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The Inhibitory Effect of Common Edible Mushrooms on Hepatic Cancer Cell Invasion

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ABSTRACT

Prevention of liver cancer cell metastasis significantly contributes to the lowering of mortality incidence. Moreover, a daily diet rich in natural ingredients has been reported to play important roles in prevention of metastatic development. This study, therefore, focused to screen and identify the potential edible mushrooms that possess the inhibitory effect on the invasion of hepatic cancer cells. The edible mushrooms were extracted by distilled water under temperature of 100°C for 3 h. The wound healing assay was used to investigate the cancer cell migration. The cytotoxicity of extracts (100-1000 µg/ml) on Hep-3B cells was examined by MTT assay. The expression levels of genes controlling migration process was assessed by real time PCR. Among different edible mushrooms, the extracts of *Lentinula edodes* (LE) and *Volvariella volvacea* (VV) have been found to be potential agents for inhibition of Hep-3B cell invasion. This inhibition was identified due to induction of apoptosis and regulation of genes involved in migration process. In particular, the treatment of *L. edodes* and *V. volvacea* extracts increased the expression of apoptosis-activating molecules, including caspase-3, -8, -9, and Bax. Moreover, these extracts caused suppression of enzyme matrix metalloproteinases, including MMP-2, MMP-9, and MMP-13, and up-regulation of tissue inhibitors of metalloproteinases, including TIMP-1 and TIMP-2. Notably, the activities of *L. edodes* and *V. volvacea* extracts were not due to cytotoxic effect. These results indicated that LE and VV extracts possess the promising anti-metastatic activity, supporting them as potential functional foods for prevention of metastasis of hepatic cancer cells.

***Keywords*:** Hep-3B, Anticancer, Metastasis, MMPs, TIMPs.

Introduction

Metastasis is defined as the invasion of cancer cells from the initial tumor site to nearby or distant site via bloodstream and lymph system in human body.[1, 2] Cancer cells can spread to any part of the body, especially bone, liver, and lung sites. The liver is the most common organ for the metastasis of cancer cells, accounting for high ratio as compared with others.[3] The fact that the metastatic cancer is usually detected at the late phage of the disease due to minimal or no symptoms. Thus, metastasis was estimated to cause high incidence of cancer related deaths.[2] Numerous studies have been conducted to identify and understand the mechanism under tumor cell invasion, showing various potential therapeutic targets for limiting tumor progression, thus reducing mortality of cancer patents.[4] Up to now, cancer treatment faces numerous problems due to requiring several types of drugs, therapies, and surgeries and lasting for long-term medication.[5] However, these therapies can also cause the damage for healthy tissues, leading to decline the overall health status. Therefore, alternative therapeutics with higher safety and efficiency and lower cost for long-term use are always necessary for study and development.
Dietary habit with high natural product components has been considered to be effective in prevention and/or treatment of various diseases.[6] Among them, edible mushrooms have been widely used in cuisine across the world as delicious and nutritious foods. These mushrooms have been found to contain numerous bioactive agent such as polysaccharides, phenolics, carotenoids, triterpenes, alkaloids, carbohydrate-binding proteins, vitamin, and organic acids.[7] Therefore, mushrooms possess various medicinal properties such as anticancer, antioxidant, anti-inflammation, anti-diabetes, immunomodulation, hepatoprotection, and anti-obesity.[8] As the result, the consumers of mushrooms can provide beneficial effects due to promotion of health and prevention of disease-related risks.[9] Especially, the anticancer activity of edible mushrooms has been emphasized in various *in vitro* studies due to their inhibition on proliferation and induction of apoptosis in various cancer cell lines.[10] However, the inhibitory activity of them on suppression of cancer cell invasion is not reported. Therefore, the present study is mainly focused to screen and evaluate the anti-invasion activity of common edible mushrooms and emphasized their potential role in prevention of cancer metastasis.

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Materials and Methods

Materials

The common edible mushrooms, including *Lentinula edodes* (LE), *Volvariella volvacea* (VV), *Agaricus bisporus* (AB), *Pleurotus eryngii* (PE), *Pleurotus ostreatus* (PO), *Flammulina velutipes* (FV), and *Auricularia auricula-judae* (AA) were purchased from Nam Thao Nguyen Xanh Company, Thu Duc district, Ho Chi Minh City in Feb 2022, Vietnam. Hep-3B cells were donated by NTT Hi-Tech Institute, Nguyen Tat Thanh University, Vietnam. Reagents for real time PCR were purchased from Qiagen (Hilden, Germany). Primers for qPCR were purchased from Intergrated DNA Technologies, Iowa, USA. The other one was purchased from Sigma-Aldrich (MO, USA).

Extraction
The fresh mushrooms were dried under the temperature of 65^{o}C for five days. The dried mushrooms were grinded and extracted with hot water at a ratio of 1/6 (m/w) for 3 hours. The water extract was then lyophilized to achieve a power form with the humidity of less than 13%. The samples were stored at 4^{o}C until use and dissolved in distilled water for the evaluation.

Wound healing assay
The inhibitory effect of the extract on cell invasion was conducted as previous described by Kwak and Ju.[11] Briefly, Hep-3B cells were cultured on 6-well plates until the cell density up to 90% confluence. The monolayer of cells was then scratched to form a straight gap using a while pipette tip. The culture medium was changed to remove the floating cells completely before treatment with 200 µg/ml of extract for 24h. Gap area was visualized under an inverted microscope (Oxion, Euromex, Netherlands). The untreated group was considered as blank. Cell-free areas was captured by an inverted microscope (Oxion, Euromex, Netherlands) and measured by ImageJ (NIH, Bethesda, MD, USA). The gap closure rate was expressed as follows:

The gap closure rate (%) = [1 – (G1/G0)]*100

Where, G1: Gap area at 24 h treatment; G0: Gap area at 0 h treatment

The cytotoxic assay
The cytotoxic effect of extract on Hep-3B cells was examined by MTT method.[12] Briefly, Hep-3B cells were cultured into 96-well plates at a density of 2 × 10^{5} cells/ml before treatment with the extract (100-1000 µg/ml) for 24 h. Afterward, the medium was completely removed, and MTT solution (100 µl, 0.5 mg/ml) was immediately added. The supernatant was carefully removed after 4 h incubation, and the formed formazan salt was solubilized by 100 µl of DMSO. The optical density was measured by a microplate reader (BioTek Instruments, USA) at 570 nm. The untreated group was considered as blank. The cytotoxicity of the extract was identified as compared with the blank group.

Real Time PCR
Hep-3B cells were treated with 100 or 200 µg/ml of the extract for 24 h. Total RNA was isolated by a commercial kit (Qiagen, Hilden, Germany) and cDNA synthesis was performed according to protocol of NEB (MA, USA). Each cycle of qPCR was conducted under the conditions of denaturation of 94°C (30 s), annealing of 60°C (30 s), and extension of 72°C (30 s). The sequences of the primers were designed as showed in Table 1. The expression level of genes was calculated according to the method of Livak and Schmittgen.[13]

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 $p < 0.05$.

Results and Discussions

The suppressive effect of mushroom extracts on cell invasion
The metastatic process is initiated by invasion activity of cancer cells that is required to pass through the basement membrane and extracellular matrix.[14] Therefore, the suppression of cancer cell invasion contributes to the obstacle of metastatic progression that may reduce cancer mortality. In order to investigate the invasion of cancer cells, wound healing assay can be used to probe the migration of cancer cells. These assays can show the information due to the gap closure rate, thence indicating the speed of the cell invasion.[11] As the result, the suppressive effect of edible mushroom extracts on Hep-3B cell invasion has been identified as shown in Figure 1. It was observed that the cell-free area was significant reduced in the untreated group after 24 h culture. Moreover, the treatment of mushroom extracts caused different inhibition on Hep-3B cell invasion. The lowest gap closure rate was observed by *V. volvacea* (18.7%) and *L. edodes* (23.9%), followed by *P. ostreatus*, *P. eryngii*, *F. velutipes*, *A. auricula-juda*e, and *A. bisporus* (71-83%).

Table 1: The sequences of primers

No.	Name	Sequence
1	MMP-2	F: TGGCACCCATTTACACCTAC and R: GATCTCAGGAGTGACAGGG
2	MMP-9	F: GCAACGTGAACATCTTCGAC and R: TCCTCAAAGACCGAGTCCAG
3	MMP-13	F: AACTTGTTTCTTGTTGCTGC and R: GCCGGTGTAGGTGTAGATAG
4	TIMP-1	F: CAGCGAGGAGTTTCTCATTG and R: AGTGTAGGTCTTGGTGAAGC
5	TIMP-2	F: CACAGAGAAGAACATCAACGG and R: CGATGTCGAGAAACTCCTGC
6	TIMP-4	F: CATCACTACCATCTGAACTGTG and R: TTTCGTTCCAACAGCCAGTC
7	Caspase-3	F: TCGCTTTGTGCCATGCTGAA and R: ACTCAAATTCTGTTGCCACC
8	Caspase-8	F: AATGGAACACACTTGGATGC and R: GCTCTACTGTGCAGTCATCG
9	Caspase-9	F: TTGAGGACCTTCGACCAGCT and R: CAACGTACCAGGAGCCACTC
10	Bax	F: CTGACGGCAACTTCAACTGG and R: CCAATGTCCAGCCCATGATG
11	GAPDH	F: ATCATCAGCAATGCCTCCTG and R: TGAGTCCTTCCACGATACCA

The rate of gap closure in the cells treated with *L. edodes* or *V. volvacea* extracts was significantly lower as compared with others. Meanwhile, the free-cell area in the cells treated with other mushroom extracts was markedly reduced. These results indicated that *L. edodes* or *V. volvacea* possess potential inhbition on the invasion of hepatic cancer cells. Numerous studies have reported that the cytotoxic effect and apoptosis induction can suppress the invasion of cancer cells.[15-18] Moreover, the down-regulation of matrix metalloproteinase expression was determined to inhibit cancer cell invasion.[19-21] Therefore, the roles of *L. edodes* or *V. volvacea* extracts on cytotoxic effect, apoptosis progression as well as matrix metalloproteinase (MMP) expression were further evaluated in Hep-3B cells. The images of cell invasion were captured by a microscope (10x magnification). (B) The gap closure rate after 24 h in each group was measure by ImageJ. (C) Different letters indicate significant differences among groups at $p<0.05$. *Lentinula edodes* (LE), *Volvariella volvacea* (VV), *Agaricus bisporus* (AB), *Pleurotus eryngii* (PE), *Pleurotus ostreatus* (PO), *Flammulina velutipes* (FV), and *Auricularia auricula-juda*e (AA).

The cytotoxic effect of L. edodes and V. volvacea extracts on Hep-3B cells
MTT assay has been used as an *in vitro* standard method for evaluation of cytotoxic effect of natural products.[22] In this study, Hep-3B cells was treated with various concentrations of *L. edodes* or *V. volvacea* extracts for 24 h, and their fifty percent inhibitory concentration (IC_{50}) values was determined. Herein, the cytotoxic effect of *L. edodes* and *V. volvacea* extracts on Hep-3B cells are shown in Table 2.
A dose-dependent manner due to the cytotoxic effect of *L. edodes* and *V. volvacea* extracts on Hep-3B cells was observed. Moreover, the IC_{50} values of *L. edodes* and *V. volvacea* extracts on Hep-3B cells was determined at 647.1 and 810.3 µg/ml, respectively. This result indicated that *L. edodes* and *V. volvacea* extracts possess low cytotoxic effect on Hep-3B cells. Therefore, the inhibition of *L. edodes* and *V. volvacea* extracts on Hep-3B cell invasion was not due to cytotoxic effect.

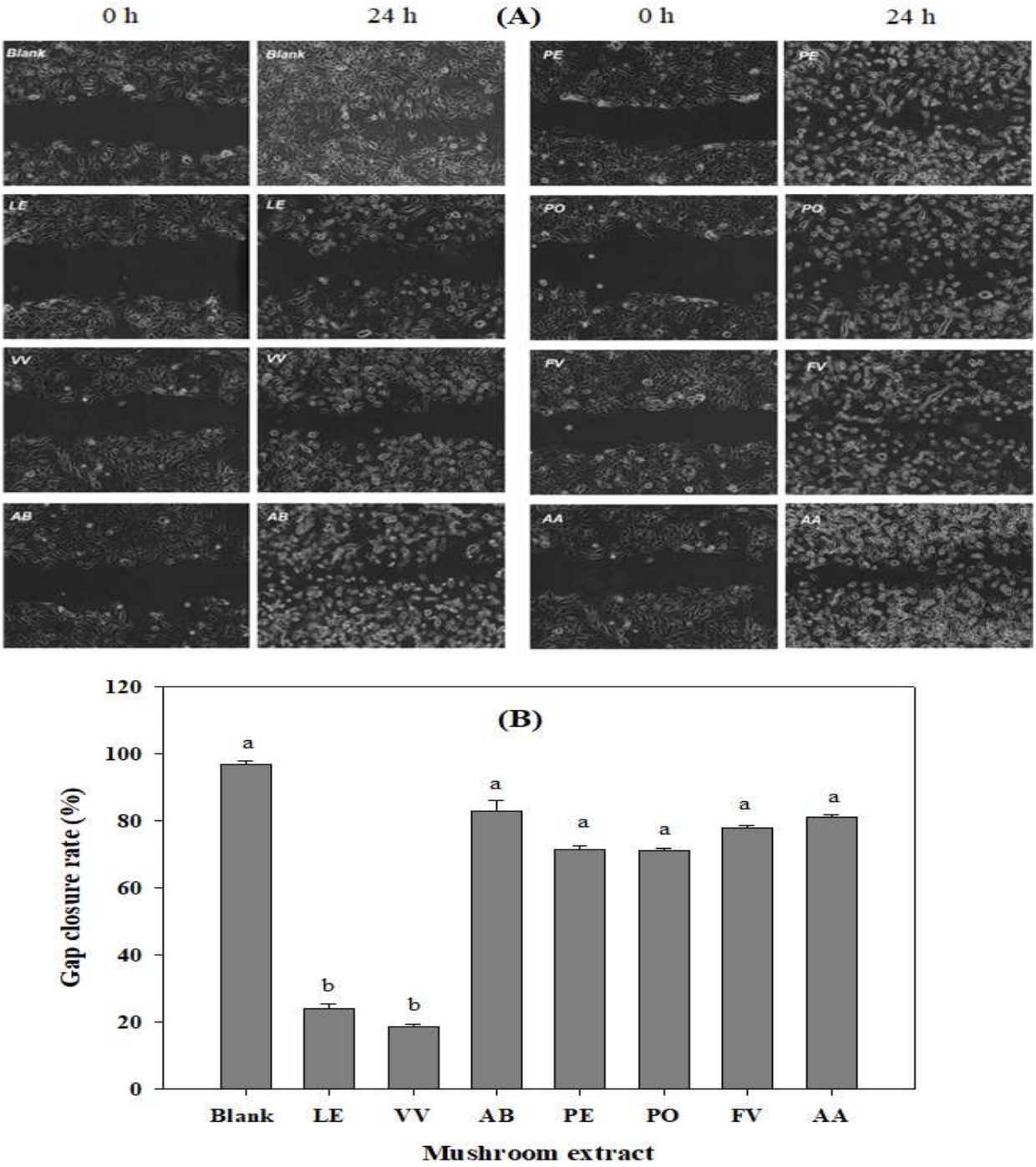

Figure 1: The inhibitory activity of edible mushroom extract on Hep-3B cell invasion.
The images of cell invasion were captured by a microscope (10x magnification). (B) The gap closure rate after 24 h in each group was measure by ImageJ. (C) Different letters indicate significant differences among groups at $p<0.05$. *Lentinula edodes* (LE), *Volvariella volvacea* (VV), *Agaricus bisporus* (AB), *Pleurotus eryngii* (PE), *Pleurotus ostreatus* (PO), *Flammulina velutipes* (FV), and *Auricularia auricula-judae* (AA).

Table 2: Cytotoxic effect of *L. edodes* and *V. volvacea* extracts on Hep-3B cells

	***L. edodes* extract**				***V. volvacea* extract**			
Concentration (µg/ml)	100	200	500	1000	100	200	500	1000
Cell death rate (%)	7.9	24.1	50.3	67.7	2.3	11.3	38.9	58.2
Regression	y = 0.0632x + 9.1058				y = 0.0619x – 0.1569			
IC_{50}	647.1 µg/ml				810.3 µg/ml			

In order to clear the inhibition of these extracts on cell invasion, the induction of apoptosis and inhibition of MMPs expression were suggested for further evaluation.

The apoptosis induction of L. edodes and V. volvacea extracts

Apoptosis is the programed cell death that naturally occurs in the cells and plays an important role in development and homeostasis. It is started by the damage and shrinkage of cell membrane, subsequently induces the fragmentation of nucleus and condensation of chromatin, and finally causes the fragmentation of DNA, leading to cell death.[23] However, this program can be blocked in the certain mutated cells, allowing those cells to survive longer and increase invasion during tumor progression.[24] Therefore, numerous anticancer agents have been developed for targeting apoptosis and inducing apoptosis in cancer cells, contributing to the suppression of cancer cell invasion. In this study, *L. edodes* and *V. volvacea* extracts have been found to suppress the invasion of Hep-3B cancer cells. Whether this suppressive effect was due to the induction of apoptosis, Hep-3B cancer cells were treated with *L. edodes* and *V. volvacea* extracts for 24 h and the expression level of molecules under apoptotic process were investigated by qPCR assay. It was found that the extract treatment remarkably activated caspase pathway via increasing the expression level of caspase-8, caspase-9, caspase-3 and Bax in Hep-3B cells (Figure 2). *L. edodes* treatment caused the highest expression level of caspase-3, followed by a similar expression level of caspase-8, -9, and Bax. Likewise, the highest expression level of caspase-8 was observed in the present of *V. volvacea* extract, followed by a similar expression level of caspase-3, -9, and Bax. The fact that the overexpression of caspases can be considered as an important signal of the initiation of apoptotic process in the cells.[25,26] As the result, the increase in the expression level of caspase-8, -9, -3 and Bax in the cells exposed to *L. edodes* and *V. volvacea* extracts indicate the role of these extract on apoptotic induction. In the similar trend, numerous studies also reported the role of various plant extracts in apoptotic induction via upregulation of caspase-3, -8, -9, and Bax in cancer cells.[27] Accordingly, the induction effect of *L. edodes* and *V. volvacea* extracts on apoptosis may partly contribute to the suppression of the invasion of Hep-3B cells. However, the effect of *L. edodes* and *V. volvacea* extracts on MMPs expression is also requred for further evaluation of their anti-invasion activity.

Effect of L. edodes and V. volvacea extracts on MMPs and TIMPs expression

Matrix metalloproteinases (MMPs) are a group of endopeptidases that can catalyze for the disruption of basement membrane and extracellular matrix molecules. Moreover, MMPs also have the role in wound healing and angiogenesis via activation and release of different growth factors and adhesion molecules.[28] Hence, MMPs play a critical function in initial phase of metastasis of cancer cells, making them as a desirable target in anticancer therapeutics.[29] For further understanding the anti-invasion activity of *L. edodes* and *V. volvacea* extracts, Hep-3B cells were treated with 200 µg/ml of the extract, and the expression levels of MMP-2, MMP-9, and MMP-13 were examined by qPCR assay. The results showed that the treatment of the extract caused a significant inhibition of MMP-2, MMP-9, and MMP-13 expression (Figure 3A). *L. edodes* extract exhibited a similar inhibition on MMP-2, MMP-9, and MMP-13, while *V. volvacea* extract possesed the strongest inhibition on MMP-9, followed by MMP-2 and MMP-13. Likewise, Lv and colleagues have evidenced that nanoplatform, a inhibitor of MMPs (MMP-2, -9, -3, -7 and -10), efficiently inhibits tumor metastasis and angiogenesis.[30] Moreover, numerous MMP inhibitors have been discussed for anticancer therapeutics by Winer and colleagues.[31] So far, tissue inhibitors of metalloproteinase (TIMPs) have been evidenced as a naturally occurring inhibitor of MMPs and invasion.[32] Notably, the treatment of *L. edodes* and *V. volvacea* extracts can enhance the gene expression of TIMP-1 and TIMP-2 in Hep-3B cells (Figure 3B). It was observed that the expression level of TIMP-2 was higher than that of TIMP-1. Moreover, *V. volvacea* extract induced higher TIMPs expression level as compared with that of *L. edodes* extract. The upregulation of the extracts on TIMP-1 and TIMP-2 expression may partly contribute to the inhibition of MMPs expression.

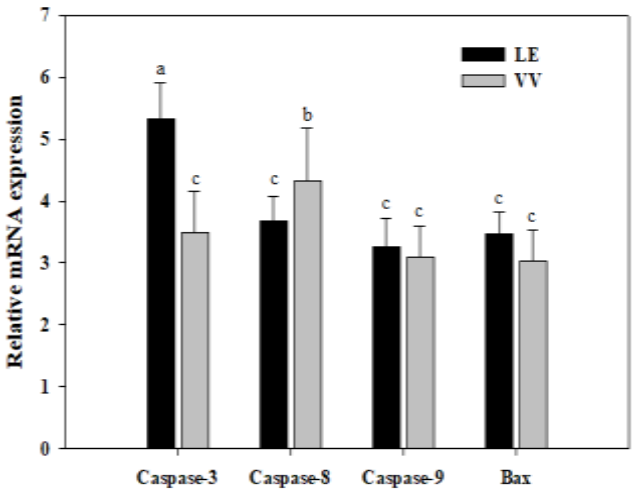

Figure 2: Apoptosis induction of *L. edodes* and *V. volvacea* extracts on Hep-3B cells.
Different letters indicate significant differences among groups at $p < 0.05$. LE: *L. edodes*; VV: *V. volvacea*.

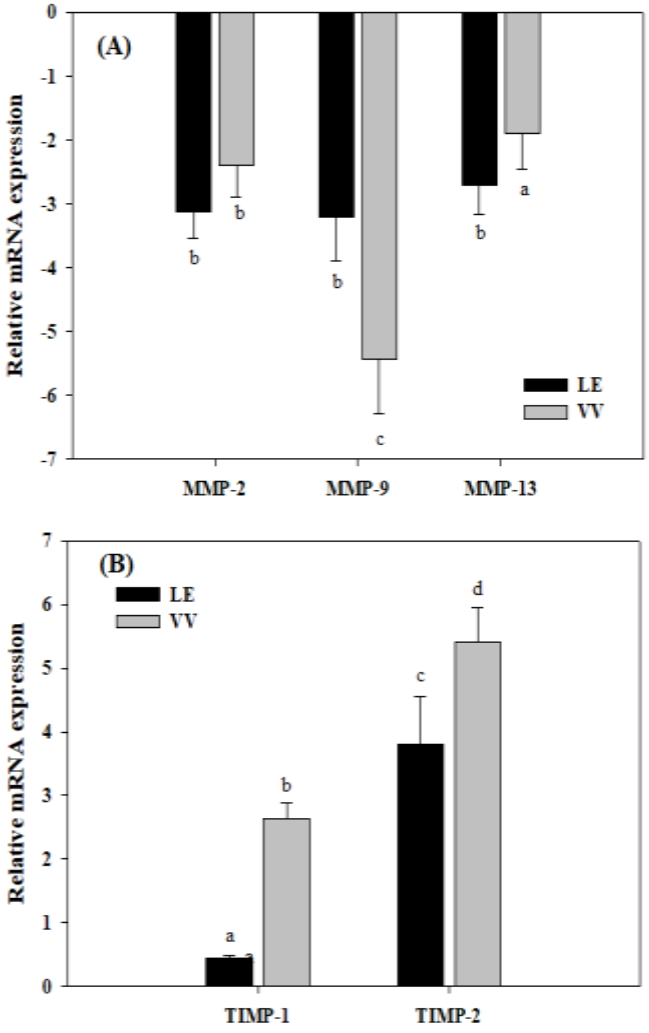

Figure 3: Effect of *L. edodes* and *V. volvacea* extracts on MMPs (A) and TIMPs (B) expression in Hep-3B cells.
Different letters indicate significant differences among groups at $p<0.05$. LE: *L. edodes*; VV: *V. volvacea*.

These results indicate that the inhibitory activity of *L. edodes* and *V. volvacea* extracts on MMPs expresion may cause the blockage of the invasion of Hep-3B cells. Several studies identified different polysaccharides from *L. edodes* and *V. Volvacea* via various extract methods.[33, 34] The anticancer activity of these polysaccharide were also reported.[35] As the result, polysaccharide components from *L. edodes* and *V. Volvacea* are suggested to be responsible for their anti-invasion activity in Hep-3B cells.

Conclusion

The present study has determined the inhibitory effect of *L. edodes* and *V. Volvacea* extracts on the invasion of Hep-3B cells. Their anti-invasion activity have been shown due to blocking cell migration, down-regulating MMP-2, -9, and -13 expression, and increasing TIMP-1 and TIMP-2 expression without cytotoxic effect. These results indicate the potential suppression of *L. edodes* and *V. Volvacea* on metastasis of tumor progression. However, the mechanism of action of *L. edodes* and *V. Volvacea* on intracellular signaling pathway regulating MMPs and TIMPs expression should be further evaluated.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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