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Original Article

In vivo potential hypoglycemic and *in vitro* vasorelaxant effects of *Cecropia glaziovii* standardized extracts

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ABSTRACT

The aim of this study is to investigate the effect of *Cecropia glaziovii* Snethl, Urticaceae, extracts on the oral glucose tolerance curve, on glycemia in alloxan-induced diabetic rats and vasorelaxant effect after the extraction process, and to standardize the extractive solutions. The effects of the process variables and their interactions were calculated in relation to dry residue, pH, total phenolic results and chemical marker content. Furthermore, the effect of the extracts (400 mg/kg), chlorogenic (2 or 15 mg/kg) and caffeic acids (2 mg/kg) were investigated on the oral glucose tolerance curve and on glycemia in alloxan-induced diabetic rats. Oral administration of ethanol extracts 4d20 and 8d20 significantly improved glucose tolerance in the hyperglycemic rats. Chlorogenic and caffeic acids, as well as the association of the compounds were able to significantly reduce glycemia after oral gavage treatments. On the other hand, the aqueous extracts did not alter the glycemia. The aqueous extracts (8020 and 9030) and only the higher dose of chlorogenic acid presented a significant effect on serum glucose lowering in diabetic rats. Additionally, the IC_{50} reveals that the ethanol extracts presented more potent vasodilator effects than the aqueous extracts in aortic rings. This study shows that *C. glaziovii* standardized extracts exhibits antihyperglycemic action, is able to improve glucose tolerance and has a potent vascular relaxing effect. These results are probably linked to concentrations of the main phenolic compounds of the extracts.

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Introduction

Leaves of *Cecropia glaziovii* Snethl, Urticaceae, are used in the traditional medicine, mainly in the form of tea. Pharmacological studies have shown that extractive solutions of this species produce anxiolytic (Rocha et al., 2002), hypotensive (Lima-Landman et al., 2007), antiasthmatic (Delarcina et al., 2007), antidepressant (Rocha et al., 2007), antacid, antiulcer (Souccar et al., 2008) effects, as well as in vitro antiviral effects against human herpes virus types 1 and 2 (Silva et al., 2010).

Some *Cecropia* species are used in popular medicine to treat diabetes and hypoglycemic effects have been reported for the extractive solutions of *Cecropia obtusifolia* Bertol., Urticaceaae, *Cecropia peltata* L., Urticaceae (Andrade-Cetto and Wiedenfeld, 2001; Nicasio et al., 2005; Andrade-Cetto et al., 2007; Alonso-Castro et al., 2008; Andrade-Cetto and Vázquez, 2010) and also *Cecropia pachystachya* Trécul., Urticaceae (Aragão et al., 2010). Based on the hypoglycemic potential described to *Cecropia* species and considering that there are no studies on the hypoglycemic effect of *C. glaziovii*, the present study sought to evaluate the effect of extracts prepared from *C. glaziovii* leaves on oral glucose tolerance curve and on glycemia in alloxan-induced diabetic rats.

In the preparation of the extractive solutions, the applicability of the maceration and decoction methods was determined for optimizing the extraction conditions of phenolic acids substances from *C. glaziovii* leaves. Some extraction parameters were analyzed using an experimental design. This work therefore determines the most appropriate extraction method, and the best conditions for promoting an extraction with the highest contents of chemical markers (chlorogenic and caffeic acids). Chlorogenic (CGA) and caffeic (CFA) acids were used as chemical markers for standardization due to high concentration present in the extracts and also to be related to

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the hypoglycemic/antihyperglycemic effects in the literature (Hsu et al., 2000; Rodriguez de Sotillo and Hadley, 2002; Jung et al., 2006; Genta et al., 2010). These initial results can be used to guide further standardization and application of *C. glaziovii* leaf extract in the development of phytopharmaceutical preparations.

Materials and methods

Materials

Chemicals and reagents were obtained from the following commercial sources: chlorogenic acid and caffeic acid (Sigma–Aldrich, St. Louis, MO, USA), Folin–Ciocalteu (Fluka, Sigma–Aldrich, St. Louis, MO, USA), methanol and acetonitrile (HPLC grade) were purchased from J.T. Baker (Phillipsburg, NJ, USA), acetic acid (Qhemis, São Paulo, Brazil), LC-grade water obtained in Milli-Q system (Millipore, Bedford, MA, USA). All samples and solutions were prepared from bidistilled water. All other reagents and solvents were of analytical grade.

Raw material characterization: plant material

The leaves of *Cecropia glaziovii* Snethl, Urticaceae, were collected by the Ílio Montanari Jr. researcher at Pluridisciplinary Center of Chemical, Biological and Agronomic Studies (CPQBA) of the University of Campinas, SP, Brazil. A specimen voucher is deposited at the CPQBA herbarium (number 78). The dry leaves were ground in a knife mill (Macmont) using a 3 mm mesh. The milled vegetal material was characterized by total ash content, loss on drying, particle size distribution and microbiological quality of the raw plant, as described below.

Total ash content (TA)

The total ash content was determined after bumming the milled leaves. The procedure was done gravimetrically by weighing 3.0 g of milled leaves in a porcelain crucible. The samples were submitted to calcination in an oven at 600 °C for 2 h. The results were expressed as percentage of remaining weight (w/w) by the mean of three measurements (Farmacopéia Brasileira, 2010).

Loss on drying (LOD)

The loss on drying analysis was carried out to determine the amount of water and volatile matter in the plant drug material. The LOD was determined gravimetrically by weighing 2.0 g of milled leaves in a weighing bottle and submitting the samples to a heated stove at 105 °C for 2 h. The procedure was repeated until constant weight was achieved (variation < 5 mg). The results were expressed as percentage of weight lost (w/w) by the mean of three measurements (Farmacopéia Brasileira, 2010).

Particle size distribution

Particle size distribution was evaluated by a standard sieving method, for a period of 15 min (Sieve Shaker Bertel 1400), with 30 g of the dried milled plant material, using a series of sieves with screen sizes corresponding to 180, 355, 500, 710, 1000 and 1700 μm. The average particle size was calculated by means of Probito's evaluation (Vila-Jato, 1997; Pasqualoto et al., 2005).

Assessment of microbiological quality of raw plant

The total viable aerobic count of the plant material was determined, as specified in the test procedure below, using the plate count method. Aerobic bacteria and fungi (molds and yeasts) were determined in this test.

Pretreatment of the test plant material

Plant material (10 g) was diluted in phosphate buffer pH 7.2, total volume adjusted to 100 ml. The treated sample resulted in a dilution of 10^{-1}.

Total viable aerobic count

To determine the total bacteria, the pre-treated herbal material was prepared in duplicate using decimal dilution until 10^{-9} dilution. Samples were incubated at 30–35 °C for 7 days in nutrient agar. Similarly, for fungi, pre-treated plant material was prepared in duplicate using decimal dilution until 10^{-9} dilution. Samples were incubated at 20–25 °C for 7 days in Sabouraud agar (WHO, 1998).

Extract preparation: experimental design

Two full factorial screening designs (2^2) was chosen to investigate the effects of the extraction conditions in the two extraction methods: maceration and decoction. In the case of maceration, the independent variables were ethanol concentration (20, 50 and 80%; v/v) (factor A) and extraction time (4, 6 and 8 days) (factor B), while for the decoction method the variables were temperature (70, 80 and 90 °C) (factor A) and extraction time (10, 20 and 30 min) (factor B). All data are given as means ± standard deviations of two independent batches. One central point was proposed in each design and these experiments were performed in duplicate. The effects of the process variables and their interactions on the dry residue, pH, total phenolic and chemical marker content (CGA and CFA) were calculated as the differences between the means on the high and the low levels, respectively. The significance of the effects was evaluated by comparing their values to the confidence intervals based on the mean standard deviation for the respective response variables (Montgomery, 2005). The experiments were carried out randomly and the effects were calculated presuming a linear model with interaction among the factors. The established screening design and the results were analyzed by Design-Expert® software (Version 8.0.6, StatEase, and Minneapolis, MN). The effects of each factor tested in these experiments were demonstrated using analysis of variance (regression coefficients and significant *p* values) and Pareto chart, which were a useful tool for showing the statistically significant effects evaluated in study. The function of the Pareto chart is to provide an additional chart used to display the *t*-values of the effects. Another use of the Pareto chart is to check "one more significant effect" that was not easily visible in the other chart. Analysis of variance (ANOVA) was performed to identify the significance of single factors and their binary interactions in terms of their influence on the responses analyzed (significant when the p value < 0.05). The interaction terms that were not significant were removed from the model.

Characterization of the extractive solutions (ethanol and aqueous extracts)

Determination of dry residue

The dry residue was determined by drying 20 g of each extractive solution in an oven at 105 °C for 2 h. After complete drying, the residual solid matter was weighed and the dry residue was expressed as a percentage (w/w) by the mean of three measurements (Farmacopéia Brasileira, 2010).

Total phenolic content (TPC)

Percentages of total phenolic of the ethanol and aqueous extracts were determined according to the method reported by Yu et al. (2002). Briefly, the reaction mixture contained extractive solution (10 μl of ethanol extract or 20 μl aqueous extract), 500 μl of the Folin–Ciocalteu reagent, and 1.5 ml of 20% sodium carbonate. The final volume was made up to 10 ml with purified water. After 2 h of reaction, the absorbance at 765 nm was measured and used to calculate the phenolic contents using gallic acid as standard. Triplicate reactions were conducted.

Quantitative analysis of CGA and CFA (HPLC)

The chlorogenic (CGA) and caffeic (CFA) acids content in the extractive solutions were determined by HPLC analysis using a previously validated method (Arend et al., 2011) on a Perkin-Elmer apparatus equipped with an autosampler Series 200, interface 600 Series LINK, binary pump Series 200, UV-Vis detector Series 200, and vacuum degasser Series 200. A Zorbax C HP C18 column (5 mm, 150 mm × 4.6 mm, Agilent Technologies) was used. The gradient of elution consisted of acetonitrile (A) 1.0% acetic acid (B) with a flow rate of 1 ml/min, and was programmed as follows: 0–15 min, 87% B; 15–25 min, 87–60% B; 25–34 min, 60% B. Detection was set at 330 nm. The injection volume was 20 μl.

The quantification of chemical markers, CGA and CFA was carried out by comparison of their retention times and co-injection of standard solutions. Two standard curves were plotted: chlorogenic acid (2.5–200 μg/ml) and caffeic acid (2.5–100 μg/ml). Quantification of the individual compounds was performed using a validated regression curve ($r > 0.9999$). Three milliliters of each extractive solutions were diluted to 10 ml using a methanol:water solution (50:50; v/v). The samples were filtered through a 0.45 mm HVLP membrane (Millipore) before injection.

Vascular reactivity in thoracic rat aorta

Pharmacological studies related to hypotensive activity have already been described for this plant (Lima-Landman et al., 2007; Ninahuaman et al., 2007). In an attempt to evaluate the potency of these extractive solutions, the potential cardiovascular effect was demonstrated through vascular reactivity in thoracic rat aorta following the steps below.

Animals

Male Wistar rats (250–300 g) were maintained in a 12 h light/dark cycle with free access to water and standard rat chow. On the day of the experiments, rats were euthanized under anesthesia with a mixture of ketamine (80 mg/kg) and xylasine (15 mg/kg) given intraperitonially and the aorta was removed. The experiments were performed after approval of the protocol by the local ethical committee for animal use (Protocol CEUA/UFSC PP00482).

Tissue preparation

The thoracic aorta was isolated as described previously by Andriambeloson et al. (1999). In brief, after removal of fat and connective tissue, aortic rings (3–4 mm in length) were mounted in an isolated organ bath on isometric force transducers connected to an amplifier and chart recorder (Soft and Solutions/KITCAD8, Brazil) for isometric tension recordings as described elsewhere. The rings were bathed with physiological salt solution with the following composition in mM: 130.0 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.6 $CaCl_2$, 14.9 $NaHCO_3$, 0.03 EDTA and 5.5 glucose, maintained at 37 °C and bubbled with 95% O_2–5% CO_2. The aortic rings were stretched applying tension of 1 g applied for 1 h with periodical washes every 15 min. After this period, the rings were contracted with phenylephrine (1 μM) and challenged with acetylcholine (1 μM) to test the tissue viability and integrity of the endothelium, respectively.

Evaluation of the vasorelaxant effect

Endothelium-intact aortic rings were contracted with phenylephrine (1 μM) until the plateau of the contraction was reached (around 15 min) and then exposed to increasing concentrations of the extractive solutions (0.1–100 μg/ml) given at 5 min intervals. A concentration–response curve of acetylcholine-induced vasodilatation (1 nM to 3 μM) was used as a positive control. The results were expressed as mean ± standard error of the mean (SEM) of percentage of relaxation. Statistical comparisons between groups were carried out using one-way analysis of variance (ANOVA) followed by the Tukey test. The results were also expressed as the geometric mean (IC_{50}) values, accompanied by their respective 95% confidence limits.

Antihyperglycemic activity

Fasted rats for antihyperglycemic activity measurements, from the same animal facility, were deprived of food for at least 16 h but allowed free access to water. All the animals were monitored and maintained in accordance with the local ethical committee for animal use (Protocol CEUA/UFSC PP00223).

Oral glucose tolerance curve

Animals were divided into groups of five animals for each treatment: hyperglycemic group: fasted rats that received glucose (4 g/kg; 8.9 M); hyperglycemic treated group: fasted rats that received glucose (4 g/kg) plus different ethanol or aqueous extracts (400 mg/kg) or tolbutamide (100 mg/kg) or chemical marker alone, chlorogenic acid (CGA 2 or 15 mg/kg) or caffeic acid (CFA at a dose of 2 mg/kg) or in combination (CGA plus CFA at a dose of 2 mg/kg). All treatments were carried out by oral gavage. Blood samples were collected just prior to and at 0, 15, 30, 60 and 180 min after the glucose loading. After centrifugation, serum was used to determine the glycemia by the glucose oxidase method (Folador et al., 2010). Data were expressed as mean ± S.E.M. One-way analysis of variance (ANOVA), followed by the Bonferroni post-test or non-paired Student's *t* test, was used to identify significantly different groups. Differences were considered to be significant at the $p \leq 0.05$ level.

Diabetic animals

Rats were made diabetic by a single intravenous injection of alloxan monohydrate 5% (w/v) in a saline solution at a dose of 40 mg/kg body weight. Blood samples were collected 3 days later, and glucose levels were determined as indicative of the development of diabetes. Diabetic rats received ethanol or aqueous extracts (400 mg/kg) or chemical marker alone, chlorogenic acid (CGA 2 or 15 mg/kg) or caffeic acid (CFA at dose 2 mg/kg) or in combination (CGA plus CFA at dose 2 mg/kg). Blood samples from the tail vein were collected and centrifuged and the serum was used to determine the glycemia by the glucose oxidase method. A serum glucose range of 22–29 mmol/l was used for the experiment (Folador et al., 2010).

Determination of the serum glucose concentration

Blood samples were collected and centrifuged, and the blood glucose levels were determined by the glucose oxidase method (Varley et al., 1976; Folador et al., 2010).

Table 1
Dry residue, pH, total phenolic content, and CGA and CFA contents for the maceration extraction method.

Extractive solution	Ethanol (%)	Time (days)	Dry residue (%)	pH	Total phenolic (mg/ml)	CGA (μg/ml)	CFA (μg/ml)
4d20	20	4	1.06	5.78	3.64	131.09	36.93
4d20	20	4	1.05	5.81	3.96	128.81	36.67
4d80	80	4	1.02	5.88	3.48	150.99	3.25
4d80	80	4	1.01	5.88	3.37	150.69	3.27
8d20	20	8	1.14	5.79	3.95	73.36	58.35
8d20	20	8	1.10	5.83	3.69	75.62	57.61
8d80	80	8	1.06	5.90	3.47	154.80	3.78
8d80	80	8	1.05	5.93	3.43	153.99	3.67
6d50	50	6	1.08	5.92	3.88	167.11	11.94
6d50	50	6	1.07	5.96	3.89	167.65	12.18

Results and discussion

Raw material characterization

Plants are complex mixtures that present a problem in terms of standardization and quality control. Therefore, protocols were developed from the raw material to establish their previous treatment and quality control in order to characterize them properly. Determination of the total ash content is particularly important for evaluating the cleanliness of the plant material, for example, the presence or absence of inorganic matter such as sand and dirt adhered to the surface of the drug (Jain et al., 2010). Similarly, the presence of water can promote degradation reactions and proliferation of microorganisms. This contamination compromises the integrity of pharmacologically active substances. Total ash content and loss on drying of the plant drug were determined by the procedures given in the Brazilian Pharmacopoeia (2010). The results were $7.92 \pm 0.03\%$ and $13.10 \pm 0.06\%$, respectively. The mean diameter of the crushed leaves was determined as 0.78 ± 0.41 mm. This information is important for standardizing the reproducibility of the extraction process, a homogenous sample might to improve the kinetics of the chemical compound extraction.

The presence of microorganisms can be a natural consequence of the agricultural practices and processing conditions of plant materials (Kolb, 1999). Thus, raw material plant may be associated with a broad variety of microbial contaminants, and this microbiological background has an important impact on the results of qualitative and quantitative analyses carried out for quality control (Wolfgang et al., 2002; Kosalec et al., 2009). The results of the standard aerobic bacteria count were 1.04×10^3 UFC/ml, moreover, the mean values for mold and yeast were from 8.25×10^4 UFC/ml. Therefore, the raw material did not exceed the value recommended by the WHO (1998). The data obtained suggest that the raw material was characterized and deemed appropriate for continuing studies.

Preparation and standardization of extractive solutions

In the standardization of herbal preparations various aspects of analysis must be performed, requiring the use of scientific proof and clinical validation with chemical standardization, biological assays and clinical trials (Ong, 2004). The choice of the technique for the extraction plant material is the most important step in the development of analytical methods (Benthin et al., 1999; Ong, 2004), particularly considering that herbal extracts are complex mixtures, and appropriate techniques are needed to allow a better extraction of their constituents of interest (Jacques et al., 2007). Decoction and maceration methods were investigated as an alternative to traditional extraction methods used in the extraction of chemical markers (CGA and CFA), searching for improved extraction efficiency. The conditions for the extraction of *C. glaziovii* using maceration and decoction methods were evaluated on dry residue, total phenolic values and CGA and CFA acids content. Tables 1 and 2 show the results of the experimental designs used for the maceration and decoction techniques, respectively.

Dry residue

In the ethanol extracts obtained by maceration, both factors – ethanol concentration (factor A) and extraction time (factor B) – were significant (Table 3; Fig. 1c). Increasing the percentage of ethanol causes a decrease of dry residue. On the other hand, the increase in extraction time resulted in an increased amount of dry residue (Fig. 1a).

In the case of the aqueous extracts obtained by decoction, only the factor temperature was significant (Table 4; Fig. 1d). The increase in temperature caused an increase in dry residue. In the decoction method, significant curvature was observed, indicating there is no linearity for the dry residue response, as shown in Fig. 1b.

pH

The pH values for the ethanol extracts were in the range 5.78–5.96. In the extracts obtained by maceration, only the factor ethanol concentration was significant (Table 3; Fig. 2c). Lowering the ethanol concentration also lowered the pH values (Fig. 2a). The same occurred with increasing temperature for the aqueous extracts (Table 4; Fig. 2b and d), which seems to indicate the extraction of substances with acidic character in those conditions. The

Table 2
Dry residue, pH, total phenolic content, and CGA and CFA contents for the decoction extraction method.

Extractive solution	Temperature (°C)	Time (min)	Dry residue (%)	pH	Total phenolic (mg/ml)	CGA (μg/ml)	CFA (μg/ml)
7010	70	10	0.82	5.88	2.09	179.09	7.75
7010	70	10	0.76	5.94	1.65	177.67	7.72
9010	90	10	0.91	5.72	2.19	190.17	7.59
9010	90	10	0.93	5.72	2.15	192.99	8.02
7030	70	30	0.87	5.85	2.21	185.41	8.06
7030	70	30	0.83	5.84	2.13	186.65	8.18
9030	90	30	0.91	5.70	2.19	191.55	8.70
9030	90	30	0.95	5.71	2.35	193.04	8.63
8020	80	20	0.77	5.65	2.15	220.87	4.86
8020	80	20	0.76	5.66	2.06	221.30	4.86

Table 3
Analysis of variance (regression coefficients and significant *p* values) for dry residue, total phenolic, CGA and CFA responses for maceration extraction method.

Polynomial term	Dry residue (%)		pH		Total phenolic		CGA (μg/ml)		CFA (μg/ml)	
	Coefficient	*p* value	Coefficient	*p* value	Coefficient	*p* value	Coefficient	*p* value	Coefficient	*p* value
Model	–	0.0023	–	0.0018	–	0.0153	–	<0.0001	–	<0.0001
Intercept	+1.06	–	5.85	–	3.62		127.42	–	25.44	–
(A) Ethanol (%)	−0.026	0.0024	0.048	0.0007	−0.19	0.0053	+25.20	<0.0001	−21.95	<0.0001
(B) Time (days)	+0.026	0.0024	0.013	0.1466 (NS)	0.011	0.8057 (NS)	−12.98	<0.0001	+5.41	<0.0001
(AB) Interaction	–	NS	–	NS		NS	+14.75	<0.0001	−5.18	<0.0001
Curvature	–	NS	–	0.0017		0.0370	–	<0.0001		<0.0001

NS, not significant.

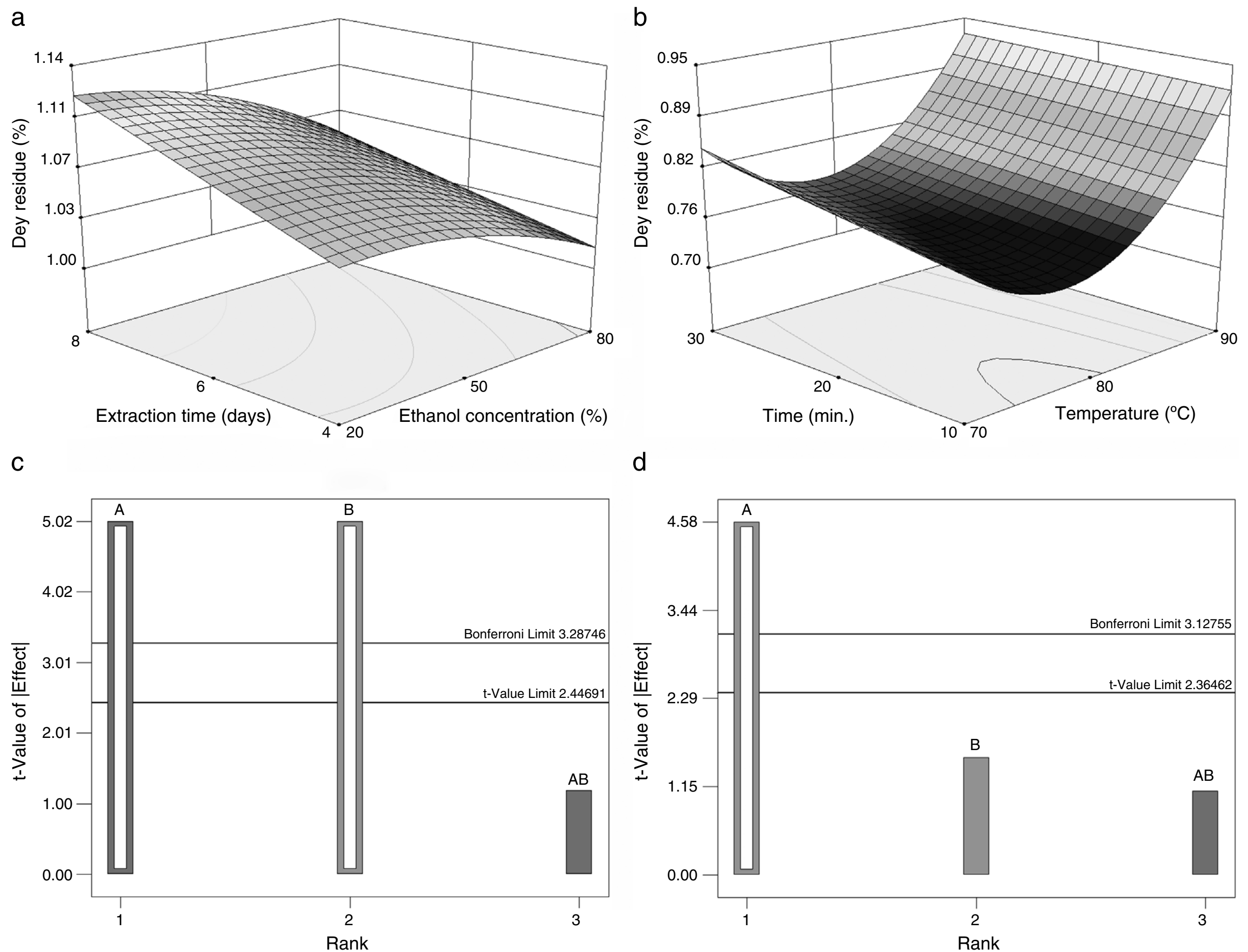

Fig. 1. Graph of ethanol concentration (A) and extraction time (B) versus dry residue for maceration (a); and temperature (A) and time (B) for decoction (b), and significance of the effects (*) on Pareto chart: Maceration (c) Decoction (d); positive effects (#); negative effects (##).

Table 4
Analysis of variance (regression coefficients and significant *p* values) for dry residue, pH, CGA and CFA responses for decoction extraction method.

Polynomial term	Dry residue (%)		pH		CGA (μg/ml)		CFA (μg/ml)	
	Coefficient	*p* value	Coefficient	*p* value	Coefficient	*p* value	Coefficient	*p* value
Model	–	0.0046	–	0.0001	–	0.0002	–	0.0044
Intercept	+0.85	–	5.79	–	+13.91	–	+7.44	–
(A) Temperature (°C)	+0.053	0.0020	−0.082	<0.0001	+0.18	<0.0001	+0.15	0.0289
(B) Time (min.)	+0.017	0.1340 (NS)	−0.020	0.0498	+0.077	0.0038	+0.31	0.0017
(AB) Interaction	–	NS	–	NS	−0.064	0.0081	–	NS
Curvature	–	0.0031	–	0.0003	–	<0.0001		<0.0001

NS, not significant.

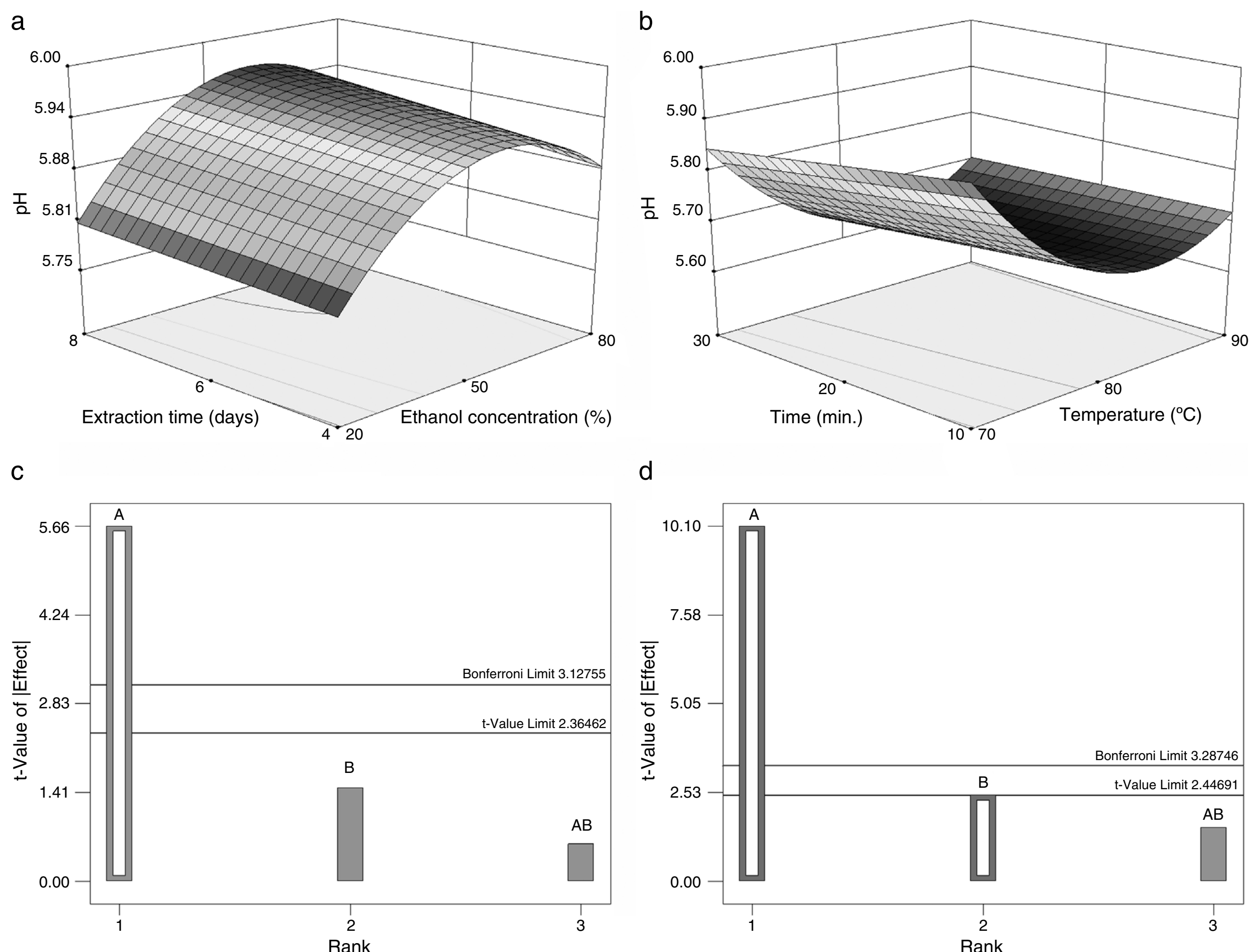

Fig. 2. Graph of ethanol concentration (A) and extraction time (B) versus pH for Maceration (a) and temperature (A) and extraction time (B) for decoction (b), and significance of the effects (*) on Pareto chart: Maceration (c); Decoction (d); positive effects (#); negative effects (##).

significant curvature values for both extraction methods show that this increase is not linear, a behavior also shown in Fig. 2a and b.

The pH values appear to reach a steady state at a higher ethanol concentration for the ethanol extracts and at a higher temperature for the aqueous extracts. However, although there are statistical differences, the small variation between the pH values (5.78–5.96) may not be relevant from technological standpoint.

Total phenolic content

Several researchers have investigated the ability of some phenolic compounds present in plant material to act as antioxidants (Seeram et al., 2006; Zhang et al., 2008; Işık et al., 2011). This activity may be correlated with the content of their phenolic compounds (Velioglu et al., 1998; Cheung et al., 2003). Determination of total phenolic content has been achieved using the Folin–Ciocalteu procedure (Yu et al., 2002). This colorimetric method is based on the extraction of phenolic compounds (acidic solution) after oxidation of the reduced molecules by a mixture of the two strong inorganic oxidant phosphotungstic and phosphomolybdic acids (Işık et al., 2011). The Folin method has poor specificity, as it can give an overestimated value for total phenol content in extracts. For example, between some non-phenolic substances this method can potentially interfere in the determination of total phenolic content by increasing the absorbance value of the phenolic compounds (Stevanato et al., 2004; Işık et al., 2011). Even with this potential interference, due to the complexity of the sample, this technique is easy and fast to use, and is convenient for routine use for quality control tests on the plant material.

Total phenolic content was expressed as gallic acid equivalents. A calibration curve was constructed with different concentrations of gallic acid as standard. The amount of phenolic compounds in the ethanol extracts was higher than that of the aqueous extracts (Table 1). The total phenolic content of ethanol extracts gradually decreased with increasing ethanol concentration, and was not influenced by extraction time (Table 3; Fig. 3a and c). This indicates that at 4 days of maceration, a steady state may have already been reached in the extraction of these compounds. The higher total phenolic content in the ethanol extracts than in the aqueous extracts might be explained by the different extractor liquid used in the extraction. In addition, the extraction method may contribute to the difference in content (Yu et al., 2002). Normally, the efficiency of the extraction of polyphenolics compounds is lower using pure solutions like water, ethanol or methanol. The yield of total phenolic compounds extracted from these extracts can be higher when solvents containing some water are used in the extraction process (Katsube et al., 2009).

As observed in Fig. 3b, the increases in temperature and extraction time tend to increase the total phenolic concentration in the aqueous extracts, although no statistical significance has been observed in the factors evaluated for these extracts (Fig. 3d). The yield of phenolic compounds may depend significantly on extraction temperature and time (Herrera and Luque de Castro, 2005). Heating may soften the plant tissue, weaken the cell wall

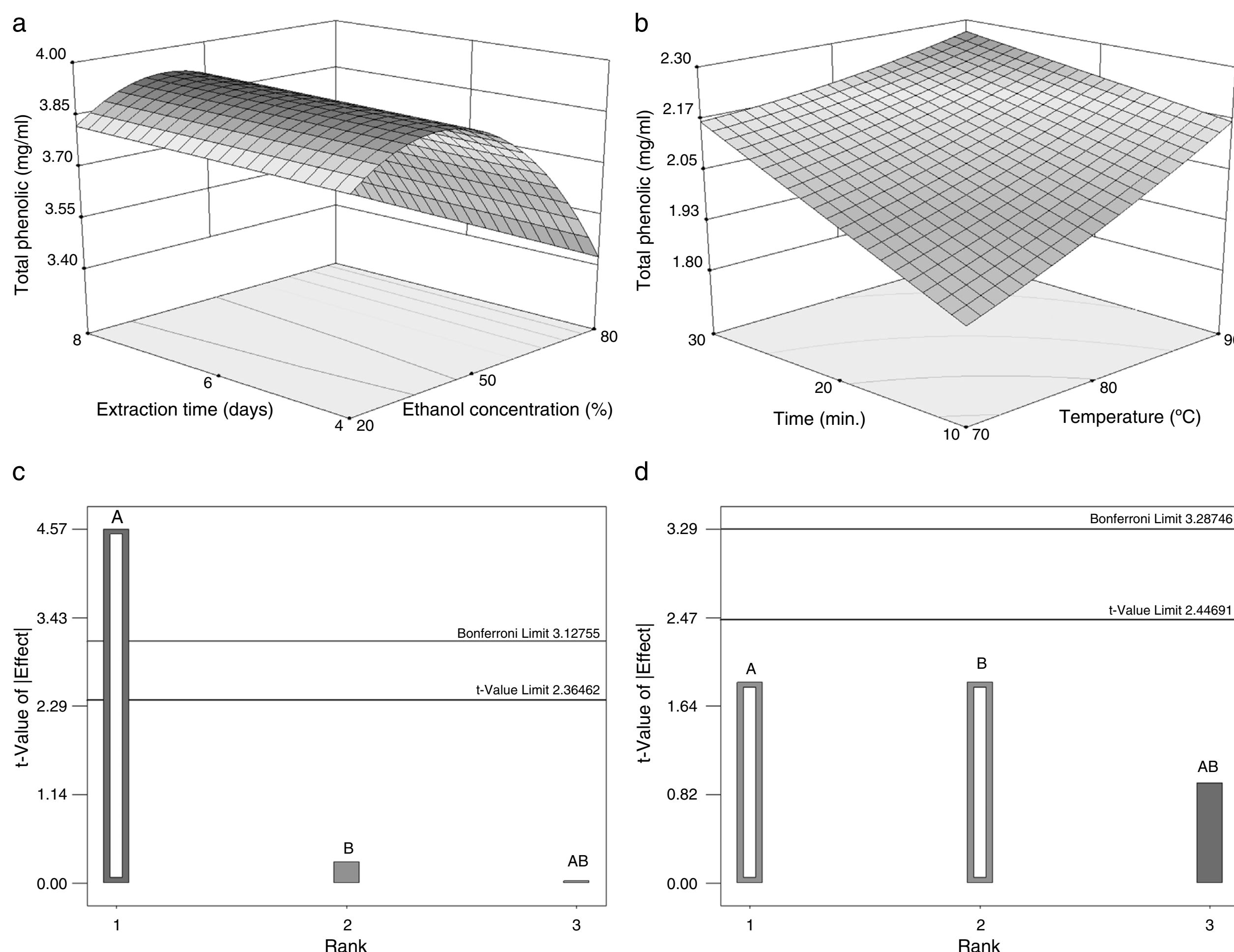

Fig. 3. Graph of ethanol concentration (A) and extraction time (B) versus total phenolic for Maceration method (a) and temperature (A) and time (B) for decoction method (b), and significance of the effects (*) on Pareto chart: Maceration (c); Decoction (d); positive effects (#); negative effects (##).

integrity, hydrolyze the bonds of bound phenolic compounds (phenol–protein or phenol–polysaccharide) and enhance phenolic solubility. As a result, phenolic compounds would disperse in the solvent (Juntachote et al., 2006; Li et al., 2006; Spigno et al., 2007; Chan et al., 2009). However, even though increased temperatures increase the solubility of the chlorogenic and caffeic acids, it should be taken into account that higher temperatures promote instability of the phenolic compounds (Herrera and Luque de Castro, 2005; Ma et al., 2009). This may indicate a degradation of some phenolic acids (Ma et al., 2009) which is a crucial influence in the extraction of phenolic acid yields.

CGA and CFA content

According to the experimental design, the extraction of CGA from maceration had a significant influence on ethanol concentration and time. However, an interaction between the factors was observed. Significant curvature was also observed, indicating there is no linearity for this response (Table 3; Fig. 4a and c).

Ethanol extracts containing 80% ethanol showed the same concentration of the CGA for all extraction times (4, 6 and 8 days). These data suggest that the steady state between drug and solvent occurred from the fourth day. On the other hand, extracts prepared with 20% ethanol presented significantly lower levels of CGA over time (Fig. 4a). The CFA content in extracts containing 20% ethanol was different according to the extraction time (Table 3; Fig. 4b and d). Between the fourth and eighth days, an increase was observed in the amount of this compound, from 36 to 58 μg/ml, respectively. In the same period, CGA concentration decreased from 130 to 75 μg/ml. This decrease in CGA content was previously investigated by our group (Arend et al., 2011). In order to check the occurrence of thermal or enzymatic degradation of CGA during the maceration time, the influence of temperature and the presence of a preservative on the content of this acid in the extractive solutions were evaluated. These results showed that CGA concentration does not alter in presence of a preservative, suggesting that the decrease in CGA content in extractive solutions is not due to microbiological degradation during the extraction period. This effect might be related to a chemical degradation, considering that chlorogenic acid is an ester of caffeic acid and quinic acid (Olthof et al., 2001; Arend et al., 2011). A higher CGA concentration was observed in extracts containing 50% ethanol, followed by with 80% ethanol (independent of extraction time) and 20% ethanol, 4 days and 8 days of extraction time, respectively.

CGA extraction on aqueous extracts appears to be influenced by time and temperature and an interaction between the factors was observed (Table 4; Fig. 5a and c). Maximum CGA content was observed at 30 min and an extraction temperature of about 80 °C, showing non-linearity in this response (Table 4). The same occurs for the amount of CFA, but in the opposite direction, with the smallest amount occurring at around 80 °C (Table 4; Fig. 5b and d). However, CFA content in the aqueous extracts was very low, particularly when compared CGA content in these extractive solutions.

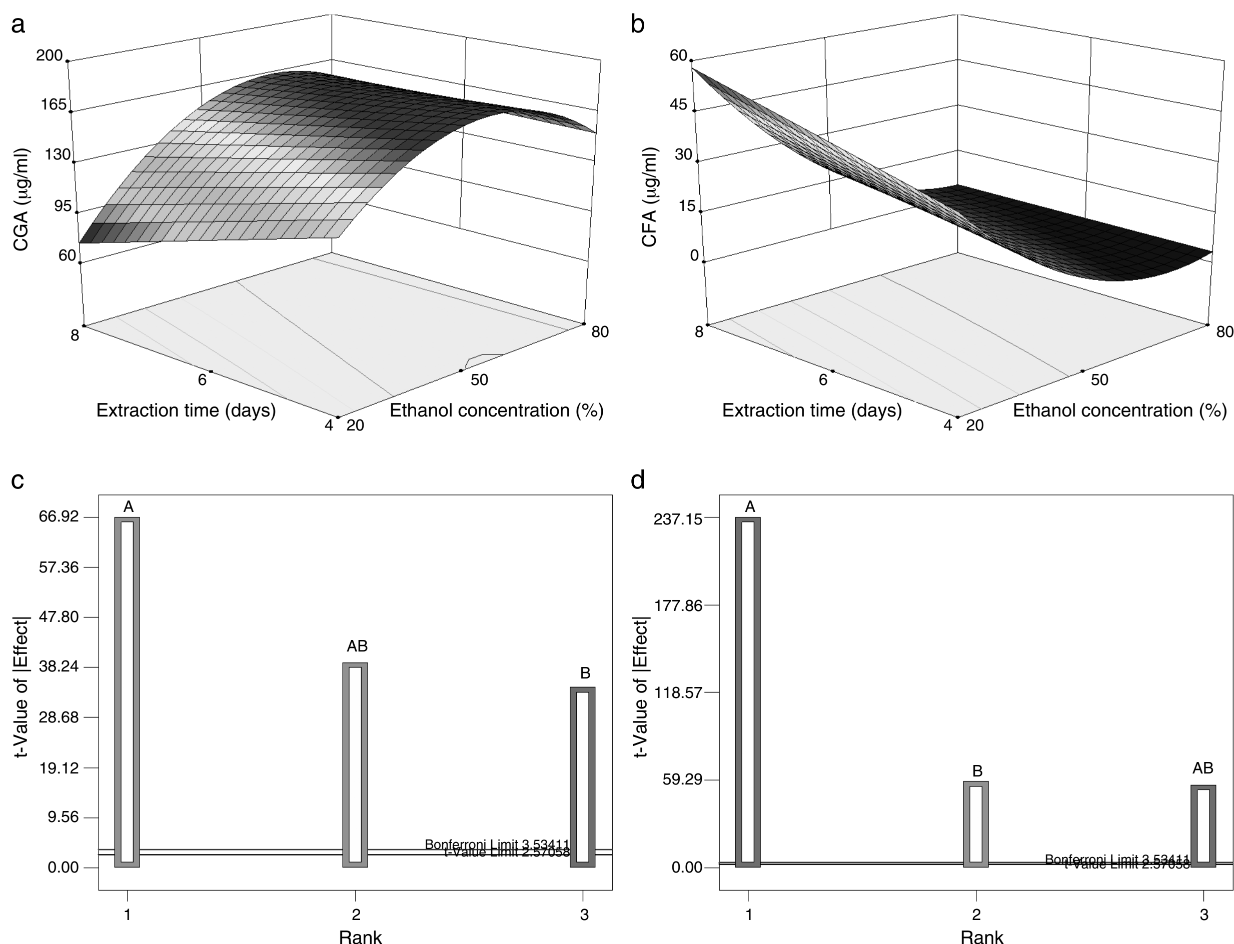

Fig. 4. Graph of ethanol concentration (A) and extraction time (B) versus CGA (a) and CFA (b) in the maceration method, and significance of the effects (*) on Pareto chart: CGA (c); CFA (d); positive effects (#); negative effects (##).

Vascular reactivity in vitro *in thoracic rat aorta*

Infusion of *C. glaziovii* leaves has been used in traditional medicine as an antihypertensive, cardiotonic and antiasthmatic (Rocha et al., 2002). Lima-Landman et al. (2007) described an antihypertensive effect for aqueous extract and butanolic fraction of *C. glaziovii*, with satisfactory results. Based on these results, this work provides evidence of the ability of the extractive solutions prepared by maceration (4d20, 8d20) and decoction (9030 and 8020) to relax rat aorta, and evaluate the potency of the extracts for this purpose. Fig. 6 shows the concentration–effect curves to samples in rat aortic rings pre-contracted with phenylephrine (1 μM). Cumulative dosing of extracts produced a concentration-dependent relaxation of aortic rings. The IC_{50} values with the 95% respective confidence intervals for the extractive solutions were: 4d20, 0.85 (0.74–0.96); 8d20, 1.48 (1.01–1.89); 9030, 2.62 (2.26–2.98); and 8020, 2.41 (1.99–2.81) μg/ml. Analyzing the IC_{50} values, we verified that the ethanol extracts presented more potent vasodilator effects than the aqueous extracts in aortic rings.

Antihyperglycemic activity

Acute antihyperglycemic effect of C. glaziovii

Fig. 7a and b shows the effect of different ethanol and aqueous extracts from *C. glaziovii* on the glucose tolerance curve. The extracts were selected based on the concentration of the chemical markers, chlorogenic acid (CGA) and caffeic acid (CFA), in the extracts. Oral administration (400 mg/kg) of ethanol extracts 4d20 and 8d20 improved glucose tolerance, and this effect was significant at 15 min for 4d20 extract and at 15, 30, 60 and 180 min for 8d20 extract when compared with the hyperglycemic control group. On the other hand, the oral treatments with 6d50 ethanol extract as well as the 8020 and 9030 aqueous extracts were not able to reduce the blood glucose levels. As expected, after starting the glucose tolerance test the serum glucose concentration was significantly increased when compared with zero time. Tolbutamide (100 mg/kg) an oral hypoglycemic agent of sulfonylurea class was used as a positive control and produced a typical serum glucose lowering at 15, 30 and 60 min compared to the hyperglycemic group (Fig. 1C).

Considering the glucose homeostasis, some species of *Cecropia* have been described to improve glucose metabolism. The hypoglycemic effect of methanol leaf extracts of *C. obtusifolia* and *C. peltata* were evaluated in healthy mice. Both extracts produced a significant hypoglycemic effect at 2 and 4 h after oral administration. However, *C. peltata* showed a better effect than *C. obtusifolia*, which may be correlated to the difference in chlorogenic acid content between them (Nicasio et al., 2005). Additionally, an investigation of the *C. pachystachya* methanol extract confirmed a hypoglycemic effect that can be explained by the presence of chlorogenic acid and *C*-glycosylated flavones as the major constituents.

Based on these results, the chemical markers were evaluated on the glucose tolerance curve and the results are shown in Fig. 8. It can be observed that CGA at 15 mg/kg was not able to reduce the glycemia, however at a lower dose (2 mg/kg) it presented a

Fig. 5. Graph of temperature (A) and time (B) versus CGA (a) and CFA (b) in the decoction method, and significance of the effects (*) on Pareto chart: CGA (c); CFA (d); positive effects (#); negative effects (##).

significant effect at 15 and 30 min after the treatment. Also, CFA at the dose (2 mg/kg) showed a rapid effect at 15 min compared to the control group. In order to evaluate if these compounds have an additive effect they were studied together in the oral glucose tolerance curve. At the dose of 2 mg/kg CGA and CFA were able to significantly reduce the glycemia at between 15 min and 60 min after oral gavage treatment.

Caffeic and chlorogenic acids have already been demonstrated to reduce blood glucose levels in hyperglycemic as well as in diabetic rats acting via several mechanisms against hyperglycemia (Meng et al., 2013; Dhungyah et al., 2014). They regulate beta cell function and increase insulin secretion, up-regulate adipocytes GLUT-4 content and increase glucose uptake in adipocytes and in muscles. Additionally, CFA and CGA inhibit alpha-amylase

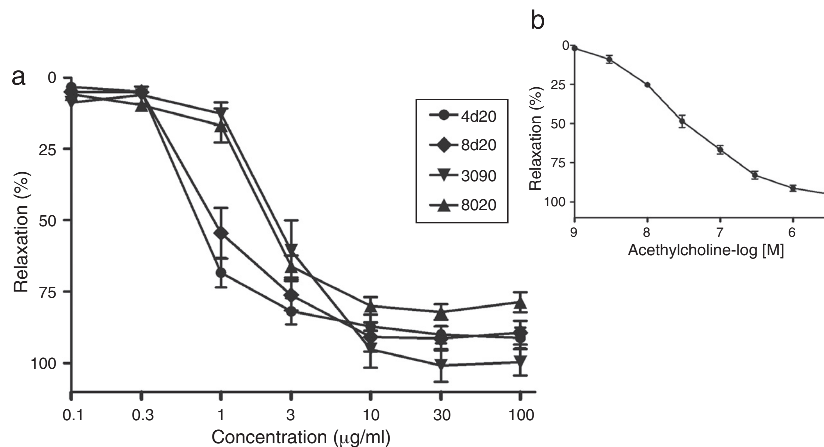

Fig. 6. (A) Concentration–response curve of 4d20, 8d20, 9030 and 8020 induced vasodilatation (0.1–100 mg/ml) in rat isolated thoracic aorta rings pre-contracted with Phe (1 mM) with intact endothelium. (B) Concentration–response curve of acetylcholine (positive control) induced vasodilation (1 nM to 3 μM) in rat isolated thoracic aorta rings pre-contracted with Phe (1 mM) with intact endothelium. Values represent the mean ± S.E.M. of 6 independent experiments.

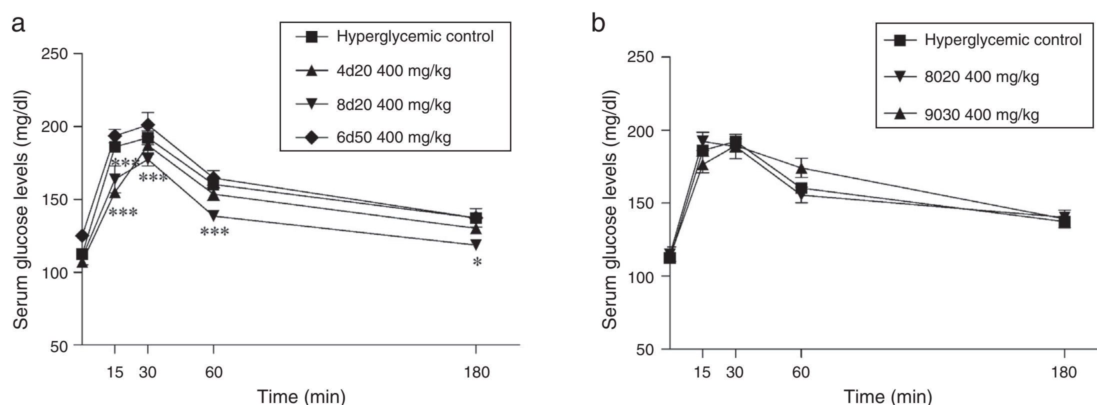

Fig. 7. Effect of maceration (A) and decoction (B) extracts from Cecropia glaziovii Snethl on glucose tolerance curve. Values are expressed as mean ± S.E.M; $n = 6$ in duplicate for each treatment. Statistically significant difference from the corresponding hyperglycemic group; $^{*}p \leq 0.05$; $^{***}p \leq 0.001$).

and alpha-glucosidase activity in the gastrointestinal tract and increases glucokinase activity in the hepatocytes (Jung et al., 2006; Tousch et al., 2008; Bassoli et al., 2008; Karthikesan et al., 2010; Ong et al., 2012, 2013; Meng et al., 2013; Dhungyah et al., 2014). Caffeic acid and chlorogenic acid also lower glucose-6-phosphatase, glucose-6-translocase and phosphoenolpyruvate carboxykinase activities, components of gluconeogenesis and glycogenolysis in rat liver resulting in a reduction of the hepatic glucose production (Schwab et al., 2001). Considering that CGA and CFA are the major constituents of *C. glaziovii* extracts, it can be suggested that the antihyperglycemic and the hypoglycemic effect observed herein to *C. glaziovii* extracts and to CFA and CGA could be mediated through different targets of action involving hepatic glucose output, glucose uptake in insulin target tissues, intestinal glucose absorption and insulin secretion.

Based on the content of the CGA and CFA on each of the *C. glaziovii* extractive solutions and based on the literature, it is suggested that the antihyperglycemic effect of the different extracts may be related with the content/concentration of the chemical markers and also to the ratio between them. The best results on glycemia were observed with the extracts that contained the lowest concentrations of CGA and CFA (8d20 and 4d20) and also the ratio close to 1:1 (Fig. 7a and b/Table 1). This result is in line with those observed for CGA and CFA on the glucose tolerance curve, in which the lowest dose of CGA as well as the association of both, CGA and CFA presented the most pronounced effect (Fig. 8) showing a dose-dependent pattern of action on the glucose tolerance curve.

Additionally, in order to complement the glycemic profile, the different extracts of *C. glaziovii* and the chemical markers were studied *in vivo*, using an alloxan-induced diabetic rat model (unable to secret insulin). Fig. 9 shows the effect of ethanol and aqueous extracts as well as CGA on glycemia in alloxan-induced diabetic rats. The aqueous extracts, 8020 and 9030, were able to reduce significantly the glycemia, at 1 h and at 2 h, respectively, after the oral treatments (400 mg/kg), when compared with the diabetic control group. Also, it can be observed that the ethanol extract 8d20 presented a slight reduction in glycemia at 2 h after the treatment. Additionally, when the chemical markers, CGA and CFA were evaluated, just the higher dose of CGA (15 mg/kg) presented a significant effect on reducing the blood glucose levels at 2 and 3 h after the treatment. The CFA, CGA as well as the association of both at 2 mg/kg were ineffective in this approach (data not shown). Contrary to that observed in hyperglycemic normal rats, the absence of and/or the slight effect of the ethanol extracts in alloxan-induced diabetic rats may be related to the insufficient concentration of CGA and CFA in the extracts, since when these compounds were evaluated only the higher dose of CGA was effective in this approach. Also, the aqueous extracts that presented a higher concentration of CGA were effective in the diabetic animals. It is possible that to exert its effect as an insulinomimetic agent in diabetic rats, *C. glaziovii* extracts/compounds must have higher concentrations of the main constituents, CGA and CFA.

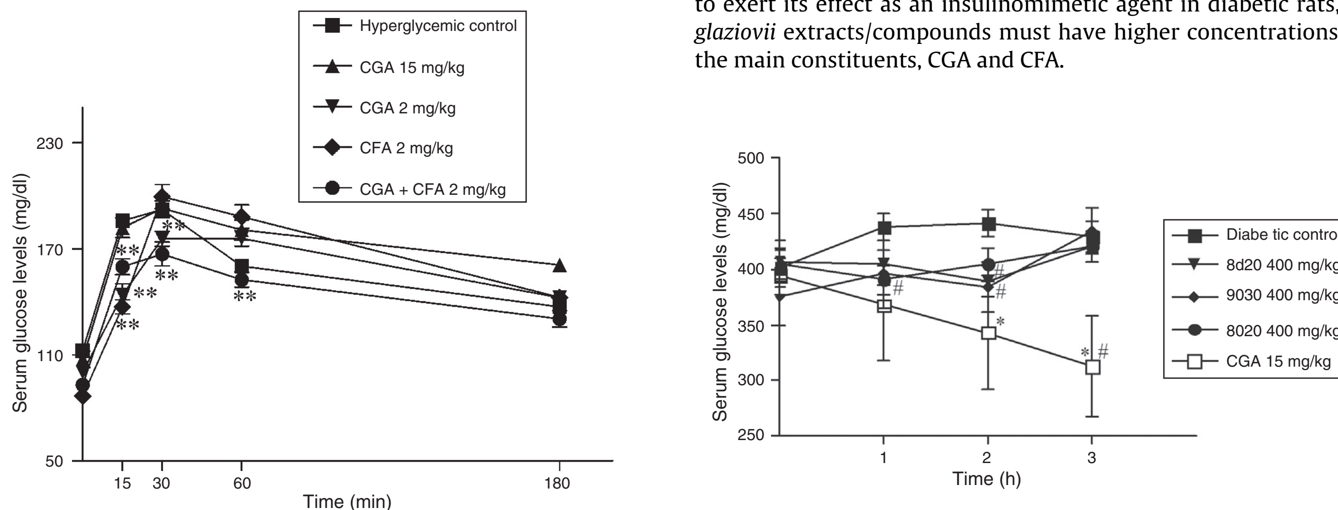

Fig. 8. Effect of the chemical markers chlorogenic acid (CGA) and caffeic acid (CFA), and the association on glucose tolerance curve. Values are expressed as mean ± S.E.M; $n = 6$ in duplicate for each treatment. Statistically significant difference from the corresponding hyperglycemic group; $^{**}p \leq 0.01$.

Fig. 9. Effect of the ethanolic and aqueous extracts and the chlorogenic acid (CGA) on the serum glucose level in alloxan-induced diabetic rats. Values are expressed as mean ± S.E.M; $n = 6$ in duplicate for each treatment. Statistically significant difference from the corresponding zero time value of each group; $^{*}p \leq 0.05$. Statistically significant difference from the corresponding diabetic group; $\#p \leq 0.05$.

Recent studies have shown the hypoglycemic effect of *Cecropia* species in diabetic animal models. Aqueous and butanol extracts prepared from *C. obtusifolia* and *C. peltata* were evaluated in streptozotocin diabetic rats and the results showed a significant decrease in serum glucose at 3 h after the oral treatment as well as on the oral glucose tolerance curve. Moreover, it is suggested that chlorogenic acid is involved in the hypoglycemic effect of *C. obtusifolia* and *C. peltata* because it was one of the major constituents of the extracts (Andrade-Cetto and Wiedenfeld, 2001; Andrade-Cetto and Vázquez, 2010). The methanol extract from the leaves of *C. pachystachya* also produced a significant hypoglycemic effect in alloxan-induced diabetic rats, probably due to the chlorogenic acid content in this species (Aragão et al., 2010; Karthikesan et al., 2010).

In conclusion, this study provides the first report on the antihyperglycemic action of the extracts of *C. glaziovii* leaves improving glucose tolerance. In addition, the potent relaxing effect of ethanol extracts on thoracic aorta was demonstrated. Independent of the exact mechanism of action involved, these results can probably be associated with the concentrations of the phenolic constituents of the extracts. The results presented here give initial experimental support for future experiments, as they link this effect to more classes of secondary compounds as well as evaluation of the isolated compounds present in the extractive solutions.

Authors' contributions

DPA (MSc student) contributed in running the laboratory work, chromatographic analysis, analysis of the data and drafted the paper. TCS contributed in drafting the manuscript. LHC and MAH contributed to biological studies. DS, ALGS, RMRV and FRMBS contributed to critical reading of the manuscript. AMC designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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