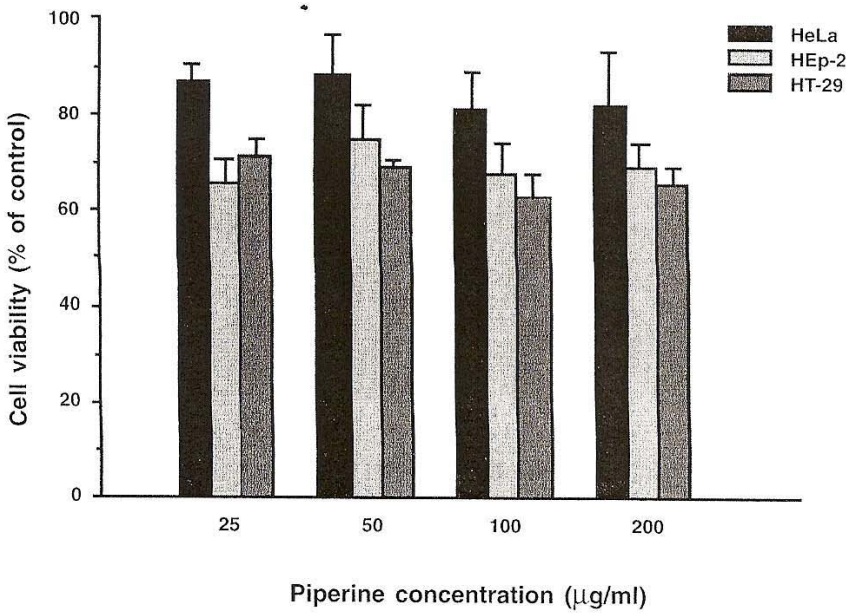


**Fig. 2** Effect of piperine on the proliferation of HeLa, HEp-2 and HT-29 tumor cells. Cells grown in 96-well plates were cultured in various concentrations of the compound for 72 h. Cells growth was assessed by MTS assay. The results are presented as mean ± S.D.

Interestingly, piperine at low concentration (40 μg/mL) markedly inhibited H9 proliferation where cell viability remained at only 36% ($p < 0.05$). Our result demonstrated that piperine provided differential anti-proliferative property on the cervix, larynge, colon and leukemia cell lines. Among these cell lines, H9 was the most susceptible to piperine treatment while HeLa was the least. Moreover, piperine was shown to inhibit H9 proliferation in a concentration-dependent manner (Figure 3), and the concentration found to reduce 50% proliferation ($IC_{50}$) was about 17 μg/mL (Figure 4).

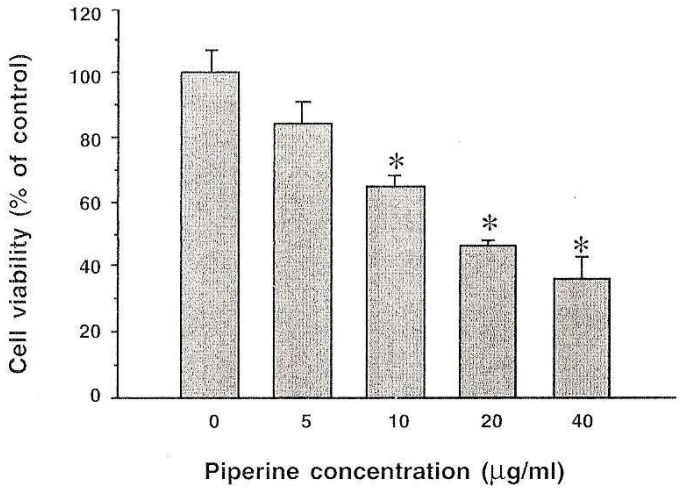


**Fig. 3** Effect of piperine on the proliferation of H9. Cells grown in 96-well plates were cultured in various concentrations of the compound for 72 h. Cell growth was assessed by MTS assay. The results are presented as mean ± S.D. * $P < 0.05$.

**Cytotoxicity of piperine towards H9 cell in culture**

Piperine was found to be cytotoxic towards H9 in culture. The compound demonstrated to be toxic was at a concentration of 10 μg/mL and piperine at a concentration of 200 μg/mL (maximal dose tested) induced the cell death 90% (data not shown).

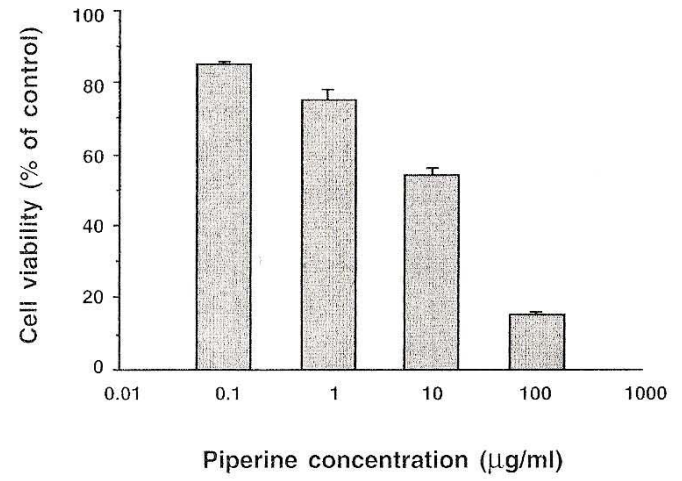


**Fig. 4** Effect of piperine on the proliferation of H9. The percentage of cell viability was assessed by MTS. The results are presented as mean ± S.D.

## Discussion

Although many of herbal products have been used traditionally for cancer treatment for a long time, few scientific reports are available about their efficacies, active principles, modes of action, side effects and possible adverse interactions with conventional anti-tumor drugs. Therefore, investigation the effects of herbal compounds *in vitro* on cancer cell lines is the preliminary study to help understanding their mechanisms of action and maybe helpful for further study using animal model.

The main objective of this study was to focus on the *in vitro* anti-tumor activity of piperine. We found that piperine ineffectively inhibited proliferation of HeLa, HEp-2 and HT-29 tumor cells even at a highest concentration tested (200 μg/mL). In our studies, the maximal concentration of piperine was limited due to its solubility. Also, percentage of vehicle (DMSO) solubilizing the compound could not exceed 1% for all concentrations tested. To overcome this limitation, we have tested tumor cells with piperine in numerous types of solvent. None of them gave a better solubility with non-toxicity to the cells themselves. Therefore, the maximal dosages of piperine selected in our model were on the basis of its solubility. Higher concentration of piperine (>200 μg/mL) might efficiently inhibit the three tumor cell proliferation or maybe toxic to those cells.

Researcher reported that the *in vitro* inhibitory effect of fagopyrum cymosum (fago-c) from Fagopyrum cymosum (Trev.) on tumor growth is selective.[19] The growth of cancer cells from lung, liver, colon, leukocytes and bone is inhibited by Fago-c. However, cancer cells derived from prostate, cervix, ovary and brain are not sensitive to Fago-c. Current studies show that piperine inhibits the growth of cell line derived from specific origin, leukocyte. On the other hand, cancer cells from cervix, larynge and colon are less sensitive to piperine. Therefore, the mechanism and the cellular target of piperine should be further investigated.

As shown in Figure 3, piperine inhibits H9 proliferation in a dose-dependent manner with the concentration required to reduce 50% of the growth is about 17 μg/mL. In studies the compound's cytotoxicity, treatment of the H9 cells using piperine at 10 μg/mL and 20 μg/mL overnight resulted in the cell death 18% and 24%, respectively, indicating this compound is in fact cytotoxic to H9.

These results may be beneficial for the search of new herbal product for T cell lymphoma treatment. Moreover, additional studies on other tumor cells derived from leukocyte and immunomodulatory activity of piperine such as the effect on lymphocyte proliferation, cell-mediated cytotoxicity and cytokine production is undergoing in our laboratory.

## Conclusion

In conclusion, our experimental evidence shows that piperine exhibits interesting anti-proliferative effect on several human tumor

cells. Our works showed differential anti-proliferative effect on tumor cells derived from different organs; cervix, larynge, colon and leukemia cell lines, with the highest activity on H9 lymphoma. The exact mechanism of action responsible for the anti-tumor proliferation and the effects of a substance targeted specific organs in the body should be studied further. In summary, these findings suggest that piperine may be useful as a cancer chemopreventive and chemotherapeutic agent, and may justify further investigation of other possible beneficial biological properties.

## Acknowledgement

This work was supported by Thailand Research Fund 2005. (grant number MRG 4880027) and partly supported by Grant for Development of New Faculty Staff, Chulalongkorn University. The authors would like to thank Dr. Tewin Tencomnao for his helpful discussion. We would also like to express our gratitude to the Halal Science Center and Faculty of Allied Health Sciences, Chulalongkorn University for allowing us to use their facilities.

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# IN VITRO INHIBITORY ACTIVITY OF BLACK PEPPER EXTRACTS AND PIPERINE ON HUMAN LEUKEMIC CELL PROLIFERATION

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## ABSTRACT

Black pepper (*Piper nigrum*) is one of the most common spices highly consumed worldwide. Peppers have been used traditionally due to their various biological activities. A major alkaloid and active component of most *Piper* spp. is piperine. Black pepper and piperine were reported to exhibit CNS depression, anti-pyretic and anti-inflammatory activity. Several studies showed their immunomodulatory and anti-tumor activity towards mouse carcinomas. In our study on various human tumor cells evaluated using MTS assay, anti-proliferative effect of tested cell lines was revealed by piperine at different degree of inhibition. Interestingly, the proliferation of human leukemic cell lines including H9, Jurkat, Molt-4 and K562 were inhibited by piperine with 50% inhibition ($IC_{50}$) of proliferation at 17, 19, 33 and 57 µg/mL, respectively. These cell lines were more susceptible to piperine as compared to HEp-2, HeLa and HT-29 ($IC_{50}$ > 200 µg/ml). Additionally, crude extracts of black pepper were investigated for their anti-proliferative effect towards human tumor cells. We found that methanol and diethylether extracts inhibited proliferation of Molt-4 more effectively as compared to K562. In conclusion, certain tested compounds were evident to suppress all human leukemic cells. Further analysis on the progression of the cell cycle and apoptotic mechanism is underway. Importantly, thorough investigation of the modulatory role of piperine in human PBMCs is ongoing.

## OBJECTIVE

To investigate an *in vitro* anti-proliferative activity of piperine and black pepper extracts on various human leukemic cell lines.

## MATERIALS AND METHODS

**Piperine**
was dissolved in DMSO as 2 mg/mL stock solution, and further diluted to the desired concentrations with culture medium.

**Black pepper extraction**
Black pepper seeds from Mueng Mai Market, Chaing Mai, Thailand, were grounded and subsequently macerated with water, methanol, dichloromethane, hexane or diethylether for 24 h. The filtrates were collected and maceration process were repeated twice. Finally, all filtrates were collected and evaporated to result in crude extracts.

**Figure 2:** Pepper plants

**Cell lines and culture conditions** Jurkat, K562 and Molt-4 were maintained in RPMI-1640 (GIBCO, Invitrogen, NY), supplemented with 10% FBS, 2 mM glutamine, 50 units/ml of penicillin, 50 µg/ml of streptomycin and anti-mycotic in a 5% $CO_2$ humidified incubator at 37 °C.

**MTS assay** were performed following the method described in CellTiter 96® Non-Radioactive Cell Proliferation Assay Technical Bulletin #TB112 (Promega Corp.; Madison, WI) with only minor modifications. Cells were seeded into 96-well plates (Corning Costar; Corning, NY) and exposed to DMSO (0.5%, as vehicle) or with different concentrations of piperine and Black pepper extracts for 72 h. After treatment, MTS tetrazolium compound 20 µl were directly added to 100 µl culture wells and incubate for 4 h in a 5% $CO_2$ humidified incubator at 37 °C. The colored formazan is correlative with the metabolic state of the cells and cell viability. Absorbance was recorded at 492 nm by ELISA reader (Anthos, zenyth 340).

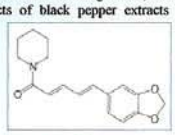
## ACKNOWLEDGEMENTS

This work was supported by Thailand Research Fund 2005 (grant number MRG 4880027) and partly supported by Grant for Development of New Faculty Staff and Research Grant from Faculty of Allied Health Sciences, Chulalongkorn University.

## INTRODUCTION

Piper plants and piperine have been reported to exhibit a variety of biological activities including central nervous system depression, anti-pyretic activity, anti-inflammatory activity, anti-oxidant activity.[1-4] Piperine (1-piperoyl piperidine, as shown in **Figure 1**) is a major alkaloid found in *Piper nigrum* (black pepper), *Piper longum* (long pepper) and several plants in the Piperaceae family. Several studies showed immunomodulatory and anti-tumor activity of piperine and black pepper towards mouse carcinomas.[4] Sunila and Kuttan showed that piperine at the dose of 1.14 mg could inhibit the solid tumor development in mice induced with Dalton's lymphoma ascites (DLA) cells and increase the life span of mice bearing Ehrlich ascites carcinoma (EAC) tumor to 37.3% and 58.8%, respectively.[5] In addition, they reported that piperine could inhibit the metastasis induced by B16F10 melanoma cells.[6,7] Our previous study showed the anti-proliferative effect of piperine on various human tumor cells.[8] Interestingly, the proliferation of human leukemic cell line, H9 was markedly inhibited by piperine ($IC_{50}$ = 17µg/mL). In this study, we performed a Non-Radioactive Cell Proliferation Assay to investigate piperine's effect on various human leukemic cells including Jurkat, Molt-4 and K562. Additionally, the anti-proliferative effects of black pepper extracts on leukemic cells were examined.

**Figure 1:** The chemical structure of piperine



## RESULTS AND DISCUSSIONS

**Anti-proliferative effect of piperine on various human leukemic cells**

As demonstrated in **Table 1**, our previous report showed that piperine slightly affected the viability of HeLa, HEp-2 and HT-29 cells even at the highest concentration (200 µg/mL) tested where cell viability still remained at about 90%, 70% and 66%, respectively. [8] On the contrary, 17 µg/mL of the compound inhibited 50% of H9 proliferation.

**Table 1: $IC_{50}$ values of piperine on a variety of human tumor cells**

| Cell lines | Piperine (µg/ml) |
|---|---|
| HT-29[8] | >200 |
| Hep-2[8] | >200 |
| HeLa[8] | >200 |
| H9[8] | 17 |
| Jurkat | 19 |
| Molt-4 | 33 |
| K562 | 57 |

In this study, we examined piperine's effect on additional human leukemic cells. As shown in **Table 1**, Jurkat, Molt-4 and K562 were treated with piperine. Proliferations of tested cell lines were observed by MTS assay. We demonstrated that the compound at 19, 33 and 57 µg/mL inhibited 50% proliferation of Jurkat, Molt-4 and K562, respectively.

**Table 2: $IC_{50}$ values of black pepper extracts on human leukemic cells**

| Black pepper extracts | $IC_{50}$ (µg/ml) | |
|---|---|---|
| | Molt-4 | K562 |
| Methanol | 10 | 46 |
| Di-ethylether | 23 | 42 |

We also investigated inhibitory effect of black pepper extracts on human leukemic cells. As shown in **Table 2**, methanol and diethylether extracts inhibited proliferation of Molt-4 more effectively as compared to K562.

In conclusion, our experimental evidence shows that piperine and black pepper extracts exhibit interesting anti-proliferative effect on human leukemic cells. The exact mechanism of action responsible for the anti-tumor proliferation should be studied further. These findings suggest that piperine and black pepper extracts may be useful as cancer chemopreventive and chemotherapeutic agents, and may justify further investigation of other possible beneficial biological properties. Further analysis on the progression of the cell cycle and apoptotic mechanism is underway. Importantly, thorough investigation of the modulatory role of piperine in human PBMCs is ongoing.

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## ABSTRACTS FOR POSTER SESSION

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**Chulabhorn Research Institute**

VOLUME II

## PT-24

# *IN VITRO* INHIBITORY ACTIVITY OF BLACK PEPPER EXTRACTS AND PIPERINE ON HUMAN LEUKEMIC CELL PROLIFERATION

**Siriporn Chuchawankul[1], Nantaka Khorana[2], Siratchana Ajsonjorn[3] and Yong Pooworawan[4]**

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Black pepper (*Piper nigrum*) is one of the most common spices highly consumed worldwide. Peppers have been used traditionally due to their various biological activities. A major alkaloid and active component of most *Piper* spp. is piperine. Black pepper and piperine were reported to exhibit CNS depression, anti-pyretic and anti-inflammatory activity. Several studies showed their immunomodulatory and anti-tumor activity towards mouse carcinomas. In our study on various human tumor cells evaluated using MTS assay, anti-proliferative effect of tested cell lines was revealed by piperine at different degree of inhibition. Interestingly, the proliferation of human leukemic cell lines including H9, Jurkat, K562 and Molt-4 were inhibited by piperine with 50% inhibition ($IC_{50}$) of proliferation at 17, 19, 33 and 57 μg/mL, respectively. These cell lines were more susceptible to piperine as compared to HEp-2, HeLa and HT-29 ($IC_{50}$ > 200 μg/mL). Additionally, crude extracts of black pepper were investigated for their anti-proliferative effect towards human tumor cells. We found that methanol and diethylether extracts inhibited proliferation of Molt-4 more effectively as compared to K562. In conclusion, certain tested compounds were evident to suppress all human leukemic cells. Further analysis on the progression of the cell cycle and apoptotic mechanism is underway. Importantly, thorough investigation of the modulatory role of piperine in human PBMCs is ongoing.

# ภาคผนวก 3

# INHIBITORY EFFECT OF PIPERINE ON PROLIFERATION OF HUMAN TUMOR CELLS


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## ABSTRACT


Piperine, an amide isolated from piper species, was reported to display central nervous system depression, antipyretic and anti-inflammatory activity. Several studies showed immunomodulator and antitumor activity of piperine towards mouse carcinomas. Our study is the first to investigate whether piperine has those effects on human tumor cells. Human cancer cells derived from different organs were employed, and their growths affected by piperine were determined by MTS assay. We found that cancer cells derived from colon (HT-29) and larynx (HEp-2) were less sensitive to piperine even at a high concentration (200 μg/ml) where cell viabilities were remained at about 70% and 66%, respectively. Interestingly, the proliferation of cancer cells derived from leukocyte, H9 and Jurkat, were markedly inhibited by piperine even at a low concentration (20 μg/ml). In addition, piperine was shown to inhibit H9 proliferation in a concentration dependent manner, with 50% inhibition ($IC_{50}$) of proliferation at 17 μg/ml. In contrast, cell viability of Jurkat was remained at about 43% even at a higher concentration (100 μg/ml). This study suggests the effect of piperine on inhibiting the growth of cell lines derived from specific origin, indicating that the inhibition by piperine is selective. Further experiments should be studied to explore the mechanism of anti-proliferative effect of piperine on H9 and Jurkat.


## INTRODUCTION

Cancer is one of the major cause of death in Thailand. The number of people with cancer is increasing everyday. There are various cancer treatment but they all have side effects that decrease the quality of life of patients. Current research has concentrate on extraction of medicinal plants to prevent and treat cancer because those plants are easy to find, inexpensive and have less side effects when compare to traditional therapy. Piperine ($C_{17}H_{19}NO_3$, as shown in figure 1) is an amide isolated from *Piper nigrum* (black pepper), *Piper longum* (long pepper) and some plants in Piperaceae family. Piperine stimulates the body's natural ability to generate heat, assists in the absorption of selenium, vitamin B and β-carotene and enable more efficient absorption of needed or targeted nutrients during the digestive process.[1,2] Piperine was reported to display central nervous system depression, antipyretic and anti-inflammatory activity. Several studies showed immunomodulator and antitumor activity of piperine towards mouse carcinomas.[3] Furthermore, piperine could inhibit the metastasis induced by B16F-10 melanoma cells.[4,5] Our study is the first to investigate whether piperine has those effects on human tumor cells.

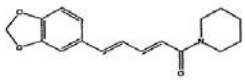

Figure 1. The chemical structure of piperine

## OBJECTIVE

To study inhibitory effect of piperine on proliferation of human tumor cells.

## MATERIALS AND METHODS

**Reagent**

Piperine was obtained from Fluka (Buchs, Switzerland). 0.25% Trypsin-EDTA (Hyclone Laboratories, Inc.; Logan, UT), $Ca^{2+}Mg^{2+}$ -free Dulbecco's phosphate-buffered saline (Promega Corp.; Madison, WI)

**Cell culture**

H9 and Jurkat (Human T cell lymphoma) were cultured in RPMI-1640 (GIBCO, Invitrogen, NY), while HT-29 (colon carcinoma) and HEp-2 (larynx carcinoma) were cultured in DMEM (Hyclone Laboratories, Inc.; Logan, UT). All media were supplemented with 10% FBS (Hyclone Laboratories, Inc.; Logan, UT), 100 U/ml of penicillin, 100 μg/ml of streptomycin and antimycotic (GIBCO, Invitrogen, NY) at 37°C in 5%$CO_2$ incubator.

**MTS assay**

The procedure followed the method described in CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega Corp.; Madison, WI). Cells were seeded into 96-well plates (Corning Costar; Corning, NY) and adherent cells were allowed to attach overnight. Then cells were exposed to DMSO (1%, as vehicle) or different concentration of piperine (5-200 μg/ml) and incubated for 72 h. After incubation, MTS tetrazolium compound 20 μl were added to 100 μl culture wells and incubate for 4 h at 37 °C in 5%$CO_2$ incubator. The MTS tetrazolium compound was bioreduced by metabolically active cells into a colored formazan product that directly proportional to the number of viable cells in the cultures and analysed on a microplate reader at a wavelength of 490 nm.

**Statistical analysis**

The experiments were repeated at least three times and the results were presented as mean ± S.D. The Student's unpaired *t*-test was used to compare the means of two groups. Differences were considered statistically significant when $P < 0.05$.

## RESULTS

As shown in Figure 2, cancer cells derived from colon (HT-29) and larynx (HEp-2) were less sensitive to piperine even at a high concentration (200 μg/ml) where cell viabilities were remained at about 70% and 66%, respectively. The results were presented in percentage of cell viability in treated cells to that of untreated cells.

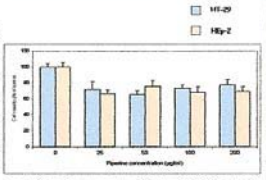


Fig. 2. Effect of piperine on the proliferation of HT-29 and HEp-2 tumor cells. Cells grown in 96-well plates were cultured in various concentration of the compound (25-200 μg/ml) for 72 h. Cells growth was assessed by MTS assay. The results are presented as mean ± S.D.

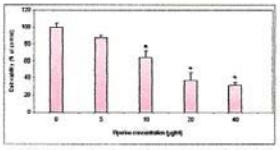

Fig. 3. Effect of piperine on the proliferation of H9 tumor cells. Cells grown in 96-well plates were cultured in various concentration of the compound (5-40 μg/ml) for 72 h. Cells growth was assessed by MTS assay. The results are presented as mean ± S.D. *$P < 0.05$.

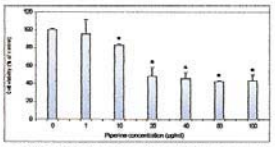

Fig. 4. Effect of piperine on the proliferation of Jurkat tumor cells. Cells grown in 96-well plates were cultured in various concentration of the compound (1-100 μg/ml) for 72 h. Cells growth was assessed by MTS assay. The results are presented as mean ± S.D. *$P < 0.05$.

Interestingly, the proliferation of cancer cells derived from leukocyte, H9 and Jurkat, were markedly inhibited by piperine even at a low concentration (20 μg/ml). In addition, piperine was shown to inhibit H9 proliferation in a concentration dependent manner (Figure 3), with 50% inhibition ($IC_{50}$) of proliferation at 17 μg/ml. In contrast, cell viability of Jurkat was remained at about 43% even at 100 μg/ml (Figure 4).

## DISCUSSION & CONCLUSION

Medicinal herbs are increasingly being used for cancer therapy, therefore investigations into the effects of herbal compounds on tumor cells and their mechanism will be beneficial. The objective of this study is to focus on the in vitro antitumor activity of piperine on human tumor cells. We found that HT-29 and HEp-2 tumor cells were less sensitive to piperine even at a highest concentration (200 μg/ml) which was limited by its solubility. In our research, the percentage of vehicle (DMSO) could not exceed 1% for all test because higher concentrations may be toxic to cells.

Researcher reported that the in vitro inhibitory effect of fagopyrum cymosum (fago-c) from Fagopyrum cymosum (Trev.) on tumor growth is selective.[6] The growth of cancer cells from lung, liver, colon, leukocytes and bone is inhibited by Fago-c. However, cancer cells derived from prostate, cervix, ovary and brain are not sensitive to Fago-c. Currently, our studies show that piperine inhibits the growth of cell line derived from specific origin, leukocyte. In contrast, cancer cells from colon and larynx are less sensitive to piperine.

As shown in figure 3, piperine inhibits H9 proliferation in a concentration-dependent manner and a concentration of 17 μg/ml reduced growth by 50%. In contrast to Jurkat cell viability which remained at about 43% in a higher concentration (100 μg/ml) of piperine. This study suggests the selective effect of piperine on inhibiting the growth of cell lines derived from specific origins. Further experiments should be studied to explore the mechanism of anti-proliferative effect of piperine on H9 and Jurkat.

## ACKNOWLEDGEMENTS


This work was supported by Thailand Research Fund 2005. (grant number MRG 4880027) The authors sincerely thank Dr. Pornthep Tiensiwakul for providing H9 and HEp-2 tumor cells, Dr. Weera wongkham for providing HT-29 tumor cells, Dr. Apiwat Mutirangura for providing Jurkat tumor cells, Dr. Nantaka Korana and Dr. Tewin Tencomnao for their kind help and valuable suggestions.


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# รวมบทคัดย่อ

## การประชุมเสนอผลงานวิจัยระดับบัณฑิตศึกษาแห่งชาติ ครั้งที่ 6

## The 6th National Symposium on Graduate Research

ณ อาคารมหิตลาธิเบศร จุฬาลงกรณ์มหาวิทยาลัย

วันที่ 13-14 ตุลาคม 2549

บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย

ร่วมกับ

ที่ประชุมคณบดีบัณฑิตวิทยาลัย
มหาวิทยาลัยของรัฐ(ทคบร.)

| | |
|---|---|
| **Title** | **PIPERINE'S INHIBITION ON PROLIFERATION OF HUMAN TUMOR CELLS** |
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| **Keywords** | *Piperine, H9, Jurkat* |

**Abstract**

Piperine, an amide isolated from piper species, was reported to display central nervous system depression, antipyretic and anti-inflammatory activity. Several studies showed immunomodulator and antitumor activity of piperine towards mouse carcinomas. Our study is the first to investigate whether piperine has those effects on human tumor cells. Human cancer cells derived from different organs were employed, and their growths affected by piperine were determined by MTS assay. We found that cancer cells derived from colon (HT-29) and larynx (HEp-2) were less sensitive to piperine even at a high concentration (200 µg/ml) where cell viabilities were remained at about 70% and 66%, respectively. Interestingly, the proliferation of cancer cells derived from leukocyte, H9 and Jurkat, were markedly inhibited by piperine even at a low concentration (20 µg/ml). In addition, piperine was shown to inhibit H9 proliferation in a concentration dependent manner, with 50% inhibition ($IC_{50}$) of proliferation at 17 µg/ml. In contrast, cell viability of Jurkat was remained at about 43% even at a higher concentration (100 µg/ml). This study suggests the effect of piperine on inhibiting the growth of cell lines derived from specific origin, indicating that the inhibition by piperine is selective. Further experiments should be studied to explore the mechanism of anti-proliferative effect of piperine on H9 and Jurkat.

# บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย

มอบเกียรติบัตรฉบับนี้ให้ไว้เพื่อแสดงว่า

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ได้รับรางวัลการนำเสนอผลงานวิจัยด้วยโปสเตอร์ ระดับดี

กลุ่มสาขาวิชาวิทยาศาสตร์และเทคโนโลยี

ในการประชุมเสนอผลงานวิจัยระดับบัณฑิตศึกษาแห่งชาติ ครั้งที่ ๖

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