.8*	87.3 ± 12.2*	53.4 ± 10.6	54.8 ± 4.4	50.1 ± 2.1	59.0 ± 5.1	46.7 ± 7.6
Creatinine (mg/dL)	0.27 ± 0.02	0.48 ± 0.06	0.53 ± 0.04	0.65 ± 0.09*	0.65 ± 0.14*	0.4 ± 0.06	0.48 ± 0.06	0.57 ± 0.05	0.49 ± 0.04	0.6 ± 0.06

Data are expressed as mean ± standard error of the mean (n=6-8 males and 6-8 females per group). *Significantly different from their respective gender control group ($p < 0.01$) by ANOVA/Bonferroni. AST: aspartate aminotransferase; ALT: alanine aminotransferase

TABLE II - Oxidative stress biomarkers evaluated in male and female Wistar rats after 28 days of oral treatment with a mixture of *p*-synephrine, ephedrine, salicin and caffeine (10:4:6:80 w/w)

Biomarkers	MALE					FEMALE				
	Control	50 mg/kg	75 mg/kg	100 mg/kg	150 mg/kg	Control	50 mg/kg	75 mg/kg	100 mg/kg	150 mg/kg
MDA (Mm)	10.5 ± 0.6	11.2 ± 0.9	13.1 ± 0.8	15.9 ± 0.9*	17.1 ± 1.2*	13.6 ± 0.7	15.4 ± 1.0	14.1 ± 1.2	16.5 ± 1.2	17.5 ± 1.1
GSH (µmol/mL erythrocytes)	6.14 ± 0.66	4.38 ± 1.1*	3.6 ± 0.65*	3.33 ± 0.27*	3.51 ± 0.47*	4.21 ± 0.55	3.13 ± 0.3	4.32 ± 0.61	4.22 ± 0.65	3.77 ± 0.5
GPx (mol NAD/min/mL)	10.9 ± 0.9	8.1 ± 0.5	9.8 ± 2.1	12.4 ± 1.4	15.5 ± 1.1	12.0 ± 1.2	13.5 ± 1.2	19.0 ± 1.6*	16.1 ± 1.1	13.8 ± 1.3

Data are expressed as mean ± standard error of the mean (n=6-8 male and 6-8 female per group). *Significantly different from their respective gender control group ($p < 0.01$) by ANOVA/Bonferroni. MDA: malondialdehyde; GSH: reduced glutathione; GPx: glutathione peroxidase.

a hallucinogenic amphetamine) free radicals can be generated (Carvalho *et al.*, 2004) and cause liver damage. Custodio *et al.* (2010) studied mitochondria involvement in MDMA and 4-methylthioamphetamine (MTA) produced liver toxicity and found that, depending on dose, both amphetamine derivatives (especially MTA in high concentrations) cause mitochondrial dysfunction, which is another mechanism for hepatotoxicity.

Hepatic enzyme alterations in female groups were opposite to those in male groups; their AST values significantly decreased in the 100 and 150 mg/kg treated groups related to controls ($p < 0.01$). However, the values were within normal limits for female Wistar rats (63-175 U/L) (Giknis, Clifford, 2008).

There are many reports of gender being a critical factor in drug-induced liver injury (Hemieda, 2007; McConnachie *et al.*, 2007; Dever, Elfarra, 2008). Moreover, most of those reports demonstrate that males were more susceptible to hepato toxin-induced liver injury than females. Studies have reported that bioactivation and estrogen concentration are mainly responsible for the gender-dependent discrepancy in liver injury (McConnachie *et al.*, 2007; Dever, Elfarra, 2008; Liang *et al.*, 2011). Also, gender differences in pharmacokinetics and pharmacodynamics characterize many drugs and contribute to individual differences in drug efficacy and toxicity. Drug metabolism is the primary cause of gender-dependent pharmacokinetics and reflects underlying gender differences in the expression of hepatic enzymes active in the metabolism of drugs, steroids, fatty acids, and environmental chemicals, including cytochrome P450 enzymes (P450s), sulfotransferases, glutathione transferases, and UDP-glucuronosyl transferases. Studies in rat and mouse liver models have identified more than 1000 genes whose expression is gender-dependent; together, these genes impart substantial sexual dimorphism on liver metabolic function and pathophysiology (Waxman, Holloway, 2009).

Arbo *et al.*, (2009a) tested *Citrus aurantium* extracts (400, 2000, or 4000 mg/kg corresponding to 30, 150, and 300 mg/kg of *p*-synephrine, respectively) and *p*-synephrine (30 and 300 mg/kg) in male mice daily for 28 days and found no changes in biochemical parameters ALT, AST, or creatinine. This result can in fact corroborate that associating *p*-synephrine with others substances promotes physiological alterations, as the doses of the tested mixture were 50, 75, 100, and 150 mg/kg, corresponding to only 5, 7.5, 10, and 15 mg/kg of *p*-synephrine.

Oxidative stress evaluations are presented in Table II. Male rats treated with 100 and 150 mg/kg showed significant increases ($p < 0.05$) of 51% and 62% respectively in MDA values and a decrease in GSH levels in all treated groups. GPx activity in females showed a significant increase of 58% in the 75 mg/kg treated group compared to controls.

Oxidative stress biomarker evaluation indicated the occurrence of lipid peroxidation which is characterized by increased MDA (Grotto *et al.*, 2009). It is known that amphetamines and analogues increase the production of reactive species such as free radicals (Giovanni *et al.*, 1995; Colado *et al.*, 1997; Fleckenstein *et al.*, 1997; Yamamoto, Zhu, 1998). Evidence shows that reactive oxygen species (ROS) directly or indirectly contribute to amphetamine toxicity mechanisms (Kovacic, Cooksy, 2005), and although the toxic effects caused by abusive consumption of these substances is already known, the molecular factors that contribute to these effects are not completely understood (Frey *et al.*, 2006).

Under basal physiological conditions, mitochondria are the primary source of intracellular ROS and their generation from mitochondria originates from complexes in the electron transport chain located on the inner mitochondrial membrane and from monoamine oxidase on the outer mitochondrial membrane. Thus, amphetamine may enhance ROS formation by promoting these basal mitochondrial functions. Since amphetamines cause the release of dopamine, another potential source of these ROS is from the autoxidation of dopamine via the Fenton reaction which uses iron as a cofactor (Graham, 1978). It has also been speculated that increases in intracellular

dopamine could lead to the formation of reactive dopamine quinones, which can in turn generate ROS (Brown, Yamamoto, 2003; Frey *et al.*, 2006). Moreover, high doses of amphetamines activate glutamate receptors which induce the formation of superoxide radicals and increase calcium influx (Brown, Yamamoto, 2003) and mitochondrial dysfunction (Brown, Yamamoto, 2003; Cunha-Oliveira *et al.*, 2006; Custodio *et al.*, 2010). Both *p*-synephrine and ephedrine are structurally and pharmacologically related to amphetamine (Firenzuoli *et al.*, 2005), which could be associated to the oxidative stress seen in this study.

Caffeine-derived effects could favor the production of free radicals and a subsequent increase in oxidative stress such as the metabolic inactivation of catecholamines (Halliwell, Gutteridge, 1985; Jewett *et al.*, 1989) and the increase in oxidative metabolism (Shigenaga *et al.*, 1994; Thompson *et al.*, 2001) including its own hepatic metabolism (Vistisen *et al.*, 1992). There are also reports suggesting that caffeine is capable of inducing certain forms of oxidative damage by increasing lipid peroxidation (Dianzani *et al.*, 1991). On the other hand, caffeine has been reported as a protective substance in cellular damage (Kamat *et al.*, 2000; Krisko *et al.*, 2005) with beneficial antioxidant effects (Nikolic *et al.*, 2003) probably due to the main metabolites of caffeine, 1-methylxanthine and 1-methyluric acid, which are highly effective antioxidants (Lee, 2000). So, the real behavior of caffeine is unclear.

There are no studies focusing on oxidative stress related to salicin, but aspirin (acetylsalicylic acid, which belongs to the same salicylate family as salicin) showed direct endothelial protection from oxidative damage at antithrombotic concentrations and demonstrated antioxidant and radical scavenging properties (Podhaisky *et al.*, 1997).

In addition, the oxidative stress seen in the 100 and 150 mg/kg male treated groups is confirmed by a decrease in GSH levels in all male treated groups. One of the most important roles of the GSH antioxidant system is the detoxification of endogenously generated peroxides. GSH production is considered the first line of defense against oxidative damage and free radical generation, where GSH functions as a scavenger and co-factor in metabolic detoxification (Nozal *et al.*, 1997). GSH levels increased in mice treated orally for 28 consecutive days with *p*-synephrine (30 and 300 mg/kg) and with *C. aurantium* extracts (4000 mg/kg corresponding to 300 mg/kg of *p*-synephrine respectively) (Arbo *et al.*, 2009a).

Interestingly, oxidative stress was not apparent in female rats. There were no changes in MDA or GSH levels with the treatment received. Some studies have reported gender differences in antioxidant capacity (Yamamoto *et al.*, 2002; Ilhan *et al.*, 2004) which could account for this finding. There is substantial evidence suggesting that the female hormone estrogen has potent antioxidant properties while the male steroid testosterone possesses no antioxidant activity (Yagi, Komura, 1986). Sugioka *et al.,* (1987) reported that in contrast to other natural steroids, estrogens have a hydroxyl group in their structure which is also found in Vitamin E. Thus, estrogen may have the potential to quench peroxy radicals and terminate peroxidative chain reactions (Tiidus, 2000). According to Ayres *et al.* (1998), at near physiological concentrations *in vitro*, estrogen may indeed be able to directly inhibit superoxide production and act as a peroxidation chain breaking antioxidant for both lipids and DNA. Huang and colleagues (1999) noted the potential for estrogen to reduce oxidation of low density lipoproteins. In addition, some studies have shown that gender differences between male and female also exist in the antioxidant status of normal animals, including differences in Vitamin C and E levels in rats (Tiidus *et al.*, 1999).

Gender may also affect liver glutathione status, and male rats have approximately 50% more reduced and total glutathione than females (Tiidus *et al.*, 1999), while muscle activities of antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase appear to be similar between genders in rats (Tiidus, 2000). Nevertheless, the results of our study showed a significant decrease in male rat GSH status, whereas no alterations were seen in their female counterparts. This fact could be explained as an organism response to oxidative damage, shown by MDA levels, as the function of GSH is to defend against oxidative damage and free radical generation (Nozal *et al.*, 1997).

Alterations in GPx activity in the female 75 mg/kg group should be not considered as it did not show a linear profile. According to Lewis *et al.* (2002), some factors can be useful in differentiating between a significant change from control values and a treatment-related effect. This difference is less likely to be an effect of treatment if: there is no obvious dose response; it is due to findings in one or more animals that could be considered outliers; or it is normal biological variation (within reference value limits).

CONCLUSION

In this experimental work we evaluated a 28-day period of oral exposure to a mixture of *p*-synephrine, ephedrine, salicin, and caffeine, which has been used in weight loss formulations. Our findings indicate that the continued use of the mixture can produce changes in hepatic and kidney function and in oxidative stress

biomarkers; these are more pronounced in males than females. More studies are necessary to clarify gender differences in sub-chronic toxicity profile and the protector role of estrogen against the effect of the mixture in females.

Further investigations should include long-term toxicity studies to better explain the effects of the mixture, as well as determine other oxidative stress and biochemical (hepatic and renal) markers. Products containing this mixture should be used with caution until the toxicological profile has been fully elucidated, whereas the number of toxicological accidents related to the use of dietary supplements whose formulations often vary, is very frequent.

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CONFLICT OF INTEREST STATEMENT

The author declares there are no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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