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RESEARCH PAPER

Reaction coupling separation for isosteviol production from stevioside catalyzed by acidic ion-exchange resin

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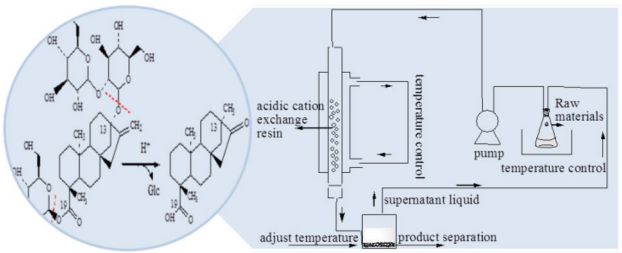
Abstract

Isosteviol, a prodrug used to be obtained via Wagner–Meerwein rearrangement from steviol with low yield and long reaction time. Herein, an in-situ separation-coupling-reaction is presented to prepare isosteviol from the natural sweetener stevioside. Simply with in-situ water-washing, the product containing 92.98% purity of isosteviol was obtained with a stevioside conversion of 97.23% from a packet bed reactor without further separation. Within the assayed inorganic acid, organic acids and acidic ionic liquids, the acidic ion-exchange resins provided higher product specificity towards isosteviol. Furthermore, comparing to 5-Fluorouracil, the product presented similar and even stronger inhibition on proliferation of the assayed human cancer cells in a time and dose-dependence by causing cell phase arrest. Isosteviol treatment caused G1 arrest on SGC-7901, HCT-8 and HCT-116 cells, S arrest on HepG2, Huh-7 and HepG3B cells, and G2 arrest on MGC-803 cells, respectively.

Graphic abstract

Reaction coupling separation for isosteviol production catalyzed by acidic ion-exchange resin.



Keywords Isosteviol · Stevioside · Hydrolysis · Cation-exchange resin · Anticancer · Ionic liquid

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Introduction

Isosteviol, (4α, 8β, 13β)-13-Methyl-16-oxo-17-norkauran-18-oic acid, was reported on its structure, stereochemistry [1] and absolute configuration in 1960s [2]. To date, isosteviol has been mostly recognizing as a popular starting material for drug synthesis [3–7]; for instance, as precursors of synthetic drugs for the treatment of cancer and inflammation [8, 9].

Recently, isosteviol itself has gained intensive interests as it possesses various biological activities [10], such as reducing vasoconstriction [11], improving glucose and insulin sensitivity, lowering plasma triglycerides and weight,

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and regulating the gene expression profile of key insulin regulatory genes for the treatment of type II diabetes [12, 13], relieving I/R injury in rat brains [14] and so on [6]. Therefore, efficient massive production of isosteviol with high purity is expected and benefit to its application.

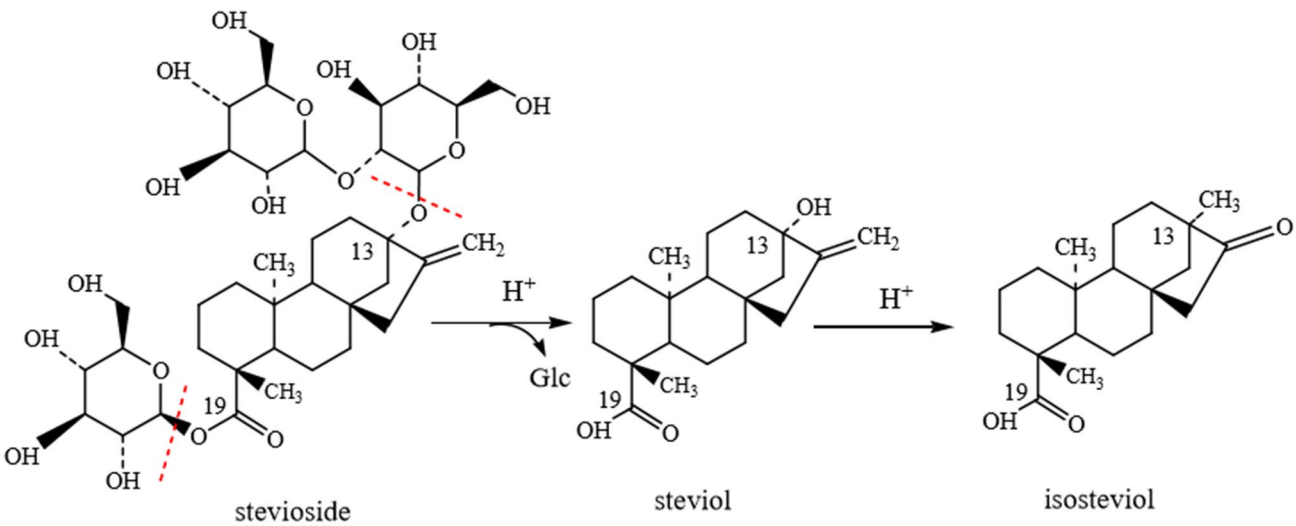
Isosteviol can be obtained by Wagner–Meerwein rearrangement from steviol [15], the latter is a product hydrolyzed from natural glycosyl steviosides such as stevioside or rebaudiosides catalyzed by acids (Scheme 1), such as hydrochloric acid, sulfuric acid and hydrobromic [16–18]. However, the acidic hydrolysis of glycosyl steviosides would simultaneously yield steviol and endocyclic isomers of steviol [19, 20]. For example, an analogous mixture of isosteviol, steviol, and Δ15-steviol was obtained using 0.7% of hydrochloric acid, and the yield of isosteviol was only 36% [16]. Cherney et al. synthesized steviol by multistep reactions, which was then converted to isosteviol by the acid-induced rearrangement [21]. In another case, an isosteviol yield of 83% along with steviol (17% yield) was obtained, assisted with $FeCl_3$ from hydrolyzing stevioside at a reflexing temperature [20]. Enzymatic hydrolysis may provide higher product specificity on isosteviol. Milagre disclosed that stevioside was hydrolyzed with the catalysis of pancreatin and offered isosteviol in a yield of 93.9% in 7 days [22].

Therefore, quick and affordable preparation of isosteviol might be still expected on acidic hydrolysis of stevioside. In this experiment, aiming at finding a practicable process to produce isosteviol in high yields, various acidic catalysts including acids, acidic ionic liquids and cation-exchange resins were investigated on their catalysis activity and selectivity to find a suitable catalyst for isosteviol production; subsequently, an in-situ separation coupling production of isosteviol from hydrolyzing stevioside was designed and conducted. Meanwhile, to elucidate isosteviol's cytotoxicity and anticancer activity, the cytotoxicity of isosteviol on several human normal cells and carcinoma cell lines was investigated as well.

Materials and methods

Chemicals

Stevioside (97% of HPLC purity) was from Qingdao Runde Biotechnology Co., Ltd (Qingdao, China). Na_2CO_3, CH_3COONa, CH_3COOH, $Na_2HPO_4{\cdot}12H_2O$, $NaH_2PO_4{\cdot}2H_2O$, NaCl, KCl, dimethyl sulfoxide (DMSO, BR) and 5-Fluorouracil (5-FU, BR) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Isosteviol (97% of HPLC purity) was from Nanjing Spring & Autumn Biological Engineering Co., Ltd (Shanghai, China). Acidic ionic liquids $[MIMPS]_3PWO_{40}$, $[C_3IMPS]_3PWO_{40}$, $[C_5IMPS]_3PWO_{40}$, $[C_7IMPS]_3PWO_{40}$, H_3PWO_{40} were provided by Dr Wei Li from School of Chemical and Materials Engineering at Jiangnan University. Resin 732 (Na^+ form, styrene–divinyl benzene copolymer, total exchange capacity of 4.6 mmol/g (Na^+ form), resin 0014 (Na^+ form, styrene–divinyl benzene copolymer, total exchange capacity of 4.5 mmol/g), resin 00112 (Na^+ form, matrix styrene–divinyl benzene copolymer, total exchange capacity of 4.0 mmol/g) and resin D113 (H^+ form, acrylic copolymer, total exchange capacity of 10.8 mmol/g) cation-exchange resins were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Trypsin–EDTA solution, propidium iodide (PI), Triton X-100, endonuclease (RNase A), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and



Scheme 1 Acid-catalyzed preparation of isosteviol from hydrolysis of stevioside

penicillin–streptomycin solution (100×) were purchased from Beyotime Institute of Biotechnology Co., Ltd (Shanghai, China). All other reagents were of analytical grade and used as received unless otherwise stated.

Cell culture

The human moderately differentiated gastric cancer cell SGC-7901, human colon adenocarcinoma cell Caco-2, human ileocecal adenocarcinoma epithelial cell HCT-8 and human colorectal cell HCT 116 were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science. The human gastric mucosal epithelial cell GES-1, human poorly differentiated gastric cancer cell MGC-803, three hepatoma cells, Huh-7 (mutated p53), HepG2 (wild-type p53) and Hep3B (p53 deleted) were purchased from the American Type Culture Collection. SGC-7901 was cultured in RPMI 1640 medium, and other cells were cultured in DMEM medium containing 10% fetal bovine serum, 1% glutamine (200 mmol/L), penicillin (100 IU/mL), and streptomycin (100 mg/L) in a humidified 5% CO_2 atmosphere at 37 °C before use.

Hydrolysis of stevioside in a batch reactor

The prepared selected cation exchange resins in Na^+ form was converted to H^+: cation exchange resins were conditioned in hydrochloric acid solution (1 mol/L HCl) and then several washings with deionized water to remove excess acid [23]. In a typical reaction, 20 mL stevioside aqueous solution (20 g/L) in a 50 mL Erlenmeyer flask was kept at 95 °C for 30 min, then mixed with the cation-exchange resin and shaken in a water bath at 95 °C for 24 h. The precipitated white powder was filtered and washed with warm DI water until no stevioside was detectable from the eluting solution. The powder was then recrystallized from 95% aqueous methanol, and white needles were obtained as the final product. The product was characterized with NMR (AVANCE III 400 MHz Digital NMR Spectrometer (Bruker, USA)) and LC–MS (BEH HILIC C18 column; mobile phase: acetonitrile and water (75:25, v/v), 0.3 mL/min; column temperature: 30 °C; collision energy: 20–55 eV; polarity: ES^-)[24, 25]. The conversion of stevioside (St) was calculated based on HPLC analysis with the calibration of a standard solution of stevioside, described as following:

$$\text{St conversion} = \frac{C_0 - C_t}{C_0} \times 100\%$$

where C_0 and C_t is the initial and real-time concentration (g/L) of stevioside in the reaction mixture, respectively. The stevioside concentration was determined with a standard calibration curve.

The yield of isosteviol or steviol was production yield, calculated based on the whole analysis of precipitated solid with mass measurement. But the yields of other byproducts, if needed, were calculated based on their corresponding HPLC chromatograph peak area (A_x) with chromatograph peak area of stevioside (A_{St}) as the reference, calculated as follows:

$$\text{Yield} = \frac{C_t \times A_x / A_{st}}{C_0} \times 100\%$$

All tests were performed in triplicate at least; all data presented were with standard deviations less than 5%.

MTT assay on cell proliferation

The effect of isosteviol on the proliferation of human carcinoma or normal cell was evaluated with MTT assay [25–27]. The cells in the logarithmic growth phase were digested with 0.25% trypsin and adjusted to 5,000 cells/well using DMEM complete medium. Prior to the isosteviol treatment, 100 μL cell suspension was pipetted into each well in 96-well plates and cultured for 24 h at 37°C in 5%CO_2. Subsequently, cells were incubated with isosteviol at 37 °C in 5% CO_2 for 48 h. The culture medium was then removed and 100 μL MTT reagent (0.5 mg/mL in culture medium) was added. Following an additional 4 h of incubation, the MTT/medium was removed and 150 µL of DMSO was added to dissolve the formazan crystals. Absorbance of the solution at 570 nm was recorded to calculate the inhibition rate on cell growth. Chemotherapy agent 5-FU was employed as a positive contrast. All measurements were performed in triplicate and recorded as means. The inhibition rate was calculated as follows:

$$\text{Cell growth inhibition rate (\%)} = \frac{A_{570}\text{ of control} - A_{570}\text{ of sample}}{A_{570}\text{ of control}} \times 100$$

Cell cycle analysis

The human carcinoma cells were plated at 2×10^5/well in 6-well plates and treated with isosteviol. After 48 h, the cells were then harvested with trypsin, washed three times for 1 min each, resuspended in cold PBS and fixed in cold (4 °C) 70% ethanol for 4 h, and kept storage at − 20 °C overnight. Next, the cells were washed three times for 1 min each again and resuspended in PBS containing 40 µg/mL PI and 0.1 mg/mL RNase (#C1052, Beyotime Institute of Biotechnology Co., Ltd, Shanghai, China), and then incubated for 30 min at room temperature. PI-stained cells were analyzed using a flow cytometer and ModFit LT 5.0 software (Verity Software House, USA) [26].

Results and discussion

Comparative study with inorganic acids and acidic cation-exchange resins

Four inorganic acids and four organic acids (Fig. 1a), five acidic ionic liquids (Fig. 1b), and five cation-exchange resins (Fig. 1c) were tested for their product specificity of hydrolyzing stevioside(Fig. 1).

As presented in Fig. 1, strong acids (Fig. 1a) mainly induced steviol in the hydrolysis products, in accordance with that reported in the literature [16] where the yield of isosteviol is only 36%; while the middle strong and weak acids did not promote hydrolysis of stevioside (Fig. 1a). The main products of the hydrolysis catalyzed by acidic ionic liquid were isosteviol and steviol, the yield ratio is about 2:1 (Fig. 1b). Similarly, weak acidic cation-exchange resins (D113) did not accelerate the hydrolysis of stevioside; but the stronger acidic resins reduced more isosteviol, especially the 732 cation-exchange resin, the yield of isosteviol and steviol is about 6:1 (Fig. 1c, Run 2). In summary, compared to the assayed inorganic acid, organic acids and acidic ionic liquids, the acidic ion-exchange resins provided high product selectivity towards isosteviol.

Therefore, 732 cation-exchange resin was then chosen for the subsequent experiments due to its higher product specific activity, in which isosteviol was the main product as the IR, LC–MS and NMR profile indicated [16, 28] (see also Supporting material, Figs. 1s, 2s, 3s). The product was recrystallized with 95% methanol twice, giving white crystal needles with a purity of 99% (HPLC). NMR profiles of the main product from hydrolysis of stevioside with 732 resin

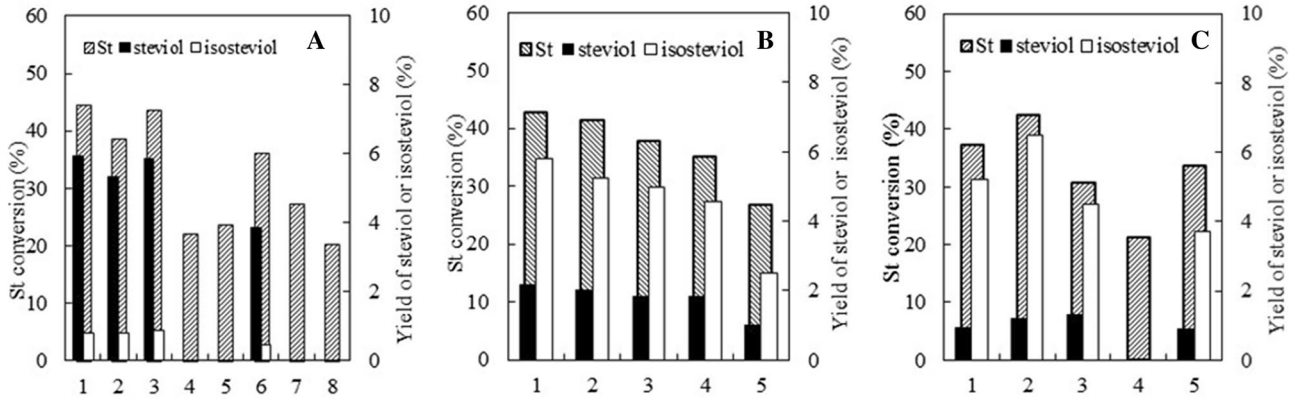


Fig. 1 Acidic hydrolysis of stevioside. 95 °C, St 50 g/L, H^+ 10 mol%/St, 3 h; catalyzed by A (acids): 1- HCl, 2- H_2SO_4, 3- HNO_3, 4 -H_3PO_4, 5- CH_3COOH, 6- NH_2SO_3H, 7- $C_2H_2O_4$, 8- $C_6H_8O_7$; B (acidic ionic liquids): 1- $[MIMPS]_3PWO_{40}$, 2- $[C_3IMPS]_3PWO_{40}$, 3- $[C_5IMPS]_3PWO_{40}$, 4- $[C_7IMPS]_3PWO_{40}$, 5- H_3PWO_{40}; C (cation-exchange resins): 1- 001×4, 2- 732, 3- 001×12, 4- D113, 5- 001×7.5

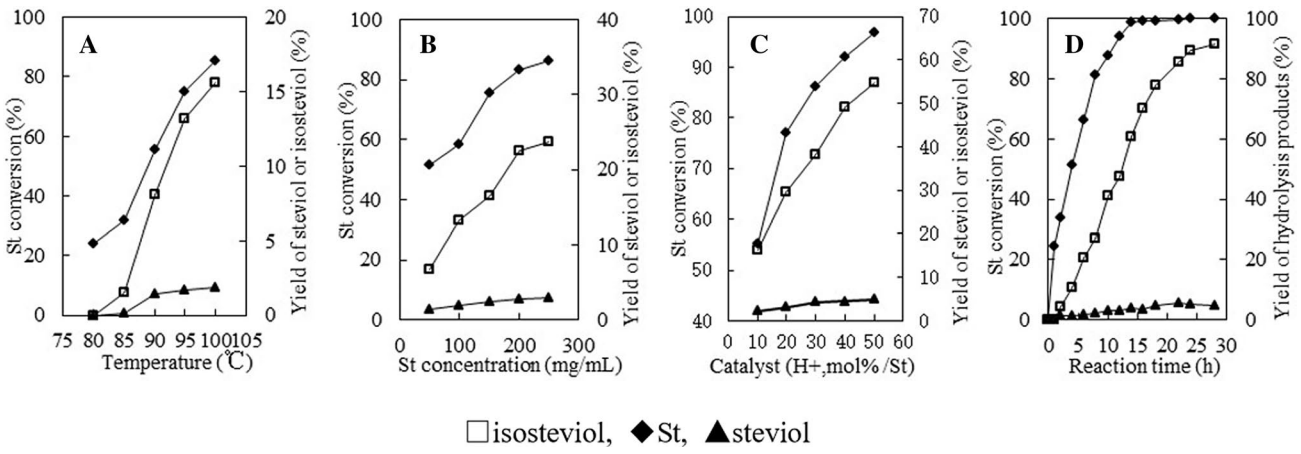


Fig. 2 Single factor test of hydrolyzing stevioside catalyzed by 732 cation-exchange resin. A: St 100 g/L, H^+ 15 mol%/St, 10 h; B: 95 °C, H^+ 15 mol%/St, 10 h; C: 95 °C, St 200 g/L, 10 h; D: 95 °C, 200 g/L, H^+ 15 mol%/St

are as follows: ^{1}H NMR: (400 MHz, Pyr) δ 14.66 (s, 1H), 2.66 (dd, J = 18.4, 3.5 Hz, 1H), 2.45 (d, J = 13.0 Hz, 1H), 2.25–2.11 (m, 1H), 2.10 – 2.01 (m, 2H), 1.78 (d, J = 18.4 Hz, 1H), 1.66–1.52 (m, 4H), 1.45 (dt, J = 11.4, 4.2 Hz, 3H), 1.35 (s, 3H), 1.34–1.24 (m, 2H), 1.24–1.03 (m, 4H), 1.02 (s, 3H), 0.96 (s, 3H), 0.87 (td, J = 13.3, 3.9 Hz, 1H).. ^{13}CNMR (101 MHz, Pyr) δ 39.34 (C-1), 19.41 (C-2), 37.21 (C-3), 43.62(C-4), 56.73 (C-5), 22.17 (C-6), 41.43 (C-7), 48.34 (C-8), 54.49 (C-9), 38.34 (C-10), 20.35 (C-11), 38.10(C-12), 39.85 (C-13), 53.98 (C-14), 48.34 (C-15), 224.5 (C-16), 20.02 (C-17), 29.15 (C-18), 180.04 (C-19), 39.34(C-20).

Reaction coupling separation for isosteviol production from stevioside with 732 acidic ion-exchange resin

First of all, using 732 acidic ion-exchange resin as the catalyst, a single factor test was applied to optimize the reaction parameters as shown in Fig. 2; where St completely converted with 91.9% of isosteviol yield and 4.8% of steviol yield in 24 h (Fig. 2d).

Subsequently, a packed bed reactor was used to utilize the resin (Fig. 3), where the reaction coupling separation

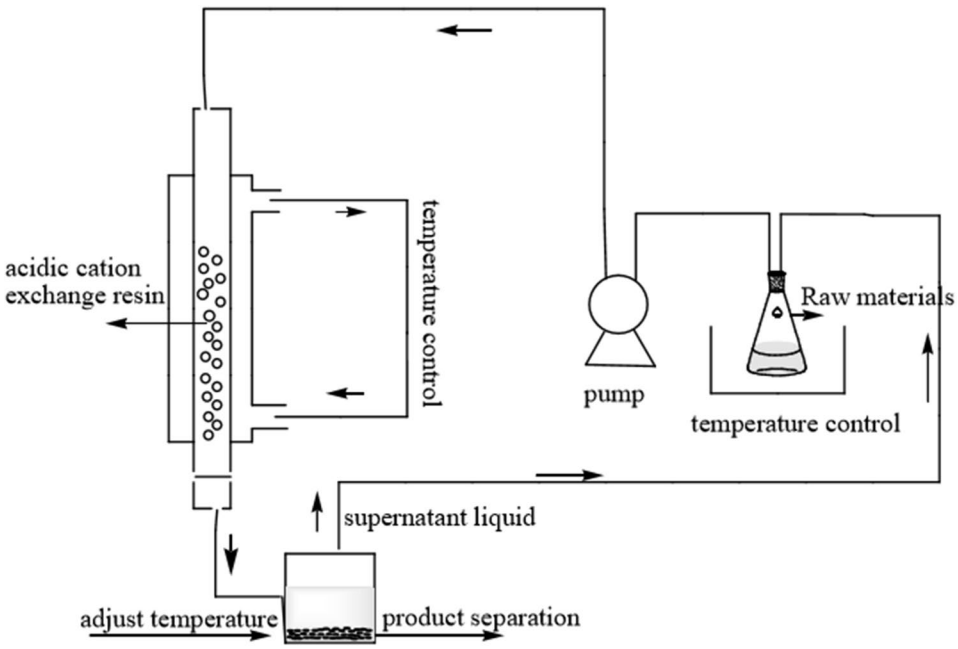


Fig. 3 Reaction coupling separation of isosteviol from stevioside with 732 acidic ion-exchange resin

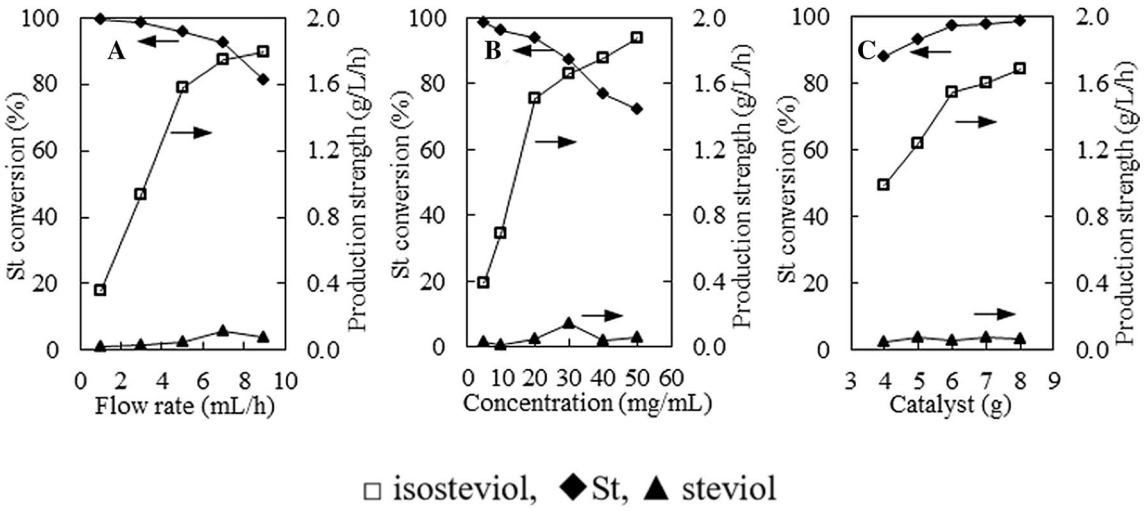


Fig. 4 Hydrolysis of stevioside with the acidic cation exchange resin in the packed bed reactor at 95 °C. 6 g ion exchange resins, **a** St 20 g/L; **b** flow rate: 5.0 mL/h; **c** St 20 g/L, flow rate: 5.0 mL/h

of isosteviol production from stevioside was realized. With an in-situ separation, the product with 92.98% purity of isosteviol was obtained with a stevioside conversion of 97.23%, simply after water washing. Considering the production strength and production yield, the suitable parameters would be obtained as (Fig. 4): 95 °C, 20 g/L of St,

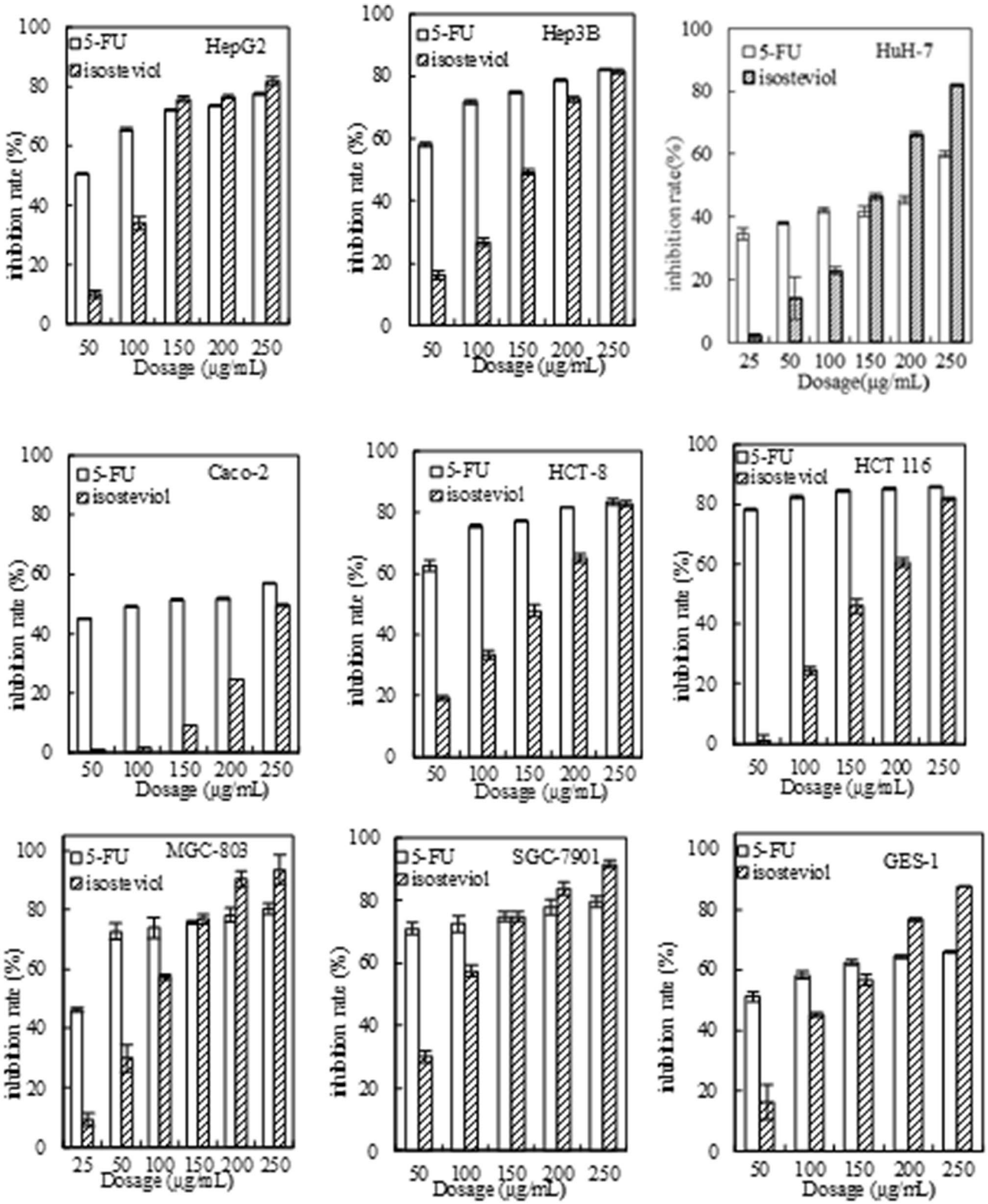


Fig. 5 Inhibition on human cancer cells proliferation with isosteviol (48 h)

5.0 mL/h of flow rate and 6 g acidic ion-exchange resin; the isosteviol production intensity was up to 1.54 g/L/h with 97.23% of St conversion. Except greatly expediting the reaction with the pure product, this process can erase the need for recrystallizing.

Anticancer potential of isosteviol

As aforementioned, there are many reports on the anticancer activity of isosteviol derivative [4, 9, 29]. Isosteviol was shown to prevent the growth of human cancer cells (human B cell acute lymphoblastoid leukemia cell BALL-1, human T cell acute lymphoblastic leukemia cell MOLT-4, and human gastric cancer cell NUGC-3) with LD_{50} values of 84–167 μM, and IC_{50} value for DNA polymerases was 64.0 μM [30, 31]. Isosteviol showed exhibited strong inhibitory effects in a two-stage carcinogenesis test using mouse skin induced by DMBA and 12-O-tetradecanoylphorbol-13-acetate (TPA), and the inhibitory was greater than that of glycyrrhizin [32].

In this study, nine human cancer cell and normal lines were tested with isosteviol treatment (Fig. 5). For comparison, 5FU (LD_{50} is 115 mg/kg, oral, mouse) was used as the positive control in this experiment. And the LD_{50} of isosteviol is 0.5 g/kg body weight (dogs, rats and mice, oral) [33], which is 3 folders of that of 5-FU.

As shown in Fig. 5, isosteviol presented similar inhibition as 5-FU, and inhibited the cells viability in a time and dose-dependent manner (see also Supporting material Fig. 4S). Moreover, isosteviol even performed stronger inhibition on some cell lines, such as Huh-7 and MGC-803.

Furthermore, flow cytometry analysis was performed to determine the cell cycle distribution and population of dead cell in isosteviol-treated cells. As it was reported, 5-FU (1000 ng/mL) inhibited SW480 and COLO320DM by a transient G1-S phase arrest [34]. Similarly, as Fig. 6 and Table 1 indicated, isosteviol also caused cell phase arrest and apoptosis. Specifically, isosteviol treatment caused G1 arrest on SGC-7901, HCT-8 and HCT-116, S arrest on HepG2, Huh-7 and HepG3B, G2 arrest on MGC-803, respectively.

Conclusions

In this work, a reaction coupled separation for preparing isosteviol was achieved, and for the first time, anticancer activity of isosteviol on human cancer cells was preliminarily explored.

With an in-situ separation, simply with water-washing, the product with 92.98% purity of isosteviol was obtained with a stevioside conversion of 97.23% in a packet bed reactor. Compared to the assayed inorganic acid, organic acids and acidic ionic liquids, the acidic ion-exchange resins provided high product selectivity towards isosteviol. Isosteviol presented similar inhibition on several human cancer cell lines as 5-Fluorouracil (5-FU) did, in a time and dose-dependent manner through inducing cell phase arrest; and it even performed stronger inhibition on Huh-7 and MGC-803 cells. Isosteviol treatment caused G1 arrest on SGC-7901, HCT-8 and HCT-116 cells, S arrest on HepG2, Huh-7 and HepG3B cells, and G2 arrest on MGC-803 cells,

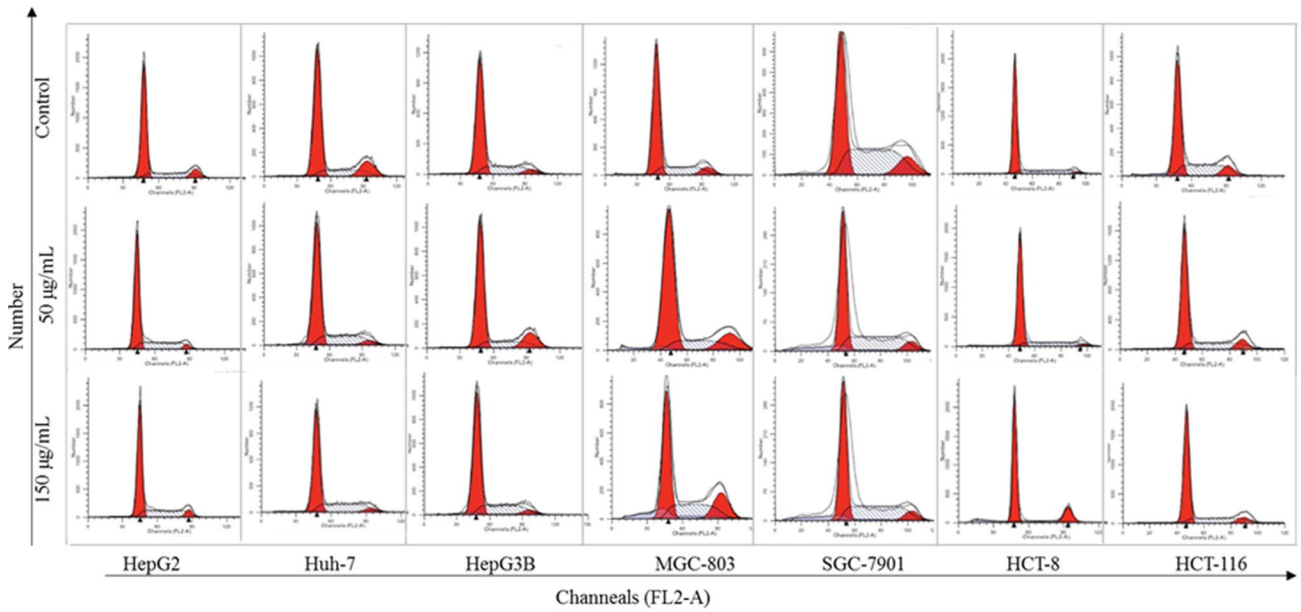


Fig. 6 Effect of isosteviol on cell cycle distribution in the human gastrointestinal and liver cancer cells

Table 1 Effect of isosteviol on cell cycle progression of the gastrointestinal and liver cancer cells

Cell	Dosage (μg/mL)	G1 (%)	S (%)	G2 (%)
HepG2	Control	69.91	22.09	8.00
	50	65.69	29.39	4.91
	150	65.96	30.47	3.57
Huh-7	Control	70.89	21.22	8.00
	50	65.69	29.39	4.91
	150	62.55	32.69	4.77
HepG3B	Control	70.81	5.02	24.17
	50	65.68	33.55	0.77
	100	59.20	39.50	1.30
MGC-803	Control	67.66	24.40	7.95
	50	64.99	19.57	15.44
	150	49.63	30.31	20.06
SGC-7901	Control	45.05	43.81	11.15
	50	60.41	39.29	0.30
	200	66.86	33.14	0.00
HCT-8	Control	73.05	24.41	2.55
	50	73.52	23.00	3.48
	150	86.05	5.95	8.00
HCT-116	Control	57.43	34.57	8.00
	50	66.77	25.23	8.00
	200	69.95	24.01	6.04

respectively. This implies isosteviol a hopeful potential on anti-cancer activity.

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Compliance with ethical standards

Conflict of interest There are no conflicts to declare.

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