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Original Research Article

Identification of the chemical components of ethanol extract of *Chenopodium ambrosioides* and evaluation of their *in vitro* antioxidant and anti-tumor activities

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Abstract

Purpose: *To determine the characteristic chemical components of the ethanol extract of Chenopodium ambrosioides and evaluate their antioxidant and anti-tumor effects in vitro.*
Methods: *The plant powder (5 g) was extracted with 1 L of 80 % ethanol at room temperature for 45 min, and then placed at 60 ^{o}C at varying microwave power and duration to obtain optimal extraction conditions. Characteristic chemical components were detected using ultra-high performance liquid chromatography quadrupole time of flight mass spectrometry (UPLC-Q-TOF-MS/MS). Kaempferitrin was isolated from the 80 % ethanol extract using a D101 macroporous resin column, and its content was assessed by high performance liquid chromatography (HPLC). The antioxidant effect of kaempferitrin was evaluated by its ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) radicals, while its anti-proliferation activity in human liver cancer cells SMMC-7721 was determined using cell counting kit-8 (CCK-8) reagent.*
Results: *Three characteristic components of ethanol extract of C. ambrosioides were obtained, namely, kaempferitrin, kaempferol-3-O-apigenin-7-O-rhamnoside and kaempferol-3-O-acetylapigenin-7-O-rhamnoside. Kaempferitrin was shown to possess strong DPPH radical and moderate ABTS radical scavenging activities. Kaempferitrin significantly inhibited the proliferation of SMMC-7721 cells at doses of 4 and 8 µg/mL, with half-maximal concentration (IC_{50}) of 0.38 µM ($p < 0.05$).*
Conclusion: *Kaempferitrin extracted from C. ambrosioides has antioxidant and anti-tumor activities. The results reported here indicate that C. ambrosioides may have potential use in herbal medicine practice.*

Keywords: *Antioxidant, Anti-tumor, Chenopodium ambrosioides, Kaempferitrin, UPLC-Q-TOF-MS/MS*

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INTRODUCTION

Chenopodium ambrosioides, a perennial plant native to tropical America, is widespread in many areas of China [1,2]. The plant has been reported to possess anti-tumor effect [3-5]. Volatile oil and ethanol extract of *C. ambrosioides* were shown to have anti-proliferation effect on human breast cancer cells MCF-7 *in vitro* [3,4]. Ethanol extract of *C. ambrosioides* reduced the number of tumor

cells in Ehrlich tumor-bearing mice, and also increased mice life span [5].

The chemical composition of volatile oil of *C. ambrosioides* has been determined in numerous studies [2,6-10]. However, the chemical components of ethanol extract, particularly characteristic components with pharmacological effects, have been unknown. Therefore, the goal of this study was to identify the main chemical compounds in the ethanol extract of *C. ambrosioides* and evaluate their *in vitro* antioxidant and anti-tumor activities.

EXPERIMENTAL

Plant materials

C. ambrosioides were collected from a garden at Xilong Village, Qingshen County, Meishan City, Sichuan Province of China (North: 29°84′64.36″, East: 103°80′58.04″).

Cell culture

Human liver cancer cell lines (SMMC-7721) was grown in RPMI-1640 medium containing 10 % fetal bovine serum, in an incubator (5 % CO_2, 37 ºC).

Microwave-ultrasonic assisted extraction of *C. ambrosioides*

The dried aerial parts of the *C. ambrosioides* plants were grounded into powder using high speed pulverizer (Yongkang Jiupin Ltd, China). The powder (5 g) was extracted with 1 L of 80 % (v/v) ethanol at room temperature for 45 min, and then placed in a microwave-ultrasonic assisted extractor (Beijing Xianghu Technology Ltd, China) at 60 ºC with different power of microwave (100, 150, 200, 250 and 300 W) and ultrasonic (100, 200 and 300 W) for various time durations (5, 10, 15, 20, 25 and 30 min). The extracts were collected, filtered through 0.45 µm membrane (Tianjin Jinteng Ltd, China) and used for analysis of kaempferitrin.

Analysis of ethanol extract

The H-class ultra-high performance liquid chromatography-mass spectrometer (UPLC-MS, Waters, USA) equipped with a diode array detector and a Waters UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 µm film thickness) was applied to analyze the main chemical compounds of ethanol extract. The parameters were: temperature of T3 column 35 ºC; flow rate 0.3 mL/min; sample amount 3 µL. Mobile phase A consisted of acetonitrile and methanol at volume ratio of 5:1, and mobile phase B was 0.1 % formic acid. The gradient elution program of mobile phase A and B: 0 - 5 % A, 0 - 2.5 min; 5 - 20 % A, 2.5 - 12.5 min; 20 - 24 % A, 12.5 - 25 min; 24 - 40 % A, 25 - 30 min; 40 - 90 % A, 30 - 35 min; 90 % A, 35 - 45 min.

The main chemical components of ethanol extract were identified using an Agilent 6540 ultra-high resolution quadrupole time of flight mass spectrometry (Q-TOF-MS/MS) and electrospray ionization (ESI) system (Waters, USA). The operating conditions of ESI-MS were: 99.999 % nitrogen gas; flow rate 8 L/min; atomization temperature 325 ºC; spray pressure 40 psi; temperature of sheath gas 350 ºC; flow rate of sheath gas 11 L/min; collision energy range 0 - 50 V; capillary voltage 4000 V. Mass ranged from *m*/*z* 100 - 200 to *m*/*z* 50 - 1250 in positive ion mode for the first-level and second-level mass spectrum scan, respectively. Data analysis was carried out using Peak View (SCIEX, USA).

Determination of kaempferitrin concentration

High performance liquid chromatography (HPLC, Shimadzu, Japan) equipped with a diode array detector and a Kromasil C18 column (250 mm × 4.6 mm, 5 µm film thickness) was used to measure kaempferitrin concentration in each sample. The parameters were: temperature of C18 column 35 ºC; flow rate 1 mL/min; volume of the test sample 10 µL. Mobile phase A consisted of acetonitrile and methanol at volume ratio of 5:1, and mobile phase B was 0.1 % formic acid. The gradient elution program of mobile phase A and B: 0 - 5 % A, 0 - 2.5 min; 5 - 20 % A, 2.5 - 12.5 min; 20 - 24 % A, 12.5 - 25 min; 24 - 40 % A, 25 - 30 min; 40 - 90 % A, 30 - 35 min; 90 % A, 35 - 45 min. Standard kaempferitrin stock solution was prepared by dissolving standard kaempferitrin (Shanghai Yuanye Biotechnology Co. China) in 5 mL of 80 % ethanol, resulting in the density of 50 µg/mL. The standard stock solution was diluted with 80 % ethanol to the concentrations of 10, 20, 30 and 40 µg/mL for calibration. Kaempferitrin concentration of each extracted sample was estimated according to the regression equation of standard calibration curve ($Y = 0.0413X + 0.7371$, in which Y is the absorbance value of the sample, X is the concentration of kaempferitrin (µg/mL), R^2 is 0.9969, linearity ranges 0 - 50 µg/mL).

Isolation of kaempferitrin from ethanol extract

Fifteen bed volume (BV) of the sample solution extracted at microwave and ultrasonic power of 300 W for 30 min was added into a D101

macroporous adsorption resin column (*Sigma*-Aldrich, USA), and then adsorbed by the resins at a flow rate of 2 BV/h. The column was eluted with 5 BV of 50 % ethanol at 2 BV/h. The eluent was frozen at -80 ºC for 3 days to obtain the crystallized product, which was then dried into powder in a vacuum drier (Beijing Boyikang Experiment Instruments Co. China).

Antioxidant activity assays

The DPPH radical scavenging effects of kaempferitrin and two control groups (rutin and quercetin) at the concentrations of 1, 2, 4 and 8 µg/mL were measured using DPPH scavenging activity test kit (Jiancheng Shengwu Gongcheng Co., Nanjing China), on the basis of the kit instructions. The choice of concentrations used to test antioxidant and anti-proliferation effects was based on outcomes from preliminary experiments. Each assay was repeated three times. The absorbance value was determined using a microplate reader (Molecular Devices, USA) at 25 °C under 517 nm wavelength. Standard trolox solution was used for calibration. DPPH scavenging activity (D) was calculated as in Eq 1.

D (%) = [1-(As-Ac/Ab) × 100 ……. (1)

where As = the absorbance value of the well, which contained kit reagent and test compound (kaempferitrin, rutin, and quercetin); and Ab = the absorbance value of the well, which contained kit reagents and 80 % methanol; Ac = the absorbance value of the control well, which contained 80 % methanol and test compound (kaempferitrin, rutin, and quercetin).

The total antioxidant activities of kaempferitrin and control groups (rutin and quercetin) at the concentrations of 1, 2, 4 and 8 µg/mL were measured using ABTS scavenging activity test kit (Jiancheng Shengwu Gongcheng Co., Nanjing China), on the basis of the kit instructions. Each assay was repeated three times. The absorbance value was determined using a microplate reader (Molecular Devices, USA) at 25 °C under 405 nm wavelength. Standard trolox solution was used for calibration. ABTS scavenging activity (A) was calculated as in Eq 2.

A (%) = (Ab-As)/Ab × 100 ……. (2)

where As = the absorbance value of the well, which contained kit reagent and test compound (kaempferitrin, rutin and quercetin), and Ab = the absorbance value of the well, which contained kit reagents

Evaluation of anti-proliferation effect

The inhibitory effect of kaempferitrin on proliferation of SMMC-7721 cells was evaluated using cell counting kit-8 (Jiancheng Shengwu Gongcheng Co., Nanjing China). 2000 cells per well were cultured in 96-well plates overnight. Kaempferitrin, rutin, and quercetin were applied individually to each well at the densities of 0, 1, 2, 4, and 8 µg/mL. Treatments lasted for 24 h and ended with 3-h incubation of CCK-8 solution. Each assay was repeated three times. The absorbance value was determined using a microplate reader (SpectraMax i3x, Molecular Devices, USA) at 25 °C under 450 nm wavelength. The cell viability (V) was determined as in Eq 3.

V (%) = (As-Ab)/(Ac-Ab) × 100 ……. (3)

where As = the absorbance value of the well, in which cells were treated individually with kaempferitrin, rutin, and quercetin; Ac = the absorbance value of the well, in which cells were not treated individually with kaempferitrin, rutin, and quercetin; and Ab = the absorbance value of the well, which contained kit reagent and medium.

Statistical analysis

Analysis of data by Student's t-test was accomplished using SPSS software (SPSS Inc, USA). $P < 0.05$ was considered statistically significant. Figures were drawn using Microsoft Excel 2010 (Microsoft, USA).

RESULTS

Optimized extraction conditions

In order to optimize the extraction conditions of *C. ambrosioides*, two solvents (methanol and ethanol) at the concentration of 80 % were used to extract *C. ambrosioides* to determine which type of solvents was optimal for production of characteristic components from the extracts. The results showed that 80 % methanol and ethanol extracts of *C. ambrosioides* contained three main characteristic components, namely components 1, 2 and 3 (Figure 1). Ethanol extract produced higher yield of these three components (Table 1) than methanol extract, and therefore was used as the solvent for extraction of *C. ambrosioides*.

Furthermore, the yield of kaempferitrin, component 1 of the three main characteristic components identified with UPLC-Q-TOF-MS later in this study, was used as the evaluation index to find the optimal microwave-ultrasonic

assisted extraction condition. The extraction condition, under which the sample was extracted with 300 W microwave power and 300 W ultrasonic power for 30 min, produced the maximal yield of kaempferitrin (27.4 ± 2.3 µg/mL, Table 2), although the extractions at the same power of microwave and ultrasonic for 20 and 25 min also produced high yield of kaempferitrin (27.0 ± 1.2 and 26.9 ± 1.2 µg/mL, respectively).

Identified three main chemical components

Mass spectrometer was more sensitive in negative ion mode than positive ion mode to detect flavonoids, which were supposed to be the main components of ethanol extract of *C. ambrosioides,* according to the literature [11]. However, the mobile-phase modifiers commonly used in negative ion mode (e.g. NH_4HCO_2 and CH_3COONH_4) suppress ionizations of some flavonoids [11]. A gradient elution with mobile-phase A and B in this study produced strong signal in positive ion mode.

The first main characteristic component (component 1) of 80 % ethanol extract of *C. ambrosioides* had first-level mass spectrum of *m/z* 579.1723 $(M + H)^+$ and second-level mass spectrum of *m/z* 433.1089 $(M\text{-}Rha+H)^+$, 287.0517 $(M\text{-}Rha\text{-}Rha+H)^+$ (Figure 2), with the molecular formula $C_{27}H_{30}O_{14}$ (Table 3).

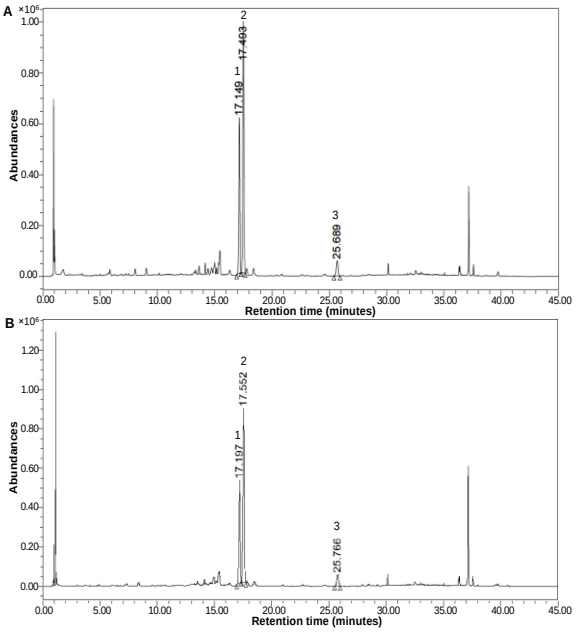

Figure 1: The UPLC spectra of *C. ambrosioides* at 265 nm UV. A: 80 % methanol; B: 80 % ethanol

Table 1: Data of UPLC analysis for three components of 80 % methanol and 80 % ethanol extracts at 265 nm UV

Solvent	Component No.	Retention Time (min)	Area (mV*s)	Area (%)	Height (mV)
80 % methanol	1	17.149	3719846	34.62	612641
	2	17.493	6370360	59.29	988415
	3	25.689	653611	6.08	59692
80 % ethanol	1	17.197	3829088	33.50	519113
	2	17.552	6946508	60.77	883371
	3	25.766	654753	5.73	56726

Table 2: Concentration of kaempferitrin in each sample extracted with different microwave and ultrasonic power at various time durations

Microwave power (w)	Ultrasonic power (w)	Kaempferitrin concentration (µg/mL) *Extraction time (min)*					
		5	10	15	20	25	30
100	100	10.3±1.5	6.2±0.8	6.3±0.5	6.4±0.7	5.8±1.3	9.8±1.1
100	200	5.2±1.1	7.0±0.8	7.0±0.7	5.7±1.1	5.5±1.1	6.3±1.3
100	300	14.7±2.2	14.1±2.0	16.2±2.1	18.1±2.0	11.7±2.2	11.5±2.6
150	100	11.5±1.2	17.5±2.6	13.6±1.7	14.6±1.6	13.8±1.1	16.4±1.4
150	200	14.3±1.9	11.1±1.5	14.6±1.6	17.4±1.1	15.9±1.5	14.7±1.3
150	300	15.2±1.2	12.0±0.8	14.0±1.0	14.2±0.8	17.9±1.2	15.6±1.7
200	100	18.3±2.0	10.7±1.0	12.5±1.1	13.7±1.0	12.6±1.3	13.0±1.1
200	200	15.8±1.1	13.3±0.7	13.0±0.8	14.0±1.2	15.2±1.2	16.5±1.1
200	300	10.9±1.1	16.8±1.3	14.3±1.8	12.4±1.5	11.4±1.3	15.4±1.2
250	100	8.4±0.8	11.2±0.6	12.8±0.7	13.4±0.8	15.2±0.9	15.8±2.8
250	200	7.0±1.7	7.6±1.8	11.1±2.5	8.4±3.3	15.9±1.6	12.4±2.6
250	300	9.9±0.8	11.1±1.1	8.9±0.9	12.0±0.4	14.3±0.6	13.9±1.9
300	100	14.0±1.2	16.9±1.9	17.5±1.5	14.1±2.2	17.6±2.4	19.6±0.5
300	200	12.7±0.6	13.3±1.0	14.3±0.7	12.3±0.9	17.8±0.8	18.5±1.0
300	300	16.8±0.8	18.9±1.4	15.7±2.3	27.0±1.2	26.9±1.2	27.4±2.3

This information was consistent with the previous report on kaempferol-3,7-O-α-L-dirhamnoside (kaempferitrin) [12]. Its content in ethanol extract of *C. ambrosioides* was 33.5 % (Table 3).

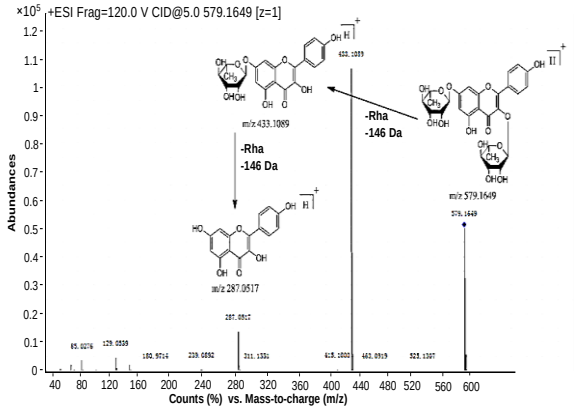

Figure 2: The mass fragmentation pathway of component 1

Component 2 had first-level mass spectrum of *m/z* 565.1572 {M+H}$^+$ and second-level mass spectrum of *m/z* 433.1091 (M-Rha+H)$^+$ and 287.0519 (M-Rha-Rha+H)$^+$ (Figure 3), with the molecular formula $C_{26}H_{28}O_{14}$ (Table 3). The *m/z* 433.1091 was very close to *m/z* 433.1089, the second-level mass spectrum of component 1, inferring that the parent nucleus of component 2 was kaempferol-3,7-O-α-L-dirhamnoside. The loss of 132 Da from *m/z* 565.1572 to 433.1091 indicated one molecule of apigenin, and the loss of 146 Da from *m/z* 433.1091 to 287.0519 indicated one molecule of rhamnose. This information inferred that component 2 was kaempferol-3-O-apigenin-7-O-rhamnoside. Its content in ethanol extract of *C. ambrosioides* was 60.77 % (Table 3).

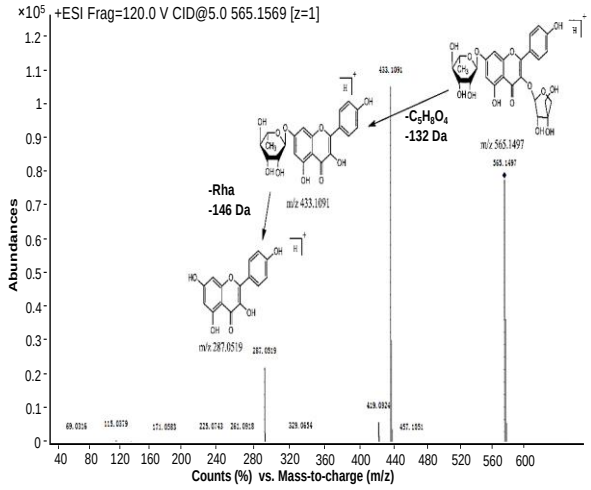

Figure 3: The mass fragmentation pathway of component 2

Component 3 had first-level mass spectrum of *m/z* 607.1677 (M+H)$^+$ and second-level mass spectrum of *m/z* 433.1080 (M-Rha+H)$^+$ and 287.0524 (M-Rha-Rha+H)$^+$ (Figure 4), with the molecular formula $C_{28}H_{30}O_{15}$ (Table 3). The *m/z* 433.1080 was close to *m/z* 433.1089, the second-level mass spectrum of component 1, inferring that the parent nucleus of component 3 was kaempferol-3,7-O-α-L-dirhamnoside. *m/z* 287.0524 represented one molecule of rhamnose, and the loss of 174 Da from *m/z* 607.1677 to 433.1080 indicated one molecule of acetylapigenin. This information inferred that component 3 was kaempferol-3-O-acetylapigenin-7-O-rhamnoside. Its content in ethanol extract of *C. ambrosioides* was 5.73 % (Table 3).

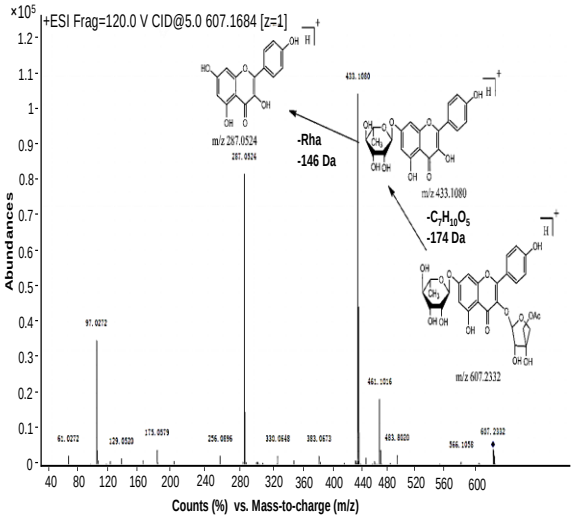

Figure 4: The mMass fragmentation pathway of component 3

Antioxidant effects

Although component 1 (kaempferitrin) accounted for 33.5 % of ethanol extract of *C. ambrosioides*, it has been reported to be a pharmaceutical flavone glycoside with multiple effects, such as antioxidant and anti-proliferation effects [13,14]. In this study kaempferitrin was identified as the major characteristic component of ethanol extract of *C. ambrosioides*, and its antioxidant effects on DPPH and ABTS radicals were detected. Kaempferitrin showed relatively high scavenging activity against DPPH with IC_{50} of 18.263 μg/mL, whereas the strong antioxidants rutin and quercetin had IC_{50} of 1.4 and 2.368 μg/mL, respectively (Figure 5). Kaempferitrin showed moderate scavenging activity against ABTS with IC_{50} of 34.21 μg/mL, whereas rutin and quercetin had IC_{50} of 1.186 and 2.792 μg/mL, respectively (Figure 6).

Table 3: The UPLC-Q-TOF-MS data for three main characteristic components of ethanol extract of *C. ambrosioides*

Component	Retention time (min)	UV_{max} (nm)	Molecular formula	Characteristic ion fragments (*m/z*)		Identification	Content (%)
				MS	MS/MS		
1	17.197	263.8, 341.1	$C_{27}H_{30}O_{14}$	579.1649 $(M+H)^+$	433.1089$(M\text{-}Rha+H)^+$, 287.0517$(M\text{-}Rha\text{-}Rha+H)^+$	kaempferol-3,7-O-α-L-dirhamnoside (kaempferitin)	33.5
2	17.552	265, 344.5	$C_{26}H_{28}O_{14}$	565.1497 $(M+H)^+$	433.1091$(M\text{-}C_5H_8O_4+H)^+$, 287.0519$(M\text{-}C_5H_8O_4\text{-}Rha+H)^+$	kaempferol-3-O-apigenin-7-O-rhamnoside	60.77
3	25.766	265, 345.7	$C_{28}H_{30}O_{15}$	607.2332 $[M+H]^+$	433.1080$(M\text{-}C_7H_{10}O_5+H)^+$, 287.0524$(M\text{-}C_7H_{10}O_5\text{-}Rha+H)^+$	kaempferol-3-O-acetylapigenin-7-O-rhamnoside	5.73

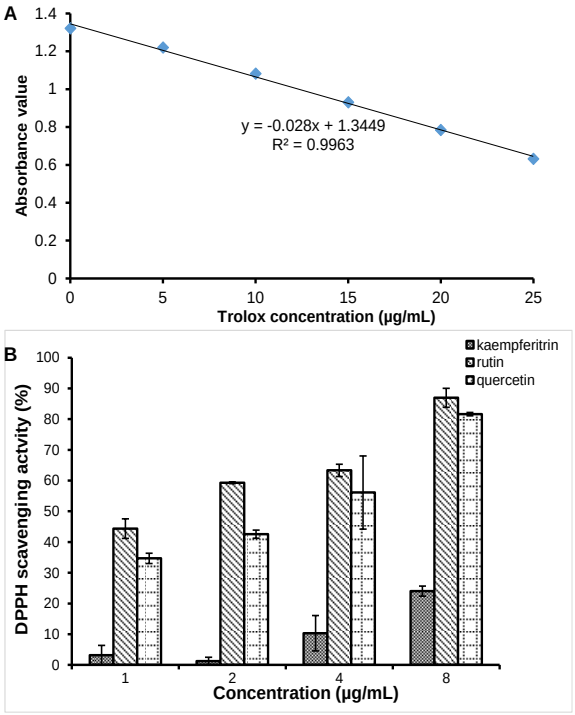

Figure 5: Antioxidant effect of kaempferitrin on DPPH radicals

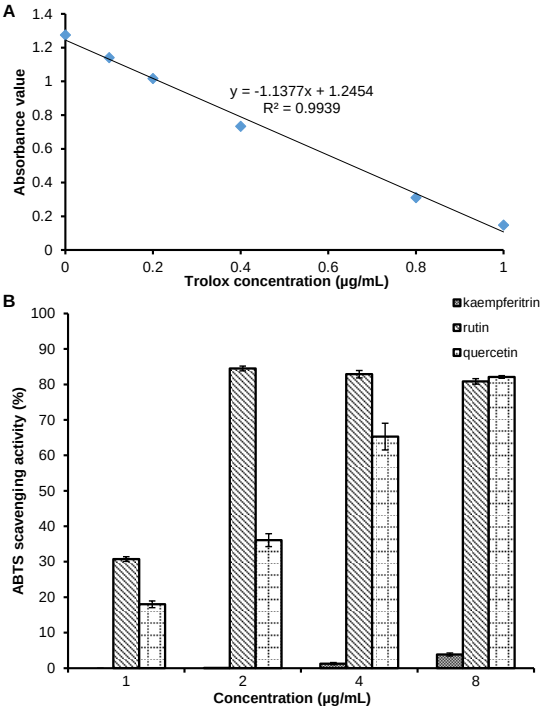

Figure 6: Antioxidant effect of kaempferitrin on ABTS radicals

Anti-proliferation activity

The viability of cells was significantly decreased at kaempferitrin treatment concentrations of 4 and 8 μg/mL ($p < 0.05$), with IC_{50} of 0.38 μM (Figure 7). However, rutin and quercetin did not inhibit proliferation of SMMC-7721 cells.

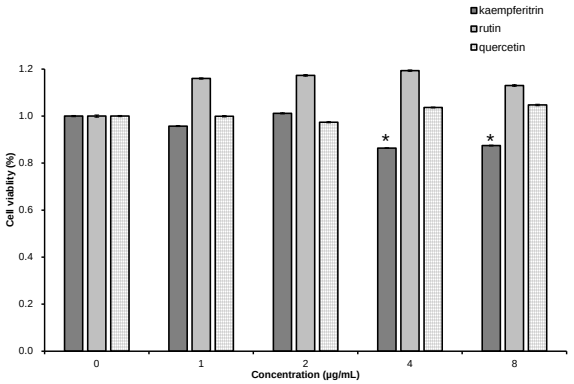

Figure 7: Anti-proliferation activity of kaempferitrin on SMMC-7721 cells *in vitro*

DISCUSSION

Medicinal plant extracts are mixtures of complex primary and secondary metabolites due to differences in many factors, e.g., plant genotype, growth environment and extraction conditions. The chemical composition of ethanol extract of *C. ambrosioides* varied among different studies [12,15]. Twelve compounds identified in 95 % ethanol extract of *C. ambrosioides* in a previous study [12] were different from five compounds (chenopodiumamines A, B, C, D and chenopodiumoside A) identified in 95 % ethanol extract in another study [15]. In this present study, three main characteristic chemical components identified in 80 % ethanol extract of *C. ambrosioides* were kaempferitrin, kaempferol-3-O-apigenin-7-O-rhamnoside and kaempferol-3-O-acetylapigenin-7-O-rhamnoside, with their contents of 33.50, 60.77 and 5.73 %, respectively. Interestingly, these three components were kaempferol derivatives.

Kaempferitrin has been reported to possess several pharmacological effects [13,14,16,17]. Kaempferitrin was shown to be able to scavenge reactive oxygen species (ROS), and its antioxidant activity varied with the type of ROS. For example, its antioxidant ability was higher in HOCl than in superoxide anion and taurine-chloramine [13]. This study enriched information on antioxidant effect of kaempferitrin by demonstrating that kaempferitrin had high ability to scavenge DPPH and moderate ability to scavenge ABTS. Kaempferitrin induced apoptosis of HeLa cells in a caspase-dependent pathway, and decreased tumor weight in tumor-bearing mice [14]. In this study kaempferitrin had significant anti-proliferation effect on SMMC-7721 cells. The relatively high content of

antioxidant and anti-tumor kaempferitrin in ethanol extract of *C. ambrosioides* explained the reason why ethanol extract of *C. ambrosioides* inhibited proliferation of MCF-7 cells *in vitro* [4] and growth of Ehrlich tumor *in vivo* [5].

CONCLUSION

Three main characteristic components of ethanol extracts of *C. ambrosioides* are kaempferitrin, kaempferol-3-O-apigenin-7-O-rhamnoside and kaempferol-3-O-acetylapigenin-7-O-rhamnoside.

Kaempferitrin has antioxidant effects on DPPH and ABTS radicals, and anti-proliferation effect on SMMC-7721 cells. The results reported here indicate that *C. ambrosioides* has potential use in herbal medicine practice.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. Jiao Li and Hong Zhang conceived and designed the study. Jiao Li, Chaojin Si, Wenjing Hong, Chaoguo Xia, Yuqi Yang, Yiting He, Min Su, Xuehua Long and Hong Zhang performed the experiments and collected the data. Jiao Li and Chaojin Si analyzed the data. Jiao Li wrote the manuscript. All authors read and approved the manuscript for publication.

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