

# *Hopea odorata* extract inhibits hepatocellular carcinoma via induction of caspase-dependent apoptosis

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**Introduction:** Cancer is a disease with a global burden and is a major and increasing threat to public health. The demand for new modalities to treat and prevent cancer is high. Given the toxic side effects of standard treatments, such as chemotherapy, there is greater research interest in naturally derived compounds due to their selective toxicity to cancer cells. This study aimed to test the anticancer activity of a crude extract of *Hopea odorata* on hepatocellular carcinoma (HCC) HepG2 cell line.

**Methods:** Methanol extracts of *H. odorata* were prepared from the bark of *H. odorata* plants (*H. odorata* extract). The in vitro cytotoxicity of *H. odorata* extracts on human HCC cell line HepG2 compared to normal human fibroblasts (HFs) was assessed by Alamar Blue assay. Caspase-3/7 was detected using a reagent that consists of DEVD peptide conjugated to a nucleic acid-binding dye. Apoptosis induction by the *H. odorata* plant extract on HepG2 was evaluated by Annexin V/7-AAD using flow cytometry. Disintegrated nuclei of plant-treated cells were observed under a fluorescent microscope using Hoechst and propidium iodide (PI) staining. In addition, using the Hoechst/PI staining technique, the ratio of dead to total cells was determined by distinguishing Hoechst and PI fluorescent signals.

**Results:** We found that the IC<sub>50</sub> value of *H. odorata* extract on HepG2 was 12.67±5 µg/mL and on HF was 44±3 µg/mL. The IC<sub>50</sub> value of doxorubicin on HepG2 was 153.3±15 ng/mL and on HF was 6.3±0.6 ng/mL. The selectivity index (SI) of *H. odorata* extract for HepG2 cells was ~3.48, while the SI of doxorubicin for HepG2 cells was ~0.04. The ratio of dead to total cells increased in a dose-dependent manner for HepG2 cells when observed under a fluorescent microscope, while the ratio of dead to total cells barely changed for HF cells. The *H. odorata* extract inhibited HepG2 cells via the activation of caspase-3/7. At 250 µg/mL concentration of the *H. odorata* extract, 35% of HepG2 cells were induced into apoptosis, and the cells exhibited disintegrated nuclei under a fluorescent microscope.

**Conclusion:** These findings demonstrate that the methanolic bark extracts of *H. odorata* plant induce apoptosis and selective cytotoxicity toward HepG2 but not HF. Therefore, purification of compounds from *H. odorata* bark extracts may be useful as anticancer agents, and thus, more studies are warranted to investigate the anticancer properties of *H. odorata*.

**Keywords:** apoptosis, caspase-3, HepG2, *Hopea odorata*, human fibroblast, methanol extract, selectivity index

## Introduction

There is a rising interest in natural product resources, particularly medicinal plants, for drug discovery and development of therapeutic agents. More than 35,000 plant species have been reported around the world for medicinal use.<sup>1</sup> Plants contain many

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biologically active compounds that have potential to be developed as anticancer therapeutic agents. Some of these compounds include curcumin (from turmeric) that has shown anticancer effects against colon cancer<sup>2–4</sup> and brain cancer,<sup>5,6</sup> apigenin (from parsley) that has been demonstrated to induce apoptosis in breast cancer,<sup>7,8</sup> and crocetin (from saffron) that has been reported to be cytotoxic against pancreatic cancer<sup>9,10</sup> and skin carcinoma.<sup>11,12</sup> By recent estimation, ~50%–60% of cancer patients in the US utilize agents from natural products exclusively or as a complement for traditional therapeutic regimens, such as chemotherapy or radiation therapy.<sup>13</sup>

Hepatocellular carcinoma (HCC) is one of the most common gastrointestinal diseases and generally carries a poor prognosis since it is highly malignant. According to the latest statistics, ~600,000 patients are diagnosed with HCC worldwide each year, and some of them die within 7–8 months after diagnosis.<sup>14</sup> Although there are many advanced options available for the treatment of HCC, these methods have shown various adverse effects, such as immune system disorders and liver toxicity. Common treatments, including surgery and even liver transplantation, only provide limited results. Chemotherapy and radiotherapy cause severe side effects such as cytotoxicity on normal cells and drug resistance.<sup>15,16</sup> Therefore, more effective alternative therapies or medicines, with fewer side effects, are critically needed for HCC. Herbal medicine has emerged as an alternative medicine with great potential for HCC therapy.

*Hopea odorata* belongs to the Dipterocarpaceae family of plants, which is widely distributed in lowland forests of Vietnam and is native to Southeast Asia.<sup>17</sup> Its local name is “sao den”. Different parts of this plant have been used as traditional medicine. In Vietnam, the extract of the *H. odorata* bark has been used as a remedy for gingivitis and for diarrhea treatment. The resin of the trunk of *H. odorata* can be applied to sores and wounds for the relief of inflammation and to stop bleeding. A recent study showed that the methanol extract of *H. odorata* strongly inhibited gene expression of proinflammatory cytokines and chemokines, such as interferon-beta, interleukin-12, and monocyte chemoattractant protein-1.<sup>18</sup> To our knowledge, there is no information of *H. odorata* as a cancer treatment, from indigenous medicine until now. Owing to its long and traditional use in Southeast Asia to treat inflammation, we questioned if it might have an effect on cancer cells since the cancer milieu is proinflammatory.<sup>19–21</sup> In this study, we aimed to determine whether methanol extracts of *H. odorata* bark showed antitumor efficacy against the HCC cell line HepG2 and human normal human fibroblast (HF) in vitro. The cytotoxic ability of the plant extract (PE) was

investigated and compared to the reference anticancer drug doxorubicin.

## Materials and methods

### Collection of plant material

The plant was collected at Ma Da forest in Dong Nai Culture and Nature Reserve, Dong Nai Province, Vietnam, in April 2014. The plant was identified by Associate PhD. Hop Tran, Institute of Tropical Biology, Ho Chi Minh City, Vietnam. The voucher sample (MCE018) is preserved at the Division of Medicinal Chemistry, Faculty of Chemistry, University of Science, Vietnam National University Ho Chi Minh City, Vietnam.

### Extraction of plant material

The dried bark of *H. odorata* plant (200 g) was cut into small pieces and extracted with MeOH (300 mL, reflux, 3 hours, ×3). The MeOH solution was evaporated under reduced pressure to give an MeOH extract (15 g).

### Cell lines

The HepG2 cell line was obtained from American Type Culture Collection (Manassas, VA, USA). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin–streptomycin in a humidified incubator with 5% CO<sub>2</sub> at 37°C; all reagents were obtained from Thermo Fisher Scientific (Waltham, MA, USA).

HF cells were isolated from foreskin, according to a previously published protocol. All patients provided written informed consent for the use of human skin tissue in this research. HF cells were isolated from human foreskin collected at Gia Dinh Hospital (Ho Chi Minh City, Vietnam). The cell isolation from human tissue was approved by the scientific ethics committee of the Stem Cell Institute, University of Science, Vietnam National University. Foreskin was stored in the PBS solution at 4°C and transferred to the laboratory for isolation and culture of fibroblasts. HF cells from passage 4 to passage 5 were used for experiments.

### Cell viability assessment of PE treatment and doxorubicin treatment

Briefly, 2,500 cells of HepG2 were seeded into each well of a 96-well tissue culture plate 1 day before treatment with PE. HF cells were used as controls and were assayed in parallel with HepG2. Cells were treated with PE in the culture medium for 48 hours at these serial extract concentrations: 1,000, 500, 250, 125, 62.5, 31, 15, and 0 µg (untreated).

Methanol solvent in the culture medium was adjusted to 0.5% in all the concentration points. Subsequently, Alamar Blue (R-7071; Sigma-Aldrich Co., St Louis, MO, USA) was added to the medium to a final concentration of 10  $\mu\text{g}/\text{mL}$  and incubated for 1 hour. The fluorescence at 560 nm was recorded using DTX 880 (Beckman Coulter, Brea, CA, USA). Each concentration was tested in triplicate. Experiments were independently repeated three times.

For doxorubicin treatment, doxorubicin (Sigma-Aldrich Co.) was evaluated at the following serial concentrations: 125, 62, 30, 15, 7, 3, and 0  $\mu\text{g}$  (untreated). Each concentration was tested in triplicate. Experiments were independently repeated three times.

### Caspase-3/7 activity

Cells were treated with *H. odorata* extract at a concentration of 250  $\mu\text{g}/\text{mL}$ . Concurrently, CellEvent Caspase-3/7 Green Detection Reagent (C10423; Thermo Fisher Scientific) was added into the culture medium at a final concentration of 5  $\mu\text{M}$  and incubated for 30 minutes. Cells were visualized by fluorescent microscopy.

### Annexin V/propidium iodide (PI) apoptosis assay

Treated and untreated cells were harvested after trypsinization and pelleted by centrifugation at  $500\times g$  for 5 minutes at  $4^{\circ}\text{C}$ . Pellets were suspended, and cells were then stained with Annexin V and PI using a FITC Annexin V Apoptosis Detection Kit I (556547; BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Stained cells were analyzed by a BD FACSCalibur flow cytometer (BD Biosciences). Data were analyzed by CellQuest software (BD Biosciences).

### Hoechst 33342 and PI staining assay

Cell viability of HepG2 (following treatment with 250  $\mu\text{g}/\text{mL}$  of extract) was evaluated by Hoechst 33342/PI staining. Skin-derived HF was used as the control cell line. Briefly, HepG2 and HF cells were treated with *H. odorata* extract at a concentration of 250  $\mu\text{g}/\text{mL}$ ; untreated cells were cultured in the normal medium complemented with a solvent. At day 2 of treatment, cells were stained with Hoechst 33342 and PI. The final concentration of Hoechst 33342, as well as PI, was 1  $\mu\text{g}/\text{mL}$  in the culture medium. Then, cells were incubated in an incubator ( $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$ ) for 5 minutes. Stained cells were observed by a fluorescent microscope.

### Statistical analysis

Data were analyzed by GraphPad Prism software (Graphpad Software, Inc., La Jolla, CA, USA) for the determination of  $\text{IC}_{50}$  values. All data were presented as the mean of triplicate experiments. Statistical significance was set at  $P < 0.05$ .

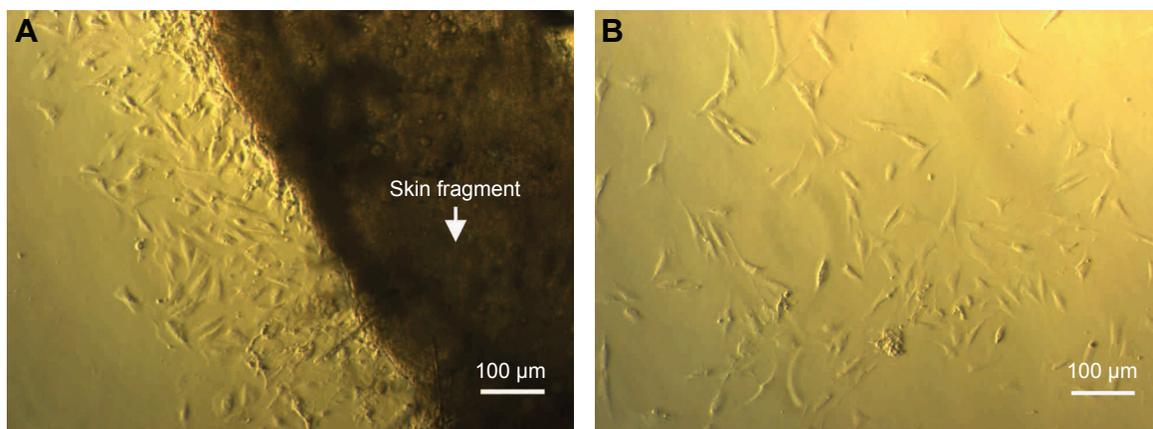
## Results

### Fibroblast isolation from skin tissue

Fat was eliminated from skin tissue and cut into small pieces with sharp scissors. Skin fragments were then cultured for 10 days. At days 9–10, cells had spread out from the edge of the skin fragment (Figure 1). At this time, cells grew slowly. At day 15, cells proliferated faster and spread out from the skin fragment and exhibited the elongated morphology of HFs. Cells from passage 4 or 5 were used for experiments.

### Selectivity cytotoxicity of *H. odorata* extract on HepG2 but not HF

The mean  $\text{IC}_{50}$  values of doxorubicin on HepG2 and HF were  $153.3 \pm 15.28$  and  $6.3 \pm 0.6$   $\text{ng}/\text{mL}$ , respectively. The  $\text{IC}_{50}$



**Figure 1** Isolation of fibroblasts from skin tissue.

**Notes:** Skin tissue after collection from hospital was cut into small pieces and laid on the surface of culture roux. After 10 days, cells were grown from the edge of the skin fragment (A). After 15 days, cells proliferated and spread out on the surface of culture roux (B).

values of *H. odorata* extract on HepG2 and HF were 12.67±5.03 and 44.2±3.85 µg/mL, respectively (Figure 2). We calculated the selectivity index (SI) of cytotoxicity of the agent by the ratio of IC<sub>50</sub> value on HF (control cells) to that on HepG2 (cancer cells). The results showed that SI of *H. odorata* is 87-fold greater than SI of doxorubicin. *H. odorata* extract is less cytotoxic to HF than to HepG2 cells. Conversely, doxorubicin is more cytotoxic against HF than to HepG2.

$$SI = \frac{IC_{50} \text{ of agent on normal cell line}}{IC_{50} \text{ of agent on target cell line}}$$

By that, SI of doxorubicin (SI<sub>doxorubicin</sub>) is

$$\frac{IC_{50} \text{ of doxorubicin on HF}}{IC_{50} \text{ of doxorubicin on HepG2}} = \frac{6.3}{153.3} = 0.04$$

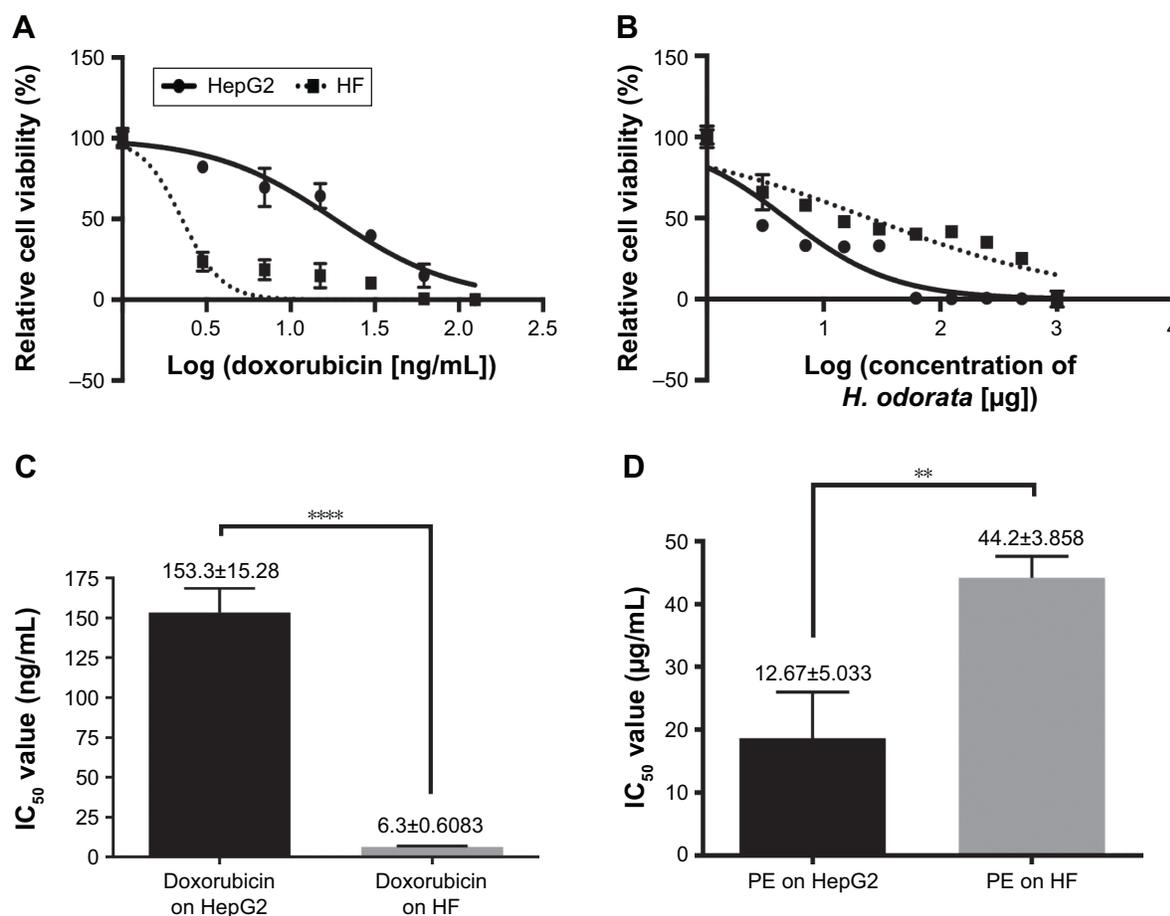
SI of *H. odorata* (SI<sub>*H. odorata*</sub>) is

$$\frac{IC_{50} \text{ of } H. \text{ odorata on HF}}{IC_{50} \text{ of } H. \text{ odorata on HepG2}} = \frac{44.2}{12.67} = 3.48$$

The ratio of (SI<sub>*H. odorata*</sub>) to (SI<sub>doxorubicin</sub>) is

$$\frac{SI_{H. \text{ odorata}}}{SI_{\text{doxorubicin}}} = \frac{3.48}{0.04} = 87$$

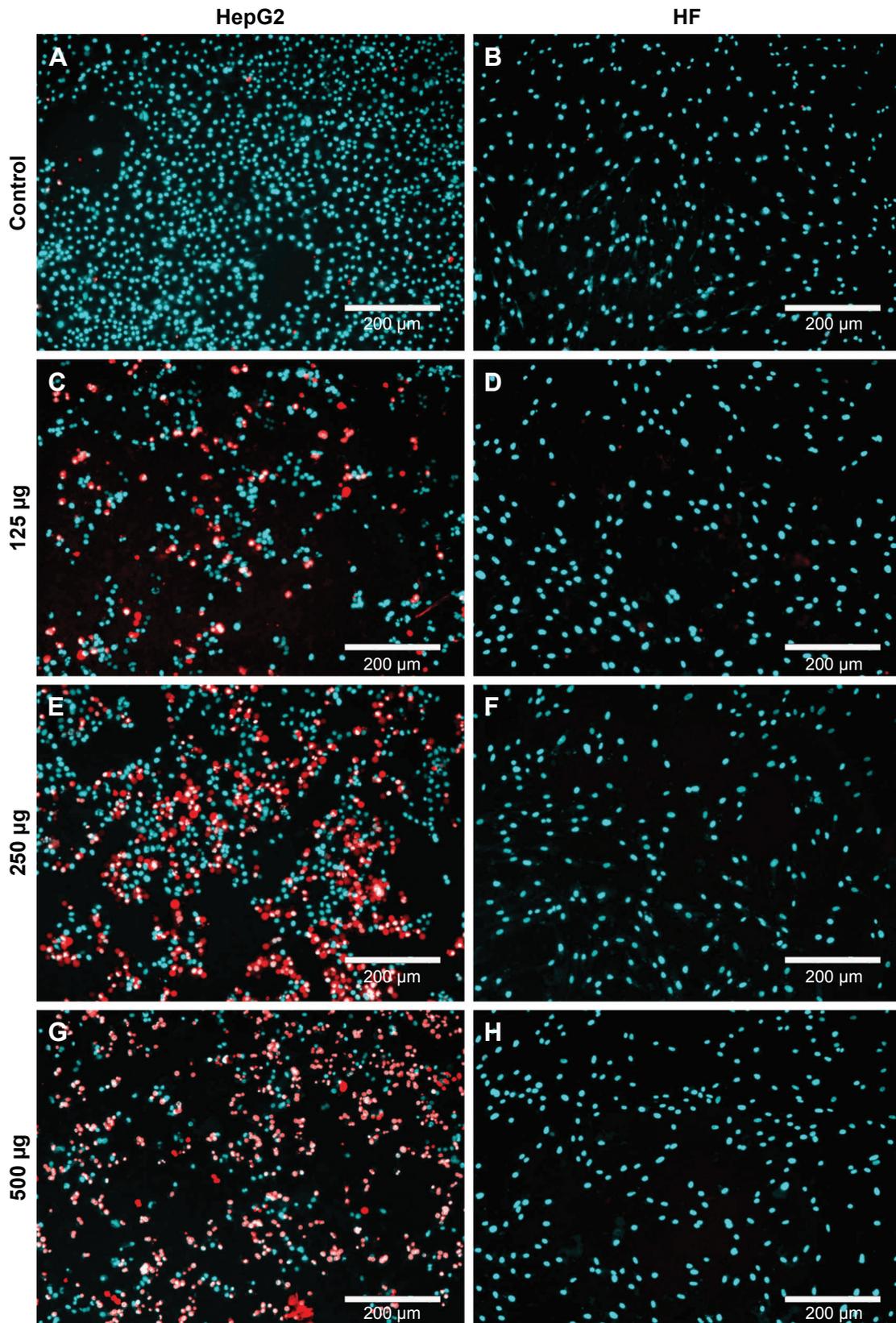
The SI of *H. odorata* extract was supported by the observation of dead cells by fluorescent microscopy. Dead cells were determined by PI-positive cells. Nuclei of total cells were visualized by Hoechst 33342 staining. The staining assays showed that *H. odorata* extract killed HepG2 cells in a dose-dependent manner (Figure 3). At concentrations of 60 µg/mL, 250 µg/mL, and 500 µg/mL of *H. odorata* extract,



**Figure 2** Cytotoxicity of doxorubicin and *H. odorata* PE on HepG2 and HF.

**Notes:** HepG2 and HF cells were treated with doxorubicin, from 0 ng/mL (untreated) to 125,000 ng/mL. Similarly, both of these cell lines were treated with *H. odorata* at concentrations of 0 µg/mL (untreated) to 2,000 µg/mL. After 48 hours of treatment, Alamar Blue was added. The fluorescence of cell samples was measured after 4 hours of incubation with dye. The experiment was conducted three independent times. **(A)** Inhibition curve of HepG2 and HF treated with doxorubicin for 48 hours. **(B)** Inhibition curve of HepG2 and HF treated with *H. odorata* extract for 48 hours. **(C)** IC<sub>50</sub> value of doxorubicin on HepG2 and HF after 24 hours or 48 hours of treatment. **(D)** IC<sub>50</sub> value of *H. odorata* extract on HepG2 and HF after 24 hours or 48 hours of treatment. Data shown as mean ± standard deviation. \*\*\*\*P<0.0001 for **C** and \*\*P=0.0053 for **D** using t-test analysis of Graphpad Prism. Mean ± SEM, n=1 independent experiment for **(A and B)** and n=3 independent experiments for **(C and D)**.

**Abbreviations:** HF, human fibroblast; *H. odorata*, *Hopea odorata*; PE, plant extract; SEM, standard error of the mean.



**Figure 3** Fluorescence of HepG2 and HF cells after 48 hours of *H. odorata* treatment.

**Notes:** HepG2 (A, C, E, G) and HF (B, D, F, H) cells were treated with *H. odorata* PE at concentrations of 500 µg/mL (G and H), 250 µg/mL (E and F), and 125 µg/mL (C and D). After 48 hours of treatment, cells were stained with fluorescent dye Hoechst 33342 and PI at final concentrations of 1 µg/mL for each dye and then incubated further for 10 minutes at 37°C, 5% CO<sub>2</sub>. Cells were then observed under a fluorescent microscope at magnification 5×. Hoechst 33342 was detected by DAPI filter, and PI was detected by PI filter.

**Abbreviations:** DAPI, 4',6-diamidino-2-phenylindole; HF, human fibroblast; *H. odorata*, *Hoepa odorata*; PE, plant extract; PI, propidium iodide.

the percentage of cell death induced was approximately 20%, 30%, and 80%, respectively. However, no dead cells were observed for HF cells even when the concentration of *H. odorata* extract was raised up to 500 µg/mL. According to our observations, while the *H. odorata* extract at a high concentration (eg, 500 µg/mL) did not kill HF cells, it appeared to reduce the proliferative capacity of HF cells.

Using ImageJ, we calculated the ratio of red signal to blue signal to determine the ratio of dead cells to live cells (data not shown). Cell death of HepG2 increased in a dose-dependent manner after treatment with *H. odorata* extract. The percentage of dead HepG2 cells following treatment with *H. odorata* extract at 60 µg/mL, 250 µg/mL, and 500 µg/mL concentrations were approximately 20%, 25%, and 80%, respectively. Conversely, there were rarely any cell deaths of HF cells after treatment with *H. odorata* extract; even the dose of *H. odorata* extract was increased.

### *H. odorata* extract-induced apoptosis of HepG2 cells

Following treatment of HepG2 cells with *H. odorata* at concentrations of 30 µg/mL, 60 µg/mL, 125 µg/mL, and 250 µg/mL, the percentage of apoptotic cells increased dose dependently (Figure 4). For instance, at 250 µg/mL concentration of extract, the percentage of dead cells significantly increased to 35.3%. Approximately 56% of cells remained alive, 13.6% of cells went on to early apoptosis, 21.8% of cells went into late apoptosis, and 7.9% of cells underwent necrosis.

With caspase-3/7 green reagent staining, cells that underwent apoptosis appeared as green fluorescent signals under a fluorescent microscope. The results showed that HepG2 cells treated with the *H. odorata* extract at 250 µg/mL concentration induced apoptosis after 30 minutes of treatment (Figure 5A). The apoptotic signals increased as the time of incubation with *H. odorata* extract increased to 2 hours, 4 hours, and 6 hours (Figure 5D, F, G, and J). In the case of

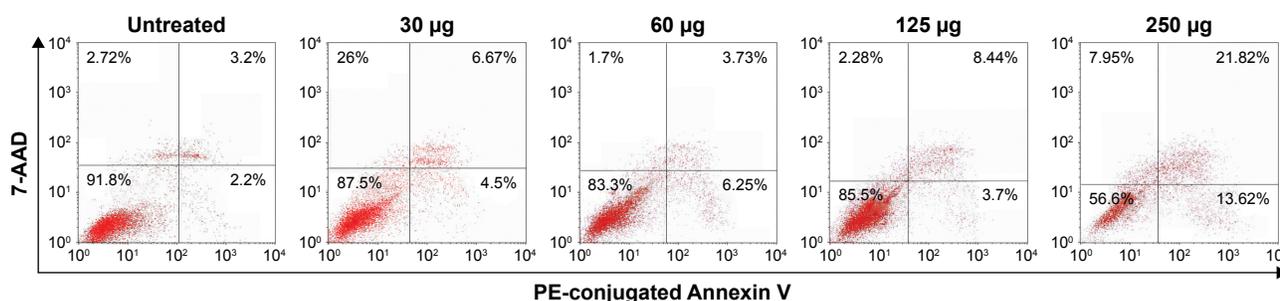
treatment with doxorubicin, there were no apoptotic signals after 30 minutes of treatment (Figure 5B). Apoptosis of doxorubicin-treated cells appeared after 4 hours and 6 hours of treatment (Figure 5E, H, and K). In those cells treated with 0.5% methanol, apoptosis was not observed at any of the time points (Figure 5C, F, I, and L). Apoptosis of HepG2 cells was discerned by shrinkage of cells into round shapes with unsmooth surfaces (Figure 5M–O). These observations indicate that *H. odorata* extract has the ability to induce apoptosis of HepG2 in a time-dependent manner through caspase-3/7 activation.

*H. odorata* extract (250 µg/mL) was used to treat HepG2 cells for 48 hours, and then, treated cells were stained with Hoechst 33342 to assess nuclei of all cells and with PI to assess nuclei of dead cells only. Control cells were treated with 0.5% methanol solvent, which is the same concentration of methanol solvent in *H. odorata* extract-treated samples. *H. odorata*-treated cells appear like dead cells with disintegration of nuclei (Figure 6A, C, and E). The cells also exhibited nuclei condensation and appeared to have brighter staining than the surrounding live cells. Conversely, control cells stained with Hoechst 33342 but not with PI; this means that there was no death cell in the control cells (HF) when compared to treated HepG2 cells (Figure 6B, D, and F).

### Discussion

Chemotherapy, radiation therapy, and surgery have been used for cancer treatment as a standard. However, these therapies have not been fully effective and the side effects are of concern. Most antitumor agents currently used in chemotherapy are toxic to normal cells and cause weakening of the immune system. Therefore, there is an increased demand to seek out new anticancer agents with lower side effects than those of current agents.

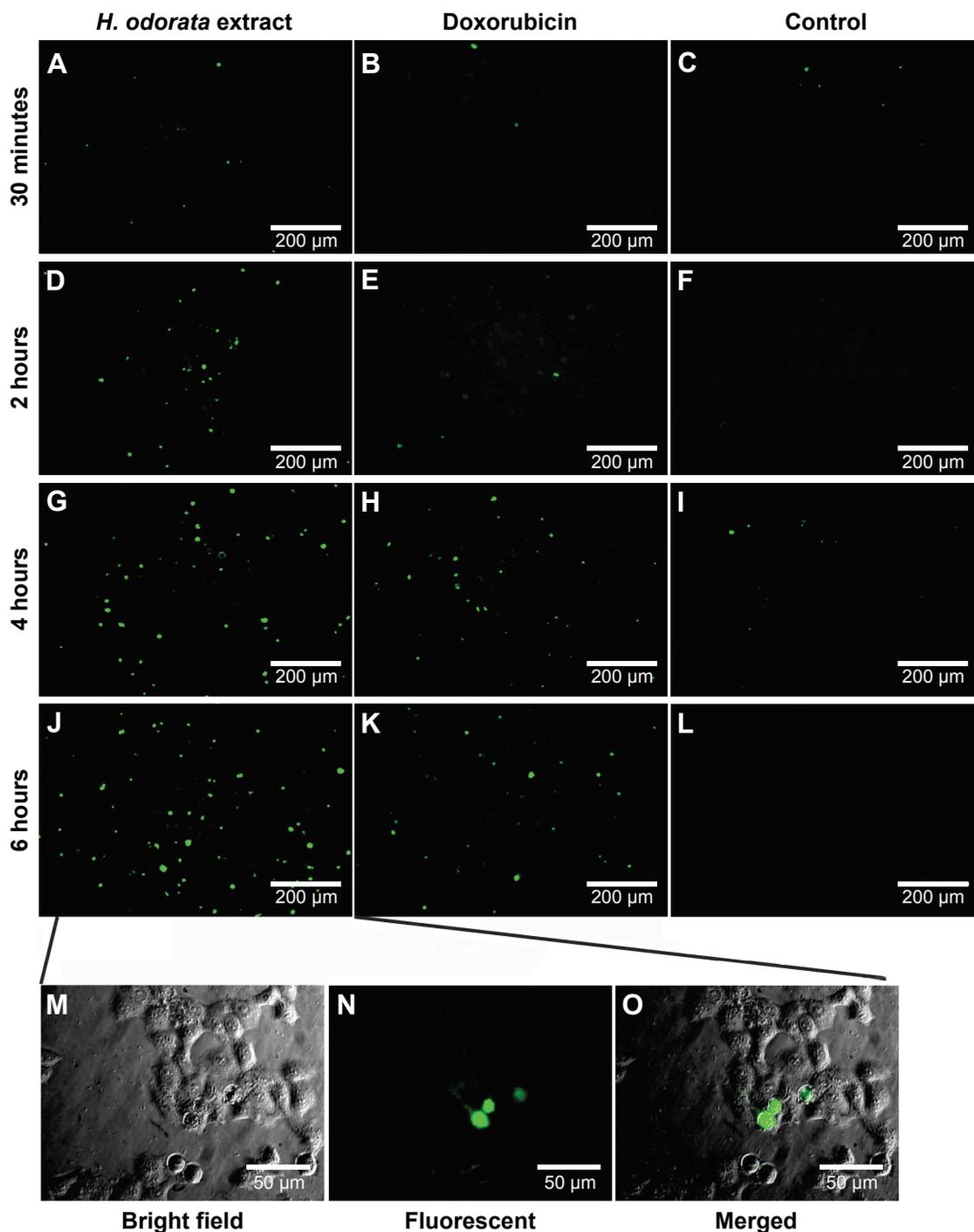
Herbal medicines have been used in many cultures for thousands of years. In fact, many plant-derived compounds



**Figure 4** Flow cytometry analysis of treated HepG2 cells.

**Notes:** HepG2 were treated with *H. odorata* extract at concentrations of 30 µg/mL, 60 µg/mL, 125 µg/mL, and 250 µg/mL. After 48 hours, cells were stained with Annexin V/PI and incubated for 15 minutes. Cells were then analyzed by CellQuest Pro using a FACSCalibur system.

**Abbreviations:** *H. odorata*, *Hopea odorata*; PE, plant extract; PI, propidium iodide.



**Figure 5** The activation of caspase-3/7 in HepG2.

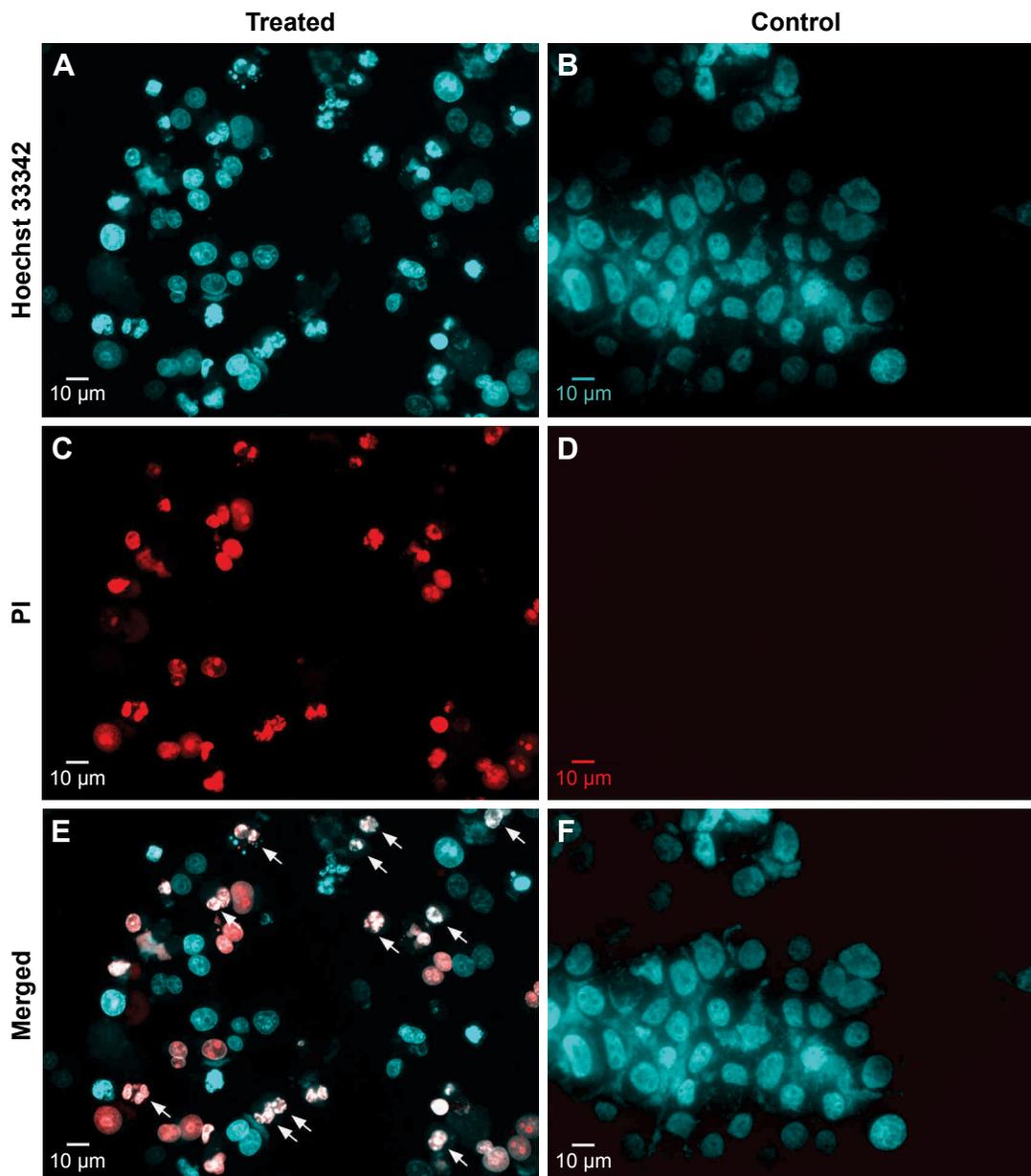
**Notes:** HepG2 cells were loaded with CellEvent™ caspase-3/7 green reagent at a concentration of 5  $\mu$ M and then treated with either 250  $\mu$ g/mL of *H. odorata* extract (A, D, G, and J), 100 mM of doxorubicin (B, E, H, and K), or 0.5% methanol solvent control (C, F, I, and L). HepG2 cells were treated for 30 minutes, 2 hours, 4 hours, or 6 hours. Cells were observed under fluorescent microscope for each time point. Untreated cells have minimal fluorescence, while treated cells show a significant increase in fluorescence (shown in green), indicating apoptosis (M, N, and O). *H. odorata*-treated cells were observed under a microscope after 6 hours of treatment at magnification 40 $\times$ . Apoptotic HepG2 cells after treatment with *H. odorata* extract shrank into a round shape. Unlike neighboring normal cells with smooth shape, the surface of apoptotic HepG2 cells was rough and shrunken.

**Abbreviation:** *H. odorata*, *Hozea odorata*.

can cure health problems, such as inflammation, virus-related disease, or even cancer. Natural resources have become increasingly popular to study. Many current studies seek the identification of new anticancer drugs from natural plants. In this study,

we aimed to investigate the in vitro anticancer activity of methanolic extract of *H. odorata* against HepG2 cancer cell line.

The cytotoxicity effect of anticancer agents was measured from the activity of the reducing environment within the



**Figure 6** Disintegrated nuclei of *H. odorata* extract-treated cells. **Notes:** After treatment with *H. odorata* extract (250 µg/mL) for 48 hours, HepG2 cells were harvested and stained with Hoechst 33342 (A and B) and PI (C and D) at a final concentration of 1 µg/mL for each dye. Nuclei of treated cells and control cells were then observed under a fluorescent microscope. Disintegrated nuclei are indicated with white arrows (E and F). **Abbreviations:** *H. odorata*, *Hopea odorata*; PI, propidium iodide.

cytosol of the live cells. Resazurin, the active compound of Alamar Blue reagent, is blue in color and virtually non-fluorescent. Upon entering cells, resazurin is reduced to resofurin, which has a red color and is highly fluorescent. Inhibition effects can be assessed by change in the fluorescent intensity. The results showed that the methanolic extract of *H. odorata* showed selective inhibition toward HepG2 but not to HF as compared to doxorubicin. The selective index (SI) of *H. odorata* extract ( $SI_{H.Odorata}$ ) was 87-fold that of doxorubicin ( $SI_{Doxorubicin}$ ). This means that *H. odorata* inhibited

HepG2 but not HF. Moreover, doxorubicin inhibited HF but not HepG2.

It is known that chemotherapy for cancer treatment causes side effects, such as hair loss, vomit, and nausea.<sup>22,23</sup> The high and selective cytotoxicity of *H. odorata* extract against HepG2 but not HF makes the extract a potential agent for cancer therapy. Previous studies have demonstrated the selectivity effect of butanolic extract of heartwood of *H. odorata* against HepG2 cells but not against normal liver cells.<sup>24</sup> The  $IC_{50}$  of *H. odorata* extract on HepG2 was  $12.67 \pm 5$  µg/mL,

which is nearly similar to the  $IC_{50}$  of 20.14  $\mu\text{g}/\text{mL}$  on HepG2 found in a previous report.<sup>24</sup> Interestingly, our study evaluates methanolic extract (not butanolic extract) from stem bark (not heartwood) of *H. odorata*. This demonstrates that different parts of *H. odorata* plant possess potential anticancer activity.

We found that HepG2 cell death induced by *H. odorata* was due to the activation of apoptosis. One of the hallmarks of apoptosis is the exposure of phosphatidylserine (PS). Since PS has an affinity for Annexin V,<sup>25</sup> we detected the apoptotic cells in our study by Annexin-positive labeling. By this principle, we found that HepG2 cells underwent apoptosis in a dose-dependent manner with increasing concentrations of *H. odorata* extract. At the 250  $\mu\text{g}/\text{mL}$  concentration, apoptosis of cells (including early and late stages) could be observed in ~35% of cells. The ability of *H. odorata* extract to induce apoptosis was strongly established in this study. However, we did not distinguish which pathway of apoptosis was activated. Methanolic extracts of *H. odorata* bark have been shown to suppress the kinase activities of enzymes Src and Syk, resulting in reduced signaling of NF- $\kappa\text{B}$  activation in Raw264.7 cells.<sup>18</sup> Other studies showed that the inhibition of NF- $\kappa\text{B}$  in HepG2 led to enhancement of caspase-3-dependent apoptosis.<sup>26,27</sup>

There are two known pathways of apoptosis. In the extrinsic apoptosis pathway, the binding of a ligand to a dead receptor initiates the activation of caspase-8. Active caspase-8 then activates executioner caspase-3/7. In the intrinsic apoptosis pathway, cellular stresses lead to cytochrome c release from mitochondria to form the apoptosome, resulting in the activation of caspase-9. Active caspase-9 then activates caspase-3/7. Therefore, caspase-3/7 is considered to be executioner caspase of both extrinsic and intrinsic apoptosis pathways. In this study, the activation of caspase-3/7 in HepG2 cells appeared in a time-dependent manner in the presence of *H. odorata* extract at 250  $\mu\text{g}/\text{mL}$  concentration. The activation of executioner caspase-3/7 triggers a cascade of proteolytic events leading to apoptotic destruction of the cell, including nuclear degradation, which is regarded as one of the hallmarks of apoptosis.<sup>28</sup>

In the study herein, HepG2 cells showed disintegrated nuclei due to the presence of *H. odorata* extract for 48 hours. Apoptotic HepG2 cells exhibited a different cell shape (and mostly shrunken) and were seen floating/detached. Morphological changes indicate the cleavage of cytoskeleton downstream of caspase activation.<sup>29</sup> Caspase-dependent cleavage of the components of the focal adhesion complex leads to detachment of apoptotic cells.<sup>30–32</sup> Taken together, the study

results demonstrate that the methanol extract of *H. odorata* possesses the ability to induce apoptosis of HepG2 cells. In this study, *H. odorata* methanol extract showed high cytotoxicity against HepG2 but not against control HFs, either alone or in comparison to doxorubicin. Therefore, compounds from *H. odorata* extracts may be useful as anticancer agents.

## Conclusion

Traditional medicine has now become more important in the modern world. We found that methanolic bark extract of *H. odorata*, a plant commonly used for anti-inflammation purposes in Southeast Asia, has selective in vitro cytotoxicity against HCC (HepG2 cells) but not against HF cells. *H. odorata* extract has the ability to induce HepG2 cells into caspase-dependent apoptosis. Thus, *H. odorata* bark extract may have great potential as a therapeutic agent for HCC. Further studies are warranted to evaluate *H. odorata* bark-derived compounds as anticancer agents.

## Acknowledgment

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## Disclosure

The authors report no conflicts of interest in this work.

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