Tropical Journal of Pharmaceutical Research July 2021; 20 (7): 1433-1439
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v20i7.16

Original Research Article

Novel effects of piperlogumine on uterine fibroid tumor: An *in vitro* mouse model study

Mei Zhang[1], Mei Ye[2], Qingxiang Hou[2], Bin Yan[3], Chao Yang[4], Yi Yang[2], Li Ma[2]*
[1]JinZhou Medical University, PLA Rocket Force Characteristic Medical Centre Graduate Training Base, Beijing 100088, [2]Department of Obstetrics and Gynaecology, [3]Department of Critical Care Medicine, [4]Blood Transfusion Department, The PLA Rocket Force Characteristic Medical Center, Beijing 100088, China

For correspondence: **Email: mali.rocketforce@gmail.com; **Tel/Fax:** 0086-01066343360*

Sent for review: 17 September 2020 *Revised accepted: 16 June 2021*

Abstract

Purpose: *To investigate the in vivo anti-tumor effect of piperlongumine (PL) in a mouse model of leiomyoma xenograft and in leiomyoma cell lines.*
Methods: *The anti-proliferative effect of PL on ELT-3 cells was determined using MTT assay. Human and rat leiomyoma cells were used for the in vitro investigations. Rat leiomyoma cell lines were treated with various PL concentrations (50 - 100 μM)) for 48 h. Immunodeficient mice were subcutaneously injected with varying doses of estrogen or progesterone, and xenografted with explanting human leiomyoma cells in in vivo experiments. Proliferation assessment, caspase-3 expression, analysis of tumour samples, insertion of pellets of oestrogen-progesterone, tissue treatment and implantation, and immuno-histochemical analyses were carried out using appropriate procedures.*
Results: *Piperlongumine (PL) produced significant and dose-dependent increase in caspase-3 activity, apoptosis and suppression of cellular proliferation ($p < 0.01$). Moreover, Western blot data demonstrated that PL decreased phosphorylation of Akt signaling pathway. The results showed significant ($p < 0.01$) inhibition of tumor growth, including in ultra-sound in vivo studies, when compared with 30-day control and animals treated with PL (100 μg/g). Immuno-histochemical studies showed that PL decreased the expression of proliferation marker in xenografted tumor tissues ($p < 0.02$).*
Conclusion: *These results suggest that piperlongumine has potentials as a therapeutic agent for the management of uterine leiomyoma. However, additional studies using human cell lines are required to understand its genetic and molecular mechanisms.*

Keywords: *Uterine fibroids, Immune-deficiencies, Xenograft animal model, Tumor, Leiomyoma cells, Estrogen, Progesterone*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Uterine leiomyomas, also known as fibroids, are abnormal growths in the smooth muscles of the uterus. These growths cause symptoms such as formation of abdominal mass, depression, abdominal pains, infertility, spontaneous abortions, dysmenorrhea, and irregular menstruation [1]. They are commonly benign tumors present in the reproductive system of females.

Piperlogumine, a natural alkaloid derived from the fruit of *Piper longum* which grows in southern regions of India and Asia, is known for its anticancer properties [2]. In addition, several studies have reported that PL exhibited cytotoxicity against cancer cell lines, and inhibited tumour growth in xenograft mice models through a variety of mechanisms such as increase in levels reactive oxygen species (ROS), and suppression of LMP-1 gene expression [3]; inhibition of the activity of telomerase reverse transcriptase [4], promotion of autophagy [5], inhibition of NF-κB expression [6], activation of the ERK pathway [7], as well as phosphorylation of AMPK [8] and C/EBP homologous proteins [9]. Piperlongumine is also known to exhibit antitumor activity in breast carcinoma [10], and in various other carcinomas e.g., ovarian carcinoma. Various findings suggest the anticancer activities of PL in cancer cell lines through many mechanisms such as induction of apoptosis [11] and induction of necrosis with autophagy [12]. *In vitro*, PL induces cell-specific cytotoxic effects without affecting other cells. Studies have revealed that PL induces anticancer activity through a mechanism involving elevation of ROS, leading to cell apoptosis, although the exact pathway through which PL induces apoptosis is yet unknown [13].

The present study investigated the *in vivo* therapeutic effect of PL in a mouse leiomyoma xenograft model, as well as its effects on tumour size and volumes.

EXPERIMENTAL

Cells

Eker rat leiomyoma cell line (ELT-3) was provided by Cancer Institute and Research Centre, China. The cells were maintained in DF8 medium after complete characterization [14]. The ELT-3 cells were incubated in a 5 % CO_2 atmosphere below 37 °C until they attained 70-80 % confluence.

Materials

Piperlongumine (PL) was obtained as a gift from Adamas Pvt Ltd (Shanghai, China). Caspase-3 assay kit and MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay kit were obtained from Merck, USA. Protease inhibitors without EGTA were purchased from Roche, USA. Monoclonal antibodies (anti-phospho Akt and anti-total Akt) as well as rabbit polyclonal antibodies (anti-Ki67) were products of Cell Signaling (USA).

Proliferation assay

The effect of PL on cell proliferation was determined using MTT assay. The ELT-3 cells were seeded in 96-well plates and cultured in the presence of PL at concentrations of 6.25, 12.5, 25, 50 and 100 μM for 48 h. The MTT assay was carried out as reported earlier [15].

Caspase-3 assay

Caspase-3 assay was carried out using quantitative fluorometric method (Thermo Scientific, Pierce), as described earlier [15]. The cells were seeded in 96-well plates. After 24 h, the culture medium (Smooth Muscle Growth Medium) was replaced with medium containing human fibroblast and epidermal growth factors (0.3 and 0.4 %), fetal bovine serum (5 %), gentamicin or amphotericin B (0.3 %) and insulin (0.2 %). These media contained PL at concentrations of 6.25, 12.5, 25, 50 and 100mμM, respectfully. After 48 h, the cells were lysed. Equal volumes of lysates added to a reaction mixture containing caspase-3 substrate (Z-DEVD-R110). Fluorometric measurement of caspase-3 activity was completed in approximately 60 min.

Studies using tumor samples

The approval of the Ethics Committee was obtained from the Human Ethics Committee of Cancer Institute and Research Center, China (approved protocol no. EC/CIRC/043_UF/20) and informed consent was obtained from patients (preferring hysterectomy) to gather (if not discarded) leiomyoma samples and shifted to the experimental laboratory immediately after sterilization [14]. Two samples of two distinct patients were collected and analyzed at two different time intervals.

Animals

The animal study using female mice was approved by the Ethics Committee for Care and Use of Animals of Medical Centre, Beijing (approval no. EC/28/013/20). The animals were treated according to the guidelines of this committee. This study used a modified animal model of xenograft leiomyoma previously described [16]. The in-house cancer center provided 6-week-old female mice with multiple immunodeficiencies (NOG). The numerous and combined immune defects make the mice suitable as xenograft models for human studies [17]. Sterility was maintained when performing each experiment, and the mice were housed in adequate environmental conditions.

Estrogen-progesterone pellet insertion

The rats were anaesthesized using isoflurane (1.5 percent). Subcutaneous injection of 1, 17β-estradiol (50 µg/kg/day in 0.2 mL of safflower oil), or progesterone (50 mg/kg/day in 0.1 safflower oil) for 21 days, was given to each mouse before 5 days of placing the xenograft leiomyoma. For pain relief, buprenorphine (50-100 µg/kg) was administered in an injection volume of 1 mL per kg via the subcutaneous route, twice daily or as required [16].

Tissue processing and implantation

Tumors (leiomyoma) obtained from patients were immediately processed under sterilized conditions. The tumor was also cut into 2 x 2 x 2 mm cylindrical slices using biopsy tool, and the slices were immersed before loading into the matrix membrane. Sterility of the leiomyoma tumor was ensured during processing. Twenty mice were injected with the tumour through subcutaneous incisions on the flanks (sides) of the skin, which were later sealed using sterile surgical staples. Two or three tumor slices were given for each mouse). The mice were continuously monitored for any symptoms of discomfort or illness during the procedure. Treatment began after a week of xenograft insertion, only when staples were removed. The mice (n = 16) were randomly assigned to either the treatment (n = 8) or control (n = 8) groups.

Animal treatments

Subcutaneous injection of PL at a dose of 100 µg/gm body weigh/day, or vehicle control, was carried out for 30 days. The mice were closely observed daily, and a caliper was used to measure the tumor size for 30 days. Three-dimensional tumor size analysis was done using a 30 - 50 MHz high-resolution ultrasonic probe (Visual Sonics, China). Tumor volume (V) was computed as shown in Eq 1.

$$V = 0.52lwh \quad \ldots\ldots\ldots\ldots \quad (1)$$

where l is length, w is width and h is height of the tumor.

The mice were euthanized with isofluorane injection after an ultrasound scan, followed by cervical dislocation. The tumors were excised through skin incision. A caliper was used to measure the weight of and size of each wet tumor. The tumours were maintained in a fixative solution of 10 % formalin at 4 °C prior to immunostaining to preserve the visibility of specific tissues. The personnel who conducted the experiments and measured the size of tumors were naïve to the treatments given to the mice groups.

Immuno-histochemical evaluation

Tumor specimens obtained from the mice were embedded in 10 % buffered formalin, and tissue sections were prepared in blocks. The morphologies of the tissue sections were determined using hematoxylin and eosin (H&E) staining. The immuno-peroxidase method using chromogen-DAB was employed to obtain immuno-stained proliferation marker Ki67, and the degree of immuno-staining was quantified using Image-Pro Plus software (Media Cybernetics, USA). After 48 h of treatment with PL, fluorometric caspase-3 activity assay was carried out on ELT-3 cells. The results were obtained after normalizing to control, following three replicated experiments.

Tissue area, staining intensities, and the percent of stained cells relative to the total number of cells, were evaluated using Image-Pro Plus software in ten isolated high-power fields (10) on each slide. The observer (pathologist) interpreting data from the slides had no idea which sample belonged to the treatment and control groups.

Statistical analysis

Results are expressed as mean ± SEM. Results from *in vitro* studies were compared amongst control and PL groups using One-way analysis of variance (ANOVA), followed by Post-hoc Bonferroni analysis (GraphPad Prism 8.0 version). Tumor sizes and immunohistochemistry values for control and PL groups were compared using Student's *t*-test. Values of $p < 0.05$ were considered indicative of significant differences.

RESULTS

Apoptosis in leiomyoma cells

Results from fluorometric assays show that PL treatment for 48 h dose-dependently caused significant differences in apoptotic cell death in ELT-3 cells, relative to control. One-way ANOVA results showed significant variations between control and each of the PL groups ($p < 0.004$). Bonferroni's post-hoc analysis revealed significant differences between the PL-treated groups (50 and 100 µM) and the control group ($p < 0.01$; Figure 1).

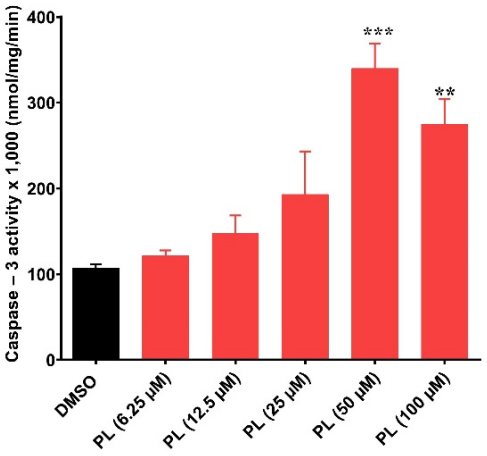

Figure 1: Effect of PL treatment on leiomyoma cells on apoptosis following fluorometric caspase-3 activity assay. The data was standardised to control and shown as the mean±SEM of three independent assays. DMSO stands for dimethyl sulfoxide, and PL stands for piperlongumine. ** P < 0.01 in comparison to control; ***P < 0.001 in comparison to control

Proliferation of leiomyoma cells

The effect of PL on the proliferation of leiomyoma cells was assessed using MTT assay. After treatment of ELT-3 cells with Pl for 48 h, results of one-way ANOVA indicated significant difference in proliferation between groups *(p* = 0.031). Bonferroni's post-hoc analysis revealed that 100 µM PL significantly suppressed cell proliferation, when compared to control group *(p* < 0.05; Figure 2).

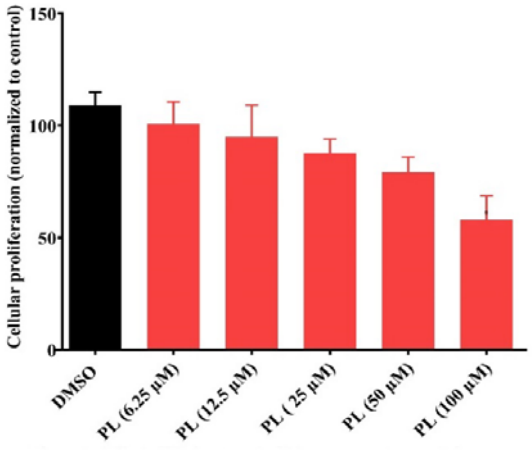

Figure 2: Effect of PL treatment on leiomyoma cells for 48 hours on cellular proliferation following a (3-[4, 5-dimethythiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay. The data was standardised to control and shown as the mean ± SEM of three independent assays. DMSO stands for dimethyl sulfoxide, and PL stands for piperlongumine. *P < 0.05 vs Control

PL Inhibited Akt signaling pathway in leiomyoma cells

Results from Western blotting showed that PL markedly and dose-dependently down-regulated the phosphorylation of Akt protein *(p* < 0.001). These results are shown in Figure 3.

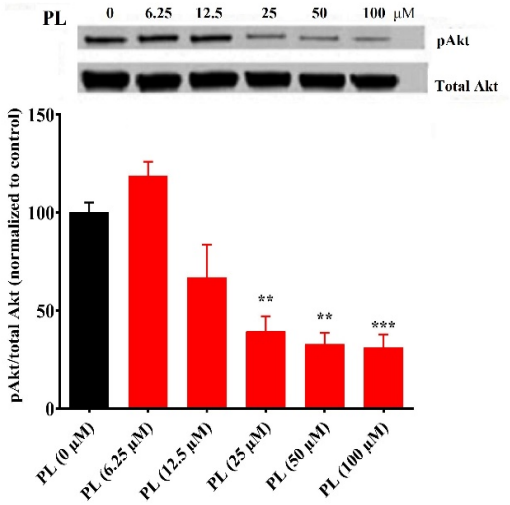

Figure 3: Effect of PL treatment on leiomyoma cells with the specified doses for 48 hours on activation of the Akt cellular signalling system, accompanied by western blot analysis of cell lysates for activated (phosphorylated) Akt and total Akt. The data was standardised to control and shown as the mean ± SEM of three independent assays. DMSO stands for dimethyl sulfoxide, and PL stands for piperlongumine. **P < 0.01 in comparison to control; ***P < 0.001 in comparison to control

Piperlongumine (PL) inhibited growth of xenograft leiomyoma in mice

The sizes of tumors in PL-treated groups were 29.4 and 40.5 % smaller than the corresponding sizes of tumors in control group at week 3 and week 4. Furthermore, the mean tumor size in the treated group was 45.7 % lower than that in the control group (*p* = 0.043; Figure 5), as recorded using ultrasound scanning. The mean weight of tumor in the treatment group was 14.5 % lower than that in the control group, although the difference was not significant (*p* = 0.14; Figure 4).

Pl Inhibited proliferation of leiomyoma cells in xenografted tumor

Figure 5 shows that PL treatment significantly reduced the expression of the proliferation marker by 47.2 percent (*p* = 0.015).

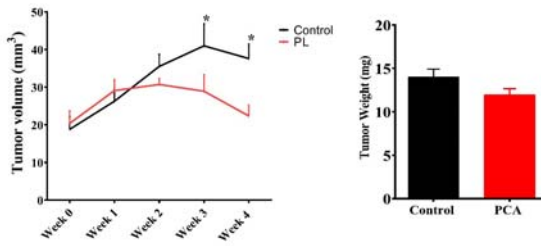

Figure 4: Effect of piperlongumine (PL) treatment on tumour volume (A) and tumour size (B) in a patient-derived leiomyoma xenograft animal model (B). For 30 days, treated animals (n = 8) were given 100 g/g body weight/day subcutaneously, while controls (n = 8) were given vehicle. Calipers were used to quantify tumour volume (mm^3) in the control and PL groups on a weekly basis. The weight of the tumours in the control and PL groups was measured shortly after euthanasia. The data is presented as a mean ± standard error of the mean (SEM)

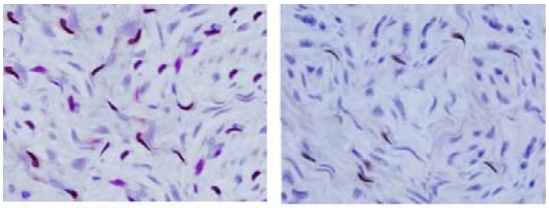

Figure 5: Photomicrographs of the control and PL-treated animals (40x magnification). Tumor tissues acquired after death are placed in a 10% formalin buffered solution and then prepared to obtain tissue slices. The proliferation marker Ki67 was then employed as a chromogen for immunostaining, with (3, 3'-diaminobenzidine) as a chromogen. The number of positively stained cells and staining intensity in high-power fields (20) in each slide were then used to grade expression using image analysis software. The results are presented as the mean SEM of 20 individual treatment and 20 individual control group slides

DISCUSSION

The results obtained in this study showed that piperlogumine exerted apoptotic effects *in vitro*, suppressed the proliferation of leiomyoma cells, and caused leiomyoma tumors in mouse xenograft models. It also inhibited the growth of leiomyoma tumor in an animal model of a patient-derived xenograft.

Mice used in this study had endogenous sex steroids, because they were not ovariectomized. It has been reported that animal models of leiomyoma xenograft showed cell necrosis only in the absence of endogenous steroids [14]. The exact therapeutic effect of PL due to estrogen and progesterone is not yet known since no studies have been reported so far. Thus, there is need for further studies in this area.

Several significant findings were made in the present study. In the first place, piperlonumine was effective in the management of uterine leiomyoma. Secondly, modification of protocols used for establishing animal model in an earlier study [14] resulted in active growth and consistently preserved uterine leiomyoma. The modification entailed substitution of oestrogen pellets with oestrogen-progesterone injection, since progesterone has been shown to be essential for the survival and development of uterine leiomyoma [14]. Another advantage is that it was easier and more accurate to carry out calculations on tumors in the subcutaneous layer. The total volume of tumors after four weeks was 195 percent of the initial volume. This is in agreement with the findings in an earlier study [14].

There are various mechanisms involved in the antitumor effect of PL [2]. A study has shown that PL inhibited tumour growth *in vivo* by activating the transcription factor fork head box O3A (FOXO3A-BIM) axis [18]. The phosphorylation and activation of Akt kinase inhibited activated FOXO3A. In a study conducted on cancer cell bladder cells, piperlogumine increased ROS concentrations and cell growth inhibition, blocked the cancer cells at G2/M phase, and also suppressed migration of the cancer cells [19]. Piperlogumine inhibited cell growth and Ras proteins. A level of P13 K which resulted in the suppression of Akt/NF-κB activation did not cause toxicity [15]. Furthermore, research has shown that PL inhibited phosphorylation of the AKT signaling pathway of growth factors, which in turn inhibited cell proliferation. Moreover, PL showed reversal of resistance to chemotherapy and regulation of gene expressions in tumor cells, along with decreased toxicity and increased activity. The PL-induced changes in Akt/Foxo3/NRF2/P-gp signaling pathway inhibited Cis efflux in A549/Cis cells. Phosphorylation of BAD was decreased by dephosphorylated Akt, which inactivated NRF2 due to reduction of Bcl-xL. The generation of the heterodimer, BAD-Bcl-xL promotes apoptosis. Other mechanisms may also operate due to the different complexities present in *in vivo* tumor microenvironment, relative to cell culture environmental conditions. As a result, further research is required in various *in vivo* leiomyoma mouse models to understand the mechanisms involved in different antitumor activities of PL.

This study has some limitations. No myometrial control was used to determine myometrial toxicity or the effect of PL on the leiomyoma-treated mice or control group. Moreover, PL levels were not investigated in the plasma and tumor. In further studies, the effect of PL on human cell lines will be investigated to correlate with aspects of the current research findings. Tumors beneath the skin are different from tumors in the usual uterine microenvironment region susceptible to steroids such as growth and inflammatory factors. Thus, development of models of orthotopic xenograft leiomyoma is of substantial benefit in leiomyoma studies.

CONCLUSION

Piperlogumine exerts therapeutic effect on uterine leiomyoma. However, further studies are required to understand the mechanism of this activity. Piperlongumine was found to be effective in regulating expression of the tumor cell gene with reversal of resistance to chemotherapy, which resulted in a decrease in toxicity.

DECLARATIONS

Acknowledgement

The work was supported by Beijing Science and Technology Program Project Foundation (no. Z171100001017228).

Conflict of interest

Authors have no conflicts of interest with respect to the publication of this study.

Contribution of authors

We 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 the authors. All authors contributed equally to the publication of this research work. The manuscript was written by Mei Zhang. The materials were collected by Mei Ye, Qingxiang Hou. Bin Yan and Chao Yang offered assistance during revision of the manuscript. All experiments were performed by Mei Zhang, Mei Ye, Qingxiang Hou, Bin Yan, and Chao Yang under supervision of Li Ma.

Open Access

REFERENCES

1. *Okolo S. Incidence, aetiology and epidemiology of uterine fibroids. Best Pract Res Clin Obstet Gynaecol 2008; 22(4): 571-588.*
2. *Bezerra DP, Pessoa C, deMoraes MO, Saker-Neto N, Silveira ER, Costa-Lotufo LV. Overview of the therapeutic potential of piplartine (piperlongumine). Eur J Pharm Sci 2013; 48(3): 453-463.*
3. *Han SS, Tompkins VS, Son DJ. Piper longumine inhibits LMP1/MYC-dependent mouse B-lymphoma cells. Biochem Biophys Res Commun 2013; 436(4): 660-665.*
4. *Duan C, Zhang B, Deng C, et al. Piper longumine induces gastric cancer cell apoptosis and G2/M cell cycle arrest both in vitro and in vivo. Tumour Biol 2016; 37(8): 10793-10804*
5. *Wang Y, Wang JW, Xiao X, et al. Piper longumine induces autophagy by targeting p38 signaling. Cell Death Dis 2013; 4(10): e824.*
6. *Xiong XX, Liu JM, Qiu XY, Pan F, Yu SB, Chen XQ. Piper longumine induces apoptotic and autophagic death of the primary myeloid leukemia cells from patients via activation of ROS-p38/JNK pathways. Acta Pharmacol Sin 2015; 36(3): 362-374.*
7. *Zheng J, Son DJ, Gu SM. Piper longumine inhibits lung tumor growth via inhibition of nuclear factor kappa B signaling pathway. Sci Rep 2016; 6:26357.*
8. *Randhawa H, Kibble K, Zeng H, Moyer MP, Reindl KM. Activation of ERK signaling and induction of colon cancer cell death by piperlongumine. Toxicol In Vitro 2013; 27(6): 1626-1633.*
9. *Ryu J, Kim MJ, Kim TO, Huh TL, Lee SE. Piper longumine as a potential activator of AMP-activated protein kinase in HepG2 cells. Nat Prod Res 2014; 28(22): 2040-2043.*
10. *Piska K, Gunia-Krzyżak A, Koczurkiewicz P, Wójcik-Pszczoła KP, Ekala E. Piper longumine (piplartine) as a lead compound for anticancer agents - Synthesis and properties of analogues: A mini-review. Eur J Med Chem 2018; 156:13-20.*
11. *Liu JM, Pan F, Li L, Liu QR, Chen Y, Xiong XX, Cheng K, Yu SB, Shi Z, Yu AC, Chen XQ. Piper longumine selectively kills glioblastoma multiforme cells via reactive oxygen species accumulation dependent JNK and p38 activation. Biochem Biophys Res Commun2013; 437(1): 87-93.*
12. *Veiga-Santos PD, Esoti VC, Miranda N. The natural compounds piperovatine and piperlonguminine induce autophagic cell death on Trypanosoma cruzi. Acta Trop 2013; 125(3): 349-356.*

13. Wang H, Wang Y, Gao H, Wang B, Dou L, Li Y. Piper longumine induces apoptosis and autophagy in leukemic cells through targeting the PI3K/Akt/mTOR and p38 signaling pathways. Oncol Lett 2018; 15(2): 1423-1428
14. Howe SR, Gottardis MM, Everitt JI, Goldsworthy TL, Wolf DC, Walker C. Rodent model of reproductive tract leiomyomata. Establishment and characterization of tumor-derived cell lines. Am J Pathol 1995; 146(6): 1568-1579.
15. Shrivastava S, Kulkarni P, Thummuri D. Piper longumine, an alkaloid causes inhibition of PI3 K/Akt/mTOR signaling axis to induce caspase-dependent apoptosis in human triple-negative breast cancer cells. Apoptosis 2014; 19(7): 1148-1164
16. VanDelden JJ, Vander G, Raaf R. Revised CIOMS International Ethical Guidelines for Health-Related Research Involving Humans. JAMA 2017; 317(2): 135-136
17. Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG. NC3Rs Reporting Guidelines Working Group. Animal research: reporting in vivo experiments: the ARRIVE guidelines. Br J Pharmacol 2010; 160(7):1577-1579.
18. Liu Z, Shi Z, Lin J, et al. Piper longumine-induced nuclear translocation of the FOXO3A transcription factor triggers BIM-mediated apoptosis in cancer cells. Biochem Pharmacol 2019; 163: 101-110.
19. Kumar S, Agnihotri N. Piper longumine, a piper alkaloid targets Ras/PI3K/Akt/mTOR signaling axis to inhibit tumor cell growth and proliferation in DMH/DSS induced experimental colon cancer. Biomed Pharmacother 2019; 109: 1462-1477.