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Apoptosis and Anti-proliferaction by *Saussurea lappa* and *Pharbitis nil* in AGS Human Gastric Cancer Cell Line

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Objectives : We performed this study to understand the molecular basis of the antitumor effect of *Saussurea lappa, Pharbitis nil, Plantago asiatica and Taraxacum mongolicum*, which have been used for cancer treatment in Korean traditional medicine.

Design: We analyzed, the effect of these medicinal herbs on proliferation and apoptosis of tumor cells and its association with gene expression. We performed semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of cell cycle- and apoptosis-related genes using a gastric cancer cell line AGS.

Results : Cell counting assay and [^{3}H]thymidine uptake analysis showed that *Saussurea lappa* and *Pharbitis nil* strongly inhibit cell proliferation of AGS in a dose-dependent manner. Interestingly, gene espression assay revealed that mRNA espression levels of c-Jun, c-Fos, c-Myc, and Cyclin D1 were markedly decreased by *Saussurea lappa* and *Pharbitis nil*. Furthermore, *Saussurea lappa* was identified to activate expression of the p53 tumor suppressor and its downstream effector $p21^{Waf1}$, which leads to G_1 cell cycle arrest and apoptosis.

These observations suggest that the anticancer effect of *Saussurea lappa* and *Pharbitis nil* might be associated with their regulatory capability of tumor-related gene expression.

Key Words: Saussurea lappa, Pharbitis nil, antitumor effect, protooncogenes, p53

I. INTRODUCTION

Saussurea lappa, Pharbitis nil, Plantago asiatica, and *Taraxacum mongolicum* are representative anticancer medicinal herbs that have been traditionally used for cancer treatment in Korea. Previous studies demonstrated that *Saussurea lappa* exhibits anti-inflammatory effect via down-regulation of tumor

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necrosis factor-α as well as anti-tumor and anti-ulcer activity (Jung JH et al., 1998; Cho JY et al., 2000, 1998; Yoshikawa M et al., 1998). *Plantago asiatica* and *Taraxacum mongolicum* have been widely used to treat for cancer and liver disease and were reported to carry immune suppressive effects (Kim SH, 1998; Yamada H et al., 1989).

It is well known that tumor development is accelerated by disruption of the balance between cell proliferation and cell death, which are maintained through various signal transduction pathways (Collins MKL et al., 1993; Williams GT et al., 1993). Active cell death, known as apoptosis or programmed cell death is caused by various physiologic and nonphysiological cell injuries including DNA damage. It has been demonstrated that various cell proliferation- and apoptosis-signal transduction pathways are built on complicated networks between oncogenes such as c-Myc and Bcl-2 and tumor suppressor genes such as p53 (Levine AJ, 1992; Lane DP, 1992; Miyashita T et al., 1994; Yonish-Rouach E et al., 1991; Wang E et al., 1994). For example, transcription of an anti-apoptosis gene Bcl-2 and a proapoptosis gene Bax is down- and up-regulated by p53, respectively (Miyashita T et al., 1994).

p53 controls various genetic expressions and plays an important role in cell proliferation and modulation of signal transduction pathway. Accumulation of p53 in cells after DNA damage leads to cell cycle arrest and apoptosis induction. In addition, p53 is involved in repair of damaged DNA and thus prevents accumulation of mutations and suppresses tumor development (Kastan MB et al., 1992; Ginsberg D et al., 1991).

It is known that the growth inhibitory function of p53 is a result of its ability to modulate transcription of several cell cycle- or apoptosis-related genes, including $p21^{Waf1}$, MDM2, and c-Myc. (Zambetti G et al., 1992; Weintraub H et al., 1991; Barak Y et al., 1993; Kern S et al., 1992; Kastan MB et al., 1992; Ginsberg D et al., 1991). p53' s DNA binding property and its ability of controlling gene transcription are usually lost by mutation in human cancers. p53 is also observed to suppress a variety of promoters including Bcl-2, interleukin-6 and MPR2 through its interaction with several transcription factors such as TATA-binding protein and SP1 or through its direct binding to a p53-dependent negative response element (Miyashita T et al., 1994; Yonish-Rouach E et al., 1991; Mack DH et al., 1993; Seto E et al., 1992; Borellini F et al., 1993; Chin KV et al., 1992). In addition, the repression of MDR1 by p53 suggests a link between p53 and chemotherapy resistance. The p53-mediated inhibition of cell cycle progression has a link with histone H3, a marker gene of cell cycle progression or with the reduction of PCNA (Mercer WE et al., 1990; Flores-Rozas H et al., 1994; Mercer WE et al., 1991). Recent studies also showed that hypoxia-induced growth arrest and apoptosis is mediated through p53 activation (El-Deiry WS et al., 1994; Harper JW et al., 1993).Transcription of the $p21^{Waf1}$ gene is highly activated by p53, and p53-induced $p21^{Waf1}$ leads to arrest of the cell cycle and/or apoptosis. Consistent with this, it was observed that cell cycle progression is re-activated when $p21^{Waf1}$ expression is suppressed by antisense $p21^{Waf1}$.

In our study, we analyzed effects of *Saussurea lappa*, *Pharbitis nil*, Plantago asiatica and Taraxacum mongolicum on proliferation and apoptosis of tumor cells using a human gastric cancer cell line AGS. Here we demonstrated first that *Saussurea lappa* and *Pharbitis nil* induce growth inhibition and apoptosis of human gastric cancer cells and these effects are accompanied with down- and up-regulation of growth-regulating protooncogenes and tumor suppressor genes, respectively, suggesting that the anticancer effects of

Saussurea lappa and *Pharbitis nil* might be associated with their regulatory capability of tumor-related gene expression.

II. MATERIALS AND METHODS

Cell culture

The AGS human gastric carcinoma cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in DMEM with 10% of fetal bovine serum. Cells were grown at 37 ℃ in a humidified 5% CO_2 atmosphere.

Preparation of medicinal herbs

Four medicinal herbs [*Saussurea lappa* (560g), *Pharbitis nil* (570g), *Plantago asiatica* (550g) and *Taraxacum mongolicum* (570g)] were added to 4.8L of distilled water, boiled for 2 h and extracted two times. The concentrated, decocted herbs were retrieved by a vacuum freeze dryer (Christ LDC-1.Alpha/4, Germany). Freeze dried [*Saussurea lappa* (115.4g), *Pharbitis nil* (14.8g), *Plantago asiatica* (82.5g) and *Taraxacum mongolicum* (71.9g)] were dissolved with cell culture medium and administered to $1x10^5$ of AGS cells for 12-72 h. Cells were harvested using a 0.1% of trypsin and extraction of DNA, RNA, and protein was performed using standard methods.

MTT assay

Five mg/*ml* of MTT were solved with PBS (pH 7.5) and 10 *μl* of MTT solution were added to 100 *μl* of cell suspension including $1x10^5$ cells and then the solution was incubated at 37℃ for 3 h. A hundred *ml* of 0.04 M HCI was added to each well, and blue formazan crystals were dissolved in the solution. After the complete dissolution, optical density was measured at 570 nm using ELISA reader.

Cell proliferation and apoptosis analysis

For cell number counting, $1x10^5$ AGS cells were treated with the medicinal herbs for 12-72 h and cell number was counted using a hemocytomer. For [^{3}H]thymidine incorporation assay, $2x10^4$ cells/well were seeded to 24-well multi plates. The cells were cultured in a 10% fetal bovine serum for 24 h and then grown with a serum-free medium before treatment. After 24 h, 1.0 Ci/*ml* of [^{3}H]thymidine (Amersham, Arlington Heights, IL) was added for 4 h, and the amount of incorporated trichloroacertic acid-precipitable radioactivity in DNA was measured using liquid scintillation counter. For apoptosis detection, 1x105 cell/*ml* cells were absorbed to a slide and stained with tryphan blue. The number of stained cells was measured using a microscope.

Quantitative RT-PCR analysis of gene expression

Total cellular RNA was extracted from cultured cells by a single-step method. One *μg* of extracted RNA was reverse-transcribed to cDNA in a 20 *μl* reaction using MoMuLV (Gibco) and random hexamer primers. Two separate cDNAs were prepared from each RNA and diluted 1:4 or 1:8 with distilled, sterile H_20 prior to PCR. For quantitative evaluation of gene expression, we initially performed PCR with exon-specific primer sets for all targets including a housekeeping standard GAPDH. The sequences of oligonucleotide primers will be provided upon request. PCR was performed with increasing cycle numbers (21, 24, 27, 30, 33, 36, 39, and 42 cycles) and diluted cDNAs (1:0, 1:2, 1:4. and 1:8). Each cycle comprised denaturation at 95℃ for 1 min, annealing at 58-62℃ for 1 min, and polymerization at 72℃ for 1 min. Ten *μl* of the PCR products were resolved on 2% agarose gels (FMC, Rockland, ME). Quantitative analysis of gene expression was confirmed through scanning of ethidium

bromide-stained gels, using laser densitometry. Measurement of signal intensity was performed using the Molecular Analyst program (version 2.0) on an IBM compatible computer.

III. RESULTS

Analysis of effect on cell viability using MTT assay

To determine treatment concentrations, we initially examined effect of the four medicinal herbs on cell viability using MTT assay. Each of the decoctions (0.1, 0.2, 0.5, 1.0, and 5.0 $\mu g/ml$) was treated to 1 x 10^5 AGS cells for 6-48 h. MTT assay was performed in triplicate and the means were calculated. As shown in Table 1, cellular viability of AGS was decreased in a time- and dose-dependent manner after treatment with the four medicinal herbs. However, reduction of cell viability was not significant in a range of 0.1-1.0 $\mu g/ml$. On this basis, 0.1-1.0 $\mu g/ml$ were selected for further cell proliferation and apoptosis assay.

Effect on cell proliferation

To evaluate the effect of the four medicinal herbs on cell proliferation, 1 x 10^5 AGS cells were treated with 0.5 and 1.0 $\mu g/ml$ for 24, 48 and 72 h. Cell number was counted using a hemocytometer in triplicate and the means were calculated. As shown in Table 2, cell proliferation was significantly inhibited by *Saussurea lappa* and *Pharbitis nil* whereas only slight growth inhibition was induced by *Plantago asiatica*, and *Taraxacum mongolicum*. Among the four medicinal

Table 1. MTT assay for qrowthinhibition of AGS cell line

Herbs (concentration)	6	12	24	48(hours)
Saussurea lappa (μg/ml)				
0.0	0.454	0.446	0.465	0.459
0.1	0.448	0.449	0.453	0.454
0.2	0.455	0.453	0.465	0.432
0.5	0.457	0.466	0.431	0.422
1.0	0.460	0.416	0.401	0.387
5.0	0.454	0.424	0.369	0.304
Pharbitis nil (μg/ml)				
0.0	0.454	0.446	0.465	0.459
0.1	0.467	0.453	0.456	0.451
0.2	0.461	0.458	0.462	0.449
0.5	0.455	0.453	0.448	0.444
1.0	0.447	0.436	0.422	0.414
5.0	0.444	0.421	0.405	0.388
Plantago asiatica (μg/ml)				
0.0	0.454	0.446	0.465	0.459
0.1	0.446	0.453	0.459	0.464
0.2	0.455	0.458	0.463	0.454
0.5	0.456	0.450	0.457	0.442
1.0	0.461	0.448	0.431	0.425
5.0	0.457	0.441	0.411	0.407
Taraxacum mongolicum (μg/ml)				
0.0	0.454	0.446	0.465	0.459
0.1	0.435	0.453	0.451	0.457
0.2	0.457	0.449	0.454	0.439
0.5	0.449	0.451	0.441	0.437
1.0	0.451	0.446	0.422	0.417
5.0	0.455	0.442	0.412	0.402

herbs we examined, Saussurea lappa was identified as exhibiting the most significant growth-inhibitory effect. An approximately 50% reduction of cell numbers was induced by 72 h treatment of *Saussurea lappa* (1.0$\mu g/ml$).

To further confirm the growth inhibitory activity, we analyzed the effect of four medicinal herbs on DNA replication, using [^{3}H]thymidine uptake assay. AGS cells were treated with each herb with 0.1, 0.2, 0.5, 1.0, and 5.0 $\mu g/ml$ for 24 h. As shown in Fig. 1, DNA synthesis of AGS cells was significantly suppressed in a dose-dependent manner by treatment with *Saussurea lappa* and *Pharbitis nil*, a result consistent with cell counting assay. In contrast, *Plantago asiatica* and *Taraxacum mongolicum* showed no detectable effect on DNA replication.

Analysis of apoptosis induction

To assess the effect of the four medicinal herbs on apoptosis, tryphan blue exclusion assay was carried out. The cells were treated with 0.1, 0.2, 0.5, and 1.0 $\mu g/ml$ for 72 h and cells showing apoptotic death were counted. The assay was performed in duplicate and the means were calculated. As summarized in Fig. 2, a marked increase of apoptotic cell death was found after treatment of *Saussurea lappa* and *Pharbitis nil*. Apoptosis induction by these two herbs occurred in a dose-dependent manner. No detectable effect on apoptosis was produced by Plantago asiatica and Taraxacum mongolicum

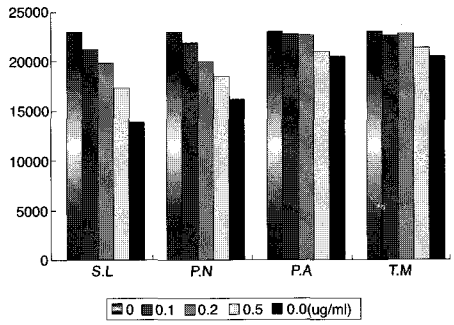

Fig. 1. [^{3}H]thymidine incorporation assay for effect on Suppressing DNA replication. The cells of 2x10^4 per well were seeded to 24-well multi plates and cultured in a 10% fetal bovine serum for 24 h and then grown with a serum-free medium before treatment. After 24 h, 1.0 Ci/ml of [^{3}H]thymidine (Amersham, Arlington Heights, IL) added for 4 h and the amount of incorporated trichloroacertic acid-precipitable radioactivity in DNA was measured using liquid scintillation counter. *S.L., Saussurea lappa; P.N., Pharbitis nil; P.A., Plantago asiatica; T.M., Taraxacum mongolicum.*

Table 2. Cell viability and survival test on AGS cell line

Herbs (concentration)	24	48	72 (hours)
Saussurea lappa (μg/ml)			
0.0	1.64	3.02	6.98 ($\times 10^5$)
0.5	1.55	2.54	4.99
1.0	1.32	2.01	3.39
Pharbitis nil (μg/ml)			
0.0	1.64	3.02	6.98 ($\times 10^5$)
0.5	1.63	2.78	5.61
1.0	1.49	2.53	4.38
Plantago asiatica (μg/ml)			
0.0	1.64	3.02	6.98 ($\times 10^5$)
0.5	1.59	2.91	5.94
1.0	1.57	2.79	5.38
Taraxacum mongolicum (μg/ml)			
0.0	1.64	3.02	6.98 ($\times 10^5$)
0.5	1.68	3.12	6.43
1.0	1.63	2.96	6.06

Effect on cell cycle- and apoptosis-related gene expression

We next examined, using semiquantitative RT-PCR assay the possibility that growth inhibition and apoptosis induction by *Saussurea lappa* and *Pharbitis nil* is associated with its regulatory effect on the cell cycle- and apoptosis-controling gene expression. As shown in Fig. 3, *Saussurea lappa* and *Pharbitis nil* strongly suppressed expression of cell cycle-promoting protooncogenes such as c-Jun, c-Fos, c-Myc, and Cyclin D1, whereas only slight inhibition of the gene expression was induced by *Plantago asiatica* and *Taraxacum mongolicum*. Interestingly, *Saussurea lappa*, which showed the most significant growth suppression and apoptosis induction effects, was identified as activating the transcription of the p53 tumor suppressor gene and its downstream effector $p21^{Waf1}$. In contrast to *Saussurea lappa*, *Pharbitis nil* also showed a strong growth arrest and apoptosis induction activity, did not activate p53 expression. No changes in p53 and $p21^{Waf1}$ expression levels were observed in cells treated with *Plantago asiatica* and *Taraxacum mongolicum*. We also analyzed mRNA expression of p16Ink4A, another tumor suppressor gene which also plays a critical role in control of cell proliferation and apoptosis, but its expression was not affected by any of the four herbal medicines tested.

Next, we examined expression of representative apoptosis-inhibiting genes, Bcl-2, Bcl-XL, and an apoptosis-promoting gene Bax. Whereas no effect on Bcl-2 and Bcl-XL expression was observed, *Saussurea lappa* and *Pharbitis nil* revealed a strong stimulating effect on Bax gene expression. Induction of Bax gene expression by both *Saussurea lappa* and *Pharbitis nil* was dependent on treatment time.

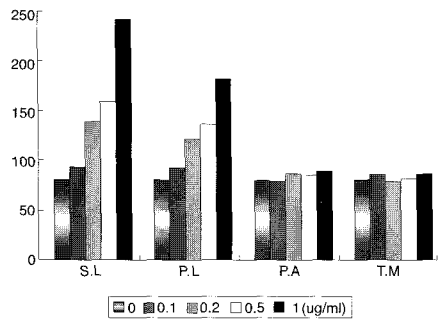

Fig. 2. Tryphan blue exclusion assay for apoptosis induction. For apoptosis detection, $1x10^5$ cell/*ml* cells were absorbed to a slide and stained with tryphan blue. The cells were treated with 0.1, 0.2, 0.5, and 1.0 *μg/ml* for 72 h and cells showing apoptotic death were counted. The number of apoptotic cells in total 2000 cells was measured using microscope. *S.L., Saussurea lappa; P.N., Pharbitis nil; P.A., Plantago asiatica; T.M., Taraxacum mongolicum.*

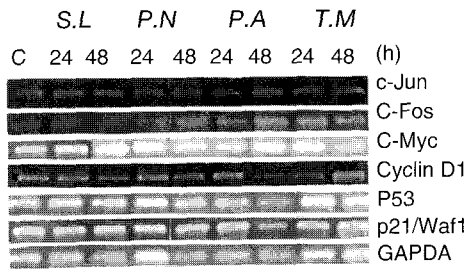

Fig. 3. A quantitative RT-PCR analysis for cell cycle?related gene expression. One *μg* of total cellular RNA extracted from untreated and treated AGS cells was converted to cDNA by reverse transcription and 1:4 diluted cDNA was subjected to PCR amplification of c-Jun, c-Fos, c-Myc, Cyclin D1, p53, $p21^{Waf1}$, $p16^{Ink4A}$ and an internal control gene, GAPDH. Ten *μl* of PCR products was resolved on a 2% agarose gel and its band intensities were scanned using a densitometry. The expression levels of each gene were determined as expression ratio, which were adjusted by intensities of GAPDH expression.

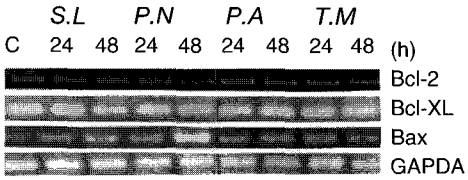

Fig. 4 A quantitative RT-PCR analysis for apoptosis-related gene expression. One μg of total cellular RNA extracted from untreated and treated AGS cells was converted to cDNA by reverse transcription and 1:4 diluted cDNA was subjected to PCR amplification of Bcl-2, Bcl-XL, Bax and an internal control gene, GAPDH. Ten μl of PCR products was resolved on a 2% agarose gel and its band intensities were scanned using a densitometry. The expression levels of each gene were determined as expression ratio, which were adjusted by intensities of GAPDH expression.

IV. DISCUSSION

Conventional medicine has usually resorted to a number of treatments such as operation, radiotherapy, and chemotherapy. The existing anti-cancer drugs, designed to eradicate cancer cells, have strong toxicities, leading to harmful side effects. Recently, a number of researches on natural products have been actively carried out in an effort to develop new treatments that can decrease side effects and increase anti-cancer effects. Researchers are proving that the efficacy of natural products as cancer-suppressors and immunity-enhancers. In particular, studies on a link between expression of natural killer cells and cell immunity are actively being carried out. Furthermore, molecular biology studies, which investigate the relations between programmed cell death and tumor cells, are moving to research on cell cycle-related gene expression.

Saussurea lappa, Pharbitis nil, Plantago asiatica, and *Taraxacum mongolicum* are representative herbal medicines that have traditionally been used for cancer treatment in Korea (Jung JH et al., 1998; Kim SH, 1998; Kim DH et al., 1995). Several previous studies have demonstrated that these herbs exhibit immune suppressive effect as well as anti-tumor activity (Jung JH et al., 1998; Cho JY et al., 2000, 1998; Yoshikawa M et al., 1993). In the present study, we analyzed effects of *Saussurea lappa, Pharbitis nil, Plantago asiatica* and *Taraxacum mongolicum* on proliferation and apoptosis of tumor cells using a human gastric cancer cell line AGS. Our data revealed that *Saussurea lappa* and *Pharbitis nil* have a strong growth inhibition and apoptosis induction activity. Moreover, we found that the anti-tumor effects of *Saussurea lappa* and *Pharbitis nil* are associated with their regulatory capability of growth-regulating protooncogenes and tumor suppressor genes such as c-Jun, c-Fos, c-Myc, Cyclin D1, p53, $p21^{Waf1}$ and Bax.

The four periods G1, S, G2, and M make up the cell division cycle. In the period of G1, no particular cell cycle progressions occur and most divided or undivided cells exit. If this phase enables the cell to grow and to produce all the necessary proteins for DNA synthesis, cells start to divide and reach a point, which is called "the first commitment point" and this phase decides eukaryote' s cell cycle. In this study, our cell proliferation assay showed that *Saussurea lappa* and *Pharbitis nil* strongly inhibit cell proliferation of AGS, whereas Plantago asiatica and *Taraxacum mongolicum* exhibit no detectable effect on cellular growth. [^{3}H]thymidine uptake analysis also demonstrated that DNA replication of AGS is suppressed in a dose-dependent manner by treatment with *Saussurea lappa* and *Pharbitis nil.* Interestingly, our semiquantitative RT-PCR analysis revealed that mRNA expression levels of c-Jun, c-Fos, c-Myc, and Cyclin D1 were markedly reduced by *Saussurea lappa* and *Pharbitis nil.* It was also observed that *Plantago asiatica* and

Taraxacum mongolicum have inhibitory effect on these growth-promoting protooncogenes, but its activity was significantly less compared to *Saussurea lappa* and *Pharbitis nil*. Saussurea lappa also activated the p53 tumor suppressor gene, which raises the possibility that down-regulation of protooncogenes including c-Myc might be mediated by activated p53, as suggested by a previous report (Levine AJ, 1992).

Apoptosis is a genetically programmed event that can be set in motion by a variety of internal or external stimuli (Wyllie AH, 1994). Recent studies have shown that tumor cells with mutational inactivation of apoptosis-inducing genes, such as the tumor-suppressor gene p53, fail to respond to chemotherapeutic agent-induced DNA damage. In other cases a tumor-promoting virus may interfere with the regulation of apoptosis, inducing immortalization of normal cells. Recently, a number of apoptosis-related genes such as Bcl-2 and Bax have been found (Sen S, 1992). Bcl-2 family members display both pro-survival and pro-apoptotic functions and exist by forming homodimers or heterodimers (Findly HW et al., 1997). Bcl-2 and Bcl-XL are representative apoptosis inhibitors, while Bax, Bak, and Bad promote apoptosis. Bax, a tumor suppressor, mediates the p53-induced apoptosis and it increases sensitivity to chemotherapy-induced apoptosis (Schlesinger PH et al., 1997; Tu Y et al., 1996; Lisovsky M et al., 1996). On the other hand, when Bcl-2 is activated, apoptosis is prohibited. Abnormal overexpression of Bcl-2 has frequently been observed in many types of human cancers, and relative expression levels of Bcl-2 to Bax were reported to determine the sensitivity to apoptosis (Itoh N et al., 1993; Strobel T et al., 1996; Yin C et al., 1997).

Our study shows that Saussurea lappa activates expression of the p53 tumor suppressor and its downstream effector $p21^{Waf1}$, which induce G1 cell cycle arrest and apoptosis, raising the possibility that $p21^{Waf1}$ that inhibits Cyclin/CDK might be directly implicated in *Saussurea lappa*-induced inhibition of tumor cell proliferation. In addition, *Saussurea lappa* also showed a strong activating effect on Bax, suggesting that its anti-tumor activity might be associated with its gene-regulating function.

Collectively, our data demonstrate that *Saussurea lappa* and *Pharbitis nil* induce growth inhibition and apoptosis of human gastric cancer cells and these effects are accompanied with down-and up-regulation of growth-regulating protooncogenes and tumor suppressor genes, respectively. This observation thus suggests that anticancer effect of *Saussurea lappa* and *Pharbitis nil* might be associated with their ability to regulate tumor-related gene expression.

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