



## ORIGINAL ARTICLE

# Synthesis and Cytotoxic Evaluation of 2-Pyrazoline Derivative on Leukemia Cancer Cell Line K562

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**ABSTRACT:** The 2-pyrazoline derivatives have a wide range of biological effects, such as anti-viral, anti-bacterial, anti-fungal, anti-depressant and anti-cancer effects. Studies have shown that compounds containing 2-pyrazoline along with another heterocycles may show more effective biological properties. In this study, a 2-pyrazoline derivative with a spiro-indenoquinoxaline ring at C3 position was synthesized by one-pot microwave-assisted method and its chemical structure was confirmed by <sup>1</sup>H NMR spectroscopy. The cytotoxic effects of the compound were evaluated on the K562 cell line and phytohemagglutinin-activated peripheral blood mononuclear cells (PHA+PBMC) by MTT assay. Additionally, the cytotoxic effects of cisplatin on these cells were investigated and compared with those of 2-pyrazoline. The IC<sub>50</sub> values obtained from the 2-pyrazoline derivative effects on the K562 cell line and PHA+PBMC cells were 45 and 55 µg/mL respectively, while cisplatin inhibited proliferation of the same cells with IC<sub>50</sub> value 1.71 and 7.8 µg/mL respectively. The results of this study showed that the synthesized derivative had a cytotoxic activity on the K562 cancer cell line at higher concentrations than cisplatin.

**INTRODUCTION**

Cancer is a progressive disease and includes a variety of malignant tumors that are called neoplasms in medical terminology. After cardiovascular disease, cancer is the second leading cause of death, accounting for around 12% of deaths worldwide. Generally, cancer-causing agents are divided into four categories: environmental factors, heritable factors, immunological factors, and age. Cancer spreads through tissue, lymph and blood in the body, and can cause tumors elsewhere in the body through metastasis [1]. According to the statistics of Iranian Cancer Society, about 85,000 cancer cases are reported annually in IRAN, including more than 30,000 deaths in this country. There is a risk of cancer occurring at different ages, and as the age increases, the risk of developing it increases.

The cancer treatment methods vary according to the type of cancer, disease progression and age of the disease, but among the existing therapies, chemotherapy, radiotherapy and surgery are the main methods of cancer treatment. The chemotherapy is widely used in cancer treatment and is the use of various medications to treat, control, or relieve the symptoms of cancer, depending on the type of cancer and its degree of progress. Most chemotherapy drugs affect cell division and proliferation via control of mitosis and DNA replication and transcription. Two goals that are considered in the synthesis of chemotherapy drugs are that the drug, in addition to having cytotoxic properties and detecting and destroying cancer cells, has the least effect on the normal cells. One of the most commonly used drugs in cancer

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chemotherapy is the cisplatin. Unfortunately, cisplatin is toxic for normal cells and kidneys in particular. One of the scientists' goals is to synthesize new alternative drugs to reduce the toxic effects of chemical drugs on normal cells and increase cytotoxic effects on cancer cells [2, 3].

In recent years, heterocyclic derivatives have been notably considered by researchers because of their diverse biological and medicinal properties. Then, a large amount of research works on organic chemistry is devoted to the synthesis, identification and evaluation of heterocycles. The most important heterocyclic compounds contain nitrogen, oxygen, or both together. Many natural compounds, as well as important pharmaceuticals such as penicillin, are heterocyclic compounds. One of the most important nitrogen-containing heterocycles is the 2-pyrazolin compound. The 2-pyrazoline derivatives are considered by synthetic and pharmaceutical chemists because of their ease of synthesis as well as the diverse biological properties. Many of the 2-pyrazoline derivatives have shown antitumor and antiproliferative properties [4-9].

Quinoxaline derivatives are cyclic compounds include two nitrogen atoms that are highly biologically important due to their diverse applications such as activity against different tumors. Also, quinoxalines act as active pharmaceuticals with antibacterial, antifungal, anticancer, antileishmanial, antimalarial and antidepressant properties [10].

The aim of this study was to synthesize 2-pyrazoline derivative containing the spiro-indenoquinoxaline ring at the C3 position of 2-pyrazoline ring, and to evaluate the cytotoxic effect on the K562 cancer cell line and PBMCs by MTT assay. The cytotoxicity of cisplatin was also studied as an anticancer drug at various concentrations and the results compared with the cytotoxicity of the 2-pyrazoline compound in the same condition.

## MATERIALS AND METHODS

### *One-pot microwave-assisted synthesis of 2-pyrazoline derivative*

Indenoquinoxaline derivative (0.04 mole or 10.4 g) and acetophenone (0.04 mole or 4.8 g) were reacted in an Erlenmeyer flask in the presence of 12 drops of dimethylamine in a solvent-free condition and at room temperature for 45 minutes and a light yellow precipitate was formed. Then, 40 mL of glacial acetic acid was poured onto

the precipitate and 1 mL of concentrated HCl was added and the reaction carried out under 100W microwave irradiation for 5 minutes. The hydrazine hydrate 99% (0.042 mole or 12 mL) was added to this mixture and the irradiation was continued for 5 minutes. The 2-pyrazoline derivative was afforded as a yellow compound and the reaction mixture was cooled and filtered and the separated compound recrystallized from an ethanol-water (30%/70%) solvent.

### *Spectral analysis*

The chemical structure of the 2-pyrazoline derivative was elucidated by  $^1\text{H}$  NMR spectroscopy. The spectrum of this compound was recorded with the NMR device (Bruker DRX-300 Avance model) at a frequency of 300 MHz and the deuterated chloroform ( $\text{CDCl}_3$ ) was used to dissolve the sample and tetramethylsilane (TMS) as internal standard was added.

### *Preparation of drug solutions*

The drug (2-pyrazolin derivative) was prepared in dimethylsulfoxide (DMSO) (from Sigma-Aldrich) at a concentration of 1000  $\mu\text{g}/\text{mL}$ . Then, in order to remove the effect of DMSO toxicity, the dilution step was performed in a DMEM (Dulbecco's Modified Eagle Medium) medium (from Gibco) with 10% fetal bovine serum (FBS, Gibco) and the samples at 100, 10, 1, 0.1, 0.01  $\mu\text{g}/\text{mL}$  concentrations were prepared and each diluted solution was filtered with a 22  $\mu\text{m}$  (Millipore) filter to remove bacterial and fungal contaminations. Stock MTT was prepared in phosphate buffer saline (pH=7.4) at a concentration of 5  $\mu\text{g}/\text{mL}$  and then filtered.

### *Cell line and cell culture*

The K562 cell line (Iranian Cell Bank, C122) were cultured in DMEM medium containing 100 U/mL penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin, 2 mM glutamine and 10% FBS. The amount of carbon dioxide of incubator was 5% and its temperature was 37°C. The PBMC cells were separated from healthy volunteer's whole heparinized blood by centrifugation with density gradient by Lymphoprep (from Nycomed Company). The isolated cells were washed three times with phosphate buffer saline, and then they were counted with a homogeneity meter and trypan blue color, and counted with contrast phase microscopy.

### Methyl thiazolyl diphenyl-tetrazolium bromide assay (MTT assay)

After examination of the cells in terms of bacterial, fungal or mycoplasma contamination, the cells were counted by trypan blue and 10,000 K562 cells in complete culture medium and 200,000 cells of PBMC in a complete culture medium containing 2.5 µg/mL phytohemagglutinin (PHA) was sprayed separately in each 96-well plate. After incubation for 2 hours, the drug solutions were added to each well. In some of the wells, as the control, only the culture medium was added, and in a number of other comparisons, the cisplatin reference drug was added. After 72 hours of incubation, the MTT color was added to each well at a concentration 0.5 µg/mL. Four hours after incubation, the purplish-colored crystals of formazan were formed by the regeneration of tetrazolium yellow salt MTT by enzymes present in the mitochondria. The supernatant was then removed and 100 µL DMSO was added to dissolve the crystalline crystals. Finally, the OD absorption rate was measured by ELISA plate reader (Stat Fax-2100 model) at wavelengths of 570 nm.

All experiments were repeated three times, and the percentage cytotoxicity and cell viability was calculated by following formula:

$$\% \text{viability} = \frac{A - B}{C - D} \times 100$$

Where;

A: absorbance of treated cells (drug)

B: absorbance of blank (media)

C: absorbance of control (untreated)

D: absorbance of blank (media)

$$\% \text{cytotoxicity} = 100 - \% \text{cell survival}$$

### Analysis of MTT assay data

The anticancer activity of 2-pyrazoline derivative was tested on the K562 cancer cell line by MTT method. In order to compare the selectivity of the compounds between healthy and cancerous cells, the effect of this compound on extracted PBMC cells from healthy individuals was evaluated. The results of MTT assay analyzed by Graph Pad Prism software version 4 and IC50 calculated.

## RESULTS AND DISCUSSION

### Reaction mechanism of 2-pyrazoline synthesis

The acetophenone **1** and the indenoquinoxaline derivative **2** were reacted in the presence of dimethylamine. Then, the reaction was continued in acetic acid and HCl by microwave irradiation and after dehydration reaction, the yellow chalcone **3** was formed. The chalcone reacted with hydrazine hydrate under microwave irradiation in a one-pot procedure and the 2-pyrazoline-spiro-indenoquinoxaline derivative **4** was produced. Figure 1 shows the synthesis steps of this spiro compound.

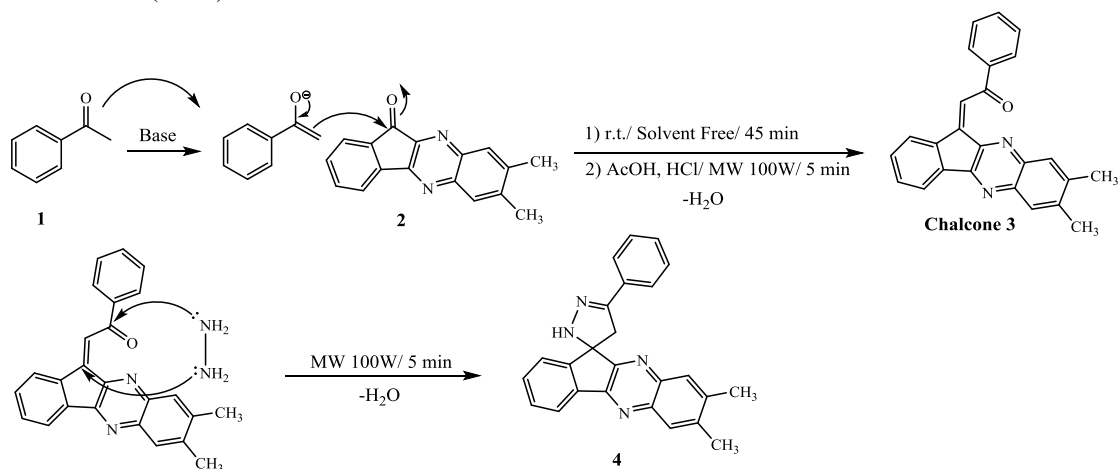
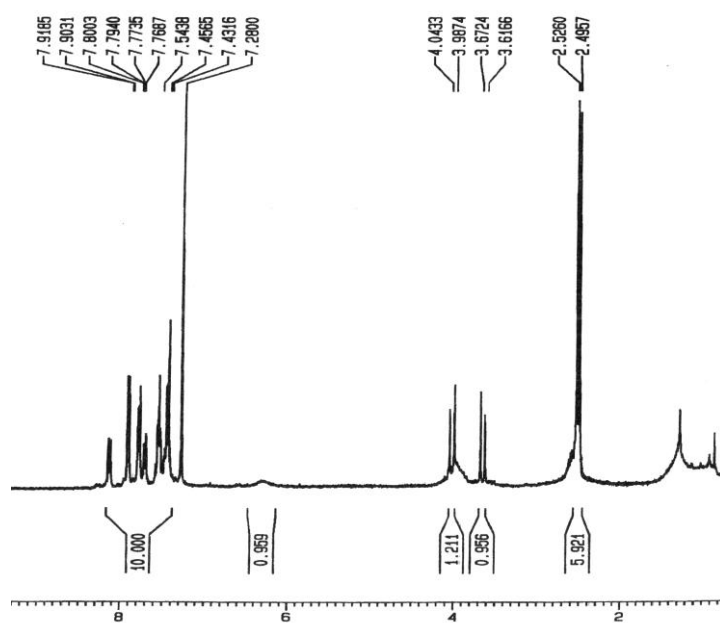


Figure 1. Reaction mechanism for the synthesis of 2-pyrazoline derivative.

**Chemical structure determination by  $^1\text{H}$  NMR spectroscopy**

In the  $^1\text{H}$  NMR spectrum (Figure 2), the diastereotopic hydrogens of the  $\text{CH}_2$  group of the 2-pyrazoline ring were appeared as AB quartet at chemical shifts 3.61, 3.67, 3.98 and 4.04 ppm. On the other hand, the hydrogen of N-H bond of the 2-pyrazoline ring has a peak at chemical shift 6.30

ppm. The hydrogen of the N-H group has been less absorbed due to the exchange with the deuterium of  $\text{CDCl}_3$ , and the peak appears broadly. The chemical shifts at the range of 7.43 to 8.20 ppm are related to hydrogens of aromatic rings. The hydrogens of two methyl groups have peaks at chemical shifts 2.49 and 2.52 ppm.



**Figure 2.**  $^1\text{H}$  NMR spectrum of the synthesized 2-pyrazoline

**Cytotoxicity evaluations**

The results of cytotoxic studies of 2-pyrazoline derivative on the K562 human cancer cell line and also on the

peripheral blood mononuclear cells of a healthy person stimulated with PHA (PBMC + PHA) are summarized in Table 1 as IC<sub>50</sub> values in comparison with cisplatin as a standard anticancer drug.

**Table 1.** The IC<sub>50</sub> results from the test of 2-pyrazoline and cisplatin on two cell lines in  $\mu\text{g}/\text{mL}$ .

Compound\Cell Line	K562	PBMC+PHA
2-pyrazoline 4	45	55
Cisplatin	1.71	7.8

The half maximal inhibitory concentration (IC<sub>50</sub>) is the concentration of compound at which 50% of biochemical processes and biological functions is inhibited in comparison with control group cells. As the IC<sub>50</sub> results in Table 1 show, the new derivative of 2-pyrazoline has a 1.2-fold higher

cytotoxic effect on the K562 cancer cell line than PBMCs in healthy individuals, suggesting a lower side effect on normal cells. This compound with an IC<sub>50</sub> value 4  $\mu\text{g}/\text{mL}$  has a less cytotoxic effect on the K562 cell line in comparison with the cisplatin reference drug, but its side effect on healthy PBMC

cells is about 7 times lower than cisplatin, which can be considered as its strength point. This new compound was shown to be 2.17 times more cytotoxic than the 2-pyrazoline derivative synthesized by Bubniak et al. (Figure 3), with an

IC50 value 97.8 µg/mL [11]. This may be due to the presence of the indenoquinoxaline ring in the structure of the 2-pyrazoline derivative, which has strengthened the pharmaceutical property.

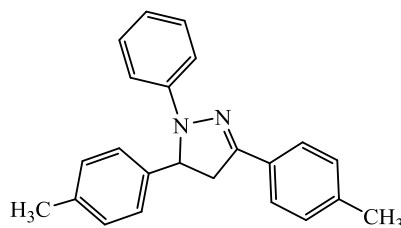


Figure 3. 1-Phenyl-3,5-diparatolyl-2-pyrazoline compound

Also, the results showed that increasing the concentration of synthesized drug from 0.001 µg/mL to 100 µg/mL causes cell death to be significantly increased. So that at 100 µg/mL

concentration of this compound, the death rate of the treated K562 cancer cells approximately reached to 75% which is acceptable level (Figure 4).

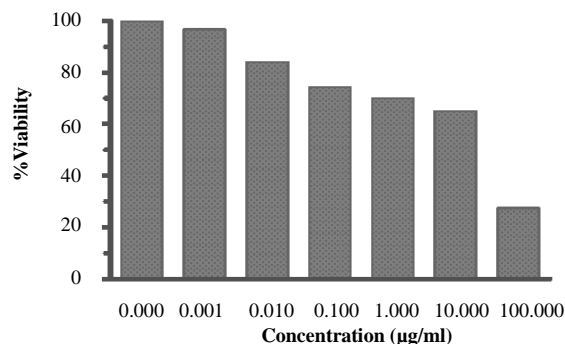


Figure 4. the viability percentage of treated K562 cells with different concentrations of 0.001 to 100 µg/mL of 2-pyrazoline derivative.

### CONCLUSIONS

The 2-pyrazoline derivative was synthesized by the reaction described in Figure 1 and its structure was confirmed by <sup>1</sup>H NMR spectroscopy. The compound showed cytotoxic activity against K562 and PBMC cells. The results show that increasing of the 2-pyrazoline concentration in range of 0.001 to 100 µg/mL due to increased induce of cell death, which the cytotoxic activity of this compound is more effective at higher concentrations. This compound has a lower cytotoxic effect on the K562 cell line than the cisplatin reference drug, and also has fewer side effects on the normal PBMC cells. The results of this research can confirm the results of previous researches in this field. For example, Lee et al showed that quinoxaline derivatives have antitumor

properties [12]. Also, these results can be related to the antiproliferative property of this compound and can inhibit

the cell cycle. Huang et al. studies on 2-pyrazoline derivatives showed that these compounds inhibit the growth and differentiation of cancer cells via inhibiting CDK2 of MCF-7 and B16-F10 cell lines [13].

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