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Original Article

In Vitro and *In Vivo* Evaluation of Antitumor Activity of *Ligustrum robustum*, A Chinese Herbal Tea*

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ABSTRACT **Objective**: To examine the effect of the aqueous extract of *Ligustrum robustum* on tumor growth *in vitro* and *in vivo* and explore the possible molecular mechanisms. **Methods**: In *in vitro* study, cell viabilities of human cervical carcinoma cells (HeLa), human breast cancer cells (MCF-7), human prostate cancer cells (PC-3), human hepatoma cells (7721) and human colon carcinoma cells (SW480) were evaluated with cell counting kit-8. For *L. robustum*-treated Hela cells, early or late apoptosis were evaluated by annexin V/PI staining. Mitochondrial membrane potential was measured by staining cells with JC-1. Apoptosis was monitored by nuclear morphology based on chromatin condensation and fragmentation by 4',6-diamidino-2-phenylinole (DAPI) staining. Caspase-3 and -8 activity levels were measured by a colorimetric assay. *In vivo*, to evaluate the possible mechanism of *L. robustum*-mediated antitumor effect, nude mouse xenograft study was also conducted. **Results**: In *in vitro* study, *L. robustum* was found to be toxic to HeLa, MCF-7, PC-3, 7721, SW480, with an half maximal inhibitory concentration value of 2–5 mg/mL ($P<0.05$). Moreover, externalization of phosphatidylserine, loss of mitochondrial membrane potential, DNA fragmentation and activation of caspase-3 and -8 were detected in *L. robustum*-treated Hela cells. Using a nude mouse model bearing Hela xenografts, we found that *L. robustum* reduced tumor volume and tumor weight ($P<0.05$), but had no effect on body weight and histological damage of important organs. Intraperitoneal injection of *L. robustum* caused a significant reduction in serum aspartate transaminase and alanine transaminase levels ($P<0.05$). Furthermore, cleaved caspase-3-positive and terminal nucleotidyl transferase-mediated nick end labeling (TUNEL)-positive cells were observed in *L. robustum*-treated tumor tissues. **Conclusions**: *L. robustum* inhibits tumor cell growth both *in vitro* and *in vivo* by inducing apoptosis in a caspase-dependent way without apparent hepatic toxicity and histological damage, which may offer partial scientific support for the ethnopharmacological claims of *L. robustum* as a herbal tea for its antitumor activity.

KEYWORDS *Ligustrum robustum*, antitumor effect, Ku-Ding-Cha, herbal tea

The human use of herbal tea is believed to have originated around 5,000 years ago in China. Nowadays, herbal tea is the most popular beverage consumed worldwide, second only to drinking water.[1] Recent reports indicate that there is an inverse relationship between tea consumption and the incidence of various diseases in humans.[2]

Ku-Ding-Cha, literally bitter spike-leaf tea, is a kind of famous herbal tea commonly used in China from many hundred years ago. People always drink it as beverage for clearing away "heat" and "toxic" materials in body and satisfy one's thirst.[3] In the southern China (e.g., Sichuan, Yunnan and Guizhou Provinces), Ku-Ding-Cha is mainly produced from the leaves of *Ligustrum robustum* (Oleaceae). *L. robustum* is a traditional herbal tea widely used in China for a long history and has been gaining in large research attention because of its potential health and economic significance.[4,5] In Chinese folk medicine, *L. robustum* is used as a diuretic and slimming agent, and for the treatment of sore throat and hypertension. It is also claimed to possess the

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antioxidative, antiinflammatory, antitumer, antidiabetic, hepatoprotective, neuroprotective as well as diuretic properties.[6.7] Modern studies tried to understand in Western medical terms the effects of *L. robustum* and have shown its benefits in some aspects, such as its antioxidative, antiinflammatory and hepatoprotective properties.[7] As an important traditional tea, *L. robustum* is consumed by local people as health-promoting beverage with many reported biological activities including antitumor activity.[6] However, the basis of its antitumor effect is largely unclear and remains to be elucidated. In order to verify some of the traditional ethnopharmacological claims of *L. robustum* and to assess its value as the tea beverage, this study looked into the efficacy of *L. robustum* extract against tumor growth, and explored the possible molecular mechanism of *L. robustum*-mediated biological effect.

METHODS

Preparation of *L. robustum* Extract

The leaves of *L. robustum* were obtained as dried plants from Julian Ku-Ding-Cha Association (Sichuan, China) with confirmation of identity by Prof. LIU Guo-ming from Hainan University of Ku-Ding-Cha Research Institute, China. For preparation of *L. robustum* extract, 50 g was ground to a fine powder with a pestle and mortar and suspended in 1500 mL distilled water. In traditional Chinese culture, the plant extract is consumed as tea after heating. Therefore, the suspension was incubated with shaking at 80 ℃ for 3 h. Afterwards, the extract was filtered, evaporated on a rotary evaporator to a concentration of 1 g/mL and stored at –20 ℃ until further use.

Cell Culture

Human cervical carcinoma cells (HeLa), human breast cancer cells (MCF-7), human prostate cancer cells (PC-3), human hepatoma cells (7721) and human colon carcinoma cells (SW480) were obtained from American Type Culture Collection. All cell lines were cultured in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mmol/L L-glutamine in a humidified atmosphere of 5% CO_2 at 37 ℃.

Evaluation of Cell Viability

Cell viability was evaluated colorimetrically with the cell counting kit-8 (CCK-8) from Dojindo Laboratories (Tokyo, Japan) according to the manufacturer's instruction. Briefly, cells were seeded into 96-well plates at a density of 10^4 cells/well in 0.1 mL medium. The following day, the cells were treated with serial concentrations of *L. robustum* for 4 h or with 5 mg/mL of *L. robustum* for different periods of time. Control cells were incubated with the equal solvent concentration. At the end of the treatment, 10 μL of CCK-8 solution was added to each well, and the plates were incubated for an additional 2 h at 37 ℃. The absorbance of each well was measured with a microplate reader at 450 nm. CCK-8 solution with solvent (without cells) acted as a blank control in microplate reading, while the solvent-treated cells served as a control of 100% survival. The cell viability was calculated as $[A_{450}\ (\text{test})–A_{450}\ (\text{blank})]/[A_{450}\ (\text{control})–A_{450}\ (\text{blank})] \times 100\%$. All experiments were done in triplicate and repeated at least 3 times.

Detection of Apoptosis by Annexin V/PI Staining

After incubation with *L. robustum* for 4 h, apoptosis of HeLa cells was observed under a fluorescence microscope using the fluorescein isothiocyanate (FITC)-annexin V/PI kit (Invitrogen) as described by the manufacturer. For flow cytometry analysis, HeLa cells (2.5×10^5 in 300 μL solution) were treated with 0, 5, 10 mg/mL of *L. robustum* for 4 h and then double stained by annexin V and PI. With this method, annexin V-/PI- population indicates vital cells; annexin V+/PI- or annexin V+/PI+ population represents cells undergoing early or late apoptosis, respectively.

Detection of Mitochondrial Membrane Potential by JC-1 Staining

Mitochondrial membrane potential was measured by staining cells with JC-1 (Invitrogen, USA). A decrease in the ratio of red to green fluorescence reflects depolarization of mitochondria. For flow cytometry analysis, cells (2.5×10^5 in 300 μL solution) were treated with 10 mg/mL of *L. robustum* for 4 h and incubated with JC-1. Both red and green fluorescence were analyzed by flow cytometry after JC-1 staining. To observe the mitochondria using a fluorescence microscope, the cells were double stained with blue fluorescent dye 4',6-diamidine-2-phenylindole (DAPI, showing nuclei).

Detection of Apoptosis by Nuclear Morphology

Apoptosis was monitored by nuclear morphology based on chromatin condensation and fragmentation. Staining of cell nuclei was performed with the blue fluorescent dye, DAPI. After treatment with *L. robustum* for 0–24 h, cells were stained with DAPI and

observed by fluorescent microscope.

Detection of Caspase Activation

Caspase-3 and -8 activity levels were measured by a colorimetric assay using the caspase activation kit (Invitrogen), according to the protocol recommended by the manufacturer. Briefly, after treated with various concentrations of *L. robustum* for 4 h, the cells (5×10^6) were harvested and lysed with provided lysis buffer for 30 min on ice. The lysed cells were centrifuged at 16,000 × *g* for 10 min, and 100 μg of the protein were incubated with 50 μL of caspase-3 or -8 specific substrate. This mixture was incubated for 2 h at 37 ℃, and then absorbance was measured with a microplate reader at 405 nm. The data were normalized to the caspase activities of the control cells and represented as "fold of control".

In Vivo Nude Mouse Xenograft Study

All animal studies were approved by the University Animal Care and Use Committee. HeLa cells (3×10^6) suspended in 100 μL of phosphate buffered saling (PBS) were subcutaneously injected into the flank of 6-week-old nude female mice. When tumors became palpable (about 50 mm^3), mice were randomized into 3 groups (*n*=10 for each group). Mice in the treatment groups were injected with *L. robustum* intraperitoneally (2 g/kg in 100 μL of PBS) every day for 5 days or intratumorally (1 g/kg in 50 μL of PBS) on day 1, 7, 11, 13, 15 and 17 (totally 5 administrations). Body weight and tumor mass were measured every day. Tumor volume was calculated as length × $width^2$ × 0.5. At the end of the experiment, blood was drawn and centrifuged at 3,000 r/min for 10 min to obtain serum samples. The levels of serum aspartate transaminase (AST) and alanine transaminase (ALT) were measured as described.[7] All animals were sacrificed and the tumor tissues were removed and weighed. Simultaneously, the histological architecture of major organs from mice injected with intratumoral injection (IT) or intraperitoneal injection (IP) were examined by hematoxylin-eosin (HE) staining and compared with that of mice in the control group.

To explore the apoptosis *in vivo*, 6 mice bearing HeLa xenografts (volume: 1000 mm^3) were intratumorally injected with 40 mg of *L. robustum* in 100 μL of PBS. For the control tumor, only PBS was administered. At 24 h postinjection, the animals were sacrificed and the tumor tissues were excised, stored at –80 ℃ for detection of Bax, Bcl-2 and β-actin by Western blot analysis or paraffin-embedded, sectioned and stained with HE to reveal the histologic architecture. Primary rabbit polyclonal antibody against human cleaved caspase-3 (Asp175, Cell Signaling, USA) was used for the detection of activated caspase-3. Terminal nucleotidyl transferase-mediated nick end labeling (TUNEL) staining (Invitrogen) was used to evaluate the apoptosis of tumor cells according to the manufacturer's instructions.

Statistical Analysis

All data were analyzed using 19.0 (SPSS Inc., USA) and expressed as mean ± standard deviation ($\bar{x}\pm s$) of at least 3 independent experiments. Statistical analysis of the data was performed using ANOVA and Student's *t*-test. *P* values less than 0.05 were considered statistically significant.

RESULTS

Inhibition of Tumor Cell Growth by *L. robustum*

As shown in Figure 1A, after treatment with various concentrations of *L. robustum* for 4 h, HeLa cells became round and shrunken, and gradually detached from the bottom of culture plates. The CCK-8 assay revealed that 1.25–10 mg/mL of *L. robustum* dose-dependently reduced cell viability by 14%–97% compared to control cells (Figure 1B). The half maximal inhibitory concertration (IC_{50}) of *L. robustum* in HeLa cells was approximately 5 mg/mL. As shown in Figure 1C, 5 mg/mL of *L. robustum* caused gradually reduced cell viability with increased incubation time. As shown in Figure 1D, *L. robustum* (2.5 and 5 mg/mL) induced approximately 32%–74% and 57%–88% cell death respectively in different tumor cells. The cell viability in the treatment group exhibited significantly difference from the control group ($P<0.05$).

L. robustum Induces Apoptosis

After treatment with *L. robustum* and double staining with annexin V and PI, many annexin V+/PI- (indicating early apoptosis) and annexin V+/PI+ (indicating late apoptosis) cells were observed under a fluorescence microscopy (Figure 2A). Simultaneously, flow cytometry analysis also detected numerous undergoing apoptotic cells. After treatment with 0, 5 and 10 mg/mL of *L. robustum*, the percentage ratio of early/late apoptotic cells was 0.1/0.2, 11.5/79.4 and 92.3/7.1, respectively (Figure 2A). Loss of mitochondrial membrane potential was assessed using the fluorescent

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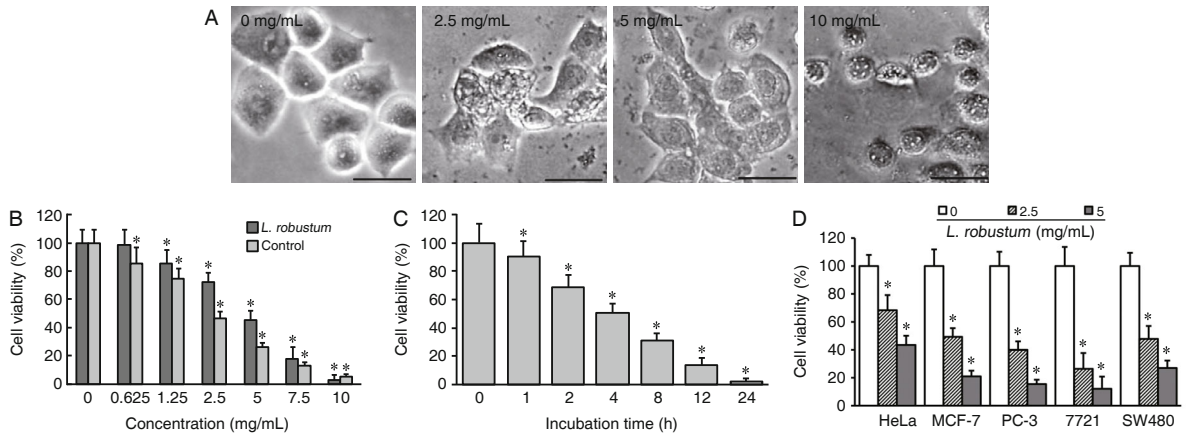

Figure 1. *L. robustum* Inhibits Growth of Tumor Cells Determined by CCK-8 Assay

Notes: A: morphological changes of HeLa cells by different concentration of *L. robustum* observed using contrast microscope (bar=25 μm). B: HeLa cells viability treated with *L. robustum* (0–10 mg/mL) for 4 h or doxorubicin (0–20 μg/mL) for 24 h. C: HeLa cells viability treated with 5 mg/mL of *L. robustum* for 0–24 h. D: different cells viability treated with 5 mg/mL of *L. robustum* for 4 h. **P*<0.05 *vs*. control cells

probe, JC-1. *L. robustum*-free cells exhibited punctate red staining, however, cells exposed to 10 mg/mL *L. robustum* lost punctate red staining (Figure 2B). Further flow cytometry analysis also detected decreases in the red/green fluorescence ratio from 98/2 to 16/84 (indicating mitochondrial membrane depolarization, Figure 2B). In addition, nuclear fragmentation characteristic of apoptosis was also observed in the *L. robustum*-treated cells (Figure 2C). As shown in Figure 2D, *L. robustum* treatment significantly induced the activation of caspase-3 in HeLa cells.

L. robustum Suppresses Tumor Growth *In Vivo*

L. robustum administration was initiated on day 3 after implantation, when the tumor volume was approximately 50 mm^3. As shown in Figure 3A, from day 6 after injection, the mean tumor volume of mice in IT group was significantly different (P<0.05) from the control group. IP injection also exhibited a tumor inhibitory effect. The mean tumor volume of mice in IP group was significantly different (P<0.05) from the control group beginning on day 12. At the end of this experiment, *L. robustum* decreased tumor weight by 28% in IP group and 62% in IT group compared with control (Figure 3B). However, *L. robustum* had no effect on body weight in the animals (Figure 3C). Moreover, IP of *L. robustum* caused a significant reduction in serum AST and ALT levels (Figure 3D). No obvious histological injury was observed in the heart, liver, spleen, lung and kidney of mice injected with *L. robustum* (data not shown).

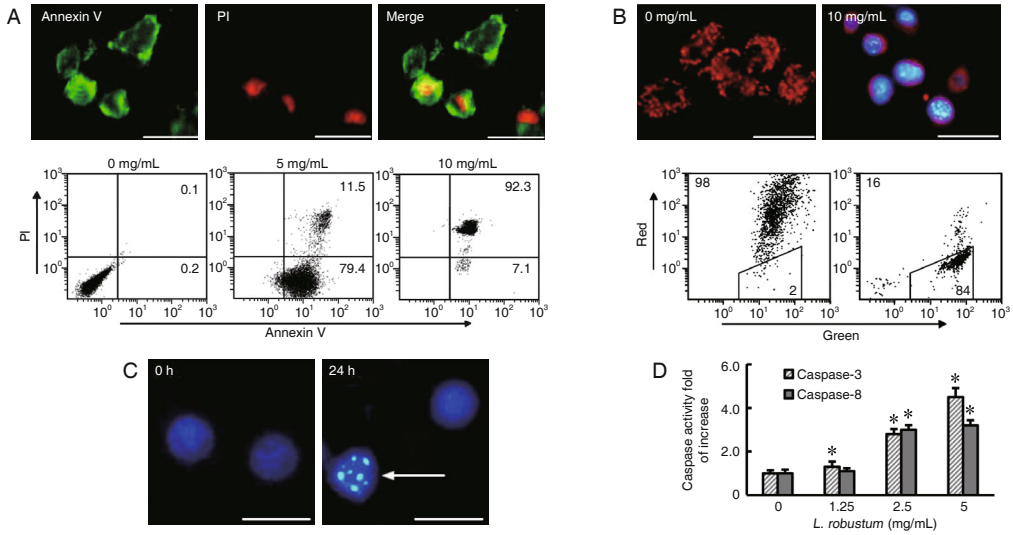

Figure 2. *L. robustum* Induces Apoptosis in Tumor Cells

Notes: A: tumor cells apoptosis detected by annexin V/PI staining and flow cytometry. B: mitochondrial membrane potential detected by JC-1 (red) and DAPI (blue) staining. C: apoptosis nuclear morphology detected by DAPI staining. D: caspase activation. **P*<0.05 *vs*. control cells

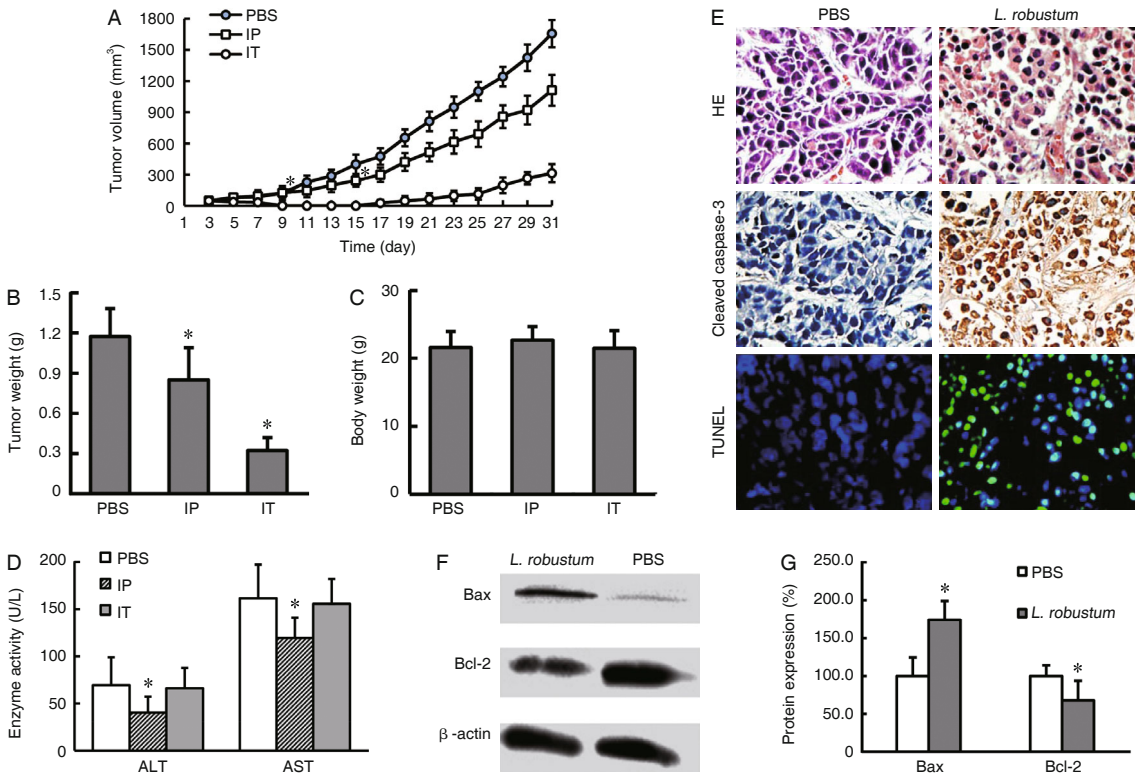

Figure 3. *L. robustum* Suppresses Tumor Xenografts Growth *In Vivo*

Notes: Detection of tumor volume (A), tumor weight (B), body weight (C) and serum levels of AST and ALT (D). E: Detection of HE staining, antibody against cleaved caspase-3 and TUNEL in tumor tissues. E, F: protein levels of Bax and Bcl-2 detected by Western blot. $^{*}P<0.05$ *vs*. control group

Further histological analysis showed that *L. robustum* caused extensive necrosis in tumor tissues. Cleaved caspase-3-positive staining was observed in the cell residues at the injection site. Tumors from mice treated with *L. robustum* exhibited numerous TUNEL-positive cells (green) as compared with treatment with PBS in control animals (Figure 3E). Western blot analysis showed that *L. robustum* treatment significantly reduced the expression of Bcl-2 but increased that of Bax in tumors (Figure 3F, 3G).

DISCUSSION

Studies on the constituents of *L. robustum* showed that compounds isolated from *L. robustum* are mainly phenylethanoid glycosides, terpenes and flavonoids, some of which have been demonstrated to exhibit strong antitumor activity, such as quercetin, apigenin, rutin, luteolin, oleanolic acid, etc.[5,8,9] Taking into account herbal tea is ordinarily prepared by water and consumed as mixture in traditional Chinese culture, we focused on *L. robustum* as it is used in traditional practice. Here, we found the aqueous extract of *L. robustum* was toxic to several tumor cell lines. These results may offer partial scientific support for the ethnopharmacological claims of *L. robustum* as herb tea as a relief for its antitumor activity.

Cancer cells are characterized by defects in apoptosis leading to the uncontrolled proliferation of cells. Therefore, drugs designed to restore apoptosis have the potential for effective treatment of cancer. One of the characteristics of apoptotic pathway is the externalization of phosphatidylserine (PS) to the outer leaflet of the plasma membrane. Detection of PS exposure can be achieved by the phospholipid-binding protein annexin V.[10] In the experiment, after double staining of annexin V and PI, we detected many early (annexin V+/PI-) and late (annexin V+/PI+) apoptotic cells in *L. robustum*-treated HeLa cells by fluorescence microscope and flow cytometry analysis. Mitochondrial membrane permeabilization is a key event in apoptotic cell death associated with activation of caspases and the loss of mitochondrial membrane potential.[11,12] Bcl-2 family proteins could regulate mitochondrial membrane permeabilization, functioning as either promoters such as Bax, or suppressors such as Bcl-2.[13,14] In this study, we found that treatment with *L. robustum* led to the loss of mitochondrial membrane potential, the activation of caspase-3, as well as the increased Bax expression

and decreased Bcl-2 expression. Moreover, apoptosis is also accompanied by a characteristic change of nuclear morphology, including nuclei condensation and DNA fragmentation.[10,14] After treatment with *L. robustum*, we detected nuclei condensation and DNA fragmentation in cell nuclei. Thus, our studies suggest that *L. robustum* induces apoptosis in a caspase-dependent way in tumor cells.

Modern studies showed that *L. robustum* possesses antiinflammatory effects on mice. *L. robustum* is also reported to have a hepatocyte protective role by inhibiting liver damage induced by CCl_4 in rats.[7] This protective role is supposed to be related with significant antioxidant activity of *L. robustum* by scavenging superoxide radicals and inhibiting lipid peroxidation. Therefore, the study of its antitumor effect and the possible molecular mechanism may provide partial support to the traditional uses of *L. robustum* as functional beverage. Here, we prepared the aqueous extract of *L. robustum*. *In vitro* cytotoxic assays showed that *L. robustum* was toxic to tumor cells, with an IC_{50} value of 2–5 mg/mL. *In vivo*, *L. robustum* inhibited human cervical carcinoma growth, without apparent hepatic toxicity and histological damage. These results suggest that *L. robustum* may be a health-promoting beverage with antitumor efficacy.

In conclusion, we demonstrate that *L. robustum could* inhibit tumor growth both *in vitro* and *in vivo* and induce caspase-dependent apoptosis. These results may offer partial scientific support for the ethnopharmacological claims of *L. robustum* as herb tea as a relief for its antitumor activity.

Conflict of Interest

The authors declare that there is no conflict of interest in this study.

Author Contributions

Zuo HJ and Liu S contributed equally to this work as co-first authors. Liu S reviewed literature, determined the antitumor activity *in vivo*, and wrote the paper. Zuo HJ detected the antitumor activity *in vitro* and prepared the extract. Yan C and Li LM tested the apoptosis. Pei XF planned and guided the study and all the experiments.

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