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Research paper

Camellia euphlebia flower extract inhibits oleic acid-induced lipid accumulation *via* reduction of lipogenesis in HepG2 cells

Dongye He[a,b], Ping Zhang[a,b], Xuan Sai[a,b], Xiaoyu Li[a,b], Lili Wang[a,b], Yongping Xu[a,b,*]

[a] School of Life Science and Biotechnology, Dalian University of Technology, Dalian, 116024, China
[b] Ministry of Education Center for Food Safety of Animal Origin, Dalian University of Technology, Dalian, 116620, China

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ABSTRACT

Introduction: *Camellia euphlebia* is a medicinal and edible plant used to treat hepatic disease in Southern China. However, there has been no report on the anti-obesity and hypolipidemic effects of *Camellia euphlebia* flower. This study evaluated the hepatic lipid-lowering potential of different preparations of *Camellia euphlebia* flower extracts using *in vitro* models.

Methods: A model of hepatic steatosis in the HepG2 cells was induced by oleic acid. HepG2 cells were divided into control group, oleic acid group, oleic acid plus aqueous extract group and oleic acid plus ethanol extract group. The cytotoxicity was quantitatively assessed by methyl-thiazolyl-tetrazolium and lactate dehydrogenase release assay. Lipid accumulation and intracellular triglyceride level were also evaluated by oil red O staining and a commercially available assay kit respectively. Expressions of 3-hydroxy-3-methylglutaryl CoA reductase, fatty acid synthase, acetyl-CoA carboxylase and glycerol-3-phosphate acyl transferase genes in the HepG2 cells were examined by reverse transcription polymerase chain reaction.

Results: Treatment of HepG2 cells with 100 μg/mL aqueous or ethanol extract significantly reduced lipid accumulation and the level of triglyceride in the cells. Furthermore, pre-treatment of HepG2 cells with 100 μg/mL aqueous extract effectively down-regulated the mRNA levels of fatty acid synthase, 3-hydroxy-3-methylglutaryl CoA reductase and glycerol-3-phosphate acyl transferase, whereas 150 μg/mL ethanol extract only down-regulated the mRNA level of 3-hydroxy-3-methylglutaryl CoA reductase.

Conclusion: These results provided support for the potential preventive effect of *Camellia euphlebia* flower on nonalcoholic fatty liver disease and could partly explain the basis of using *Camellia euphlebia* for the treatment of fatty liver.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is characterized by hepatic fat accumulation in the absence of significant ethanol consumption. Approximately 20–30% of adults are estimated to have excess liver fat accumulation in a normal population [1]. A recent study estimated that in North America, Europe, Australia, and Asia, over 30% afflicted with obesity, 50% with type 2 diabetes, and nearly 100% with morbid obesity suffer from NAFLD [2]. It has been considered that increased free fatty acids supplied to the liver play a major role in the early stage of NAFLD [3]. Currently, some drugs such as vitamin E and obeticholic acid, have demonstrated efficacy in randomized clinical trials, although a drug has not yet to be approved by the US Food and Drug Administration (FDA) for the treatment of NAFLD [4]. Nevertheless, vitamin E use may be associated with increased risks of prostate cancer and hemorrhagic stroke [5,6]. Likewise, concerns were raised about safety due to obeticholic acid's adverse effect of pruritus and unfavorable effects on the lipid profile [4]. Thus, there continues to be an unmet need for developing more effective therapies with fewer (or no) adverse effects in patients with NAFLD.

Recent studies on fatty liver in medicine have focused on the searching for functional food ingredients or herbal extracts that can suppress the accumulation of hepatic lipid. Examples of these plants include *Cichorium intybus* L. [7], *Camellia sinensis* [8], Blueberry [9], *Morus alba* L. [10], *Avena sativa* L. [11] and *Sida rhomboadea* [12]. Thus, functional food ingredients or herbal extracts may provide an effective alternative treatment for NAFLD.

Camellia euphlebia Merr. ex Sealy (Theaceae), called "the Giant Panda of the Plant Kingdom", is an evergreen shrub with a natural distribution limited to North Vietnam and Southwest China, especially

* Corresponding author at: School of Life Science and Biotechnology, Dalian University of Technology, Dalian, 116024, China.
E-mail addresses: 912799291@qq.com (D. He), 821399157@qq.com (P. Zhang), 714942452@qq.com (X. Sai), 6888544@qq.com (X. Li), 1042363178@qq.com (L. Wang), hehe2287@sina.com, hechen2287@sina.com (Y. Xu).

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in the Guangxi Zhuang Autonomous Region. *Camellia euphlebia* (*C. euphlebia*) appeared in the Chinese medical classics Ben Cao Gang Mu about 400 years ago and has been widely used to treat tumor, nephritis, hepatitis with jaundice, urinary tract infection, dysentery, hypertension, diarrhea, faucitis and irregular menstruation. Extracts prepared from *C. euphlebia* have been reported to possess anticancer, antioxidant, anxiolytic, antidepressant and hypoglycemic properties [13–16]. *C. euphlebia* contains many biologically active substances such as flavonoids, polysaccharides, saponins, polyphenols, sterols, and peptides. However, the potential inhibitory effect of extract prepared from *C. euphlebia* flower on fatty liver disease has not yet been supported by pharmacological data.

The present study investigated the effect of different concentrations of aqueous or ethanol extract of *C. euphlebia* on oleic acid (OA)-induced lipid accumulation in human hepatocarcinoma HepG2 cells.

2. Materials and methods

2.1. Plant material

Fresh flowers of *C. euphlebia* were obtained in Guangxi Zhuang Autonomous Region during its flowering period in the winter of 2014. The flowers were authenticated by Dr Zhonghui Ma at the Department of Botany Sciences, College of Agriculture, Guangxi University, China. A voucher specimen with the number 8109255 has been deposited in the herbarium of the Guangxi Insitute of Botany, Chinese Academy of Sciences, China.

2.2. Preparation of C. euphlebia flower extracts

The fresh flowers were washed three times with tap water and then dried at 55 °C for 6 h in a forced air oven. The dried flowers were ground into a coarse powder with a pulverizer (HC-150T2, Yongkang Lv Ke Food Machinery Company, Zhejiang, China) at room temperature, and then further sieved through a 60-mesh sieve to obtain a fine powder. To prepare an aqueous extract of *C. euphlebia* flower, 20 g of the fine powder was extracted with 400 mL distilled water (1:20) for 2 h at 100 °C using an electrical heating jacket (ZNHW, Gongyi Zi Hua Instrument Company, Henan, China). The extract was then cooled to room temperature and filtered through a 0.45-μm PVDF membrane filter. The extraction was repeated and the two filtered extracts were combined and A lyophilized powder with a yield of 36.25% (w/w) was prepared using a freeze dryer (FD-1A-50, Beijing Bo Kang Experimental Medical Instrument Company, Beijing, China) and stored in a sealed bag at −20 °C until use. It was labeled as AEC. An ethanol extract of *C. euphlebia* flower was also prepared using the Soxhlet extraction method [17]. Briefly, 20 g of the dry flower powder was placed in the Soxhlet extraction thimble and extracted with 300 mL anhydrous ethanol for 24 h. The extract was cooled to room temperature and filtered through a 0.45-μm polyvinylidene fluoride (PVDF) membrane, evaporated to dryness at 40 °C using a rotatory evaporator, and then stored at −20 °C until use. It was labeled as EEC. The yield of EEC was 21.11% (w/w). Subsequently, 0.1 g AEC and EEC were dissolved in 10 mL distilled water and anhydrous ethanol, respectively, and used as stock solutions (10 mg/mL) of two extracts.

2.3. Determination of phytochemicals in C. euphlebia flower extracts

The contents of total flavonoids, polysaccharides, polyphenols and saponins in AEC and EEC were determined by the Aluminum chloride [18], Phenol-sulfuric acid [19], Folin-Ciocalteu [20] and Vanillin-glacial acetic acid-perchloric acid colorimetric methods [21], respectively. Rutin, glucose, gallic acid and oleanolic acid were used to generate the calibration curves.

2.4. Cell line culture

HepG2 cells were purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, penicillin (100 unit/mL) and streptomycin (100 μg/mL) (Invitrogen Carlsbad, CA) at 37 °C in a 5% CO_2 incubator.

2.5. Cell viability assay

Cell viability was determined by methyl-thiazolyl-tetrazolium (MTT) assay [22]. Briefly, HepG2 cells were seeded at a density of 5×10^3 cells/well in a 96-well microplate for 24 h, and the cells were then treated with different concentrations (50, 100, 150, 200, 300 and 400 μg/mL) of AEC or EEC for 24 h. At the end of the treatment, the medium was carefully removed and fresh medium containing 0.5 mg/mL MTT was then added to the cells followed by 4-h incubation at 37 °C. After that, the culture medium was replaced with an equal volume of dimethyl sulphoxide (DMSO) followed by 10-min incubation at room temperature. The absorbance of the plate was then measured at 570 nm by using a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific, Vantaa, Finland). Cell viability was expressed as percentage of control (non-treated) cells.

2.6. Lactate dehydrogenase (LDH) release assay

Cytotoxicity was quantitatively assessed by measuring the activity of lactate dehydrogenase (LDH) in the culture medium [23]. HepG2 cells were seeded at a density of 5×10^3 cells/well in a 96-well microplate for 24 h, and the cells were then exposed to different concentrations (50, 75, 100 and 150 μg/mL) of AEC or EEC for 48 h. At the end of the treatment, the plate was centrifuged at 4000 × *g*/4 °C for 5 min to collect the supernatant fraction. The cell pellet was lysed with cell lysis buffer containing 1% Trition X-100. LDH activity in the supernatant and cell lysate was measured with an LDH assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol. The rate of LDH release was calculated using the formula: (Supernatant value − blank value)/[(Supernatant value − blank value) + (lysates value − blank value)] 100%.

2.7. Induction of lipid accumulation in HepG2 cells

HepG2 cells were seeded at a density of 5×10^3 cells/well in a 96-well microplate for 24 h. After that, the cells were incubated with different concentrations (0.1, 0.2, 0.3, 0.4 and 0.5 mM) of OA for 24 h. At the end of incubation, the cells were stained with oil red O to observe the accumulation of intracellular lipid as described below. Additionally, to quantitate lipid content, 100 μL of isopropanol (100%) was added to each sample shaken at room temperature for 10 min, and samples were read spectrophotometrically at 510 nm [6].

2.8. Oil red O staining

HepG2 cells were seeded in a 24-well microplate at a density of 8×10^4 cells/well for 24 h, and then treated with 0.5 mM OA, 0.5 mM OA plus AEC (50, 75 and 100 μg/mL) and 0.5 mM OA plus EEC (50, 75, 100 and 150 μg/mL), respectively, for another 24 h. The cells were then washed twice with phosphate buffered saline (PBS) and fixed in 75% ethanol for 10 min. After they were rewashed with PBS, the cells were stained using working 0.5% Oil Red O in isopropanol for 30 min. For the optical microscopy observation, the cells were further washed with distilled water to remove the unbound dye. Subsequently, after removal of all water, 500 μL of isopropanol (100%) was added to each well shaken, incubated for 10 min and then transferred to another 96-well plate and was read at 510 nm using a Multiskan GO microplate