Orbital
The electronic journal of chemistry

the electronic journal of chemistry

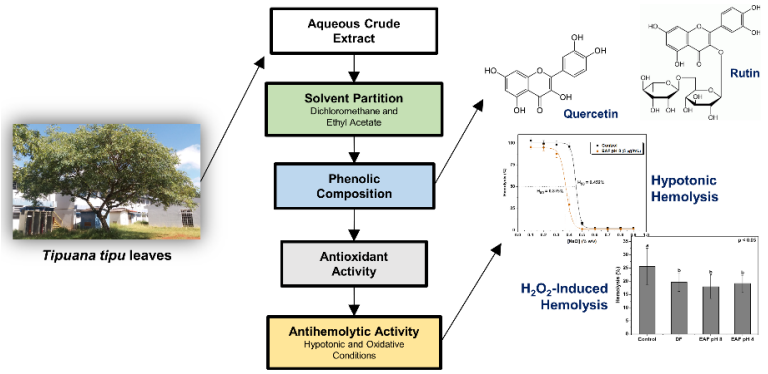
Full Paper | http://dx.doi.org/10.17807/orbital.v17i2.20191

Phenolic Composition and Antioxidant and Antihemolytic Activities of the Dichloromethane and Ethyl Acetate Fractions from *Tipuana tipu* (Benth.) Leaves

Thiago Mendanha Cruz* [a], Ana Laiza Kutzmy [a], Yasmin Stelle [a], Jânio Sousa Santos [b], Daniel Granato [c], Domingos Savio Nunes [a], and Mariza Boscacci Marques* [a]

The *Tipuana tipu* is a leguminous tree employed in popular medicine for its healing and anti-inflammatory properties. This study aimed to assess the antioxidant activity and bioactivity of fractions of the crude extract from the *T. tipu* leaves. The chemical composition, antioxidant activity, lipid peroxidation inhibition and antihemolytic activity were assessed on the dichloromethane and ethyl acetate (pH 4 and 8) fractions of the crude extract obtained from *T. tipu* leaves. The fractions presented significant antioxidant activity (IC_{50} DPPH = 246, 185 and 244 mg/L for dichloromethane, ethyl acetate at pH 8 and ethyl acetate at pH 4, respectively), protection against lipid peroxidation (inhibition of 10-81%) and hemoprotective properties in hypotonic conditions ($H_{50, Control}$ = 0.45%; $H_{50, dichloromethane}$ = 0.41%; $H_{50, ethyl\ acetate\ pH\ 8}$ = 0.36%; $H_{50, ethyl\ acetate\ pH\ 4}$ = 0.37%), as well as inhibiting hemolysis in oxidative conditions (between 23-30%).

Graphical abstract



Keywords

Antioxidants
Bioactive compounds
Flavonoids
Hemolysis
Lipoperoxidation inhibition

Article history

Received 06 Feb 2024
Revised 10 Jun 2024
Accepted 17 Jun 2024
Available online 17 May 2025

Handling Editor: Sergio R. Lazaro

1. Introduction

The *Tipuana tipu* (Benth.) (Figure 1), the only species of the *Tipuana* genus (Fabaceae/Leguminosae: Faboideae, Tribe Dalbergieae), is a leguminous tree common in South America, especially in Brazil and Argentina. It is a plant used in popular medicine for wound-healing and to treat uterine inflammations, abdominal pains, hemorrhoids and gastritis,

[a] Department of Chemistry, State University of Ponta Grossa, Av. Carlos Cavalcanti, 4748, 84030-900, Ponta Grossa, Brazil. [b] Faculdade Integrada Carajás, Redenção, Brasil. Rodovia BR 155, Km 03, Parque dos Buritis III, Redenção, Brazil. [c] Department of Biological Sciences, Faculty of Science and Engineering, University of Limerick, V94 T9PX Limerick, Ireland. *Corresponding author. E-mail: marizaboscacci@yahoo.com.br

with such properties being commonly associated to the leaves of this plant [1], which also presents a high nutritional value [2].

Fig. 1. *Tipuana tipu* specimen located in the State University of Ponta Grossa's campus.

Studies have reported the identification and isolation of some secondary metabolites of *T. tipu* leaves, such as *β*-sitosterol, alpinumisoflavone, protocatechuic acid, protocatechuic aldehyde [3], and an acylated kaempferol tetraglycoside [4], which are compounds that present analgesic, anti-inflammatory [4–6], antioxidant [7], and antitumor [3,8] properties. However, no studies were found in the literature that corroborate these properties of *T. tipu* leaves in folk medicine.

To investigate the bioproperties of this plant and identify the compounds responsible for each bioactivity, the fractioning of the crude extract is usually carried out. It is not rare that the several fractions of the same crude extract present different biological properties [9–13], given that the chemical composition of each fraction is different. Compounds with a low polarity, such as fatty acids, esters and steroids, are present in nonpolar fractions, e. g. the hexane fraction. Methoxylated flavonoids, sesquiterpenes and coumarins are often present in fractions with intermediary polarity, for instance, the dichloromethane fraction. Whereas polar fractions, obtained with ethyl acetate and *n*-butanol contain flavonoids, phenolic acids and saponins [14].

In view of this, this study aimed to evaluate: the antioxidant capacity in chemical and biological media, the antihemolytic property in osmotic and oxidative stress conditions of dichloromethane (DF) and ethyl acetate (EAF) pH 4 and pH 8 fractions of the aqueous crude extract of *Tipuana tipu* leaves.

2. Material and Methods

2.1 Chemicals

The chemicals used in this study are listed below, followed by the name and location of their suppliers: absolute ethanol, sodium hydroxide and hexahydrate aluminum chloride from Dinâmica (Indaiatuba, Brazil); Sodium azide, dichloromethane and hydrochloric acid 37% from Emaia (Indaiatuba, Brazil); hexane and isobutanol from Nuclear (São Paulo, Brazil); 2,2-diphenyl-1-picrylhydrazyl (DPPH), methanol, ethyl acetate, and ascorbic and acetic acids from Vetec (Rio de Janeiro, Brazil); $NaH_2PO_4.H_2O$, $Na_2HPO_4.12H_2O$, sodium chloride and Folin–Ciocalteu reagent from Biotec (Curitiba, Brazil); dimethyl sulfoxide (DMSO) and hydrogen peroxide from Synth (Diadema, Brazil); anhydrous sodium hydroxide from Quimex (Uberaba, Brazil); potassium hexacyanoferrate, and hexahydrate ferrous and pentahydrate copper sulfates from Neon (São Paulo, Brazil); sodium nitrite, hexahydrate ferric chloride, 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), protocatechuic, chlorogenic, and thiobarbituric acids, as well as quercetin, (+)-catechin, rutin, ferrozine, pyrocatechol violet from Sigma-Aldrich (São Paulo, Brazil); formic acid from Reagen (Colombo, Brazil); and anhydrous sodium acetate from Anidrol (São Paulo, Brazil). All assays were carried out with ultrapure water.

2.2 Vegetable material and extraction procedure

The leaves were collected from a *T. tipu* specimen located at the Uvaranas Campus of the State University of Ponta Grossa (UEPG), in the municipality of Ponta Grossa, Paraná, Brazil (25º5'23"S 50º6'23"W), on March 10th, 2018, at 10 AM (exsiccate n° 19120/2012 from UEPG's herbarium). The leaves were manually removed from the branches and dried at ambient temperature away from direct sunlight until constant mass was obtained.

Three litters of ultrapure water and 270 g of leaves were utilized (Figure 2). Following the 1-hour hydrodistillation and simple filtration, a liquid-liquid extraction was carried out with 300 mL hexane. Subsequently, another liquid-liquid extraction was performed, with 300 mL dichloromethane, for 3 times,

resulting in the dichloromethane fraction (DF). The aqueous phase was alkalinized up to pH 8 with 6 mol/L NaOH, followed by a liquid-liquid extraction with 300 mL ethyl acetate, thrice, to obtain the pH 8 ethyl acetate fraction (EAF pH 8). Furthermore, the aqueous phase was acidified with HCl 1:1 until pH 4 and a new extraction employing 300 mL ethyl acetate was performed three times again, to obtain the pH 4 ethyl acetate fraction (EAF pH 4).

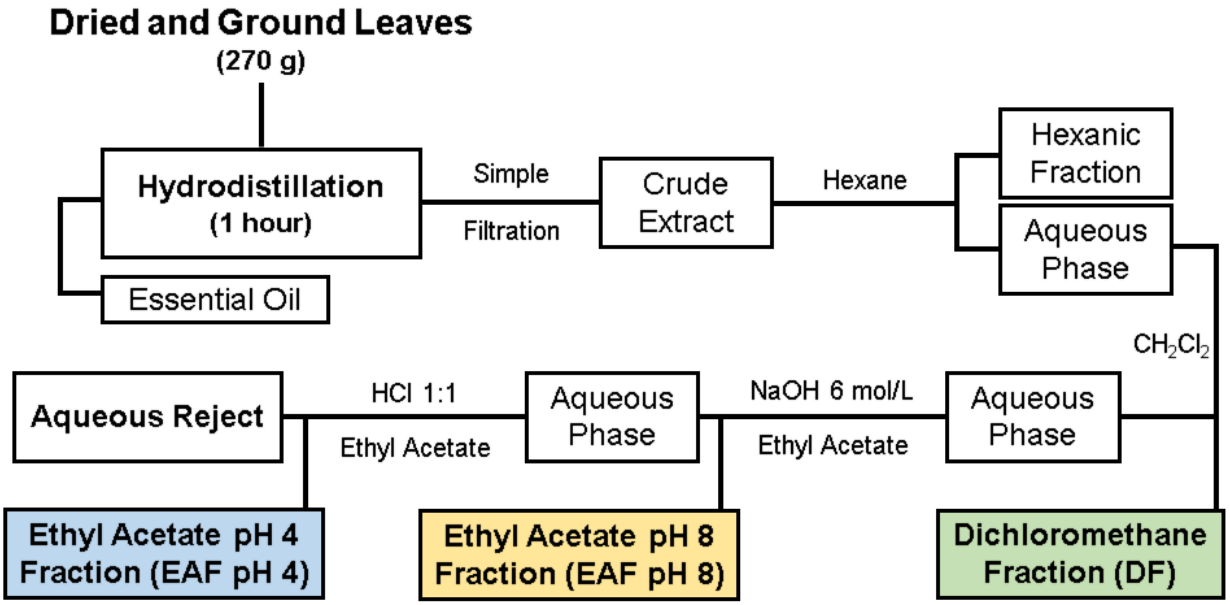


Fig. 2 Flowchart for the obtention of *Tipuana tipu* leaves' crude extract and its fractions.

DF, EAF pH 8, and EAF pH 4 were dried with anhydrous Na_2SO_4 and rotoevaporated. The extraction sequences were carried out to obtain a polarity gradient of the phenolic compounds, being DF the one with the least polarity, while EAF pH 4 presented the highest one [15].

2.3 Phenolic Composition

2.3.1 Total phenolics and flavonoids determination

The total phenolic content (TPC) was quantified using the Prussian Blue method [16], starting from a gallic acid analytical curve (5-35 mg/L, R^2 = 0.9979), with the results shown as mg of gallic acid equivalent per each gram of fraction (mg AGE/g). The total flavonoid content (TFC) (Herald et al. 2012) was quantified from a (+)-catechin calibration curve (25-250 mg/L, R^2 = 0.9964) with results shown as mg of catechin equivalent per gram of fraction (mg CE/g).

2.3.2 Phenolics Identification by HPLC/DAD/UV

The quercetin, rutin, protocatechuic and chlorogenic acid content was quantified using High-Performance Liquid Chromatography (HPLC) in a Shimadzu LC-20T, equipped with a Diode Detector Array (DAD; 210, 270, 320, and 360 nm), degasser system, and auto sampler. The chromatographic separation took place in a reverse phase column (C_{18}, 150 mm x 4.6 mm, particle size: 3.5 µm) maintained at 40 °C. The fractions were filtered in a 0.45 µm nylon membrane, where 10 µL of the samples were thrice injected with an eluent flow rate of 500 µL/min. The compounds were chromatographically separated following elution gradients according to the literature [17], in which the mobile phase corresponds to water acidified with formic acid 0.2% (v/v) and acetonitrile. The phenolic compounds were quantified using calibration curves ($R^2 \geq 0.9970$) with the results shown as mg per fraction gram (mg/g).

2.4 Antioxidant activity assays

The antioxidant capacity of the fractions was tested by the two main oxidation protection mechanisms employed by non-enzymatic antioxidants: electron donating to reactive species and chelation of transition metals, in this case, Fe^{2+} and Cu^{2+} [18]. The Ferric Reducing Antioxidant Power (FRAP) [19] was determined by means of an ascorbic acid calibration curve (10-90 mg AAE/g, R^2 = 0.9986) with results expressed as mg of ascorbic acid equivalent per gram of fraction (mg AAE/g). The DPPH reducing antioxidant capacity [20] was evaluated, with results shown as inhibition percentage ($\%_{Inhibition}$). The Total Reducing Capacity (TRC) (Berker et al. 2013) was quantified using a quercetin calibration curve, 50-500 mg/L and R^2 = 0.9990, with results expressed as mg of quercetin equivalent per gram of fraction (mg QE/g). The Cu^{2+} Chelation Ability and the Fe^{2+} Chelation Ability were analyzed [21], with results shown as chelating percentage ($\%_{Chelation}$) and refer to the fractions with a concentration of 1000 mg/L.

2.5 Inhibition of lipoperoxidation

The lipid peroxidation inhibition capacity was assessed in two biological matrices: white-shelled eggs' yolk [22] and cerebral tissue from Wistar rats [17], employing a methodology approved by the Animal Usage Ethics Committee (Protocol 047/2017). The analysis was carried out with fractions in a 50 µg/mL concentration and the control was obtained through the substitution of the sample by a 0.1 mol/L phosphate buffer. The lipid peroxidation inhibition was calculated by means of equation 1, where $A_{Control}$ is the control absorbance and A_{Sample} is the sample absorbance, both at 532 nm, and the results are expressed as inhibition percentage ($\%_{Inhibition}$).

$$\%_{Inhibition} = [(A_{Control} - A_{Sample})/A_{Control}] \times 100 \quad \text{(Eq. 1)}$$

2.6 Antihemolytic activity

All the analysis employing erythrocytes were carried out thrice, with blood samples from three O+ donors, obtained from the University Regional Hospital Wallace Thadeu de Mello e Silva. The procedures were previously approved by the Ethics Committee of the State University of Ponta Grossa (Certificate of Presentation for Ethical Consideration - CAAE 94830318.1.0000.0105). The erythrocytes were isolated [23] and resuspended in PBS until hematocrit were at 12.5% (v/v), and stored. During the assays, the volumes of the solution to be employed were chosen as to obtain a final hematocrit of 0.8% (v/v).

2.6.1 Isotonic condition

The hemolytic activity in isotonic conditions [24] was assessed with the fractions in their final concentrations of 1, 5 and 9 µg/mL. Total Hemolysis (TH) was measured by the substitution of the sample by ultrapure water. Mechanical Hemolysis (MH) was measured by substituting the fractions by PBS. The hemolysis percentage ($\%_{Hemolysis}$) was calculated according to equation 2, where A_{Sample} is the sample absorbance and A_{TH} the total hemolysis absorbance, both at 576 nm.

$$\%_{Hemolysis} = (A_{Sample}/A_{TH}) \times 100 \qquad \text{(Eq. 2)}$$

2.6.2 Hypotonic and H_2O_2-induced hemolysis

The hemoprotective potential of the fractions (5 µg/mL) was studied in hypotonic conditions [23, 25]. The assay in H_2O_2-induced oxidative stress was performed according to [26]. In both assays, TH was obtained by the substitution of the fractions with ultrapure water and the hemolysis rate was calculated according to equation 2.

2.7 Statistical analysis

All the analysis were carried out in triplicates, with the results expressed in means and standard deviation. The statistical analysis employed the Statistica v.13.3 software (Statsoft, USA), with a significance level of 0.05. The normality and homoscedasticity of the data were analyzed with the Shapiro-Wilk and Brown-Forsythe tests, respectively, whereas the difference between the values obtained was evaluated through the one-way variance analysis (ANOVA). Pearson correlation matrices were utilized to statistically correlate the chemical composition of the fractions with their antioxidant and antihemolytic activities.

3. Results and Discussion

3.1 Phenolic composition

The fractions' yield (Table 1), is a result of the use of water as solvent to obtain the crude extract, extracting the most polar compounds. Hence the yields increased from DF to EAF pH 4. The pH value of the fractions in ethyl acetate were selected because, at pH 8, phenolic acids are deprotonated. As a result, there is greater interaction with the aqueous phase and they are not extracted by ethyl acetate. Values of pH greater than that might lead to the capture of the phenolic hydroxyl hydrogen, turning the extraction less specific. The subsequent acidification of the aqueous phase to pH 4 protonates the previously deprotonated phenolic compounds, allowing their extraction by ethyl acetate [15].

Table 1. Yield and quantification of compound classes in the fractions of *Tipuana tipu* leaves.

Sample	Yield (mg)	TPC (mg AGE/g)	TFC (mg CE/g)
DF	115	81 ± 1^{b}	121 ± 10^{b}
EAF pH 8	200	97 ± 1^{a}	144 ± 8^{a}
EAF pH 4	580	80 ± 1^{b}	128 ± 3^{ab}
***p*-Value (Homoscedasticity)**	NA	0.8900	0.7461
***p*-Value (one-way ANOVA)**	NA	< 0.05	< 0.05

Note: DF = Dichloromethane fraction; EAF = Ethyl Acetate fraction; TPC = Total Phenolic Content; TFC = Total Flavonoid Content. Different letters on the same column represent statistically significative differences ($p \leq 0.05$). NA = Non-Appliable.

All fractions presented significant amount of total phenolic compounds, mainly EAF pH 8 (97 mg AGE/g), when compared to values found within the aqueous extract of *Medicago minima* L. leaves (16.65 mg AGE/g) [27], which also belongs to the Fabaceae family. The highest value of total flavonoids (Table 1) was detected on EAF pH 8 (144 mg CE/g). The fact that the fractions presented a lower content of total phenols than that of total flavonoids is possibly due to the low accuracy of the methods employed. The vegetable matrix presents a large number of interferents that may maximize or minimize the assessed response, therefore, other compounds might react in the same way as the target molecules or inhibit their action [16].

Table 2. Quantification by HPLC-DAD of individual phenolic compounds in the fractions of *Tipuana tipu* leaves.

Sample	Quercetin (mg/100 g)	Rutin (mg/100 g)	Protocatechuic acid (mg/100 g)	Chlorogenic acid (mg/100 g)
DF	ND	137 ± 11^{c}	ND	ND
EAF pH 8	98 ± 7^{a}	550 ± 13^{b}	ND	ND
EAF pH 4	88 ± 6^{a}	2225 ± 119^{a}	ND	ND
***p*-Value (Homoscedasticity)**	0.3080	0.1427	NA	NA
***p*-Value (one-way ANOVA)**	< 0.05	< 0.05	NA	NA

Note: DF = Dichloromethane fraction; EAF = Ethyl Acetate fraction. Different letters on the same column represent statistically significative differences ($p \leq 0.05$). ND = Not Detected; NA = Non-Appliable.

This study sought to quantify quercetin, rutin, and chlorogenic and protocatechuic acids, since these compounds were previously reported in *T. tipu* extracts [3,28] (Table 2). Rutin was detected in all three fractions and quercetin only in the ethyl acetate fractions. Factors such as geographical location, climate and seasonality might have interfered in the phenolic compositions of the fractions [27,29], which might justify the absence of protocatechuic and chlorogenic acids.

3.2. Antioxidant activity

The antioxidant activity of the fractions, assessed by Ferric Reducing Antioxidant Power (FRAP) Assay, varied between 120-134 mg AAE/g, these values are over the ones found in the aqueous extracts of *Aspalathus linearis* Burm leaves, (29-35 mg AAE/g) [24]. The fractions also exhibited a total reducing capacity between 215 and 280 mg QE/g, and EAF pH 8 was the most efficient one.

The fractions obtained were able to capture DPPH$^{\cdot}$ in a dose-dependent manner (Figure 3), and EAF pH 8 was the most efficient in all the tested concentrations. In low concentrations, DF presented a radical capture efficiency similar to that of EAF pH 8, while in high concentrations, EAF pH 4 and 8 exhibited a similar reducing capacity. Table II shows the IC_{50} values, i.e. the antioxidant concentration capable of reducing 50% of the DPPH$^{\cdot}$ initially added to the sample. The fraction which presented the lowest IC_{50} value, and, therefore, the highest efficiency in capturing the radical, was EAF pH 8 (185 mg/L). The IC_{50} of these samples were greater than those found in extracts with a binary methanol-water mixture (1:1) from *Parkia roxburghii* G. Don leaves (16 mg/L) [30].

The antioxidant activity responses were statistically correlated ($p \leq 0.05$) to the samples' total phenolic content (TPC) ($r_{FRAP} = 0.8535$; $r_{CRT} = 0.8170$; $r_{DPPH} = -0.9951$), in addition, the FRAP and DPPH results were correlated ($p \leq 0.05$) to the flavonoids content in the fractions ($r_{FRAP} = 0.7180$; $r_{DPPH} = -0.8138$). This confirms the report that demonstrated the correlation between the content of phenolic compounds and the capacity of reducing reactive species detected in extracts of 30 plants with potential industrial usage [31]. Furthermore, the analysis showed that EAF pH 8 was the most efficient at reducing reactive species among the fractions.

The DF and EAF pH 8 were the most efficient fractions in the chelation of Fe (II) and Cu (II) (Table 3). The chelating capacity of the fractions was different to each metal, with chelation rates for Fe^{2+} between 17% (EAF pH4) and 23% (EAF pH 8), and for Cu^{2+} between 10% (EAF pH 4) and 14% (DF). The chelation of transition metals is an important mechanism of antioxidant action, because if such metals are free in a biological medium, they can take part in reactions that generate free radicals [32], and, eventually, be involved in an array of cellular injuries [33–35].

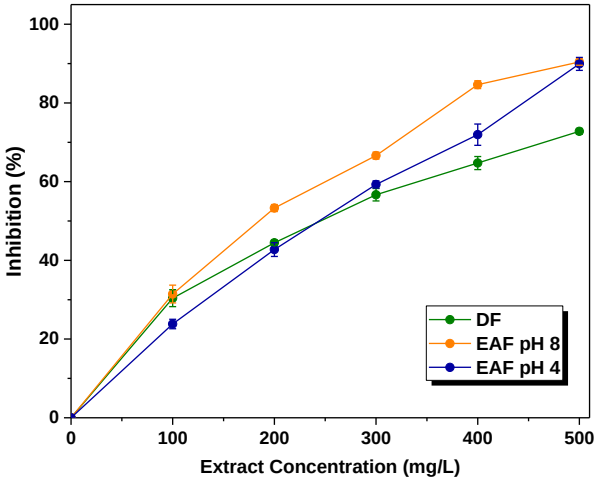


Fig. 3 Dose-dependent inhibition of the DPPH radical (0.10 mmol/L) in the presence of the fractions of *Tipuana tipu* leaves in: dichloromethane (DF) and ethyl acetate (EAF) at pH 8 and 4.

3.3 Inhibition of lipoperoxidation

The effects of the samples presented different intensities on each tissue, being more efficient on the egg yolk than on the brain, which agrees with the literature (Table 4) [17]. Such divergence may be ascribed to the different lipid composition of such matrices [36,37], which, in fact, influences the sample induced protection.

Table 3. Antioxidant activity of *Tipuana tipu* leaves fractions.

Sample	FRAP (mg AAE/g)	TRC (mg QE/g)	DPPH IC_{50} (mg/L)	Fe^{2+} Chelating Ability (%)	Cu^{2+} Chelating Ability (%)
DF	120 ± 4^{b}	242 ± 8^{b}	246 ± 3^{a}	22 ± 1^{a}	14 ± 1^{a}
EAF pH 8	134 ± 5^{a}	280 ± 20^{a}	185 ± 2^{b}	23 ±1^{a}	12 ± 1^{ab}
EAF pH 4	120 ± 3^{b}	215 ± 10^{b}	244 ± 4^{b}	17 ± 1^{b}	10 ± 1^{b}
***p*-Value (Homoscedasticity)**	0.8463	0.4872	NA	0.9880	0.8801
***p*-Value (one-way ANOVA)**	< 0.05	< 0.05	NA	< 0.05	< 0.05

Note: DF = Dichloromethane Fraction; EAF = Ethyl Acetate Fraction; FRAP = Ferric Reducing Antioxidant Power; TRC = Total Reducing Capacity. The chelating percentages of Fe^{2+} e Cu^{2+} correspond to 1000 mg/L of the fractions. Different letters in each column represent statistically different values ($p \leq 0.05$).

EAF pH 8 was the most efficient fraction at IPL in both matrices (81% for egg yolk and 70% for Wistar rats' brains). These results were similar to those found in the presence of quercetin 10 µg/mL. Despite presenting a similar antioxidant activity in chemical medium (FRAP, DPPH, and Fe^{2+} and Cu^{2+} Chelation Activity), EAF pH 4 and DF displayed a lipid peroxidation inhibition capacity statistically different in both matrices. Such difference may be ascribed to changes in the environment, from a solely chemical medium to a biological matrix, which presented interferents that might optimize or minimize the fraction action, reinforcing the importance of carrying out analysis employing biological models, especially *in vivo* assays [38]. Additionally, DF interacts in a more efficient manner with lipids because it contains substances with a lower polarity than that of ethyl acetate pH 4, which favours the protection against lipid peroxidation.

3.5 Antihemolytic activity

The analysis in an isotonic condition evidenced the non-toxicity of the fractions regarding erythrocytes (Figure 4A). DF, in 1, 5 and 9 µg/mL concentrations did not favour hemolysis in this osmotic condition. However, it did not significantly decrease the hemolysis rate when compared to the control sample (0 µg/mL). As regards EAF pH 8 and 4, they minimized the hemolysis in a dose-dependent manner. Such results are similar to those reported for fractions of dichloromethane and ethyl acetate (1-2%) of *Rhynchosia pseudo-cajan* (Camb.) leaves [39]. The decreased hemolysis rate is due to the reduction in oxygen reactive species generated in the medium, even without the oxidizing agent addition, by the reaction between hemoglobin iron and the oxygen present in the sample [40].

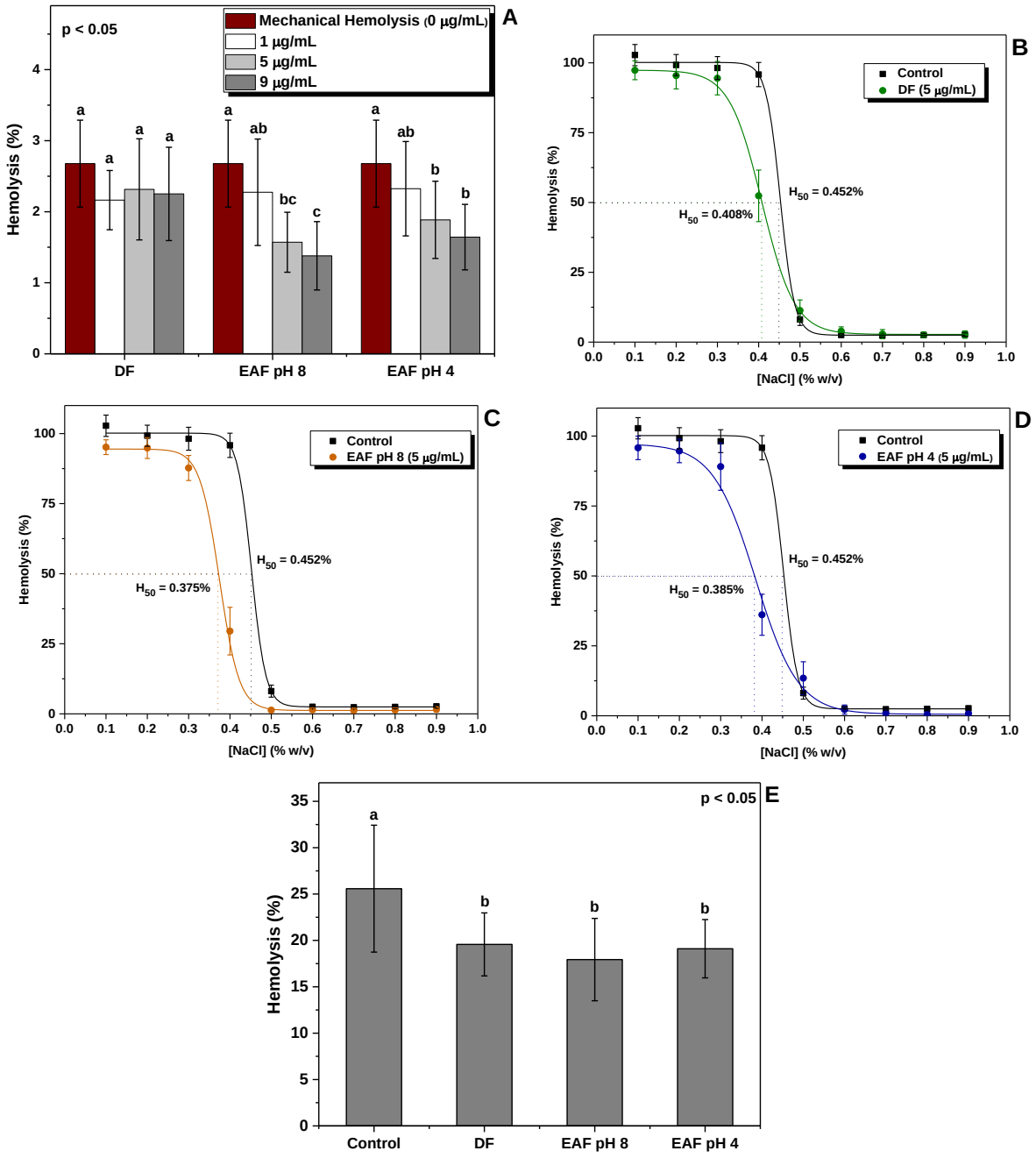


Fig. 4 Hemolysis in erythrocytes O⁺ (hematocrit 0.8%) in isotonic conditions and pre-incubated with the fractions at 1, 5 and 9 µg/mL (A); in hypotonic conditions and pre-incubated at 5 µg/mL of the fractions DF (B), EAF pH 8 (C) and EAF pH 4 (D); In oxidative condition induced by H_2O_2 incubated with the fractions at 50 µg/mL (E). Note: Different letters represent statistically different results ($p \leq 0.05$). On (A), the values were compared between the different concentrations of each extract and not between the extracts themselves.

Table 4. Lipid peroxidation inhibition potential of the fractions (50 µg/mL) in two biological matrices.

Sample (50 µg/mL)	Egg Yolk Homogenate ($\%_{Inhibition}$)	Wistar Rat Brain Homogenate ($\%_{Inhibition}$)
DF	80 ± 1[a]	32 ± 3[b]
EAF pH 8	81 ± 2[a]	70 ± 1[a]
EAF pH 4	69 ± 6[b]	10 ± 1[c]
Quercetin 10 µg/mL	79 ± 7[a]	65 ± 3[a]
***p*-Value (Homoscedasticity)**	0.0627	0.1993
***p*-Value (one-way ANOVA)**	< 0.05	< 0.05

Note: DF = Dichloromethane Fraction; EAF = Ethyl Acetate Fraction; Different letters on the same column indicate statistically different values ($p \leq 0.05$).

All three fractions displayed hemoprotective activity in hypotonic conditions (Figure 4), that is, in susceptible conditions of the erythrocyte membrane. The control curve, built only with erythrocytes in hypotonic PBS ([NaCl] = 0.0-0.9% m/v), presented a H_{50} of 0.452%. Such value indicates the NaCl concentration in which 50% hemolysis is obtained. The fractions provided lower H_{50} values, namely, 0.408% for DF, 0.375% for EAF pH 8, and 0.385% for EAF pH 4, validating the hemoprotective profile of the samples, given that under a gradual decrease in osmotic pressure, even in low concentrations, the compositions of the fractions hinder erythrocyte rupture.

Total flavonoid (r = -0.7220) and quercetin (r = -0.9146) contents were statistically correlated ($p \leq 0.05$) with H_{50}

values. The flavonoids of the fractions were capable of decreasing membrane fluidity through hydrogen bonds with the polar heads of phospholipids and, therefore, protecting the erythrocytes from osmotic stress [41]. The more polar these flavonoids are, the more efficient the protection is [42], which explains the EAF pH 4 higher efficiency in membrane protection when compared to DF, considering that both fractions presented similar flavonoid contents.

In H_2O_2 induced oxidative stress conditions, the responses obtained also evidenced a significant protection from hemolysis (Figure 4E). However, there was no statistical difference between the percentages of hemolysis in the presence of the fractions, even though all those values were statistically different from the hemolysis value in the absence of antioxidants (23-29% hemolysis inhibition). The results obtained under oxidative stress were higher than those found with the methanolic extract of *Carica papaya* L. leaves (7-15% inhibition) [43]. This protection is probably due to the antioxidant potential shown by the fractions, thus preventing the oxidation of membrane lipids and proteins, and, consequently the occurrence of cellular rupture.

4. Conclusions

The presence of quercetin and rutin was detected in the tested fractions. Those samples were rich in phenolic compounds and flavonoids, presenting results that suggest their efficiency regarding antioxidant activity (FRAP, DPPH, TRC, and Iron and Copper Chelation Abilities), antihemolytic activity (isotonic, hypotonic and oxidative conditions), and lipid peroxidation inhibitive activity (white-shelled egg's yolk and Wistar rats' brain). EAF pH 8 was the most efficient fraction. Hence, *T. tipu* leaves can be considered an important source of antioxidant substances with *in vitro* properties related to membrane protection.

Supporting Information

Table S1 – Analytical curves for the quantification of individual phenolic compounds by HPLC-DAD and Figure S1 – Chromatograms obtained in the HPLC-DAD analysis for each sample: (A) DF, phenolic acids method; (B) DF, flavonoids method; (C) EAF pH8, phenolic acids method; (D) EAF pH8, flavonoids method; (E) EAF pH4, phenolic acids method; (F) EAF pH4, flavonoids method.

Acknowledgments

The authors acknowledge: CAPES (Superior Education Personnel Enhancement Coordination) for the master's and doctorate scholarships, the State University of Ponta Grossa (UEPG) and c-LABMU (Multi-users laboratories complex of UEPG) for its structure.

Author Contributions

Domingos Savio Nunes, Daniel Granato, and Mariza Boscacci Marques conceived and planned the experiments. Thiago Mendanha Cruz, Ana Laiza Kutzmy, and Yasmin Stelle carried out the experiments and analyzed the data. Jânio Sousa Santos provided the statistical analysis. Thiago Mendanha Cruz e Mariza Boscacci Marques wrote the article.

References and Notes

[1] Quiroga, R.; Meneses, L.; Bussmann, R. W. *J. Ethnobiol. Ethnomed*. **2012**, *8*. [Crossref]

[2] Norton, B. W.; Waterfall, M. H. *Small Ruminant Res*. **2000**, *38*, 175. [Crossref]

[3] Amen, Y. M.; Marzouk, A. M.; Zaghloul, M. G.; Afifi, M. S. *J. Am. Sci*. **2013**, *9*, 334.

[4] Amen, Y. M.; Marzouk, A. M.; Zaghloul, M. G.; Afifi, M. S. *Nat. Prod. Res*. **2015**, *29*, 511. [Crossref]

[5] Nirmal, S. A.; Pal Subodh, C.; Subhash, C. M.; Anuja, N. P. *Inflammopharmacology* **2012**, *20*, 219. [Crossref]

[6] Wei, G.; Guan, Y.; Yin, Y.; Duan, J.; Zhou, D.; Zhu, Y.; Quan, W.; Xi, M.; Wen, A. *Inflammation* **2013**, *36*, 592. [Crossref]

[7] Sroka, Z.; Cisowski, W. *Food Chem. Toxicol*. **2003**, *41*, 753. [Crossref]

[8] Yin, M. C.; Lin, C. C.; Wu, H. C.; Tsao, S. M.; Hsu C. K. *J. Agric. Food Chem*. **2009**, *57*, 6468. [Crossref]

[9] Pinto, N. C. C.; Cassini-Vieira, P.; de Souza-Fagundes, E. M.; Barcelos, L. S.; Castañon, M. C. M. N.; Scio, E. *J. Ethnopharmacol*. **2016**, *19*, 131. [Crossref]

[10] Pinto, N. C. C.; dos Santos, R. C.; Machado, D. C.; Florêncio, J. R.; Fagundes, E. M. S.; Antinarelli, L. M. R.; Coimbra, E. S.; Ribeiro, A.; Scio, E. *Pharmacologyonline* **2012**, *3*, 63.

[11] Pinto, N. C. C.; Duque, A. P. N.; Pacheco, N. R.; Mendes, R. F.; Motta, E. V. S.; Bellozi, P. M. Q.; Ribeiro, A.; Salvador, M. J.; Scio, E. *Pharm. Biol*. **2015**, *53*, 1780. [Crossref]

[12] Pinto, N. C. C.; Machado, D. C.; Da Silva, J. M.; Conegundes, J. L. M.; Gualberto, A. C. M.; Gameiro, J.; Chedier, L. M.; Castañon, M. C. M. N.; Scio, E. *J. Ethnopharmacol*. **2015**, *173*, 330. [Crossref]

[13] Pinto, N. C. C.; Maciel, M. S. F.; Rezende, N. S.; Duque, A. P. N.; Mendes, R. F.; da Silva, J. B.; Evangelista, M. R.; Monteiro, L. C.; da Silva, J. M.; da Costa, J. C.; Scio, E. *J. Pharm. Pharmacol*. **2020**, *72*, 1. [Crossref]

[14] Monção, N. B. N.; Araújo, B. Q.; Do Nascimento Silva, J.; Lima, D. J. B.; Ferreira, P. M. P.; Airoldi, F. P. S.; Pessoa, C.; Citó, A. M. G. L. *Molecules* **2015**, *20*, 4204. [Crossref]

[15] Nunes, D. S.; Besten, M. A. In: Análises Químicas, Propriedades Funcionais e Controle Da Qualidade de Alimentos e Bebidas: Uma Abordagem Teórico-Prática. Granato, D.; Nunes, D. S., eds Amsterdam: Elsevier, 2016, chapter 1.

[16] Margraf, T.; Karnopp, A. R.; Rosso, N. D.; Granato, D. *J. Food Sci*. **2015**, *80*, C2397. [Crossref]

[17] Fidelis, M.; Santos, J. S.; Escher, G. B.; do Carmo, M. V.; Azevedo, L.; da Silva, M. C.; Putnik, P.; Granato, D. *Food Chem. Toxicol*. **2018**, *120*, 479. [Crossref]

[18] Thomas, M. J. *Nutrition* **2000**, *16*, 716. [Crossref]

[19] Granato, D.; Karnopp, A. R.; van Ruth, S. M. *J. Sci. Food Agric*. **2015**, *95*, 1997. [Crossref]

[20] Brand-Williams, W.; Cuvelier, M. E.; Berset, C. *LWT-Food Sci. Technol*. **1995**, *28*, 25. [Crossref]

[21] Santos, J. S.; Brizola, V. R. A.; Granato, D. *Food Chem*. **2017**, *214*, 515. [Crossref]

[22] Margraf, T.; Santos, É. N. T.; de Andrade, E. F.; van Ruth, S. M.; Granato, D. *Food Res Int*. **2016**, *82*, 145.

[Crossref]

[23] Zhang, L.; Santos, J. S.; Cruz, T. M.; Marques, M. B.; do Carmo, M. A. V.; Azevedo, L.; Wang, Y.; Granato, D. *Food Res. Int*. **2019**, *125*, 108516. [Crossref]

[24] Santos, J. S.; Deolindo, C. T. P.; Esmerino, L. A.; Genovese, M. I.; Fujita, A.; Marques, M. B.; Rosso, N. D.; Daguer, H.; Valese, A. C.; Granato, D. *Food Res. Int*. **2016**, *89*, 476. [Crossref]

[25] Escher, G. B.; Borges, L. C. C.; Santos, J. S.; Cruz, T. M.; Marques, M. B.; Do Carmo, M. A. V.; Azevedo, L.; Furtado, M. M.; Sant'ana, A. S.; Wen, M.; Zhang, L.; Granato, D. *Antioxidants* **2019**, *8*. [Crossref]

[26] Cruz, T. M.; Santos, J. S.; do Carmo, M. A. V.; Hellström, J.; Pihlava, J. M.; Azevedo, L.; Granato, D.; Marques, M. B. *Food Chem*. **2021**, *361*, 130078. [Crossref]

[27] Kabtni, S.; Sdouga, D.; Bettaib Rebey, I.; Save, M.; Trifi-Farah, N.; Fauconnier, M. L.; Marghali, S. *Sci. Rep*. **2020**, *10*, 8293. [Crossref]

[28] Afifi, M. S.; Elgindi, O. D.; Bakr, R. O. *Nat. Prod. Res*. **2014**, *28*, 257. [Crossref]

[29] Ben Ghorbal, A.; Leventdurur, S.; Agirman, B.; Boyaci-Gunduz, C. P.; Kelebek, H.; Carsanba, E.; Darici, M.; Erten, H. *J. Food Comp. Anal*. **2018**, *74*, 1. [Crossref]

[30] Dubey, R. K.; Upadhyay, G.; Singh, V.; Pandey, S. *South African J. Botany* **2020**, *131*, 454. [Crossref]

[31] Dudonné, S.; Vitrac, X.; Coutiére, P.; Woillez, M.; Mérillon, J. M. *J. Agric. Food Chem*. **2009**, *57*, 1768. [Crossref]

[32] Winterbourn, C. C. *Toxicol. Lett*. **1995**, *82–83*, 969. [Crossref]

[33] Lloyd, D. R.; Phillips, D. H. *Mutat. Res., Fundam. Mol. Mech. Mutagen.* **1999**, *424*, 23. [Crossref]

[34] Adachi, T.; Nonomura, S.; Horiba, M.; Hirayama, T.; Kamiya, T.; Nagasawa, H.; Hara, H. *Sci. Rep*. **2016**, *6*, 1. [Crossref]

[35] Cherrak, S. A.; Mokhtari-Soulimane, N.; Berroukeche, F.; Bensenane, B.; Cherbonnel, A.; Merzouk, H.; Elhabiri, M. *PLoS One* **2016**, *11*, 1. [Crossref]

[36] Lehninger, A. L. *Mol. Neurobiol*. **1968**, *60*, 1069. [Crossref]

[37] Li, S. C.; Chien, J. L.; Wan, C. C.; Li, Y. T. *Biochem J*. **1978**, *173*, 697. [Crossref]

[38] da Cruz, R. G.; Beney, L.; Gervais, P.; de Lira, S. P.; Vieira, T. M. F. S.; Dupont, S. *Food Chem*. **2019**, *277*, 698. [Crossref]

[39] Abbasi, M. A.; Irshad, M.; Aziz-Ur-Rehman; Riaz, T.; Siddiqui, S. Z.; Shahid, M. *Pak J. Pharm. Sci*. **2019**, *32*, 2003.

[40] Çimen, M. Y. B. *Clin. Chim. Acta* **2008**, *390*, 1. [Crossref]

[41] Sato, Y.; Yamakosi, H.; Suzuki, Y. *Biol. Pharm. Bull*. **1993**, *16*, 506. [Crossref]

[42] Phan, H. T. T.; Yoda, T.; Chahal, B.; Morita, M.; Takagi, M.; Vestergaard, M. C. *Biochim. Biophys. Acta Biomembr*. **2014**, *1838*, 2670. [Crossref]

[43] Okoko, T.; Ere, D. *Asian Pac. J. Trop. Biomed*. **2012**, *2*, 449. [Crossref]

How to cite this article

Criz, T. M.; Kutzmy, A. L.; Stelle, Y.; Santos, J. S.; Granato, D.; Nunes, D. S.; Marques, M. B. *Orbital: Electronic J. Chem*. **2025**, *17*, 171. DOI: http://dx.doi.org/10.17807/orbital.v17i2.20191