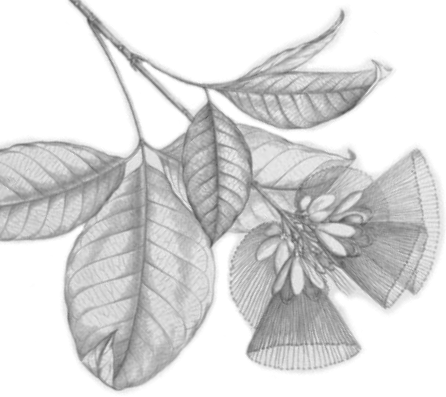

Antioxidant Activity and Flavonoids Identification by LC-MS/MS Analysis in Leaf Extract from *Trattinnickia rhoifolia* Willd

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ABSTRACT

The objectives of this work were to evaluate the antioxidant and hepatoprotective activities in the oxidative stress model induced by paracetamol in male *Swiss* mice, to evaluate the hypoglycemic and hypolipidemic action and to identify flavonoids in the leaves ethanolic extract from *Trattinnickia rhoifolia*. By the LC-MS / MS method, eight flavonoids were identified in the hydromethanolic (HM) and ethyl acetate (EA) fractions, except for Amentoflavone, the flavonoids Apigenin, Canferol, Luteolin, Quercetin, Quercetin-3-β-d-glucoside, Rutin and Taxifoline were identified for the first time in this species. These fractions were evaluated for antioxidant capacity (DPPH$^{\bullet}$ test) and their protective effect *in vivo* through the analysis of superoxide dismutase, catalase, glutathione-s-transferase, non-protein thiols, ascorbic acid, TBARS and carbonylated proteins. The data showed that EA has antioxidant capacity and superior oxidative stress repair in chemical and biological analyzes, besides hypoglycemic and hypolipidemic action. Thus, the present work contributes significantly to the literature, since it is the first study that identifies the chemical constituents and pharmacological properties of leaves extract from *T. rhoifolia*.

Keywords: *Trattinnickia*; Flavonoids; Antioxidant; Acetominophen.

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Plants are natural sources for treatment and prevention of diseases due to the presence of secondary metabolites, such as the polyphenols they possess. Among its properties, there is the capacity to act as an antioxidant (Coulibaly et al. 2014; Salgueiro et al. 2016). Among these phenolic compounds are flavonoids, present in several parts of plants, with the potential to reduce free radicals and the ability to chelate metal ions, which cause health damage (Heim et al. 2002).

Trattinnickia rhoifolia (Willd) (*T. rhoifolia*) is a tree species with angiosperm plants, present in the neotropical regions, belonging to the Burceraceae family. Known popularly in Brazil as *breu-sucuruba* (Silva et al. 2016), it is classified in the Canarieae tribe (Daly 1989). It has its resin and leaves popularly used to cure throat diseases, skin lacerations, treatment and prevention of tumors and leukemia (Salazar-Silvera & Rosquete 2010).

Previous studies on the phytochemistry of the genus *Trattinnickia* report the presence of lichenxantone, triterpenes and sterols in the bark of *T. peruviana* (Marques & Ribeiro 1994). The triterpenes and a sesquiterpene lactone (Aregullin et al. 2002) were isolated from the exuded resin of the *T. áspera* trunk. From the leaves of *T. glaziovii* the biflavonoid podocarpusflavone A was identified (Siani & Ribeiro 1995).

In the target species of this study, trunk bark, resins of *T. burserifolia* and *rhoifolia* triterpenes and monoterpenes were isolated (Lima et al. 2004), determined the presence of volatile monoterpenes in the resin oil removed from the *T. rhoifolia* tree (Ramos et al. 2003), amentoflavone and sterols were isolated as main constituents of fresh leaves of *T. rhoifolia* and sesquiterpene lactones isolated from *T. rhoifolia* resin (Rosquete et al. 2010).

Besides the phytochemical characterization, the evaluation of biological effect of its constituents is very important for the study of plants. The oxidative stress model induced by paracetamol has been widely used with the intention of investigating the effects of extracts and/or fractions of plant extracts rich in phenolic compounds on enzymatic and non-enzymatic biological activities, oxidative stress markers and biochemical parameters (Hodgman & Garrard 2012; Olaleye et al. 2014). Paracetamol (PCM) is a drug used as analgesic and antipyretic, but in high doses causes toxicity and depletion of the organs, causing liver damage, leading the body to oxidative stress and altering the antioxidant system (Mirochnitchenko et al. 1999). On the other hand, antioxidants act by inhibiting and protecting the body against the oxidative stress process caused by free radicals and reactive oxygen species (Nijveldt 2001).

Flavonoids have become the target of many studies because of their diverse benefits to human health. There are reports that some of them, such as quercetin, luteolin, apigenin, canferol and rutin, present anti-inflammatory, antioxidant, anti-diabetic and anticancer action (Duan et al. 2011; Ramirez et al. 2016; Shanmugam et al. 2016). In this context, this is the first study aiming to quantify the total phenolic and flavonoid content present in the leaves of *T. rhoifolia* and its correlation with the possible antioxidant potential as well as to identify and characterize the presence of flavonoids by LC-MS/MS. After evaluating the hepatoprotective effect in mice of the fractions of *T. rhoifolia* against hepatotoxicity induced by paracetamol and hypoglycemic and hypolipidemic action.

MATERIAL AND METHODS

CHEMICAL COMPOUNDS

Solvents: ethanol, ethyl acetate, hexane, dichloromethane and methanol (PA grade). Flavonoids: amentoflavone, apigenin, canferol, luteolin, quercetin and quercetin-3-β-d-glioside, rutin and taxifoline. Reagents: Folin-Ciocalteau, Gallic acid, ascorbic acid, ketamine, xylaxine, acepromazine, bovine serum albumin, Triton X-100, hydrogen peroxide (H_2O_2), reduced glutathione, malondialdehyde (MDA), 2-thiobarbituric acid (TBA), 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), 2,4-dinitrophenylhydrazine (DNPH). Bradford's reagent, trichloroacetic acid (TCA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium phosphate monobasic and dibasic, sodium phosphate monobasic and dibasic, ethylenediamine tetra-acetic acid (EDTA), disodium salt, trisaminomethane (Tris) and Tween 80. All these products were purchased from Sigma-Aldrich and Merck. The total cholesterol, triglyceride, glucose and alanine aminotransferase (ALT) dosage kits were purchased from Labtest®, Diagnostic S.A, Minas Gerais, Brazil.

PLANT MATERIAL AND EXTRACT PREPARATION

The *T. rhoifolia* leaves were collected around UFMT - Sinop - MT, located at 11°52'00"S and 55°28'58"W and identified by Professor Juliano de Paulo Santos. The exsicata was deposited in the Herbarium Center North - Mato Grossense (CNMT), Biological Collection of the Southern Amazon (Abam) of the Federal University of Mato Grosso - Campus of Sinop, under the number 6320.

The leaves were oven dried with forced ventilation at a temperature of 45 °C and submitted to exhaustive extraction by maceration (3x) for 7 days at ambient temperature with ethanol (PA). The crude extract was obtained after filtration and evaporation of the solvent under vacuum at 45 °C. Then, resuspended in methanol/water (1:1 v/v), filtered to remove chlorophyll and subjected to partition

with solvents by polarity gradient, resulting in Hydromethanolic (HM) and ethyl acetate (EA) fractions. Due to the high and medium polarity, these fractions were used to perform this study.

QUANTIFICATION OF PHENOLS AND TOTAL FLAVONOIDS

Analysis of the HM and EA fractions were determined by UV-Vis spectrometry. The total phenol content was determined by the Folin-Ciocalteu method at 760 nm (Woisky & Salatino 1998), using gallic acid as the standard for the calibration curve at concentrations 1.5; 2.0; 2.5; 3.0; 3.5 and 4.0 μg mL^{-1} and expressed in milligrams of gallic acid equivalent per gram of extract (mg EAG g^{-1}). The total flavonoid content was measured at 425 nm by the colorimetric method with aluminum chloride ($AlCl_3$) (Woisky and Salatino 1998). For the calibration curve, the quercetin standard used was (0.25, 0.50, 1.0, 1.5, 2.5, 3.0, 3.5, 4.0 and 4.5 μg mL^{-1}) and the result expressed in milligrams of quercetin equivalent per gram of extract (mg EQ g^{-1}).

DETERMINATION OF ANTIOXIDANT POTENTIAL *IN VITRO* DPPH

The evaluation of antioxidant potential (Coulibaly et al. 2014), with adaptations, consists in determining via UV-Vis spectrophotometry at 515 nm the absorbance of the gradual change of coloration by action of the antioxidant potential of the extracts with sequestering capacity of the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) free radical. The percentage of DPPH remaining in the reaction medium was determined from the comparison with standard ascorbic acid and rutin, determining the effective concentration (EC_{50}) as the amount of antioxidant needed to decrease the initial concentration of DPPH in 50%.

LC-MS/MS ANALYSIS

The identification of flavonoids in the HM and EA fractions of the crude ethanolic extract of T. rhoifolia leaves was performed according to the methodology below, with modifications (Duan et al. 2011). It was characterized by comparing the retention time with the authentic external standards prepared in methanol, filtered and injected separately: Amentoflavone, Apigenin, Canferol, Luteolin, Quercetin, Quercetin-3-β-d-glucoside, Routine and Taxifolin. LC-MS/MS analysis was performed on Agilent 1290 Infinity UHPLC equipment (Agilent Technologies, USA). Chromatographic separations were obtained with an Agilent Eclipse AAA column (4.6 x 150 mm, 3.5μm) at 25 °C with a sample injection volume of 20 μL. Gradient elution of phases A (0.1% formic acid in water) and phase B (0.1% formic acid in ACN) were carried out under the following conditions: 0-30 min 5-95% B; 30-32 min 100% B; 32-33 min 95-5% B with a flow rate of 0.5 mL min^{-1}. Mass spectrometric detection was

performed on an Agilent 6460 Triple Quad with electrospray ionization source (ESI) using nitrogen gas under the following conditions: gas temperature of 300 °C; flow 5 L min^{-1}; nebulizer pressure 45 psi; sheath gas temperature 250 °C; flow 11 L min^{-1}; capillary voltage -3500 V and scanning range of m/z 120-900 units.

ANIMALS AND TREATMENT

Male *Swiss* mice with mean weight 32 ± 4 g were obtained from the Central Biotério of the Federal University of Mato Grosso, Campus of Cuiaba. The animals were divided into 06 groups of 07-08 animals, acclimated for 10 days with photo control of 12 hours (light/dark), temperature of 24 ± 1 °C, relative humidity of 51 ± 2%, ration and filtered water in free demand. The treatments were administered orally (gavage) and extracts (HM and EA, 100 mg kg^{-1}) dissolved in water and Tween 80 (0.1%), dose determined by Malone Hippocratic test by means of a dose curve (50 to 1000 mg kg^{-1}). Paracetamol (PCM 250 mg kg^{-1}) was diluted in water (Olaleye & Rocha 2008). According to the treatments, the groups were divided into: Group I- Control (water); Group II - PCM; Group III - PCM + HM; Group IV - PCM + EA; Group V - HM; Group VI - EA. The animals of groups II, III and IV received a single dose of PCM as an inducer of injury 03 hours before the start of daily treatment. Fractions were given in groups III, IV, V and VI, and groups I and II received water + tween 80 (0.1%) during the 07 days of treatment.

After 24 hours of the last dose of treatment, the animals were submitted to 06 hours of fasting and anesthetized intraperitoneally (ketamine 50 mg kg^{-1}, xylaxine 2 mg kg^{-1} and acepromazine 2 mg kg^{-1}) for blood collection by cardiac puncture and sacrificed for dislocation of the organs. Liver and kidney tissue were rapidly removed and frozen at -85 °C. This research was certified according to protocol no. 23108.701484/14-2 of the Ethics Committee on Animal Research (CEPA), UFMT/Cuiaba.

BIOCHEMICAL TESTS

For biochemical tests, plasma obtained from the centrifugation (1000 x g for 10 min) of the blood collected and tissue samples (liver and kidney) were homogenized in buffer solution specific to each technique.

ANALYZIS OF ENZYMATIC ANTIOXIDANT ACTIVITIES

The following analyzes were performed: superoxide dismutase (SOD), in the liver (1:20 m/v), based on the inhibition of auto-oxidation of epinephrine by the enzyme, measured at 480 nm in UV-Vis and expressed in μmol min^{-1} mg $protein^{-1}$ (Misra & Fridovich 1972). The catalytic adsorption of

H_2O_2 by catalase (CAT) was determined by the change in absorbance at 240 nm, expressed in μmol H_2O_2 decomposed per minute per milligram of protein (μmol min^{-1} mg $protein^{-1}$), in the liver (1:30 m/v) and kidney (1:20 m/v) (Nelson & Kiesow 1972). The activity of glutathione-S-transferase (GST) was measured in a spectrophotometer at 340 nm using 1-chloro-2,4-dinitrobenzene as substrate in the liver (1:20 m/v), and expressed in μmol GS-DNB min^{-1} mg $protein^{-1}$ (Habig et al. 1974).

Analysis of Non-Enzymatic Antioxidant Activities

Non-protein thiols (GSH) were measured in the liver (1:40 m/v) and kidney (1:30 m/v) with absorbance measurements read at 412 nm, the GSH concentration was calculated through a standard reduced glutathione curve and the results expressed in μmol of GSH mg $protein^{-1}$ (Sedlack & Lindsay 1968). The levels of ascorbic acid (ASA) in the liver (1:15 m/v) were determined, expressed in μmol ASA g^{-1} weight (Roe 1954).

Protein concentration in the assays (except for ASA) was measured in a spectrophotometer at 595 nm using bovine albumin as standard, with the appropriate modifications according to dilution and analysis in the tissues used (Bradford 1976).

Oxidative stress Markers

Lipid damage in the liver (1:8 m/v), with some modifications (Buege & Aust 1978). The readings were performed in a UV-Vis spectrophotometer at 535 nm and the values for thiobarbituric acid reactive substances (TBARS) were expressed by nmol MDA mg $protein^{-1}$. The determination of carbonylated proteins (CP), in the liver and kidney (1:80 m/v), absorbance read at 370 nm, and CP levels expressed as nmol carbonyl mg $protein^{-1}$ (Yan et al. 1995).

For analysis of the hepatic damage marker and plasma metabolic parameters, the activity of the enzyme alanine aminotransferase (ALT), expressed in UL^{-1}, as well as the quantification of plasma glucose, total cholesterol and triglycerides expressed as mg dL^{-1}, using commercial kits.

Statistical Analyzes

All values were expressed as means ± standard deviation (SD). To determine the differences between the means of the experimental groups, the statistical analysis of variance (ANOVA) of one way was used and, as post hoc, the Tukey test, with $p < 0.05$ as indicative of significance level. Values for EC_{50} were estimated graphically by linear regression analysis.

RESULTS AND DISCUSSION

YIELD OF PHENOLS, TOTAL FLAVONOIDS AND ANTIOXIDANT POTENTIAL

According to Table 1, the total phenol values in the EA and HM fractions did not show any difference, since the total flavonoids content was higher in the EA fraction. The results of the antioxidant potential through the remaining % DPPH, the EC_{50} of the fractions were similar to standard ascorbic acid and rutin, with EC_{50} 38 and 43 μg mL^{-1}, respectively.

Table 1. Values of phenols, total flavonoids and potential antioxidants of HM and EA fractions of *T. rhoifolia*.

Analisys	HM	EA
Total phenols (mgEAG g^{-1})	436 ± 2.96	437 ± 6.05
Total flavonoids (mgEQ g^{-1})	12 ± 0.18	40 ± 0.88
DPPH• (μg mL^{-1})	EC_{50} 44	EC_{50} 35

Source: The Author

LC-MS/MS ANALISYS

The multiple reaction monitoring (MRM) method was used in the negative ionization mode for the identification of flavonoids, with the parameters described in Table 2 and Figure 1.

Table 2. LC-MS/MS Parameters of 08 compounds identified in the crude ethanolic extract of *T. rhoifolia*. Fragmentation stress (F.S.), collision energy (C.E.) and retention time (R.T).

	Compound	$[M - H]^-$	F.S./ C.E. (eV)	R.T. (min)	Transition MRM
1	Amentoflavone	537.46	10 / 30	17.4	537.46→375.00
2	Apigenin	269.24	10 / 30	16.6	269.24→ 116.80
3	Kaempferol	285.24	10 / 30	17.1	285.24→ 93.00
4	Luteolin	285.24	10 / 25	14.9	285.24→133.00
5	quercetin	301.24	10 / 25	15.0	301.24→151.00
6	quercetin-3-β-d-glucoside	463.38	10 / 30	10.8	463.38→300.00
7	Rutin	609.27	10 / 30	10.4	609.27→300.20
8	Taxifoline	303.25	10 / 15	12.1	30.25→125.00

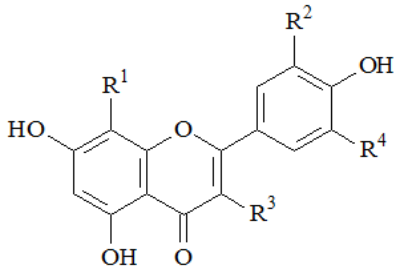
Source: Authors.

The analysis of the crude ethanolic extract presented eight flavonoids: the compounds 1, 2, 3, 4, 5, 6, 7 and 8 in the HM fraction and the compounds 1, 2, 3, 4, 5 and 6 in the EA fraction. The scheme shows the chemical structure of the eight flavonoids identified in the crude ethanolic extract of *T. rhoifolia* and Figure 2 shows the chromatogram with retention time of the flavonoids found in the HM fraction.

Among the various classes of natural bioactive products, in the group of polyphenolic substances are the flavonoids, which are prominent, and widely distributed by the vegetable kingdom, been notable for its diversified biological actions (Coutinho et al. 2009). Flavonoids may have different

concentrations, depending on the plant organ (Simões et al. 2004). In relation to *T. rhoifolia*, there are few studies on the ethanolic extract of leaves of the plant, which makes the present study unpublished in relation to the presented results. It is known that this species belongs to a family known to produce aromatic oils, resin for treatment of various diseases and that the wood of its tree is used in the construction and for the manufacture of artifacts and furnitures (La Cruz-Cañizares et al. 2005; Salazar-Silvera & Rosquete 2010). In the present study, the ethyl acetate (EA) fraction had flavonoid content higher than the hydrometanic fraction (HM). This may be associated with the fact that, to improve extraction and obtain greater yield, the selection of conditions and solvents influences the ability to have a good solubility of the substituted radicals of flavonoids (Al-Saeedi & Hossain 2015).

Figure 1. Chemical structure of the eight flavonoids present in the ethanolic extract of *T. rhoifolia*.



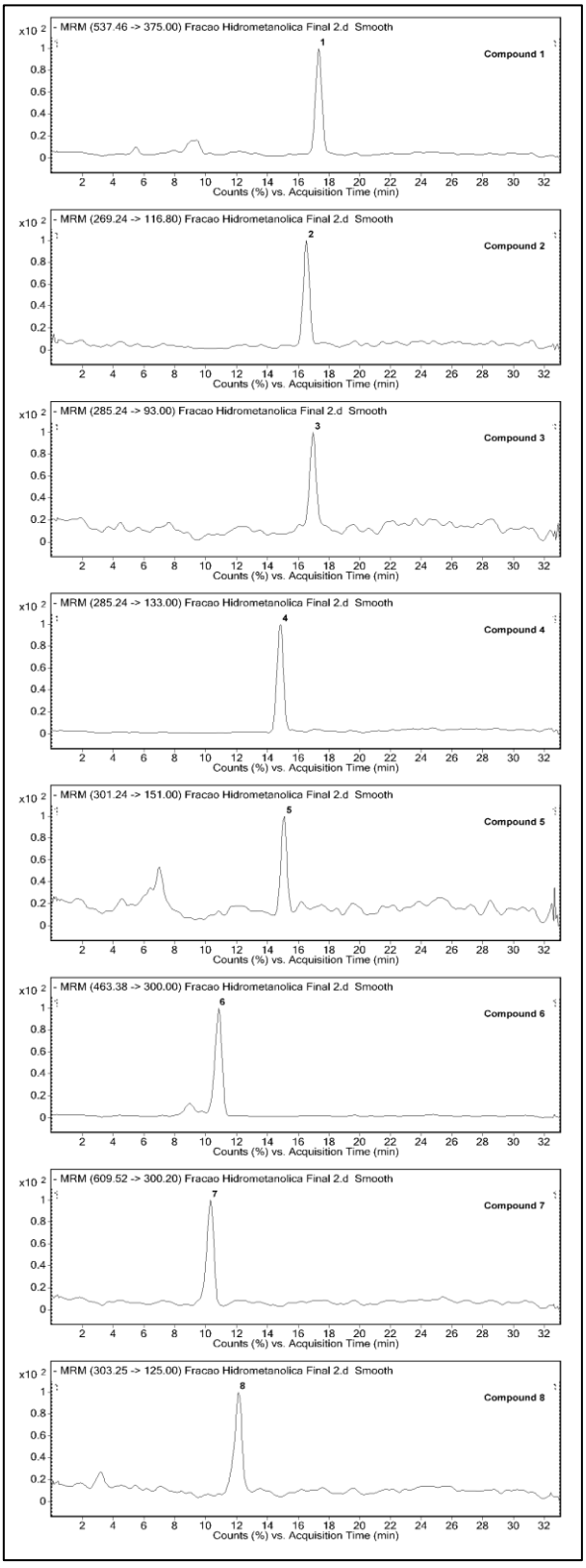
Compound	R^1	R^2	R^3	R^4
1	apigenin	H	H	H
2	H	H	H	H
3	H	H	OH	H
4	H	OH	H	H
5	H	OH	OH	H
6	H	H	O-glucoside	OH
7	H	H	O-(rhamnosyl-glucoside	OH
8	H	H	OH	OH

Source: Authors.

Liquid chromatography coupled to mass spectrometry (LC/MS) represents a powerful, fast and reliable tool for the analysis and identification of compounds (Cuyckens & Claeys 2004). The use of a mobile phase containing acetonitrile and a 0.1% concentration of formic acid provides greater sensitivity, lower background noise and increases the efficiency of electrospray ionization (ESI) for phenolic compounds (Duan et al. 2011). In order to identify the flavonoids in the fractions, the multiple reaction monitoring (MRM) method was used, that had its precursor ions and product ions initially characterized according to their mass spectra (Qiu et al. 2015).

The results showed that negative ion-mode ESI was more sensitive to phenolic compounds in the present study, as the optimized MS/MS parameters and the MRM transitions were consistent with the previously described investigations (Biesaga & Pyrzynska 2009; Duan et al. 2011; Faccin et al. 2016; Tang et al. 2014; Xie et al. 2011). When analyzed by LC-MS/MS the presence of flavonoids in the fractions were confirmed by their molecular ions and fragments.

Figure 2. LC-MS/MS from *T. rhoifolia* HM fraction. Chromatograms with retention time of the identified compounds 1 to 8, mass of the deprotonated molecular ion, mass of the main ion and breaks of the chemical structures of identified flavonoids.



Source: Authors.

Compound 1 presented molecular ion 537.46 (m/z -1) and fragment 375.00 (m/z - 162.46), representing the loss of the fragment located in C-ring, which shows the phenol, ether and ketone functions. Compound 2 showed the molecular ion 269.24 (m/z -1) and fragment 116.80 (m/z - 158.44), representing the C-ring breaking between carbons 2 and 3. Compound 3 showed molecular ion 285.24 (m/z -1) and fragment 93.00 (m/z - 192.25), corresponding to the loss of a phenol group. Compound 4 presented molecular ion 285.24 (m/z - 1) and fragment 133.00 (m/z - 152.24), representing the loss of benzene diol ring. Compound 5 presented the molecular ion 301.24 (m/z - 1) and fragment 151.00 (m/z - 150.24), which also corresponds to the breakage of C-ring between carbons 2 and 3, having three hydroxyls. Compound 6 presented molecular ion 463.38 (m/z - 1) and fragment 300.00 (m/z - 163,38) representing the breaking of a sugar. Compound 7 presented molecular ion 609.52 (m/z - 1) and fragment 300.20 (m/z - 309.32), corresponding to the loss of two sugars. Compound 8 presented molecular ion 303.25 (m/z - 1) and fragment 125.00 (m/z - 178.25), representing the C-ring break between carbon 2 and carbonyl. The analyzes presented similar fragments already described in the literature (Xie et al. 2011; Biesaga & Pyrzynska 2009; Tang et al. 2014; Orčić et al. 2014; Touati et al. 2017).

Analysis of Enzymatic Antioxidant Activities of Hepatic Tissue

Figure 3A shows SOD activity did not change in the groups exposed to PCM, but there was an increase in the HM group, presenting a significance level of $p<0.05$ when compared to the control group. Figure 3B shows that the enzyme CAT decreased in the PCM group (11.98 ± 1.75 μmol min^{-1} mg $protein^{-1}$), compared to the control group (16.28 ± 1.88 μmol min^{-1} mg $protein^{-1}$) and the PCM+HM and PCM+EA groups were able to restore the levels of this enzymatic activity (18.43 ± 3.26, 16.83 ± 2.62 μmol min^{-1} mg $protein^{-1}$, respectively; $p<0.001$). Liver GST (Figure 3C) decreased in the PCM group (0.79 ± 0.06 μmol GS-DNB min^{-1} mg $protein^{-1}$) versus control group (1.03 ± 0.11 μmol GS-DNB min^{-1} mg $protein^{-1}$) and the treatment was able to reestablish this decrease at the control levels (PCM+EA group 1.03 ± 0.11 μmol GS-DNB min^{-1} mg $protein^{-1}$; $p <0.01$).

Analyzis of Non-Enzymatic Antioxidant Activities of Hepatic Tissue

In GSH (Figure 4A) there was a decrease in the PCM, PCM+HM and PCM+EA groups (47.58 ± 5.64, 46.12 ± 10.57, 46.98 ± 9.25 μmol of GSH mg $protein^{-1}$, respectively) compared to the control group (65.22 ± 7.3 μmol of GSH mg $protein^{-1}$) with $p <0.0001$. Ascorbic acid (ASA) decreased in the PCM group (0.83 ± 0.05 μmol ASA g^{-1}weight) compared to the control group (1.11 ± 0.17 μmol ASA g^{-1} weight). On the other hand, the PCM+HM (1.13 ± 0.15 μmol ASA g^{-1} weight) and PCM+EA

(1.09 ± 0.14 μmol ASA g^{-1} weight) groups, both $p<0.01$, were able to restore these levels, as shown in Figure 4B.

Figure 3. Effect of HM and EA fractions on liver of mice submitted to paracetamol-induced oxidative stress on enzyme activity: A. SOD *p <0.05 compared to control group; B. p<0.001 compared to the control group. **p <0.001 compared to the PCM group and; C. GST *p <0.01 compared to the control group. Values are mean ± SD. (N = 8).

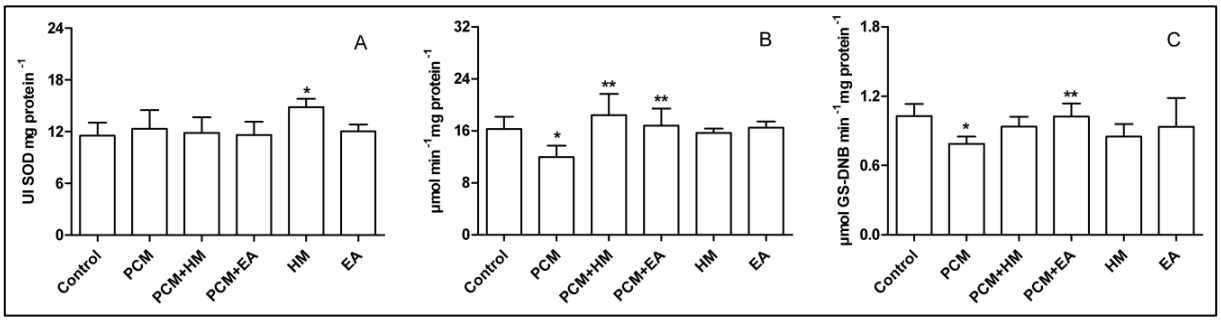


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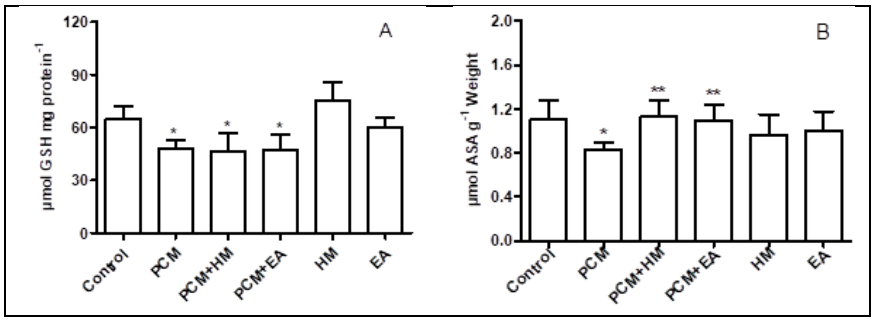
Figure 4. Effect of HM and EA fractions on liver of mice submitted to paracetamol-induced oxidative stress in the concentration of: A. GSH *p<0.0001 compared to the control group and; B. ASA *p<0.01 compared to the control group. **p<0.01 compared to the PCM group. Values are mean ± SD. (N = 8).

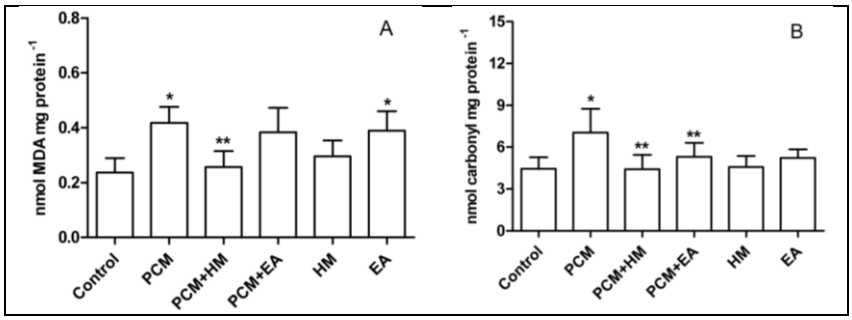


Source: Authors.

OXIDATIVE STRESS MARKERS OF LIVER TISSUE

Figure 5A shows an increase in lipid peroxidation (TBARS) in the PCM group (0.42 ± 0.06 nmol MDA mg $protein^{-1}$), when compared to the control group (0.24 ± 0.05 nmol MDA mg $protein^{-1}$), which was reduced for treatment with HM (PCM+HM group 0.26 ± 0.06 nmol MDA mg $protein^{-1}$, $p<0.0001$). However, the same did not occur for treatment with EA (0.39 ± 0.07 nmol MDA mg $protein^{-1}$). In Figure 5B, it was observed that PCM induced a significant increase in the levels of carbonylated proteins (7.06 ± 1.69 nmol carbonyl mg $protein^{-1}$) when compared to the control group (4.45 ± 0.82 (PCM+HM 4.41 ± 1.04 and PCM+EA 5.31 ± 0.99 nmol carbonyl mg $protein^{-1}$; $p<0.0001$).

Figure 5. Effect of HM and EA fractions on liver of mice submitted to paracetamol-induced oxidative stress in the concentration of: A. TBARS and; B. PC, both *p<0.0001, compared to the control group. **p<0.0001, compared to the PCM group. Values are mean ± SD. (N = 8).

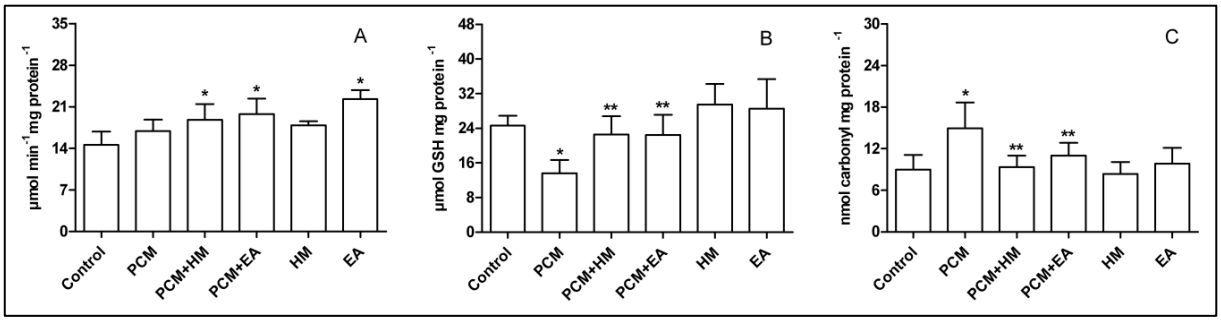


Source: Authors.

EFFECTS ON ANTIOXIDANT PARAMETERS AND RENAL OXIDATIVE STRESS

CAT increased its activity in the PCM+HM, PCM+EA and EA groups (18.79 ± 1.94, 19.75 ± 2.64 and 22.30 ± 4.19 μmol min^{-1} mg $protein^{-1}$, respectively), related to the control group (14.56 ± 2.27 μmol min^{-1} mg $protein^{-1}$), presenting a level of significance p<0.0001, as shown in Figure 6A. In the GSH of kidney (Figure 6B) there was a reduction of their concentration in the PCM group (13.62 ± 3.07 μmol of GSH mg $protein^{-1}$), when compared to the control group (24.58 ± 2.31 μmol of GSH mg protein-1). The PCM+HM groups (22.53 ± 4.29 μmol of GSH mg $protein^{-1}$) and PCM+EA (22.46 ± 4.62 μmol of GSH mg $protein^{-1}$) reversed this decrease with a significance level of p<0.0001.

Figure 6. Effect of HM and EA fractions on kidneys of mice submitted to oxidative stress induced by paracetamol. A. CAT enzyme activity; B. concentration of GSH and C. concentration of PC. *p <0.0001 compared to the control group. **p <0.0001 compared to the PCM group. Values are mean ± SD. (N = 8).



Source: Authors.

Figure 6C shows the effects on carbonylated proteins where the PCM group had their levels increased (14.95 ± 3.73 nmol carbonyl mg $protein^{-1}$), compared to the control group (9.01 ± 2.10 nmol carbonyl mg $protein^{-1}$), that was significantly restored (p<0.0001) by both the PCM+HM group (9.34 ± 1.67 nmol carbonyl mg $protein^{-1}$) and the PCM+EA group (10.99 ± 1.87 nmol carbonyl mg $protein^{-1}$).

Enzymatic Activity Related to Liver Function and Metabolic Parameters of Plasma

The analysis of plasma ALT enzymatic activity showed a significant increase ($p < 0.0001$) in the PCM group of approximately 68.0% and in the HM group of 90.0% in relation to the control group. The PCM+EA group was able to reduce this ALT increase by around 52.0%. The HM and EA fractions showed a capacity to decrease glycemic levels (hypoglycemic activity) in 30.0% and 24.5%, respectively, when compared to the control group. Total cholesterol did not present significant differences in the groups of interest. In the dosage of triglycerides both fractions had their levels significantly reduced: HM (32.5%) and EA (28.4%) in relation to the control group ($p < 0.0001$) (Table 3).

Table 3. Effect of the hydromethanolic and ethyl acetate fractions of *T. rhoifolia* leaves on biochemical analyzes of plasma.

Group	Treatment	ALT (U L^{-1})	Glucose (mg dL^{-1})	Cholesterol (mg dL^{-1})	Triglycerides (mg dL^{-1})
I	Control	39.96 ± 3.52	250.6 ± 19.71	40.31 ± 9.06	268.8 ± 53.30
II	PCM	67.05 ± 11.16*	237.2 ±24.42	49.74 ± 3.16	251.4 ± 24.88
III	PCM+HM	64.17 ± 8.35	275.7 ± 30.06	46.32 ± 6.49	210.3 ± 35.03
IV	PCM+EA	34.80 ± 6.18**	221.6 ± 48.12	40.18 ± 7.74	146.6 ± 31.37
V	HM	75.94 ± 13.76*	175.4 ± 36.31*	41.45 ± 7.69	181.5 ± 41.71*
VI	EA	46.02 ± 10.13	189.0 ± 29.19*	48.29 ± 5.24	192.5 ± 41.07*

Source: Authors.
Values are mean ± SD. (N = 7); * $p < 0.0001$ compared to the control group, ** $p < 0.0001$ compared to the PCM group.

To the flavonoids identified in the leaves of *T. rhoifolia* are attributed mainly the antioxidant property, which provides protection to the renal, cardiovascular and hepatic systems (D'Andrea 2015). They act by inhibiting the process of free radical formation, by interacting with superoxide anions, the formation of hydroxyl radicals produced by the Fenton or Harber-Weis reaction on metal ion chelation and lipid peroxidation, by reacting with peroxyl and alkoxyl radicals. They can also act as pro-oxidants, depending on the number of hydroxyl groups present in their structure (Afanas'ev et al. 1989; Heim et al. 2002).

The human body produces free radicals from normal metabolism or it is induced by physical-chemical factors in the environment (Bouterfas et al. 2016). It is important to note that the use of DPPH is a stable free radical widely used in solutions for a simple estimation of the antioxidant capacity of phenolic compounds present in plants (Achat et al. 2016). The EA fraction had a higher capacity to kidnap this free radical, because it has a higher concentration of flavonoids, suggesting an increase in the antioxidant protective effect, favoring the biological system (Behling et al. 2004). Some

studies report that free radicals react with nitric oxide, which at high concentrations cause oxidative damage. The flavonoids quercetin and kaempferol present in the *T. rhoifolia* fractions act to inhibit the production of nitric oxide by regulating the inducible activity of the iNOS enzyme (Coutinho et al. 2009; Marković et al. 2014).

Oxidative stress caused by the imbalance of the antioxidant system generates excess of free radicals that can cause damage to cells and tissues. The imbalance between oxidative and antioxidant compounds favors the installation of oxidative stress process, which leads to the oxidation of biomolecules, generating excess of free radicals with consequent loss of their biological functions, whose manifestation is potential oxidative damage against cells and tissues (Halliwell & Whiteman 2004). Cells and body tissues are continually threatened by damage from free radicals and reactive oxygen species, which are produced during normal oxygen metabolism, or induced by exogenous damage, as in the case of paracetamol (Sánchez 2016).

The body's antioxidant defense mechanisms include two lines, the enzymatic ones, such as superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST), and non-enzymatic ones, such as reduced glutathione (GSH) and ascorbic acid (ASA) (Urso & Clarkson 2003). When the production of free radicals and/or reactive species exceeds the antioxidants' ability to act, oxidation of biomolecules occurs, generating specific metabolites, markers of oxidative stress that can be identified and quantified, such as TBARS and carbonylated proteins (PC) (Mayne 2003).

The PCM is a widely used and safe analgesic and antipyretic drug when administered in therapeutic doses. When ingested at high doses it may cause hepatic necrosis, following its conversion by the cytochrome P450, which covalently binds to proteins and reacts rapidly with glutathione, causing hepatic depletion of GSH (Olaleye et al. 2014; Shanmugam et al. 2013). Oxidative stress caused by excess of N-acetyl-p-benzoquinone imine (NAPQI) and increased production of reactive oxygen species during tissue damage has been shown to affect the body's antioxidant system (Olaleye & Rocha 2008; Silva et al. 2012).

The results obtained in this study show that PCM caused the reduction of CAT, GST, GSH and ASA and the increase of TBARS and PC in the liver. In the kidney, decreased the GSH and raised the PC, which proves the damage caused by PCM to the antioxidant defense system. In a leaf extract study, indicated a possible function of the compounds luteolin and quercetin-3-β-d-glucoside to alleviate liver damage of the liver against intoxication (Shanmugam et al. 2016).

GSH is the most prevalent, important and multifunctional intracellular antioxidant present in all living organisms. It is characterized by a reactive thiol group and γ-glutamyl bond which proves to be a natural capture agent in the detoxification of free radicals as well as reactive electrophiles (Suke et al. 2013; Sentellas et al. 2014). In the present study, the GSH of the liver was reduced in the PCM groups and treated with the fractions. This may be associated with the fact that flavonoids, such as quercetin, can conjugate with thiols and reduce their peroxynitrite elimination capacity and antioxidant potential (Pollard et al. 2006). In contrast, in the kidney, despite the decrease in activity by PCM, the concentration of GSH was normalized by the treatments, suggesting that it may be due to the lower availability of GSH found in this tissue, compared to the liver (Limón-Pacheco & Gonsebatt 2009).

An important antioxidant enzyme involved in the process of detoxification and excretion of xenobiotics and drugs, GST, had its levels decreased by treatment with PCM in the liver, as expected. The EA fraction reversed this reduced activity by PCM indicating that the compounds present in this fraction have antioxidant constituents, which facilitate the rapid and efficient consumption of reactive oxygen species, contributing to the metabolization of toxic compounds to non-toxic compounds (Singh et al. 2015).

Biological systems self-protect against reactivated species by means of endogenous SOD and CAT antioxidant enzymes that are responsible for the dismutation of superoxide radicals in H_2O_2 and detoxification of H_2O_2 in water, respectively, providing protection against tissue damage (Olaleye et al. 2014). The reduction of CAT in the liver is linked to enzyme depletion, as result of the oxidative stress caused by PCM. The treatments performed in this experiment increased the activity of this enzyme by demonstrating the antioxidant properties of oxygen free radical fractions (Srinivasan et al. 2007). On the other hand, it may suggest that reversion of the damage and increase of CAT activity by the EA fraction in the kidney may be related to the ability of flavonoids to increase the function of endogenous antioxidants (Nijveldt 2001; Olaleye et al. 2014).

The non-enzymatic defense system includes, in particular, antioxidant compounds of dietary origin, which are vitamins, minerals and phenolic compounds (Shinagawa et al. 2015). Ascorbic acid (ASA) is an excellent antioxidant, soluble in water, essential to man for its action as a redox agent in biological systems (Suke et al. 2013). It is commonly found in our body in the form of ascorbate which has the property of converting reactive oxygen and nitrogen species into harmless species, acting as an antioxidant *in vivo* (Halliwell 1999). The hepatic reversion of the toxic effect of PCM on the ascorbate pathway may be a result of the flavonoid ability present in *T. rhoifolia* fractions acting as antioxidants,

inactivating free radicals in both cellular and hydrophilic and lipophilic compartments (Teixeira et al. 2005).

Lipid peroxidation is a process induced by free radicals and can lead to cell membrane rupture by the hydrogen sequestration of polyunsaturated fatty acids (Puntel et al. 2007). In this study, animals poisoned with PCM showed an increase in TBARS and treatment with the HM fraction reversed this effect. The animals treated with the EA fraction suffered similar intoxication. This fact may be due those phenolic compounds, among them flavonoids, which in addition to antioxidant can act as pro-oxidant, depending on conditions and tissues self-oxidizing. The auto-oxidation products may react or reduce the concentrations of the antioxidant defense system (Pereira et al. 2013). The fact that the HM fraction has restored lipid damage suggests the glycosylation and methylation of OH groups of flavonoids, such as rutin, to attenuate the pro-oxidant behavior of flavonoids (Heim et al. 2002).

Another oxidative marker, protein carbonylation (PC) had its PCM toxicity abolished in both liver and kidney by the HM and EA fractions of *T. rhoifolia* because they contain compounds able to contributing to the maintenance of redox balance of antioxidant enzymatic activities (Carvalho et al. 2013). According to specific structural characteristics, flavonoids can chelate a wide range of metal ions of biological interest (iron, copper and zinc) to form complexes that exhibit new pharmacological activities or improve their activity (Imessaoudene et al. 2016).

The PCM group showed an increase in plasma ALT activity, indicating that the dose tested may cause liver damage, as already been demonstrated in several studies. Reversal by EA fraction may be indicative of non-toxicity to hepatocytes. The ability to restore the activity of this enzyme suggests the regulatory influence of antioxidant compounds present in the extract (Olaleye et al. 2014).

The reduction of glucose and triglycerides suggests that *T. rhoifolia* has a hypoglycemic and hypolipidemic effect. The studied species presented, in its constitution, phenolic compounds and flavonoids that, in addition to the innumerable properties, have the possibility to modify several physiological functions through the interaction with enzymes, such as cytochrome P450, and membrane transporters, such as glucose, transport proteins (Dornas et al. 2007). Quercetin and rutin have anti-diabetic and antioxidant potential, but their magnitude of action varies and both can be considered as a dietary supplement with potential for the prevention and treatment of type 2 diabetes and to suppress the damage mediated by oxidative stress in diabetic pathophysiology (Dhanya et al. 2014). The ability to reduce triglycerides can occur by suppressing oxidative modification of lipids and lipoproteins due to the presence of phenolic compounds in extracts of plants with potential

antioxidants (Ramchoun et al. 2012). Thus, this study and its results suggest that the concentration and form of the flavonoid, related to type of conjugate and its location in the skeleton, influences the rate of absorption, the capacity for inhibition of enzymes, the antioxidant activity and protective action against endogenous and exogenous agents (PCM), which can attack the body.

ACKNOWLEDGMENT

The authors thank to UFMT-Sinop for the laboratory structure, to FAPEMAT for the financial support and to EMBRAPA Agrossilvopastoril-Sinop for the LC-MS/MS analysis.

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Atividade Antioxidante e Identificação de Flavonoides por Análise LC-MS/MS em Extrato de Folhas de *Trattinnickia rhoifolia* Willd

RESUMO

Os objetivos deste trabalho foram avaliar as atividades antioxidante e hepatoprotetora no modelo de estresse oxidativo induzido por paracetamol em camundongos *Swiss* machos,avaliar a ação hipoglicemiante e hipolipidêmica e identificar flavonoides no extrato etanólico de folhas da *Trattinnickia rhoifolia*. Pelo método de LC-MS/MS foram identificados oito flavonoides nas frações hidrometanólica (HM) e acetato de etila (EA), com exceção da Amentoflavona, os flavonoides Apigenina, Canferol, Luteolina, Quercetina, Quercetina-3-β-d-glicosídeo, Rutina e Taxifolina, foram identificados pela primeira vez na espécie. Estas frações foram avaliadas quanto à capacidade antioxidante (teste de DPPH$^{\bullet}$) e seu efeito protetor *in vivo* através das análises de superóxido dismutase, catalase, glutationa-*s*-transferase, tiois não-proteicos, ácido ascórbico, TBARS e proteínas carboniladas. Os dados mostraram que EA possui capacidade antioxidante e reparo do estresse oxidativo superior nas análises químicas e biológicas, além de ação hipoglicemiante e hipolipidêmica. Assim, o presente trabalho contribui significativamente para a literatura, uma vez que é o primeiro estudo que identifica os constituintes químicos e propriedades farmacológicas do extrato de folhas de *T. rhoifolia*.

Palavras-Chave: *Trattinnickia*; Flavonoides; Antioxidante; Paracetamol.

Submission: 29/03/2018
Acceptance: 03/12/2018