Journal of Biotechnology **18**(4): 671-678, 2020

PRODUCTION OF NANOLIPOSOMES WITH PIPERINE FROM BLACK PEPPER *(Piper nigrum)* AND ITS IMPROVED GROWTH INHIBITORY ACTIVITY ON COLORECTAL CANCER CELLS

Le Nhat Minh[1], Tran Thi Minh Anh[1], Tran Van Loc[2,4], Phung Thi Kim Hue[1,4], Do Thi Thao[3,✉]

[1]*Hung Vuong High School for the Gifted, Gia Lai, Pleiku, Vietnam*
[2]*Institute of Chemistry, Vietnam Academy of Science and Technology*
[3]*Institute of Biotechnology, Vietnam Academy of Science and Technology*
[4]*Institute of Human Health Research and Education Development in Central Highlands*

✉To whom correspondence should be addressed. E-mail: thaodo@ibt.ac.vn

Received: 07.11.2020
Accepted: 28.12.2020

SUMMARY

Black pepper (*Piper nigrum*) is an autoicous and decorous vine cultivated in many local regions of Gia Lai. Black pepper is one of the most commonly consumed spices, and its pungency is due to the presence of alkaloids, such as piperine. This compound represents diverse biological activities, including anti-inflammatory, anticancer, antiviral, anti-larvicidal, pesticide, anti-alzheimer's activities, *etc*. However, due to its poor solubility as well as its toxic effects at high use concentration, piperine is still in limit of pharmaceutical applications. In this study, we have used black pepper seed collected at Chu Se - Gia Lai to extract piperine. The compound extracted efficiency was approximately 18% with 96.7% of purity. Based on the obtained pure piperine, the hybrid nanopiperine-CD133 monoclonal antibody (mAb^CD133) complexes were fabricated with the nanoparticle size of about 170 nm, the polydispersity index (PDI) of 0.23 and the zeta potential of -9.4 mV. The nanocomplex was subjected for growth inhibitory activities against cancer colorectal cells (HT-29 cell line). The results showed that the nanopiperine-mAb^CD133 complex exhibited significant *in vitro* growth inhibition HT-29 colorectal cancer cells (46.56 ± 2.78%), while the viability of healthy cells remained unaffected (17.77 ± 0.82 %). The nanocomplex could also label 12.17% of HT-29 cells, which was rather higher than 3.83% from mAb^CD133 conjugated phycoerythrin (PE) as positive control. The fabricated nanopiperine-mAb^CD133 complex has proved the enhanced cytotoxic activities against colorectal cancerous cells as well as promising biopharmaceutical potency.

Keywords: Cancer colorectal cells, CD133 monoclonal antibody, HT-29, nanoliposome, piperine, polydispersity index, zeta potential

INTRODUCTION

Pepper is the second staple crop in Central Highlands of Vietnam besides coffee. As reported, black pepper (*Piper nigrum* L.) contains a high content of an active compound which is piperine (Zarai *et al*., 2013). Piperine is an alkaloid and a main chemical component of black pepper. Piperine was discovered to inhibit the enzymes which were involved into different drugs' metabolisms. As reported, piperine attends into phase I of drug metabolism (such as oxidative reaction through cytochrome P-450 forms). Besides, piperine also involves in phase II of metabolisms (called as conjugation or biotransformation reactions). Thus, piperine

could help to increase drug accumulation and therapeutic potency (Stojanovic *et al*, 2019). As such, piperine combined with theophylline, a drug that has been clinical used for inhibition of phosphodiesterase isoenzymes, antagonism of adenosine, enhancement of catecholamine secretion, and modulation of calcium fluxes, could increase 1.5 times higher concentration of theophylline in patients' serum than using the drug itself, as well as reduce the elimination process considerably. Therefore, nowadays, piperine is often used in combination with other drugs as a factor to reduce excretion process, increase the accumulation of drugs in targeted site which help to enhance the medical properties of drugs (Stojanovic *et al*, 2019). Piperine has also been used globally with curcumin in a category of functional foods such as "TURMERIC" (Doctor Formulate); "ENZEST-DHA" (Enomark Healthcare) to support illness treatment in cancer, hepatitis, senescence etc. Moreover, piperine increased the absorption and bioavailability of different kinds of drug molecules (Khatri *et al.*, 2016). However, due to its poor solubility in water and its toxicity, pharmaceutical applications of piperine are still in limit.

On the other hand, nanoliposomes were recently applied popularly in medicine since they could amplify drug distribution, improve performance features of the products, prevent early degradation of encapsulated drugs, and effective treatment by decreasing toxicity (Deshpande *et al.*, 2013). Moreover, drugs that conjugated into liposomes have pharmacokinetic characteristics changed explicitly compared to free drugs in solution (Malam *et al*., 2009). Recently, liposomes have been often used as effective carriers for many kinds of bioactive agents including drugs, vaccines, cosmetics, and nutraceuticals (Deshpande *et al*., 2013). Besides, CD133 antigen, the most commonly surface marker of cancer stem cell (CSC) population from various gliomas to carcinomas, has become a new cancer treatment targeting. Moreover, there has not been any research to entrap piperine into nanoliposomes in combination with monoclonal antibody for the pharmaceutical improvement. Thus, this study had tried to conjugate into liposomes together with anti-CD133 monoclonal antibodies (mAb^CD133) to produce nanopiperine - antibody complex. Piperine would be isolated and purified from black pepper harvested in Chu Se – Gia Lai. The nanopiperine-mAb^CD133 conjugation will be tested on colorectal cancer cells (HT29) to assess its cancer targeted inhibitory effects.

MATERIALS AND METHODS

Materials

Black pepper was harvested at Chu Se area in Gia Lai province.

Colorectal cancer cells (HT-29), human colon normal cells (CCD-18Co) were kindly provided by Dr. Chi-Ying Huang, Yang-Ming National University, Taiwan;

DMEM, Fetal Bovine Serum (FBS), human CD133 monoclonal antibody conjugated with PE (CD133-PE), human CD133 monoclonal antibody were purchased from Miltenyi (Germany). All other chemicals including pure piperine (> 97%) as standard compound, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG2000-Maleimide) were from Sigma Aldrich (St. Louis, M.O., USA).

Extract and purify piperine from black pepper

Supersonic extraction: 50 g black pepper powder was extracted with 150 mL ethanol (EtOH) 96^o at the temperature of 40 ^{0}C (three times). After 1 hour of solvent evaporation, the collected sediment was extracted continuously with EtOH two more times.

Purification of piperine: 94 g of the above extracted sediment was mixed in 150 mL of 10% NaOH solution in EtOH 96%, stirred at room temperature (RT) for 1 h. Then, 250 mL distilled water was added and refined the precipitation to collect rough piperine in solid form. This solid piperine was mixed with EtOH, added activated

carbon and stirred at 40°C in 1 hour. After that, the mixture was filtered out activated carbon, eliminated EtOH and stored overnight for obtaining crystals before filtering to yield pure piperine.

Piperine quantitization was carried out by using HPLC. Exactly 0.25 g of pepper powder was put into 100-mililitre glass flask and further adding with 80 mL of EtOH. The mixture was supersonic extracted in 30 minutes before adding just enough EtOH to the mark and mixed well. The mixture was filtered through a 0.45 µm filter before analyzing by HPLC.

HPLC specifications: C18 (4.6 x 250 mm; 5 µm); UV detector: 343 nm; dynamic phase: MeOH-water (77:23, v/v); 1 mL/min flow rate; Sample injection volume: 10 µL. Sample injection volume is 8.0 µL. Detector DAD: wavelength 345 nm.

Produce nanopiperine-antibody complex

Lipids including phosphatidylcholine (PS), cholesterol and DSPE-PEG2000-maleimide were dissolved in dichloromethane solvent. Piperine was then mixed with this lipid mixture according to the different molar ratios of PS:Cholesterol:DSPE-PEG-Mal:Piperine. The solution was then vacuum evaporated to remove solvent and to create thin lipid layer. The thin lipid layer contained piperine was hydrated completely by using PBS (phosphate buffer saline) at 40 ^{0}C for 10 minutes. The PBS buffer was supplemented with anti-CD133 monoclonal antibody (mAb^CD133) at a suitable concentration (1 µg/mL). Solution was sonicated at 2 atm for a 20 second cycle and rested for 10 seconds, then repeated 10 times. Next, the solution was centrifuged at 12000 rpm for 30 minutes to collect nanoliposome residue (**LP**).

Assess the physical properties of nanoliposomal complex

The nanoparticle size, polydispersity index (PDI) and zeta voltage were determined by using a DLS light scattering equipment named as Zetasizer Nano-Z meter (Malvern Instruments, UK).

In vitro cell culture

HT-29, CCD-18Co cells were cultured in DMEM medium containing 2 mM L-glutamine, 10 mM HEPES and 1.0 mM sodium pyruvate, 10% fetal bovine serum (FBS). Cells were sub-cultured every 3-5 days at the ratio of 1: 3 and incubated at 37 ^{0}C, 5% CO_2.

Determine the ability of LP to label cancer cells

HT29 and CCD-18Co cells were seeded into wells of a 6-well plate and cultured in 24-hour with DMEM medium in an incubator of 37 ^{0}C, 5% CO_2. Then, the samples including either blank-liposome or liposome-piperine-mAb^CD133 (**LP**) or CD133-PE was added into cell-seeded-wells and further incubated for 3 hours in the incubator. After the incubation period, culture medium was removed, washed with PBS. Cells were detached from the well bottom by using Trypsin-EDTA 0.04%. Cells were then collected into eppendorf tubes and analyzed for sample labeling capacity using using Novocyte flowcytometry system and NovoExpress software (ACEA Bioscience Inc.).

MTT anti-proliferative assay

The *in vitro* cellular viability measurement using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye was strictly followed as described by Mosmann *et al*. (1983). In the assay, this tetrazolium salt was used as colorimetric reagent to assess cellular survival. The number of surviving cells were calculated using the formula: % cell survival = [OD (reagent) – OD(blank)]/[OD(DMSO) – OD(blank)]. The concentration to inhibit cell survival at 50% (IC_{50}) was determined using TableCurve 2Dv4 software (Systat Software Inc., US).

RESULTS AND DISCUSSION

Pipirine extraction and purification from black pepper collected at Chu Se area

In this study, piperine was efficient isolated from black pepper using EtOH with up to 18.37 % yield. This percentage was calculated based on

the amount of obtained pure piperine over the total quantity of collected sediment. It has been reported that piperine content is up to 9% higher in black pepper (Chopra *et al*., 2016). Piperine content may be affected by changes in farming conditions such as climate or drying conditions and origin (Sozzi *et al.*, 2012).

By using 94 g of the sedimental extract to isolate piperine, we obtained crude piperine solids (28 g). Raw piperine product was further purified as mentioned in the method. The results showed that purified product reached 96.7% purity (Fig.2) . This piperine extraction and purification method of the study was based on the reported protocols from other previous researchers with small modification to optimize the efficiency (Han *et al*., 2016; Ikan, 1991).

According to Hien *et al.* (2014), piperine extracts from black pepper purchased in Ha Dong, Hanoi reached 96.6% of purity. Thus, the black pepper seed in Chu Se - Gia Lai and Ha Dong, Hanoi have similar piperine content in the same extraction conditions. With this result, the piperine extracted and purified from black pepper collected at Chu Se area, Gia Lai province was used as material for our further researches.

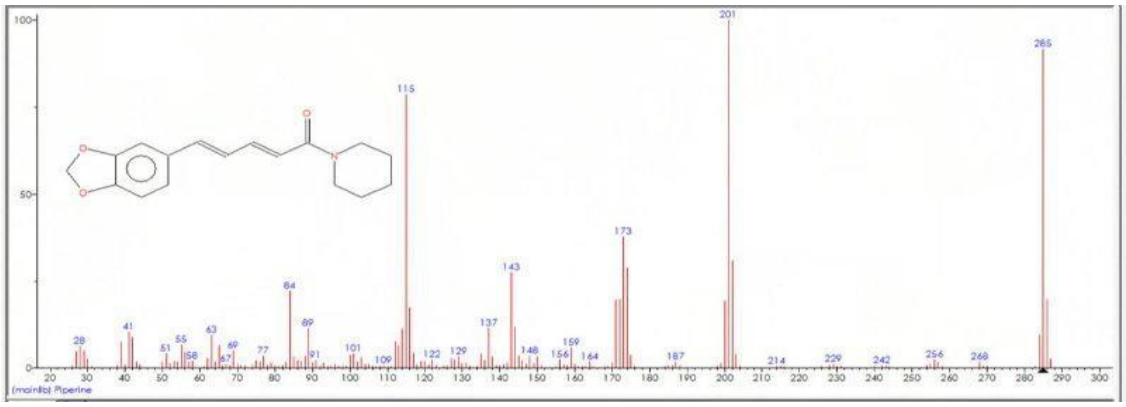


Figure 1. Gas Chromatography Mass Spectroscopy of piperine

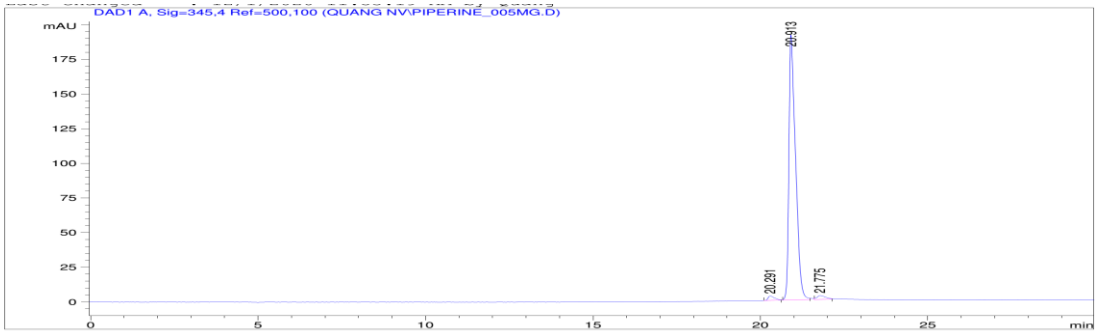


Figure 2. HPLC chromatogram of purified piperine showing approximately 96.7% of purity

Characterization of the nanopiperine-mAb^CD133 complex (LP)

Characteristics of nanoliposomal piperine-antibody LP such as particle size, polydispersity index (PDI) and zeta potential were determined and shown in Table 1 and Figure 3. Several basic physical properties of LP complex were measured. The results showed that the nanoparticle size was about 170 nm, the PDI reached 0.23 and the zeta potential was -9.4 mV. This is the right size for nanoliposomes to be well distributed in blood

vessels as well as increase circulation time of drugs in the circulatory system.

The nanopiperine-mAb^CD133 complex (LP) capacity to label HT-29 cells

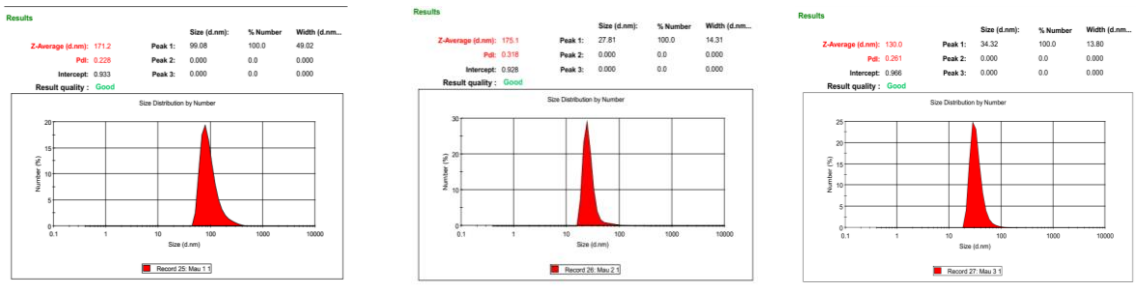
As reported, CD133 is a typical surface antigen of CSCs. Thus, CD133 monoclonal antibody was selected to conjugate onto LP complex with the CSC targeting purpose. In this research, HT29 colorectal cancer cells were employed since this cell line were reported to contain a sub-population of CSCs (Yeung *et al*., 2010). Thus, we will be able to clarify the CSC labeling capacity of LP. After piperine and anti CD133 monoclonal antibodies incorporated to liposomes, the obtained LP complex were evaluated the ability to label HT-29 colorectal cancer cells using flow cytometry technique. Results presented that LP could distinguish up to 12.17% HT29 cells, which was better than that of the control CD133-PE with 3.83% labeled cells (Fig.4). It seemed that LP complex with piperine incorporated component holding the improved cellular labeling and uptaking capacity.

The results also showed that number of labeled cells by nanoliposome-blank was only 1.81%. In addition, the HT-29 morphology under the effect of the research samples has had certain changes (Figure 5).

Table 1. Effects of active ingredient ratio on liposome creaty ability.

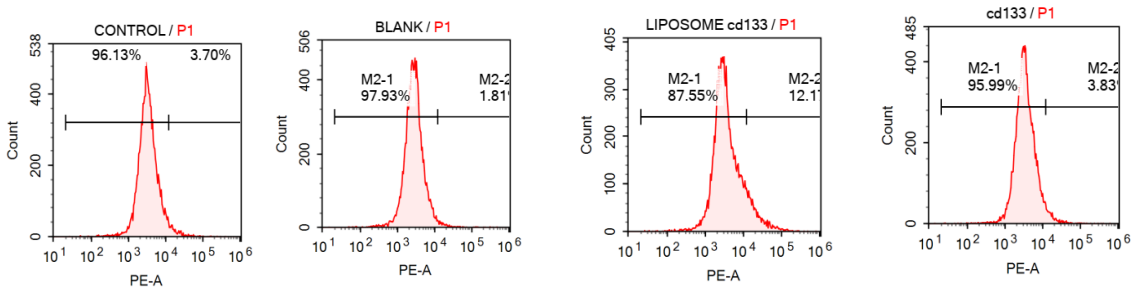
Complex liposome	Size (nm)	PDI	Zeta (mV)
Liposome-piperine-mAb^CD133	171,2	0,23	-9,4
Liposome -piperine	175,2	0,32	-13,8
Blank liposome*	130,0	0,26	-12,2

Note: *Blank liposome contained neither piperine nor mAb^CD133



Nanoliposome-piperine-CD133 *Nanoliposome-piperine* *Nanoliposome-blank*

Figure 3. Distribution and size of liposomes



HT-29 control *Liposome-blank* *LP complex* *CD133-PE*

Figure 4. The ability of LP to label HT29 cells.

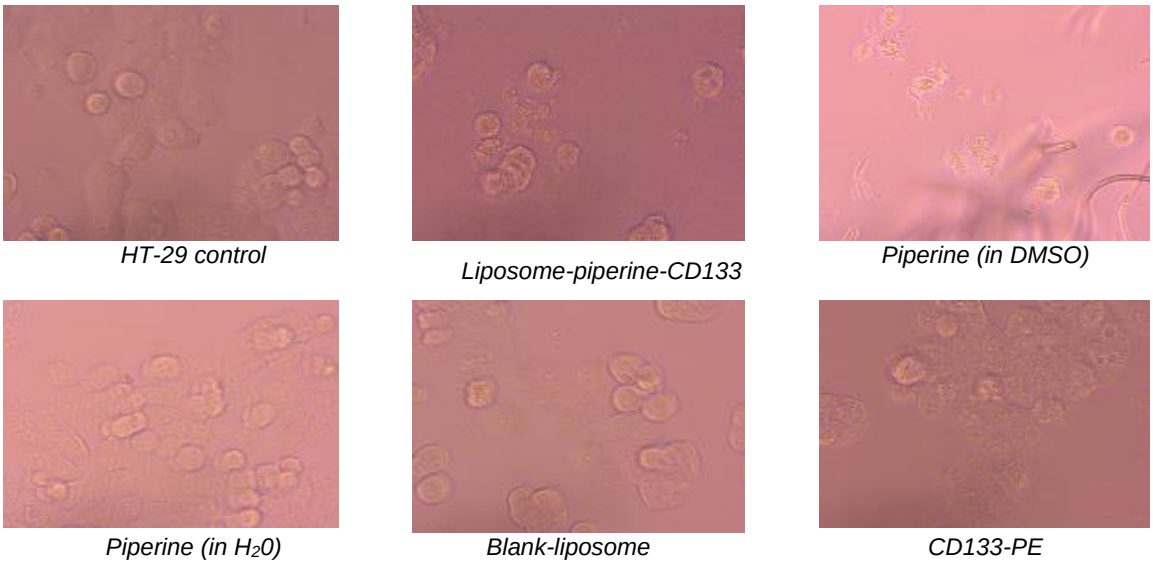


Figure 5. Image of HT-29 cells under impact of study samples observed from the reversed microscope (20X magnification, transmitted light).

The growth inhibitory effects of LP on HT-29 colon cancer cells

The cytotoxicity of the study samples presented in Table 2 showed that piperine dissolved in water could not inhibit HT-29 cells to growth, even at the highest concentration (2.27%), while it affected much stronger on healthy cells (11.79%) at the same tested concentration. This result exhibited that piperine itself was not cancerous targeting and the bioavailability of piperine was rather poor. In contrast, piperine which was dissolved in dimethyl sulfoxide (DMSO), could inhibit HT29 cells' survival at the IC_{50} = 391.09 ± 24.04 µM, showing that the compound had very low solubility in water but only in polar solvent.

The result also noted that the relative target of piperine to HT-29 cells is relatively high. In the case that piperine was nanoliposomal conjugated and combined with CD133 monoclonal antibody, the complex LP improved significantly its inhibitory activity on HT-29 surviving rate. The survival percentage of HT29 cells under LP treatment was 46.56%, in compared with that on healthy cells was 17.77%. This result again reflected that the LP complex had better target activity on cancer cell (HT-29) than on healthy CCD-18Co cells.

Table 2. Inhibitory effect of sample on cancer cells.

Conc. (µM)	LP		Piperine - DMSO		Piperine –H_2O	
	CCD-18Co	HT29	CCD-18Co	HT29	CCD-18Co	HT29
500	17.77	46.56	40.41	74.71	11.79	2.27
100	-0.47	11.29	5.82	4.44	4.72	-0.17
20	-2.04	5.08	2.04	2.80	1.94	-3.63
4	-9.86	-7.81	-2.04	-3.46	-9.30	-4.38
IC_{50}	>500	>500	>500	391.09 ± 24.04	>500	>500

Thus, piperine in black pepper seeds from Chu Se- Gia Lai, after conjugated in LP complex initially showed their positive signals, in terms of bioavailability and cancer cell targeting. Some other reports showed that piperine suppressed tumor growth by modulating reactive oxygen species (ROS)-induced apoptosis and cell cycle regulation (Siddiqui *et al*., 2017; Rather and Bhagat, 2018). In this study, the LP complex could inhibit HT-29 cells' proliferation significantly. That effect might come from piperine as main component active compound of LP, while anti-CD133 monoclonal antibody should be the factor to improve the targeting capacity of the complex. The result implies further researches should be conducted on this issue.

CONCLUSION

The piperine purification process has been established to achieve 96.7% purity from black pepper collected at Chu Se - Gia Lai. This study has also fabricated a complex nanopiperine - antibody (LP) successfully, with a size of about 170 nm, the PDI about 0.23 and the zeta potential at neutral form (-9.4 mV). The LP complex has been shown its influence on HT-29 cell morphology. The LP complex also exhibited 46.56% inhibition of HT-29 cells' growth, while the level of inhibition of healthy cells was only about 17.77%. The nanocomplex could also distinguish up to 12.17% of CSC subpopulation from HT-29 cells, which was better than 3.83% labeled cells of the mAb^CD133 conjugated phycoerythrin (PE) as positive control.

Acknowledgments: *The authors acknowledge the financial support from Institute of Biotechnology (VAST) under the grant No. CS21-12.*

REFERENCES

Chopra B, Dhingra AK, Kapoor RP, Prasad DN (2016) Piperine and its various physicochemical and biological aspects: A review. *Open Chem J*3(1).

Deshpande PP, Biswas S, Torchilin VP (2013) Current trends in the use of liposomes for tumor targeting. *Nanomedicine* 8(9): 1509-1528.

Ikan, R. *Natural Products, A Laboratory Guide*, 2nd ed.; Academic Press: New York, 1991.

Malam Y, Loizidou M, Seifalian AM (2009) Liposomes and nanoparticles: nanosized vehicles for drug delivery in cancer. *Trends Pharmacol Sci* 30(11), 592-599.

Nguyen Van Han et al. (2016) Study on piperine extraction from pepper (*Piper nigrum* L). *J MedResDrug Inform* 1: 17-21.

Pham Thi Mai Hien *et al*. (2014) Building up the process of extracting piperine by solvent ethanol from pepper (piper nigri l.). *J Milit Pharm - Med* 3: 19-23.

Siddiqui S, Ahamad MS, Jafri A, Afzal M, Arshad M (2017) Piperine triggers apoptosis of human oral squamous carcinoma through cell cycle arrest and mitochondrial oxidative stress. *Nutr Can* 69(5): 791-799.

Sozzi GO, Peter KV, Babu KN, Divakaran M (2012) Capers and caperberries. In *Handbook of herbs and spices* (pp. 193-224). Woodhead Publishing.

Stojanović-Radić Z, Pejčić M, Dimitrijević M, Aleksić A, V Anil Kumar N, Salehi B & Sharifi-Rad J (2019) Piperine-A Major Principle of Black Pepper: A review of its bioactivity and studies. *Appl Sci* 9(20), 4270.

Yeung TM, Gandhi SC, Wilding JL, Muschel R, Bodmer WF. Cancer stem cells from colorectal cancer-derived cell lines (2010) *Proceedings of the National Academy of Sciences of the United States of America* (PNAS USA) 107(8): 3722-3727.

Zarai Z, Boujelbene E, Salem NB, Gargouri Y & Sayari A (2013) Antioxidant and antimicrobial activities of various solvent extracts, piperine and piperic acid from *Piper nigrum*. *Lwt-Food Sci Technol* 50(2): 634-641.

CHẾ TẠO NANOLIPOSOME CHỨA PIPERINE CHIẾT XUẤT TỪ HẠT HỒ TIÊU VÀ KHẢO SÁT HOẠT TÍNH ỨC CHẾ SINH TRƯỞNG TẾ BÀO UNG THƯ RUỘT KẾT

Lê Nhật Minh[1], Trần Thi Minh Anh[1], Trần Văn Lộc[2,4], Phùng Thị Kim Huệ[1,4], Đỗ Thị Thảo[3]

[1]*Trường THPT chuyên Hùng Vương Gia Lai*
[2]*Viện Hóa học, Viện Hàn Lâm Khoa học và công nghệ Việt Nam*
[3]*Viện Công nghệ sinh học, Viện Hàn Lâm Khoa học và công nghệ Việt Nam*
[4]*Viện nghiên cứu sức khỏe và phát triển Giáo dục Tây Nguyên*

TÓM TẮT

Hạt tiêu đen được trồng nhiều tại Gia Lai là một trong những loại gia vị được tiêu thụ phổ biến nhất, và vị cay của nó là do sự hiện diện của một alkaloid được gọi là piperine. Piperine đại diện cho các hoạt động sinh học đa dạng, chẳng hạn như chống viêm, chống ung thư, kháng vi rút, chống ấu trùng, thuốc trừ sâu, chống bệnh alzheimer v.v. Tuy nhiên, do tính kém tan và có độc tính, những ứng dụng của piperine trong lĩnh vực y dược còn bị hạn chế. Trong nghiên cứu này, chúng tôi đã sử dụng hạt tiêu đen thu hái tại Chư Sê - Gia Lai để chiết tách và phân lập piperpine. Hiệu suất phân lập hoạt chất này là khoảng 18% với độ tinh sạch đạt 96.7%. Hoạt chất piperine phân tách được sử dụng để chế tạo phức hợp lai nanopiperin-kháng thể kháng CD133 (mAb^CD133) với đặc tính kích thước khoảng 170 nm, độ đồng đều hạt đạt 0,23 và thế zeta ở khoảng trung tính (-9.4 mV). Phức hợp được đánh giá hoạt tính ức chế tế bào ung thư ruột kết dòng HT-29. Kết quả cho thấy các tế bào ung thư HT-29 thể hiện bị ức chế tăng trưởng đáng kể trong thực nghiệm *in vitro* khi được ủ với phức hợp nanopiperin-kháng thể, đạt tới 46,56 ± 2,78%. Trong khi đó, tác động của tổ hợp tới tế bào lành ở người (dòng CCD-18Co) vẫn chỉ là 17,77 ± 0,82%. Phức hợp cũng cho thấy khả năng đánh dấu tiểu quần thể tế bào gốc ung thư trên dòng HT-29 đạt 12.17%, cao hơn so với 3.83% của đối chứng là kháng thể kháng CD133 cộng hợp phycoerythrin (PE). Phức hợp nanopiperine-mAb^CD133 đã cho thấy sự tăng cường hoạt tính kháng tế bào ung thư ruột kết và tiềm năng ứng dụng trong y sinh.

Từ khóa: Tế bào ung thư ruột kết, kháng thể kháng CD133, HT29, nanoliposome, piperine, chỉ số PDI, thế zeta