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# Effect of extracts from araticum (*Annona crassiflora*) on $CCl_4$-induced liver damage in rats

*Influência dos extratos etanólicos de araticum (Annona crassiflora) na atividade das enzimas hepáticas de ratos intoxicados por $CCl_4$*

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**Abstract**

The influence of ethanolic extracts of *Annona crassiflora* on the activities of hepatic antioxidant enzymes was examined. Extracts of *A. crassiflora* seeds and peel were administered orally (50 mg of galic acid equivalents.kg$^{-1}$) to Wistar rats for 14 consecutive days followed by a single oral dose of carbon tetrachloride ($CCl_4$, 2 g.kg$^{-1}$). Lipid peroxidation and the activities of hepatic catalase (CAT), cytochromes P450 (CP450) and b5, glutathione peroxidase (GPx), glutathione reductase (GRed), superoxide dismutase (SOD), and the content of glutathione equivalents (GSH) were evaluated. The treatment with $CCl_4$ increased lipid peroxidation, the level of GSH equivalents and the content of cytochrome b5 by 44, 140 and 32%, respectively, with concomitant reductions of 23, 34 and 39% in the activities of CAT, SOD, and CP450, respectively. The treatment with *A. crassiflora* seeds and peel extracts alone inhibited lipid peroxidation by 27 and 22%, respectively without affecting the CP450 content. The pretreatment with the *A. crassiflora* extracts prevented the lipid peroxidation, the increase in GSH equivalents and the decrease in CAT activity caused by $CCl_4$, but it had no effect on the $CCl_4$-mediated changes in CP450 and b5 and SOD. These results show that *A. crassiflora* seeds and peel contain antioxidant activity in vivo that could be of potential therapeutic use.
***Keywords:*** *Annona crassiflora; antioxidant activity; lipid peroxidation; $CCl_4$; liver enzymes.*

**Resumo**

Neste estudo, a influência dos extratos etanólicos de *Annona crassiflora* na atividade das enzimas hepáticas de ratos foi avaliada. Extratos de casca e sementes de *A. crassiflora* (50 mg de equivalentes de ácido gálico.kg$^{-1}$) foram administrados por gavagem a ratos Wistar por 14 dias consecutivos seguidos por uma dose oral única de tetracloreto de carbono ($CCl_4$, 2 g.kg$^{-1}$). A peroxidação lipídica, conteúdo de citocromo P450 (CP450) e b5 e a atividade das enzimas hepáticas catalase (CAT), superóxido dismutase (SOD), glutationa peroxidase (GPx), glutationa redutase (GRed) e equivalentes de glutationa (GSH) foram avaliados. O tratamento com $CCl_4$ aumentou a peroxidação lipídica, o nível de equivalentes de glutationa e o conteúdo de citocromo b5 em 44, 140 e 32%, respectivamente, bem como reduziu em 23, 34 e 39% a atividade das enzimas CAT, SOD e conteúdo de CP450, respectivamente. O tratamento com extratos de sementes e casca de *A. crassiflora* em ratos não intoxicados por $CCl_4$ inibiu a peroxidação lipídica em 27 e 22%, respectivamente, sem alterar o conteúdo de CP450. Em ratos intoxicados por $CCl_4$, os extratos de *A. crassiflora* preveniram a peroxidação lipídica, o aumento de equivalente de GSH e o decréscimo na atividade de CAT, mas não tiveram efeito significativo sobre as mudanças induzidas pelo $CCl_4$ no CP450, b5 e SOD. Os resultados demonstram que extratos de sementes e casca de araticum possuem atividade antioxidante in vivo que pode ser de uso potencial para fins terapêuticos.
***Palavras-chave:*** *Annona crassiflora; atividade antioxidante; peroxidação lipídica; $CCl_4$; enzimas hepáticas.*

## 1 Introduction

Reactive oxygen species (ROS) are products of oxidative metabolism, and their production can be stimulated by radiation and xenobiotic agents derived from air pollution or chemicals such as carbon tetrachloride, paraquat, and cigarette smoke. In the absence of adequate endogenous antioxidant defenses, the propagation of free radical-producing events can lead to the co-oxidation of nucleophilic cellular constituents, and the reaction of secondary lipid autoxidation products with nucleophilic macromolecules such as membrane constituents, enzymes, and DNA (YUAN; KITTS, 1996).

Oxidative stress has been associated with the development of chronic and degenerative diseases including cancer, heart disease, and neuronal degeneration such as in Alzheimer's disease, as well as being involved in aging (AMES; SHIGENAGA; HAGEN, 1993; 1995; DIAZ; FREI; KEANEY, 1997; LANG; LOZANO, 1998; CHRISTEN, 2000). The potential of antioxidant plants to contribute to human health and to protect against heart diseases and cancer has attracted considerable interest among scientists, food manufacturers, and consumers, and it has led to the development of functional foods with specific health effects (LOLINGER, 1991). Typical compounds with antioxidant activity include phenols, phenolic acids, and their derivatives, flavonoids, tocols, phospholipids, amino acids and peptides, phytic acid, ascorbic acid, pigments, and sterols. Phenolic compounds are primary antioxidants that act as free-radical scavengers (XING; WHITE, 1996).

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*Annona crassiflora*, commonly known as araticum, is a tropical fruit consumed mainly by native people of the Brazilian cerrado (the second biggest biome of Brazil). In previous studies, the seeds and peel of slightly ripe fruit were found to have significant antioxidant activity in various models in vitro (ROESLER et al., 2006). Electrospray ionization mass spectrometry (ESI-MS) also revealed the presence of important bioactive components widely recognized as antioxidants including malic acid, ascorbic acid, caffeic acid, quinic acid, ferulic acid, xanthoxylin, rutin, caffeoyltartaric acid, caffeoyl glucose, and [quercetin + hexose + pentose - H]-1 (ROESLER et al., 2007). The aim of the present study was to assess the protective effect of an ethanolic extract of *A. crassiflora* seeds and peel in Wistar rats treated with carbon tetrachloride ($CCl_4$). The protective effect of the extract was assessed by quantifying the level of lipid peroxidation and the activities of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), cytochromes P450 and b5, glutathione peroxidase (GPx), glutathione reductase (GRed), and glutathione (GSH) equivalents in liver homogenates.

## 2 Material and methods

### *2.1 Reagents and standards*

All solvents and reagents were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA).

### *2.2 Plant material*

Araticum fruits (*A. crassiflora*) were obtained from Fazenda Erlow, Km 7, Br 070 in the State of Goiânia, Central-West Region of Brazil. The fruits were harvested on two occasions, with special care to avoid damaging the flesh and were transported to UNICAMP (State University of Campinas) where they were stored at 5 °C until use (usually no longer than two months).

### *2.3 Preparations of the ethanolic extraction*

Undamaged fruits were selected and the peel, pulp, and seeds were manually separated. An amount around 100 g of each part of the fruit (peel and seeds) was cut into small parts, mixed, and extracted twice (for 20 minutes each) with 300 mL of aqueous ethanol (5:95, v/v, water:ethanol) using a household mixer. The extracts were filtered through cotton membranes, and the residue was re-extracted under the same conditions. The residues were kept for additional evaluations. The ethanol was removed from the resulting material by vacuum rotary evaporation at 40 °C. The concentrated ethanolic extracts were lyophilized and stored at –18 °C in amber glass bottles until used.

### *2.4 Determination of the total phenol content*

The total polyphenol content of the extracts was determined by the Folin-Ciocalteu method, which involves the reduction of the reagent by phenolic compounds and the concomitant formation for a blue complex; the resulting absorbance at 760 nm increases linearly with the concentration of phenols in the reaction medium (SWAIN; HILLIS, 1959). Briefly, ethanolic extracts were dissolved in methanol to a concentration of 0.5 $mg.mL^{-1}$. The samples (0.5 mL) were passed through a 0.45 μm membrane filter and mixed with 2.5 mL of 10 fold diluted Folin-Ciocalteu reagent and 2.0 mL of 7.5% sodium carbonate solution. After incubation for 5 min at 50 °C, the resulting absorbance was measured at 760 nm. Galic acid was used as the spectrophotometric standard and the total phenolic content of the fruit extracts was expressed as galic acid equivalents (GAE.100 $g^{-1}$). The content of phenolic compounds was assayed in triplicate.

### *2.5 Experimental procedure*

Male Wistar rats (230-265 g) were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB) at UNICAMP and were housed under a 12 hours light/dark cycle at 23 °C with free access to food and water. The rats were allocated to one of six groups containing seven rats each. The first group (G1) served as the control, the second group (G2) received $CCl_4$ alone, the third (G3) and fifth group (G5) received an aqueous solution of *A. crassiflora* peel extract (50 mg of galic acid equivalents.$kg^{-1}$), and the fourth (G4) and sixth group (G6) received an aqueous solution of *A. crassiflora* seed extract (50 mg of galic acid equivalents.$kg^{-1}$) for 14 consecutive days. The dosage of the extract administered was based on the $LD_{50}$ of polyphenols (BOMBARDELLI; MORAZZONI, 1995). The groups G1 and G2 were given saline solution (0.9%) until the $14^{th}$ day, whereas the groups G2, G5, and G6 received a single oral dose of CCl4 (2 $g.kg^{-1}$) in olive oil (1:1, v/v) 6 hours after the last administration of extract or saline solution (0.9%) on the $14^{th}$ day. All of the rats were killed with an overdose of anesthetic halotano 24 hours later. The liver was excised and microsomal and cytosolic fractions were prepared by differential centrifugation (OMURA; SATO, 1964) and stored in aliquots at –80 °C until analysis. The protein concentrations of the microsomal and cytosolic fractions were measured by the Lowry method (LOWRY et al., 1951) with bovine serum albumin as the protein standard.

### *2.6 Lipid peroxidation*

Thiobarbituric acid reacts with malondialdehyde (MDA) to form a diadduct (pink chromogen) that can be detected spectrophotometrically at 532 nm (VAN DER SLUIS et al., 2000). The assay used was modified for ELISA microtiter plates, which allowed the analysis of a large number of samples per run. Microsomes were thawed on ice, diluted to a concentration of 1 mg of protein.$mL^{-1}$ with 50 mM Tris-HCl (pH 7.4) containing 150 mM KCl, and then centrifuged (100.000 g, 60 minutes, 4 °C). The pellet was resuspended in 1 mL of Tris buffer and diluted to a final concentration of 0.5 $mg.mL^{-1}$, unless stated otherwise (OMURA; SATO, 1964; VAN DER SLUIS et al., 2000; SINGH et al., 2002).

Microsomes (aliquots of 240 μL) were preincubated in a 48-well plate for 5 minutes at 37 °C, after which the lipid peroxidation (LPO) was induced by adding 15 μL of 4 mM ascorbic acid and 15 μL of 0.2 mM $FeCl_3$. After incubation for 60 minutes at 37 °C, the reaction was stopped by adding 0.5 mL of 0.83% thiobarbituric acid dissolved in

a solution of trichloroacetic acid (TCA)-HCl (16.8%, w/v, TCA in 0.125 N HCl). LPO was quantified by measuring the thiobarbituric acid reactive species (TBARS) after heating the plates for 15 minutes at 80 °C followed by centrifugation (2.500 rpm, 15 minutes). A 250 µL sample of each incubation was transferred to 96-well plates and the absorption was read at 540 nm (color) versus 620 nm (turbidity correction) using a SpectroMax 340 ELISA reader (VAN DER SLUIS et al., 2000; SINGH; CHIDAMBAR MURTHY; JAYAPRAKASHA, 2002). The results were calculated using an extinction coefficient of $1.56 \times 10^5$ $M^{-1}.cm^{-1}$ and were expressed as MDA equivalents. One unit of lipid peroxidation activity was defined as the amount of TBA that was converted to TBARS, and the specific activity was expressed as units.$mg^{-1}$ of protein.

### *2.7 Enzyme assays*

The total microsomal cytochrome P450 and b5 content was quantified as described by Omura and Sato (1964). Catalase was assayed as described by Aebi (1984); glutathione peroxidase (GPx) and glutathione reductase (GRed) were estimated by the method of Albrecht Wendel (1981). The content of glutathione equivalents was determined by the method of Theodorus, Sies and Akerbooom (1981), and the superoxide dismutase (SOD) was assayed as described by Misra and Fridovich (1972).

### *2.8 Statistical analysis*

The results were expressed as the mean ± S.E.M., and the statistical comparisons were done using Student´s *t*-test. A value of $p \leq 0.05$ indicated significance.

## 3 Results

### *3.1 Body and organ weight and general observations*

The oral administration of the extracts did not produce any clinical signs or gross alterations attributable to hepatic toxicity or other organ damage. However, rats that received the seed extract showed some anxiety and were restless, perhaps because of the phenolic acids and their derivatives (caffeic acid, caffeoyl glucose, and caffeoyl tartaric) present in this extract (ROESLER et al., 2007). There were significant differences among the body weight gain of the groups that received seed extract (G4 and G6) compared with the control group (G1). Groups 4 and 6 showed reduction in body weight of approximately 28 and 23% compared with the control. Table 1 shows the body weight, hepatic index, and microsomal and cytosolic protein concentrations of the different groups.

### *3.2 Influence of A. crassiflora seed and peel extracts on hepatic lipid peroxidation*

Enhanced lipid peroxidation associated with a depletion of tissue antioxidants is characteristic of CCl4-treated rats (WEBER; BOLL; STAMPFL, 2003). In agreement with this, $CCl_4$ significantly increased the level of hepatic TBARS (by 44%) of G2 in this study compared to the control group (G1). The *A. crassiflora* seed extract (G6) significantly decreased the levels of TBARS compared to CCl4-treated rats (–41%), as shown in Figure 1. The *A. crassiflora* peel (G3) and seed extracts (G4) alone inhibited TBARS formation by 22 and 27%, respectively, compared to the saline controls.

### *3.3 Influence of A. crassiflora seed and peel extracts on the hepatic content of cytochrome P450 and b5*

The recognition of clinically significant interactions between plant-based drugs and conventional medicines has raised concerns regarding the potential of herbal products to interfere with the metabolism of medications such as kava-kava and echinacea (GORSKI et al., 2003; ZOU et al., 2004). However, as shown in this study (Figure 2), the extracts alone did not alter the basal levels of cytochrome P450. $CCl_4$ decreased the content of cytochrome P450, and the *A. crassiflora* seed and peel extracts were unable to prevent this reduction. However,

**Table 1.** Modulatory influence on the two investigated extracts of *Annona crassiflora* on weight gain profiles, protein levels, and toxicity related parameters.

| Groups & treatment | Body weight gain profile | Liver wt × 100/ Final body wt | Protein ($mg.mL^{-1}$) | |
|---|---|---|---|---|
| | | | Microsome | Cytosol |
| Group 1 | 50.43 ± 7.91 | 4.78 ± 0.17 | 22.90 ± 0.43 | 47.10 ± 0.93 |
| Control | | 100.00* | 100.00* | 100.00* |
| Group 2 | 63.53 ± 10.06 (1) | 4.72 ± 0.09 | 20.55 ± 0.98 | 48.20 ± 0.63 |
| $CCl_4$ (negative control) | | 98.49* | 89.74* | 102.33* |
| Group 3 | 46.00 ± 7.92 | 4.43 ± 0.15 (1) | 24.40 ± 0.36 (1) | 46.70 ± 0.75 |
| Peel extract | | 92.58* | 106.55* | 99.15* |
| Group 4 | 36.29 ± 12.00 (1) | 4.95 ± 0.19 | 21.00 ± 1.42 | 42.70 ± 1.92 |
| Seed extract | | 103.53* | 91.70* | 90.66* |
| Group 5 | 62.43 ± 15.06 | 4.38 ± 0.07 (1) | 17.92 ± 0.87 (1.3) | 46.50 ± 0.42 |
| Peel extract + $CCl_4$ | | 91.35* | 78.25* | 98.73* |
| Group 6 | 38.70 ± 5.88 (1.2) | 4.46 ± 0.08 (1.4) | 17.30 ± 1.16 (1.4) | 46.20 ± 0.79 |
| Seed extract + $CCl_4$ | | 93.05* | 75.55* | 98.09* |

Values are expressed as mean ± SD of 6-7 animals. (*) Represents relative changes in parameters assessed (i.e., levels of parameter assessed in livers of rats receiving test substance to that of control rats). (1) Represents significant difference from group 1 at $p < 0.05$; (2) represents significant difference from group 2 at $p < 0.05$; (3) represents significant difference from group 3 at $p < 0.05$, and (4) represents significant difference from group 4 at $p < 0.05$.

there was no additive effect in rats treated with the extracts and $CCl_4$ (Figure 2). On the other hand, both extracts induced cytochrome b5, even under $CCl_4$ effect.

### *3.4 Influence of A. crassiflora seed and peel extracts on hepatic catalase activity*

Catalase converts $H_2O_2$ to water and oxygen and also converts hydroperoxides to the corresponding alcohols in the presence of a hydrogen donor (AEBI, 1984). As shown in Figure 3, a single dose of $CCl_4$ produced a reduction of catalase activity by 23% (G2). Both of the *A. crassiflora* extracts prevented the decrease in catalase activity caused by $CCl_4$ (G5 and G6).

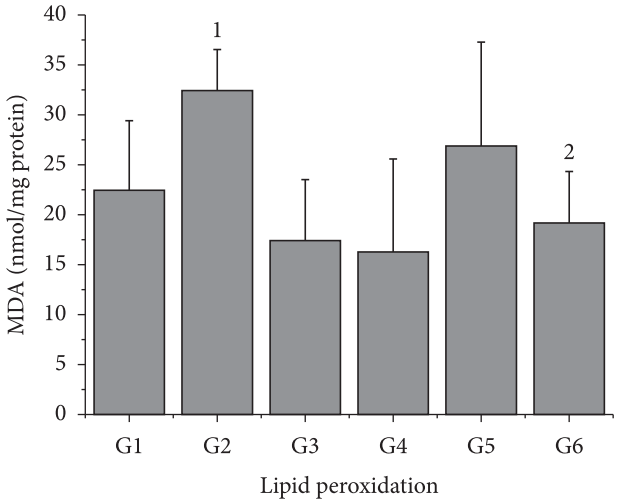


**Figure 1.** Hepatic lipid peroxidation in rats treated with $CCl_4$ in the absence or presence of *A. crassiflora* seed and peel extracts. The columns represent the mean + S.E.M. of at least 5 rats. (1) Represents significant difference from group 1 at $p < 0.05$; (2) represents significant difference from group 2 at $p < 0.05$.

### *3.5 Influence of Annona crassiflora seed and peel extracts on hepatic glutathione peroxidase (G-Px) and glutathione reductase (GRed) activities*

Enzymes involved in the detoxification of ROS and lipid hydroperoxides include the glutathione redox cycling enzymes, glutathione peroxidase (GPx), and glutathione reductase (GRed). GPx catalyzes the inactivation of $H_2O_2$ and its activity is the greatest when the enzyme is in its reduced form. Other enzymes, notably catalase and glutathione-S-transferase, also influence the activity of GPx. While catalase competes with GPx for $H_2O_2$, glutathione-S-transferase competes with GPx only for hydroperoxides (YUAN; KITTS, 1996). In our study, there were no significant changes in the GPx and GRed activities among the different groups including the rats treated with $CCl_4$ (Figure 4), possibly because of competition with enzymes such as catalase.

### *3.6 Influence of A. crassiflora seed and peel extracts on the level of glutathione equivalents (GSH equivalents)*

Under physiological conditions, GSH occurs in its reduced form with oxidation leading to the formation of glutathione disulfide (GSSG). There has been considerable interest in the redox state of GSH and GSSG (THEODORUS; SIES; AKERBOOM, 1981). In the rats, treatment with the peel extract increased the GSH levels, whereas the seed extract had no effect. $CCl_4$ significantly increased the level of GSH equivalents, and this increase was partially prevented by both *A. crassiflora* extracts (Figure 5). Since the oxidation of GSH in vivo can lead to the formation of GSSH and hydrogen peroxide, the

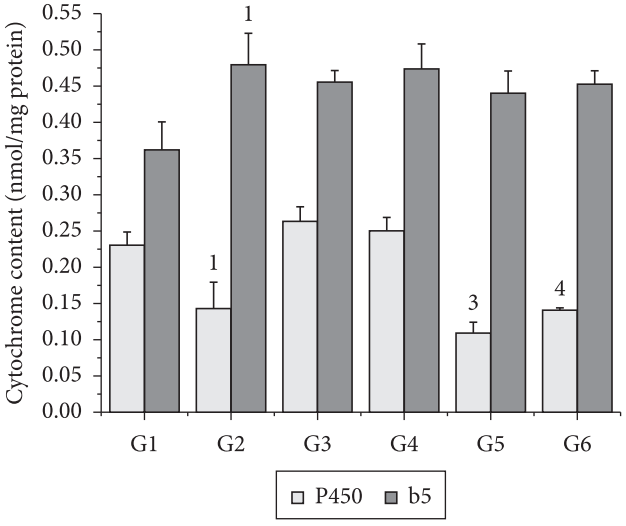


**Figure 2.** Hepatic content of cytochrome P450 and b5 in rats treated with $CCl_4$ in the absence and presence of *A. crassiflora* seed and peel extracts. The columns represent the mean + S.E.M. of at lest 5 rats. (1) Represents significant difference from group 1 at $p < 0.05$; (3) represents significant difference from group 3 at $p < 0.05$, and (4) represents significant difference from group 4 at $p < 0.05$.

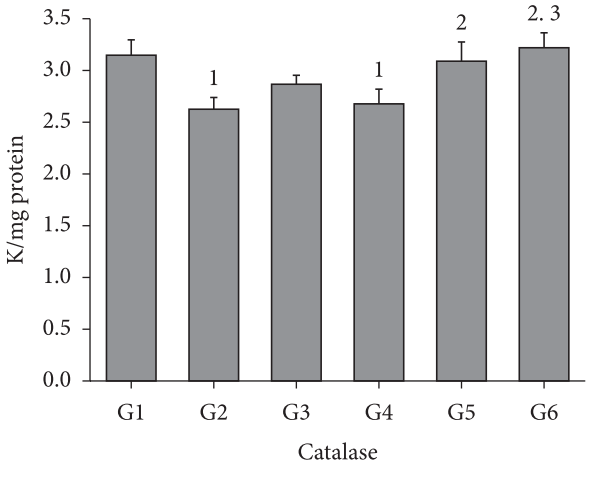


**Figure 3.** Hepatic catalase activity in rats treated with $CCl_4$ in the absence and presence of *A. crassiflora* seed and peel extracts. The columns represent the mean+S.E.M. of at least 5 rats. (1) Represents significant difference from group 1 at $p < 0.05$; (2) represents significant difference from group 2 at $p < 0.05$, and (3) represents significant difference from group 3 at $p < 0.05$.

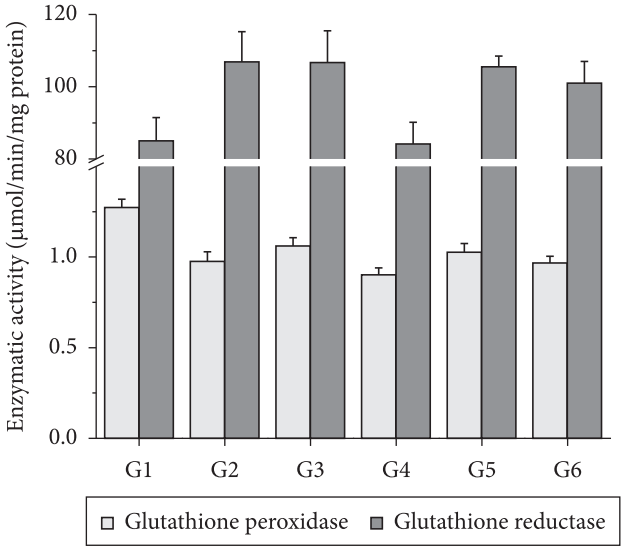


**Figure 4.** Hepatic glutathione peroxidase and glutathione reductase activities in rats treated with $CCl_4$ in the absence and presence of *A. crassiflora* seed and peel extracts. The columns represent the mean+S.E.M. of at least 5 rats.

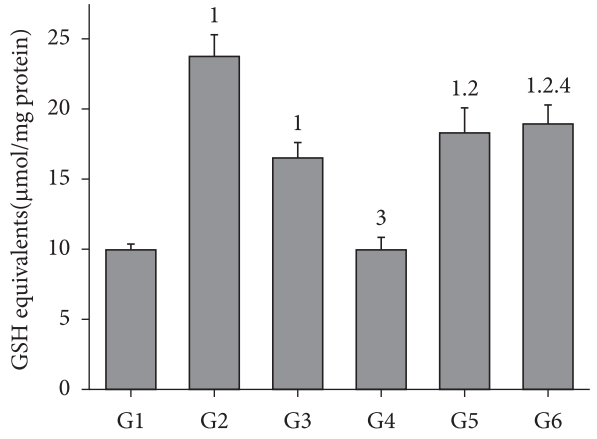


**Figure 5.** Hepatic glutathione (GSH) equivalents in rats treated with $CCl_4$ in the absence and presence of *A. crassiflora* seed and peel extracts. The columns represent the mean+S.E.M. of at least 5 rats. (1) represents significant difference from group 1 at $p < 0.05$; (2) represents significant difference from group 2 at $p < 0.05$; (3) represents significant difference from group 3 at $p < 0.05$, and (4) represents significant difference from group 4 at $p < 0.05$.

increased level of GSH equivalents caused by $CCl_4$ probably corresponded to GSSH.

### *3.7 Influence of A. crassiflora seed and peel extracts on hepatic SOD activity*

The ability of superoxide dismutase to inhibit the autoxidation of epinephrine at pH 10.2 has been used as the basis of a convenient and sensitive assay for this enzyme. SOD was determined by measuring the inhibition of the adrenochrome formation rate (MISRA; FRIDOVICH, 1972) in the reaction medium containing 1mM-adrenaline (epinephrine) and 50 mM-glycine/NAOH, pH 9.6 (BOVERIS et al., 1983). The treatment with $CCl_4$ induced the autoxidation of epinephrine; therefore, the inhibition of autoxidation had a reduction of 34% compared with the control group. As shown in Figure 6, the two *A. crassiflora* extracts alone significantly decreased the SOD activity and consequently increased the autoxidation of the epinephrine. The *A. crassiflora* peel (G3) and seed (G4) inhibited the autoxidation of epinephrine by only 48.1 and 28.9%, respectively, while the control (G1) showed an inhibition of approximately 74%. The profile of SOD activity seen with the extracts was unaltered by concomitant treatment with $CCl_4$.

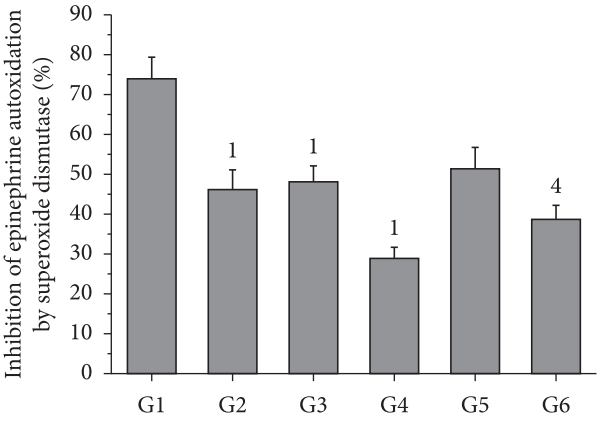


**Figure 6.** Hepatic SOD inhibition of autoxidation of epinephrine in rats treated with CCl4 in the absence and presence of *A. crassiflora* seed and peel extracts. The columns represent the mean+S.E.M. of at least 5 rats. (1) Represents significant difference from group 1 at $p < 0.05$ and (4) represents significant difference from group 4 at $p < 0.05$.

## 4 Discussion

Araticum (*A. crassiflora*) is a tree that bears a typical fruit known as araticum of cerrado or cerradão. Its fruits are consumed extensively "in natura" but have no commercial value in Brazil. The seeds in oil are used against scalp infections and in the folk medicine. The leaves and seeds infusion is used against diarrhea and as antitumoral agent (LORENZI, 1988; ALMEIDA et al., 1994). Many members of annonaceae are used in folk medicine for antiparasitic or antitumoral treatment of intestinal diseases. Previous studies have shown the in vitro free radical scavenging potential of *Annona crassiflora* peel and seed extracts at different concentrations by the DPPH method, and the seed and peel presented $IC_{50}$ of 31.14 and 48.82 $\mu g.mL^{-1}$, respectively. The inhibition of lipid peroxidation using rat liver microsomes as an oxidative system was evaluated, and it was found that fifty percent inhibition of lipid peroxidation of microsomes to TBARS requires 1.72 $\mu g.mL^{-1}$ of seed ethanolic extract, 4.44 $\mu g.mL^{-1}$ of peel ethanolic extract, and 8.62 $\mu g.mL^{-1}$ of pulp etanolic extract (ROESLER et al., 2006).

In the present study, an experimental model of acute hepatotoxicity in Wistar Rats was induced by oral administration of $CCl_4$. $CCl_4$ has been extensively used in experimental models to elucidate the cellular mechanisms behind oxidative damage (BASU, 2003). $CCl_4$ is activated by cytochrome P450 (CYP) 2E1, 2B1/B2, and possibly 3A to form trichloromethyl radicals ($CCl_3$*)

and trichloromethyl peroxy radicals ($CCl_3OO^*$) that cause lipid peroxidation and subsequent tissue damage (WEBER; BOLL; STAMPFL, 2003). Enhanced lipid peroxidation associated with a depletion of tissue antioxidants is a characteristic of $CCl_4$-treated rats (SIPES; KRISHNA; GILLETTE, 1977).

Direct evidence of this hepatotoxicity was noted through alterations in hepatic parameters in the second group (G2), such an increase in hepatic lipid peroxidation (MDA concentration), decreased phase I cytochrome P450 content, decreased phase II CAT and SOD activity, and increased GSH-equivalent level. The present results agree with previously reported data (JAYAKUMAR; RAMESH; GERALDINE, 2006; SRINIVASAN et al., 2005; SINGAB et al., 2005; CHIDAMBARA MURTHY; JAYAPRAKASHA; SINGH, 2002; ZHU et al., 1999). Since free radicals play an important role in $CCl_4$- induced hepatotoxicity, it seems logical that compounds that neutralize such radicals may have a hepatoprotective effect.

The oral administration of *A. crassiflora* peel (G3) and seed extract (G4) inhibited the TBARS formation by 22 and 27%, respectively. *A. crassiflora* seed extract (G6) significantly decreased the levels of TBARS compared with the $CCl_4$ treated group (G2) by 41%. Thus, the maintenance of normal levels of hepatic MDA in group 6 (administrated *A. crassiflora* seed extract + $CCl_4$) is of great interest since it provides evidence to suggest a hepatoprotective effect. The lipid peroxidation control in vivo is important for several reasons, in particular because it contributes to the development of atherosclerosis. The products of lipid peroxidation, particularly the citotoxic aldehydes such as malondialdehyde (MDA), are important because they can also cause damage DNA (HALLIWELL; ARUOMA, 1960). Thus, the maintenance of normal levels of hepatic MDA is of great interest since MDA is a major aldehyde resulting from the peroxidation of biological tissue and it is an indicator of tissue damage. Lipid peroxidation may be prevented at the initiation stage by free radical scavengers, whereas the chain propagation reaction can be intercepted by peroxy-radical scavengers such as phenolic antioxidants (TAKAHAMA, 1983). The investigation by direct infusion electrospray ionization mass spectrometry (ESI-MS) provided important information of biactive components of araticum extracts mainly phenolic antioxidants such as caffeic acid, quinic acid, ferulic acid, xanthoxylin, caffeoyltartaric acid, caffeoyl glucose, [quercetin + hexose + pentose – H]-, and rutin (ROESLER et al., 2007).The phenolic compounds act by scavenging free radicals and quenching the lipid peroxides. The hydroxyl and phenoxy groups of phenolic compounds donate their electron to the free radicals and neutralize them forming phenolic radical and quinine methide intermediate, which is excreted via bile (PAN et al., 1999).

The consumption of vegetables and fruits is known to reduce the risk of cancer (WATTENBERG, 1992). In general, the induction of phase 2 drug-metabolizing enzymes, the suppression of phase 1 enzymes, or the combination of these actions have been reported to be the mechanisms responsible for the protection against toxic and neoplastic effects of carcinogens (TALALAY, 1989; 2000).

The phase 1 enzyme cytochrome P450 dependent monooxygenase system is responsible for the oxidative and reductive metabolism of a variety of drugs, carcinogens, pesticides, and steroid hormones. The monooxygenase system is subject to the inductive and inhibitory effects of environmental and genetic factors, which may play an important role in determining the biological fate of foreign chemicals that require P450–mediated detoxication or bioactivation. *A. crassiflora* extracts preserve the cytochrome P450 content which is comparable with the control values of the enzyme. The pre-treatment with the *A. crassiflora* extracts was unable to alleviate the inhibition of the cytochrome P450 activity of the $CCl_4$ treated group. On the other hand, both extracts induced cytochrome b5, even under $CCl_4$ effect. The role of this enzyme includes electron transfer, coupled catalysis, and allosteric regulation of cytochrome P450 during the metabolism of endogenous and xenobiotic compounds (PORTER, 2002). Cytochrome b5 is required in several reactions, such as fatty acids and hormones synthesis (SCHENKMAN; JANSSON, 2003). Sheweita, El-Gabar and Bastawy (2001) described cytochrome b5 induction after concomitant administration of $CCl_4$ and antioxidants, but no changes were observed with single compound treatment. The mechanism of induction and the role of cytochrome b5 under $CCl_4$ and antioxidants exposure still requires further investigation.

Phase 2 enzymes play important roles in the detoxification of xenobiotics, and thus their up-regulation gives protection against potentially harmful insults from the environment (KONG et al., 2001). Living tissues are endowed with innate antioxidant defense mechanisms, such as the presence of the enzymes CAT, SOD, and GPx. A reduction in the activities of enzymes associated with the accumulation of highly reactive free radicals, leading to deleterious effects such as loss of integrity and function of cell membranes (REEDY; LOKESH, 1992; KRISHNAKANTHA; LOKESH, 1993; SHEELA; ANGUSTI, 1995). The administration of $CCl_4$ leads to the generation of a peroxy radical, which is associated with the inactivation of CAT and SOD enzymes. This probably explains the significantly reduced activities of CAT and SOD in the rats challenged with $CCl_4$ (G2). The extracts of *A. crassiflora* are capable of enhancing/maintaining phase 2 enzymes' activity. The only exception was observed for SOD phase 2 enzyme, whose activity was unexpectedly depleted by *A. crassiflora* extracts as much as for $CCl_4$.

Nutritional factors and dietary habits have a significant effect on the regulation of antioxidant enzyme activity. According to L´Abbe, Trick and Beare-Rogers (1991), the SOD activity can be depleted in the liver and heart by fish oil diet. In addition, the decrease in the SOD activity in the heart has been already associated with weight loss (WOHAIEB; GODIN, 1978). As reported in Table 1, there was a significant difference between the body weight gain profile between the control group and the seed extract group (-23%). For the peel extract group, there was also a tendency for body gain profile reduction (-9%). In adittion, araticum seeds have approximately 16% of lipids (ROESLER et al., 2006). The lipids from the seeds were not removed before preparing the extracts and the solvent used for the extraction process also removed lipids; therefore, it is possible to affirm that the seed extract has approximately 16% of lipids as well. The composition of araticum oil had already

been reported by Zuppa (2001). It contains approximately 15% of saturated fatty acids (palmitic and estearic acids), 51% of monounsaturated fatty acid (oleic acid), and 32% of polyunsaturated fatty acid (linoleic and linolenic acids). Fish oil has approximately 32% of saturated fatty acids (miristic and palmitic acids), 25% of monounsaturated fatty acids (oleic and palmitoleic acids), and 40% of polyunsaturated fatty acids (20:5 n-3 and 22:6 n-3) (GAMBOA; GIOIELLI, 2006). The araticum oil has quantities of polyunsaturated fatty acid as high as those of fish oil', but fish oil has more fatty acids with long chain. Therefore, further studies are needed for better understanding of *A. crassiflora* seed and peel extracts, SOD activity, and nutrition and metabolic factors that can regulate antioxidant enzyme activity.

Glutatione is a major non-protein thiol present in living organisms and performs as a key role in coordinating antioxidant defense mechanisms. The main role of GSH (L-y-glutamyl-L-cysteinyl-glycine) in vivo is to be a primary agent involved in deactivating electrophilic free radicals. The oxidation of GSH as an antioxidant can occur in vivo with oxidized glutathione (GSSH) (YUAN; KITTS, 1996). Treatment with $CCl_4$ induced the GSH-equivalents by 140%. Since the oxidation of GSH in vivo can lead to the formation of GSSH and hydrogen peroxide, the increased level of GSH equivalents caused by $CCl_4$ probably corresponded to that of the GSSH. This increase in GSH-equivalents, probably GSSH, was partially prevented by both *A. crassiflora* extracts.

The treatment of rats with a single dose of $CCl_4$ at 2.0 $g.kg^{-1}$ of body weight and the pre-treatment with the *Annona crassiflora* seed and peel extracts at 50 $g.kg^{-1}$ (in terms of galic acid equivalents) were not able to alter significantly the level of glutathione peroxidase (G-Px) and glutathione redutase (G-red).

## 5 Conclusions

In conclusion, our results show that ethanolic extracts of *A. crassiflora* can enhance or maintain the activity of hepatic antioxidant enzymes, except for SOD, which was unexpectedly depleted by the extracts to a similar extent as that seen with $CCl_4$. The pretreatment with *A. crassiflora* extracts protected against $CCl_4$ toxicity, as shown mainly by the lipid peroxidation assay, catalase activity, and level of GSH equivalents. In contrast, the treatment with $CCl_4$ and the pretreatment with *A. crassiflora* extracts did not significantly alter the level of glutathione peroxidase and glutathione reductase. It is also important to emphasize that the *A. crassiflora* extracts preserved the cytochrome P450 content which can be compared to the control values of the enzyme. Hence, there is probably no need to be concerned about interactions between plant-based drugs and conventional medicines. The antioxidant activity of the *A. crassiflora* extracts in vitro (ROESLER et al., 2006) and in vivo, as demonstrated in this study, may be mediated by compounds such as ascorbic acid, caffeic acid, quinic acid, ferulic acid, xanthoxylin, caffeoyltartaric acid, caffeoyl glucose, [quercetin + hexose + pentose-H], and rutin. These compounds are widely reported as potent antioxidants and can probably explain the antioxidant activity of the *A. crassiflora* extracts (GÜLÇIN, 2006; KIM et al., 2006; ROCHE et al., 2005; KWEON; HWANG; SUNG, 2001). The present study represents the first attempt in determining whether the *Annona crassiflora* extracts can protect Phase 1 and Phase 2 hepatic enzymes against CCL4 induced liver damage. Further studies are needed to elucidate the mechanisms involved in this hepatic protection and to explore possible synergism among the components of the extracts.

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## References

AEBI, H. Catalyse in vitro. **Methods Enzymology**, v. 105, p. 121-125, 1984.

AMES, B.N.; SHIGENAGA, M. K.; HAGEN, T. M. Oxidants, antioxidants, and degenerative diseases of aging. **Proceeding of the National Academy of Sciences U.S.A.**, v. 90, n. 17, p. 7915-7922, 1993.

BASU, S. Carbon tetrachloride-induced lipid peroxidation: eicosanoid formation and their regulation by antioxidant nutrients. **Toxicology**, v. 189, n. 1-2, p. 113-27, 2003.

BOMBARDELLI, E.; MORAZZONI, P. *Vitis vinifera*. L. **Fitoterapia**, v. 65, n. 4, p. 291-317, 1995.

BOVERIS, A. et al. Increased chemiluminescence and superoxide production in the liver of chronically ethanol-treated rats. **Archives of Biochemistry and Biophysics**, v. 227, p. 534-538, 1983.

CHIDAMBARA MURTHY, K. N.; JAYAPRAKASHA, G. K.; SINGH, R. P. Studies on antioxidant activity of pomegranate (*Punica granatum*) peel extract using "in vivo" models. **Journal of Agricultural Food Chemistry**, v. 50, n. 17, p. 4791-4795, 2002.

CHRISTEN, Y. Oxidative stress and Alzheimer's disease. **American Journal of Clinical Nutrition**, v. 71, n. 2, p. 621S-629S, 2000.

DIAZ, M. N.; FREI, B.; KEANEY, J. R. Antioxidants and atherosclerotic heart disease. **New England Journal of Medicine**, v. 337, n. 6, p. 408-416, 1997.

GAMBOA, O. W. D.; GIOIELLI, L. A. Comportamento de cristalização de lipídios estruturados obtidos a partir de gordura de palmiste e óleo de peixe. **Quimica Nova**, v. 29, p. 646-653, 2006.

GORSKI, J. C. et al. The effect of echinacea (*Echinacea purpurea* root) on cytochrome P450 activity in vivo. **Clinical Pharmacoly & Therapeutics**, v. 75, n. 1, p. 89-100, 2004.

GÜLÇIN, I. Antioxidant activity of caffeic acid (3,4-dihydroxycinnamic acid). **Toxicology**, v. 217, n. 2-3, p. 213-220, 2006.

HALLIWELL, B.; ARUOMA, I. O. Free radicals and antioxidants: the need for in vivo markers of oxidative stress. In: ARUOMA, I. O.; CUPPETT, L. S. **Antioxidant methodology in vivo and in vitro concepts**. Champaign, Illinois: AOCS Press, 1960. p. 1-22.

JAYAKUMAR, T.; RAMESH, E.; GERALDINE, P. Antioxidant activity of the oyster mushroom, *Pleurotus ostreatus,* on CCl4-induced liver injury in rats. **Journal Food Chemistry and Toxicology**, v. 44, n. 12, p. 1989-1996, 2006.

KIM, H. J. et al. Vanillic Acid Glycoside and Quinic Acid Derivatives from *Gardeniae Fructus*. **Journal of Natural Products**, v. 69, n. 4, p. 600-603, 2006.

KONG, A. N. et al. Induction of xenobiotic enzymes by the MAP kinase pathway and the antioxidant or electrophile response element (ARE/EpRE). **Drug Metabolism Reviews**, v. 33, n. 3-4, p. 255-271, 2001.

KRISHNAKANTHA, T. P.; LOKESH, B. R. Scavenging of super oxide anion spice principles, **Indian Journal of Biochemistry & Biophysics**, v. 30, n. 1, p. 133-134, 1993.

KWEON, M.; HWANG, H.; SUNG, H. Identification and Antioxidant Activity of Novel Chlorogenic Acid Derivatives from Bamboo (*Phyllostachys edulis*). **Journal of Agricultural and Food Chemistry**, v. 49, n. 10, p. 4646-4655, 2001.

L`ABBE, M. R.; TRICK, K. D.; BEARE-ROGERS, J. L. Dietary (n-3) fatty acids affect rat heart, liver and aorta. **Journal of Nutrition**, v. 121, p. 1331-1340, 1991.

LANG, A. E.; LOZANO, A. M. Parkinson's disease. First of two parts. **New England Journal of Medicine**, v. 339, n. 16, p. 111-114, 1998.

LOWRY, O. H. et al. Protein measurement with the Folin phenol reagent. **Journal of Biological Chemistry**, v. 193, n. 1, p. 265-275, 1951.

MISRA, H. P.; FRIDOVICH, I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. **Journal of Biological Chemistry**, v. 247, n. 10, p. 3170-3175, 1972.

OMURA, T.; SATO, R. The carbon monoxide-binding pigment of liver microsomes. Evidence for its hemoprotein nature. **Journal of Biological Chemistry**, v. 239, n. 7, p. 2370-2378, 1964.

PAN, G. X.; SPENCER, L.; LEARY G. J. Reactivity of ferulic acid and its derivative towards hydrogen peroxide and peracetic acid. **Journal of Agriculture and Food Chemistry**, v. 47, n. 8, p. 3325-3331, 1999.

PORTER, T. D. The roles of cytochrome b5 in cytochrome P450 reactions. **Journal of Biochemical and Molecular Toxicology**, v. 16, n. 6, p. 311-316, 2002.

REEDY, A. C.; LOKESH, B. R. Studies on spice principle as an antioxidant in the inhibition of lipid peroxidation of rat liver microsomes. **Molecular and Cellular Biochemistry**, v. 111, p. 117-124, 1992.

ROCHE, M. et al. Antioxidant activity of olive phenols mechanistic investigation and characterization of oxidation products by mass spectrometry. **Organic and Biomolecular Chemistry**, v. 3, p. 423-430, 2005.

ROESLER, R. et al. Antioxidant activity of *Annona crassiflora*: characterization of major components by electrospray ionization mass spectrometry. **Food Chemistry**, v. 104, n. 3, p. 1048-1054, 2007.

ROESLER, R. et al. Evaluation of the Antioxidant Properties of the Brazilian Cerrado Fruit *Annona crassiflora* (Araticum). **Journal of Food Science**, v. 71, n. 2, p. 102-107, 2006.

SCHENKMAN, J. B.; JANSSON, I. The many roles of cytochrome b5. **Pharmacology and Therapeutics**, v. 97, n. 2, p. 139-152, 2003.

SHEELA, C. G.; ANGUSTI, K. Antiperoxidase effects of S-allyl cysteine sulphoxion isolated from *Allium sativum Linn* and gugulipid in chlosterol diet fed rats. **Indian Journal of Experimental Biology**, v. 33, p. 337-341, 1995.

SHEWEITA, A. S.; EL-GABAR, M. A.; BASTAWY, M. Carbon tetrachloride changes in the activity of cytochrome P450 in liver of male rats: role of antioxidants. **Toxicology**, v. 169, n. 2, p. 83-92, 2001.

SINGAB, A. N. B. et al. Hepatoprotective effect of flavonol glycosides rich fraction from egyptian vicia calcarata desf against CCl4 – induced liver damage in rats. **Archives of Pharmacal Research**, v. 28, n. 7, p. 791-798, 2005.

SINGH, R. P. J.; CHIDAMBAR MURTHY, K. N.; JAYAPRAKASHA, G. K. Studies on antioxidant activity of pomegranate (*Punica granatum*) peel and seed extracts using in vitro models. **Journal of Agricultural and Food Chemistry**, v. 50, n. 1, p. 81-86, 2002.

SIPES, I. G.; KRISHNA, G.; GILLETTE, J. R. Bioactivation of carbon tetrachloride, chloroform and bromotrichloromethane: role of cytochrome P-450. **Life Science**, v. 20, n. 9, p. 1541-1548, 1977.

SRINIVASAN, M. et al. Ferulic acid, a natural protector against carbon tetrachloride-induced toxicity. **Fundamental and Clinical Pharmacology**, v. 19, n. 4, p. 491-496, 2005.

SWAIN, T.; HILLIS, W. E. The phenolic constituents of *Prunus domestica*. The quantitative analysis of phenolic constituents. **Journal of Science of Food and Agriculture**, v. 10, n. 1, p. 27-38, 1959.

TAKAHAMA, U. Suppression of lipid photoperoxidation by quercetin and its glycosides in spinach chloroplasts. **Photochemistry and Photobiology**, v. 38, p. 363-367, 1983.

TALALAY P. Mechanisms of induction of enzymes that protect against chemical carcinogenesis. **Advances in Enzyme Regulation**, v. 28, p. 337-250, 1989.

TALALAY, P. Chemoprotection against cancer induction of phase 2 enzymes. **Biofactors**, v. 12, p. 5-11, 2000.

THEODORUS, P. M.; SIES, H.; AKERBOOM, T. P. M. assay of glutathione, glutathione disulfide, and glutathione mixed disulfides in biological samples. In: JAKOBY, W. B. **Methods in enzymology** - Detoxication and drug metabolism: conjugation and related systems. Academic Press, 1981. vol. 77.

VAN DER SLUIS, A. A. et al. An improved, rapid in vitro method to measure antioxidant activity. application on selected flavonoids an apple juice. **Journal of Agriculture and Food Chemistry**, v. 48, n. 9, p. 4116-4122, 2000.

WATTENBERG, L. W. Inhibition of carcinogenesis by minor dietary constituents. **Cancer Research**, v. 52, n. 7, p. 2085-2091, 1992.

WEBER, L. W. D.; BOLL, M.; STAMPFL, A. Hepatotoxicity and mechanism of action of haloalkanes: Carbon tetrachloride as a toxicological model. **Critical Reviews in Toxicology**, v. 33, n. 2, p. 105-136, 2003.

WOHAIEB, S. A.; GODIN, D. V. Alterations in free radical tissue-defense mechanisms in streptozocin-induced diabetes in rat: effects of insulin treatment. **Diabetes**, v. 36, p. 1014-1018, 1987.

XING, Y.; WHITE, P. J. antioxidants from cereals and legumes. In: SHAHIDI, F. **Natural antioxidants chemistry**, **health effects, and applications**. Champaign, Illinois: AOCS Press, 1996.

YUAN, Y. V.; KITTS, D. D. endogenous antioxidants: role of antioxidant enzymes in biological systems. In: SHAHIDI, F. **Natural antioxidants chemistry, health effects, and applications, champaign**. Illinois: AOCS Press, 1996.

ZHU, M. et al. Evaluation of the protective effects of *Schisandra. chinensis* on Phase I drug metabolism using a CCl4 intoxication model. **Journal of Ethnopharmacology**, v. 67, n. 1, p. 61-68, 1999.

ZOU, L. et al. Effects of kava (Kava-kava, 'Awa, Yaqona, Piper methysticum) on c-DNA-expressed cytochrome P450 enzymes and human cryopreserved hepatocytes. **Phytomedicine**, v. 11, n. 4, p. 285-294, 2004.

ZUPPA, T. O. **Avaliação das potencialidades de plantas nativas e introduzidas no Cerrado na obtenção de óleos e gorduras vegetais**. 2001. 116 p. Dissertação (Mestrado em Química)-Instituto de Química, Universidade Federal de Goiás, Goiâna, 2001.