

Xao Tam Phan (*Paramignya trimeria*) methanol extract induced apoptosis in hepatocellular carcinoma HepG2 cell line *in vitro*

Sinh Truong Nguyen^{1,2,3}, Nghia Minh Do^{1,2,3}, Phuc Vo Hong^{1,2,3}, Trinh Thi – Tu Nguyen^{1,3}, Kiet Dinh Truong⁴, Phuc Van Pham^{1,2,3,5,*} 



Use your smartphone to scan this QR code and download this article

¹Stem Cell Institute, University of Science Ho Chi Minh City, Viet Nam

²Cancer Research Laboratory, University of Science Ho Chi Minh City, Viet Nam

³Viet Nam National University Ho Chi Minh City, Viet Nam

⁴Medical Genetic Institute, Ho Chi Minh City, Viet Nam

⁵Laboratory of Stem Cell Research and Application, University of Science Ho Chi Minh City, Viet Nam

Correspondence

Phuc Van Pham, Stem Cell Institute, University of Science Ho Chi Minh City, Viet Nam

Cancer Research Laboratory, University of Science Ho Chi Minh City, Viet Nam

Viet Nam National University Ho Chi Minh City, Viet Nam

Laboratory of Stem Cell Research and Application, University of Science Ho Chi Minh City, Viet Nam

Email: pvphuc@hcmuns.edu.vn; phucpham@sci.edu.vn

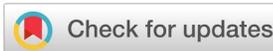
History

• Received: 15 Dec 2019

• Accepted: 03 Mar 2020

• Published: 31 Mar 2020

DOI : 10.32508/stdj.v23i1.2013



Copyright

© VNU-HCM Press. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license.



ABSTRACT

Introduction: Xao Tam Phan (*Paramignya trimeria*) has long been used in Viet Nam as an herbal medicine for the treatment of Hepatitis, hepatocellular carcinoma, and diabetes. This study aimed to determine the anti-proliferation effect of *Paramignya trimeria* extract (*P. trimeria* extract) on HepG2 hepatocellular carcinoma cells. **Methods:** AlamarBlue assay was used to determine the IC₅₀ values of *P. trimeria* extract on HepG2 cells. Adipose-derived stem cells (ADSCs) was used as normal cell control. For apoptosis examination, *P. trimeria* extract-treated HepG2 cells were incubated with Annexin V/Propidium iodide (PI). Then they have been analyzed their expression of Annexin-V and PI by flow cytometry. The cell nuclear degradation also was evaluated by PI/Hoechst 33342 staining assay. **Results:** Doxorubicin and *P. trimeria* extract IC₅₀ values on HepG2 cells were 55.13 ± 2.028 ng/ml and 582.533 ± 16.521 μg/ml, respectively. Those on ADSCs were 5.96 ± 0.56 ng/ml and 268.976 ± 19.325 μg/ml, respectively. Side effect index value (SEI) of *P. trimeria* extract was 2.175 ± 0.12, and the SEI of doxorubicin was 8.71 ± 0.36. Flow cytometry analysis indicated significant apoptosis on *P. trimeria* extract-treated HepG2 cells at a dose of 500 μg/ml (32.39 ± 2.28% apoptotic cells, and 14.63 ± 1.59% necrotic cells). Nuclear aggregation and degradation was seen on 500 μg/ml *P. trimeria* treated HepG2 cells. **Conclusion:** *P. trimeria* extract could inhibit HepG2 hepatocellular carcinoma cell proliferation by inducing apoptosis.

Key words: Paramignya trimeria, HepG2, side index effect, apoptosis, flow cytometry

INTRODUCTION

According to Globcan 2018 statistics, 841.080 people were diagnosed with liver cancer worldwide; an estimated 781,631 people died from the disease¹. Among the most common types of fatal cancer in the world, liver cancer has the fourth highest mortality rate and accounts for the second-highest death rate for men². Among the regions, Asia is the most alarming region. Asia has consistently led the way in terms of new cases of diagnosis and mortality, as well as the proportion of people living with liver cancer. The highest is in West Asia and then Southeast Asia¹.

According to IARC data in 2018, Viet Nam had 25335 new cases, 25404 deaths, and 21055 people living for five years with liver cancer³. Liver cancer accounts for the highest percentage of all common diseases, as well as the leading cause of death in Viet Nam. There have been many developed methods to treat cancer. Among them, five routine methods are commonly used: tumor removal surgery, chemotherapy, radiation therapy, hormone therapy, and immunotherapy⁴. Depending on the type of cancer, the patient's condition that the doctor chooses a suitable treatment or a combination of treatments to bring the best effect.

In particular, chemotherapy is still an important method to treat this disease. However, the cases of drug resistance are getting a problem. Besides, chemotherapy also causes many side effects, the possibility of cancer recurrence. Research on developing new drugs is still being promoted, including the use of compounds derived from nature, especially medicinal plants from ancient times. Up to date, folk remedies still play an essential role in the treatment and protection of human health. Vinca alkaloid, saponins, flavonoids, and their derivatives are anti-cancer compounds isolated from plants and scientifically proven to treat cancer⁵⁻⁷.

Vietnam is located in the tropics, with abundant biological resources, including many herbs that, according to folk medicine points, can cure cancer. However, most are used according to folk experience or used as a functional food, without precise mechanism. In Vietnam and Thailand, *P. trimeria* extract is considered a folk medicine to treat hepatitis, liver cancer, and diabetes⁸. However, studies on *P. trimeria* in general and their medicinal properties are quite limited. Xao Tam Phan is a small tree with a climbing body; body more than 4 m long, about 10 cm diameter;

Cite this article : Truong Nguyen S, Minh Do N, Vo Hong P, Thi – Tu Nguyen T, Dinh Truong K, Van Pham P. Xao Tam Phan (*Paramignya trimeria*) methanol extract induced apoptosis in hepatocellular carcinoma HepG2 cell line *in vitro*. *Sci. Tech. Dev. J.*; 23(1):484-489.

body and branches hairless, spiky, and to 7 to 8 cm long, slightly curved down. Leaves grow in a cluster or three-fold pattern; thick slab; oblong to extended narrow circles; size 10 – 18 x 1 – 2.5 cm; The whole cover is slightly bent; tendon filler 8 – 10 pairs; petioles short, 2 - 3 mm long. The parts of the plant have the essential oils, and their roots are at most⁸. In Vietnam, the *P. trimera* tree grows mainly in tropical forests, especially in the central provinces such as Khanh Hoa, Ninh Thuan, and Binh Thuan, in addition to areas adjacent to Laos and Cambodia.

According to folk experience, *P. trimera* roots are used as medicine. *P. trimera* root has a slightly bitter taste, slightly acrid, slightly sweet. According to some analyzes, *P. trimera* extract contains saponin (267.15 mg EE/g dry sample), proanthocyanidin (3.98 mg CE/g dry sample), phenolic (11.27 mg GAE/g dry sample), flavonoid (19.88 mg RE/g dry sample)⁹. Besides, *P. trimera* extract has also been shown to contain coumarins with potent anti-inflammatory properties, including ostruthin and ninhvanin¹⁰. Moreover, there are also studies proving *P. trimera* has many antioxidants. In another recent study, it was shown that *P. trimera* extract has a strong ability to inhibit MCF-7 breast cancer cells in both 2D and 3D culture conditions¹¹.

However, there have not been many studies on the anti-proliferation of this plant on HepG2 hepatocellular carcinoma cells. Therefore, this study aimed to assess the effects of *P. trimera* extract on HepG2 hepatocellular carcinoma cells.

MATERIAL — METHODS

Cell culture

HepG2 hepatocellular carcinoma cells were obtained from ATCC (Manassas, VA). They were cultured in DMEM/F12 medium (Thermo Fisher Scientific, Waltham, MA) containing 10% fetal bovine serum (FBS) and 1% antibiotic-mycotic (Gibco, Thermo Fisher Scientific, MA). HepG2 cells were cultured at 5% CO₂ humidified atmosphere at 37°C. Cells were passed at 80% confluency by trypsin/EDTA (0.025%) (Gibco, Thermo Fisher Scientific, MA).

Adipose-derived stem cells (ADSCs) were isolated from adipose tissues with consent from the donor as the published protocol¹². Adipose tissues were kept in cool temperature in PBS solution containing 1% antibiotic-antimycotic solution, and transferred to the laboratory for subsequent processing. The stromal vascular fraction (SVF) was extracted from the adipose tissue by the Cell Extraction Kit (Regenmedlab, Ho Chi Minh City, VN). Then SVF was cultured to collect ADSCs per a previously published protocol. All assays were performed per the published study¹².

Cytotoxicity assay

HepG2 and ADSC cells were seeded in 96 wells plate. The cell density was 2500 cells per well. After 24h, cells were treated with *P. trimera* extract at concentration 2000, 1000, 500, 250, 125, 60,30,15,7 μg/ml. Parallely, HepG2 cells also treated with doxorubicin at concentrations of 2000, 1000, 500, 250, 125, 60, 30, 15,7 ng/ml. After 48h incubation, cells were evaluated viability by Alamar Blue assay.

Side Effect Index (SEI) is calculated using the formula:

$$SEI_{P. trimera} = \frac{IC_{50} \text{ of } P. trimera \text{ extract on HepG2 cells}}{IC_{50} \text{ of } P. trimera \text{ extract on ADSCs}}$$

$$SEI_{Doxorubicin} = \frac{IC_{50} \text{ of Doxorubicin HepG2 cells}}{IC_{50} \text{ of Doxorubin on ADSCs}}$$

Apoptosis assay

HepG2 cells were seeded in 6 wells plate. The cell density was 200.000 cells per well. Cells were treated with *P. trimera* extract at concentrations of 60, 125, 250, 500, and 1000 μg/ml and incubated in 48h. Cells were labeled with 3 μL Annexin V-FITC (BD Biosciences, Franklin Lakes, NJ) (and 3 μl of Propidium Iodide (PI) in 500 μl of binding buffer for 15 mins to detect apoptotic and necrotic cell death using a FAC-SCalibur Flow Cytometer. Data were analyzed by CelQuest software (BD Biosciences).

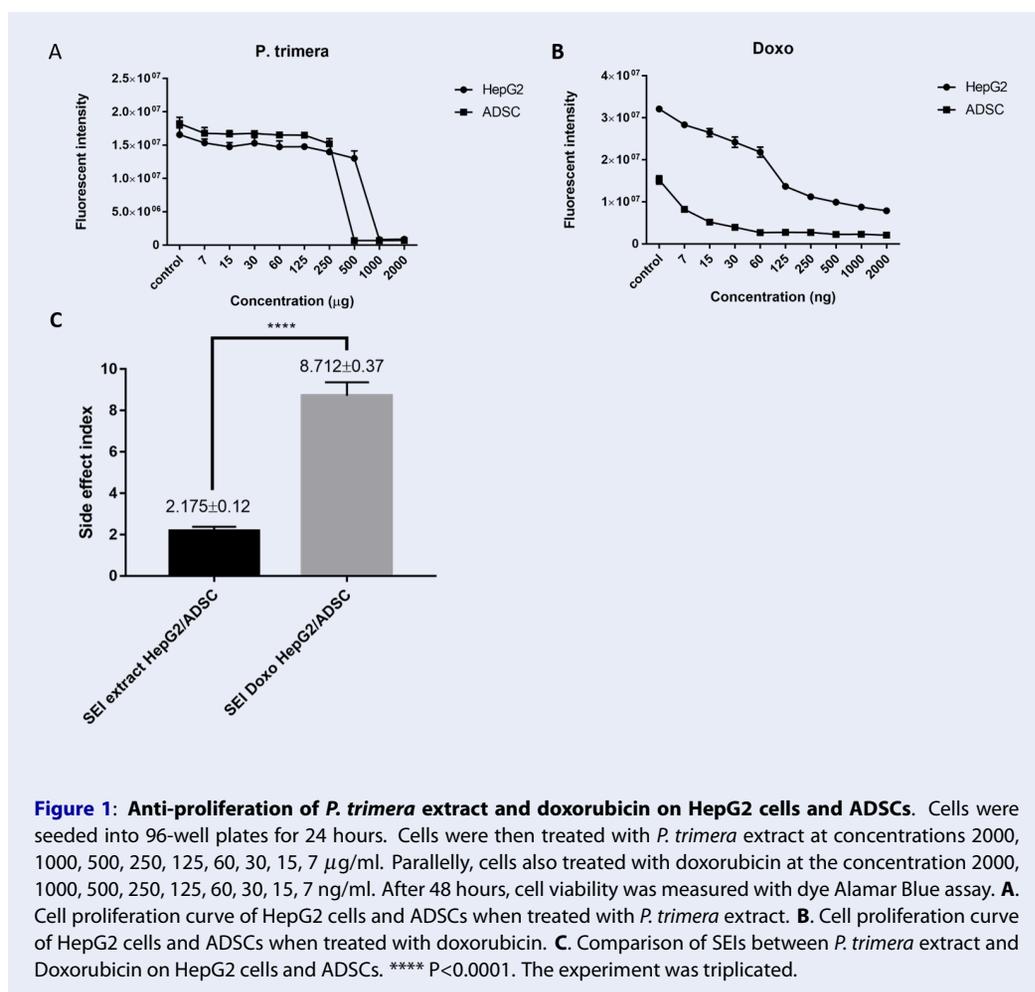
Nuclei staining assay

HepG2 cells were added into a 6-well plate with a density of 200000 cells/well. HepG2 cells were treated with *P. trimera* extract at concentrations of 2000 μg/ml, 500 μg/ml, 125 μg/ml, and control for 48 hours. Similarly, doxorubicin was added at concentrations of 300 ng/ml, 60 ng/ml, 15 ng/ml, and control for 48 hours. Then, cells mixed with 50 ml Hoechst 33342 and 10 ml Propidium Iodide, incubated for 10 minutes. Finally, the cell suspension was dropped in the lame, observed under the microscope fluorescence objective 40X (Carl-Zeiss, Germany).

RESULTS

Cytotoxicity of *P. trimera* extract and doxorubicin on HepG2 cells

The results showed that IC₅₀ values of *P. trimera* extract on ADSCs and HepG2 cells were 268.98 ± 19.33 μg/ml and 582.53 ± 16.52 μg/ml, respectively (Figure 1). The IC₅₀ values of doxorubicin on ADSCs and HepG2 cells are 5.96 ± 0.56 ng/ml and 55.13 ± 2.028 ng/ml, respectively. We performed the side effects index (SEI) of *P. trimera* and doxorubicin. SEI is considered as a method to assess the toxicity of a substance on a normal cell line. The result showed that SEI value of *P. trimera* was 2.175 ± 0.12, and SEI value of doxorubicin was 8.71 ± 0.36.



Apoptosis of HepG2 cells after treatment of *P. trimera* extract

Results showed that the total percentage of late apoptosis increased in dose-dependent manner. Late apoptosis population at concentrations of *P. trimera* extract 60, 125, 250, 500, 1000 µg/ml increased by $6.687 \pm 0.78\%$, $7.28 \pm 0.85\%$, $9.77 \pm 1.35\%$, $7.743 \pm 2.67\%$, $32.82 \pm 2.28\%$, $49.28 \pm 2.43\%$ respectively (Figure 2). At the concentration of 500 µg/ml, the total cell death counted almost 50%. When the concentration increased up to 1000 µg/ml, cell death was at 80%. This result proves that *P. trimera* extract can induce apoptosis in HepG2 cells.

Cell nuclei fragmentation of HepG2 cells after treatment of *P. trimera* and doxorubicin extracts

PI and Hoechst 33342 staining results showed in both *P. trimera* extract treatment group, and doxorubicin treatment group, the number of dead cells increased

in the manner of concentration dependence. These cells have DNA compressed or fragmented. In control, cells were mainly in blue fluorescent with contact cell nuclei. When cells were treated with *P. trimera* extract at the concentration of IC₅₀ value and at 1/4 of IC₅₀ value, dead cells were significantly increased compared to that in control ($582.533 \pm 16.521 \mu\text{g/ml}$) at IC₅₀, and $49.33 \pm 2.50\%$ at $\frac{1}{4}$ of IC₅₀ compared to $7.00 \pm 1.00\%$ at control). Particularly, when cells were treated with *P. trimera* extract at 4 x IC₅₀ concentration, most cells were no longer intact ($94.00 \pm 6.25\%$), stained with red fluorescent (Figure 3). This result proves that *P. trimera* extract can cause HepG2 cell death and nuclear fragmentation.

DISCUSSION

According to the World Cancer Research Organization (IARC), Vietnam is in an epidemiological area with a high rate of liver cancer. In 2018, liver cancer led to the number of new cases and deaths in the country. Studies looking for extracts or compounds to

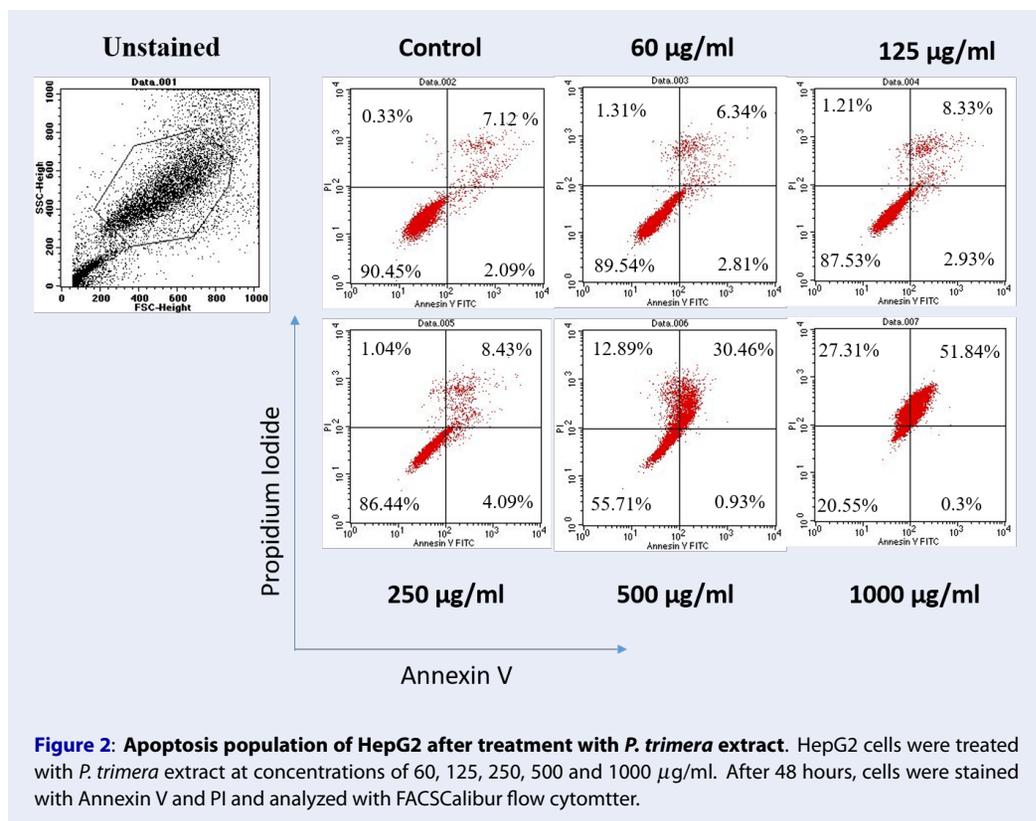


Figure 2: Apoptosis population of HepG2 after treatment with *P. trimera* extract. HepG2 cells were treated with *P. trimera* extract at concentrations of 60, 125, 250, 500 and 1000 µg/ml. After 48 hours, cells were stained with Annexin V and PI and analyzed with FACSCalibur flow cytometer.

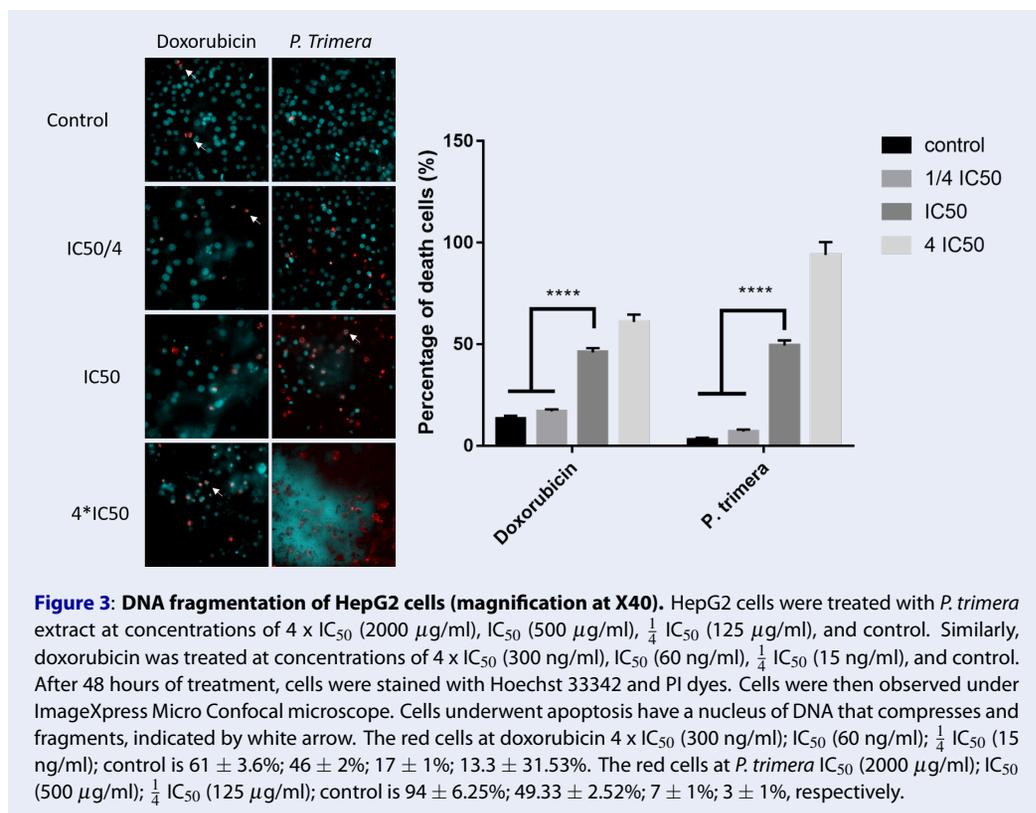


Figure 3: DNA fragmentation of HepG2 cells (magnification at X40). HepG2 cells were treated with *P. trimera* extract at concentrations of 4 x IC₅₀ (2000 µg/ml), IC₅₀ (500 µg/ml), 1/4 IC₅₀ (125 µg/ml), and control. Similarly, doxorubicin was treated at concentrations of 4 x IC₅₀ (300 ng/ml), IC₅₀ (60 ng/ml), 1/4 IC₅₀ (15 ng/ml), and control. After 48 hours of treatment, cells were stained with Hoechst 33342 and PI dyes. Cells were then observed under ImageXpress Micro Confocal microscope. Cells underwent apoptosis have a nucleus of DNA that compresses and fragments, indicated by white arrow. The red cells at doxorubicin 4 x IC₅₀ (300 ng/ml); IC₅₀ (60 ng/ml); 1/4 IC₅₀ (15 ng/ml); control is 61 ± 3.6%; 46 ± 2%; 17 ± 1%; 13.3 ± 31.53%. The red cells at *P. trimera* IC₅₀ (2000 µg/ml); IC₅₀ (500 µg/ml); 1/4 IC₅₀ (125 µg/ml); control is 94 ± 6.25%; 49.33 ± 2.52%; 7 ± 1%; 3 ± 1%, respectively.

treat the disease are always promoted¹³. About 80% of cancer medicines approved by the US Food and Drug Administration (FDA) are derived from natural compounds, mainly from medicinal plants¹⁴. The rich and diverse biological resource of Vietnam is an abundant source of medicinal materials. There are many types of plants used as medicines. However, there is no scientific evidence of a transparent effect. Among them, Xiao tam phan (*P. trimera*) is a plant belonging to the Citrus family (Rutaceae). In our previous study, *P. trimera* extract showed a strong inhibitory effect on MCF-7 breast cancer cell lines in 2D culture conditions ($260.8 \pm 16.54 \mu\text{g/ml}$) and 3D ($\text{IC}_{50} 168.9 \pm 11.65 \mu\text{g/ml}$). It was shown that *P. trimera* has a full inhibitory effect on the invasion of MCF-7 cells¹⁴. To clarify the impact of *P. trimera* extract on liver carcinoma line, we conducted a test to determine the impact of this extract on HepG2 cells. The IC_{50} value of *P. trimera* extract was established on two lines of HepG2 cells and ADSCs, and doxorubicin used as a positive agent. The results showed that the average IC_{50} values of *P. trimera* and doxorubicin on HepG2 cells were $582.53 \pm 16.52 \text{ ng/ml}$ and $55.13 \pm 2.03 \text{ ng/ml}$. For ADSCs, *P. trimera* extract had an average IC_{50} value of $268.98 \pm 19.33 \mu\text{g/ml}$. The IC_{50} value of doxorubicin on ADSCs was $5.96 \pm 0.56 \text{ ng/ml}$. Besides, the side effects index SEI values of *P. trimera* and doxorubicin were 2.175 ± 0.12 and 8.71 ± 0.36 . This result proves that in addition to the inhibitory effect on the MCF-7 cell line published in the previous study¹¹, *P. trimera* extract also has anti-proliferation effects on the HepG2 cells and *P. trimera* has less side effects than doxorubicin. This could be a promising potential for cancer treatment.

Doxorubicin is one of the most commonly used drugs in cancer chemotherapy. The study showed that doxorubicin was removed from the body by reducing two electrons by carbonyl reductase, which is most important in the liver¹⁵. Doxorubicin resistance is also due to over-expression of the adenosine triphosphate-binding cassette (ABC) family, typically ABCB1 (MDR1; MDR1) or ABCC1 (MDR1-related gene 1; MRP1)^{16,17}. These may be the cause of higher IC_{50} in HepG2 than those in ADSC.

Apoptosis is an important process for getting rid of cells that lose function in the body. Cancer cells, though not functioning, still exist due to their ability to evade programmed death. The anticancer drug, therefore, aims to resist cell division or kill cancer

cells through apoptosis or necrosis¹⁸. Flow cytometry result showed that after treatment of *P. trimera* extract, apoptosis population cells increased by $6.687 \pm 0.78\%$, $7.28 \pm 0.85\%$, $9.77 \pm 1.35\%$, $7.74 \pm 2.67\%$, $32.82 \pm 2.28\%$, $49.28 \pm 2.43\%$ correspond to concentrations of 60, 125, 250, 500 and 1000 $\mu\text{g/ml}$. In which the concentration of 500 $\mu\text{g/ml}$ showed a significant increase in cyclic cell death rate (31%). This result showed that *P. trimera* can activate the programmed pathway of death. Nuclear fragmentation, an important feature of programmed cell death, is biological damage at the molecular biology level. Results showed that cells treated with *P. trimera* have nuclei compressed, divided into small pieces, the cell membrane remains intact. This result provides further evidence that *P. trimera* extract has the ability to inhibit HepG2 cells through the programmed cell death.

CONCLUSION

Cancer is the leading cause of death worldwide. Live cancer is high rate of death in Vietnam. Traditional medicine plays the critical role in drug development. Vietnam possesses tropical forest with resources of potential herbal medicine. *P. trimera* is popularly used in primarily treatment of some disease. In this study, *P. trimera* has shown that it can anti-proliferate HepG2 cells at $\text{IC}_{50} 268.98 \pm 19.33 \mu\text{g/ml}$, while *P. trimera* has less side effect on ADSCs compared to doxorubicin. This implicated that *P. trimera* is the potential herbal in further investigation of drug development for cancer.

ABBREVIATIONS

ADSC: adipose-derived stem cell

P. trimera: Paramignya trimera

SEI: side effects index

COMPETING INTERESTS

All authors equally contributed in this work. All authors read and approved the final version of the manuscript for submission.

AUTHORS' CONTRIBUTIONS

The authors report no conflicts of interest in this work.

ACKNOWLEDGMENTS

This work was supported by the Vietnam National University, Ho Chi Minh City, Vietnam, under grant A2015-18-01.

REFERENCES

1. 11 liver fact sheet [http://gco.iarc.fr/today/data/factsheets/cancers/11-Liver-fact-sheet.pdf].
2. Facts in pictures/Detail/Cancer [https://www.who.int/news-room/facts-in-pictures/detail/cancer].
3. 704 Vietnam fact sheet [http://gco.iarc.fr/today/data/factsheets/populations/704-viet-nam-fact-sheets.pdf].
4. Sudhaka A. History of cancer, ancient and modern treatment methods. *Journal of cancer science & therapy*. 2009;1(2):1. PMID: 20740081. Available from: <https://doi.org/10.4172/1948-5956.100000e2>.
5. Man S, Gao W, Zhang Y, L H, C L. Chemical study and medical application of saponins as anti-cancer agents. *Fitoterapia*. 2010;81(7):703–714. PMID: 20550961. Available from: <https://doi.org/10.1016/j.fitote.2010.06.004>.
6. Batra P, Sharma AK. Anti-cancer potential of flavonoids: recent trends and future perspectives. *3 Biotech*. 2013;3(6):439–459. PMID: 28324424. Available from: <https://doi.org/10.1007/s13205-013-0117-5>.
7. Newman DJ, Cragg GM. Natural products as sources of new drugs from 1981 to 2014. *Journal of natural products*. 2016;79(3):629–661. PMID: 26852623. Available from: <https://doi.org/10.1021/acs.jnatprod.5b01055>.
8. Hộ PH: Cây cỏ Việt Nam, tập 1, 2, 3. Nxb trẻ, Thành phố Hồ Chí Minh 1999.
9. Nguyen T, Pham NMQ, Vuong Q, Bowyer M, van Altena I A, Scarlett C. Phytochemical retention and antioxidant capacity of Xao tam phan (*Paramignya trimera*) root as prepared by different drying methods. *Drying Technology*. 2015;34(3):324–334. Available from: <https://doi.org/10.1080/07373937.2015.1053566>.
10. HL TA, D-C K, W K, TM H, NX N, PH Y, et al. Anti-inflammatory coumarins from *Paramignya trimera*. *Pharm Biol*. 2017;55(1):1195–1201. PMID: 28245363. Available from: <https://doi.org/10.1080/13880209.2017.1296001>.
11. Nguyen-Thi LH, Nguyen ST, Tran T, Phan-Lu CN, Van TT, Pham PV. Anti-cancer Effect of Xao Tam Phan *Paramignya trimera* Methanol Root Extract on Human Breast Cancer Cell Line MCF-7 in 3D Model. *Advances in experimental medicine and biology* .2018;2018((First Online)). Available from: https://doi.org/10.1007/5584_2018_148.
12. P P, NB V, NL-C P, DM L, NC T, NH T, et al. Good manufacturing practice-compliant isolation and culture of human adipose derived stem cells. *Biomedical Research and Therapy*. 2014;4(1):1–9. Available from: <https://doi.org/10.7603/s40730-014-0021-6>.
13. Saxena M, Saxena J, Nema R, Singh D, Gupta A. Phytochemistry of medicinal plants. *Journal of pharmacognosy and phytochemistry*. 2013;1(6).
14. DJ N, GM C. Natural products as sources of new drugs over the 30 years from 1981 to 2010. *Journal of natural products*. 2012;75(3):311–335. PMID: 22316239. Available from: <https://doi.org/10.1021/np200906s>.
15. N K, K H, H-J M, U GA, A M, I M, et al. Carbonyl reductase 1 is a predominant doxorubicin reductase in the human liver. *Drug Metabolism and Disposition*. 2008;36(10):2113–2120. PMID: 18635746. Available from: <https://doi.org/10.1124/dmd.108.022251>.
16. G L, X C, Q W, Z X, W Z, L Y. The roles of four multi-drug resistance proteins in hepatocellular carcinoma multidrug resistance. *Journal of Huazhong University of Science and Technology*;27(2):173–175. PMID: 17497289. Available from: <https://doi.org/10.1007/s11596-007-0217-8>.
17. J W, JYW C, CC F, CH T, KP F, M Y. Transcriptional analysis of doxorubicin-induced cytotoxicity and resistance in human hepatocellular carcinoma cell lines. *Liver International*. 2009;29(9):1338–1347. PMID: 19627484. Available from: <https://doi.org/10.1111/j.1478-3231.2009.02081.x>.
18. Mahassni SH, Al-Reemi RM. Apoptosis and necrosis of human breast cancer cells by an aqueous extract of garden cress (*Lepidium sativum*) seeds. *Saudi journal of biological sciences*. 2013;20(2):131–139. PMID: 23961228. Available from: <https://doi.org/10.1016/j.sjbs.2012.12.002>.