

Inhibition of cell proliferation by *Houttuynia cordata* extract on gastric cancer cells via induction of apoptosis

Abstract

Houttuynia cordata Thunb. is an edible and medicinal plant that belongs to the family of Saururaceae and widely distributes in Eastern Asia countries. *H. cordata* have been reported as a promising anticancer agent against various cancer cells. This study was to determine anti-proliferative effect of *H. cordata* extract on gastric cancer cells using an in vitro experimental model. The cytotoxicity was examined via the 2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, while cell migration was conducted by wound healing assay. The gene expression level was measured by real time PCR. The result showed that butanol extract exhibited highest anti-proliferative activity against BGC-823 cells with IC₅₀ value of 193 µg/ml. Moreover, the wound healing assay determined the suppressive capacity of butanol extract on cell migration at concentration of 50 µg/ml. Notably, butanol extract treatment (50 µg/ml) was able to up-regulate the mRNA expression of apoptosis-mediated signaling molecules, including caspase-8, Bax, caspase-9, and caspase-3 in gastric cancer cells. These results indicate that butanol extract of *H. cordata* possesses inhibitory activity on tumor development and metastasis via induction of the process of programmed cell death.

Keywords: *Houttuynia cordata*, gastric cancer cells, BGC-823 cells, apoptosis, caspase-9

1. INTRODUCTION

Gastric cancer is among the most common cancer with high mortality worldwide. A high incidence of gastric cancer has been detected in East Asian countries such as Korea, Japan, China [1]. The risk factors affecting the development of gastric cancer were suggested due to diet, stomach diseases, and age. Gastric cancer is usually diagnosed at the late stage of metastasis due to the delay of symptoms such as indigestion and pain [2]. Therefore, the treatment of gastric cancer is mainly involved in surgical excision, followed by radiotherapy and chemotherapy [3]. However, these therapies still remain to be unsatisfactory and exhibit several adverse effects, causing various health problems for patients. As the result, novel approaches for improvement of efficacy and reduction of side effects are necessary for treatment of gastric cancer patients.

Dietary products have been considered as alternative therapeutics for prevention and management of gastric cancer [4]. *Houttuynia cordata* Thunb. is an edible and medicinal plant that belongs to the family of Saururaceae and widely distributes in Eastern Asia countries such as China, Japan, India, Thailand, Taiwan, and Vietnam [5]. *H. cordata* has been applied as a traditional medicine for treatment of skin diseases, cholera, hemorrhoids, blood deficiency, cancer, coughs, urination promotion, dysentery, toxin release, enteritis, and fever [6]. So far, *H. cordata* was found to contain various medicinal ingredients such as volatile oils, aristolactams, 5,4-dioxoaporphines, oxoaporphines, flavonoids, benzenoids, and steroids. Hence, numerous biological activities of *H. cordata* was evidenced in different experimental models [6]. Notably, *H. cordata* was also risen as a promising anticancer agent against the growth of various cancer cells such as colorectal cancer cells [7, 8], hepatic cancer cells [9], leukemia cells [10], lung cancer cells [11], breast cancer cells [12], and cervical cancer cells [13]. In the same trend, the

inhibitory activity of *H. cordata* on gastric cancer cells was further investigated in the present study on BCG-823 cells-mediated model.

2. MATERIALS AND METHODS

2.1 Materials

H. cordata was purchased from local organic greengrocer in Ho Chi Minh city, Vietnam. BGC-823 cells are human gastric cancer cells donated by Chinese Academy of Sciences (Shanghai, China). Reagents for real time PCR were purchased from Qiagen (Hilden, Germany). Primers for qPCR were purchased from Intergrated DNA Technologies, Iowa, USA. The others were purchased from Sigma-Aldrich (MO, USA).

2.2 Extraction

Dried powder of *H. cordata* was extracted by different solvents, including hexane, CH₂Cl₂, EtOAc, ethanol, and butanol at a solid-to-liquid ratio of 1:8, (w/v) for 24h at room temperature. The process was repeated three times and the extracted solution was combined and subsequently evaporated to remove solvent under vacuum machine at 40 °C. The extracts of *H. cordata* were obtained with the humidity level of less than 12% and stored at 4°C for further investigation.

2.3 Cytotoxic assay

The cytotoxic effect of extracts on human gastric cancer cells was examined via MTT assay [14]. Briefly, BGC-823 cells were treated with 200 µg/ml of the extract for 24h. The MTT solution (0.5 mg/ml) was added and incubated for 4 h in CO₂ incubator. Finally, DMSO was added to solubilize formazan salt, and optical density was measured by a microplate reader (BioTek Instruments, USA) at 540 nm.

2.4 Inverted microscope assay

BGC-823 cells were treated with 200 µg/ml of extract for one day. Afterward, the treated cells were washed by PBS and the morphology was visualized under an inverted microscope with magnification of 10X (Oxion, Euromex, Netherlands).

2.5 Cell migration assay

The migration of the cells was examined according to Kwak and Ju [15]. Briefly, a monolayer of BGC-823 cells were scratched by a yellow tip. Subsequently, the cells were treated with extract at concentration of 50 µg/ml for 24 h. Gap area were visualized under an inverted microscope (Oxion, Euromex, Netherlands). Moreover, the relative gap area between 24h and 0 h in each group was measure by ImageJ and expressed as following formula:

$$\text{The relative gap area (\%)} = \frac{\text{Gap area of 24h}}{\text{Gap area of 0h}} * 100\%$$

2.6 Real time PCR

BGC-823 cells were treated with 50 µg/ml of extract for 24 h. Total RNA from the treated cells was isolated by a commercial kit (Qiagen, Hilden, Germany). Subsequently, cDNA synthesis was performed according protocol of NEB (MA, USA). Each cycle of qPCR was conducted by using a master mix containing SYBR® Green as a detector of DNA under the conditions of denaturation of 94 °C (30 s), annealing of 60 °C (30 s), and extension of 72 °C (30 s). The mRNA expression level of relative gene in the treated cells was respectively compared with that of the blank (without treatment). The sequences of the primers were designed as following Genecards and NCBI gene database. Primer for caspase-3 (4q35.1): F = TCGCTTTGTGCCATGCTGAA and R = ACTCAAATTCTGTTGCCACC; for caspase-8 (2q33.1): F = AATGGAACACACTTGGATGC and R = GCTCTACTGTGCAGTCATCG; for caspase-9 (1p36.21): F = TTGAGGACCTTCGACCAGCT and R =

CAACGTACCAGGAGCCACTC); for Bax (19q13.33): F = CTGACGGCAACTTCAACTGG and R = CCAATGTCCAGCCCATGATG); for PARP-1 (1q42.12): F = GTCTGATGATAGCAGCAAGG and R = ATACTTCCTGATGATCTCGG; for GAPDH (12p13.31): F = GGGCTCTCCAGAACATCATC and R = GGTCCACCACTGACACGTTG.

Experiments were performed in triplicate and repeated three times with similar results. The expression level of genes was calculated as following formula (1) and (2):

$$\Delta Cq = Cq (\text{Tar}) - Cq (\text{Ref}) \quad (1)$$

$$\Delta \Delta Cq = \Delta Cq (\text{Exp}) - \Delta Cq (\text{Con}) \quad (2)$$

Where, Cq = quantification cycle; Tar = Target gene; Ref = Reference gene (GAPDH); Exp = Experimental; Con = Control

Statistical analysis

The ANOVA test of SPSS was used for analysis of data. Tukey's multiple range test was further assessed to identify statistical differences among groups at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Cytotoxic effect of *H. cordata* extracts

In vitro assay has been developed as a useful, reliable and rapid method for identifying the cytotoxic activity of natural products toward different target cancer cells [16]. In this study, the cytotoxic effect of *H. cordata* extracts on human gastric cancer cells (BGC-823) was examined by MTT assay. The result of this assay showed that the rate of cell death was identified in a range of 28.2 – 55.1% as the cells treated with 200 µg/ml of hexane, CH₂Cl₂, EtOAc, ethanol, and butanol extracts (Fig. 1A). Among them, butanol extract exhibited the highest cytotoxic effect against BGC-823 cell proliferation. Accordingly, butanol extract with high anti-proliferation effect was preferred to be used for the further investigation. The inverted

microscopy assay showed that the morphological changes occurred in butanol-treated cells (Fig. 1B). The cells were observed to be round-shaped, reduced cell size, disrupted boundaries, and irregular surfaces as compared with the blank cells. Moreover, the inhibition of cell proliferation in dose-dependent manner was also investigated by butanol extract (Figure 1C). Whereby, IC_{50} value of butanol extract on cell growth inhibition was determined up to 193 $\mu\text{g/ml}$. Notably, the suppression of butanol extract of *H. cordata* on gastric cancer cell growth is higher than that of aqueous extracts of ziziphora, ginger, saffron, aloe vera [17], dandelion (*Taraxacum* spp.) [18], and *Curcuma manga* [19]. So far, *H. cordata* has been determined as a rich source phenolic compounds such as quercetin, rutin, hyperin, afzelin, isoquercitrin, apigenin, kaempferol, vanillic acid, and protocatechuic acid [20]. Numerous studies have reported that phenolic compounds possess the inhibitory activity on tumor generation, and thus have capacity for the prevention and treatment of cancer [21, 22]. As the result, the high inhibition of butanol extract of *H. cordata* on the proliferation of gastric cancer cells could be suggested due to its phenolic content.

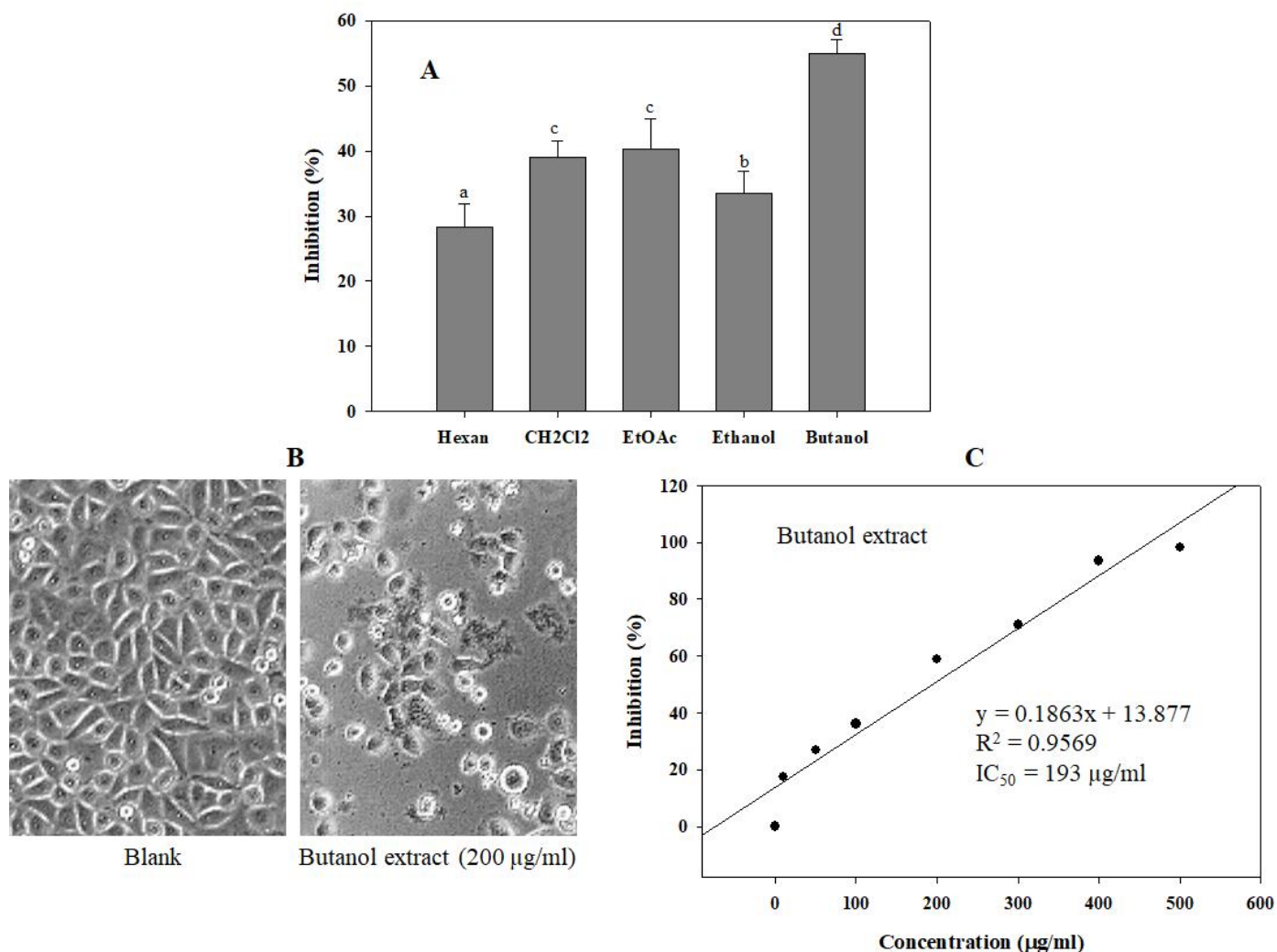


Fig. 1. The cytotoxic effect of *H. cordata* extracts on gastric cancer cells. Each determination was made in three independent experiments, and the data are shown as means \pm SD. Different letters (a–d) indicate significant difference among groups ($p < 0.05$) by Tukey's multiple range test.

3.2 The inhibition of butanol extract on cancer cell migration

A crucial capacity of cancer cells is known as metastasis that is due to the migration of cancer cells from the primary tumor to secondary sites, causing mortality for many patients [23]. Thus, the inhibition of cell migration is considered to be as important as suppression of cell

proliferation. Herein, a wound-healing assay was conducted to determine the inhibitory activity of butanol extract on gastric cancer cell migration [24]. It was shown that the treatment of *H. cordata* extract at concentration of 50 µg/ml significantly suppressed the spreading of gastric cancer cells to the gap area as compared with untreated group (Fig. 2). These results supported that butanol extract suppressed not only cell proliferation but also cell migration of gastric cancer cells, suggesting its role in prevention of cancer cell growth and metastasis. However, the effect of *H. cordata* extract on anti-proliferative activity-related intracellular signaling should be further investigated in gastric cancer cells.

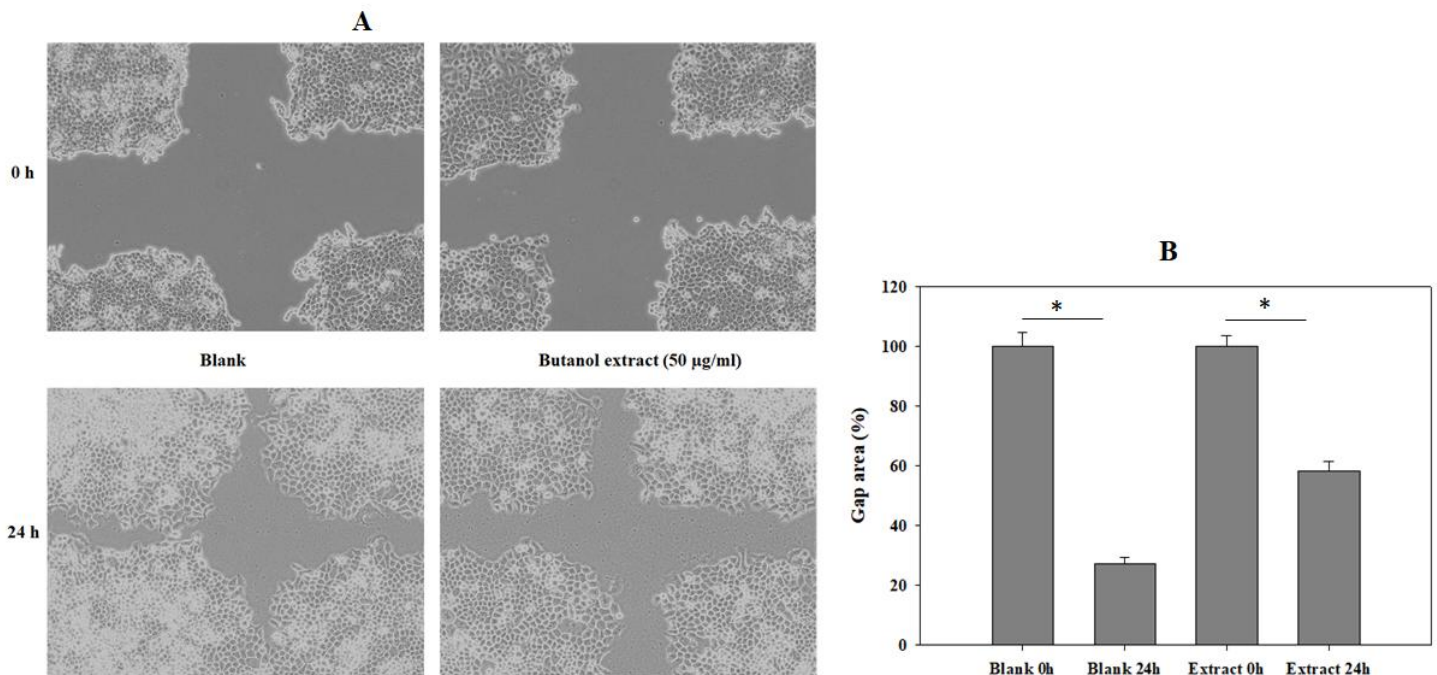


Fig. 2. The suppressive effect of butanol extract on cell migration. BGC-823 cells were seeded on 6-well plates before a gap line was made by a yellow pipette tip. The cells were then treated with butanol extract for 24 h. (A) The images were captured at 0 h and 24 h treatment by an inverted microscope (10x magnification). (B) Moreover, the relative gap area between 24h

and 0 h in each group was measure by ImageJ. Differences compared to the blank group were considered significant at * $p < 0.05$.

3.3 Effect of butanol extract on apoptotic signaling molecules

It is the fact that butanol extract of *H. cordata* caused cytotoxicity on gastric cancer cells, bring about suppression of cell growth and migration. It should be clear that whether its cytotoxic effect was due to necrosis or apoptosis on gastric cancer cells. Certainly, a natural product is considered as a potential anticancer agent as it has capacity of apoptotic induction in the cancer cells [25, 26]. Apoptosis is a caspase-mediated programmed cell death, leading to chromosome condensation, nuclear fragmentation, and membrane blebbing [27]. It has been evidenced that apoptotic induction triggers the up-regulation of caspase-8, Bax, caspase-9, and caspase-3 activation in cancer cells, leading to the process of cell death. In this study, the apoptotic effect of *H. cordata* butanol extract was investigated via measuring the expression levels of caspase-8, Bax, caspase-9, and caspase-3 in BGC-823 cells. Notably, butanol extract treatment significantly up-regulated the gene expression of apoptotic signaling molecules including caspase-8, Bax, caspase-9, and caspase-3 (Fig. 3). The gene expression levels of caspase-8, Bax, caspase-9 and caspase-3 in the treated cells were up to 2.8-fold, 1.8-fold, 2.3-fold, and 2.2-fold higher than that of the untreated cells, respectively. Likewise, several anticancer agents derived from plant extracts, such as *Brucea javanica*, *Camellia sinensis*, *Cinnamomum kanehirai* Hayata, *Corni Fructus*, *Cucurbita ficifolia*, *Cyperus rotundus* L. have been evidenced as apoptotic inducers due to up-regulating caspase-8, Bax, caspase-9, and caspase-3 in cancer cells [28]. This result indicated that *H. cordata* butanol extract possessed anticancer activity via induction of apoptosis, leading to the inhibition of proliferation and migration of gastric cancer cells.

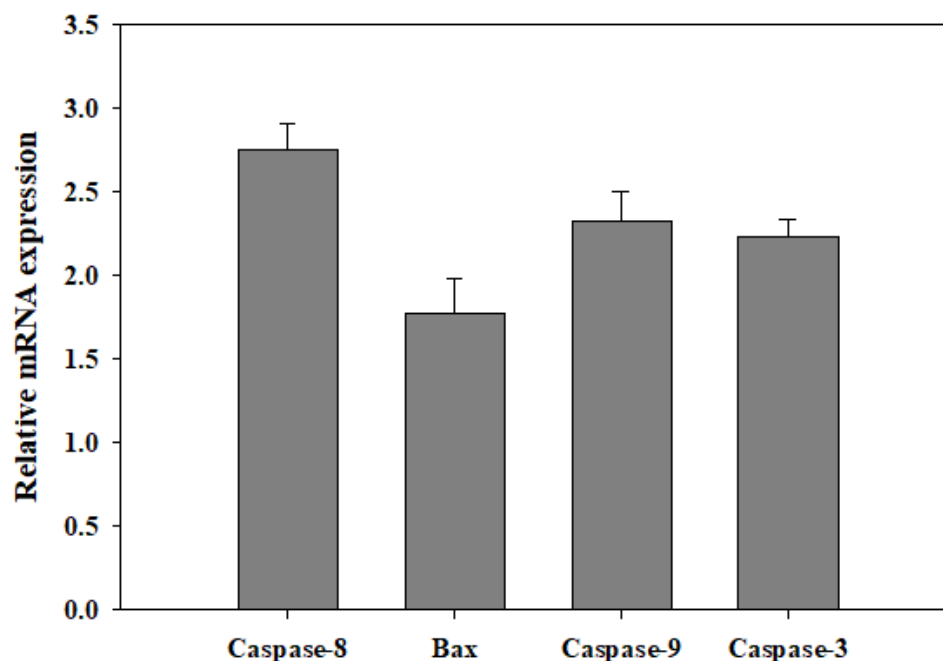


Fig. 3. Butanol extract of *H. cordata* induced apoptosis in BGC-823 cells. Each determination was made in three independent experiments, and the data are shown as means \pm SD.

4. CONCLUSION

H. cordata butanol extract can be concluded to possess a selective antitumor agent for gastric cancer due to inhibiting cell proliferation, inducing apoptosis and suppressing cell migration. These results suggested that crude extract of *H. cordata* can be developed as a novel component of potential products that is used for the treatment of gastric cancer. However, the mechanism of action of *H. cordata* extract on inhibition of cell migration and metastasis should be further investigated in the next studies.

Ethical Approval:

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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