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Full Length Research Paper

Cytotoxic activities of *Ferulago angulata* extract on human leukemia and lymphoma cells by induction of apoptosis

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***Ferulago angulata* (FA), also known as Chovir, is one of the indigenous plants used in Persian medicinal herbs. Although, recent studies have demonstrated anti-tumor activities on cancer cells *in vitro*, but the exact mechanism is not completely clarified. Furthermore, the objective of this study was to examine the *in vitro* cytotoxic activities of methanol extract of *F. angulata* on B-cell lymphoma (Raji), human leukemic monocyte lymphoma (U937), human acute myelocytic leukemia (KG-1A) and** human umbilical vein endothelial (HUVEC) **cell lines by a micro-culture tetrazolium test (MTT) cytotoxicity and trypan blue assays. Cell death enzyme linked immunosorbent assay (ELISA) was applied to quantify the nucleosome production subsequent nuclear DNA fragmentation during apoptosis and to test whether the mechanism involves induction of apoptosis or not. Results showed that methanol extract at 50 to 800 µg/ml time and dose-dependently suppressed the proliferation of Raji, U937 and KG-1A cells by more than 80% ($p < 0.01$), with ascending order of IC_{50} values in 24 h: Raji (121.404 µg/ml), U937 (208.019 µg/ml) and KG-1A (211.011 µg/ml). The extract did not exert any significant cytotoxic effect on normal cell line HUVEC (IC_{50} > 800 µg/ml). Nucleosome productions in Raji, U937 and KG-1A cells were significantly increased upon the treatment of *F. angulata* extract. *F. angulata* extract was found to selectively time and dose-dependently inhibit the proliferation of lymphoma and leukemic cells probably via an apoptosis-dependent pathway.**

Key words: *Ferulago angulata*, cytotoxic, apoptosis, antitumor, lymphoma, leukemia.

INTRODUCTION

Historically, medicinal plant has been used for maintaining heath, boosting immune system function, prevention, therapy and remission of cancer in Persian traditional medicine. By introducing successful bioactive component of anticancer drug with minor side effect on normal cells and high tumor selectivity, natural product could serve as chemopreventive and chemotherapeutic agent (Mehta et al., 2010). Many traditional medicines have remarkable biological activities with potential therapeutic application. Recently, plant-derived chemo-therapeutic drugs such as taxol from *Taxus brevifolia* L., camptothecin from *Camptotheca acuminate*, Decne, vinca alkaloids from *Catharanthus roseus* G. Don and podophyllotoxin from *Podophyllum peltuturn* L. are extensively used in clinical trial (Taran et al., 2010). Ferulago is a main species of Apiaceae family. There are 35 species of this plant in the world, seven of which are

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found in Iran, including *contracta*, *angulata*, *macrocarpa*, *galbanifera*, *trachycarpa*, *phialocarpa* and *carduchrom*. Except for Iran, *Ferulago angulata* (FA) grows in other countries like Turkey, Greece, Serbia and Macedonia. *F. angulata* extract composed of ferulagone, β-hydroxy-13-epi-manoyl oxide, α-pinene, 2,5-dimethoxy-p-cymene, p-cymene, methyl carvacrol, transchrysanthenyl acetate, γ-terpinene (Z)-β- ocimene, α-pinene, myrcene, (Z)-β-ocimene, terpinolene, 2,4,5 trimethylbenzaldehyde and α-phellandrene predominantly (Ghasempour et al., 2007). This plant has been used ethnically as sedutive, tonic, and remedy of digestive pains, aphrodisiac properties, chronic ulcers, snakebites and headache (Sodeifian et al., 2011).

Apoptosis is a physiological mechanism of cell death that rapid reductions in the cellular volume followed by chromatin condensation associated with characteristic inter nucleosomal DNA cleavage. This results in the production of nucleosomes of DNA fragments complexes with core histones, which are discrete multiples of a 180 to 200 bp subunit (Elmore, 2007). One of the major modes of action of chemotherapeutic anti-cancer drugs on malignant cells is via the induction of apoptosis (Reed and Pellecchia, 2005).

Taking these into considerations, the traditional usage suggested that *F. angulata* is a potential candidate as a cytotoxic agent for anti-cancer therapy. The objective of this study was to examine the *in vitro* cytotoxic activity of a crude methanol extract of *F. angulata* on the in lymphoma and leukemic cell lines, using a 3-(4, 5-dimethylthiazolyl)-2, 5-diphenyl-tetrazolium bromide (MTT) cytotoxicity assay. This study also tested whether the mechanism of action involves induction of apoptosis or necrosis. Cell death enzyme linked immunosorbent assay (ELISA) was used to quantify the nucleosome production resulting from nuclear DNA fragmentation during apoptosis.

MATERIALS AND METHODS

Preparation of plant extract

F. angulata (FA) plants were collected from the western parts of Iran (Kermanshah). Mr Ajani from the Department of Botany, Institute of Medicinal Plants (IMP) of Karaj, Iran identified the plant. A voucher specimen was deposited in the herbarium of the Department of Botany, Institute of Medicinal Plants (IMP) of Karaj, Iran. The aerial parts of the plant were separated, shade dried and ground into powder using mortar and pestle. The prepared powder was kept in tight containers protected completely from light. Extraction of methanolic extract was carried out by macerating 100 g of powdered dry plant in 500 ml of 70% methanol for 48 h at room temperature. Then, the macerated plant material was extracted with 70% methanol solvent using percolator apparatus (2 L volume) at room temperature. The plant extract was removed from percolator, filtered through Whatman filter paper (NO.4) and dried under reduced pressure at 37°C with rotator evaporator before being added to methanol as the solvent. The methanol extract was filtered and concentrated by a rotary evaporator and then evaporated to dryness. Briefly, the concentrated plant extracts were dissolved in dimethyl sulphoxide (DMSO) (SIGMA, USA) to get a stock solution of 10 mg/ml. The sub-stock solution of 0.2 mg/ml was prepared by diluting 20 µl of the stock solution into 980 µl serum-free culture medium, RPMI 1640 (the percentage of DMSO in the experiment should not exceed 0.5). The stock and sub-stock solutions were both stored at 4°C.

Cell cultures

Burkitt's lymphoma B-cell line (Raji), human leukemic monocyte lymphoma cell line (U937), human acute myelocytic leukemia cell line (KG-1A) and human umbilical vein endothelial cells (HUVEC) were purchased from Pasteur Institute of Iran (BANK CELL). The cells were grown and maintained in a humidified incubator at 37°C and in 5% CO_2 atmosphere. RPMI-1640 medium (SIGMA, USA) supplemented with 0.01 mg/ml heat inactivated fetal calf serum (FCS), 100 µg/ml penicillin, and 100 µg/ml streptomycin (ALL PREPARED FROM INVITROGEN GIBCO) was used for cell cultures. Upon reaching appropriate confluence, the cells were passaged. After being harvested from sterile T75 culture flasks (NUNC, DENMARK), the cells were counted using a hemocyto-meter and cell viability was determined by trypan blue exclusion. Ten thousand cells from log phase cultures were seeded in 100 µl of RPMI medium supplemented with 10% fetal bovine serum per well of 96-well flat-bottom culture plates (NUNC, DENMARK). Cells were incubated with the *F. angulata* extract for a defined time (12, 24 and 48 h). Proliferative response and cell death of the *F. angulata* extract-treated cells were determined using MTT assay and cell death ELISA and cell viability assay, respectively (Phelan, 1998).

MTT cell viability assay

The assay detects the reduction of MTT (SIGMA, USA; a colorimetric technique) by mitochondrial dehydrogenase to blue formazan product, which reflects the normal function of mitochondria and hence for measuring the cytotoxicity cell and viability. 1×10^4 viable cells/well were plated into the 96-well tissue culture plates (NUNC, DENMARK), and then incubated at 37°C overnight. The next day when cells reached >80% confluence, the media were replaced with 200 ml of fresh complete medium containing 0 (as control), 50, 100, 200, 300, 400, 500, 600, and 800 µg/ml concentrations of crude extract; no extract was added to the negative control well. After 12, 24, or 48 h, the supernatants were removed and cell layers were washed with phosphate buffered saline (PBS, INVITROGEN GIBCO) and incubated with MTT (50 ml and 0.5 mg/ml) in RPMI 1640 without FCS for 4 h in a humidified atmosphere at 37°C according to the manufacturer's protocol. The cell cultures were centrifuged at 1000 g for 5 min and the supernatants were discarded. Subsequently, 200 ml of DMSO (SIGMA) and 25 ml Sorenson buffer were added to dissolve the formazan crystals formed. The optical density (OD) colored solution was quantified at 570 nm wavelengths by using an ELISA reader (BIO-RAD). The absorbance of untreated cells was considered as 100%. Each extract and control was assayed in triplicate in three independent experiments. The concentration of the crude extract that killed 50% of the cells (IC_{50}) was calculated by EXCEL software and the percentage of cell viability was calculated according to the following equation:

The percentage of cell viability = OD of treated cells/OD of control cells×100

or

Viability (%) = 100 - % Cytotoxicity (Mosmann, 1983).

Table 1. Concentration producing 50% growth inhibitions (IC_{50}) of *F. angulata* extract on the 3 cell lines in 12 h.

Cell line	IC_{50} (µg/ml)
Raji	188.383
U937	297.051
KG-1A	293.351

Table 2. Concentration producing 50% growth inhibitions (IC_{50}) of *F. angulata* extract on the 3 cell lines in 24 h.

Cell line	IC_{50} (µg/ml)
Raji	121.404
U937	208.019
KG-1A	211.011

Table 3. Concentration producing 50% growth inhibitions (IC_{50}) of *F. angulata* extract on the 3 cell lines in 48 h.

Cell line	IC_{50} (µg/ml)
Raji	96.814
U937	158.684
KG-1A	128.475

IC_{50} values were expressed as the mean SD, determined from the results of MTT assay in triplicate experiments.

Dye exclusion assay

Cellular cytotoxicity induced by the *F. angulata* extract treatment was measured by trypan blue exclusion assay. Briefly, 1×10^4 cells were seeded into 96-well plates and treated with or without (as control) crude extract at specified doses for 12, 24 and 48 h. After the incubation period, the cultures were harvested and wash twice with PBS. The cell pellet was then suspended with 0.5 ml PBS. Then, 20 µl of cell was mixed with equal volume of 0.4% trypan blue (SIGMA, USA) and was counted using Neubauer haemocytometer (WEBER, ENGLAND) by clear field microscopy (NIKON, JAPAN). The number of viable cells was calculated according to the following formula:

The unstained cell count (viable cells) × the dilution of the cell suspension × 10^4]/the number of hemacytometer squares that were counted.

Percent viability was calculated as: [viable cells/the total cell count] × 100.

Cell death detection

Cell death detection ELISA^{PLUS} (ROCHE APPLIED SCIENCE) was used to quantify histone-complexed DNA fragments (nucleosomes) in cytoplasm of the apoptotic cells after induction of apoptosis. Briefly, after incubation with the methanol extract (at concentrations determined by MTT assay) for 24 h cells were pelleted and lysed. Mouse monoclonal antibodies against single-strand DNA and histones (H1, H2a, H2b, H3 and H4) specifically detected and bound mononuclesomes and oligonucleosomes derived from cells undergoing apoptosis. Biotinylated anti-histone antibodies were then fixed in the antibody-nucleosome complexes to the streptavidin-coated microtiter plate. The anti-DNA antibodies were conjugated with a peroxidase that reacted with the substrate 2,2V-azino-di (3- ethylbenzthiazolin-sulfonate) (ABTS) to form a colored product. The remaining steps were carried out according to the instructions supplied by the manufacturer. The resulting color development, which was proportional to the amount of nucleosomes captured in the antibody sandwich, was measured at 405 nm wavelength by a Benchmark microtiter plate reader (BIO-RAD). Results were expressed as the apoptotic index, calculated from the ratio of absorbance of treated (apoptotic) sample to that of the untreated (control) sample (Frankfurt and Krishan, 2001).

Statistical analysis

The data are expressed as mean ± standard deviation (SD) for at least three independent determinations in triplicate for each experimental point. The data were analyzed using IBM SPSS Statistics 20 software. For all the measurements, Two-way analysis of variance (ANOVA), followed by Duncan's New Multiple Range Test ($P\leq0.05$) were used to assess the statistically significance of difference between control and *F. angulata* treated.

RESULTS

Effects of methanolic *F. angulata* extract on proliferation of leukemia and lymphoma cells

The cytotoxic effects of *F. angulata* methanol extracts on the growth of Raji, U937, and KG-1A cells were determined by MTT. As shown in Figure 1, the treated cells with *F. angulata* extracts in comparison with the untreated control cells exhibited significant drop in cell viability. *F. angulata* extract at 50 to 800 µg/ml exhibited significant time and dose-dependent inhibitory effects on the proliferation of Raji (A), U937 (B), and KG-1A cells (C), with more than 80% suppression. However, the extract induced no significant suppression on the proliferation of normal HUVEC cells (D).

Data analysis of cytotoxicity assay as shown in Tables 1, 2 and 3 revealed the concentrations producing 50% growth inhibition (IC_{50}) of the *F. angulata* extract on the 3 cell lines in 12, 24 and 48 h; Raji proliferation was most potently suppressed with the lowest IC_{50} value. Similar inhibitory effects were found in U937 and KG-1A cells after incubation with the *F. angulata* extract. Proliferation of Raji cells was most significantly reduced by *F. angulata* extract at 100 to 300 µg/ml, resulting in a lower IC_{50} value; while U937 and KG-1A cell growth was most significantly reduced from 200 to 400 µg/ml with higher IC_{50} values.

Direct counting for dead and viable cells using the trypan blue exclusion test displayed that the percentage of viable Raji cells decrease sharply (from 84 to 18.3%, 68 to 10.6% and 65 to 9%). However, the percentage of

viability of U937 (from 87 to 24%, 77 to 20% and 70 to 14%) and KG-1A cells (from 85 to 16%, 81 to 12.6% and 67 to 9.6%) declined at 12, 24 and 48 h from 50 to 800 µg/ml concentration, respectively.

Effects of *F. angulata* extract on cell death of lymphoma and leukemia

As determined by MTT assay, *F. angulata* extract at 100, 200 and 300 µg/ml at concentrations derived from IC_{50} in 24 h were chosen for each cell line in cell death detection ELISA. The possible mechanism was via induction of apoptosis, as evidenced by the significant increase in nucleosome production at 100 to 300 µg/ml of FA extract after incubation for 24 h, but the ratio of apoptosis was constant approximately 40±3% (Figure 2).

DISCUSSION

Historically, ethno-medicine has been used for maintaining heath, boosting immune system function, moreover prevention, therapy and remission of cancer. Natural product can serve as chemoperventive and chemotherapeutic agent, because of minor side effects on normal cells in clinical trial (Mehta et al., 2010). Recently, extensive studies have been dedicated to the apoptosis and the role of this process in intervening the lethal properties of anti-neoplastic agents in cancer cells. Anticancer agents induce apoptosis, so that disruption of apoptotic cell death will reduce treatment sensitivity. Extensive varieties of natural compounds possess significant cytotoxic as well as chemoperventive activity, which act via apoptosis.

This study has demonstrated that methanol extract of *F. angulata*, a commonly used Persian medicinal herb in natural form, could significantly suppress the proliferation of Raji, U937, and KG-1A *in vitro* by means of the MTT assay. Such antiproliferative activity of *F. angulata* extract was characterized by the time and dose-dependent and tumor-selective manner, as reflected by the comparatively low IC_{50} values and the absence of significant effects on normal HUVEC cells, respectively (Figure 1).

In Raji, U937 and KG-1A, cell lines viability were decreased (from 72 to 12%, 78 to 19% and 77 to 12.7%) by increasing treatment concentration from 50 to 800 µg/ml and (from 48 to 37%, 56 to 45% and 56 to 41%) treatment time at 12, 24 and 48 h, respectively.

Direct counting of dead and viable cells using the trypan-blue exclusion test showed that the percentage of viable Raji cells decrease sharply (from 84 to 18.3%, 68 to 10.6% and 65 to 9%). Furthermore, the percentage of viable U937 (from 87 to 24%, 77 to 20% and 70 to 14%) and KG-1A cells (from 85 to 16%, 81 to 12.6% and 67 to 9.6%) declined at 12, 24 and 48 h from 50 to 800 µg/ml concentration, respectively.

In order to determine whether the antiproliferative activity of *F. angulata* extract was induction of apoptosis or not, cell death detection ELISA was used to quantify the nucleosome production during nuclear DNA denaturation of apoptotic cells. For Raji cells, the sharp increase in percentage of dead cells via apoptosis had partly constant variation about 40±3%. Similar results were seen in KG-1A and U937 cells (Figure 2).

This study demonstrated that *F. angulata* extract, did not display any inhibitory effect on proliferation of normal HUVEC cell line after 48 h incubation; so results suggest that the extract exert tumor-specific activity on these leukemia and lymphoma cells via an apoptosis-dependent pathway. To our knowledge, there was no previous study on cytotoxic effect of *F. angulata* methanol extract against lymphoma and leukemic cells. Nevertheless, in previous studies on leukemia and lymphoma cells, Sheng et al. (1998) reported that water extract of *Selaginella tamariscina* induced apoptosis mediated cytotoxicity in human leukemic cell lines HL- 60, K-562 and human EBV-transformed B-lymphoma cell line Raji. Amirghofran et al. (2006) reported that medicinal herbs *Linum persicum* and *Euphorbia cheiradenia* showed anticancer effect and induction of apoptosis on leukemia cell lines including K562 and Jurkat cell lines. Roya et al. (2008) reported the apoptogenic activity of Litchi chinensis leaf extract (LCLE) against three human leukemic cell lines-U937, K562 and HL-60.

They showed the LCLE inhibition cell growth and metabolic activity of the leukemic cells. In our study, the nucleosome concentration of apoptotic cells was directly measured by antibody-antigen interactions using ELISA that is a quantitative method with high specificity and sensitivity (Drexler et al., 1995). Therefore, further investigations are required to evaluate whether cell cycle arrest or apoptosis induction contributes more to the *F. angulata* extract-induced cytotoxic effect on panel cells. In order to clarify the cytotoxic activities of *F. angulata* extract on the growth of leukemia and lymphoma cells of other sub-types, acute T-cell leukemia cell line and Hodgkin's lymphoma may become the target cells used in our future studies. In addition, mechanistic studies on cell cycle arrest and early apoptotic events may be conducted to define other possible antitumor mechanisms of the *F. angulata* extract. Besides, future *in vivo* antitumor studies will be performed in order to confirm these *in vitro* results.

Conclusion

This study provides the evidence which confirm *in vitro* cytotoxic activity of methanol crude extract from wild *F. angulata*. Methanol crude extract of *F. angulata* demonstrated tumor-selectively, dose-dependent and time-dependent inhibitory effect on proliferation of lymphoma and leukemic cells possibly via an apoptosis- dependent pathway.

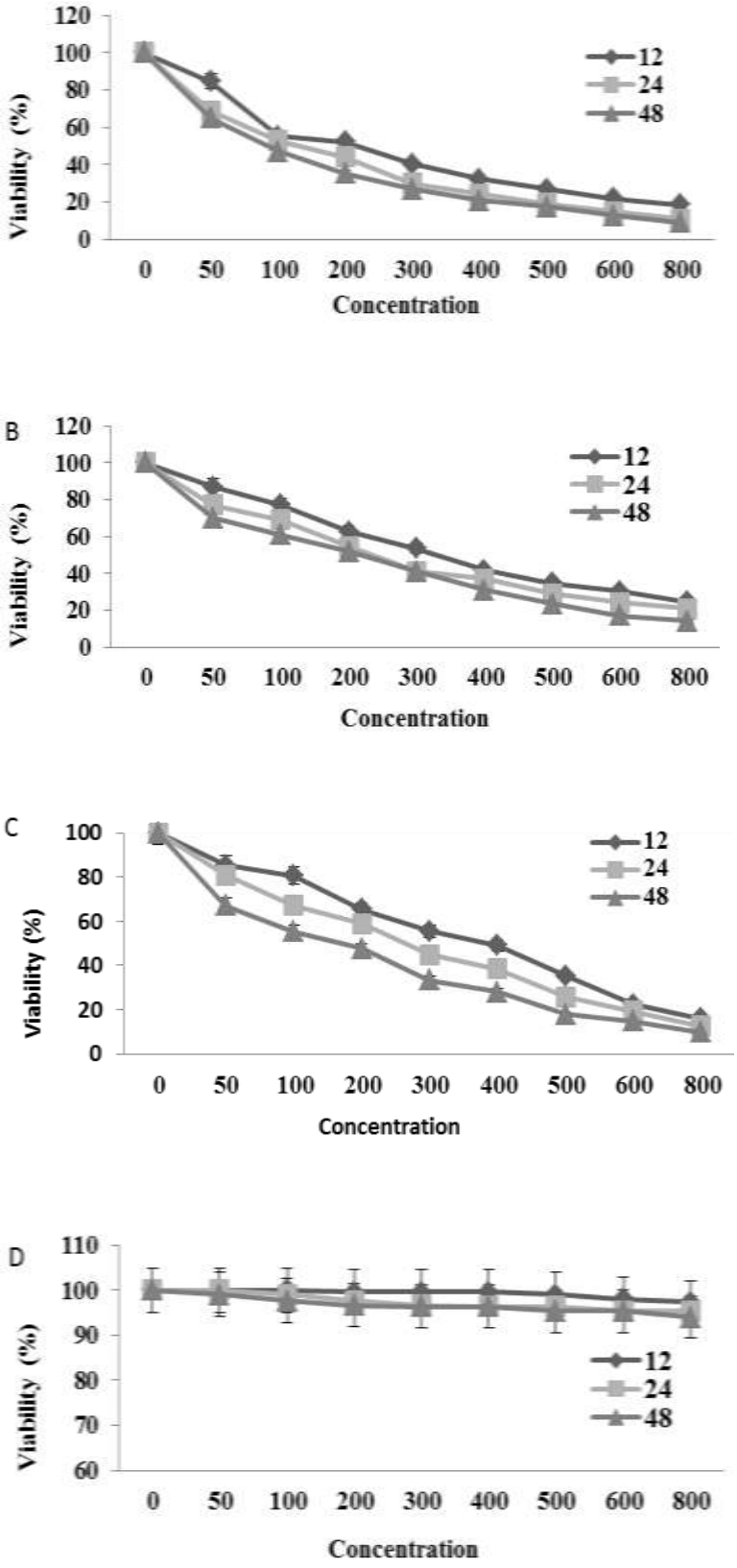

Figure 1. Effect of different concentrations of *F. angulata* (A) Raji, (B) U937, (C) KG-1A and (D) HUVEC cell lines in 12, 24 and 48 h. Values represent the mean of three experiments. Standard deviations were less than 0.8.

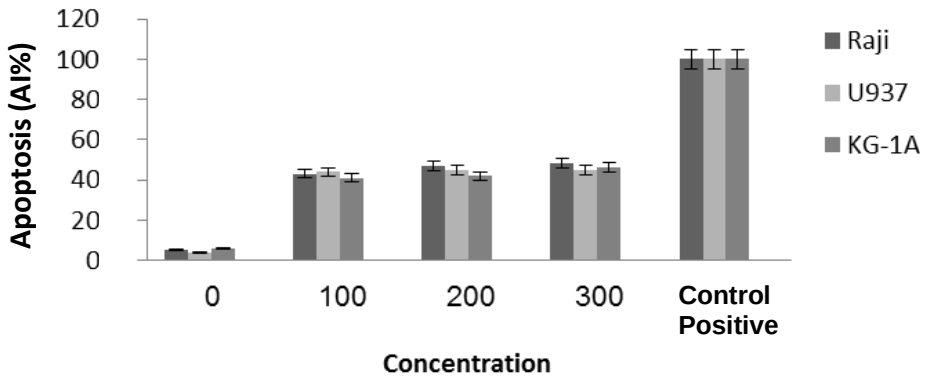

Figure 2. Effects of FA extract on cell death of Raji, U937 and KG-1A. Cells were incubated with the *F. angulata* extract in culture medium at concentrations derived from IC_{50}, 100, 200 and 300 µg/ml, or culture medium alone for 24 h.

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