ระเบียบวิธีวิจัยและผลการทดลอง

1. การเพาะเลี้ยงเซลล์ HepG2

Human hepatocelluair carcinoma, HepG2 cells ซึ่งสั่งซื้อจาก the American Type Culture Collection (ATCC) จะใช้เป็นเซลล์ทดลองในการทดลองนี้ โดยเซลล์จะเพาะเลี้ยงใน 37°C a humidified incubator with 5% CO_2/95% air atmosphere โดยใช้ media ชนิด DMEM ซึ่ง supplement ด้วย 10% fetal bovine serum, 3 mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin. โดยเซลล์จะถูก subculture ประมาณ 2 ครั้ง/สัปดาห์

2. Cell proliferation assay

เพื่อพิสูจน์ผลของสาร piperine ต่อการยับยั้ง Cell proliferation ของ HepG2 cells ทำการวิเคราะห์โดย 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (sigma) assays วิธีการคือ adding 15 μl MTT (5 mg/ml) ในแต่ละ well เป็นเวลา 4 h ที่ room temperature ถึง 37°C Supernatants จะถูก removed หลังจากนั้นการ form ตัวของ formazan จาก tetrazolium salt จะสามารถ solubilized ได้โดยใส่ 100 μl/well dimethyl sulfoxide (DMSO, Amresco) และ shake เป็นเวลา 10 นาที ค่า absorbance value (OD) ของแต่ละ well จะทำการวัดด้วย microplate photometer ที่ 540 nm

ผลการทดลองพบว่า proliferation ของ HepG2 cells จะถูกยับยั้งเมื่อได้รับ piperine ตั้งแต่ 100 μmole/L จนถึง 1 mmole/L ที่ 24 48 และ 72 ชม. โดยที่ the highest inhibitory rate มีค่าประมาณ 44% เมื่อได้รับ piperine 1 mmole/L ที่ 24 ชม. ($p<0.05$) และมีค่าประมาณ 62% เมื่อได้รับ piperine 1 mmole/L ที่ 48 ชม ($p<0.001$) และมีค่าประมาณ 78% เมื่อได้รับ piperine 1 mmole/L ที่ 72 ชม ($p<0.001$) ดังแสดงใน table 1 และ figure 1

Table 1 : Impact of piperine treatment for 24, 48 and 72 h on HepG2 cell viability analyzed by MTT assay

Piperine (µmol/L)	**%cell viability**		
	24 hr.	**48 hr.**	**72 hr.**
0	100±29.34	100±23.78	100±5.612
10	97.31±28.91	101.55±25.59	94.95±1.393
100	88.31±29.61	84.41±28.55	73.69±2.465
200	73.77±17.06	62.99±15.82 *	38.54±1.160 **
300	69.91±15.57	53.42±8.58 *	30.04±1.042 **
400	65.02±10.12 *	47.58±6.09 **	28.48±1.283 **
500	61.09±9.55 *	42.42±3.47 **	27.52±0.674 **
1000	56.91±0.48 *	38.66±0.34 **	22.98±1.034 **

Data are represented as mean ± SEM, n=3.

*p<0.05 and **p<0.001, significantly different from control, untreated group.

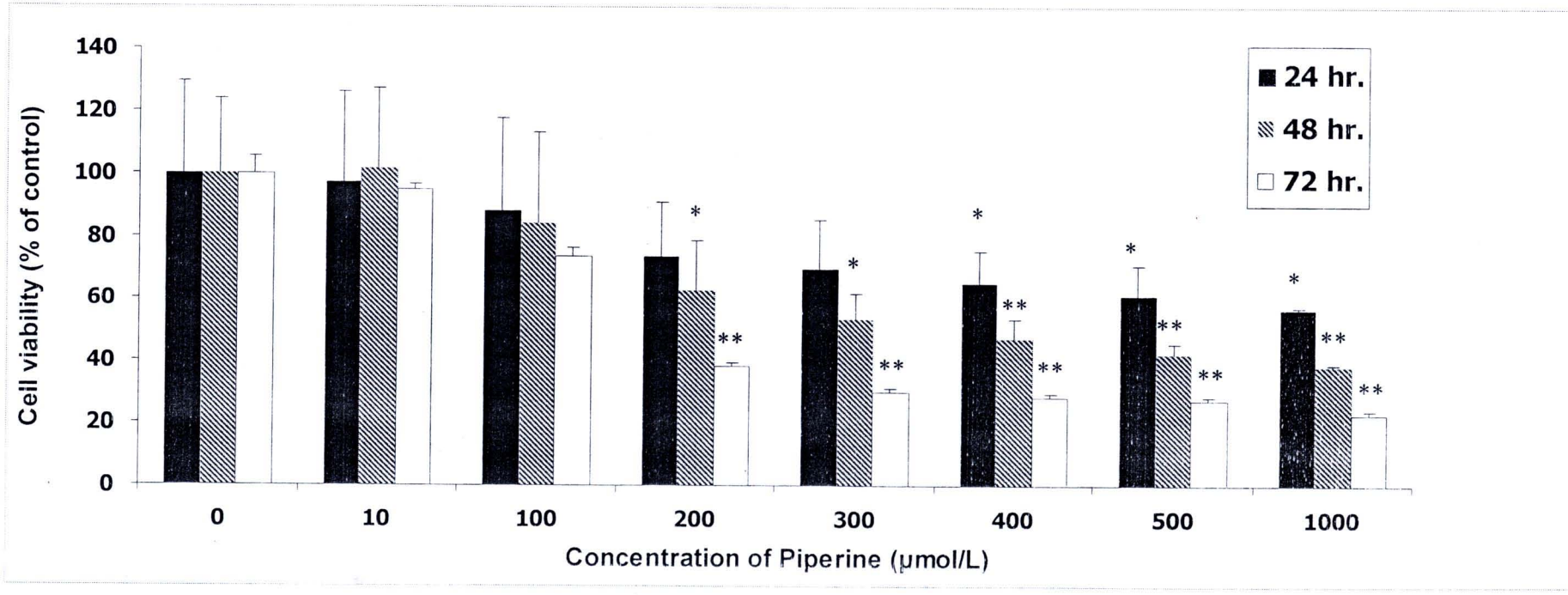


Figure 1 Impact of piperine treatment for 24. 48 and 72 h on HepG2 cell viability. HepG2 cells were treated with piperine at 10 to 1000 µmole/L for 24, 48, and 72 h. At the indicated time points after piperine treatment, cells were analyzed by MTT assay. Data shown are representative for three independent experiments; columns indicate means; bars are the standard error. *p<0.05 and **p<0.001, significantly different from control, untreated group.

6. FASN expression by immunoblot analysis

เตรียม Cell extract โดยใช้ M-PER lysis buffer หลังจากนั้นทำการวัด Protein concentrations โดยการ diluted samples ด้วยวิธีการของ a bicinchoninic acid procedure ตาม protocol (Pierce Biochemical Company, Rockford, IL) ทำ Western analysis โดยใช้ triplicate experiments ทำการ load Equal amounts of protein ให้แยกด้วย NuPage Tris-acetate gel (Invitrogen) และ electrotransferred ไปที่ polyvinylidene difluoride membranes (Roche, Mannheim, Germany) หลังจากนั้น incubate Membranes ด้วย rapid blocking solution (Amresco, USA) และทำการ probed ด้วย antibodies against FASN (Abcam) Horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody ด้วย Novex® ECL, HRP Chemiluminescent substrate reagent kit (Invitrogen) เพื่อจะ detect the immunoreactive protein ด้วย chemiluminescence (ImageQuant™ LAS 4000, GE Healthcare BIO-Science AB, Sween) วิธีการทดลองนี้ทำเพื่อ determine the effect ของ piperien ต่อการ expression ของ FASN protein โดยที่พบว่าการ expression ของ FASN protein ในกลุ่มที่ได้รับการ treat ด้วย piperine โดยเฉพาะที่ piperine ความเข้มข้น 200, 300, 400, 500 และ 1,000 μmole/L มีผลให้การ expression ของ FASN ลดลงอย่างมาก เมื่อเปรียบเทียบกับกลุ่ม control cells ผลการทดลองนี้แสดงให้เห็นว่า piperine มีผลต่อการ expression ของ FASN protein ซึ่งมีบทบาทในการ regulates intracellular de novo fatty acid synthesis ใน HepG2 cells

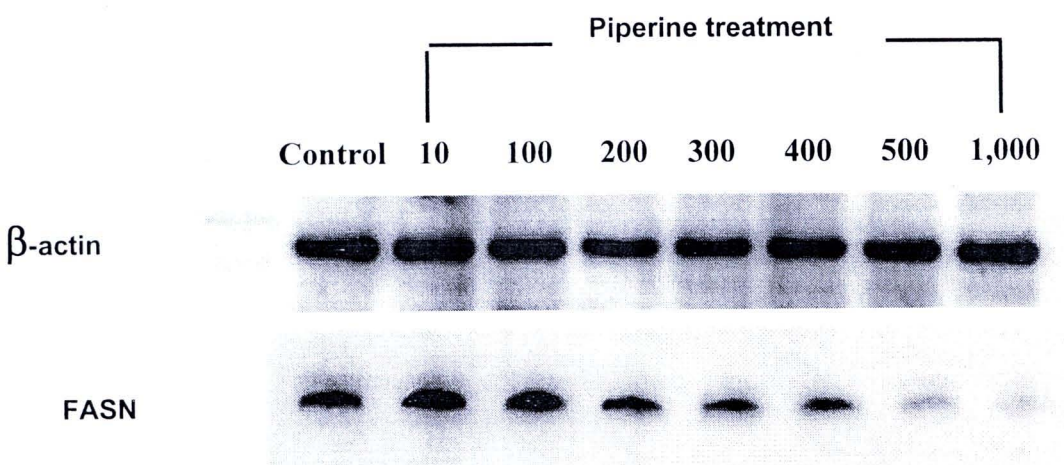


Figure 2 Impact of piperine on FASN expression. HepG2 cells were treated with piperine at 10 to 1000 μmole/L for 24, 48, and 72 h. Protein extracts were made and FASN protein levels were determined by Western blot analysis. 42 kD β-actin expression was used as internal control.

7. Analysis on externalization of phosphatidylserine (PS) , the early stage of apoptosis

In order to examine whether the cytotoxic effect of piperine was related to the induction of apoptosis, HepG2 cells apoptosis was analyzed by flow cytometry. Annexin V/propidium iodide staining was assessed to measure the externalization of PS during early apoptosis. The attached and detached cells were harvested from culture. Apoptosis induced by piperine was determined with an Alexar Flour® 488 Annexin V and propidium iodide (PI) apoptosis detection kit (Invitrogen) according to manufacturer's instructions. Briefly, cells were washed and subsequently incubated for 15 min at room temperature in the dark in 300 µl of 1X binding buffer containing 5 µl of Alexar Flour® 488 Annexin V and 10 µl of PI. Afterward, apoptosis was analyses by a flow cytometer (BD FACSCaliber, BECTON DICKINSON, California, CA). The cells in the Annexin V-positive and PI-negative fraction were regarded as an early apoptotic cells. Numerous studies have reported that advanced DNA fragmentation is preceded by alteration in the plasma membrane, such as PS externalization. During early apoptosis, the plasma membrane asymmetry is lost due to the externalization of PS. In Table and Figure below, untreated cells showed low or negative with both Annexin V and PI, indicating the viable cells. The early apoptotic cells were approximately 11% , 18%, and 25% for 24 h of 10, 300, and 500 µmole/L piperine treatment, respectively. Notably, a considerable decreasing of early apoptotic and increasing of late apoptotic cells, approximately from 0.76% to 89%, was visible at 24 h of 1 mmole/L piperine treatment. The early apoptotic cells were approximately 19% , 20%, and 12% for 48 h of 10, 300, and 500 µmole/L piperine treatment, respectively. Notably, a considerable decreasing of early apoptotic and increasing of late apoptotic cells, approximately from 8% to 81%, was visible at 48 h of 1 mmole/L piperine treatment.

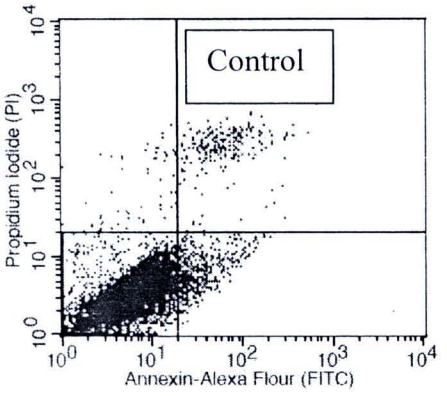
Table 2 : Impact of piperine treatment for 24 h on HepG2 cell apoptosis analyzed by flow cytometry with an Alexar Flour® 488 Annexin V and propidium iodide (PI) staining

	Control	10 µmol/L	300 µmol/L	500 µmol/L	1 mmol/L
Viability	91.99±6.10	79.49±13.10	71.83±12.19	49.34±4.37	3.42±3.03
Early	2.01±0.22	11.03±11.52	18.78±11.57	25.28±10.96	0.76±0.21
Late	4.28±3.97	8.35±2.20	8.58±1.53	24.52±9.27	89.00±4.56

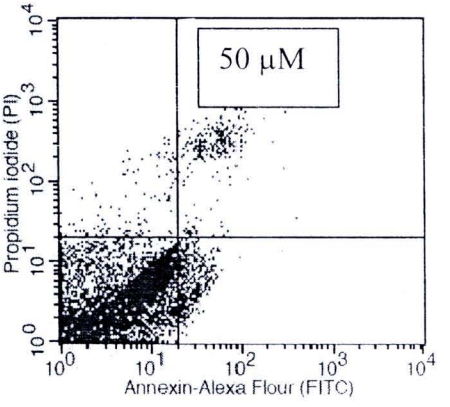
Data are represented as mean ± SEM, n=3.

*p<0.05 and **p<0.001, significantly different from control, untreated group.

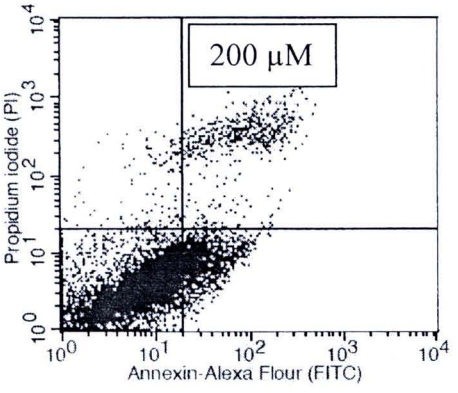
A.



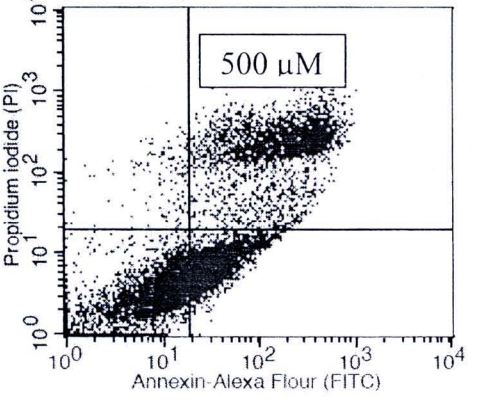
B. Piperine treatment for 24 h



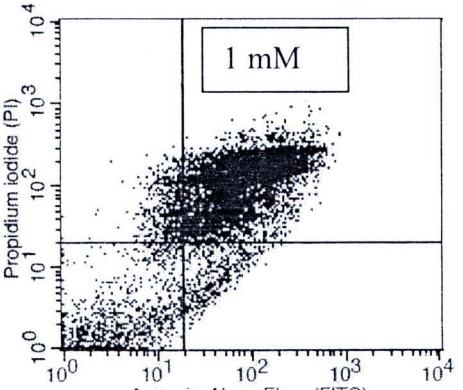
C. Piperine treatment for 24 h



D. Piperine treatment for 24 h



E. Piperine treatment for 24 h



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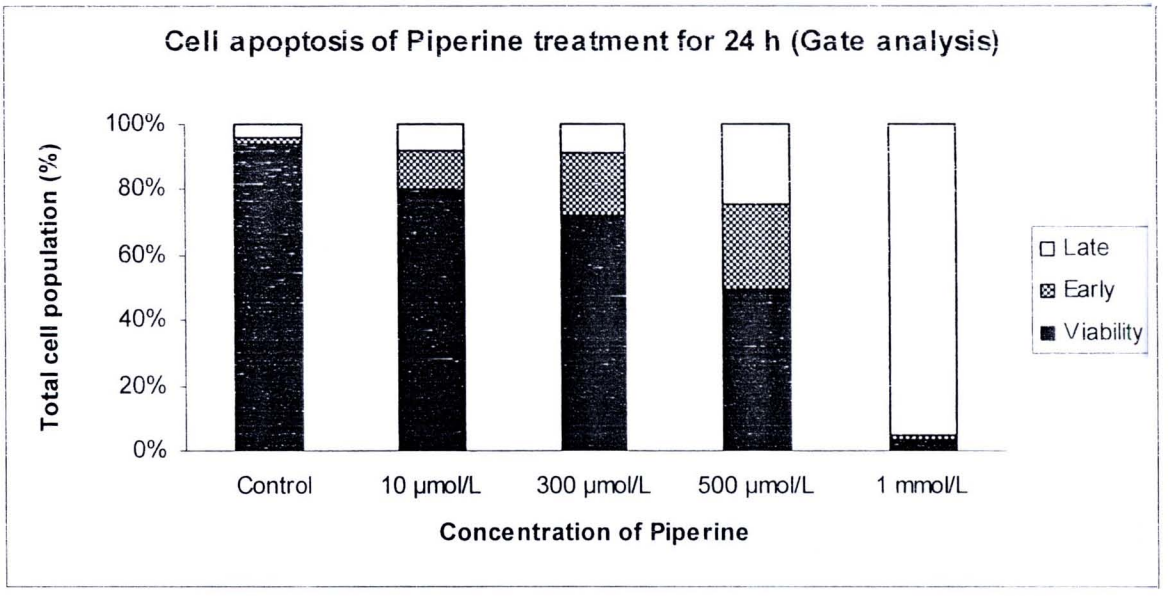


Figure 3 Effect of 24 h piperine-induced an externalization of PS, the early stage of apoptosis of HepG2 cells. At the indicated time points after treatment, cells were collected and double stained with Alexar Flour® 488 Annexin V and propidium iodide and analyzed by flow cytometry as described above (no gated analysis). A. to E. represents dual parametric dot plots of propidium iodide fluorescence (y-axis) versus Alexar Flour® 488 Annexin fluorescence (x-axis). D. bar charts showing the propotion of viable, early apoptotic, and late apoptotic cells. Early apoptotic cells were defined as Annexin V-positive, PI-negative cells. Values are mean ± S.E.M. from three experiments.

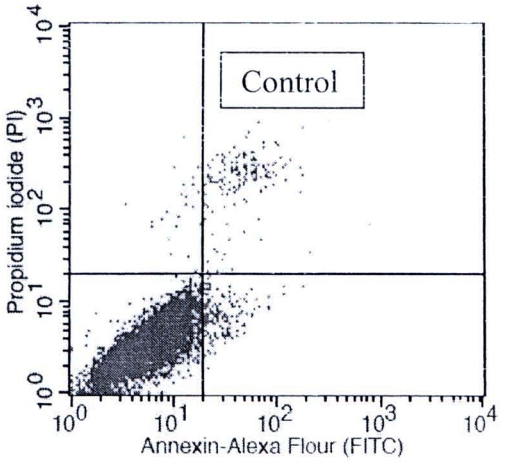
Table 3 : Impact of piperine treatment for 48 h on HepG2 cell apoptosis analyzed by flow cytometry with an Alexar Flour® 488 Annexin V and propidium iodide (PI) staining

	Control	**10 µmol/L**	**300 µmol/L**	**500 µmol/L**	**1 mmol/L**
Viability	91.31±2.7	67.31±19.13	65.22±20.89	56.74±13.09	5.33±2.11
Early	2.44±1.32	19.45±15.88	20.85±16.15	12.95±9.89	8.08±2.68
Late	5.52±1.93	10.63±8.29	12.7±7.70	28.53±5.06	81.22±4.14

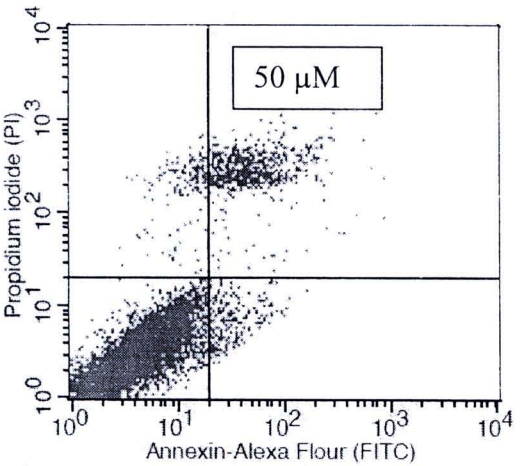
Data are represented as mean ± SEM, n=3.

*p<0.05 and **p<0.001, significantly different from control, untreated group.

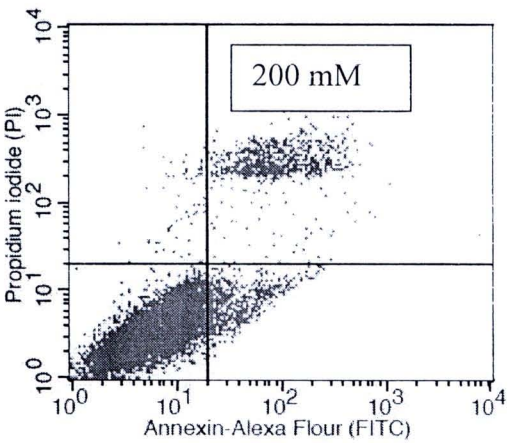
A.



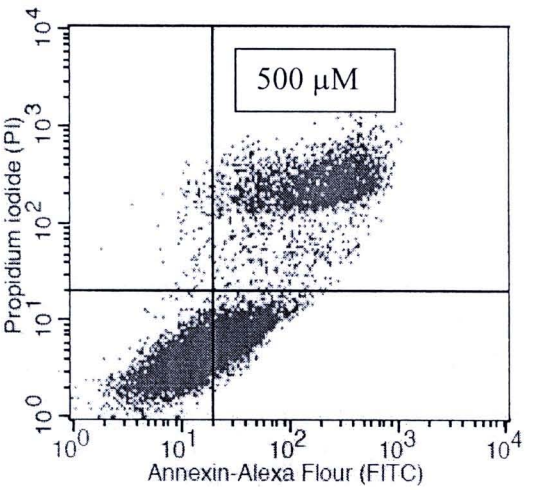
B. Piperine treatment for 48 h



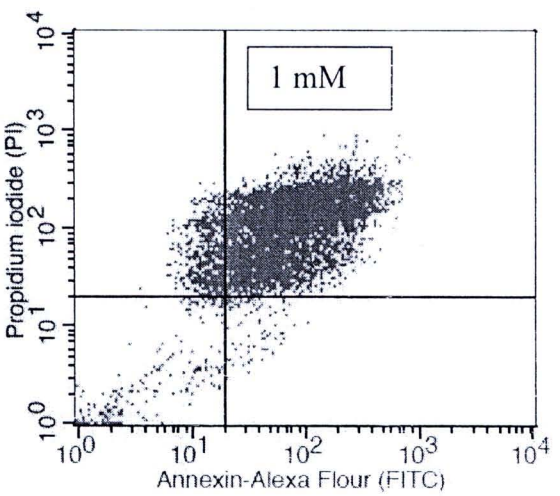
C. Piperine treatment for 48 h



D. Piperine treatment for 48 h



E. Piperine treatment for 48 h



F.

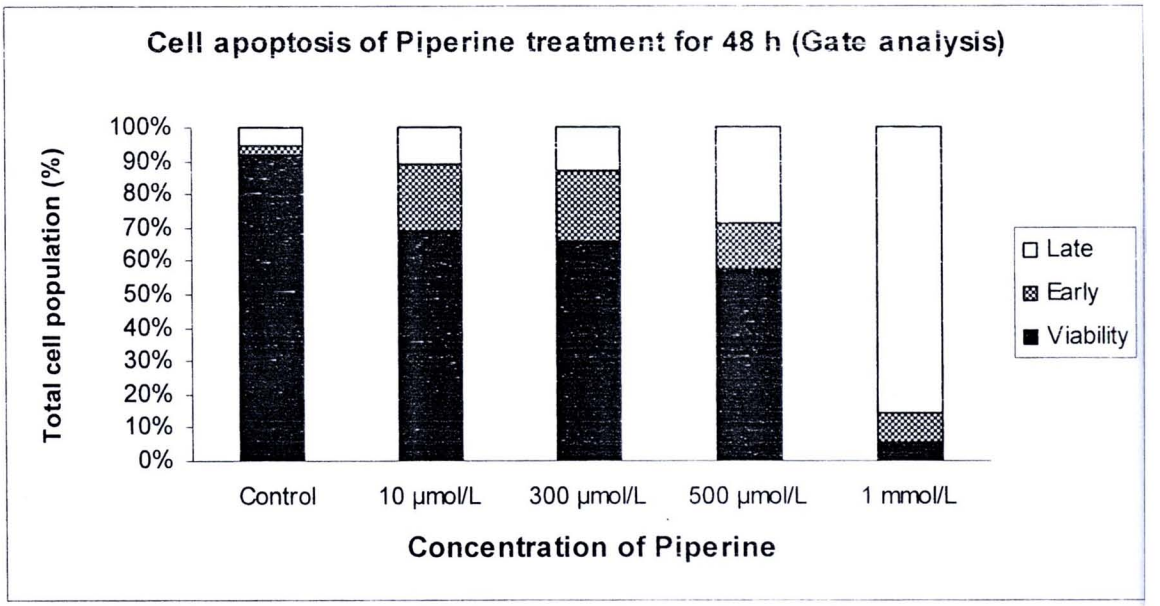


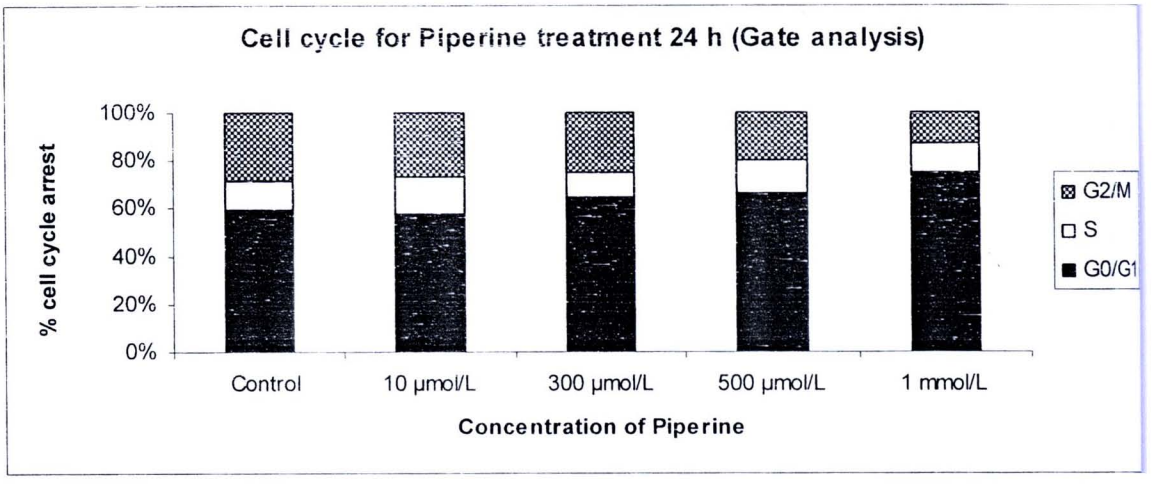
Figure 4 Effect of 48 h piperine-induced an externalization of PS, the early stage of apoptosis of HepG2 cells. At the indicated time points after treatment, cells were collected and double stained with Alexar Flour® 488 Annexin V and propidium iodide and analyzed by flow cytometry as described above (no gated analysis). A. to E. represents dual parametric dot plots of propidium iodide fluorescence (y-axis) versus Alexar Flour® 488 Annexin fluorescence (x-axis). D. bar charts showing the propotion of viable, early apoptotic, and late apoptotic cells. Early apoptotic cells were defined as Annexin V-positive, PI-negative cells. Values are mean ± S.E.M. from three experiments.

8. Cell cycle analysis

Induction of programmed cell death by piperine treatment was detected using flow cytometry analysis. After 24 and 48 h of treatment, cells were harvested by trysinization for 5 min, washed with ice-cold PBS twice, and fixed with 75% cold ethanol at 4°C overnight. Propidium iodide (PI) at concentration of 20 µg/ml and RNase A at concentration of 200 µg/ml was used to stain the nuclei to monitor the phases of cell cycle for 6 h. The fluorescence of DNA-bound PI in cells was measured with a flow cytometer (BD FACSCaliber, BECTON DICKINSON, California, CA).. This experiment was performed to further examine the effects of NaCT silencing on the cell cycle of HepG2 cells.

As shown in Figure below, piperine treatment for 24 and 48 h resulted in a higher number of cells in G1/G0 phase compared to control cells. At control group, only 59.4 % of cells were in G1/G0 phase , 12.87% of cells were in S phase and 28.20% of cells were in mitosis (M) phase. After 24 h of piperine treatment at 300, 500, and 1,000 μmole/L, the percentage of HepG2 cells in the G1/G0 phase increased to 65.21% , 66.83% , and 74.86%, respectively. The percentage in the S phase seemed not changed to much and the percentage in the M phase decreased to 24.77% , 19.62%, and 13.35% in 300, 500, and 1,000 μmole/L, respectively in piperine treatment compared with control cells. After 48 h of piperine treatment, the percentage of HepG2 cells in the G1/G0 phase increased as shown in 24 h treatment, and the percentage in the S phase decreased to 11.05% , 10.67%, and 9.75% in 300, 500, and 1,000 μmole/L of piperine treatment, respectively compared with control cells. These data suggest that piperine arrests HepG2 cells in the G1/G0 phase.

A.



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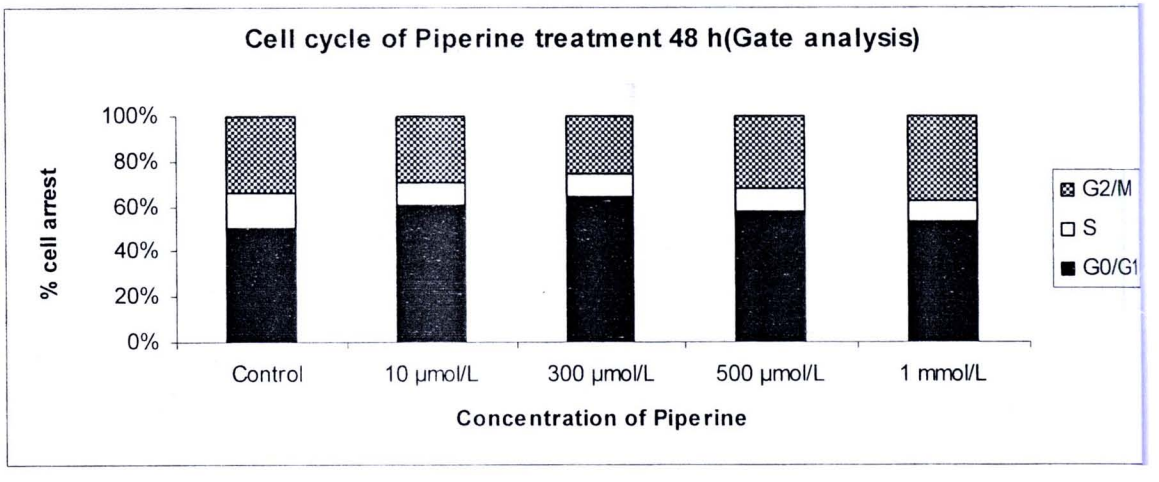


Figure 6 A and B represents the effect of piperine treatment on cell cycle progression. After 24 and 48 h of treatment, cells were fixed with 75% ethanol overnight and incubated with RNase A and propidium iodide for 6 h. DNA content was assessed by flow cytometry. Each point represents the mean±SEM of three independent experiments.

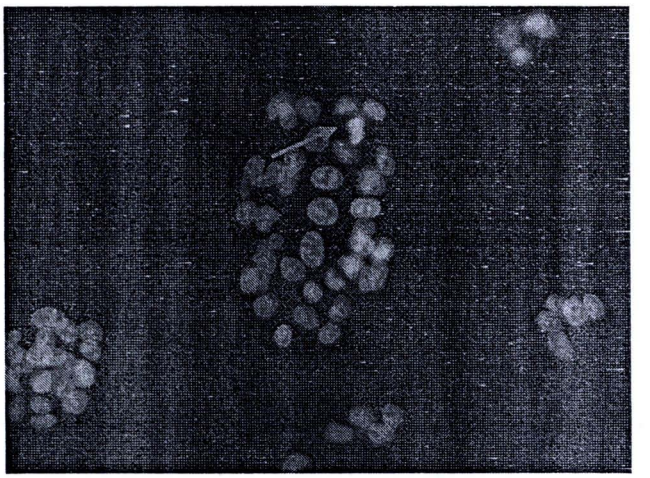
9. Detection of apoptosis by fluorescence microscopy using DAPI apoptosis assay

Cells were treated with piperine for24 and 48 h., the attached and detached cells were harvested from the culture and washed with PBS, followed by fixation of cells in 75% ethanol at 4°C overnight. The fixed cells were washed with PBS and the changes in nuclear morphology during apoptosis were stained with 4',6'-Diamidino-2-phenylindole (DAPI) for 30 min in the room temperature. The cells with nuclear

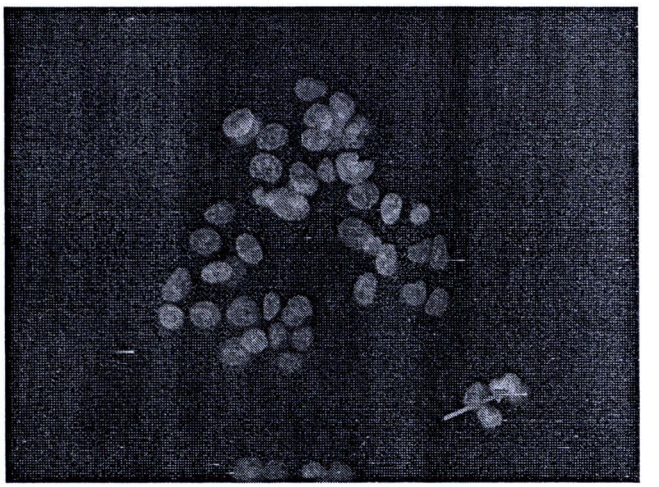
breakdown were manually counted and capture the photographs by fluorescence microscopy. Fragmentation of the nucleus into oligonucleosomes and chromatin condensation was detected by fluorescence microscopy using a filter for DAPI.

Apoptosis is initially characterized by morphological features, such as chromatin condensation, nuclear fragmentation, and membrane blebbing. As shown in the Figures below, cells underwent unclear changes upon piperine treatment. In the control untreated cells, the nuclei were intact, round and uniformly stained with DAPI. However, after silencing, cells manifested apoptosis occurrences.

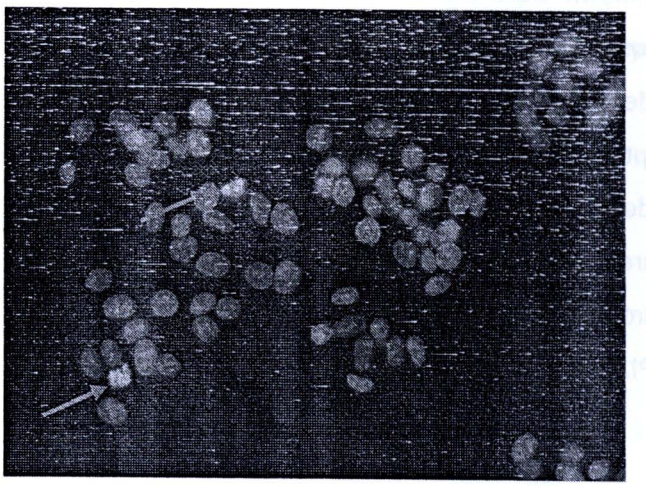
A. Control , untreated cells for 24 h



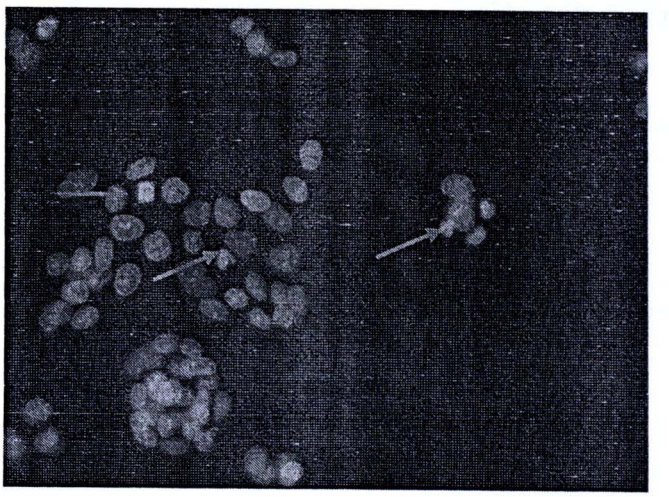
B. Piperine 10 μmole/L for 24 h treated cells



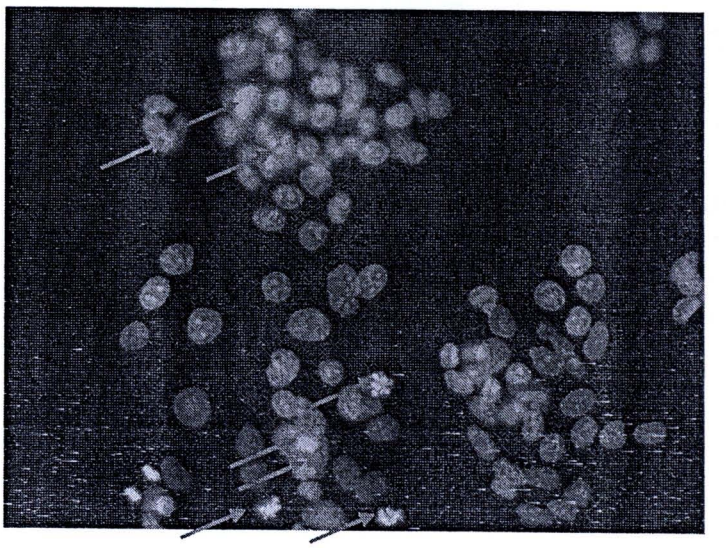
C. Piperine 300 μmole/L for 24 h treated cells



D. Piperine 500 μmole/L for 24 h treated cells



E. Piperine 1,000 μmole/L for 24 h treated cells



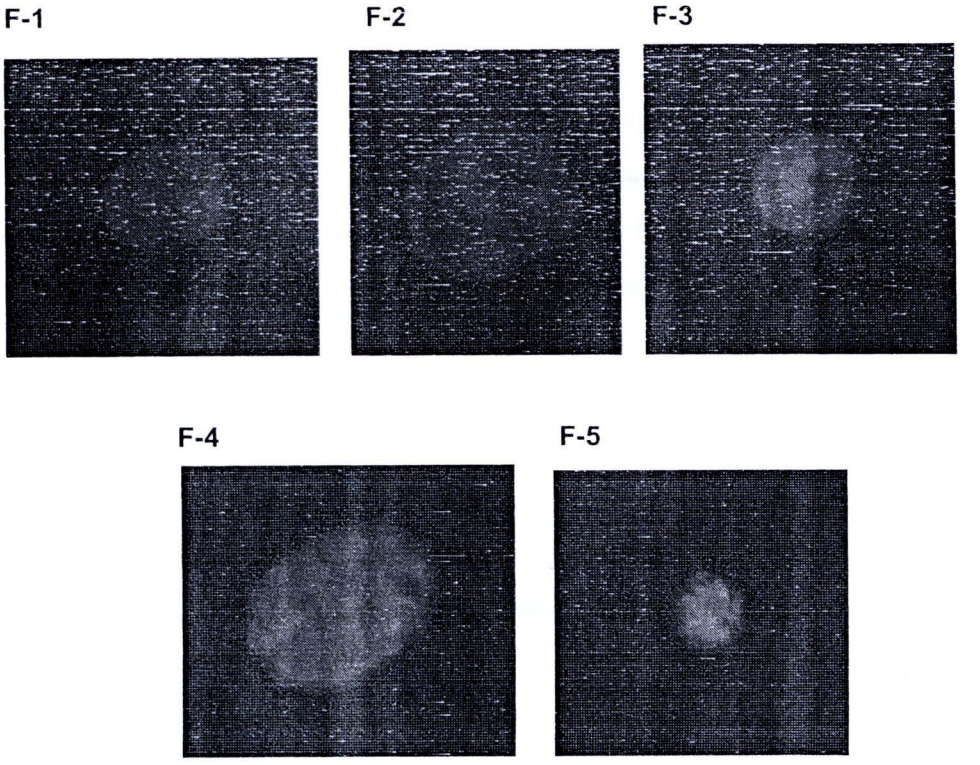


Figure 6 Effect of piperine on nuclear alterations in HepG2 cells. Cells were stained with DAPI and visualized by fluorescence microscopy. The arrows in A to F indicate nuclear shrinkage, condensation, or fragmentation with 100x magnification.

10. Data analysis

All measurements will be performed with at least three independent experiments. Data will be shown as means ± SEM. Significant difference from control value will be assessed using Student *t*-test or one-way ANOVA for multiple comparisons. Significant will be accepted at $p<0.05$ or $p<0.001$.