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Research Paper

Cecropia pachystachya extract attenuated the renal lesion in 5/6 nephrectomized rats by reducing inflammation and renal arginase activity

Claudia C. Maquiaveli [a,*], Edson R. da Silva [b], Lara C. Rosa [b], Heloísa D.C. Francescato [a], João Francisco Lucon Júnior [b], Cleonice G.A. Silva [a], Dulce E. Casarini [c], Fernanda A. Ronchi [c], Terezila M. Coimbra [a]

[a] Departament of Physiology, School of Medicine of Ribeirão Preto, University of São Paulo, Av. Bandeirantes, 3900 Monte Alegre, Ribeirão Preto, São Paulo, Brazil
[b] Department of Veterinary Medicine, School of Animal Science and Food Engineering, University of São Paulo, Av. Duque de Caxias Norte, 225, Pirassununga, São Paulo, Brazil
[c] Department of Medicine, Nephrology Division, Federal University of São Paulo, Rua Botucatu, 740, Vila Clementino, São Paulo, Brazil

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ABSTRACT

Ethnopharmacological relevance: The plant *Cecropia pachystachya* Trécul has been used in Brazilian folk medicine to treat hypertension, bladder and kidney inflammation and renal diseases. The aim of this study was to evaluate the potential of the aqueous fraction from the ethanolic extract of *Cecropia pachystachya* (FCP) in the management of hypertension, inflammation and progressive renal disease in rats submitted to 5/6 nephrectomy.
Materials and methods: Thirty male Wistar rats submitted to 5/6 nephrectomy (5/6 NE) were untreated (NE) or treated (NE+FCP) with the FCP (0.5 g/kg/day). The treatment started 15 days after surgery, and the rats were followed for a period of 60 days. Systolic blood pressure (SBP) and albuminuria were evaluated from 15–60 days after the surgical procedure. Function and estructural renal changes, TGF-β (transforming growth factor β), MCP-1 (monocyte chemoattractant protein-1) and nitric oxide (NO) urinary excretion were analyzed. Expression and activity of the renal enzymes arginase (ARG), angiotensin converting enzyme (ACE), and MAP kinase p-JNK expression also were analyzed.
Results: The nephrectomized rats developed progressive albuminuria and increased SBP that was less intense in the treated group. There was a reduction in the glomerular filtration rate (GFR) in the nephrectomized rats, which was attenuated by treatment with FCP extract. The treatment with FCP also attenuated the histological changes, reduced the expression and activity of renal arginase, the number of macrophages (ED-1 positive cells) and the p-JNK expression in the renal cortex of the rats submitted to 5/6 NE. The urinary excretion of TGF-β was less intense in the treated group and was associated with the reduction of the expression and activity of the renal arginase.
Conclusions: These results suggest that the reduction of renal arginase activity, p-JNK and TGF-β expression can explain the mechanism by which the treatment with *C. pachystachya* reduced the inflammation and improved renal function. This study presents the potential use of *Cecropia pachystachya* in the treatment of chronic renal diseases.

1. Introduction

The *Cecropia* genus has extensive use in folk medicine. The plants are native from South and Central America and are known as embauba (Lorenzi and Matos, 2002). *Cecropia pachystachya* Trécul is traditionally used in folk medicine to treat asthma, bronchitis (Soraru and Bandoni, 1978) and diabetes (Lorenzi and Matos, 2002). Ethnobotanical studies realized in Brazil have shown that *Cecropia pachystachya* has been used traditionally to the treatment of the renal diseases (Alves and Povh, 2013) and as analgesic to renal pain (Bessa et al., 2013).

We recently also reported that *Cecropia pachystachya* aqueous extract attenuated hypertension and renal lesion in rats subjected to 5/6 nephrectomy (5/6 NE) by decreasing angiotensin converting enzyme (ACE) activity in the renal cortex (Maquiaveli et al., 2014).

* Corresponding author. Tel.: +55 19 3565 4371; fax: +55 19 3565 4117.
E-mail addresses: cmaquiaveli@usp.br (C.C. Maquiaveli), edsilva@usp.br (E.R. da Silva).

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The 5/6 NE is a model of chronic renal failure (CRF) characterized by hypertension, proteinuria, inflammation, loss of renal function and histological changes similar to those observed in many renal human diseases (Kleinknecht et al., 1995). One of the main features observed in this experimental model is an increase in renin–angiotensin–aldosterone system (RAAS) activity, which results in renal hemodynamic changes and inflammation (Taal and Brenner, 2000).

Several studies have shown that an increase in RAAS activity associated with a reduction in L-arginine availability contributes to the loss of renal function in this model (Ashab et al., 1995; Sabbatini et al., 2003). L-arginine is an amino acid endogenously synthesized in renal proximal cells from L-citrulline and is then degraded through distinct enzymatic routes that may affect renal function and morphology (Reyes et al., 1994). In rats subjected to 5/6 NE the treatment with captopril associated with L-arginine supplementation was more effective than the treatment only with an ACE inhibitor (Ashab et al., 1995). The improved renal function and renal plasmatic flow (RPF) observed in these animals were associated with a reduction of the inflammation induced by angiotensin II (AII) and an increase in nitric oxide (NO) production (Ashab et al., 1995).

Arginase is a key enzyme in the regulation of the production of NO because this enzyme shares the L-arginine substrate with nitric oxide synthase (NOS). The increase in arginase activity not only reduces NO production but also increases the synthesis of proline, a precursor amino acid of collagen production. Therefore, arginase can contribute to an increase of the interstitial fibrosis in inflammatory processes (Schnorr et al., 2008) that are observed in chronic renal diseases. The inhibition of arginase by flavanoids present in cocoa was recently reported (Schnorr et al., 2008).

Cecropia pachystachya leaves are rich in polyphenols mainly flavonoids that can inhibit ACE (Lacaille-Dubois and Wagner, 2001) and arginase in vitro. Therefore, the objective of the present study was to evaluate the effect of the aqueous fraction from ethanolic extract of *Cecropia pachystachya* (FCP) on hypertension, inflammation, arginase and ACE activity in model experimental of renal disease.

2. Materials and methods

2.1. Plant material, preparation and administration of the FCP

Cecropia pachystachya Trécul (URTICACEAE) was collected at Ribeirão Preto (-21.167925, -47.859927, altitude 546 m), state of São Paulo, Brazil. A voucher specimen (ESA 120140) from the plant was identified by MSc. Gerson Oliveira Romão and deposited in the Herbarium of the ESALQ, University of São Paulo. The dried leaves of *Cecropia pachystachya* were ground in a mill (type Wyllie, Tecnal, Piracicaba, São Paulo, Brazil) to obtain a powder, which was used to prepare ethanolic extract. A Soxhlet system was used in hot and standard extraction to extract lipids from *Cecropia pachystachya* powdered leaves using hexane as a solvent. After lipid extraction, ethanolic extract (EE) was prepared by a maceration process at 2% w/v (weight of powdered leaves/volume of ethanol). The maceration process was performed for 72 h at room temperature. After extraction, the EE was filtered and the solvent was removed by rotary evaporation under reduced pressure (~100 mmHg) at 55 °C. After this, the water soluble fraction of the EE (FCP) was obtained by adding 500 mL of distillate water at 42 °C to the resulting EE dry extract from 4 L. The samples were centrifuged at 4500*g* and supernatant (FCP) was frozen at −20 °C until its administration. Samples in triplicate of 25 mL of each bottle were lyophilized to verify the concentration of FCP. Based on an evaluation of water consumption of the animals over a 24 h period, the FCP was defrosted and diluted for administration to achieve a minimum dose of 0.5 g/kg/day. The dose of FCP was determined from a previously pilot experiment based on the effect in systolic blood pressure (SBP) after 15 days of treatment (Maquiaveli et al., 2014). The FCP was administered in amber glass drinkers given daily to the animals. The consumed volume of the FCP per cage was determined, and the mean dose was evaluated during the treatment.

2.2. Characterization of the active constituents from C. pachystachya

The profile of the constituents from *Cecropia pachysatachya* FCP extract was performed using liquid chromatography–mass spectra (LC–MS) using Esquire 3000 Plus (Bruker Daltonics) as described previously (Cruz et al., 2013).

2.3. Animals and experimental protocols

Thirty male Wistar rats (180–200 g) were utilized in this study. The animals were maintained 3 rats per cage at a temperature of 22 °C with a light–dark cycle of 12 h and fed with standard diet ad libitum. The experiments were performed in accordance with the ethical principles for animal experimentation adopted by the Brazilian College of Animal Experimentation, and the Animal Experimentation Committee of the University of São Paulo at Ribeirão Preto School of Medicine approved the study protocol (COBEA/CETEA/FMRP-USP, Protocol no. 050/2010). The animals were subjected to 5/6 NE according to the technique described previously (Chanutin and Ferris, 1932). The animals were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) per intramuscular injection. The right kidney was removed, and an infarction was provoked in two-thirds of the left kidney by ligation of two branches of the left renal artery. In the control group, a sham (S) operation was performed by laparotomy and manipulation of the renal pedicles without causing ischemia or destruction of renal tissue. The animals were divided into 3 groups ($n=10$ per group): untreated animals that were subjected to sham surgery(S); untreated animals that were subject to 5/6 nephrectomy (NE); and animals treated with FCP that were subjected to 5/6 NE (NE+FCP). The treatment with FCP started 15 days after surgery. After the surgical procedure, all groups were followed for a period of 60 days. Body weight (BW) and systolic blood pressure (SBP) measured by the tail-cuff method (CODA System, Kent Scientific, Torrington, Connecticut, USA) were evaluated from 15–60 days after surgery. The animals were placed in metabolic cages for 24 h, and urine samples were collected to quantify albumin excretion (AE) and NO production.

2.4. Renal function studies

The albumin in urine samples was quantified by electroimmunoassay and the glomerular filtration rate (GFR) was evaluated 60 days after surgery by inulin clearance. The rats were anesthetized with sodium thiopental (40 mg/kg, intraperitoneally). After tracheostomy, the femoral artery and vein were cannulated to collect blood samples and to inject fluids, respectively. The ureters were cannulated to collect urine. Urine samples were collected before the administration of inulin solution to analyze TGF-β and MCP-1. After urine sample collection, the rats received a priming inulin dose (12 mg/100 g diluted in PBS solution, pH=7.4), followed by a maintenance inulin dose of 0.5 mg/min/100 g. After 60 min of stabilization, urine was collected for 1 h, and the blood was sampled at 30 and 60 min. The kidneys were perfused through the aorta with PBS solution and removed for histological and immunohistochemical studies. The animals were euthanized via

anesthesia overdose. Inulin was quantified in the plasma and urine samples by the method described by Fuhr et al., 1955.

2.5. Quantification of TGF-β and MCP-1 in urine

The urine samples collected by the ureter were immediately treated with 1 mM phenylmethylsulfonil fluoride (PMSF, Sigma Chemical Co, St. Louis, Missouri, USA) and stored at −70 °C until analysis. The TGF-β and MCP-1 quantification was performed by enzyme immunoassay (ELISA) using commercial kits (Promega Corporation, Madison, Missouri, USA and Pierce, Rockford, USA, respectively). The median values of TGF-β and MCP-1 were expressed in pg of TGF-β or MCP-1 per mg creatinine.

2.6. Determination of urinary NO levels

The samples of urine collected in 24 h from control and nephrectomized groups were aliquoted and deproteinized with 95% ethanol (4 °C) 1:2 (lysate:ethanol) and centrifuged at 4000*g* for 5 min. The supernatant was utilized to determine NO content by the NO/Ozone technique described previously (Hampl et al., 1996) using a Sievers analyzer (Sievers 280 NOA, Sievers, Boulder, CO, USA).

2.7. Light microscopy

Histological sections (3 μM) were stained with Masson's trichrome and analyzed by a light microscope. The incidence of segmental glomerulosclerosis was determined by scoring 50 glomeruli in a section of each kidney. The scores reflected changes in the extension of the lesion (0=0–5%, 1=5–25%, 2=25–50%, 3=50–75%, and 4= > 75%). Tubulointerstitial injury in the cortical area was defined as inflammatory cell infiltrates, tubular dilation and/or atrophy or interstitial fibrosis. Injury was graded on a scale of 0–4: 0=normal, 0.5=focal and discrete changes, 1=changes involving less than 10% of the renal cortex, 2=involvement of 10–25% of cortex, 3=involvement of 25–75% of cortex and 4=extensive damage involving more than 75% of the renal cortex.

2.8. Immunhistochemical analysis

The sections were incubated at 4 °C overnight with 1/30 anti p-JNK monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA), 1/200 anti-AII polyclonal antibody (Peninsula Laboratories, San Carlos, USA) or 1/500 anti-Arginase I polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or for 1 h with 1/1000 anti-ED1 monoclonal antibody that only reacts with cytoplasmic antigen present in macrophages and monocytes (Serotec, Oxford, UK) and 1/500 anti-vimentin monoclonal antibody (Dako, Glostrup, Denmark). The reaction product was detected with an avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, California, USA). Negative controls were created by replacing the primary antibody with normal mouse immunoglobulin G (IgG) or rabbit IgG for monoclonal or polyclonal antibodies, respectively, at equivalent concentrations. The color reaction was developed with immunoglobulin G (Sigma Chemical Company, St. Louis, Missouri, USA). The sections were counterstained with methyl green or with Harris hematoxylin, dehydrated and mounted. Scores for p-JNK and Arginase I (ARG 1) were obtained through of the analize of 50 glomeruli and 30 fields in the tubulointerstitial area (measuring 0.200 mm^2), and the mean score per kidney was calculated. Vimentin was used as a marker of tubular cell lesions and was assessed only in the tubulointerstitial area (Coimbra et al., 2000). The scores mainly reflected changes in the extent, rather than the intensity, of staining and depended on the percentage of glomeruli or grid field showing positive staining: 0=0–5%, 1=5–25%, 2=25–50%, 3=50–75% and 4= > 75%. The number of ED1 (macrophages/monocytes)-positive cells (glomeruli and renal cortical tubulointerstitium) and of AII-positive cells (cortical tubulointerstitium) were counted through the examination of 50 glomeruli and 30 grid fields (measuring 0.200 mm^2), respectively, and the mean counts per kidney were calculated.

2.9. Arginase inhibition assay

Rat liver arginase was prepared by blending 30 g of tissue with 300 mL of 100 mM Tris–HCl buffer (pH 7.4) containing 1 mM PMSF. The homogenate was centrifuged at 20,000*g*, and the supernatant was fully activated by incubation with 10 mM $MnSO_4$ at 60 °C for 10 min. The extract was newly centrifuged at 20,000*g* for 10 min, and the supernatant was submitted to chromatography on a Chelating Sepharose® (GE Healthcare) column charged with Ni^{+2} to remove blood pigments. The eluate was then used to test the inhibition of arginase in vitro. Renal cortex arginase was prepared by homogenizing 0.2 g of tissue with 500 μL of 100 mM Tris–HCl buffer (pH 7.4) containing 1 mM PMSF, centrifuged at 20,000*g* for 10 min. The supernatant was fully activated with 10 mM $MnSO_4$ at 60 °C for 10 min. The arginase activity was evaluated in reactions performed in 50 mM CHES buffer (pH 9.5) and 50 mM L-arginine (pH 9.5) and arginase enzyme in a final reaction volume of 100 μL. The reaction mixtures were incubated at 37 °C for 15 min and 60 min, respectively for liver and renal arginase samples. In the inhibition test in vitro, FCP or orientin (Sigma-Aldrich) was added to the reaction mixture to a final concentration of 0.33 mg/mL. The control experiments were performed under the same conditions but in the absence of the inhibitor. All arginase assays were performed in duplicate in two independent experiments. Arginase activity was measured by urea production determined by the Berthelot method. The activity of arginase in the renal cortex was determined by the amount of urea formed in nmol/mg of protein.

2.10. ACE inhibition assay

The catalytic activity of ACE was determined by the fluorimetric method assay (Friedland and Silverstein, 1976). The renal cortex (1 g) was homogenized with 10 mL of 100 mM borate buffer (pH 7.2) containing 340 mM sucrose, 300 mM NaCl and 100 mM PMSF. The homogenate was centrifuged at 5000*g*, 4 °C for 10 min and the supernatant was conditioned at −70 °C until analysis. An aliquot of 10 μL plasma or renal homogenate was incubated with 200 μL of assay solution (1 mM Z–Phe–His–Leu, 100 mM sodium borate buffer, pH 8.3, 300 mM NaCl and 0.1 mM $ZnSO_4$) for 10 min at 37 °C. The reaction was stopped by adding 1.5 mL of 280 mM NaOH. Then, 100 μL of o-phtaldialdehyde at 20 mg/mL diluted in methanol was added to the reaction, and after 10 min the reaction was acidified with 200 μl of 3 N HCl. The mixture was centrifuged at 3000*g* for 10 min at room temperate. The HL (His–Leu) peptide released was measured by fluorimetry (360 nm of excitation and 465 nm of emission) in a Tecan infinity F200 system (Oliveira et al., 2000). The median values of the renal and plasmatic ACE activity were expressed in nmol/mg of protein and mU/mL of HL produced, respectively. The ACE activity assay was performed in vitro replacing the tissue with rabbit lung ACE (EC 3.4.15.1, Sigma-Aldrich) in the presence of the 10 μM captopril or 0.33 mg/mL FCP. The captopril and FCP were pre-incubated with rabbit lung ACE for 30 min at 37 °C. ACE activity in the presence of inhibitors was expressed in mU/mL of produced HL.

Table 1
Systolic blood pressure (SBP), urinary albumin excretion (AE) and body weight (BW) in rats from the control group (S) and in rats subjected to 5/6 NE, untreated (NE) or treated (NE+FCP) with FCP 15–60 days after surgery.

Days (d) after surgery	15 d	30 d	60 d
SBP, mmHg			
S	134 ± 12	125 ± 6	131 ± 4
NE	182 ± 9*	190 ± 11**	176 ± 14*
NE+FCP	172 ± 11*	160 ± 12*,****	166 ± 14
AE, mg/24 h			
S	0.28 (0.15; 0,72)	0.34 (0.01; 0,72)	0.51 (0,33; 0,63)
NE	59.20 (30.78; 79.84)*	104.1 (23.75; 188.8)**	126.8 (48.75; 213.0)**
NE+FCP	23.80 (17.80; 106.0)*	20.00 (10.20; 121.9)	60.60 (24.33; 154.5)
BW, g			
S	330 (315; 335)	460 (449; 483)	516 (470; 647)
NE	300 (276; 314)	404 (359; 441)	501 (458; 552)
NE+FCP	282 (256; 405)*	393 (367; 434)	432 (408; 517)

Data are presented as means ± SEM (SBP) and median values and quartiles (25; 75%) (AE and BW).

* $p < 0.05$.
** $p < 0.01$ vs S.
**** $p < 0,05$ vs NE.

2.11. Statistical analysis

One-way analysis of variance (ANOVA) with Newman–Keuls was applied for the data that presented normal distribution and homogeneity of variance (SBP and arginase activity in vitro). Those data were expressed as the means and standard errors of the mean (SEM). For data related to other studied parameters, we used a nonparametric Kruskall–Wallis test with Dunn's post-test. Those data were expressed as the median values and interquartile. Statistical analyses were performed using Graph Pad Prism version 5.0 for Windows, Graph Pad Software, San Diego, California, USA. The level of statistical significance was set at $p < 0.05$.

3. Results

3.1. Determination of the FCP extract dose and body weight (BW)

The daily dose of FCP consumed by the treated nephrectomized group (NE+FCP) was 0.50 ± 0.02 g/kg. The body weight (BW) did not differ between the groups except before treatment when the NE+FCP rats presented lower BW when compared to the control group S (Table 1).

3.2. Characterization of the active constituents from C. pachystachya

In an attempt to identify the major constituents of FCP, we performed LC–MS analysis using standard flavonoids (Sigma-Aldrich). The compound orientin was identified by RT and UV/VIS spectra that matched with the authentic sample.

3.3. Determination of the systolic blood pressure (SBP), albuminuria (AE) and glomerular filtration rate (GFR)

The untreated rats subjected to 5/6 NE (NE group) presented an increase in the systolic blood pressure (SBP) and albuminuria (AE) when compared to the control group S. However, there was a significantly reduction in the SBP in the NE+FCP group when compared with NE group after 15 days of treatment with FCP, which corresponds to 30 days after surgery (Table 1) ($p < 0.05$). The increase of SBP and AE was also attenuated by treatment with FCP 60 days after surgery (Table 1). The glomerular filtration rate (GFR) was 0.43 mL/min/100 g (median value) in the control group and fell to 0.17 mL/min/100 g in the untreated NE group ($p < 0.01$), representing a fall of 60.5% in the GRF in comparison to control group. The decrease of the GFR was less intense in the treated NE+FCP group, in which a reduction of 46.5% was observed (0.23 mL/min/100 g) (Fig. 1).

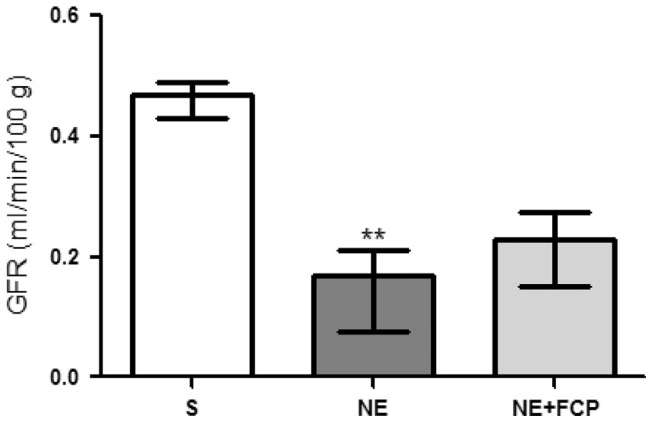

Fig. 1. Glomerular filtration rate (GRF) of rats from control group (S) and nephrectomized groups treated (NE+FCP) and untreated (NE) with FCP, 60 days after surgery. The bars represent median values and interquartiles (25–75%). **$p < 0.01$. Groups with $n = 5$.

3.4. Quantification of TGF-β, MCP-1 and NO in urine

There was an increase of the urinary rate of TGF-β in the NE group in comparison to control group S ($p < 0.01$). This increase was also less intense in the NE+FCP group (Table 2). The urinary excretion of the MCP-1 and NO did not differ between groups (Table 2).

3.5. Light microscopy

The rats subjected to 5/6 NE presented morphological features that are characteristic of this model: glomerulosclerosis, tubular lumen dilation with flattening of proximal tubular cells, tubular atrophy and increased interstitial area with inflammatory cell infiltrates [Fig. 2(a)–(c)]. These histological changes were less intense in the NE+FCP group [Fig. 2(a)–(c) and (g)–(h)].

3.6. Immunohistochemical analysis

The expression of the vimentin protein in the tubulointerstitial area was higher in the renal cortex of the rats subjected to 5/6 NE, and this increase was attenuated by treatment with FCP [Fig. 2(d)–(f) and (i)]. The increased p-JNK expression in the renal cortex

tubulointerstitium and increased ED-1 expression in the glomeruli observed in the nephrectomized rats were also lower in the NE+FCP rats (Fig. 3). The number of the AII positive cells in the tubulointerstitium did not differ significantly between the groups ($p > 0.05$) [Fig. 4(a)–(c) and (g)]. Arginase 1 (ARG 1) expression was more intense in the glomeruli of rats from untreated NE group when compared with control group S ($p < 0.05$), and the treatment with FCP also attenuated the ARG 1 expression in the glomeruli [Fig. 4(d)–(f) and (i)].

Table 2
Transforming growth factor β (TGF-β), monocyte chemoattractant protein-1 (MCP-1) and nitric oxide (NO) urinary excretion in the control group (S) and rats subjected to 5/6 NE, untreated (NE) or treated (NE+FCP) with FCP 60 days after surgery.

	TGF-β(pg/mg urinary creatinine)	MCP-1(pg/mg urinary creatinine)	NO(μmol/mg urinary creatinine)
S	127 (66; 148)	1740 (1110; 2432)	0.94 (0.77; 1.35)
NE	4888 (2425; 5788)**	1305 (551; 14160)	1.08 (0.71; 1.22)
NE+FCP	1946 (500; 2454)	1554 (584; 5541)	1.00 (0.44; 1.52)

Data are presented as median values and interquartiles (25; 75%).
** $p < 0.01$ vs S.

3.7. ACE and ARG activity

A tendency of increased ACE activity in the renal cortex of the untreated NE groups was observed. However, the treatment with FCP did not interfere with ACE activity in the renal tissue and plasma (Table 3). The FCP fraction did not inhibit ACE activity in vitro at the concentration of 0.33 g/mL (Fig. 5(a)), while methanolic extracts obtained from *Cecropia pachystachya* leaves at 0.33 g/mL showed 42% of ACE inhibition (Lacaille-Dubois and Wagner, 2001). On the other hand, the arginase activity was increased in the renal cortex in the untreated NE group when compared to control group S ($p < 0.01$), and this increase was prevented by treatment with FCP (Table 3). We observed also that ARG 1 activity in vitro was inhibited in 45% by FCP fraction (Fig. 5(b)). ARG 1 inhibition should be related to other compounds present in FCP that were not identified because the orientin flavonoid was not able to inhibit the enzyme in vitro (Fig. 5(b)).

4. Discussion

The results of this study showed that treatment with *Cecropia pachystachya* fraction (FCP) reduced hypertension and inflammation, as well the functional and structural renal changes in 5/6 NE rats. Our results corroborated with those of others studies that have shown the anti-hypertensive activity of the plants of the *Cecropia* genus (Lima-Landman et al., 2007; Maquiaveli et al., 2014).

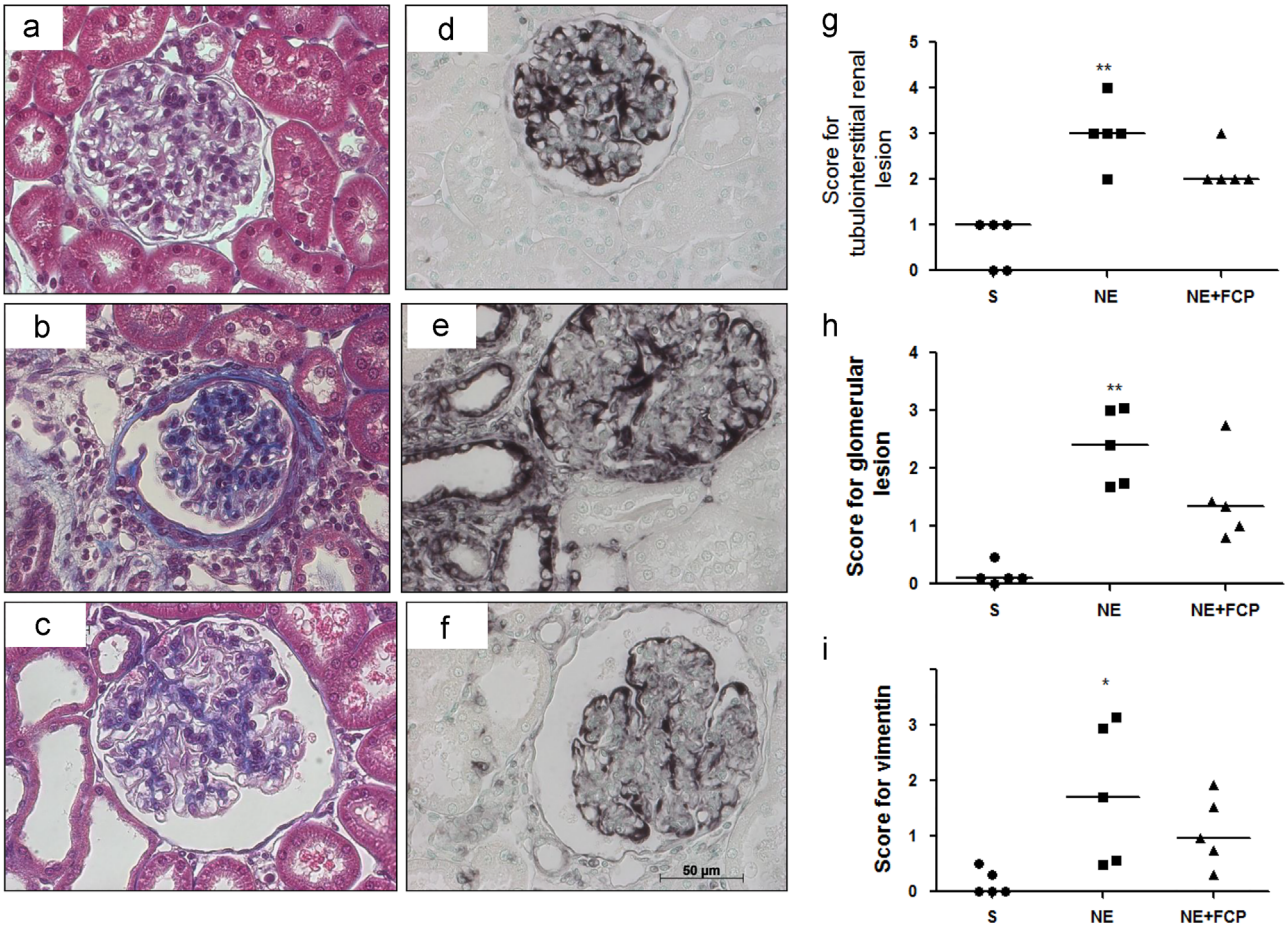

Fig. 2. Representative Masson's trichrome-stained histological sections of renal cortex (a–c) and vimentin staining (d–f) of rats from (a, d) control, (b, e) 5/6 nephrectomy (NE) and (c, f) NE+FCP groups 60 days after surgery. Note that glomerular sclerosis, interstitial lesions and the expression of vimentin in tubule cells in (b) and (e) are more intense than (c) and (f), respectively. Bars represent 50 μm. Score for tubulointerstitial lesion (g) and glomerular sclerosis (h), and vimentin (i) in the renal cortical tubulointerstitium per area of renal cortex measuring 0.200 mm^2 in control rats (S) and rats subjected to 5/6 NE treated (NE+FCP) or untreated (NE) with FCP 60 days after surgery. Horizontal lines represent the median values.*$p < 0.05$, **$p < 0.01$ vs S. Groups with $n = 5$.

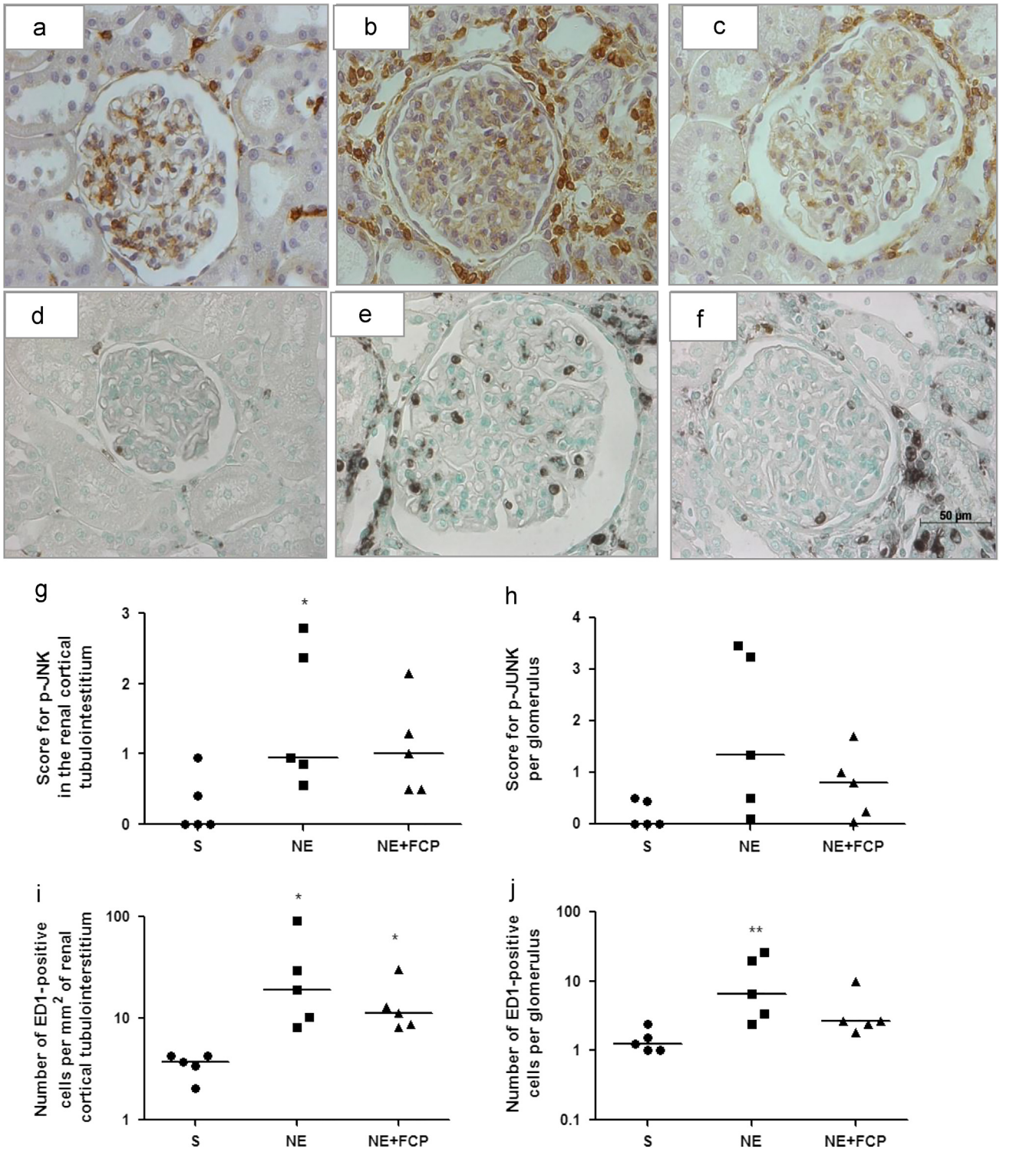

Fig. 3. Representative immunostaining in the renal cortex for (a–c) p-JNK and (d–f) ED1-positive cells (macrophages) from rats from control (a, d), NE (b, e) and NE+FCP (c, f) groups, 60 days after surgery. Note that the expression of p-JNK in the tubular and interstitial cells and number of ED1-positive cells in glomerulus are higher in (b) and (e) than in (c) and (f). Bars represent 50 μm. Score for p-JNK (g, h) and number of ED1-positive cells (i, j) in the renal tubulointerstitium (g, i) and glomerulus (h, j), in control rats (S) and rats subjected to 5/6 NE treated (NE+FCP) or untreated (NE) with FCP 60 days after surgery. Horizontal lines represent median values. $^{*}p < 0.05$, $^{**}p < 0.01$ vs S. Groups with $n = 5$.

Albuminuria and hypertension are important risk factors for the loss of kidney function, and reductions in both have been associated with slower progression of the renal disease (Levey et al., 2005).

The reduction of the vimentin expression associated with the histological studies indicated that FCP can reduce glomerular and tubulointerstitial lesions in rats subjected to 5/6 NE. Vimentin is an important marker of recent tubular lesion because cells only express vimentin when they are proliferating (Coimbra et al., 2000).

Recently we showed that aqueous extract of *Cecropia pachystachya* (CP extract) reduced renal inflammation in the rats subjected to 5/6 NE via reduction of p-JNK expression and macrophage recruitment. These effects were associated with presence of chlorogenic acid in the

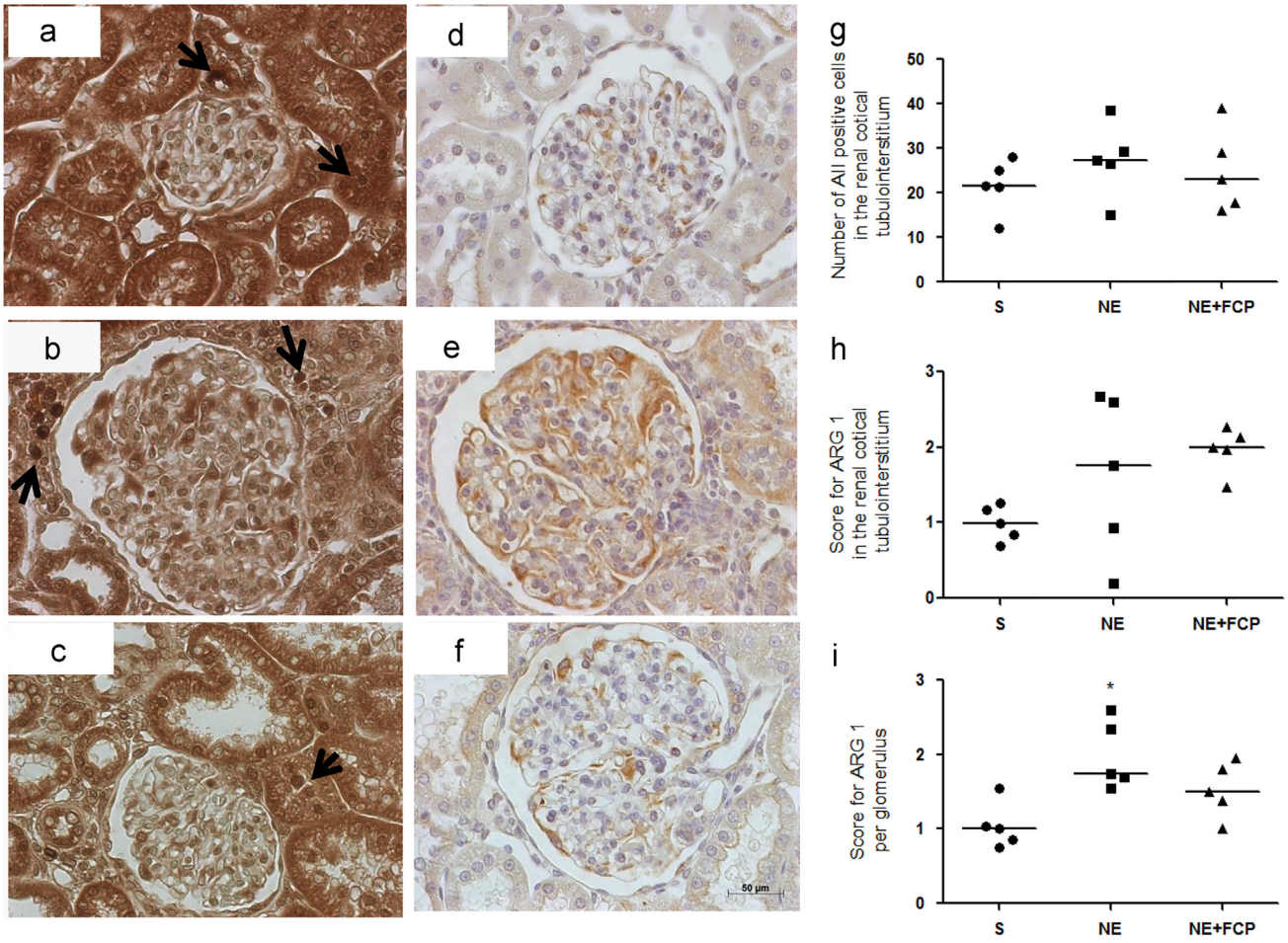

Fig. 4. Representative immunostaining cells positive for angiotensin II (AII) in the renal cortex (a–c) and for cells positive for arginase 1 in the glomerulus (ARG 1) (d–f) of rats from control (a, d), NE (b, e) and NE+FCP (c, f) groups 60 days after surgery. Note that the expression ARG 1 in the glomerulus is more intense in (e) than in (f). Bars represent 50 µm. The number of cells positive AII (g) and ARG 1 in the renal tubulointerstitium (h) and glomerulus (i) in control rats (S) and rats subjected to 5/6 NE treated (NE+FCP) or untreated (NE) with FCP 60 days after surgery. Horizontal lines represent median values. *$p < 0.05$ vs S. Groups with $n=5$.

Table 3
Angiotensin converting enzyme (ACE) and renal arginase activity in the control groups (S) and rats subjected to 5/6 NE, untreated (NE) or treated (NE+FCP) with FCP 60 days after surgery.

	Renal ACE activity (nmol/mg of protein)	Plasma ACE activity (nmol/mg of protein)	Renal arginase activity (nmol/mg of protein)
S	0.59 (0.45; 1.02)	68.25 (45.85; 101.1)	160.4 (126.7; 183.7)
NE	1.01 (0.34; 1.54)	57.20 (49.30; 89.58)	236.5 (198.0; 509.0)**
NE+FCP	0.85 (0.34; 1.39)	79.30 (54.18; 86.90)	183.0 (136.8; 266.2)

Data are presented as median values and interquartiles (25; 75%).
The assays were performed in duplicate in two independent experiments.
** $p < 0.01$ vs S.

CP extract (Maquiaveli et al., 2014). In this study, we used the ethanolic extract from *Cecropia pachystachya* (FCP), which did not have chlorogenic acid. The main compound identified in FCP was the orientin flavonoid, which has been previously shown to have important anti-inflammatory effects (Aragão et al., 2013; Ku et al., 2014). Ku et al. (2014) verified that orientin attenuated vascular inflammation induced by high glucose (HG) via reduction of both nuclear factor Kappa B (NFk-B) and adhesion molecules (CAMs) expression, resulting in the reduction of the monocyte adhesion. Therefore, the presence of orientin FCP also could be associated with reduction of p-JNK expression and macrophage recruitment in the renal cortex. This result supports anti-inflammatory effect by the FCP. MAPK c-Jun N-terminal Kinase (JNK) pathway has been implicated in apoptosis, inflammation and synthesis of inflammatory mediators (Maquiaveli et al., 2014). The use of the *Cecropia pachystachya* leaves by Brazilian people in renal diseases (Alves and Povh, 2013; Bessa et al., 2013), in part, could be explicated by reduction of the inflammation. The anti-inflammatory effect of *Cecropia pachystachya* also was demonstrated in other experimental model of inflammation (Aragão et al., 2013).

Macrophages and renal tubular cells producing the TGF-β during the inflammatory process induced by 5/6 NE. TGF-β is one of the major fibrogenic cytokines and enhances the synthesis of matrix components and blocks matrix degradation, thereby promoting extracellular matrix (ECM) accumulation. Therefore, the protective effect of the treatment with FCP in reducing interstitial and glomerular fibrosis may be related a decreased renal production of this fibrogenic cytokine. The increase of TGF-B is related with increase of the RAAS activity in this renal failure model. Previously, we showed that CP extract reduced ACE activity and AII expression as well as TGF-β production, in the renal cortex of rats subjected to 5/6 NE, 90 days after nephrectomy (Maquiaveli et al., 2014). However, in the present study we verified that AII expression and ACE activity did not differ between control and nephrectomized groups, 60 days after surgical procedure. Therefore, the treatment with FCP attenuated TGF-β production, inflammation, and renal lesion independent of changes in the ACE activity.

On the other hand, we verified that arginase 1 expression in the glomerulus and renal arginase activity were decreased in the neprectomized rats treated with FCP. ARG 1 is higher in the liver

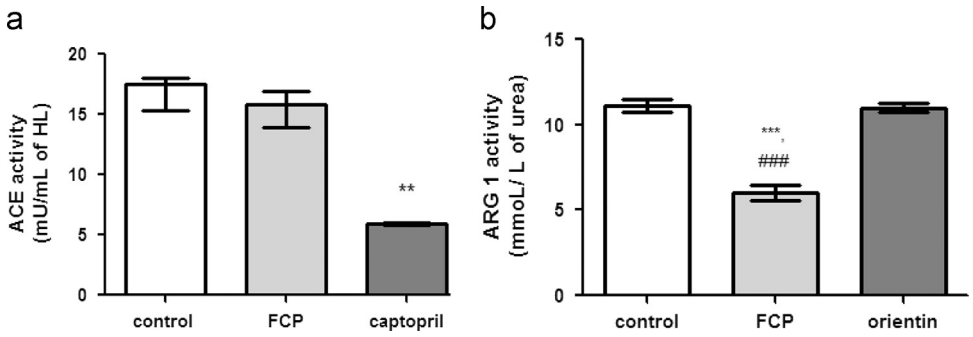

Fig. 5. (a) Angiotensin converting enzyme (ACE) activity in vitro in the presence of the inhibitors captopril (10 μM) and the aqueous fraction of the ethanolic extract of *Cecropia pachystachya* (FCP) at 0.33 mg/mL. The positive control of the reaction was performed with ACE in the presence of substrate (Z–Phe–His–Leu) without inhibitor. The median values of the ACE activity were expressed as mU/mL of HL (His–Leu) produced. (b) Arginase 1 (ARG 1) activity in vitro in the presence of FCP and orientin, both at 0.33 mg/mL. The positive control of the reaction was performed with ARG 1 in the presence of the L-arginine substrate. The mean values of ARG 1 activity were expressed as mmoL/L of urea formed. The bars represent the median values and interquartiles for the ACE activity (a) and means ± SEM for the ARG 1 activity (b). The assays were performed in duplicate in three independents experiments.

and arginase 2 (ARG 2) is more abundant in the kidney (Schnorr et al., 2008). However, after the development of hypertension, increases the expression of ARG 1 and ARG 2 and L-arginine transporters in afferent arterioles are observed (Hultström et al., 2009). In inflammatory diseases, ARG 1 expression increases in macrophages (Schnorr et al., 2008). Therefore, arginase inhibition may offer an alternative target to treat endothelial dysfunction in hypertension (Michell et al., 2011) and renal failure (Sabbatini et al., 2003). ARG 1 expression is also induced in cardiac disease, and its inhibition regulates blood pressure (Bagnost et al., 2008) and mediates cardioprotection during ischemia-reperfusion (Jung et al., 2010).

In children with progressive renal disease, a significant increase in the levels of assimetric dimethylarginine (ADMA) and simetric dimethylarginine (SDMA), analogous of the L-arginine that blocks eNOS activity by competitive inhibition, was observed (Kari et al., 1997; Vallance et al., 1992). Plasma levels of L-arginine were also decreased in these patients. Other molecules that accumulate in uremia such as IL-8 also inhibit NOS (Vallance et al., 1992). Despite the fact that urinary excretion of NO did not change in this experiment, the reduced expression of ARG 1 in the glomerulus may be an indication that *Cecropia pachystachya* components could be interfering with the metabolism of the amino acid L-arginine. TGF-β has been shown to increase the expression of the arginase in macrophages (Boutard et al., 1995). The reduction of renal arginase activity could have occurred by reduction in expression of the arginase enzyme due to reduced production of TGF-β. For the first time we verified that FCP extract from *Cecropia pachystachya* reduced ARG 1 expression and ARG activity in the renal cortex of 5/6 NE rats, which may have contributed to the reduction renal inflammation and fibrosis. In addition we showed for the first time that FCP inhibited ARG 1 in vitro. The production of L-ornithine by arginase is the first step in the polyamine pathway, and L-ornithine is also a substrate for proline biosynthesis that is involved in cell differentiation and the synthesis of collagens (Reyes et al., 1994). Therefore, the reduction in the activity/expression of renal arginase and the synthesis of renal TGF-β may contribute to the reduction of the glomerular and tubulointerstitial fibrosis observed in the treated neprectomized rats with FCP extract.

5. Conclusion

The FCP extract from *Cecropia pachystachya* reduced inflammation and renal arginase activity in rats subjected to 5/6 NE. These results suggest that reduction of renal arginase activity, p-JNK and TGF-β expression can explain the mechanism by which this plant to reduce inflammation and renal lesion. These mechanisms not are only associated with the reduction of the ACE activity. *Cecropia pachystachya* extract can act through multiple mechanisms to improve renal function, including decreases in ARG activity and expression, as well as through anti-inflammatory effects.

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