OncoTargets and Therapy

Dovepress
open access to scientific and medical research

Open Access Full Text Article

ORIGINAL RESEARCH

The total flavonoid of *Eucommia ulmoides* sensitizes human glioblastoma cells to radiotherapy via HIF-α/MMP-2 pathway and activates intrinsic apoptosis pathway

This article was published in the following Dove Press journal:
OncoTargets and Therapy

Yongsheng Wang[1,*]
Xiangru Tan[2,*]
Song Li[1]
Shuilu Yang[1]

[1]Department of Neurosurgery, Caoxian People's Hospital of Shandong Province, Heze, Shandong 274400, People's Republic of China; [2]Department of Neurology, Caoxian People's Hospital of Shandong Province, Heze, Shandong 274400, People's Republic of China

*These authors contributed equally to this work

Background: As one of the most common and lethal malignant primary brain tumors, glioblastomas (GBMs) are identified as grade IV neoplasms, the most severe grade, according to WHO classification systems. The outcome of surgery against GBMs is limited since its frequent relapse. Radiotherapy is a crucial and widely used treatment after surgery, while the strong radioresistance of GBM cells still becomes a severe problem of radiotherapy. *Eucommia ulmoides Oliv.* is used for the treatment of various diseases, such as lower blood pressure and inflammation.
Purpose: To explore the anti-tumor effect of *Eucommia ulmoides Oliv.* against GBMs.
Methods: Dose-viability assays were performed to examine the anti-tumor effect. Would-healing and transwell assays were carried out to evaluate the migration and invasion ability of GBMs. Cell apoptosis was detected by 33, 258 staining, and the expressions of key proteins were examined by western blot.
Results: In this study, we confirmed that the inhibitory effect of the total flavonoid of *Eucommia ulmoides* on proliferation, migration and invasion of human GBM cells. Its favorable effects inspired us to explore the potential ability in enhancing radiosensitivity of GBM cells. The results demonstrated that it could further induce apoptosis during radiotherapy via intrinsic apoptosis pathway. Besides, it could significantly reduce the malondialdehyde level after radiotherapy, which suggested it inhibited tumor cell and protected normal neuronal cells. By examining the expression of important genes of radioresistant pathway, we found a significant decrease of HIF-α/MMP-2 when using the total flavonoid of *Eucommia ulmoides* during radiotherapy.
Conclusion: This result suggests that the enhancement of radiotherapy may be mediated by modulating glucose metabolism of GBMs in HIF-α/MMP-2 pathway.
Keywords: *Eucommia ulmoides*, glioblastoma, radiotherapy, apoptosis, HIF-α/MMP-2 pathway

Introduction

Glioblastoma (GBM) is one of the most frequent and aggressive malignant primary brain tumors in adults.[1–4] There were around 3.5 cases of GBM diagnosed in 100,000 people per year worldwide.[5] According to the classification of WHO for malignant gliomas, which based on their histological and immunohistochemical features, GBMs were identified as grade IV neoplasms, the most severe grade. This was partly due to its high lethality, and patients usually survived for only about 15 months on average. Ninety-seven percent of patients who were diagnosed with GBMs survived shorter than 5 years.[6] GBMs were uncurable and had strong

Correspondence: Shuilu Yang
Department of Neurosurgery, Caoxian People's Hospital of Shandong Province, 1 Fumin Avenue, Heze, Shandong 274400, People's Republic of China
Email shandongjinan2639@163.com

submit your manuscript | www.dovepress.com
DovePress
http://doi.org/10.2147/OTT.S210497

aggressiveness. Tumor cells could easily invade surrounding structures, which made no clear margins between tumors and normal organizations for surgical resection. Thus, normally, GBMs relapsed and developed rapidly at 8–9 months after surgery.[1] The subsequent radiotherapy could help achieve a better outcome, while the GBM was on the list of the most resistant tumors against radiation.[3,4]

Previous studies have reported that GBMs were strongly related to hypoxia, in which glucose metabolism played an important role in tumor growth and radioresistance.[2,7] Hypoxia-inducible factor-1α (HIF-1α) was widely considered as one of the primary mediators in hypoxic responses, and it was shown that HIF-1α was involved in the radioresistance of GBMs to radiotherapy by regulating the glucose metabolism.[8–10] HIF-1α promoted the invasion, migration and survival of GBMs in hypoxic conditions.[11,12] Recent studies suggested that this process was mediated by matrix metalloproteinase-2 (MMP-2).[12–16] MMP-2 was able to degrade various extracellular matrix and induces angiogenesis, which contributed to tumor invasion and metastasis.[17–19] Besides, it was reported that, in hepatocellular carcinoma, the expression of MMP-2 was positively correlated with HIF-1α at both transcriptional and translational levels.[20]

The main strategy of radiotherapy against tumor cells was inducing apoptosis.[21–23] Apoptosis was a kind of programmed cell deaths, which was strictly regulated and involved various biological functions.[24] Both Bcl-2 and Bax were famous apoptosis-mediating factors that were well characterized in the intrinsic apoptosis pathway.[25–28] As an anti-apoptotic protein, Bcl-2 controlled the release of pro-apoptotic factors, such as cytochrome c, as well as mitochondrial membrane potential.[29] Bax, a pro-apoptotic factor, played an important role in cell death by translocating from the cytoplasm to the mitochondrial membrane.[30] The Bcl-2/Bax ratio was suggested to reflect the sensitivity of cell to apoptosis, in which low Bcl-2/Bax ratio was related to stronger sensitivity to radiotherapy and more favorable clinical outcome.[25]

Eucommia ulmoides Oliv. is a traditional medical plant in People's Republic of China, Japan and Korea. It was widely used for its antidiabetic, anti-inflammatory, antihypertensive and diuretic effects.[31–34] However, its antitumor activity still remains unexplored in GBMs.

In this study, we investigated the inhibitory effect of the total flavonoid of *Eucommia ulmoides* on GBMs and reported it could effectively reduce GBMcells growth, migration and invasion. We also found it significantly sensitized GBMs to radiotherapy by inducing intrinsic apoptosis, and reduced malondialdehyde (MDA) level and activity of superoxide dismutase (SOD) after radiotherapy. Furthermore, it was also shown that the enhancement of radiosensitivity was mediated by HIF-α/MMP-2 pathway, which briefly revealed its mechanisms.

Materials and methods

Materials, reagents and antibodies

The leaf of *Eucommia ulmoides* was purchased from Pingxiang city, Jiangxi Province. Rutin was purchased from Sinopharm Chemical Reagent Co., Ltd. DMEM was purchased from Gibco company (CA, USA). Antibody against blc-2 was purchased from ZYMED (CA, USA). Antibodies against BAX and Wee1 were purchased from Sigma (Saint Louis, MO, USA). And antibodies against HIF-1α, MMP-2 and GAPDH were purchased from Abcam (Shanghai, People'sRepublic of China).

The extraction of total flavonoid of *Eucommia ulmoides*

On the basis of the method of Yuan et al,[35] an improved method was developed for extraction of total flavonoid of *Eucommia ulmoides*. The dehydrated leaf of *Eucommia ulmoides* was pulverized and then sifted by 40 mesh sieve. The powder was extracted by ultrasonic traction with 65% ethanol. The extract was cleaned up by petroleum ether and adsorbed by resin for 2 hrs after collection and concentration. Then, the extract was eluted with deionized water, and passed through 90% isopropyl alcohol and formaldehyde in turn.

Cell culture

GBMs cells lines U251, U87, HS683 and A172 and human normal cell HA were purchased from China Center for Type Culture Collection (Wuhan, People'sRepublic of China). GBMs cells lines were cultured at 5% CO_2 and 37°C with AM medium (ScienCell, CA, USA) containing 1% FBS.

Cell viability assay

Cell viability was measured by ATPlite assay (Perkin Elmer, Waltham, MA, USA), and the experiments were carried out according to the instruction. 96-well plates were used to culture GBMs cells. After being cultured for 24 hrs, GBMS cells were treated for 72 hrs with the total flavonoid of *Eucommia ulmoides* and radiotherapy. Each plate was examined immediately on a microplate

reader (Expire Technology, Perkin Elmer, Waltham, MA, USA). The half-maximal effective concentration (EC50) and the half-maximal lethal concentration (LC50) values calculated using Compusyn software from the dose–response curves.

Wound-healing migration assay

U251 and U87 cell (5×10^5 cells) grew in six-well tissue culture plates and wounded with a sterile 10-μL pipette tip. The cells were washed by phosphate-buffered saline to remove loosely attached cells. Then, serum-free medium was added. The cells were cultured and photographed every 6 hrs. The number of cells migrated into the scratched area was calculated.

Transwell invasion assay

24-transwell chamber (BD Falcon, Corning-Costar, New York, NY, USA) was employed. Cells were suspended in serum-free medium containing BSA after washed by phosphate-buffered saline. Cell suspension was added to 24-well invasion chamber. Cell invasion chamber was incubated overnight in incubator at 37°C, 5% CO_2 atmosphere. Migrated cells were fixed by formaldehyde for 10 mins and stained by crystal violet. Then, the percentage of average cell number per filed was recorded.

Clonogenic assay

Cells were cultured to 70% confluence and were treated with irradiation combined with the total flavonoid of *Eucommia ulmoides*. One thousand cells were re-seeded into a new 100 mm tissue culture dish and incubated for 9 days. Fresh media were added at day 5. The numbers of colonies were scored by Cristal Violet staining. The dose of irradiation was 6 Gy, and the concentration of total flavonoid of *Eucommia ulmoides* was 2 μg/mL.

Cell apoptosis and Hoechst 33,258 staining

Cell suspension was fixed by acetic acid and ethanol for 10 mins or more and washed by phosphate-buffered saline twice for 3 mins. Then, the cell was stained by Hoechst 33,258 for 5 mins. The results were observed under a fluorescence microscope. The dose of irradiation was 6 Gy, and the concentration of total flavonoid of *Eucommia ulmoides* was 2 μg/mL.

Western blotting analysis

Total proteins were extracted with NP40 lysis buffer. After SDS-PAGE, proteins were transferred to PVDF membrane for 2 hrs. And membrane was closed by TBST solution containing 5% skim milk powder for 1 hr and incubated overnight with primary antibodies. Then, TBST was used to wash immunocomplexes three times, and each time 5 mins. Secondary antibody and the membrane were incubated for 1 hr. The data were operated to quantify by Uvitec Alliance software (Eppendorf, Hamburg, Germany). GAPDH was selected as the positive control. The dose of irradiation was 6 Gy, and the concentration of total flavonoid of *Eucommia ulmoides* was 2 μg/mL.

Detection of MDA content and SOD activity

The content of MDA and activity of SOD was detected by the kit (Changchun Insititute of Biological Products Co., Ltd.) in U251 according to the instruction manual. The dose of irradiation was 6 Gy, and the concentration of total flavonoid of *Eucommia ulmoides* was 2 μg/mL.

Statistical analysis

For each experiment, at least three times of repeats were performed. The data were processed by SPSS 25.0 statistical software. Parametric data were analyzed with two-tailed *t*-test and F-test ANOVA, while non-parametric data were analyzed by Kruskal–Wallis one-way ANOVA on ranks test, in which $*p<0.05$ and $**p<0.01$.

Results

The total flavonoid of *Eucommia ulmoides* selectively inhibits GBMs cells

We performed cell viability assays with the total flavonoid of *Eucommia ulmoides* in different concentrations (from 0 to 32.0 μg/mL) to analyze its inhibitory effect on GBMs cells. As shown in Figure 1, the viability rate of GBMs cells was reduced in dose-dependent manner. In concentrations ranging from 0 to 4.0 μg/mL, the inhibitory effect of the total flavonoid of *Eucommia ulmoids* on GBMs cells was relatively strong after treating for 72 hrs. And the viability rate of normal human HA cells remained stable. This result suggested that the GBMs cells were more sensitive to the total flavonoid of *Eucommia ulmoides* than normal cells. To further confirm this result, we analyzed the half-maximal effective concentration (EC50) and the half-maximal lethal concentration (LC50) of all cell lines (Table 1). The EC50 average value of all GBMs cells lines was 2.76 μg/mL, and the LC50 average value was 3.15 μg/mL. Compared to EC50 (17.97 μg/mL) and LC50

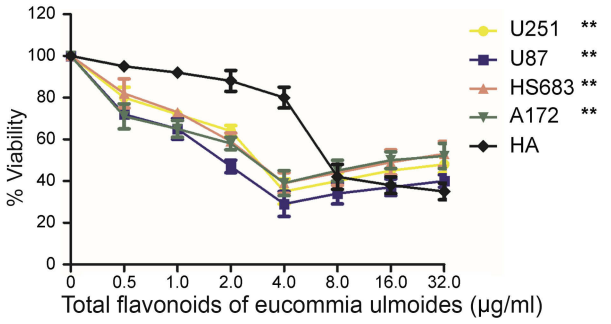

Figure 1 The viability rate of HA and GBMs cells lines treated with the total flavonoids of *Eucommia ulmoides*. At least three repeats were carried out, $^{**}p<0.01$.

Table 1 The EC50 and LC50 of GBMs cells

	U251	U87	HS683	A172	HA
EC50 (μg/mL)	2.93	2.29	2.99	2.83	17.97
LC50 (μg/mL)	3.36	2.66	3.31	3.27	4.71

Abbreviations: EC50, the half-maximal effective concentration; LC50, the half-maximal lethal concentration; GBMs, glioblastomas.

(4.71 μg/mL) values of HA, the total flavonoid of *Eucommia ulmoides* was obviously more effective to the GBMs cells.

The total flavonoid of *Eucommia ulmoides* impairs the abilities of GBMs cells to migrate and invade

Migration and invasion were the most essential features of malignant tumors as well as the main cause of death of cancer patients. To investigate the inhibitory effect of the total flavonoid of *Eucommia ulmoides* on migration ability of GBMs cells, wound-healing assays were used. In this test, we selected the GBMs cells lines U251 and U87 with the concentration of 2.0 μg/mL for subsequent functional analysis. The results showed that the migration ability of GBMs cells was inhibited by the total flavonoid of *Eucommia ulmoides* after 24 hrs (Figure 2A). The effect of the total flavonoid of *Eucommia ulmoides* on invasion ability of GBMs cells was examined with the concentration of 2.0 or 1.0 μg/mL as well. In transwell invasion assays, it was shown that the total flavonoid of *Eucommia ulmoides* could significantly reduce cell invasion ability in both U251 and U87 (Figure 2B). All those results above demonstrated that the total flavonoid of *Eucommia ulmoides* impaired the abilities of GBMs cells to migrate and invade.

The apoptosis is promoted in intrinsic pathway during radiotherapy in combination with the total flavonoid of *Eucommia ulmoides*

Radiotherapy was among the major strategies against cancers. In order to explore the application value of the total flavonoid of *Eucommia ulmoides*, we performed the clonogenic assays with irradiation to analyze the inhibitive effects of this combination in GBMs. As a result, the inhibitory effect of radiotherapy in combination with the total flavonoid of *Eucommia ulmoides* on U251 and U87 was most prominent, followed by radiotherapy and the total flavonoid of *Eucommia ulmoides* (Figure 3A). The number of normalized colonies treated with radiotherapy in combination with the total flavonoid of *Eucommia ulmoides* was about a fifth of that in control. Since radiotherapy kills cancer cells by inducing apoptosis, we analyzed the apoptotic rate in U251. The proportion of apoptotic cells treated with radiotherapy in combination with the total flavonoid of *Eucommia ulmoides* was significantly higher than that of control (Figure 3B). These results suggested that the total flavonoid of *Eucommia ulmoides* could significantly increase apoptotic of GBMs cells induced by radiotherapy. To further investigate it, we analyzed the expression of Bcl-2 and BAX in different treatments. As expected, the ratio of Bcl-2/Bax was the lowest when treated with radiotherapy in combination with the total flavonoid of *Eucommia ulmoides* (Figure 3C). This suggested that the apoptosis was promoted in intrinsic pathway during radiotherapy in combination with the total flavonoid of *Eucommia ulmoides*.

In addition, serum MDA content and SOD activity were detected in U251. The result showed the MDA content was decreased dramatically by using irradiation and the total flavonoid of *Eucommia ulmoides* together, while SOD activity decreased slightly (Table 2). This suggested that the total flavonoid of *Eucommia ulmoides* not only strongly suppressed GBMs cells, but also had protective effect for normal cells.

Human GBMcells are sensitized by the total flavonoid of *Eucommia ulmoides* via HIF-α/MMP-2 pathway

It was shown that the apoptosis induced by the total flavonoid of *Eucommia ulmoides* was limited, while it could significantly increase the apoptotic rate of GBMs cells induced during radiotherapy. Thus, we hypothesized that the total flavonoid of *Eucommia ulmoides* can enhance

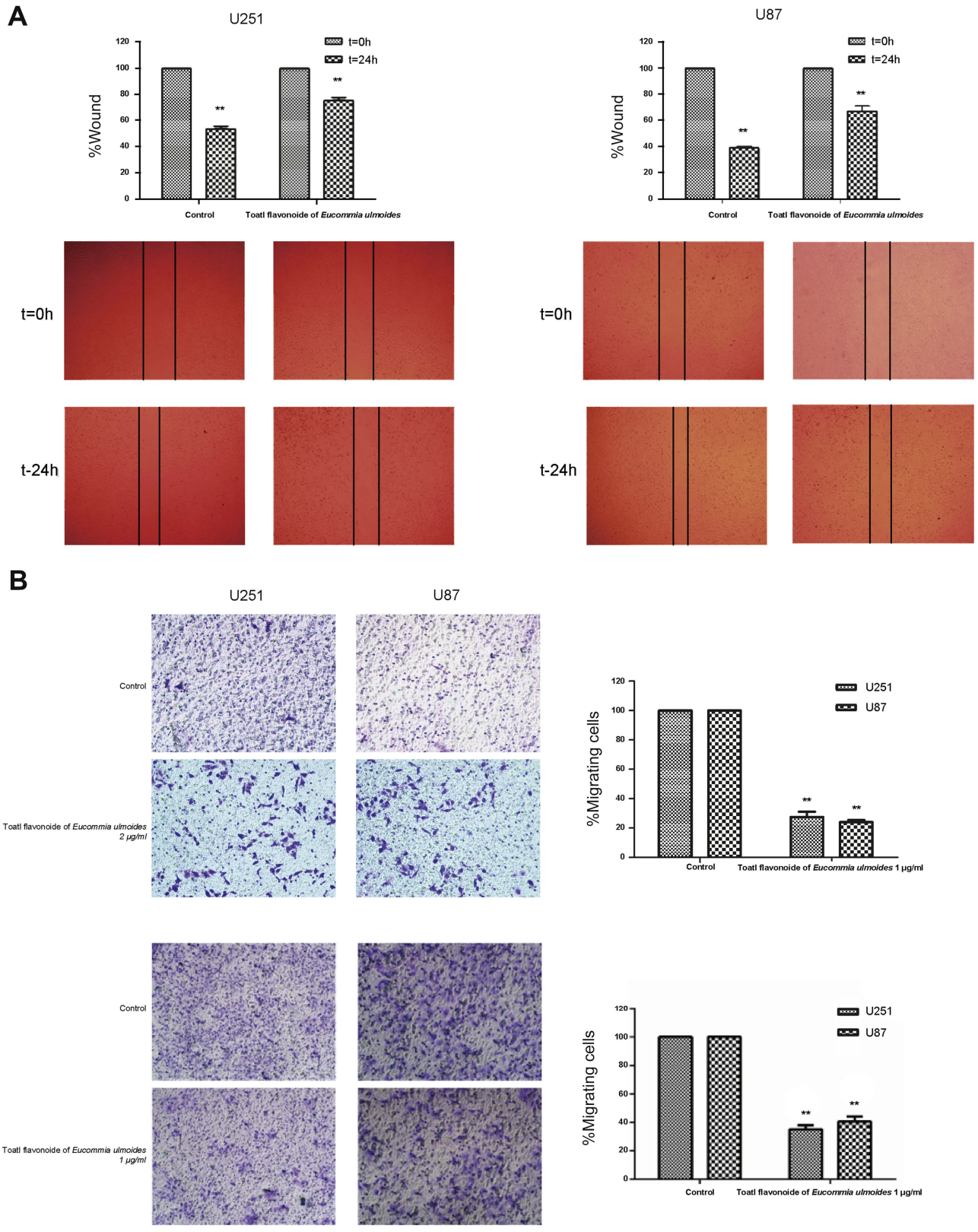

Figure 2 The migration and invasion of GBMs treated with the total flavonoids of *Eucommia ulmoides*. The total flavonoids of *Eucommia ulmoides* was used in wound healing assays (**A**) (2 μg/mL) and transwell invasion assays (**B**) (2 or 1 μg/mL). Each treatment has three repeats. The error bars represent mean±SD, $^{**}p<0.01$.

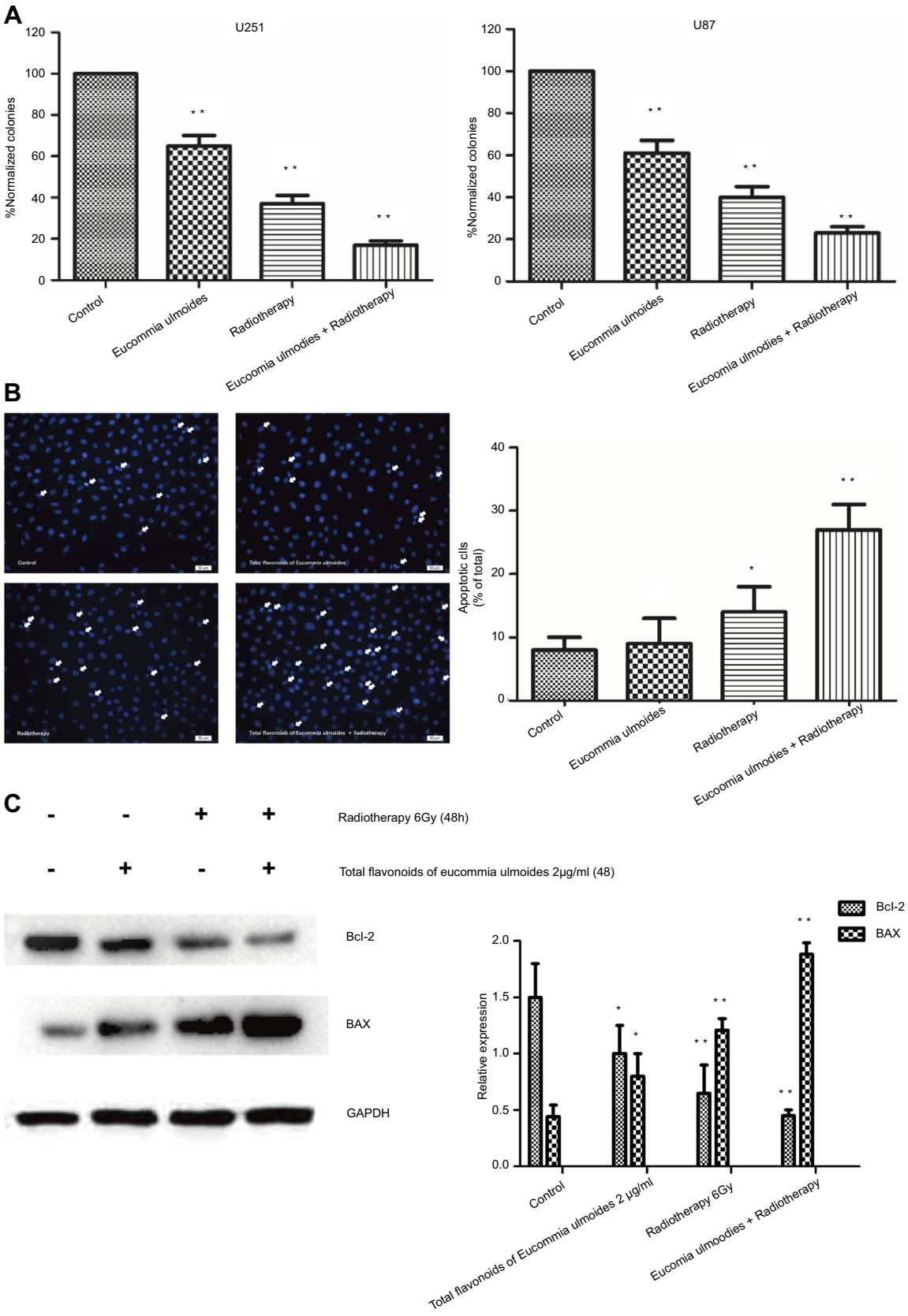

Figure 3 The total flavonoid of *Eucommia ulmoides* significantly increases the apoptotic rate of GBMs cells during radiotherapy in intrinsic pathway. Radiotherapy in combination with the total flavonoid of *Eucommia ulmoides* was used in clonogenic assays (**A**) and cell apoptotic test (**B**). The error bars represent mean±SD, **p*<0.05; ***p*<0.01. (**C**) Western blot analysis and the relative expression of Bcl-2 and BAX. The error bars are presented as mean±SD, **p*<0.05; ***p*<0.01.

Table 2 Detection of MDA content and SOD activity in U251

Group	MDA (μmol/L)	SOD (U/L)
Control	1.093±0.093	0.636±0.061
The total flavonoid of *Eucommia ulmoides*	0.834±0.049	0.529±0.065
Radiotherapy	0.464±0.047	0.491±0.052
Radiotherapy+the total flavonoid of *Eucommia ulmoides*	0.175±0.033	0.475±0.056

Abbreviations: MDA, malondialdehyde; SOD, superoxide dismutase.

the sensitivity of GBMs cells in radiotherapy. To confirm this hypothesis, we performed cell viability assays in GBMs cells lines U251 and U87. As shownin Figure 4A, the viability of GBMs cells was significantly decreased when GBMs cells were treated with radiotherapy in combination with the total flavonoid of *Eucommia ulmoides*. We also analyzed the EC50 and LC50 of radiotherapy in U251 and U87 (Table 3). With the total flavonoid of *Eucommia ulmoides*, the average EC50 and LC50 was 5.59 and 4.365, respectively. Compared to EC50 (7.22) and LC50 (5.675) values of the control, the total flavonoid of *Eucommia ulmoides* increased the sensitivity of GBMs cells to radiotherapy.

By examining resistance-related proteins of GBMs, we found the expressions of HIF-1α and MMP-2 were further reduced during radiotherapy in combination with the total flavonoid of *Eucommia ulmoides* (Figure 4B). However, Wee1, the cell cycle-related protein, remained stable and failed to show a synergistic effect when the total flavonoid of *Eucommia ulmoides* was used in radiotherapy (Figure 4B). This result suggested that the total flavonoid of *Eucommia ulmoides* might radiosensitize GBMs via HIF-1α/MMP-2 pathway.

Discussion

GBM is widely considered as one of the most aggressive and lethal malignant tumors, which becomes a great threat to human health. To achieve a better outcome, the radiotherapy is usually employed after surgical resection, while the effect of radiotherapy is limited by the strong radioresistance of GBMs. *Eucommia ulmoides Oliv.* is a useful medical plant, which was used for several diseases in East Asia and may perform the anti-cancer activity against GBMs. Thus, in this study, we investigated anti-cancer effect of the total flavonoid of *Eucommia ulmoides* and explore its application in radiotherapy, which may provide a new way against GBMs.

According to our results, the total flavonoid of *Eucommia ulmoides* could significantly reduce the viability rate of GBM cells. Moreover, the normal cells exhibited a less sensitivity to the total flavonoid of *Eucommia ulmoides*, which suggested its killing effect was selective and might be used in the treatment for GBMs. However, the viability rate of GBMs cells rose when the concentration of the extract was over 4 μg/mL. It may be because of the strong screening effect of high concentration of the total flavonoid of *Eucommia ulmoides*, in which drug-resistant GBMs cells proliferate rapidly, or other adaptive mechanism of GBMs cells induced by the high concentration. This result also suggests that the dose of the total flavonoid of *Eucommia ulmoides* should be strictly controlled during practical application. The data of migration assays indicated that the total flavonoid of *Eucommia ulmoides* could impair the migration ability of GBM cells, and this inhibitory effect varied according to different lines of GBMs. As for the inhibitory effect to invasion of GBM cells, the total flavonoid of *Eucommia ulmoides* drastically reduced the rate of migrating cells both in U251 and U87.

We also explored its application in GBMs by investigating the effect of the total flavonoid of *Eucommia ulmoides* in combination with radiotherapy. By examining the number of colonies in different treatments, we concluded that the inhibitive effect of irradiation was stronger than that of the total flavonoid of *Eucommia ulmoides*, and combining radiotherapy with the total flavonoid of *Eucommia ulmoides* could inhibit the formation of colonies more effectively. Since the killing effect of irradiation was mainly because of inducing apoptosis, we detected the apoptotic rate in U251. By analyzing with Hoechst 33258 staining, we found that the apoptotic rate was drastically increased in radiotherapy using the total flavonoid of *Eucommia ulmoides* though it was slightly promoted with the total flavonoid of *Eucommia ulmoides* only. The results were further demonstrated by detecting the expression of Bcl-2 and BAX, two key proteins in intrinsic apoptotic pathway. The significantly decreased Bcl-2/Bax ratio suggested the promoted apoptosis was in mitochondrial pathway, and radiosensitivity of GBMs was enhanced. It was also demonstrated by the viability rate, the EC50 and LC50 of U251 and U87 with increasing doses of irradiation in combination with the total flavonoid of *Eucommia ulmoides*. By detecting the expression of HIF-1α, MMP-2 and Wee1, it was shown that the HIF-1α/MMP-2 pathway was markedly inhibited, by which the

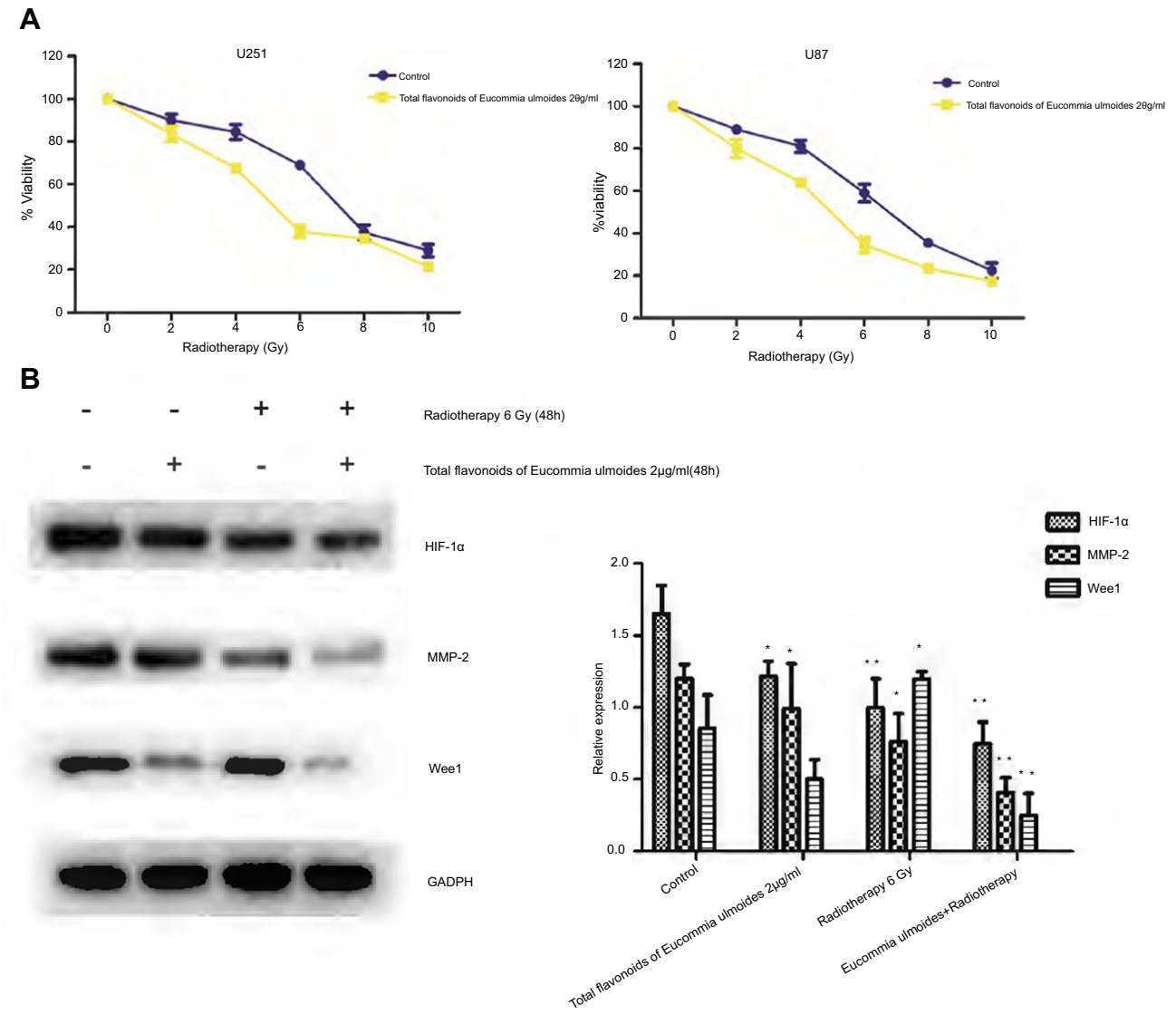

Figure 4 The total flavonoid of *Eucommia ulmoides* radiosensitizes GBMs cells via HIF-1α/MMP-2 pathway. (**A**) The viability of U251 and U87 treated with radiotherapy in combination with the total flavonoid of *Eucommia ulmoides*. There were three independent experiments performed at each treatment. (**B**) Western blot analysis and the relative expression of HIF-1α and MMP-2. Dates are presented as mean±SD, *p<0.05; **p<0.01.

Table 3 The EC50 and LC50 of radiotherapy in U251 and U87

	U251		U87	
	Radiotherapy EC50 (Gy)	**Radiotherapy LC50 (Gy)**	**Radiotherapy EC50 (Gy)**	**Radiotherapy LC50 (Gy)**
Control	7.71	7.19	6.73	4.18
The total flavonoid of *Eucommia ulmoides* (2μg/mL)	6.24	5.42	5.66	3.31

Abbreviations: EC50, the half-maximal effective concentration; LC50, the half-maximal lethal concentration.

movability and metastasis of GBMs were reduced, and the radiosensitivity of GBMs was increased via modulating the glucose metabolism. Besides, Wee1 failed to exhibit certain relationships in the change of radiosensitivity.

Altogether, our study investigated the anti-cancer activity of the total flavonoid of *Eucommia ulmoides* in GBMs, in which it could significantly inhibit the proliferation, migration and invasion of GBMs.

Moreover, it was demonstrated in our study that the total flavonoid of *Eucommia ulmoides* could be used as adjuvant drug during radiotherapy to sensitize GBM cells via HIF-1α/MMP-2 pathway and promoted apoptosis in mitochondrial apoptotic pathway, which might provide a useful treatment against GBMs.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Qazi MA, Vora P, Venugopal C, et al. Intratumoral heterogeneity: pathways to treatment resistance and relapse in human glioblastoma. *Ann Oncol*. 2017;28(7):1448–1456. doi:10.1093/annonc/mdx169
2. Talasila KM, Rosland GV, Hagland HR, et al. The angiogenic switch leads to a metabolic shift in human glioblastoma. *Neuro-Oncol*. 2017;19(3):383–393. doi:10.1093/neuonc/now175
3. Zorzan M, Giordan E, Redaelli M, Caretta A, Mucignat-Caretta C. Molecular targets in glioblastoma. *Future Oncol*. 2015;11(9):1407–1420. doi:10.2217/fon.15.22
4. Cloughesy TF, Cavenee WK, Mischel PS. Glioblastoma: from molecular pathology to targeted treatment. *Annu Rev Pathol*. 2014;9:1–25. doi:10.1146/annurev-pathol-011110-130324
5. Ohgaki H, Kleihues P. Population-based studies on incidence, survival rates, and genetic alterations in astrocytic and oligodendroglial gliomas. *J Neuropathol Exp Neurol*. 2005;64(6):479–489. doi:10.1093/jnen/64.6.479
6. Preusser M, de Ribaupierre S, Wöhrer A, et al. Current concepts and management of glioblastoma. *Ann Neurol*. 2011;70(1):9–21. doi:10.1002/ana.22425
7. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science*. 2009;324(5930):1029–1033. doi:10.1126/science.1160809
8. Ahmed EM, Bandopadhyay G, Coyle B, Grabowska A. A HIF-independent, CD133-mediated mechanism of cisplatin resistance in glioblastoma cells. *Cell Oncol*. 2018;41(3):319–328. doi:10.1007/s13402-018-0374-8
9. Shen H, Hau E, Joshi S, Dilda PJ, McDonald KL. Sensitization of glioblastoma cells to irradiation by modulating the glucose metabolism. *Mol Cancer Ther*. 2015;14(8):1794–1804. doi:10.1158/1535-7163.MCT-15-0247
10. Meijer TW, Kaanders JH, Span PN, Bussink J. Targeting hypoxia, HIF-1, and tumor glucose metabolism to improve radiotherapy efficacy. *Clin Cancer Res*. 2012;18(20):5585–5594. doi:10.1158/1078-0432.CCR-12-0858
11. Soeda A, Park M, Lee D, et al. Hypoxia promotes expansion of the CD133-positive glioma stem cells through activation of HIF-1alpha. *Oncogene*. 2009;28(45):3949–3959. doi:10.1038/onc.2009.252
12. Jing SW, Wang YD, Kuroda M, et al. HIF-1alpha contributes to hypoxia-induced invasion and metastasis of esophageal carcinoma via inhibiting E-cadherin and promoting MMP-2 expression. *Acta Med Okayama*. 2012;66(5):399–407. doi:10.18926/AMO/48964
13. Adachi N, Kubota Y, Kosaka K, et al. Low-dose radiation pretreatment improves survival of human ceiling culture-derived proliferative adipocytes (ccdPAs) under hypoxia via HIF-1 alpha and MMP-2 induction. *Biochem Biophys Res Commun*. 2015;463(4):1176–1183. doi:10.1016/j.bbrc.2015.06.079
14. Ai P, Shen B, Pan H, Chen K, Zheng J, Liu F. MiR-411 suppressed vein wall fibrosis by downregulating MMP-2 via targeting HIF-1α. *J Thromb Thrombolysis*. 2018;45(2):264–273. doi:10.1007/s11239-017-1596-8
15. Ou M, Sun X, Liang J, et al. A polysaccharide from Sargassum thunbergii inhibits angiogenesis via downregulating MMP-2 activity and VEGF/HIF-1alpha signaling. *Int J Biol Macromol*. 2017;94(Pt A):451–458. doi:10.1016/j.ijbiomac.2016.10.046
16. Hu Y, Li Y, Wu C, et al. MicroRNA-140-5p inhibits cell proliferation and invasion by regulating VEGFA/MMP2 signaling in glioma. *Tumour Biol*. 2017;39(4):1010428317697558. doi:10.1177/1010428317697558
17. Lukaszewicz-Zajac M, Mroczko B, Guzinska-Ustymowicz K, et al. Matrix metalloproteinase 2 (MMP-2) and their tissue inhibitor 2 (TIMP-2) in gastric cancer patients. *Adv Med Sci*. 2013;58(2):235–243. doi:10.2478/ams-2013-0018
18. Xiang ZL, Zeng ZC, Fan J, Tang ZY, Zeng HY, Gao DM. Gene expression profiling of fixed tissues identified hypoxia-inducible factor-1alpha, VEGF, and matrix metalloproteinase-2 as biomarkers of lymph node metastasis in hepatocellular carcinoma. *Clin Cancer Res*. 2011;17(16):5463–5472. doi:10.1158/1078-0432.CCR-10-3096
19. Li X, Deng D, Xue J, Qu L, Achilefu S, Gu Y. Quantum dots based molecular beacons for in vitro and in vivo detection of MMP-2 on tumor. *Biosens Bioelectron*. 2014;61:512–518. doi:10.1016/j.bios.2014.05.035
20. Liu WB, Xu GL, Jia WD, et al. Prognostic significance and mechanisms of patterned matrix vasculogenic mimicry in hepatocellular carcinoma. *Med Oncol*. 2011;28(Suppl 1):S228–S238. doi:10.1007/s12032-010-9706-x
21. Azria D, Riou O, Castan F, et al. Radiation-induced CD8 T-lymphocyte apoptosis as a predictor of breast fibrosis after radiotherapy: results of the prospective multicenter french trial. *EBioMedicine*. 2015;2(12):1965–1973. doi:10.1016/j.ebiom.2015.10.024
22. Li MY, Liu JQ, Chen DP, et al. Radiotherapy induces cell cycle arrest and cell apoptosis in nasopharyngeal carcinoma via the ATM and Smad pathways. *Cancer Biol Ther*. 2017;18(9):681–693. doi:10.1080/15384047.2017.1360442
23. Dewey WC, Ling CC, Meyn RE. Radiation-induced apoptosis: relevance to radiotherapy. *Int J Radiat Oncol Biol Phys*. 1995;33(4):781–796. doi:10.1016/0360-3016(95)00214-8
24. Que FG, Gores GJ. Cell death by apoptosis: basic concepts and disease relevance for the gastroenterologist. *Gastroenterology*. 1996;110(4):1238–1243.
25. Falleni M, Pellegrini C, Marchetti A, et al. Quantitative evaluation of the apoptosis regulating genes Survivin, Bcl-2 and Bax in inflammatory and malignant pleural lesions. *Lung Cancer*. 2005;48(2):211–216. doi:10.1016/j.lungcan.2004.10.003
26. Mei JM, Niu CS. Effects of CDNF on 6-OHDA-induced apoptosis in PC12 cells via modulation of Bcl-2/Bax and caspase-3 activation. *Neurol Sci*. 2014;35(8):1275–1280. doi:10.1007/s10072-014-1700-1
27. Mohseni M, Mihandoost E, Shirazi A, Sepehrizadeh Z, Bazzaz JT, Ghazi-Khansari M. Melatonin may play a role in modulation of bax and bcl-2 expression levels to protect rat peripheral blood lymphocytes from gamma irradiation-induced apoptosis. *Mutat Res*. 2012;738–739:19–27. doi:10.1016/j.mrfmmm.2012.08.006
28. Guo XZ, Shao XD, Liu MP, et al. Effect of bax, bcl-2 and bcl-xL on regulating apoptosis in tissues of normal liver and hepatocellular carcinoma. *World J Gastroenterol*. 2002;8(6):1059–1062. doi:10.3748/wjg.v8.i6.1059
29. Shimizu S, Narita M, Tsujimoto Y. Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. *Nature*. 1999;399(6735):483–487. doi:10.1038/20959
30. Hartmann A, Michel PP, Troadec JD, et al. Is Bax a mitochondrial mediator in apoptotic death of dopaminergic neurons in Parkinson's disease? *J Neurochem*. 2001;76(6):1785–1793.
31. He X, Wang J, Li M, et al. *Eucommia ulmoides Oliv.*: ethnopharmacology, phytochemistry and pharmacology of an important traditional Chinese medicine. *J Ethnopharmacol*. 2014;151(1):78–92.
32. Sugawa H, Ohno R, Shirakawa J, et al. *Eucommia ulmoides* extracts prevent the formation of advanced glycation end products. *Food Funct*. 2016;7(6):2566–2573. doi:10.1039/C5FO01563D

33. Wang JY, Yuan Y, Chen XJ, et al. Extract from *Eucommia ulmoides Oliv.* ameliorates arthritis via regulation of inflammation, synoviocyte proliferation and osteoclastogenesis in vitro and in vivo. *J Ethnopharmacol*. 2016;194:609–616. doi:10.1016/j.jep.2016.10.038
34. Kim BH, Park KS, Chang IM. Elucidation of anti-inflammatory potencies of *Eucommia ulmoides* bark and Plantago asiatica seeds. *J Med Food*. 2009;12(4):764–769. doi:10.1089/jmf.2008.1239
35. Yuan DX, Hussain T, Tan B, Liu Y, Ji P, Yin Y. The evaluation of antioxidant and anti-inflammatory effects of *Eucommia ulmoides* flavones using diquat-challenged piglet models. *Oxid Med Cell Longev*. 2017;2017:8140962. doi:10.1155/2017/8140962