

The HepG2 cells growth inhibitory capacity from the leaves of *Peristrophe bivalvis* (L.) Merr. a natural dye in food culture

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Abstract



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There is increasing interest in medicinal use of *Peristrophe bivalvis* (L.) Merr (PM) in traditional medicine practical applications. The *in vitro* HepG2 cells growth inhibitory capacity from ethanol 70% extract (E70) of the leaves of *Peristrophe bivalvis* (L.) Merr was firstly investigated. The results indicated that E70 extract inhibited the proliferation of HepG2 cells. When the effect of E70 on intracellular signaling was measured, E70 stimulated the apoptotic factors bax, caspase-3 and caspase-9 in HepG2 cells. The results demonstrated that E70 induces programmed cell death (apoptosis) in HepG2 cells. The further research to determine pharmacokinetics of the main components, the definitive mechanism and clinical trial should be carried out more to firm evidence.

Keywords: *Peristrophe bivalvis* (L.) Merr, natural dyes, food coloring, HepG2.

INTRODUCTION

Many natural dyess are used as both food coloring and substances that protect health and wellbeing by precluding or even treating diseases⁴. Several colored molecules including anthocyanins, carotenoids, apocarotenoids, anthraquinones, flavonoids, etc., were reported with antitumorigenic inhibitory capacity³. Significant epidemiological evidence has suggested that the presence of natural colorants in the vegetables and fruits can decrease the risk of different types of cancer¹.

Peristrophe bivalvis (L.) Merr. called Cam in Vietnam, is consumed as both a herbal medicine and for dying foods as sticky rice and other dishes by the ethnic minorities in Vietnam traditional food culture⁷. The PM is used in traditional medicine for the treatment of pulmonary tuberculosis, haemoptysis, congestion, bronchitis, and wrench. PM compounds and their bioactivities were reported in some former works. It is reported that the herb contained peristrophine, perisbivalvine A¹¹, anthocyanin⁵. This herb shows versatile pharmacological effects for blood problems as assisted therapy of high blood pressure, high levels of lipids; inhibiting development of fungi and bacteria⁷, and

correcting the hyperlipidemia and hypoglycemia², anti-snake venom¹⁰.

Recently, in Vietnam, PM has also been used to detoxify the body and treat liver cancer. Until now, inhibiting growth capacity of HepG2 cells of the PM leaves extracts has not been reported. Hence, the present work was efforded to evaluate anticancer effect on HepG2 cells of the PM leaves extracts.

MATERIALS AND METHODS

Chemicals and reagents

Ethanol (95%) was purchased from Echo ChemicalCo., LTD. (Taiwan). Phosphate buffer, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), tris-HCl, NaCl, tween 20 and nonfat dry milk were procured from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS) and penicillin G-streptomycin were obtained from GIBCO Laboratories (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO) was purchased from J.T. Baker (Avantor Performance Inc, USA), and other chemicals and reagents.

Preparation of PM ethanol extracts

Samples of PM leaves were harvested at herbal farm in Thai Nguyen University of Agriculture and Forestry, Vietnam. The fresh parts of this herb were washed by distilled water and dried in oven at 45 °C for 24 hours (h). The dry materials were crushed into 50-mesh sieve powder. Extracts were obtained as follows: 5 grams of each herb was separately extracted twice with 70% ethanol in circulator bath for 2 h at 80 °C and then was filtered. The filtrates were combined and concentrated in a vacuum evaporator at 45 °C. The dehydrated fractionation was weighted to calculate yield, then dissolved in dimethyl sulfoxide (DMSO) to a regular concentration and the the activity was measured.

Cell culture

HepG2 cells (Human hepatocellular carcinoma cell lines) were cultured in DMEM supplemented with 10% FBS, 100U/ml penicillin, and 100 µg/mL streptomycin with a change of medium every 3 days in a humidified atmosphere of 5% CO₂ incubator at 37 °C.

Analysis of Cell Morphology Change

HepG2 cells (5x10⁴ cells/dish) were seeded in 10 cm dishes for analysis of cell morphology. After 24 h, cells were treated with serial concentrations (62.5 and 750 µg/mL) with rate of 50% volume for E70 extract. Cells treated with 0.2 % DMSO was used as control. After 48 h of incubation at 37 °C, cells' morphological change was analyzed by light microscopy camera was used to take cell image as result.

Cell Viability Assay

HepG2 (5x10³ cells/well) were seeded in 96 - well plates for MTT assay. After 24 h, cells were treatment with serial concentrations (62.5, 125, 250, 500 and 750 µg/mL) with rate of 50% volume for E70 extract sample. Cells treated with 0.2% DMSO were used as control. After 24, 48, 72 h, cells were subjected to MTT assay. MTT stock solution and Serum-free media were mixed in ratio of 1:4, respectively, just before conducting assay, followed by MTT assays, as described previously⁹.

Western blot analysis

Whole cells extract was prepared using 1xRIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with complete protease inhibitor cocktail. Lysates were cleared by centrifugation (12000 x g, 4 °C, 20 minutes (min)) and supernatants were collected. After the

quantification of protein using Bradford assay, proteins were mixed with 2x sample buffer, incubated at 95 °C for 5 min and loaded onto 12% polyacrylamide gels using the Mini Protean 3 Cell (Bio-Rad). Proteins separated on the gels were transferred onto nitrocellulose filters. Blots were blocked for 2 h at room temperature in 5% non-fat milk/TBST (10 mM tris-HCl, pH 7.5, 150 mM NaCl, 0.1% tween 20), and then incubated at 4 °C overnight with primary antibodies. After washing with blocking buffer three times for 30 min, membranes were probed with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) and anti-rabbit IgG (Cell Signaling Technology) for 2 h. The membranes were washed three times for 1 h with Tris-buffered saline Tween 20 solution. The protein bands were visualized using an Enhanced Chemiluminescence Detection System, an advance Western Blotting Detection Kit using a LAS-3000 luminescent image analyzer. The following antibodies and dilutions were used for Western blots: beta-actin (1:3000); caspase-3, caspase-9 (1:500), and bax (1:1.000)⁶.

Statistical analysis

The data obtained from this study were subjected to one-way analysis of variance (ANOVA) to test for least significant differences (LSD), and all analyses were performed using the SAS statistical package (SAS Institute 1990).

RESULTS AND DISCUSSION

MTT assay

Antiproliferation activities were studied *in vitro* using HepG2 cells (adenocarcinomic human alveolar basal epithelial cell line). Under those experimental conditions, a dose-dependent inhibition of cell viability in this cell line was observed with E70. The effect of E70 on HepG2 cell growth at different concentrations (62.5-750 µg/mL for 24 to 72 h) showed decrease of the viability of cells. In the concentration of 750 µg/mL were showed 34.61±0.75%, 16.9±0.76, 14.8±0.16 of cell viability of HepG2 at 24, 48 and 72 h, respectively. These results indicated that E70 extract had ability to kill HepG2 line. The IC₅₀ was 371.57 µg/mL at 24 h, 207.52 µg/mL at 48 h, and 137.79 µg/mL at 72 h. E70 extract of PM leaves which maybe become a functional food is efficient in inhibiting cancer cell growth due to the presence of phytochemical potential.

Table 1: The E70 induced growth inhibition in HepG2

Cell line	Concentration (µg/mL)	Cell viability (% of control)		
		24h	48h	72h
HepG2	62.5	85.68±2.57	79.84±0.19	64.19±1.39
	125	69.54±2.72	63.71±0.28	57.54±1.13
	250	56.76±2.33	45.34±2.63	34.79±0.13
	500	47.5±1.77	27.85±2.27	21.42±0.55
	750	34.61±0.75	16.9±0.76	14.8±0.16

Cell morphology

HepG2 cell morphology before and after extract exposure different concentrations (control, 62.5 $\mu\text{g/mL}$, 750 $\mu\text{g/mL}$) after 48 h are showed in Figure 1. The comparison between Figure 1A, 1B and 1C, cells tend to

shrinkage and decrease in cell density when increasing concentration rate. The shrinkage of cell by extract is due to dead cells which ruptured and underwent a lytic process. Afterward, cell lost its ability to adhere on the plate of the dish and float in the media.

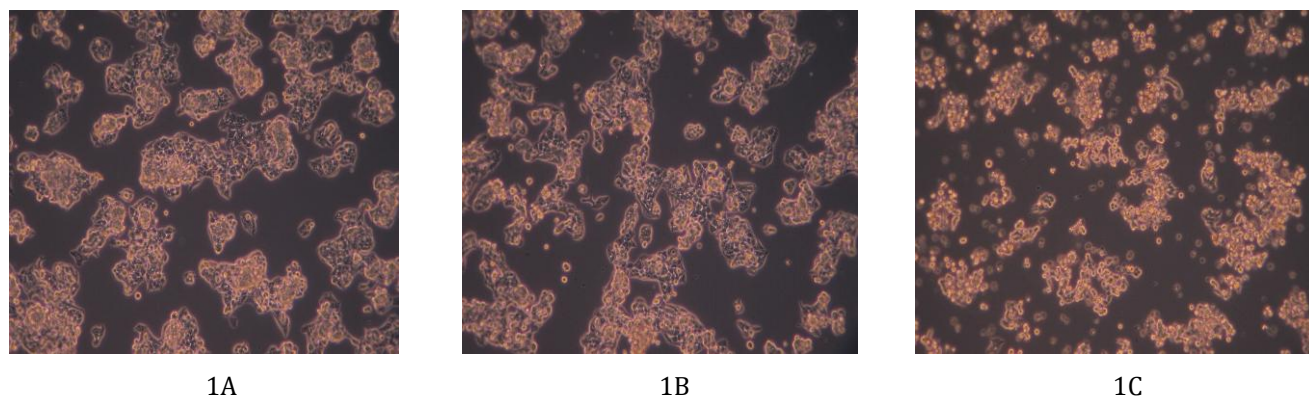


Figure 1: Morphology of HepG2 cell by different concentrations (A: Control, B: 62.5 $\mu\text{g/mL}$, C: 750 $\mu\text{g/mL}$)

The E70 altered the expression of apoptosis-related proteins in HepG2 cells

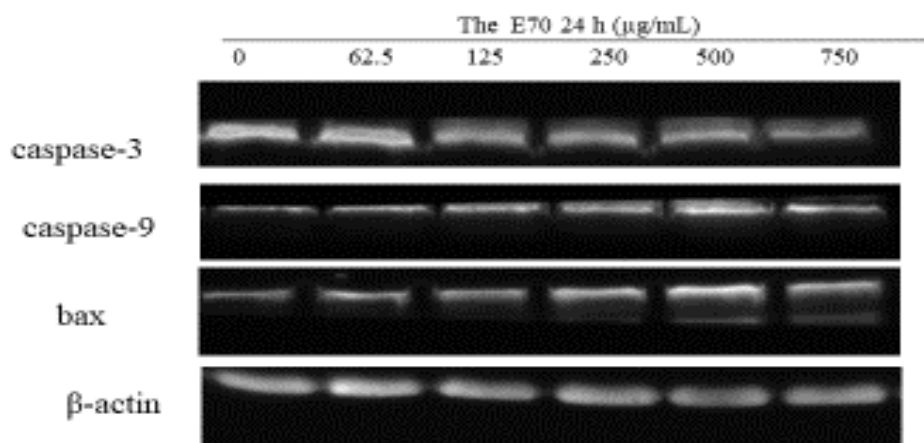


Figure 2: The E70 altered the expression of apoptosis-related proteins: caspase-3, caspase-9 and bax in HepG2 after 24 h

The expression of apoptosis-related proteins in HepG2 cells was recorded by western blotting (Figure 2). The results indicated that E70 extract induced bax and caspase-3 and caspase-9 proteins activity in HepG2. Especially, Bax is cleaved when activated. Thus cleavage of Bax during apoptosis may contribute to increase the intrinsic cytotoxic properties of this proapoptotic molecule and enhance its cell death function at the mitochondria¹². The effector caspases are responsible for the controlled degradation process that is characteristic of apoptotic cell death⁹. Caspase-3 keeps a important role in the terminal and execution phases of apoptosis induced by varied stimuli. Caspase-9 is an initiator closely associated with pro-apoptotic signals⁶. These discoverings more strongly evidenced that the E70 extract caused apoptotic cell death in HepG2 cells via activating apoptotic factors which are reliable proofs of the anticancer properties of the leaves of *Peristrophe bivalvis* (L.) Merr as well as is scientific basis to confirm for applying the PM leaves to suppress tumor growth in many clinical situations in Vietnamese ethnic medicine.

CONCLUSION

The leaves of *Peristrophe bivalvis* (L.) Merr. were applied widely to process into coloring sticky rice and other dishes to look more attractively. When the supplementation of the PM in food, it not only has culinary significance but also has health benefits. The present study results revealed that this herb may emerge as natural anticancer. In this regard, components, molecular mechanisms and more clinical trials by the PM leaves extracts induce HepG2 cell apoptosis with longer research periods are required to provide a strong insight for creating new guidelines as part of a healthy diet.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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