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# 歸朮破癥湯 추출물의 인간 유방암세포에 대한 성장억제 효과

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## ABSTRACT

**Anti-proliferative effects of Guichulpajing-Tang extract on MCF-7 cells**

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이 연구는 MCF-7 인간 유방암 세포주에 대한 歸朮破癥湯 추출물의 증식억제효과 세포독성효과 세포사 유발효과를 확인하기 위하여 이루어졌다.

MCF-7 인간 유방암 세포주는 Dulbecco's modified Eagle's medium/F12 (DMEM/F12)에 10% fetal bovine serum(FBS;Gibco) 와 항생제를 가하여 만든 배지를 이용하여 배양하였고, MCF-7 세포를 96- well plate에 접종한 후 다양한 농도(0~2000 g/ml)의 歸朮破癥湯이 든 배지로 처리하고 다양한 시간(48, 96, 192)동안 배양하여 현미경으로 관찰하고 각각 MTS assay kit를 이용하여 세포생존율을 측정하였다. 세포독성은 Sulforhodamine B assay 방법을 이용해 측정하였고 세포사 과정에서 MCF-7세포에서의 caspase 활성화를 측정하기 위해 Western blotting을 수행하여 poly ADP ribose polymerase(PARP)의 절단을 확인하였다.

실험결과 歸朮破癥湯 추출물에 의한 세포성장 및 독성효과는 시간 및 농도에 비례하는 것으로 나타났고 세포고사과정에서 작용하는 caspase의 전 기질인 PARP 절단량이 歸朮破癥湯 처리 농도와 시간에 비례하여 증가하였다. 이것은 caspase-3가 MCF-7 세포의 성장을 억제하는데 중요한 역할을 수행함을 의미한다. 따라서 歸朮破癥湯은 다양한 기전에 의해서 유방암 세포에 대한 억제효과를 가진다는 것을 인식할 수 있다.

중심단어 : 유방암, MCF-7, 歸朮破癥湯, 세포성장억제, 세포독성, 세포사

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# Ⅰ. Introduction

Breast cancer is the most common in Korean women. The incidency of breast cancer is around 25 per 100,000 women, and more than 5,500 women are diagnosed as breast cancer annually[1]. Despite remarkable improvements in treatment strategies against various cancer during the past 40 years, breast cancer still remains as one of the main cause of cancer mortality among women in the whole world[2].

Gwichulpajing-Tang(GPT) originated from 《Uibang-yuchjip》 written by Gwak gam has been used to treat abdominal mass, amenorrhea etc[13]. Analyzing composition of GPT, it has effect on clearing the liver heat and relieving constraint, regulating *Qi*, promoting blood flow. By recent work, phalmacological effectiveness of GPT are analgesic, anti-pyretic, anti-imflammatory effects etc[14,15]. It may be suggested that GPT has potential effects in breast cancer. But it hasn't been reported about GPT's effects on breast cancer so far.

In this paper we have reported the anti-proliferative activities of GPT extract on breast cancer cells(MCF-7 cells) and effects of different concentration and treatment time of GPT extract on cell proliferation and activation of caspase-3 has been investigated.

# Ⅱ. Materials and methods

## 1. Materials

### 1) Chemicals and laboratory wares

Unspecified, chemicals and laboratory wares used in this study were purchased from Sigma Chemical Co.(St. Louis, MO, USA) and Falcon Labware(Becton-Dickinson, Franklin Lakes, NJ, USA).

Cell Titer 96 aqueous ane solution cell proliferation assay was from Promega(CA, USA). Media were purchased from GIBCO BRL(Life Technologies, CA, USA). PARP were purchased from Cell Signaling Technology, and beta-actin antibody was purchased from Sigma.

### 2) Extract of GPT

An aqueous extract of GPT was prepared in combination of the next herbs. These were ground in approximately 10 volumes of distilled water by homogenate for 30 packs and lyophilized. The appropriate amount of the brown powder obtained was dissolved in PBS and stored at 4℃ until use.

Table 1. Composition of GPT

| Name of herb | Scientific name | weight(g) |
|---|---|---|
| 香附子 | Cyperi rhizoma | 6.0 |
| 三棱 | Sparganii rhizoma | 4.0 |
| 蓬朮 | Zedoariae rhizoma | 4.0 |
| 赤芍藥 | Paeonia rubra radix | 4.0 |
| 白芍藥 | Paeoniae radix | 4.0 |
| 當歸 | Angelicae gigantis | 4.0 |
| 靑皮 | Aurantii immatri pericarpium | 4.0 |
| 烏藥 | Linderae radix | 2.8 |
| 紅花 | Carthami flos | 2.0 |
| 蘇木 | Caesalainiae lignum | 2.0 |
| 肉桂 | Cinnamomi cortex spissus | 2.0 |
| **Total** | | 38.8 |

2. Methods

1) Cell culture and treatment

MCF-7 cells were cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) supplemented with 10% fetal bovine serum(FBS; Gibco) and antibiotics. $1\times10^6$cells were seeded in 60-㎜ culture and 5000 cells/well were seeded in 96-well flat-bottomed plates, respectively. At varying times after extract treatment, cells were harvested with scraper and processed for analysis of protein expression, proliferation, cytotoxicity and apoptosis.

2) The assessment of proliferation

MCF-7 cells were seeded in 96-well flat-bottomed plates. After 24hours incubation cells were treated with varying concentration of extract a. After appropriate time, 20㎕/well of Cell Titer 96 Aqueous One Solution Reagent (Promega Corp.) was added. After 3hours at 37℃ in a humidified, 5% $CO_2$ atmosphere, the absorbance at 490㎚ was recorded using an ELISA plate reader. A reference wavelength at 65㎚ was used to subtract background contributed by excess cell debris, fingerprints and other nonspecific absorbance.

3) The assessment of cytotoxicity

The cellular cytotoxic effect of extract was measured using In vitro Toxicology Assay Kit(SIGMA). MCF-7 cells were seeded in 96-well flat-bottomed plates. After 24hours incubation cells were treated with varying concentration of extract a. After appropriate time, fixed the cells by gently layering 1/4 volume of cold 50% TCA on top of the growth medium. After 1 hour incubation at 4℃, rinsed cells with water several times to remove TCA and air dried. Sulforhodamin B solution(0.4%) was added onto dried cells in an amount sufficient to cover the culture surface area and then allowed to stain for 20minutes. After being rinsed with 1% acetic acid several times, the cells were dried completely. The incorporated dye was then solubilized in a volume of Sulforhodamine B Assay Solubilization Solution(10mM Tris) equal to the original volume of culture medium. Allowed cultures to stand for 5minutes at room temperature with gentle stirring in a gyratory shaker and then measured absorbance at a wavelength of 565㎚.

4) The assessment of apoptosis

Aliquots of MCF-7 cells in 60㎜ culture dishes were treated with extract for varying times and varying concentrations and then attached and floated cells were harvested altogether. Cleavage of poly(ADP-ribose) polymerase(PARP) facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis so the PARP cleavage was checked by western blot analysis using PARP antibody.

5) Western blot analysis

Added 50㎕ NP-40 lysis buffer(50mM Tris-Cl (pH7.4), 150M NaCl, 50mM NaF, 0.5% NP-40, 1mM EGTA, 1mM EDTA, 1mM PMSF) to 60㎜ plate scale and scraped cells with cell scraper. Transferred the

supernatant to eppendorf tube and vortexed moderately. Centrifuged the tube for 30minutes. At 14,000 rpm at 4℃ and then transferred the suppernant to new eppendorf tube. Cell lysates that contained 35 ㎍/㎖ of total protein were subjected to 7.5-12% SDS-PAGE and transferred by electroblotting to nitrocellulose membranes. The membranes were blocked with 50mM Tris(pH 7.5) containing 500mM NaCl, 5% non-fat dried milk. The blots were probed with specific primary antibodies and visualized using an enhanced chemiluminescence(ECL) kit(Amersham Pharmacia Biotech, Buckinghamshire, UK), according to the manufacturer's instructions. Polyclonal antibodies specificfor PARP and MAPK were purchased from Cell Signaling Technology and monoclonal antibodies for beta-actin were purchased from Sigma.

6) Statistical analysis

The data are expressed as the mean±SD. The differences between groups were analyzed by Student's t-test. The significance level was set at $p<0.05$.

## Ⅲ. Results

1. The effect of GPT on the proliferation of MCF-7 cells

The effect of extract on the proliferation of MCF-7 human breast cancer cells was examined. As shown in Fig. 1, extract of GPT induced cell growth inhibition and morphological change of MCF-7 cells and inhibited proliferation of MCF-7 cells in a dose dependent manner(Fig. 2). In MCF-7 cells, the inhibition of cell growth by GPT was so obvious that one could observe the difference easily under a microscope(Fig. 1). This inhibitory effect was dose-dependent. Treatment with 500㎍/㎖ for 72 h could result in a nearly 50% inhibition(Fig. 2). Similarly, GPT inhibited proliferation of MCF-7 cells in a time-dependent manner(Fig. 3). When the cells were treated with 2,000㎍/㎖ of GPT for 96 hours, more than 60% inhibition of MCF-7 cell growth was observed. These findings indicate that GPT strongly inhibits the proliferation of MCF-7 cells in a dose-dependent and time-dependent manner.

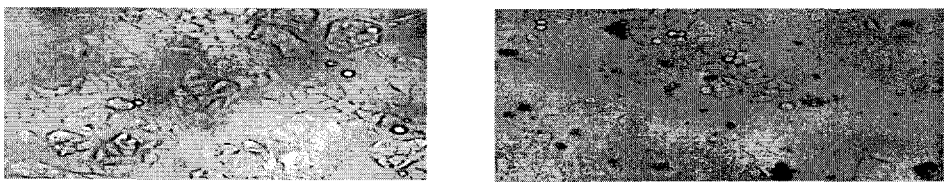


Fig. 1. Microphotographs showing the inhibitory effect of Gwichulpajing-Tang on cell growth.
MCF-7 cell lines were plated onto 6-well plates and treated with drug-free media (control) or media containing 500 ㎎/㎖ of Gwichulpajing-Tang for 72 hrs. The photographs were taken directly from culture plates using a phase microscope (×100 magnification).

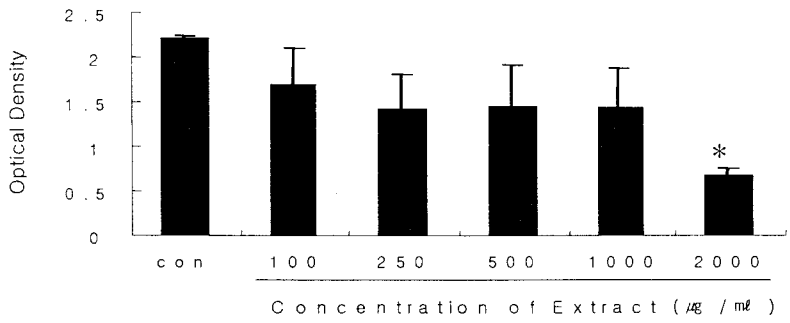


Fig. 2. Dose-dependent effects of Gwichulpajing-Tang on cell growth. MCF-7 cells were plated onto 96-well plates and treated with or without (control, con) varying concentrations (100, 250, 500, 1000 and 2000 g/㎖) of Gwichulpajing-Tang extract for 72 hrs. The number of viable cells in each well was quantified by using MTS assays. Data are representative of at least three independent experiments. Error bars represent mean±SD. significantly different when compared with control (con) ($p<0.05$).

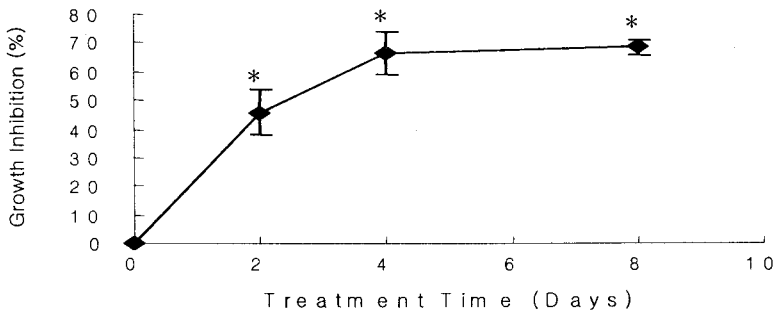


Fig. 3. Time-dependent effects of Gwichulpajing-Tang on cell growth. MCF-7 cells were plated onto 96-well plates and treated with without of Gwichulpajing-Tang extract for 48, 96 and 192 hrs. The number of viable cells in each well was quantified by using MTS assays. Results (optical densities) were calculated as the percentage of unexposed control cultures. Data are representative of at least three independent experiments. Error bars represent mean±SD. *, significantly different when compared with zero point of treatment ($p<0.05$).

2. The cytotoxic effect of GPT on MCF-7 cells

To characterize the mechanism by which GPT inhibits the proliferation of MCF-7 cells, we examined whether GPT exerted a cytotoxic effect on breast cancer cells by using an Sulforhodamine B assay, a means of measuring total biomass by staining cellular proteins with the Sulforhodamine B. As shown in figure 4, Sulforhodamine B showed that the addition of GPT extract reduced the viability of MCF-7 cells in a dose-dependent manner.

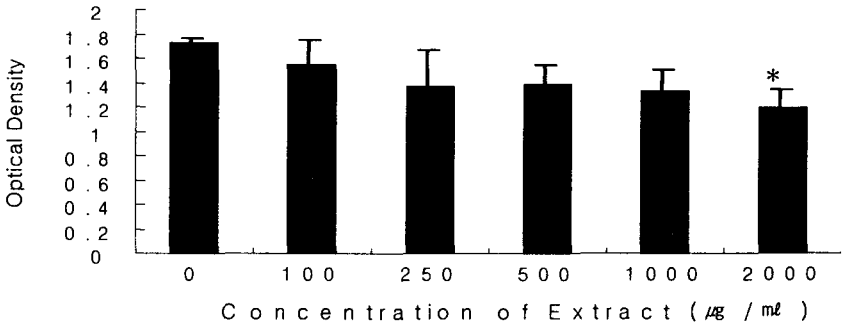


Fig. 4. Cytotoxic effects of Gwichulpajing-Tang in MCF-7 cells.
MCF-7 cells were treated with the indicated concentrations of Gwichulpajing-Tang for 48 hrs. The cells were then processed for Sulforhodamine B assay. Each bar represents the mean±SD values of three separate experiments. *, significantly different when compared with extract untreated group (0) ($p<0.05$).

3. The effects of GPT on apoptosis

To assess the potential role of caspases in the GPT extract-induced apoptosis, whole cell lysates were prepared after the treatment of the cells with the herb extract. Caspase-3 activity in the whole-cell lysates was examined by Western blot assay on one of its major substrate, poly[ADP (ribose)] polymerase (PARP) and the metabolite.

Fig.5. shows the cleavage of the full-length PARP (116kd) to generate the 89-kd cleaved PARP fragment, indicating the activation of caspase-3.

Fig. 6. represents the concentration-dependency of GPT extract-treatment.

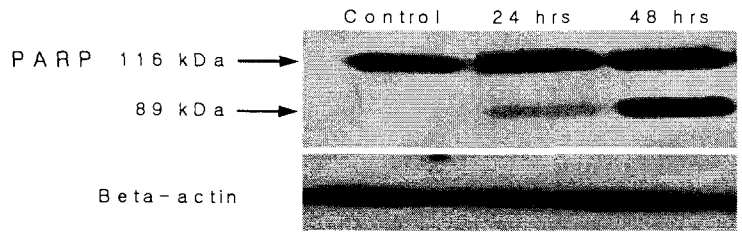


Fig. 5. Caspase activation in Gwichulpajing-Tang extract treated MCF-7 cells.
The whole cell lysate from treated cells with Gwichulpajing-Tang was assayed by Western blotting after a 24- and 48-hour exposure period. Poly[ADP(ribose)] polymerase (PARP), a major substrate for caspase-3 was extensively cleaved in the treated cells with Gwichulpajing-Tang, while control cells failed to show PARP cleavage.

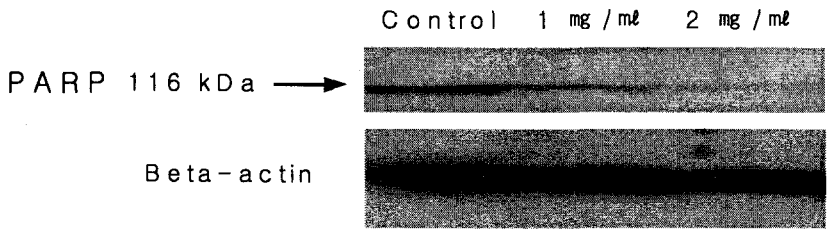


Fig. 6. Caspase activation in Gwichulpajing-Tang extract treated MCF-7 cells.
The whole cell lysate from treated cells with Gwichulpajing-Tang with 1 ㎎/㎖, 2 ㎎/㎖ concentration or without (control) extract was assayed by Western blotting after a 24 hour exposure period. Poly[ADP(ribose)] polymerase (PARP).

## Ⅳ. Discussion

Breast cancer is one of the main cause of cancer mortality among womon in the whole world, and has rapid increasing rate annually. Though breast cancer was 8.7% among cancer of womon in 1984, it was reported that breast cancer was 15.6% in 2004. At present, western cancer theraphy by chemotherapeutic agents, surgery and radiation have not been fully effective against the high incidence or low survival rate of breast cancer[16,17,18].

Despite the fact that many tumors initially respond to therapy, cells can subsequently survive and gain resistance to these treatments. An cancer theraphy by chemotherapeutic a has many side effect. The development of new therapeutic approach to breast cancer remains one of the most challenging area in cancer research. In recently, many interests are given to herbal medcine on cancer treatment[3-8]. Many herbal medicine researches of anti-proliferation, cytotoxicity, and

apoptosis of human breast cancer has been already done by many scientists[9-12]. Until now, it is researched herbal medicine effect on cancer in example Scutellaria Baicalensis[20-22], Euonymus Alatus[3], soybean and brown rice[9], Thesium chinense[10], beevenom[11], Gamissangwhatang[12], Coriolus versicolor[21], Glycyrrhiza glabra[22], Gossypium hirsutum[23], Scutellaria Barbatae[3,24] etc. In this study we have use GPT extracts to investigate its effects on cancer cell proliferation.

In traditional korean medicine, breast cancer called Yu-Am, Beon-Hwa-Nae, Seok-Ong[19]. The symptoms of Yu-Am which is the tumor of breast are no pain, no itching, bigger and pus by time. *Yu-Am* in traditional korean medicine was caused by stagnation of liver Qi, imbalance between liver and spleen, deficiency of Qi and blood, external affections etc[19].The treatment principle of *Yu-Am* is to promote blood flow eliminating blood stasis, checking the liver and calming the wind. The herbal medicine of Yu-Am are *Sibyungmiyugieum* medicinal beverage for making Qi flow of sixteen ingredients, *Cheongganhaeultang* decoction for clearing the liver of heat and relieving depression, and *Yongdamsagantang* decoction for purging the liver of fire with Radix Gentianae etc[19].

GPT originated from 《Uibang-yuchji p》 written by Gwak-gam tastes pungent, bitter and has warm nature and reates to liver, spleen, heart meridians[13]. GPT is the decoction for Breaking Abdominal Mass with Radix Angelicae Gigantis and Rhizoma Zedoariae. It is composed of Radix Angelicae Gigantis and Rhizoma Zedoariae, Rhizoma Cyperi, Rhizoma Sparganii, Rhizoma Zedoariae, Radix Paeoniae Rubra, Radix Paeoniae Alba, Radix Angelicae Gigantis (tail), Pericarpium Citri Reticulatae Viride, Radix Linderae, Flos Carthami, Lignum Sappan, and Cortex Cinnamomi(Table 1.). This medicine is composed of herbs for checking the liver and calming the wind, regulating the flow of Qi(Rhizoma Cyperi, Pericarpium Citri Reticulatae Viride) and dispelling blood stagnation(Radix Paeoniae Rubra, Radix Paeoniae Alba, Radix Angelicae Gigantis, Flos Carthami etc). Even if the traditional uses of GPT focused on abdominal mass treatment, it may be suggested that GPT has potential effects in breast cancer by considering pathogenesis of breast cancer.

To investigate the effect of GPT against breast cancer, MCF-7 cells were treated by GPT at varying time-concentration. In MCF-7 cells, the inhibition of cell growth by GPT was so obvious that one could observe the difference easily under a microscope. The inhibition of cell growth was dose-response and time-relation. Treatment with 500㎍/㎖ for 72hours could result in a nearly 50% inhibition. Sulforhodamine B showed that the addition of GPT extract reduced the

viability of MCF-7 cells in a dose and time dependent manner. It is observed that The PARP, a major substrate for caspase-3, was extensively cleaved in the treated cells with GPT, while control cells failed to show PARP cleavage. The treated cells with GPT 2㎎/㎖ concentration had more the 89-kd cleaved PARP fragment than 1㎎/㎖ concentration. The cleavage of the full-length PARP(116kd) to generate the 89-kd cleaved PARP fragment was also increased with dose depent manner. We assumed that the activation of caspase-3 could play a significant role in minimizing the MCF-7 cells proliferation.

Consequently, Our results show that GPT may play a role in anti-breast cancer agent by means of inhibiting of proliferation and inducing apoptosis. In this study, teatment with 2000㎍/㎖ for 96 hrs could result in a nearly 60% inhibition. We have to consider both a medication dose and a period for clinical application. It is likely that anti-breast cancer agent has keep more than 2000㎍/㎖ consentration of GPT extract. The activity of caspase-3 by GPT extract indicates that anti-breast cancer effect of GPT related to apoptosis. Our opinion is that GPT has good effect on breast cancer at early stage. However more research needs to be done prior to incorporating these findings into clinical recommendation. So, it should be further study in breast cancer and other cancer by GPT. And then, many Traditional Korean Herbs will be studied in Oncology.

# V. Conclusion

In conclusion the anti cancer activities of GPT extract on MCF-7 cells were observed. Our results showed that GPT extract inhibit the proliferation of MCF-7 cells in dose and time dependant manner. Sulforhodamine B showed that the addition of GPT extract reduced the viability of MCF-7 cells in dose and time dependent manner. PARP which serves as a marker of cells undergoing apoptosis, was extensively cleaved in the GPT-treated cells.

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