Anti-hyperuricemic potential of stevia (*Stevia rebaudiana* Bertoni) residue extract in hyperuricemic mice

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Preparation of STVRE

The Stevia leaves powder was extracted twice with 18 volumes of distilled water at 65°C for 1.5 hr and then filtrated. The filtrate was mixed with ferrous sulfate (0.9% w/v, final concentration) and adjusted to pH 9.0–10.0 with calcium hydroxide. After flocculation at 50°C for 30 min, the mixture was filtrated to obtain the Stevia residue. The Stevia residue was then extracted three times with acidified 95% (v/v) acetone (pH 1.5) at 45°C for 1 hr and filtrated. The filtrate was evaporated under reduced pressure to remove acetone, and thereafter purified by extraction with ethyl acetate. The partially purified extract was applied to an HZ841 macroporous resin column, and then eluted it with 2 bed volume (BV) of distilled water, followed by 2 BV of alkaline water (pH 9.5) at a flow rate of 1 BV/hr to remove impurities. Subsequently, the adsorbed components were eluted with 3 BV of 17% (v/v) ethanol, followed by 1.5 BV of 50% (v/v) ethanol. The eluents were concentrated and lyophilized to obtain SRE1 and SRE2, respectively. The resulting STVRE powder was stored at −20°C prior to use (1-2, 5).

Qualitative and quantitative analysis of the major bioactive compounds in STVRE

The major bioactive compounds in SREs were identified by HPLC– ESI–QTOF–MS/MS (6530 HPLC-MS system; Aglient Technologies, Santa Clara, CA, USA) and quantified by HPLC (1290 HPLC system; Aglient Technologies, Santa Clara, CA, USA). The HPLC parameters were as follows: chromatographic column, Agilent Eclipse Plus C18 column (50 mm × 2.1 mm i.d., 1.8 μm; Aglient Technologies, Santa Clara, CA, USA); mobile phase, 0.1% formic acid in water (A) and methanol (B); flow rate, 0.1 mL/min; elution procedure, 0–10 min, 10%–50% B; 10–12 min, 50%–10% B; 12–15 min, 10% B; detection wavelength, 320 and 360 nm; column temperature,

30°C; volume of sample injection, 1 μL. Authentic compounds (3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, caffeic acid, quercetin, 3,4-O-dicaffeoylquinic acid, 3,5-O-dicaffeoylquinic acid, 4,5-O-di-caffeoylquinic acid and quercetin-3-O-rhamnoside) were dissolved in methanol; the concentrations were adjusted to 6.25, 12.5, 25, 50, 100, 200 and 400 μg/mL, respectively, and the calibration curves were established under the same determination conditions. Mass spectrometric detection was set as follows: Negative ion mass spectra of the column eluate were recorded in the range of m/z 100–1500 (1-2, 5).

Supplementary Table 1. Retention times (Rt), MS and MS2 fragmentation ions of the phenolic compounds in stevia residue extract (STVRE).

Compounds	Rt (min)	Pseudomolecular ion $[M-H]^-$ (m/z)	MS2 (m/z)	Tentative identification	mg/ml
1	3.8	353.0878	191.0571 (100), 179.0357 (72), 135.0451 (9)	3-O-Caffeoylquinic acid	1.68 ± 0.75
2	4.2	353.0878	191.0567 (100), 179.0353 (76), 173.0431 (4), 135.0442 (13)	4-O-Caffeoylquinic acid	3.79 ± 0.41
3	4.4	179.0350	135.0456 (100)	Caffeic acid	8.54 ± 0.82
4	7.9	515.1195	353.0916 (100), 335.0823 (1), 191.0575 (12), 179.0361 (9), 135.0443 (2)	3,4-O-Dicaffeoylquinic acid	232.75 ± 2.17
5	8.0	515.1195	353.0914 (100), 335.0771 (1), 191.0564 (5), 179.036 (14), 173.0467 (24), 135.0440 (2	3,5-O-Dicaffeoylquinic acid	92.43 ± 0.97
6	9.1	515.1195	353.0921 (100), 191.0587 (4), 179.0365 (21), 173.0462 (19), 135.0459 (2)	4,5-O-Dicaffeoylquinic acid	265.94 ± 2.63
7	9.3	447.0933	301.0373 (100)	Quercetin-3-O-rhamnoside	125.97 ± 1.13
8	11.0	301.0354	107.0139 (8), 121.0301 (11), 151.0042 (100), 178.9994 (72)	Quercetin	3.05 ± 0.63
				Total chlorogenic acids	605.13 ± 5.66
				Total flavonoids	129.02 ± 20.61

Adopted from (1-2, 5)

Supplementary Table 2. Experimental plan

Treatments	Dosage Information
NC	Normal diet + 0.5% (w/v) CMC-Na
MC	Normal diet + potassium oxonate (100 mg/kg bw) + fructose in drinking water (10 %) + yeast extract (100 mg/kg bw)
PC	Normal diet + potassium oxonate (100 mg/kg bw) + fructose in drinking water (10 %) + yeast extract (100 mg/kg bw) + allopurinol (5 mg/kg bw)
STVRE-1	Normal diet + potassium oxonate (100 mg/kg bw) + fructose in drinking water (10 %) + yeast extract (100 mg/kg bw) + stevia residue extract (75 mg/kg bw)
STVRE-2	Normal diet + potassium oxonate (100 mg/kg bw) + fructose in drinking water (10 %) + yeast extract (100 mg/kg bw) + stevia residue extract (150 mg/kg bw)
STVRE-3	Normal diet + potassium oxonate (100 mg/kg bw) + fructose in drinking water (10 %) + yeast extract (100 mg/kg bw) + stevia residue extract (300 mg/kg bw)

Eight weeks

All drugs and plant material used in animal study were dispersed in 0.5% CMC-Na. NC: Normal control, MC: Model control: PC: Positive control, STVRE-1: Stevia residue extract 1, STVRE-2: Stevia residue extract 3, STVRE-3: Stevia residue extract 3

Supplementary Table 3: List of primers used in this study

Genes	Primer	Primer sequences	Reference
UART1	Forward	GCTACCAGAATCGGCACGCT	4
	Reverse	CACCGGGAAGTCCACAATCC	
GLUT9	Forward	GAGATGCTCATTGTGGGACG	
	Reverse	GTGCTACTTCGTCCTCGGT	
ABCG2	Forward	TAAATGGAGCACCTCAACCT	
	Reverse	GAGATGCCACGGATAAACTG	
OAT1	Forward	GCCTATGTGGGCACCTTGAT	
	Reverse	CTTGTTTCCCGTTGATGCGG	
OAT3	Forward	AAGAACATCTCTGTGAGGGTG	
	Reverse	GGCAAGATGAACCAAAACTGG	
OCT1	Forward	ACATCCATGTTGCTCTTTCG	
	Reverse	TTGCTCCATTATCCTTACCG	
OCT2	Forward	ACAGGTTTGGGCGGAAGT	
	Reverse	CACCAGAAATAGAGCAGGAAG	
OCTN1	Forward	AGGAGAGGTGGAAACATGCG	
	Reverse	TCCTTCGTCTCCAAGGGGA	
OCTN2	Forward	CTTATTCCCATACGGGCGCT	
	Reverse	TTTCTGAGGCACCTGTCGTC	
NF-κB	Forward	5′-CTCACCGGCCTCATCCACAT-3′	3
	Reverse	5′-TGGCTAATGGCTTGCTCCAG-3′	

Supplementary Table 4. Effect of STVRE on body, liver and kidney weight of hyperuricemic mice (n = 10)

Group	Body Weight (g)	Liver Weight (g)	Kidney Weight (g)	Liver Index (%)	Kidney Index (%)
NC	55.56 ± 2.63	2.59 ± 0.30	0.58 ± 0.07	4.66 ± 0.3	1.04 ± 0.02
MC	52.56 ± 2.08	2.39 ± 0.23	0.53 ± 0.08	4.54 ± 0.23	1.00 ± 0.03
PC	53.56 ± 1.99	2.62 ± 0.21	0.59 ± 0.02	4.89 ± 0.21	0.97 ± 0.01
STVRE-1	51.24 ± 1.85	2.35 ± 0.17	0.52 ± 0.06	4.58 ± 0.17	1.01 ± 0.03
STVRE-2	53.02 ± 2.35	2.56 ± 0.15	0.56 ± 0.03	4.82 ± 0.15	1.05 ± 0.01
STVRE-3	56.58 ± 2.21	2.60 ± 0.19	0.56 ± 0.05	4.59 ± 0.19	0.98 ± 0.02

Non-significant ($p>0.05$) effect on general characteristic such as body weight, kidney weight, liver weight, kidney index, and liver index were observed among all groups.

NC: normal control group, MC: model control group, PC: positive control group, STVRE-1: Stevia residue extract 75 mg/kg bw, STVRE-2: Stevia residue extract 150 mg/kg bw, STVRE-3: Stevia residue extract 300 mg/kg bw

$^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compare with normal control, $*p<0.05$, $**p<0.01$, $***p<0.001$ compare with model control

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