

Publication state: Japan
ISSN: 2423-8287

Publisher: J-INSTITUTE
Website: <http://www.j-institute.jp>

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Peer reviewer
E-mail: editor@j-institute.jp

<http://dx.doi.org/10.22471/sport.2016.1.2.17>

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Composition Information of Allium Tuberosum Juice Extract and Its Anticancer Effect in C6 GLIOMA CELLS

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Abstract

Cancer is one of the leading causes of death worldwide. Cancer motility plays a central role in malignant tumorigenesis. Hence, several studies have been focused on investigating effective treatments for this disease. Natural compounds are among the potential therapeutic agents against cancer. *Allium tuberosum* has been used in Korean traditional medicine to improve stamina, and has been recently reported to possess anti-cancer properties. In the present study, we aimed to investigate the effect of *A. tuberosum* juice extracts (ATS) on migration and proliferation of glioma cells. The composition of ATS was determined using gas chromatography-mass spectrometry (GC/MS). Cell viability and proliferation were determined using MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and cell migration was assessed using scratch wound-healing assay. The expression levels of proteins were determined using western blot with specific antibodies such as the extra cellular signal-regulated kinase 1/2 (ERK1/2) and MMP2. Our data showed that ATS did not affect cell viability at a concentration of 300 µg/mL in C6 glioma cell lines. However, ATS significantly reduced the migration of C6 glioma cells. Moreover, ATS significantly suppressed ERK1/2 phosphorylation and MMP2 expression at dosages of 100 and 300 µg/mL. A total of 34 constituents of ATS were detected using GC/MS. Therefore, we suggest that ATS is a potential agent against glioma.

[Keywords] *Allium Tuberosum, Juice, Natural Compounds, Glioma, Anti-Cancer*

1. Introduction

In the last few decades, western nations have had a sizable number of patients affected with cardiovascular disorders, stroke, and cancer[1][2]. Cancer, in particular, has been the leading cause of death worldwide[3][4][5].

World Health Organization (WHO) recommends individuals to exercise for health. Some researchers claim that the risk of developing cancer is decreased with nutrition and exercise. Therefore, research is required to combine exercise and scientific methodology for effective treatment.

Glioma, a malignant tumor of the brain, accounts for approximately 50% of primary brain diseases[6]. Glioma entails a very high mortality rate because the tumor has peculiar and abnormal characteristics, such as rapid cell migration into normal tissues that cannot be controlled by surgery or radiation[7][8]. Thus, in the case of glioma, a patient may suffer a fatal condition within a year from the developmental stage, because therapy against the mortality pattern, such as migration, is still unclear[9].

A recently influential medical report showed that cancer motility has a central role in malignant tumorigenesis[10]. The malignant process of cancer has been reported to

*Funding Agency: This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education(2015R1D1A4A01019776), ACE(Advancement of College Education) Project fund and the Dongguk University Research fund of 2016.

be caused by the activation of mitogen-activated protein kinase(MAPK)[11]. In particular, ERK1/2 phosphorylation is involved in cell invasion, migration, and motility, coupled with the progression of most types of cancer cells[12].

A Korean medical encyclopedia, called “Donguibogam”, provides extensive information on medicinal plants having side effects, but does not provide sufficient clinical data up until the present. *Allium tuberosum* has been used in Korean traditional medicine to improve stamina. In this study, we aimed to establish the potential efficacy of *A. tuberosum* juice(ATS) against cancer motility. The results indicated that ATS may be a potent chemo-prevention agent that acts by inhibiting the migration of glioma cells.

2. Methods

2.1. Materials

The cell culture materials and MTT were purchased from Sigma, Gibco BRL(Gaithersburg, MD, USA), and Daeil Lab Service(Seoul, Korea), respectively. Antibodies—NOX-1, Cu/Zn SOD, MMP-2, Cleaved caspase-3, Bax, Bcl-2, anti-ERK1/2, anti-phospho-ERK1/2, and anti-GAPDH—were purchased from Santa Cruz Biotechnology(Santa Cruz, CA, USA). All other chemicals were purchased from Sigma(St. Louis, MO, USA). Rat C6 glioma cells were obtained from the Korean Cell Line Bank(Seoul, Korea).

2.2. Design of experiments

We performed the MTT assay to evaluate cell viability, the scratch wound-healing assay to test cell migration, western blot to test the expression levels of proteins, and gas chromatography-mass spectrometry(GC/MS) assay to determine the composition of ATS.

2.3. Procedure

2.3.1. Cell culture and MTT assay

Cells were cultured in Dulbecco’s modified Eagle’s medium(DMEM) containing 10% fetal bovine serum(FBS) and 1% penicillin-streptomycin at 37°C in a 5% CO₂ atmosphere. Rat

C6 glioma cells were seeded at 2×10⁴ cells/well in a 96-well microplate containing DMEM and incubated for 24 h. Cells were incubated with different concentrations of ATS(10–1000 µg/mL) in FBS-free or FBS-containing medium for 24 h. Cell viability was then determined using an MTT reduction method. The cell viability of ATS-treated cells was determined relative to that of control cells by measuring the absorbance at 540 nm.

2.3.2. Scratch wound-healing assay

After culturing the cells, a scratch wound was made by scratching the center of each well with a 200-µl sterile pipette tip. This was followed by incubation in the presence or absence of ATS in serum-containing medium for 24 h. Images of the cells that migrated into the cell-free scratch wound area were acquired using an inverted microscope(COOLPIX; Nikon, Japan), and analyzed using ImageJ software(NIH, Bethesda, MD, USA).

2.3.3. Western blot

Twenty micrograms of protein from each treatment group were used. After the proteins were boiled at 100°C for 10 min, they were separated using electrophoresis on 12% acrylamide gels, and transferred onto polyvinylidene difluoride(PVDF, Amersham Pharmacia Biotech, Piscataway, NJ, USA) membranes in transfer buffer at 4°C for 2 h. The membrane was blocked in 5% bovine serum albumin in Tris-buffered saline(TBS) at room temperature for 1 h, and then washed in TBS with 0.1% Tween 20(TBS/T). The membrane was incubated overnight at 4°C with antibodies. The membranes were washed with TBS/T, followed by incubation with a 1:5000 dilution of IgG secondary antibody conjugated to horseradish peroxidase. The protein expression levels were analyzed via chemiluminescence(ECL plus kit; Amersham Pharmacia Biotech). The protein bands were visualized and quantified using ImageJ software.

2.3.4. Gas chromatography-mass spectrometry assay

GC/MS analysis was performed using an Agilent 6890N GC/5975i MS instrument(Palo

Alto, CA, USA) and DB5-MS capillary column(30 m × 250 μm, 0.25 μm film thickness). The carrier gas used was helium at a flow rate of 1 mL/min. The injector port and interface temperatures were 280 and 300°C, respectively. The gas chromatography oven was kept at 40°C for 2 min and increased to 230°C at a rate of 5°C/min, and then kept constant at 300°C for 5 min. The split ratio was 1:10. The mass ranges were from m/z 40 to 800.

2.3.5. Statistical analysis

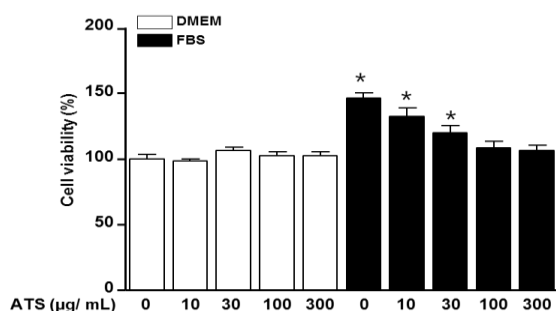
The results were expressed as the mean ± standard error(SE) of at least three independent experiments. The difference between the two groups was examined using Student's t-test. $p < 0.05$ was considered statistically significant. Statistical analysis was carried out using GraphPad Prism 4.0 software.

3. Results

3.1. Effect of ATS on the viability and proliferation of C6 glioma cells

ATS at different concentrations, ranging from 10 to 300 μg/mL, did not induce cytotoxicity in C6 glioma cells. A concentration-dependent decrease in cell density was observed at 100 and 300 μg/mL ATS <Figure 1>.

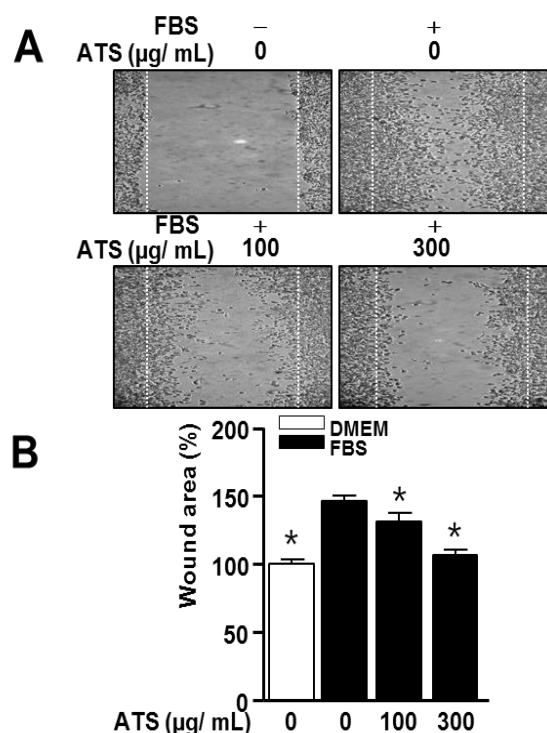
Figure 1. Effect of allium tuberosum(ATS) on FBS-induced cell proliferation of C6 glioma cells. C6 glioma cells were incubated with 10, 30, 100, and 300 μg/mL ATS in FBS-containing media for 24 h, and their proliferation rates were assessed via MTT assay. Data represented are means ± SE of at least three independent experiments(* $p < 0.05$ compared with untreated group).



3.2. Effect of ATS on scratch wound-healing in FBS-stimulated C6 glioma cells

Treatment with ATS(100 and 300 μg/mL) induced a concentration-dependent decrease in in FBS-induced cell migration <Figure 2>.

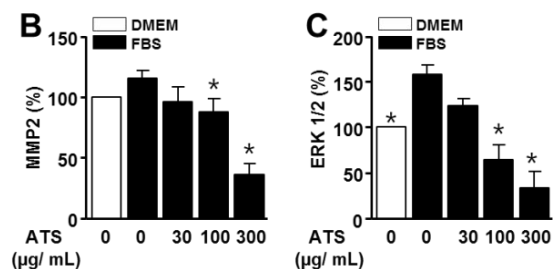
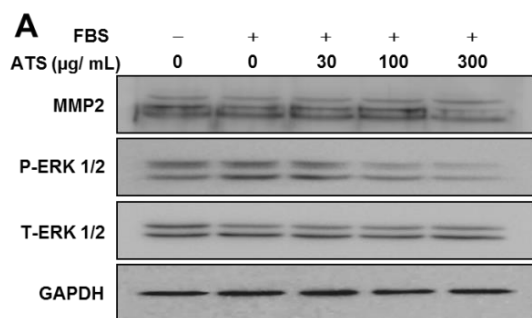
Figure 2. Effect of Allium tuberosum juice(ATS) on serum-induced migration of C6 glioma cells assessed using wound-healing assay. (A) Photographs of scratch-wound assay showed that serum-induced C6 cell migration could be inhibited by treatment with ATS. (B) The relative migration ratio of C6 glioma cells were obtained after analyzing measurements of scratch wound-healing distance. Data represented are means ± SE(* $p < 0.05$ compared with FBS-treated alone).



3.3. Effect of ATS on ERK1/2 and MMP-2 in FBS-stimulated C6 glioma cells

Data show that FBS-induced MMP2 and ERK 1/2 expression decreased upon treatment with ATS in a concentration-dependent manner <Figure 3>.

Figure 3. Effect of allium tuberosum juice (ATS) on the expression of MMP2 and P-ERK 1/2 in FBS-stimulated C6 glioma cells. (A) Expression levels of MMP2, P-ERK 1/2, and T-ERK in C6 glioma cells after ATS treatment. (B, C) Relative band intensity of MMP2 and phosphorylation of ERK 1/2. Data represented are means ± SE(* $p < 0.05$ compared with FBS-treated group).



3.4. Composition of ATS

A total of 34 constituents of ATS were detected, and these major compounds are listed in <Table 1>.

Table 1. Composition of absolute from allium tuberosum juice extracts.

Compound name	Area (%)
1-METHYL PROPYL FORMATE; Formic acid	4.89
gamma.-Hydroxycrotonic acid lactone	1.61
Glycerin	7.52
2-METHYL-1-D1-AZIRIDINE Butanal, 2-methyl-Silacyclopentane	2.3
1,4-dimethyl-Piperazine	2
1,2,2,3,3-Pentamethyl-aziridine 1-Piperidineethanol	1.67
propylamine	2.99
Thymine	2.46
2,3-dihydro-3,5-dihydroxy- 6-methyl-4H-pyran-4-one	10.16
(S)-5-Hydroxymethyl-2[5H]-furanone	1.31
Isothiourea	1.56
2,6-Difluorobenzyl alcohol	1.62
1-Acetylamino-5,5-dimethoxy-2,3-dimethyl-2(E)-pentenePropylamine	1.54
2-Methoxy-4-vinylphenol	3.29
3-Pyridinecarboxamide	1.7
Pyrrolidine	1.38
1-Octen-3-ol Cyclopentanol	1.51
1,6-Anhydro-.beta.-D-glucopyranose (levoglucosan)	1.63
2,5-dioxo-3-methylpiperazine 2(3H)-Furanone	2.35
N2-ethylguanine ETHYL N-O-TOLYL CARBAMATE	4.8
5-Chloro-2-methyl-3(2H)-isothiazolone PIMELIC ACID-CARBOXY-D2 1-Pentanol	1.3
4-Methylproline Mefloquine Pidolic Acid	2.58
(E)-(2,2,2',2'-d4)-2,2',3,3'-tetrahydro-1,1'-bi-1H-indenylidene 2(1H)-Pyridinon	1.86
3-Pyrrolidin-2-yl-propionic acid	2.7
ADENINE; 1H-Purin-6-amine	4.05
Pyrrolo[1,2-a]pyrazine-1,4-dione	6.72
1,2-Cyclopentanedione	2.6
Pyrrolo[1,2-a]pyrazine-1,4-dione	2.53

1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane	3.47
Cyclo(L-prolyl-L-prolyl)	1.56
4-Methoxy-3,5-dihydroxybenzoic acid	1.53
5-Nitroso-2,4,6-triaminopyrimidine	1.43
[1,1'-Biphenyl]-4-ol	2.48
13-Docosamide	5.89

4. Discussion and Conclusion

In this study, we focused on ATS extract, a juice based on Korean traditional medicine, as a potential treatment for human illness. Although the modernization of medicines influences the simplification of curing a disease, it also encourages drug abuse and overdosing. For these reasons, fast advancement of alternative medicines or medication is critical to counteract the effects of western medicine. We have identified 33 constituents of ATS. The major component is 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one(DDMP), which is an active molecule and antioxidant.

Many species of *A. tuberosum*, a perennial herb, are widespread in Korea, Japan, and China. This plant has been cultivated as a condiment vegetable that is highly preferred owing to its characteristic taste and fragrance. In traditional medicine, *A. tuberosum* is considered a therapeutic agent for blood circulation owing to its spicy characteristics and detoxification function. Previous studies have confirmed that *A. tuberosum* contains important nutrients including carotene, vitamin B2, vitamin C, calcium, iron, and the eight main aliphatic sulfur compounds, such as allyl sulfide, pentose and allithiamine many sulfide derivatives, adenosine, alanine, glutamic acid, aspartic acid, valine, amino acid, dimethyl disulfide, and dimethyl trisulfide. A recent research showed the biological activity of *A. tuberosum* from Gimhae and Pohang(Korea) on cancer by analyzing quinone reductase induction activity, superoxide dismutase assessment, and anti-bacterial and anti-oxidant effect. However, the effective ingredients of *A. tuberosum* and their use as cancer-preventive functional foods are worth investigating. Therefore, in this study, we sought to evaluate the application of ATS for combined prescription against C6 glioma migration.

Glioma is characterized by rapid cell growth, migration, and invasion to surrounding normal tissues. Despite advanced treatments, glioma is resistant to surgical operation, chemotherapy, and radiation therapy. It has a high relapse rate, and still has no accurate therapy. In a previous study, Lee et al. suggested blocking the migration of cancer cells as a strategy to inhibit the progression of glioma[13]. Therefore, we performed scratch wound-healing assay to understand the inhibitory effect of ATS against migration, and the results showed that it can significantly reduce the migration of C6 glioma cells.

Furthermore, Lee et al. showed that inhibition of ERK1/2 expression can significantly reduce cancer cell migration[13]. Our results also indicated that ATS significantly reduced the expression of ERK1/2 in vitro. Our data implied that the major role of ATS in cancer cell migration is to regulate the ERK1/2 signaling pathway. MMP-2, the regulating protein for cancer cell migration Migration of C6 glioma cells decreased in the presence of ATS in a dose-dependent manner.

In conclusion, the results of this study suggest that ATS may inhibit cancer cell migration by reducing ERK1/2 phosphorylation. We clearly show the in vitro anti-cancer effect of ATS. Downregulating ERK1/2 phosphorylation not only suppresses migration but also regulates cell proliferation. Therefore, ATS is a potential treatment for human health, and a promising candidate as a therapeutic agent against metastasis of glioma.

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