Cyclocarya paliurus extract alleviates diabetic nephropathy by inhibiting oxidative stress and aldose reductase

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ABSTRACT
Diabetes is the leading cause of end-stage renal disease because diabetic nephropathy (DN) develops in 30–40% of the patients. This study investigated the protective effect of the aqueous extract from leaves of Cyclocarya paliurus (Batal.) Iljinsk (ACP) on DN by inhibiting oxidative stress and aldose reductase (AR) activity. ACP was obtained by hot water extraction. The in vitro antioxidant capability and AR inhibition of ACP were investigated by employing various established systems. DN rats were used to assess the reno-protective effect of ACP. Results showed that the polysaccharide and total polyphenol contents of ACP were (479.3 ± 19.8) mg/g and (38.3 ± 2.3) mg/g, respectively. ACP exhibited strong antioxidant ability and AR inhibition in vitro and in vivo; furthermore, the inhibition mechanism of ACP in AR takes the form of uncompetitive inhibition. In addition, the animals treated with ACP showed significant amelioration of blood glucose, serum biomarkers related to renal function, urinary protein excretion, and histopathological changes in the kidney. The results suggest that ACP has a potential role in ameliorating renal damage involved in DN.

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Introduction
Diabetic nephropathy (DN) is a clinical syndrome in patients with diabetes mellitus (DM) that is characterized by persistent hyperglycemia, worsening proteinuria, hypertension, and progressive renal failure. The blood glucose levels of a diabetic can be controlled within the normal range using insulin and other oral antihyperglycemic agents, but DN develops in 30–40% of the patients.1 With the rapidly rising number of DM worldwide, DN is becoming one of the leading causes of end-stage renal disease (ESRD). The pathogenesis of DN is complex, and an increasing body of evidence shows that oxidative stress (OS) has a pivotal role in the development and progression of DN.2 The increased superoxide productions caused by the metabolic abnormalities of DM are the central and major mediators of microvascular injury in DN, and they trigger the activation of numerous pathways involved in the pathogenesis of DN, such as the polyol pathway (PP), one of the vital pathways of glucose metabolism in organisms.3,4 Aldose reductase (AR) is the key rate-limiting enzyme of PP, that is, AR inhibitors can inhibit AR activity and interfere with PP metabolism and therefore interfere with the occurrence and development of DN. Studies on OS and AR inhibition will contribute to the prevention and therapy of DN.5–7

Many chemicals work with some or other pathway in the pathogenesis of DN and are characterized by weak specificity and considerable side effect. With respect to OS, traditional herbal medicines show particular superiority and features, such as Toona sinensis extract, Ginkgo biloba extract, Pueraer panicum., which have been proven to intervene in the OS injury in DN rats.8–10 Cyclocarya paliurus (Batal.) Iljinsk (abbreviated as CP), commonly known as “sweet tea tree,” is a kind of medicinal herb that is widely used in China to treat DM. In addition, CP antihyperglycemic herbal tea has been approved by the United States Food and Drug Administration (FDA) and is the first health tea from China certificated by FDA.11 Many studies have demonstrated that CP possesses a variety of bioactivities, including antihypertensive, hypoglycemic, hypolipidemic, and antioxidant activity.12,13 However, no report is available on the effects of CP as an AR inhibitor, and no extensive research has been conducted on the effect of CP on DN. In this study, we investigate the antioxidant activity and AR inhibition of the aqueous extract of leaves of CP (ACP) in vitro and in vivo. Furthermore, the mechanism of the AR inhibition is
analyzed, and the reno-protective effect of ACP is manifested in DN model rats. We also measure the polysaccharide and total polyphenol contents of ACP because they are the major bioactive substances.14

Materials and methods
Chemicals
Epalrestat (standard, content >99.4%, National Institutes for Food and Drug Control, China), NADPH and DL-glyceraldehyde (Shanghai Yuanye Bio-technology Co., Ltd. China), STZ (Sigma Co., St. Louis, MO), rat insulin ELISA kit (EMD Millipore Co., Billerica, MA), rat IL-6 and endothelin ELISA kit (NEO Bioscience Technology, Beijing, China), and antioxidant capacity assay kits used in the study were all purchased from Jiancheng Bioengineering Institute, Nanjing, China. All the other chemicals and reagents used were of analytical grade.

Plant material and preparation of ACP
Cyclocarya paliurus (Batal.) (CP) leaves were collected in Zhangjiajie, Hunan province, China and authenticated by Dr. Chongmei Xu of the Department of Pharmacognosy of Weifang Medical University, China. A voucher specimen (No. 20131102) was deposited in the herbarium of the university. The air-dried leaves were extracted with 20 parts of pure water (2/2 h), and the ACP was used in the research. The extracts were immediately dissolved in ultra pure water before the experiments.

Animals
Male Wistar albino rats weighing between 180–220 g and New Zealand white rabbits were all provided by the Weifang Medical Experimental Animal Center and were bred in a standard animal facility. The animals were kept under controlled conditions and fed with standard pellet diet and water ad libitum. All the experiments on the animals were conducted in accordance with and were approved by the Institution Animal Ethics Committees of Weifang Medical University.

Determination of polysaccharide and total polyphenol contents
The polysaccharide contents were determined using the phenol-sulfuric acid colorimetry method with D-glucose as standard, and the total polyphenol contents were detected using the Folin-phenol colorimetry method with gallic acid as standard.

In vitro antioxidant activity of ACP
Determination of total antioxidant capability
The total antioxidant capability (T-AOC) assay kit and microplates were used for quantitative assay. Ascorbic acid was used for positive control. T-AOC of ACP was calculated by the following equation derived from the kit’s description:

\[ T - \text{AOC (IU/ml)} = \frac{[\text{A}_{\text{sample}} - \text{A}_{\text{control}}]}{\text{V}_{\text{bulk}}} \times \text{V}_{\text{sample}} \]

Determination of DPPH radical scavenging
The DPPH radical scavenging assay was conducted by means of an improved method.15 Briefly, ACP with different concentrations were mixed with the same volume of DPPH solution (0.2 mM, in absolute alcohol), and the mixtures were placed away from light for 15 min. The absorbance was detected immediately at 515 nm.

Determination of capability of scavenging superoxide anion and hydroxyl radical
Anti-superoxide anion and hydroxyl radical kits were used with ascorbic acid as standard to determine the ACP’s capability of scavenging radicals.

Inhibitory effect of ACP on AR
Preparation of AR
The rabbits were anesthetized, and the crystalline lenses were separated. Two lenses were cut in a centrifuge tube, and 1 ml of pre-cooled distilled water was added. They were sonicated in an ice bath (200 w, 9 s x 6) to homogenize them and then centrifuged at 4 °C (17,465 g x 30 min). The colorless and transparent supernatant was taken and placed at 4 °C for usage within 24 h.

Determination of the inhibitory effect of ACP and PCP on AR
A 20 mg/mL solution of ACP was precipitated three times as its volume of 95% ethanol. After filtering and freeze drying, the crude polysaccharide of CP (abbreviated as PCP) was obtained.

The reaction was conducted in a 96-well ELISA plate, and three repeated wells were set for each group with epalrestat (EP) as positive control.16,17 ACP and PCP were dissolved with ultra pure water at different concentrations. The blank reaction consisted of 10 μl of enzyme
preparation. The standard reaction and the control consisted of 80 μl of 0.16 mM NADPH and 10 mM DL-glyceraldehyde (substrate, dissolved by NADPH), respectively. The test sample consisted of all the components of the control along with 10 μl of different concentrations of sample solutions, individually. The blank sample was used to correct the absorbance of the sample solutions, and the final volume was made up of 100 μl with sodium phosphate buffer (pH 6.4). The BioTek microplate reader (Gene Co., Ltd., Chai Wan, Hong Kong) monitored the absorbance of NADPH at 340 nm for 20 successive min. The enzyme activity at 60% to 70% time point was taken to calculate the AR inhibition rate of the samples. The formulas used are as follows.

\[
\text{Enzyme activity (\%)} = \left(\frac{A_{\text{standard}} - A_{\text{control}}}{A_{\text{standard}} - A_{\text{blank}}}\right) \times 100\%
\]

\[
\text{Inhibition rate (\%)} = \left(\frac{(A_{\text{sample}} - A_{\text{blank sample}} - (A_{\text{control}} - A_{\text{blank}}))}{A_{\text{standard}} - A_{\text{control}}}\right) \times 100\%
\]

**Determination of inhibition type on AR**

Based on the method above, the concentrations of the substrate dl-glyceraldehyde were 2.0, 2.5, 3.3, 5.0, and 10 mM. A decrease in NADPH absorbance was recorded at every minute. A double-reciprocal plot was used to determine the inhibition type, and the Michaelis–Menten equation was used to calculate the kinetic constants of the inhibitory activity on AR.

**In vivo experiments**

Ten Wistar rats were selected as the normal control group (group 1). The remaining rats were fed with a high-fat diet (HFD) for 6 weeks and were injected intraperitoneally with a dose of 35 mg/kg streptozotocin (STZ) in citrate buffer (pH = 4.5). Blood was drawn from the tail vein after 72 h to measure the blood glucose levels. The animals with blood glucose fewer than 10 mM were subjected to fasting and injected again. After a period of five weeks, the rats with blood glucose >16.7 mM, urine output >1.5 times as much as the original amount, and urinary albumin excretion rate >20μg/min were used for study. The animals were then divided into the following six groups according to their blood glucose gradient: Group 2 (20 rats): DN model; Groups 3 and 4 (12 rats each): DN rats treated with ACP (0.047 and 0.094 g/kg/day, respectively; the dose used was determined by the conversion ratio of rats to human and the yield of ACP); and Group 5 (12 rats): DN rats treated with ascorbic acid (0.075 g/kg/day). The rats in Groups 1 and 2 were treated with an equal volume of distilled water. Each group was administered by gavage once a day every evening for 8 weeks. The general status, body weight, food intake, blood glucose, urine output, and urine protein excretion were determined regularly, and a metabolic cage was used to collect urine within 24 h.

At the end of the treatment, all the rats were anesthetized, and the serum was separated for biochemical and anti-oxidative analysis. An automatic biochemistry analyzer was used as an auxiliary measuring instrument to detect blood glucose (BG), serum creatinine (SCR), blood urea nitrogen (BUN), and 24 h urine protein (Upro), urine glucose (UG), urine creatinine (UCr), cystatin C(CysC), α1 microglobulin(AMG) levels. Rat insulin, endothelin, and IL-6 ELISA kits were used to determine the serum insulin (SI), endothelin-1 (ET-1), and interleukin-6 (IL-6). Several OS indicators, such as catalase (CAT), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), malondialdehyde (MDA), and T-AOC, were detected with commercial kits. The kidneys were collected and weighed. A piece of cortex renis was frozen immediately in liquid nitrogen and stored at −80°C until analysis, and the rest of the kidneys were preserved in 40% paraformaldehyde. The renal index (RI) was calculated with the following equation: RI (%) = [kidney weight (g)/body weight (g)] × 100%.

**Statistical analysis**

The experimental results were subjected to variance analysis using SPSS version 16.0 (SPSS Inc., Chicago, IL) and expressed as mean ± SD.

**Results**

**Determination of polysaccharide and total polyphenol contents**

The yield of ACP was 4.98%. The polysaccharide and total polyphenol contents of ACP were (479.3 ± 19.8) mg/g and (38.3 ± 2.3) mg/g, respectively.

**In vitro antioxidant activity of ACP**

ACP showed a property to scavenge DPPH, superoxide anion, and hydroxyl radicals in varying degrees. The IC<sub>50</sub> of ACP and ascorbic acid on three radicals are listed in Table 1, and the T-AOC of ACP at different concentrations is shown in Figure 1. The results showed that ACP had good antioxidant activity and could well scavenge DPPH and hydroxyl radicals.
Inhibitory effect of ACP on AR

Determination of rabbit lens AR activity

NADPH has a single absorption peak at 340 nm. The reaction of AR to the substrate consumes NADPH, thereby decreasing the absorption at 340 nm. Figure 2 shows that the absorbance was unchanged in the standard group because no substrate was added and that it declined in the control group. However, EP exhibited a significant inhibition on AR, which demonstrated that the reaction system was stable and reliable.

Inhibitory effect of ACP and PCP on AR

The results showed that ACP and PCP could inhibit AR in varying degrees and that the performance of ACP was remarkable with IC₅₀ = 0.13 mg/mL, which was significantly better than that of PCP (IC₅₀ = 1.13 mg/mL).

Inhibitory mechanism of ACP on AR

The results indicated that, as the concentration of the substrate increased, the reaction rate grew. The AR catalysis of the substrate decreased as the ACP concentration increased (r > 0.99), but the gradient remained unchanged, which suggested that the inhibitory effect of ACP on AR took the form of uncompetitive inhibition, that is, the inhibitors combined with the enzyme–substrate to inhibit the enzymatic reactions. The values of the Michaelis constant (Kₘ) and maximum reaction rate (Vₘₐₓ) are shown in Table 2. The Kₘ value indicated the high affinity of the enzyme substrate, and the decline in Kₘ when the inhibitor was added confirmed the uncompetitive inhibition.

General features of the experimental rats

Compared with those in the normal control, DN rats had tarnished fur and anabrotic tails with the following symptoms: polyphagia, polydipsia, polyuria-thickened urine smell, and other signs of DN. Their body weight decreased after a period of increase caused by the abundant nutrition of HFD. Treatment of ACP improved these general features and depressed their mortality rate compared with the DN group.

Effects of ACP on renal function

The results are summarized in Table 3. The increase in BG, decrease in SI, and other variations in blood and urine analysis indicated the failure of the renal function. The treated groups notably improved compared with the DN group in terms of these indicators. The effects of ACP were better than the positive control.

Effects of ACP on oxidative stress

The oxidative stress systems were determined as shown in Table 4. The variations in these OS indicators were found to be significant versus the blank control, which indicated OS in DN rats. In contrast, the OS injuries were
found to be significantly improved in the ACP- and ascorbic acid-treated groups versus in the DN group. The results suggested that the ACP might have effective antioxidative properties in vitro and in vivo.

Effects of ACP on renal AR activity

Kidney homogenate was centrifuged, and the supernatant was used for analysis. A regular reaction time was set, and the enzyme activity of the blank group was used as a reference of 100%. Figure 3 shows that the renal AR activity in DN rats was obviously enhanced compared with that in normal rats. This enhancement was markedly suppressed by ACP treatment.

Histopathological findings

Light microscopy of HE-stained sections showed the damage of the renal tissue in the DN group, which thickened much in the glomerular basement membrane, increased in the mesangial matrix material, and expanded in the mesangial. The above histopathological changes in the ACP treatment groups were attenuated in a dose-dependent manner (Figure 4).

Discussion

The STZ-induced renal pathophysiological changes and deteriorated functions that occur in animals under hyperglycemic conditions are very similar to those in

| Groups  | Normal control | DN model | ACP-L (0.047 g/kg/day) | ACP-H (0.094 g/kg/day) | Positive control |
|---------|----------------|----------|------------------------|------------------------|------------------|
| n       | 10             | 13       | 9                      | 11                     | 8                |
| BG (mM) | 3.8 ± 0.6##    | 20.1 ± 3.2*** | 7.2 ± 0.8#  | 4.2 ± 0.4#  | 10.34 ± 1.1#   |
| SL (mIU/l) | 104.69 ± 5.72## | 15.90 ± 1.21** | 18.46 ± 1.24 | 25.34 ± 1.49# | 17.04 ± 1.38 |
| SCR (µM) | 40.35 ± 4.47#  | 73.76 ± 7.42* | 60.14 ± 5.09 | 49.33 ± 4.88# | 68.52 ± 6.41 |
| BUN (mM) | 5.63 ± 0.43*   | 10.03 ± 1.07* | 8.87 ± 0.86# | 7.22 ± 0.85# | 8.88 ± 1.03#   |
| RI (%)  | 0.32 ± 0.02#   | 0.52 ± 0.05* | 0.42 ± 0.04# | 0.36 ± 0.04# | 0.41 ± 0.04# |
| UG (mM) | 12.07 ± 1.39## | 440.53 ± 56.90**  | 321.07 ± 30.87# | 271.35 ± 21.71## | 347.52 ± 28.69# |
| Uplo (mg) | 12.37 ± 1.85## | 40.11 ± 4.69*    | 20.45 ± 1.92# | 14.22 ± 1.28# | 30.45 ± 3.11   |
| UCr (mM) | 2.13 ± 0.20##  | 3.38 ± 0.26*     | 2.75 ± 0.29# | 2.36 ± 0.21# | 3.11 ± 0.31    |
| CysC (mg/L) | 0.24 ± 0.02#  | 0.37 ± 0.03*     | 0.33 ± 0.03 | 0.29 ± 0.02# | 0.33 ± 0.03    |
| α1-MG (mg/L) | 1.04 ± 0.01#  | 1.86 ± 0.02*     | 1.60 ± 0.01 | 1.00 ± 0.01# | 1.82 ± 0.02    |
| IL-6 (pg/ml) | 191.22 ± 9.87## | 376.78 ± 34.67*** | 298.66 ± 30.01# | 246.56 ± 23.44## | 369.00 ± 28.97 |
| ET-1 (ng/L) | 10.01 ± 0.89  | 27.85 ± 25.01**  | 22.61 ± 18.97# | 20.05 ± 18.88## | 29.04 ± 26.38  |

Notes: *p < 0.05 compared with the normal control group. **p < 0.01 compared with the normal control group. #p < 0.05 compared with the DN group. ##p < 0.01 compared with the DN group.
human DM. In this study, a second injection of STZ is given to the undesirable rats, and this approach has a success rate over 90% and is conducive to the extremely efficient use of experiment animals. A high level of blood glucose and low level of SI are observed in the experimental rats. The RI, BUN, SCr, and Upro all significantly increase in the DN group, together with the pathologic changes that indicate the formation of DN. Nevertheless, BUN and SCr concentrations are affected by many factors, so that there is strong evidence supporting the need for the early detection of DN when timely intervention can improve the long-term outcome.

Cys C is emerging as a better estimator of glomerular filtration rate than SCr. The increased levels of Cys C in diabetes has been reported as a possible early indicator of incipient DN, and even minimal glomerular damage results in a significant increase in serum Cys C. AMG is a glycoprotein in blood and has been studied as a sensitive marker for renal tubular dysfunction in various disorders. Furthermore, the link of the pro-inflammatory cytokines IL-6 and ET-1 to various diabetic complications has been demonstrated. A number of clinical studies have indicated a significant correlation between the serum concentration of ET-1 and the severity of albuminuria.

Animal studies have shown that ET-1 is not only more markedly expressed in the kidney of DM rats but can also mediate the effect of hyperglycemia on mesangial cell hypertrophy and synthesis of extracellular matrix proteins. IL-6 as a candidate cytokine has a major role in the beginning and progression of DN. Increased levels of IL-6 and other proteins are detected as early as stages 1–2 of the chronic kidney disease prior to significant kidney impairment. Clinical studies have also reported that serum levels of IL-6 are associated with hypertension and atherosclerosis in patients with early stages of type 2 DN. In this study, we find that several cytokines and biomarkers are significantly altered in experiment animals. Treatment with ACP decreases the blood glucose level and reduces the RI, BUN, SCr and Upro, as well as the Cys C, AMG levels and pro-inflammatory cytokines IL-6 and ET-1. These findings suggest that ACP can effectively ameliorate renal injury in DN rats and that the anti-inflammatory effect of ACP can be considered to be one of the renal protective mechanisms.

The T-AOC of a compound may serve as a significant indicator of its potential antioxidant activity, DPPH, and superoxide anion; hydroxyl radicals are all well known to be harmful to cellular components. The results show the antioxidant capability of ACP, although it is inferior to
ascorbic acid. Under normal physiological conditions, a balance exists in the generation of oxygen free radicals and antioxidant defense systems used by organisms to deactivate and protect themselves against free radical toxicity. The antioxidant defense system includes ROS degradation molecules, such as uric acid, ascorbic acid, glutathione, and antioxidant enzymes, such as CAT, GSH-Px and SOD. In addition, the increase in MDA content reflects the degree of lipid peroxides. Some authors have reported that the expression of redox enzyme decreases and the expression of oxidase increases in diabetic models. The expression of oxidative stress-related cytokines is suppressed with the intervention of polyphenols. In the present research, OS is observed in cytokines is suppressed with the intervention of polyphenols.

In the diabetic, who is affected by high glucose level in vivo, carbohydrate oxidation increases, and the PP and other pathways are activated. This in turn leads to the unbalanced oxidation and anti-oxidization of organisms and excessive active oxygen radicals, which in turn damage tissues and cells and finally result in DN and other DM complications. Influenced by sustained hyperglycemia, AR activity increases significantly and activates PP, which enables many metabolites, including sorbitol, to accumulate in cells, thereby affecting cellular structures and functions and finally triggering a series of pathological changes. In our present study, renal AR activity can be effectively reduced by ACP in vitro and in vivo, and this suggests that ACP can suppress PP and alleviate renal failure in DN. Furthermore, this research provides new insights for the investigation of the antioxidant and AR inhibition of natural herb medicine in preventing and treating of DN.

In fact, many synthetic ARIs have been developed as drug candidates, but most of them have been virtually unsuccessful in clinical trials because of adverse pharmacokinetic properties, inadequate efficacy, and toxic side effects. By contrast, ACP, as a natural product has the advantages of high efficiency and low toxicity. The results of ACP and PCP on AR indicate that PCP is not the major effective substance in ACP on AR. The presumption that some other constituents take effect in ACP should inspire us to conduct further research.

Conclusion

Iljinsk (ACP) shows significant antioxidant and AR inhibitory activity in vitro and in vivo. The protective effects of ACP on reducing STZ induce hyperglycemia, and the associated renal injury may be related to the effect of ACP on reducing oxidative stress as well as on suppressing the activation of the polyol pathway through AR inhibition. However, further investigations need to be conducted to justify the active constituents of ACP for DN treatment and to study its mechanism in depth.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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