Journal of Ethnopharmacology 198 (2017) 379–388

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Ginkgo biloba exocarp extracts induces apoptosis in Lewis lung cancer cells involving MAPK signaling pathways

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ARTICLE INFO

Keywords:
Ginkgo biloba exocarp extracts
Lewis lung cancer
Apoptosis
MAPK signaling pathways

Chemical compounds studied in the article:
Cyclophosphamide (PubChem CID: 2907)
Cis-Dichlorodiammine platinum(II) (PubChem CID: 5702198)
Rhodamine 123 (PubChem CID: 65217)
Mannose (PubChem CID: 18950)
Rhamnose (PubChem CID: 25310)
galacturonic acid (PubChem CID: 439215)
glucose (PubChem CID: 5793)
galactose (PubChem CID: 6036)
arabinose (PubChem CID: 66308)
Aspartic acid (PubChem CID: 24868)
glutamic acid (PubChem CID: 33032)
serine (PubChem CID: 5951)
glycine (PubChem CID: 750)
threonine (PubChem CID: 6288)
alanine (PubChem CID: 5950)
proline (PubChem CID: 145742)
valine (PubChem CID: 6287)
methionine (PubChem CID: 6137)
isoleucine (PubChem CID: 6306)
leucine (PubChem CID: 6106)
phenylalanine (PubChem CID: 6140)
tryptophan (PubChem CID: 6305)
lysine (PubChem CID: 5962)
Pb (PubChem CID:5352425)
Cr (PubChem CID: 23976)
Cu (PubChem CID: 23978)
As (PubChem CID: 5359596)
Hg (PubChem CID: 23931)

ABSTRACT

Ethnopharmacological relevance: A fruit of *Ginkgo biloba* L. is known as Ginkgo nuts. It is an edible traditional Chinese medicine, and could be used for the treatment of cancer thousands of years ago in China. The extracts prepared from the exocarp of *Ginkgo biloba* (*Ginkgo biloba* exocarp extracts, GBEE) has the effects of anti-cancer, immune promotion, anti-aging and etc.

Aim of study: To study the effects of GBEE inducing apoptosis in Lewis lung cancer (LLC) cells and the role of Mitogen-activated protein kinase(MAPK) signaling pathways in it.

Materials and methods: The LLC solid tumor model was established in C57BL/6J mice. The tumor-bearing mice were randomly divided into 5 groups. A normal control group without tumor cells was established additionally. There were 10 mice in each group, and they were dosed 24 h after inoculation. The GBEE (50, 100, 200 mg/kg b.w.) groups were dosed by intragastric gavage (i.g.). The mice in positive control group were intraperitoneal (i.p.) injected with cyclophosphamide (CPA) at a dose of 20 mg/kg (b.w.). The model control group and the normal control group were both given normal saline (NS) by i.g.. All the groups were dosed at a volume of 0.1 mL/10 g (b.w.), once a day for 18d. The day after the last administration, the transplanted tumors was stripped and weighed, and the inhibition rate was calculated. In vitro experiments, MTT method was applied to detect the effects of GBEE on LLC cells and primary cultured mouse lung cells. Annexin V-FITC/PI method was used to detect the apoptosis rate of LLC cells. Rhodamine 123 method was used to detect the Mitochondrial transmembrane potential (MTP). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to detect the levels of Fas mRNA. Western Blot was used to detect the expression of Bax, Bcl-2, Cyt C, cleaved Caspase-3 and MAPK proteins in the corresponding parts of LLC cells.

Results: GBEE (50–200 mg/kg) inhibited the growth of LLC transplanted tumors with a dose-effect relationship. GBEE (5–160 μg/mL) inhibited the proliferation of LLC cells in vitro with the half maximal inhibitory concentration (IC50) value of 162.43 μg/mL, while it had no significant inhibitory effects on the primary cultured mouse lung cells. After GBEE (10, 20 and 40 μg/mL) acted on the LLC cells, the apoptosis rate was increased and the MTP was decreased. The ratio of Bax/Bcl-2 was increased in the cells. Meanwhile, it also promoted the translocation of Bax/Bcl-2 in mitochondrial membrane and the release of Cyt C from mitochondria to cytosol. In addition, it up-regulated the cleaved-Caspase-3 protein expression. The mRNA levels of Fas and the protein levels of Fas, FasL and p-p38 in the cells were both increased. The levels of p-ERK1/2 and p-JNK1/2 protein were down-regulated but the p38, ERK1/2 and JNK1/2 were not significantly changed.

Conclusions: GBEE induces apoptosis in LLC cells via mitochondrial-mediated intrinsic pathway and death receptor-mediated extrinsic pathway, which may be closely relevant to the regulation of MAPK signaling pathways.

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http://dx.doi.org/10.1016/j.jep.2017.01.009
Received 30 September 2016; Received in revised form 6 January 2017; Accepted 8 January 2017
Available online 20 January 2017
0378-8741/

1. Introduction

Lung cancer is a common malignant tumor. There have been over one million persons emerging lung cancer annually in the world (Yao and Liu, 2014). The extremely high mortality has also made it become one of the worldwide concerned diseases. General chemical medicines intervened in the clinical therapy has a certain effect, but the traditional antitumor drugs mainly depending on the cytotoxic effects will inevitably affect normal cells, causing various serious adverse reactions including bone marrow suppression, immune suppression, digestive system toxicity and etc (Zhang et al., 2015a; Zhang et al., 2010; Li and Zhang, 2014). Therefore, it has become an increasingly important issue in the drug development areas to find the antitumor drugs with definite curative effects as well as less adverse reactions.

Apoptosis is the process of initiative cell suicide controlled by genes. In 1992, Hickman (1992) suggested that inducing apoptosis in tumor cells could be the main goal of tumor therapy research for the first time. The effects of natural products and their extracts on the treatment of cancer has been affirmed (Zheng et al., 2016; Moore et al., 2016). Many studies found that some polysaccharide extracts of traditional Chinese medicine can induce apoptosis in tumor cells (Razali et al., 2016; Luo et al., 2016).

Ginkgo biloba L. is a unique precious tree species in China. A fruit of *Ginkgo biloba* L. is also known as Ginkgo nuts. It is nutrient-rich and edible, which was a traditional Chinese medicine thousands of years ago (Xu and Chen, 2003). *Ginkgo biloba* leaves can be prepared into Ginkgo tea (Zhu et al., 2005). *Ginkgo biloba* leaf extracts can be used for the treatment of cardiovascular and cerebrovascular diseases. Therefore, Ginkgo nuts and *Ginkgo biloba* leaves both have an important medicinal value. "Cancer" was called "rock" or "tumor" in ancient China. "Compendium of Materia Medica" and "Renewed Materia Medica" record: Ginkgo nuts have the effects of warming the lung, boosting qi, and eliminating sore, scab, flat-abscess and tumor, which can be used to treat cancer (Li, 1982; Ye, 1919). *Ginkgo biloba* exocarp is the succulent skin outside Ginkgo nuts, often discarded when taking Ginkgo nuts. The extracts prepared by *Ginkgo biloba* exocarp (GBEE) takes the proteoglycan as the main active ingredient (Xu et al., 2010a). Studies showed that GBEE has the activities of immune promoting (Li et al., 2012), anti-aging (Xu et al., 1996b), anti-tumor (Cao et al., 2015) and delaying drug resistance (Hu et al., 2016). Shen et al. (2013) and Han et al. (2016) have reported the therapeutic effects of GBEE on mouse lung cancer. The mechanism involve inhibiting tumor cell adhesion molecules and tumor angiogenesis. However, studies on GBEE affecting apoptosis in lung cancer cells have not yet been involved. In this study, the LLC cell line was used to research the effects and mechanism of GBEE on apoptosis in lung cancer cells in vivo and in vitro.

2. Materials and methods

2.1. GBEE

Samples of Ginkgo nuts were collected in Taixing ginkgo orchard (Jiangsu, China), identified by Director of pharmacists Meng Yin in Yangzhou Food and Drug Inspection and Testing Center (Jiangsu, China) as the family plant of *Ginkgo biloba* L. The GBEE was extracted from the *Ginkgo biloba* exocarp separated from the Ginkgo nuts according to the invention patent method in our laboratory (Xu et al., 2010b). Qualitative analysis of Infrared (IR) spectra showed that the GBEE contains proteoglycan. The content of polysaccharide was 39.63%, measured by phenol-sulfuric acid method, and the content of protein was 26.77%, measured by brilliant blue method. High-Performance Liquid Chromatography (HPLC) detection made clear that the polysaccharide in GBEE contains six kinds of monosaccharides including mannose, rhamnose, galacturonic acid, glucose, galactose and arabinose. The protein contains fourteen kinds of amino acids including aspartic acid, glutamic acid, serine, glycine, threonine, alanine, proline, valine, methionine, isoleucine, leucine, phenylalanine, tryptophan and lysine. The composition analysis showed that the GBEE did not contain ginkgolic acid, flavonoids and terpene lactones. The contents of Pb, Cr, Cu, As and Hg were also in accordance with Chinese Pharmacopoeia standard. The GBEE voucher specimen was deposited at the pharmacy experimental center in Medical College of Yangzhou University.

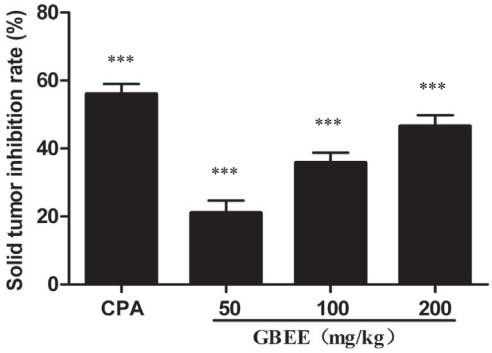

Fig. 1. The inhibitory effects of GBEE on LLC transplanted solid tumor in mice. The LLC transplanted solid tumor model was established in C57BL/6J mice, and randomly divided into 6 groups: normal control group, positive control group, model control group and GBEE (50, 100 and 200 mg/kg) groups. After giving corresponding drugs for 18 days, the tumor blocks was stripped and the inhibition rate was calculated. All data are presented as mean value ± SD and n=10 in each group. ***$P < 0.001$, vs Control.

2.2. Cells

The LLC cells were purchased from Shanghai cell bank, Chinese Academy of Sciences. The cells were maintained in DMEM medium (Gibco, USA) with 10% fetal bovine serum (FBS, Gibco, USA) in a humidified incubator with 5% CO_2 at 37 °C. The cells were subcultured according to the conventional method, and the passage time was generally 3d (Wang et al., 2014). The cells at the exponential phase of growth were used to conduct the experiments.

2.3. Animals

The C57BL/6J mice were 6-weeks-old, weighing (18 ± 2) g, provided by the Center of Comparative Medicine of Yangzhou University (Animal Certificate: SCXK Su 2012-0004; Animal use license: SYXK Su 2012-0029). They were half male and half female. The mice were raised in (22 ± 1) ℃, placed in a 12 h light/12 h dark cycle and acclimated for one week (Silva et al., 2016). All protocols were adopted by the Institutional Animal Care and Ethics Committee of Yangzhou University.

2.4. Inhibition rate of transplanted solid tumor in vivo assay

The LLC tumor-bearing mice were sacrificed by cervical dislocation, and the tumor tissues were separated under sterile conditions. The cell suspension was prepared by conventional method. The cells were counted under a microscope and the cell density was adjusted to 1×10^7 cells/mL using NS. A volume of 0.2 mL of such cell suspension was inoculated subcutaneously in the right forelimb armpit of C57BL/6J mice, and the LLC transplanted solid tumor model was established. The tumor-bearing mice were randomly divided into model control group, positive control group, and 3 dose groups of GBEE. The normal control group without tumor cells was established additionally. There are 10 mice in each group, and they were dosed 24 h after inoculating. The mice in 3 groups of GBEE 50, 100, 200 mg/kg (b.w.) were dosed by intragastric gavage (i.g.). The positive control group were intraperitoneal (i.p.) injected with cyclophosphamide (CPA) at a dose of 20 mg/kg