RESEARCH ARTICLE Agricultural Science Digest, Volume 43 Issue 6: 817-823 (December 2023)

Helicteres isora Ethanol Extract Induces Apoptotic Cell Death in HepG2 Cells

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10.18805/ag.DF-548

ABSTRACT

Background: *H. isora* is a species of the genus *Helicteres*, a medicinal plant known to be used in the treatment of liver cancer in Vietnam. However, there has been no recorded about biological activity of *H. isora* species in Vietnam. The current study aimed to study the antitumor activity through its antioxidant activity and inhibitory properties on hepatocellular carcinoma cells.

Methods: Arial parts of *Helicteres isora* were collected from Dak Nong province. Samples were extracted maceration method in ethanol solvent. The antitumor activity were identified though the DPPH radical scavenging activity (Brand-Williams *et al*., 1995) and WST-1 assay, Real-time PCR, Hoechst 33258 spectrofluorometric assay and Annexin V-FITC Apoptosis Assay on the human liver cancer cells (HepG2) and non-malignant cells of Chang's Live.

Result: The ethanol extract of *H. isora* exhibited antioxidant activity with IC_{50}= 21.16 µg/ml. *H. isora* extract also exhibited inhibitory activity on HepG2 cell division with IC_{50}= 37 µg/ml after 48 h. The expression of DNA fragmentation of the extract also increased with time (48-72 h). Apoptosis induction of extract was associated with up-regulation of pro-apoptotic *Bax* gene expression and down-regulation of anti-apoptotic *Bcl-2* gene expression. The *H. isora* extract was found to induce the apoptotic process in HepG2 cells through the up and down regulation of the *Bax*/*Bcl-2* gene.

Key words: Apoptosis, Fragmentation, *Helicteres isora*, Hepatoprotective.

INTRODUCTION

The genus *Helicteres* (Malvaceae) includes about 60 species distributed in the tropics of Asia and America (Cowie, 2011; Mabberley, 2008). This genus can be readily recognized by stamens and pistil forming an androgynophore, united sepals, oblong fruits with hairs and wingless seeds (Chantaranothai and Poompo, 2019). In Vietnam, eight species and one variety of the genus *Helicteres* have been recorded according to recent publications, inclusion: *Helicteres viscida* Bl, *H. angustifolia* L., *H. angustifolia* var. glaucoides Pierre, *H. angustifolia* var. obtusa Pierre, *H. angustifolia* glabriuscula Wall, *H. isora* L., *H. lanceolata* DC., *H. plebeja* Kurz and *H. hirsuta* Lour (Nguyen, 2003; Pham, 1999). In which, *H. isora* is one of the species of interest with active ingredients recorded, including: phenols, flavonoids, alkaloids, glycosides, phytosterols, carotenoids, tannins, neolignans, rosmarinic acid derivatives, betulic acid, daucosterol, tannnins, anthoquinones, sterols, lupeol, β-sitosterol, α and β amyrin, taraxerone, 49-O-b -D-glucopyranosyl rosmarinic acid, 4,49- O-di-b-D-glucopyranosyl rosmarinic acid and 2R-O-(49-O-bD-glucopyranosyl caffeoyl)-3-(4-hydroxyphenyl), lactic acid named as 49-O-b –D-glucopyranosyl isorinic acid together with rosmarinic acid (Gayathri *et al*., 2010; Loganayaki *et al*., 2013; Satake *et al*., 1999), *lignans,* (6)-*pinoresinol,* (2)-*boehmenan* and (2)-*boehmenan H* (Chin *et al.,* 2006). Flavones such as methyl ether, 7, 41-di-o-methyleisoscutellarein (5,8- dihydroxy-7,41-flavones) along with kaempferol-3-ogalactoside and herbacetin-8-oglucoronide from the leaves of *H. isora* (Ramesh and Yuvarajan, 1995). Various biological activities have also been identified, such as antidiabetic (Kumar *et al*., 2007), cardiac antioxidant, antiperoxidative potential and hypolipidaemic activities (Kumar *et al*., 2008) in streptozotocin (STZ) induced diabetic rats. Fruits have revealed hypolipidaemic (Raja *et al*., 2010), antioxidant (Jain *et al.,* 2014; Dayal *et al.,* 2015); cytotoxic activity against human lung carcinoma, hormone-dependent human prostate carcinoma and human breast carcinoma (Chin *et al.,* 2006) and anticancer properties (Kumar *et al*., 2012). Whole plant of *H. isora* is reported to have anticancer (Varghese *et al*., 2011), antinociceptive activities (Venkatesh *et al*., 2007) while roots have hepatoprotective activity (Chitra and Prema, 2009) against carbon tetrachloride induced liver damage in rats.

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How to cite this article: Thach, B.D., Giang, T.T.L., Nhu, L.P.Q., Ben, T.T., Linh, L.N.T., Dao, V.Q., Oanh, N.T.T., Tuan, L.H. and Chung, D.C. (2023). *Helicteres isora* Ethanol Extract Induces Apoptotic Cell Death in HepG2 Cells. Agricultural Science Digest. 43(6): 817-823. doi: 10.18805/ag.DF-548.

Submitted: 29-03-2023 **Accepted:** 16-09-2023 **Online:** 23-10-2023

However, there has been no recorded about biological activity of *H. isora* species in Vietnam. In this paper, we studied the activity *H. isora* specie, based on antioxidant activity and cytotoxicity, collected in Dak Nong province, Vietnam.

MATERIALS AND METHODS

Preparation of the extract

Arial parts of *Helicteres isora* were collected in April 2022 from Dak Nong province (Dak Nong, Dak Mil, Dak Lao, altitude 545 m above sea level, coordinates: 12°31′665″N, 107°38′240″E). The samples were wasted with water, cutted into small pieces and dried in Laboratory drying oven at 50°C. They were then crushed into a course powder using a laboratory grinding mill with ring sieve, size 0,25 mm. Maceration extraction of samples in ethanol solvent (70%) for 7 days, followed by drying at 50°C by vacuum evaporator. The extract was stored at -20°C.

Cell culture

Human liver cancer cells (HepG2) and non-malignant cells of Chang's Liver were obtained from American Type Cell Culture Collection (ATCC), Maryland, USA. All cultured cells were maintained in the logarithmic phase of growth in DMEM supplemented with 10% fetal bovine serum (GIBCO BRL), penicillin-streptomycin, fungizon and miramycin at 37°C in a humidified incubator with 5% CO_2 and 95% air. Cultures were regularly examined using inverted microscope.

Determination of 1,1, dipheny-2-picrylhydrazyl (DPPH) radical scavenging activities

The DPPH radical scavenging assay was performed using 1,1 diphenyl-2-picrylhydrazyl (DPPH) according to the method described by Brand-Williams *et al.* (1995) with some modifications. Briefly, five different concentrations of the studied plant extracts (0.005, 0.01, 0.015, 0.02 and 0.025 mg/ml) were prepared in ethanol (analytical grade). The same concentrations were also prepared for L-ascorbic acid, which was used as a standard antioxidant. 1 ml of each studied extract was transferred into a clean test tube into which 0.5 ml of 0.3 mM DPPH in ethanol was added. The mixture was shaken and left to stand in the dark at room temperature for 15 minutes. Blank solutions comprising of the studied extract solutions (2.5 ml) and 1 ml of ethanol were used as baseline.

The negative control comprised 2.5 ml of DPPH solution and 1 ml of ethanol, while L-ascorbic acid at the same concentrations as the studied extracts was used as the positive control. After incubation in the dark, the absorbance values were measured at 517 nm using a spectrophotometer. The experiments were performed in triplicate. The DPPH radical scavenging activity was estimated using the equation described by Brand-Williams *et al.* (1995).

$$\% \text{ radical scavenging activity} = \frac{A_c - A_s}{A_c} \times 100$$

Where

A_s = Absorbance of the sample.

A_c = Absorbance of the control.

The half maximal inhibitory concentration (IC_{50}) of the extracts was computed from a plot of percentage DPPH free radical inhibition versus the extract concentration.

Cell viability assay

The WST-1 assay is a cell viability assay that uses water soluble tetrazolium salt as an agent, which reacts with mitochondrial dehydrogenase and produces formazan that can be detected quantitatively by spectrometry. For the viability assay, HepG2 cells were seeded on 24-well plates at a density of 5×10^4 cells per well. After 1 day and 3 days, CDES was applied to each well. After each period, cell viability was measured using the WST-1 (EZ3000, Daeil Lab Service Co., Seoul, Korea) assay. Each well was washed with PBS, 200 µL of the 1:10 WST-1 agent diluted to the culture media was added and cultured for an hour and 100 µL of each well was transferred to a 96-well plate to read at a 450 nm wavelength ELISA plate reader (SUNRISE, TECAN, Grödig, Austria).

Real-time PCR

HepG2 cells were grown in 6 well plates and the media were exchanged with the ones including the extract in the difference concentrations. After 48 hrs incubation, the cells were washed with cold PBS once and scraped. The cells were washed again with cold PBS twice before extracting total RNA molecules using Qiagen RNeasy kit. cDNAs were constructed from 600 ng of the collected RNAs using Reverse Transcription System (Promega, Japan). cDNA was analyzed through real-time quantifying PCR using SYBR Green Realtime PCR Master Mix (Toyobo, Japan) according to the manufacturer's instructions (CFX96 Touch Real-time PCR System, BioRad). The primers used for the experiments are summarized in Table 1.

Hoechst 33258 spectrofluorometric assay

HepG2 cells were grown in a 96-well plate for 24, 48 and 72 hrs at 37°C and were centrifuged (5 min, 8000 g) at RT. Then, 70 µL of a supernatant was replaced with 70 µL of warmed phosphate-buffered saline (PBS 1 ×, 37°C) and 10 µL of H33258 solution (in PBS 1 ×) was added to a well. The final concentrations of H33258 in a well were 0.1-5 µg/mL. Then, the cells were incubated with H33258 for 60 min during optimization of the assay, or for 5 min at optimal conditions and the spectrofluorometric measurement was performed at EX/EM= 352/461 nm (EX/EM slit widths 25/25 nm) using a Tecan Spark fluorescence microplate reader (Tecan, Switzerland) while incubated at 37°C. The samples were measured at least in triplicates. After background subtraction, the fluorescence signal was presented in Relative Fluorescence Units (RFU) as mean±SEM.

Annexin V-FITC apoptosis assay

The Annexin V-FITC apoptosis detection assay was followed after the viability assay and performed according to the supplier's protocol to determine apoptosis after the treatment, followed by fluorescence-activated cell sorting (FACS) for more precise analysis. Annexin V binds to phosphatidylserine, which is located at the inner cell membrane and exposed when cells are in the apoptotic process. After harvesting the cells, the samples were washed twice with cold PBS and resuspended in 1X binding buffer. Subsequently, 5 µL of FITC-Annexin V solution and 5 µL of PI solution were added to the samples. PI exhibits red fluorescence upon binding to DNA inside the cell and is generally used to detect dying or dead cells or opening of the cell plasma membrane. After gentle vortexing, the samples were incubated for 15 min at room temperature in the dark. After incubation, 400 µL of 1X binding buffer was added to the samples and analyzed by FACS within 1 h (FACS Aria II from BD Biosciences, USA).

Statistics

All data were expressed as mean ± SD (n= 10). The data were analyzed by Student's t test and One way ANOVA using SigmaPlot version 11.0. Differences between groups were considered to be statistically significant at $P<0.05$.

RESULTS AND DISCUSSION

DPPH radical scavenging activities of ethanol extract *H. isora* and cell viability assay

The analysis results showed that the antioxidant capacity through DPPH free radical scavenging reaction showed that *H. isora* extract has the ability to neutralize free radicals with IC_{50} = 21.16 µg/ml.

Sample *H. isora* has anti-proliferative activity of HepG2 cell line with IC_{50} = 37 µg/ml. Tested on Chang liver cell line, *H. isora* L. extract with a concentration of 200 µg/ml after 48 hours has an IC_{50} value of 25%.

Real time PCR assay

Analyzed the change in expression of 2 apoptosis genes (*Bcl-2* and *Bax*), using *GAPDH* gene as internal control on HepG2 liver cancer cell line, after treatment with *H. isora* extract at 2 concentrations (50 and 100 µg/ml) after 48 hrs, using 1% DMSO as control. There was a difference between the control and the experimental treatments and also observed the difference between the two experimental treatments of 50 mg/ml and 100 mg/ml. The mRNA expression of *Bcl-2* gene was reduced by 27% in the batch treated with 50 mg/ml extract after 48 hours and by 43% in the batch treated with 100 mg/ml extract after 48 hours (Fig 1). Thus, it was shown that there was a decrease in the expression of *Bcl-2* gene when were treated with *H. isora* extract. The expression of the *Bax* gene did not change after 48 hours difference between the control group and the treatments of 50 mg/ml and 100 mg/ml (Fig 2).

Hoechst 33258 spectrofluorometric assay

To evaluate the ability to trigger apoptosis through phenotype, HepG2 cells treated with *H. isora* extract at 2 concentrations (50, 100 µg/ml) and compared with the control, with time points of 24, 48 and 72h. There was a change in the cell membrane as well as the appearance of apoptotic bodies in the extract treatment group after 24 h. However, in the groups with extraction treatment, the fragmentation of the HepG2 cell nucleus became stronger with the longer the treatment time and the higher the concentration of the treated extract (Fig 3). In addition, testing of *H. isora* extract on Chang liver cells (Fig 4) showed that at the concentrations of *H. isora* examined, there is no effect on normal liver cells.

The ability to induce apoptosis

The percentage of HepG2 cells that went into apoptosis increased in the treatment group compared with the control group after 48 and 72 h (Fig 5). Besides, in the Chang liver cell line, there was no statistical difference between experiments (Fig 6). Thus, at a concentration of 50 µg/ml after 48 hrs of treatment, *H. isora* extract was able to induce HepG2 cells to go into apoptosis and Chang liver cells showed very low activity. The test of inhibitory activity against hepatocellular carcinoma cell line (HepG2) of *H. isora* L. extract at both cellular and molecular levels showed that at a concentration of 50 µg/ml after 48 h, the extract was capable of inducing apoptosis in HepG2 cells.

Oxidative stress is a pathological state in which reactive oxygen/nitrogen species (ROS/RNS) overwhelm antioxidative defenses of the organism, leading to oxidative modification of biological macromolecules (*i.e.*, lipid, protein, DNA), tissue injury and accelerated cellular death (Trevisan *et al.*, 2001) as the foundation of many diseases. Measuring the antioxidant activity of medicinal plant is carried out for treatment of oxidative stress-associated diseases in clinical biochemistry, for meaningful comparison of foods in regard to their antioxidant content and for controlling variations within or between products. The results of testing the antioxidant activity of the extract ethanol *H. isora* showed that the extract had high antioxidant activity (IC_{50} = 21.16 µg/ml). The antioxidant capacity of extract *H. isora* has also been reported by authours: fresh and dried samples from parts of *H. isora* such as leaves, bark, fruit and roots were extracted by various solvents (water, ethanol, methanol and acetone), obtained IC_{50} of the methanol extract of fresh fruit

Table 1: The primers used for the Real time PCR.

Target gene	Primer sequence
GAPDH	F: 5′-GAAGGTCGGAGTCAACGGATTT-3′
	R: 5′-CTGGAAGATGGTGATGGGATTTC-3′
Bcl-2	F: 5′-TTCTTTGAGTTCGGTGGGG-3′
	R: 5′-CAGGAGAAATCAAACAGAGGC-3′
Bax	F: 5′-CTTTTGCTTCAGGGTTTCATC-3′
	R: 5′-CACTCGCTCAGCTTCTTGGT-3′
	R: 5′-CCAAATCCTCCAGAACCAAT-3′

reaching the lowest value of 34.37 mg/ml (Jain *et al*., 2014). Chaudhary *et al*. (2016) recorded the roots of *H. isora* species when extracted by different solvents: water, ethanol, benzene, ethyl acetate, chloroform, petroleum ether with IC_{50} of 400±120 µg/ml, 660±230 µg/ml, 730±320 µg/ml, 1730±910 µg/ml, 3280±1010 µg/ml, 7010±1240 µg/ml

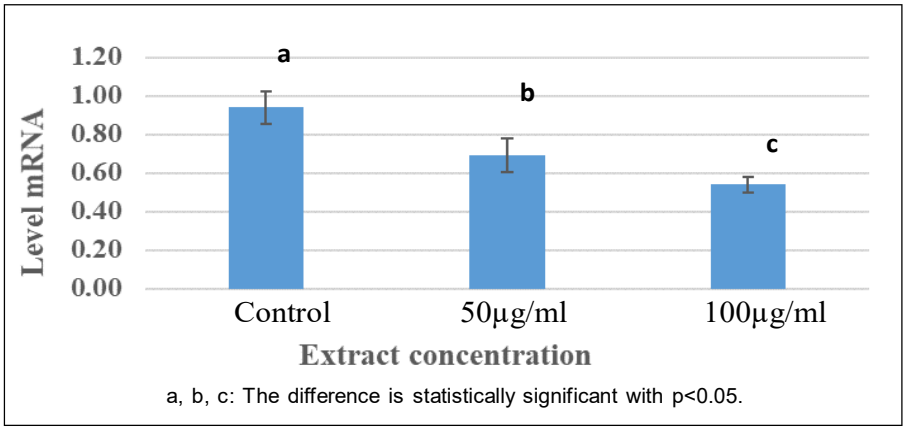

Fig 1: Effect of *H. isora* extract on *Bcl-2* expression in HepG2 cells.

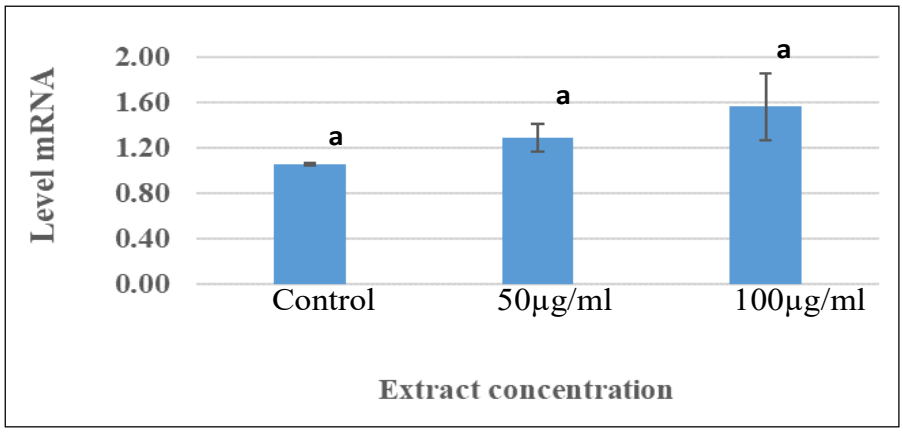

Fig 2: Effect of *H. isora* extract on *Bax* expression in HepG2 cells.

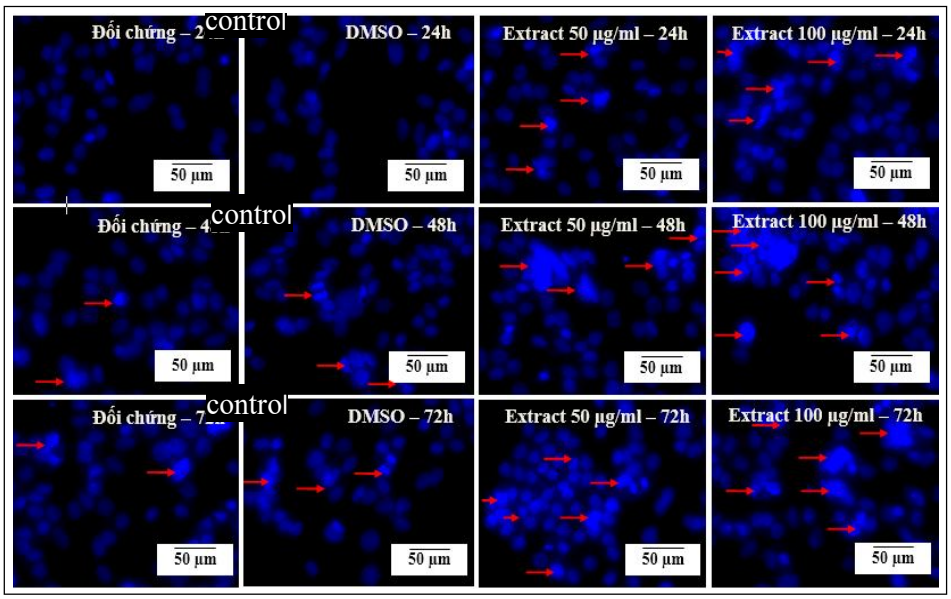

Fig 3: Image of HepG2 cells stained with Hoechst 33342 under the influence of *H. isora* extract.

respectively. The dried fruit methanol extract of *H. isora* has IC_{50} = 42.95 µg/ml; *H. isora* ether extract has IC_{50} = 89.81 µg/ml (Manke *et al*., 2015); 70% ethanol extract of *H. isora* L. stem bark has IC_{50} = 97.53±0.28 µg/ml (Shori *et al*., 2013).

The results of analysis of the expression of the anti-apoptogenic gene *Bcl-2* and the pro-apoptotic gene *Bax* at the transcriptional level showed that the mRNA transcription product of the Bax gene was expressed unchanged in all experimental groups, while the mRNA expression of *Bcl-2* gene decreased after extract treatment after 48 h. In the control group, the expression of *Bcl-2* and *Bax* genes was balanced, which prevented cells from going into apoptosis. In addition, this extract exhibits low toxicity to normal hepatocytes and is fully capable of being used to produce hepatoprotective functional foods. Similar results were also recorded by Shaikh *et al*. (2014), the ethanol extract of *H. isora* L. at a concentration of 1 mg/ml after 72 h of treatment, did not exhibit cytotoxicity on Chang liver cells and inhibited

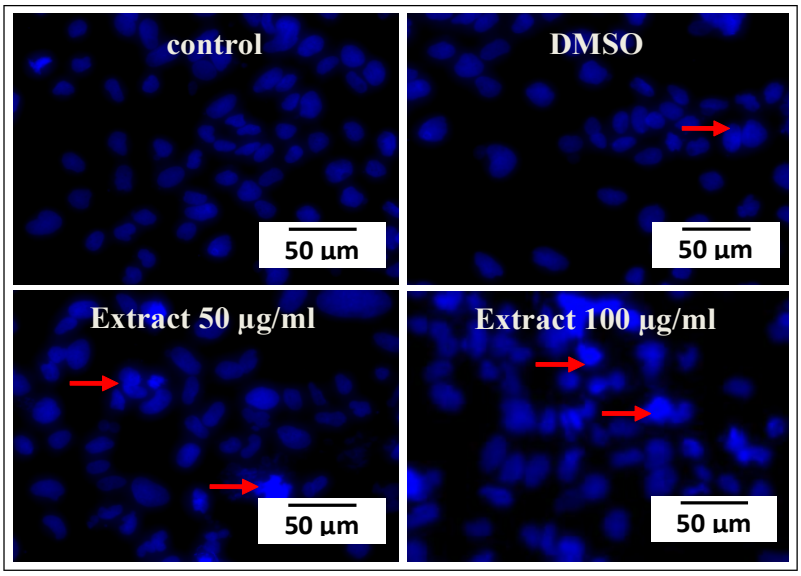

Fig 4: Image of Chang liver cells stained with Hoechst 33342 under the influence of *H. isora* extract.

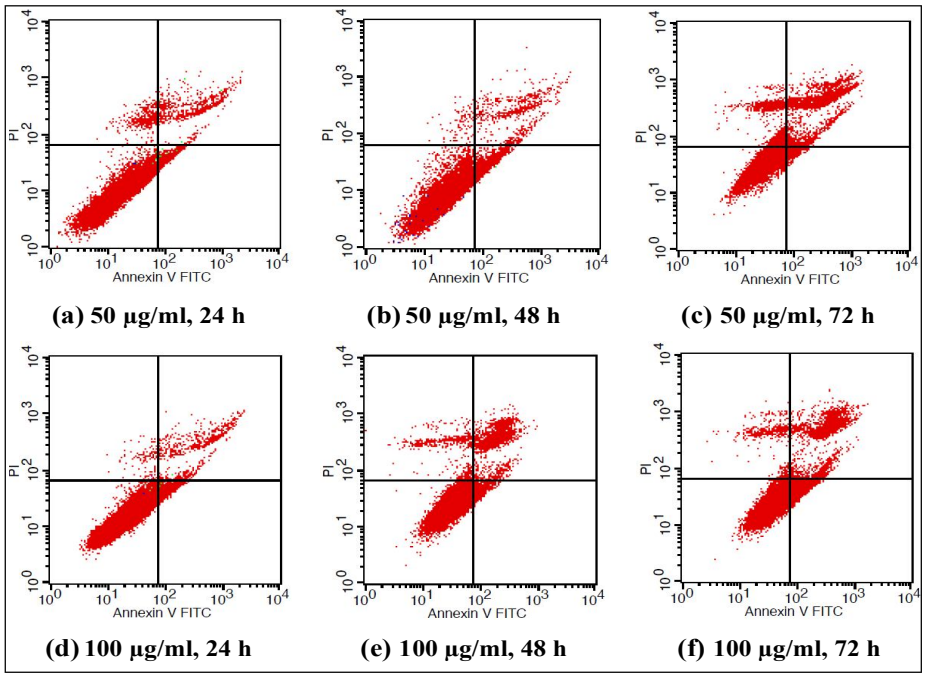

Fig 5: Effect of *H. isora* extract on apoptosis of HepG2 cells by cytometry flow extract after 24 h, 48 h and 72 h. a,b,c: Significant difference, $p<0.05$.

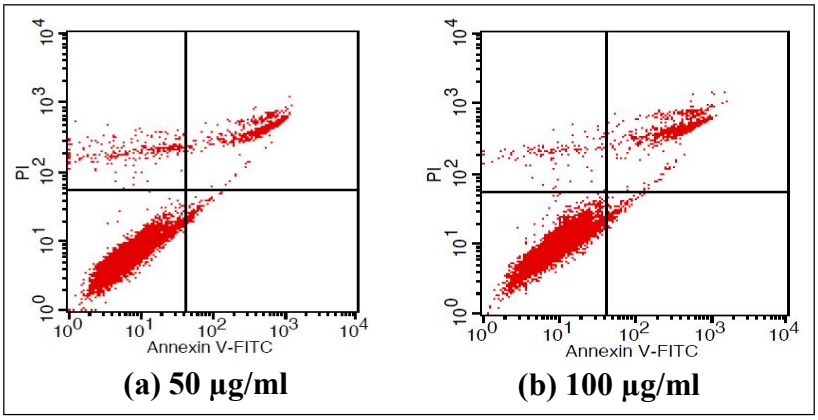

Fig 6: Effect of *H. isora* extract on apoptosis of Chang liver cells after 48 h by cytometry flow.

proliferation in the lines cancer cells (HeLa B75, 34.21%; HL 60, 30.25%; HEP 3B, 25.36%; PN 15, 29.21%) (Shaikh *et al*., 2014).

CONCLUSION

From the obtained results, it was concluded that *H. isora* induces apoptosis in HepG2 cells by inhibiting cell division in cancer cells. This inhibitory activity was caused by decreasing the level of the anti- apoptotic gene, Bcl-2 and increasing the livel of the proapoptotic gene, *Bax*. Therefore, we suggest that *H. isora* could be further investigated as an alternative treatment for human hepatoma.

ACKNOWLEDGEMENT

This work was supported by DakNong Department of Science and Technology; Vietnam Academy of Science and Technology.

Conflict of interest

All authors declared that there is no conflict of interest.

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