RESEARCH ARTICLE

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The Selective Cytotoxicity of *Quercus Brantii* Lindl. Galls on A375 and SK-MEL-3 Human Malignant Melanoma Cell Lines

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Abstract

This study aimed to find out the mechanism of cytotoxic effects of galls of *Quercus Brantii* on A375 and SK-MEL-3 melanoma and AGO-1522 normal human fibroblast cell lines for the first time. Therefore, cell viability and cytotoxic activities were evaluated. Furthermore, ROS formation, lipid peroxidation, and release of cytochrome-c were also assessed. The results revealed that the extract of these galls at a concentration of 0.05 mg/ml significantly ($P<0.001$) increased cytotoxicity, ROS formation, TBARS formation, and cytochrome-c release in A375 and SK-MEL-3 melanoma cell lines compared to AGO-1522 normal human fibroblast. These results demonstrated that these galls can be considered a promising candidate which acts in synergy with anticancer agents used in the clinical treatment of human malignant melanoma.

Keywords: Melanoma- *Quercus*- cytotoxicity- skin cancer

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Introduction

Malignant melanoma is a highly malignant and invasive skin tumor, which is the most fatal skin cancer (Habif, 2015). In recent decades, the worldwide incidence of that cancer has increased rapidly. Researches indicate that due to the aging of the population, melanoma prevalence is doubled every 10 to 20 years (Garbe and Leiter, 2009; Liu et al., 2016). Environmental factors including exposure to sunshine (especially type B ultraviolet radiation), occupational and nutritional factors, as well as multiple and abnormal nevi and immunosuppression are melanoma risk factors (Jiang et al., 2015; Noonan et al., 2012). Besides, genetic and epigenetic factors play an important role in this disease (Caini et al., 2017; Chen et al., 2015; Gandini et al., 2005). There are many efforts for reaching effective treatment for melanoma. Routine therapies include surgery, chemotherapy, hormone therapy, radiation therapy, gene therapy, and immunotherapy. Surgery is the first-line treatment for local lesions, especially in the early stage. However, in advanced cases, due to the metastatic nature of this cancer, response to treatment is weak. Melanoma is resistant to common medications such as single-drug chemotherapy regimens and radiotherapy, therefore, suggesting novel, effective, and low-risk therapies (Guy Jr et al., 2015).

The usage of medicinal plants in cancer therapy regimens has always been an interesting topic for research (Cragg and Newman, 2005). However, the herbal compounds and antioxidants in cancer therapy is still an important issue. But research has shown that nutritional advice provided to patients at the right time and under the supervision of a specialist can be somewhat helpful to treatment during the course of the disease and reduce drug side effects (Richardson, 2001; Tonkaboni et al., 2021). Researches have demonstrated the synergistic effects of natural products with various chemotherapeutic medications used to treat cancer (Guney Eskiler et al., 2019; Yurdacan et al., 2019). In a study by Abu Hazafa and his colleagues, the use of herbal compounds and especially polyphenols could be helpful in the treatment of various cancers through molecular mechanisms (Hazafa et al., 2020). Previous studies reported the effectiveness of natural products in the treatment of malignant melanoma (Gladfelter et al., 2017; Iranzadasl et al., 2021).

Quercus Brantii Lindl. (Oak), belongs to the family of Fagaceae. They are medium or short trees in size. There are galls (locally called 'mazoo') that arise on its younger branches which are caused by the gall wasp Adleria gallae-tinctoria offense (Samuelson and Bohlin, 1992). These trees are widespread in provinces like Lorestan and Kordestan in Iran. Tannins, gallic acid, syringic acid, ellagic acid, β-sitosterol, amentoflavone, ether,and isocryptomerin are some of the main gall compounds.

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(Kaur et al., 2004).

Quercus Brantii galls are used for decades in Persian medicine for treating different types of inflammation (Aghili, 2009). Previous researches have been reported that these galls have many pharmacological effects like: astringent, anti-pyretic, anti-parkinsonism (Kaur et al., 2004), local anesthetic, central nervous system depressant, analgesic (Dar et al., 1976; Kaur et al., 2004), anti-diabetic (Hwang et al., 2000; Kaur et al., 2004), anti-bacterial (Rodríguez and Rull, 1980; Sawangjaroen et al., 2004), anti-oxidant (Hamid et al., 2005; Kaur et al., 2008) and anti-inflammatory (Kaur et al., 2004). In this mechanistic cellular study, we wanted to demonstrate whether the hydroalcoholic extract of galls could be helpful in the treatment of human malignant melanoma.

Materials and Methods

Chemicals

2,4,6-Trinitrobenzene sulfonic acid (TNBS) and rhodanine from Sigma-Aldrich Chemie (GmbH, Munich, Germany), thiobarbituric acid (TBA), trichloroacetic acid (TCA), n-butanol, hexadecyl trimethyl ammonium bromide (HETAB), 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ), diphenyl-2-picryl hydrazyl (DPPH), methanol, hydrochloric acid (HCl), malondialdehyde (MDA), ethylenediamine tetra-acetic acid (EDTA), O-di anisidine hydrochloride, hydrogen peroxide, acetic acid, sodium acetate, Coomassie reagent, bovine serum albumin (BSA), ferric chloride (FeCl3 · 6 H2O),sodium sulfate (Na2SO4), sulfuric acid (H2SO4),phosphoric acid (H3PO4), potassium dihydrogen phosphate (KH2PO4), potassium hydrogen diphosphate (K2HPO4), peroxide hydrogen (H2O2) and sodium carbonate (Na2CO3) from Merck (Germany) and galls of *Quercus Brantii* were purchased from Iran.

Preparation of Plant Sample

The galls of *Quercus Brantii* were purchased from the market of medicinal plants in Tehran, Iran, in December 2020. They looked fresh and ground well (Figure 1). The document of the sample was preserved in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Science, Tehran, Iran (No. 6730-TEH).

The dried galls of *Quercus Brantii* were powdered. The ethanolic extract was prepared by mixing the 30 g of mentioned powder with ethanol/water (70:30) mixture (21.8 g dry weight corresponding to 73% w/w) (Alizade Naini et al., 2021).

Cell culture

All procedures were conducted according to the ethical standards and protocols approved by the Experimentation Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran. Human malignant melanoma, A375 cell line, and normal human fibroblast, AGO-1522 cell line, were obtained from the Iranian biological resource center (Tehran, Iran). In this study, The AGO-1522 cell line was used as a healthy control cell line. The mentioned cell lines were grown as a monolayer culture in an RPMI medium supplemented with 1% nonessential amino acids, 1% L-glutamine, 100 IU per mL penicillin, 100 IU per mL streptomycin, 20 mg/mL glutamine, and 10% bovine serum at 37°C in a 5% CO_2 humidified atmosphere and 95% air in a CO_2 incubator. Cells were passaged twice/week under sterile conditions.

Cell lines at the exponential growth phase were washed, trypsinized, and resuspended in a fresh medium. Cells were seeded at a concentration of 104 cells/well in the 96-microtitre plate. The mentioned cells were exposed to various concentrations of the galls of *Quercus Brantii* extracts for 24 h.

MTT assay

The anti-proliferation effect is the first indication to be assessed when investigating novel antitumor agents, thus the cell growth inhibitory activity of plant extract was initially assessed on the melanoma cell line and normal human fibroblast 24 h after treatment with different concentrations of the extract and IC_{50} which 50% of the cells in the plate lost their proliferation and viability, was defined by regression analysis and related models with probit regression model procedure using PRISM program. Then, the cytotoxicity effects of galls of *Quercus Brantii* extract was evaluated on the melanoma cell line in comparison with normal human fibroblast using MTT assay (Alizade Naini et al., 2021).

Lipid peroxidation assay

Cellular lipid peroxidation was investigated by measuring the amount of thiobarbituric acid-reactive substances (TBARS) formed during the decomposition of lipid hydroperoxides. The absorbance was measured spectrophotometrically (Beckman DU-7 spectrophotometer) (sadat Yousefsani et al., 2020).

Reactive oxygen species production

For determination the free radical scavenging activity of galls, dichlorofluorescin diacetate (1.6 μM) was added to cell plates. ROS were determined spectrofluorometrically (Shimadzu RF5000U, Japan) by the measurement of highly florescent DCFH. The results were expressed as fluorescent intensity per 106 cells (Kiani et al., 2017).

Cytochrome-c Release Assay

The concentration of cytochrome-c was determined by using the Cytochrome-c Immunoassay kit provided by R & D Systems, Inc. (Minneapolis, Minn) and the optical density of each well was determined by spectrophotometer set to 450 nm (Waseem et al., 2017).

Statistical Analysis Statistical

Results are reported as mean ± SD. Assays were performed in triplicate and the mean was used for statistical analysis. Statistical significance (all tests) was determined using the one-way ANOVA test followed by the post hoc Tukey. Statistical significance was set at $p < 0.05$.

Results

MTT Assay

Due to MTT assay results, normal fibroblast and melanoma cancer cell lines had acted differently when exposed to the gall extract of *Quercus Brantii* in a dose-dependent manner. The IC_{50} of the gall extract of *Quercus Brantii* for the human melanoma cell line was 0.0523 mg/ml and for the normal fibroblast cell line was 0.719 mg/ml. Therefore, all of the tests were done at 0.0523 mg/ml concentration (Figure 1).

As shown in Figure 2, extract-treated human melanoma cell line after 24 and 48 h showed a significant decrease in cell viability ($p < 0.001$). On the other hand, there was not a significant decrease in the normal fibroblast cell line at this dose and duration (Figure 2).

Lipid peroxidation assay

The addition of *Quercus Brantii* gall extract (0.0523 mg/ml) to human melanoma cell line, significantly increased MDA formation compared to their corresponding control normal fibroblast cell ($P < 0.001$) (Figure 3).

ROS formation

As shown in Figure 4, Quercus brantii extract at a concentration of 0.0523 mg/ml induced significant ROS formation in human melanoma cell line ($P < 0.001$). However, there was not a significant increase in ROS formation in normal fibroblast cell.

Determination of Cytochrome-c Release

As shown in Figure 5, the cytochrome-c release which is the result of the collapse of the mitochondrial membrane potential and disruption of mitochondrial outer membrane integrity occurred in the human melanoma cell line after treatment with *Quercus Brantii* gall extract. But, there was not a significant increase in the release of cytochrome-c in normal fibroblast cells.

Discussion

After heart diseases, cancer still is a moment driving reason for death worldwide (Millimouno et al., 2014). Therefore, this situation put a force on scientists to study and examine new medications for cancer treatment.

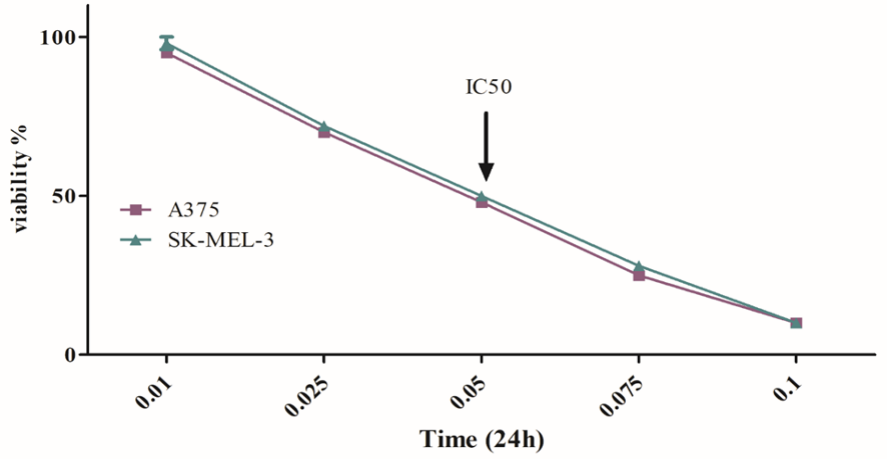

Figure 1. Measurement of IC_{50} Values of *Quercus brantii* Lindl. Gall Extract on A375 and SK-MEL-3 Melanoma Cell Lines.

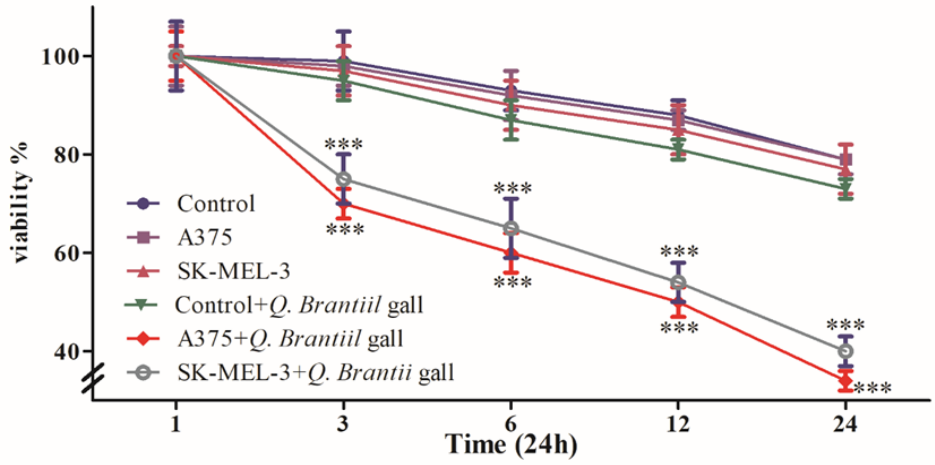

Figure 2. Effect of *Quercus brantii* Gall Extract at the Concentration of 0.05 mg/ml on Induction of Cytotoxicity Using Human Melanoma Cell Lines (A375 and SK-MEL-3) and Normal Fibroblast Cell Line (Control). Cytotoxicity was measured using MTT dye. Values are shown as mean ± SD of 3 separate experiments (n = 3). *** $p < 0.001$, significant difference in comparison with control cells.

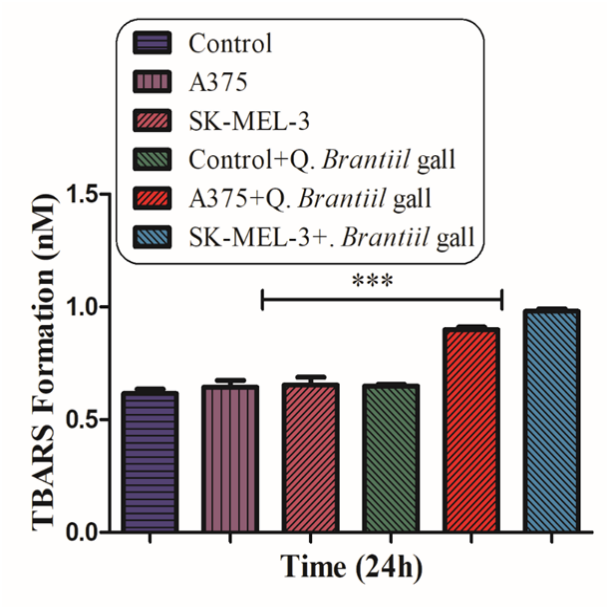

Figure 3. Effect of *Quercus brantii* Gall Extract at the Concentration of 0.05 mg/ml on Induction of Lipid Peroxidation Using Human Melanoma Cell Line (A375 and SK-MEL-3) and Normal Fibroblast Cell Lines (Control). TBARS formation was expressed as µM concentrations. Values are shown as mean ± SD of 3 separate experiments (n = 3). *** $p < 0.001$, significant difference in comparison with control cells.

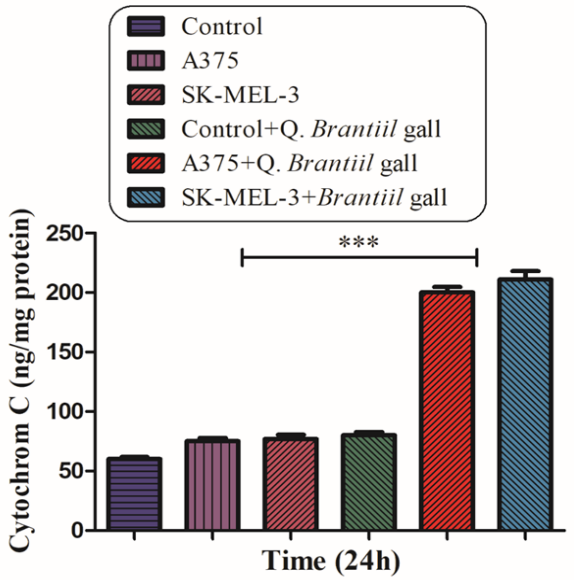

Figure 5. Effect of *Quercus brantii* Gall Extract at Concentration of 0.05 mg/ml on the Cytochrome-c Release Using Human Melanoma Cell Lines (A375 and SK-MEL-3) and Normal Fibroblast Cell Line (Control). Cytochrome-c released was assayed spectrophotometrically (450 nm) by ELISA kit. Values are shown as mean ± SD of 3 separate experiments (n = 3). *** $p < 0.001$, significant difference in comparison with control cells.

Hence, many laboratories start working on natural products and their anticancer effects. Natural products can induce anticancer effects through many mechanisms such as the modulation of cell cycle signaling, neutralizing free radicals, removal of cancerous agents, antioxidant enzyme activity, and cell cycle arrest in human beings (Ramos, 2008). Natural products can modulate the Nrf2 and NF-κB (Gopalakrishnan and Kong, 2008). An in vitro study demonstrated herbal constituents significantly affect the MAPK and PI3K cells that revealing their participation in cancer cell proliferation (Dhillon et al., 2007). Furthermore, Plant constituents like crocin, quercetin, apigenin, genistein, and luteolin can induce apoptosis in different types of malignant cells (Vauzour et al., 2010; YousefsaniBoozari et al., 2021; YousefsaniMehri et al., 2021). Oxidative stress and ROS formation and destructions in DNA are the most important cause that activates the mitochondrial apoptotic pathway, rupturing the mitochondrial membrane which results in the cytochrome-c release (Dhillon et al., 2007; sadat Yousefsani et al., 2020).

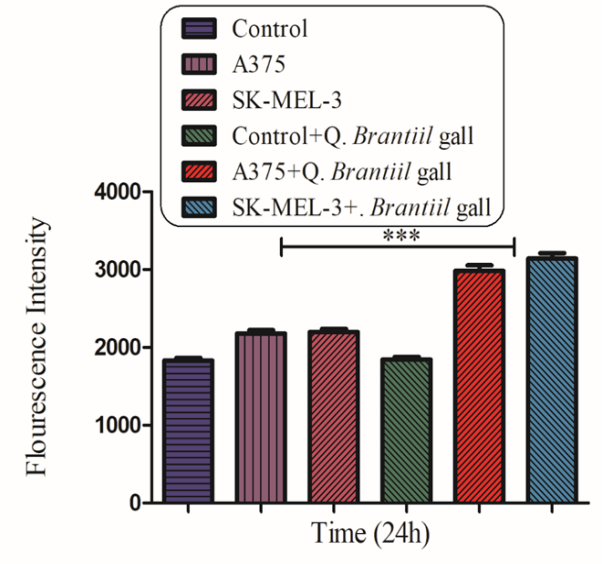

Figure 4. Effect of *Quercus brantii* Gall Extract at the Concentration of 0.05 mg/ml on the Formation of Reactive Oxygen Specious Using Human Melanoma Cell Lines (A375 and SK-MEL-3) and Normal Fibroblast Cell Line (Control). Reactive oxygen specious were evaluated by the measurement of highly fluorescent dichlorofluorescin. Values are shown as mean ± SD of 3 separate experiments (n = 3). *** $p < 0.001$, significant difference in comparison with control cells.

In this study, we evaluated the anticancer effect of *Quercus Brantii* gall extract on the human malignant melanoma. MTT study demonstrated that *Quercus Brantii* gall extract has a beneficial effect on the cytotoxicity of the human malignant melanoma cell line. Furthermore, evaluation of lipid peroxidation shows a significant increase in TBARS formation in the human malignant melanoma cell line. Besides, ROS formation in mentioned cell line showed a significant increase. Finally, the cytochrome-c release which is the result of the collapse of the mitochondrial membrane potential and disruption of mitochondrial membrane integrity was happened in the human malignant melanoma cell line. All these results indicate that the extract has a very good and abundant effect in the treatment of malignant melanoma.

On the other hand, there are some safety concerns

regarding the treatment of melanoma using natural compounds, even when these traditional medicines have been used for thousands of years. But, this study has another important finding. The normal fibroblast cell line was not affected by the *Quercus Brantii* gall extract. This golden finding means that *Quercus Brantii* extract can safely use in patients with malignant melanoma without any worries about possible damage to normal healthy cells. It is suggested that the galls of *Quercus Brantii* have a certain degree of selectivity against malignant melanoma compared with normal skin cells. However, further studies are needed.

There are few studies on the pharmacological effects on galls of *Quercus Brantii* extract. For example, Moradi et al. revealed antioxidant effects of *Quercus Brantii* gall extract (Moradi et al., 2016). Khanavi et al. evaluated the effects of *Quercus Brantii* gall extract on colitis in mice. This study showed high TNF-a, IL-6, and NO inhibitory activity, indicating that the bioactive compounds present in the extract have the potential to reduce inflammation (Khanavi et al., 2014). Furthermore, Alizade Naini et al., (2021) confirmed the curative effect of *Quercus Brantii* gall extract on decreasing ulcerative colitis in rats. The inhibitory effect on MAO and MBA indicates promising healing stimulatory and anti-inflammatory properties as well as antioxidant effects which make it an appropriate agent for the treatment of ulcerative colitis. In a study by Karimi et al. total phenolic, flavonoid, and flavonol content was identified which revealed that this plant is a rich source of these agents (Karimi and Moradi, 2015). Polyphenols including gallic acid are responsible for the main antioxidant and anti-inflammatory effect of galls of *Quercus Brantii*. The antioxidants and anti-cytokines usually prevent complications or further progress of the inflammatory disease (Karimi and Moradi, 2015).

In addition, there are certain limitations to this study. The anti-melanoma effect of galls of *Quercus Brantii* was only evaluated in one human melanoma cell line, A375, and thus other skin cancer cells will be using to further explore the therapeutic effect of galls of *Quercus Brantii* in subsequent studies.

In conclusion, natural products especially herbal products are important in cancer therapy because of their reachability, safety, innocuity, inexpensiveness, proficiency, and being hostile to malignant capacity. Phytochemicals have revealed a significant therapeutic role in various malignancies, however, natural polyphenol compounds (specially flavonoids) represent a broad and assorted group in different types of cancers and even other diseases (Hashem-Dabaghian et al., 2022; Hazafa et al., 2020; Jamshidi et al., 2020; Kenari et al., 2021). Natural polyphenols have their own anticancer efficacy through various pathways. For instance, cancer prevention agent enzymatic movement, cellular cycle arrest (S/G2, G1, S, and G2 stages), by regulation of the Nrf2 and NF-κB cells, which prevent the cellular proliferation, apoptosis, MAPK signaling pathway, etc. The anticancer effects of natural polyphenols could be changed by doses, cancer types, and cell lines. The clinical trials are limited to these natural compounds.

In conclusion, it is therefore strongly recommended to examine useful medicinal herbs like full of antioxidants galls of *Quercus Brantii* in the clinic concurrent with standard therapies to determine their benefit in patients who suffered from cancer. As it is clear, the synthesis of new formulations of natural phenolic compounds which is well-targeted and well-designed can lead to the development of clinically beneficial medications with efficiency and selectivity.

Author Contribution Statement

Jalal Pourahmad and Bahareh Sadat Yousefsani contributed to this research in formulating the research question (s), designing the study, carrying it out as thesis supervisors, analyzing the data, and writing paper. Kobra Shirani, Ali Qobadi and Elahe Aghazadeh contributed to this research in supervising some experiments and analyzing the data and giving scientific advice. Kamyar Mohajer contributed to this research in carrying out the experiments and performing statistical analysis as the thesis student.

Acknowledgements

This study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences with the ethical code: IR.SBMU.PHARMACY.REC.1399.404. The data provided in this article was extracted from the Pharm.D. thesis of Dr. Kamyar Mohajer.

Data Availability

Data presented in this study is available upon request.

Conflict of Interest

All authors declared that there is no conflict of interest.

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