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The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2021; 10(3): 884-889

www.thepharmajournal.com
Received: 12-01-2021
Accepted: 21-02-2021

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Antihyperlipidemic activity of isoflavones

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Abstract
Cyclocarya paliurus has been used commonly to treat diabetes in China. However, the effective components and the effect of plant origin remain unclear. In this study, C. paliurus leaves with different chemical compositions were selected from five geographical locations, and their effects on streptozotocin (STZ)-induced diabetic mice were evaluated with both ethanol and aqueous extracts. Glucose levels, lipid levels, and biomarkers of liver and kidney function were measured. Results showed that C. paliurus extracts with better antihyperglycemic effects were characterized by higher contents of total flavonoids, especially quercetin-3-O-glucuronide and kaempferol-3-O-glucuronide. Furthermore, significantly negative correlations were found between triterpenoids contents and lipid levels. The anti-hyperlidemic effect of flavone-rich B. chinensis leaf extract (HTP) in ICR mice fed a high-fat diet. Treatment with HTP significantly decreased peri-epididymal fat weight ($p< 0.01$ and $p< 0.05$ for 200 and 100 mg/kg, respectively), lowered serum and hepatic lipid, and decreased glucose area under curve (AUC) in oral glucose tolerance test ($p< 0.01$ for 200 mg/kg). Western blot and ELISA analysis showed that administration of HTP (200 mg/kg) significantly increased AMPK ($p< 0.05$) phosphorylation and PPARα expression in liver ($p< 0.05$).

Keywords: *Cyclocarya paliurus*, diabetes, flavonoids, triterpenoids, geographical locations, extraction solvents, *Belamcanda chinensis*, Flavone, Hyperlipidemia, Adenosine 5′-monophosphate-activated protein kinase, Peroxisome proliferator activated receptor-alpha, Glucose tolerance

Introduction
Cyclocarya paliurus (Batal) Iljinskaja belongs to the Juglandaceae family and is widely distributed in mountainous regions of sub-tropical China [8]. Leaves of this plant are traditionally used in China as an ingredient in nutraceutical tea or drug formulations for the treatment of hypertension, diabetes mellitus, and hyperliposis [9-12]. Previous studies have reported that C. paliurus leaves contain abundant bioactive components including polysaccharides, triterpenoids, flavonoids, and phenolic compounds [13-15]. Thus, chemical identification and the anti-diabetic effect of C. paliurus extracts have attracted the attention of many scholars, however the constituents responsible for anti-diabetic effect still remains controversial. For example, previous studies have revealed that the water extract of C. paliurus could reduce postprandial triglyceride level in hyperlipidemic mice, and predicted polysaccharides to be the active components [16, 17]. Polysaccharides of C. paliurus are also found to exhibit a lipid-lowering effect on high-fat-diet-induced rats [18, 19].
However, it is demonstrated that polysaccharides did not appear to be the active anti-diabetic constituent, based on the comparison of anti-diabetic effects of C. paliurus ethanol extract (without polysaccharides) and aqueous extract in streptozotocin (STZ)-induced diabetic rats [20]. In addition, several studies have shown that triterpenic acid-enriched C. paliurus fraction or extracts possess better antihyperlipidemic activities in mice fed with high-fat-diet [21-23]. Thus, further investigation of the anti-diabetic activities of C. paliurus extracts with detailed component information is required. To fill the knowledge gap mentioned above, in this study, C. paliurus leaves with different chemical compositions were selected from five geographical locations and their effects on streptozotocin (STZ)-induced diabetic mice were evaluated with both ethanol and aqueous extracts. Principal components analysis (PCA) and canonical correspondence analysis (CCA) were used to analyze the chemical variability and its relationship with anti-diabetic activities. The potential antihyperglycemic capacity of C. paliurus flavonoids and the antihyperlipidemic effect of C. paliurus triterpenoids were revealed. Taken together, this study provides essential information supporting the use of C. paliurus extract as a natural medicine for diabetic patients.
Belamcanda chinensis belongs to the family of Iridaceae and their rhizomes have been widely used as traditional medicine in China.

Many isoflavones such as tectoridin, iridin, irigenin and tectorigenin have been identified from this plant [14]. The antifebrile, antioxidant, antiinflammatory and hepato-protective activities of B. chinensis have been well-documented [15-17]. Previously, we reported the hypoglycemic and anti-hyperglycemic effects of flavone-rich B. chinensis leaf extract (HTP) in normal and STZ induced diabetic rats [18, 19]. Apart from these, no study to-date has investigated the antihyperlipidemia activity of HTP. In this study, we assessed the anti-hyperlipidemia effects of HTP on high-fat diet induced obese mice. The stimulating effects of HTP on AMPK and PPARα were also studied.

Material and Methods

Plant Materials

Five geographical locations from five provinces in China for C. paliurus sampling were identified based on the major distribution of the species. Voucher specimens were deposited in Silviculture Lab of Nanjing Forestry University (Voucher code: 2011GX, 2011SC, 2011HB, 2011ZJ, 2011HN). The detailed geographic, climatic information, and soil index of the sample locations are listed in Table 1. Leaf samples were collected from each location in September 2014. At each location, 6–30 trees (generally dominant or co-dominant tree in the stand) were selected based on tree age (over 20 years-old), stem form, and growth vigor. Number of trees for collecting leaves for each location was determined according to quantity and stand area of C. paliurus which distributed on the area (about 10% of the total). About 400 g fresh fully developed leaves were collected from the middle crown for each tree and then sealed up with silica gel for transportation. Leaves collected in each location were mixed together in the lab and then dried at 70 °C for 48 h to constant weight, and ground into fine powder before extraction. All samples were stored at room temperature prior to analysis.

Chemical reagents and References

Streptozotocin (STZ) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Metformin Hydrochloride Tablets (MHT) was bought from Sino-American Shanghai Squibb Pharma (Shanghai, China), and Xiaoke Pill (XKP) from Guangzhou Zhongyi Pharmaceutical Enterprise (Guangdong, China). Commercial test kits used for measuring TG, TC, LDL-c, HDL-c, BUN, CREA, TBIL, AST, and ALT were purchased from Jiancheng Institute of Biotechnology (Nanjing, China). The reference standards of 3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, isoquercitrin, 4,5-di-O-caffeoylquinic acid, kaempferol-3-O-glucuronide, quercetin-3-O-glucuronide, arjunolic acid, quercetin-3-O-rhamnoside, quercetin-3-O-galactoside, and kaempferol-3-O-glucoside (purity >98%) were purchased from BioBioPha Co., Ltd. (Kunming, China), and arjunolic acid, oleanolic acid (purity >98%) were purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China), whereas cyclocaric acid B, kaempferol-3-O-rhamnoside, and pterocaryoside A and pterocaryoside B (purity >98%) were isolated and purified from the laboratory of China Pharmaceutical University (Nanjing, China) [24].

Plant materials

Leaves of Belamcanda chinensis were collected from Hainan province, South China, and authenticated by Dr Hubiao Chen (Health Science Center, Peking University, China). The preparation of HTP was performed as previously reported [18, 19]. A voucher specimen has been deposited in School of Life Science, Beijing Institute of Technology (NO. HTP20120911).

Animals and experiment design

Male ICR mice (20 ± 2 g, Peking University Laboratory Animal Center, Beijing, China) were housed at 22 ± 2 ºC and 55 ± 5% relative humidity; 12 h light-dark cycle and allowed free access to water and feed. The study was carried out in accordance with International Guidelines for Care and Use of Laboratory Animals [20] and approved by Animal Ethical Committee of Beijing Institute of Technology (reg. no. 201209007/BITAEC). Seventy-two ICR mice were kept in a week. Animals were then randomly divided into 6 groups with 12 mice in each group: BC, fed a standard diet and received 0.5% sodium carboxy methyl cellulose (CMC-Na) solution only; NC, fed a humidity controlled room on a 12-h light–dark cycle with food and water available ad libitum for one high-fat diet (HFD) and received 0.5% CMC-Na solution only; PC, fed a HFD and treated with gemfibrozil (200 mg/kg); and HTP groups, fed a HFD and treated with HTP (100, 200 and 400 mg/kg, respectively). HFD consists of 10% lard, 10% sugar, 10% egg York, 1% cholesterol, 0.2% sodium cholate, and 68.8% standard diet. HTP and gemfibrozil were administrated by oral gavage. Body weight was monitored weekly. During the experiment, blood was collected from tail vein for the measurement of blood glucose. At the end of the 7th week, blood sample was collected from orbital venous plexus and the serum was prepared for biochemical analysis. Liver and peri-epididymal fat were removed, weighted and stored at -70 oC refrigerator (Thermo Fisher Scientific, Waltham, USA,). Serum and hepatic levels of triglyceride (TG) and total cholesterol (TCH) were determined by corresponding kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instruction. The hepatic PPARα level was determined by a mouse PPAR-α ELISA kit (HanKe Biotech co., Ltd, Beijing, China) according to the manufacture's instruction.

Oral glucose tolerance test (OGTT)

Animals were orally administrated corresponding regents after overnight fast. PC group was treated with acarbose (50 mg/kg). After another 2 h, glucose (2.5 g/kg) was given to each animal orally. Blood were collected from tail vein of each mouse at 0, 30, 60, and 120 min after glucose administration, and glucose levels were determined by a blood glucose meter (Roche Diagnostics, Basel, Switzerland).

Western blot

Western blot analysis was performed on liver tissue extract as previously reported [21]. Antibodies against phospho-AMPKα (Thr172) and AMPKα were from Cell Signaling Technology Inc. (Beverly, USA), and antibody against GAPDH was from Abcam, Inc. (Cambridge, USA).

Statistical analysis

Data are presented as the means ± S.D. Oneway ANOVA was used to determine significant differences among groups, after which the modified Student's t-test with the Bon ferroni correction was used for comparison between individual groups. $P < 0.05$ was considered statistically significant.

Results and Discussion

Table 1: Index of liver and kidney function in different groups of experimental mice at the end of the diabetic trial (mean ± SD). Different letters indicate significant differences ($p< 0.05$ by Duncan's test) between treatments (n = 6).

Treatment	ALT (U/L)	AST (U/L)	TBIL (µmol/L)	CREA (µmol/L)	BUN (mmol/L
DC	52.8± 2.17 a	145.6 ± 3.08 a	7.50 ± 0.89 a	25.8 ± 1.17 a	17.3 ± 0.99 a
NC	28.0 ± 1.84 e	99.8 ± 6.87 fg	1.00 ± 0.18 g	14.7 ± 1.36 e	9.80 ± 0.18 d
EL1	36.1 ± 2.63 cd	113.3 ± 8.10 def	1.20 ± 0.15 fg	20.8 ± 0.75 bcd	13.4 ± 0.86 b
EL2	35.9 ± 1.80 cd	94.8 ± 8.59 g	1.02 ± 0.02 g	19.2 ± 1.94 cd	12.6± 1.08 bc
EL3	35.1 ± 3.36 d	94.5 ± 4.45 g	1.19 ± 0.18 fg	18.2 ± 2.13 d	13.1 ± 1.26 bc
EL4	36.9 ± 3.35 bcd	95.8 ± 9.05 g	1.46 ± 0.16 fg	21.0 ± 0.89 bcd	11.0 ± 1.17 cd
EL5	36.2 ± 3.38 cd	114.0 ± 7.66 de	1.38 ± 0.18 fg	21.0 ± 1.26 bcd	12.7 ± 0.26 bc
AL1	40.3 ± 4.51 bcd	130.2 ± 4.56 b	1.47 ± 0.18 fg	21.7 ± 1.86 bc	14.7 ± 0.81 b
AL2	43.4 ± 6.36 b	128.9 ± 5.30 bc	2.03 ± 0.10 ef	22.3 ± 1.21 b	14.2 ± 0.69 b
AL3	41.6 ± 1.36 bcd	123.6 ± 6.69 bcd	2.57 ± 0.28 e	22.8 ± 0.98 b	14.3 ± 0.92 b
AL4	43.1± 5.16 b	125.8 ± 4.16 bcd	4.87 ± 0.17 bc	21.8 ± 1.17 bc	14.2 ± 1.17 b
AL5	40.0 ± 3.19 bcd	115.5 ± 6.08 cde	3.63 ± 0.21 d	21.2 ± 0.98 bc	14.2 ± 1.32 b
MHT	42.4 ± 2.06 bc	121.1± 6.06 bcde	5.60 ± 0.55 b	21.0 ± 1.67 bcd	14.3 ± 0.76 b
XKP	43.5 ± 2.39 b	108.2 ± 8.57 efg	4.41 ± 0.22 cd	22.0 ± 1.79 bc	13.9 ± 0.82 b

The development of diabetes often leads to the leakage of the circulatory system, and high levels of AST, ALT, TBIL, CREA, and BUN were often observed in STZ-induced diabetic mice [36, 37], which is in agreement with our results (Table 2). Results indicated that C. paliurus extracts improved the liver and kidney function by decreasing the serum AST, ALT, and TBIL, CREA, and BUN levels in the diabetic mice. Moreover, significantly positive correlations were found between the values of AST, ALT, CREA, BUN, and lipid index (TC, TG, and LDL-c), which is in agreement with other reports in hyperlipidemic mice [38, 39]. Overall, these results suggested that C. paliurus extracts could offer protection to liver and kidney by effectively lowering the lipid level. These findings, together with evidence collected from previous reports suggest that the composition of C. paliurus compounds might help for the design of therapeutic alternatives for the treatment of diabetes mellitus. However, geographic origins and the extraction solvents can also affect the effectiveness of the treatment as these factors influence the chemical compositions and their antihyperglycemic and antihyperlipidemic activities.

Table 2: Effect of HTP on the body weight, peri-epididymal fat weight and fat index

Group (n=12)	Body weight (g)	Peri-epididymal fat weight (g)	Fat index (mg/g BW)
BC	44.8±4.3	0.40±0.16**	8.14±2.62**
NC	46.1±3.0	0.83±0.30##	18.07±6.74##
PC	43.1±2.3*	0.55±0.18*	12.70±3.84
HTP100	44.8±3.9	0.59±0.42	9.57±3.15**
HTP 200	44.8±3.6	0.39±0.25**	8.62±5.33**
HTP 400	45.4±2.8	0.57±0.29*	11.13±5.06*

BC = fed a standard diet; NC = fed a high fat-diet (HFD); PC = fed a HFD and treated with gemfibrozil (200 mg/kg); HTPs = fed a HFD and treated with indicated dose of HTP. Values are mean ± S.D; ##$p< 0.01$ NC vs. BC; *$p< 0.05$, **$p< 0.01$ vs. NC.

Animals in all groups showed a steady increase in body weight throughout the experimental period. At the end of the study, the average body weight of NC group was significantly higher than BC group ($p< 0.05$). Treating with HTP for 7 weeks did not influence the weight gain but significantly decreased the peri-epididymal fat weight and fat index (Table 2).

Conclusion

In conclusion, based on the analysis of chemical variability and its relationship with anti-diabetic activities, our results revealed the potential antihyperglycemic capacity of C. paliurus flavonoids and the antihyperlipidemic effect of C. paliurus triterpenoids, while these potential capacities were closely related to their contents. Further studies on the antihyperglycemic effect of C. paliurus kaempferol or quercetin glycosides and the antihyperlipidemic mechanism of C. paliurus triterpenoids will be carried out in future research.

The results presented in this study suggest that the flavone-rich B. chinensis leaf extract (HTP) has good potentials for lipid management. Up regulation of AMPK and PPARα are two possible mechanisms for its antihyperlipidemic activity.

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