

## ARTICLE

# CYTOTOXIC EFFECT OF THE FLOWER AND LEAF BUD EXTRACT OF CRATAEGUS MICROPHYLLA C.KOCH ON HELA CELL LINE

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## ABSTRACT

**Background:** *Crataegus microphylla c.koch* for having divers biological compounds, used in traditional medicine to treat different diseases. The aim of this study was to investigate potential antitumor activity of etanolic extract of flower and leaf bud of *C. microphylla* on Hela cell line and also to analyze its safety on human peripheral mononuclear cells. **Material and Methods:** Different concentrations of the extracts were added to the cultured cells and incubated for 72 h. Cell viability and growth inhibition was evaluated by morphologic observation and using MTT assay. **Results:** The results showed that the highest cell growth inhibition was observed in 1.25mg/ml. The IC50 value is 0.871mg/ml in Hela. At the same time, the extracts had no inhibitory effect on the normal human mononuclear cells. **Conclusion:** Based on the results it is determined that *C.microphylla* is a significant source of biologically active substances that have cytotoxic and antiproliferative activity and least cytotoxicity on normal cells. But further study are necessary to identification of the active ingredients of this extrac.

## INTRODUCTION

**KEY WORDS**  
Cytotoxicity, *Crataegus microphylla c.koch*, HeLa cell line, MTT assay.

*Crataegus* sp. Comprises of a complex group of trees and shrubs, that is widely distributed in Asia, Europe and North America. *Crataegus* belongs to the subfamily Maloideae in the Rosaceae, with approximately 280 species [1-3].

This plants has been used as a medicinal material and food for hundreds of years both in Europe and in China. In the traditional medicine system Hawthorn(refers to the plant *Crataegus*) fruit is used for the treatment of various ailments such as heart (cardiovascular disorders), central nervous system, immune system, eyes, reproductive system, liver, kidney etc. It also exhibits wide range of cytotoxic, gastro protective, anti-inflammatory, anti-allergic, anti-diarrheal, anti-HIV, antimicrobial, antioxidant and radical scavenging activities [4].

It has been reported that in different parts of hawthorn plants, there is several groups of phenolic compounds, including procyanidins, flavanols, flavonols, C-glycosyl flavones, phenolic acids, anthocyanins and lignans [5]. The medicinal parts of this plant are leaves, flowers, fruits, and flowering tops [6]. In fruits, oligomeric procyanidins and their glycosides are the major phenolic compounds, whereas flavonols, flavones glycosides and C-glycosyl flavones dominate in leaves [5].

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Triterpene acids are another group of bioactive compounds that have been suggested to be in hawthorn species. Evidence has shown that triterpene acids have beneficial effects such as anti-cancer activity (Hsu et al., 1997; Liu, 1995) [7, 8].

In Iran *Crataegus microphylla* C. Koch generally growth in Hyrcanian forests in north of Iran [9] and use in traditional and naturopathic medicine as a digestive aid, promotes blood circulation, and reduce blood stasis [10]. It also exhibits wide range of antioxidant and radical scavenging [11].

Previous preliminary studies have revealed that different members of the family of C.SPP (*C.monogina*, *C.pinnatifida*) possess cytotoxic effects against a number of tumor cells in vitro [12-14].

Currently, there are no data on cytotoxicity of any part of *C.microphylla*. Therefore the aim of this study was to investigate cytotoxicity potential of ethanolic extracts of aerial parts (flowers and leaf bud)of *C.microphylla* on Hela cell line and also to analyze its safety on normal cells we use human peripheral mononuclear cells.

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## MATERIALS AND METHODS

### Plant material

### Collection and Identification

Flowers and leaf bud of *C. microphylla* were collected in May 2013 from the region of the kijaboor of sheykhusa in north of Babol. It was identified by Dr. Bahman Eslami.

### Drying and grinding

The collected plant parts were air-dried in darkness at room temperature (20°C) for one week. Then cutting into small pieces, the plant part were ground into a fine powder with the help of a Hammer Mill. Fine powder was stored in an airtight container and kept in a cool, dark and dry place until analysis commenced.

### Preparation of Eethanolic extract

About 80 gm of powered material was taken in a clean, flat bottomed glass container (2litres) and soaked in 1000 ml of 98% ethanol. The container with its contents was sealed and kept for a period of 3days accompanying occasional shaking and stirring. Then it was filtered through whatman filter paper and the filtrate was concentrated by using traditional spontaneous natural vaporization method at room temperature. Dry ethanolic extract were then dissolved in 100 µL DMSO .Different required dilutions were made by the cell culture medium(RPMI-1640).

### Cell line

Hela cell line was purchased from Pasture Institute (Tehran, Iran). Cells were maintained in RPMI-1640 with 10% of fetal calf serum(of PAA compony) and penicillin/streptomycin (50 IU/ml and 50 µg/ml respectively),(of PAA compony) in an incubator for 24 h (37OC, 5% CO2). Cells were harvested using 0.25% trypsin (of jibco compony) at 70 to 80% confluence in culture flasks [15]. and after a few passages cells were seeded in 96-well plate.

### Cell viability assay (MTT assay)

Hela cells and normal blood lymphocytes and monocytes cell were seeded in a 96-well plate (10000 cells per well). After 24 h of cells incubation, the medium was replaced with 100 µL medium containing various doses of ethanolic extracts of C.microphylla at different concentrations (.156, .312, , .625, 1.25,2.5,5,7.5,and 10 mg/ml ) for 72 h. The same Hela cell, cultured in medium with no treatment,served as control group. After 72 h of treatment, the cells were observed under the light inverted microscope for morphological alterations. Cell viability was determined by MTT(of sigma compony) assay [16]. This assay is based on the metabolic reduction of soluble MTT by mitochondrial dehydrogenase enzyme activity of viable cells into an insoluble colored formazan product, which can be measured spectrophotometrically after dissolving in DMSO (17). At the end of the treatment period, MTT (final concentration 5 mg/mL PBS) was added to each well, which was then incubated at 37°C in 5% CO<sub>2</sub> for 2-4 h. The colored crystals of produced formazan were dissolved in 100 µL DMSO. The absorbance was measured at 492 nm on by an ELISA plate reader.

The percentage growth inhibition was calculated using following formula,

$$\% \text{cell inhibition} = 100 - \left\{ \frac{(At-Ab)}{(Ac-Ab)} \right\} \times 100$$

Where,

At=Absorbance value of test compound

Ab=Absorbance value of blank

Ac=Absorbance value of control

The IC<sub>50</sub> (Inhibitory concentration) is the concentration of the toxic compound that reduces the biological activity by 50%.

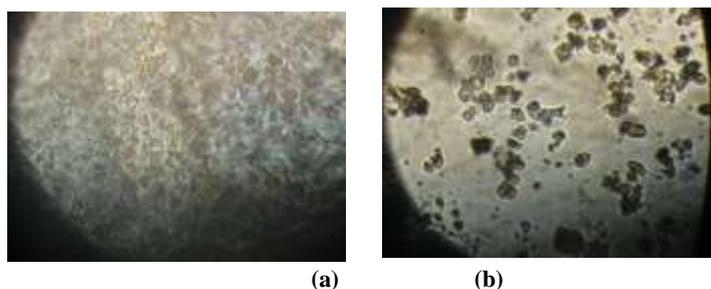
### Observation of morphological changes

Cells plated in 96-well culture plates (10000 cells per well) in RPMI supplemented with 10% FBS for 72 hours were treated with or without flower and leaf bud extracts of C. microphila at various concentrations. After 72 h, the cells were observed under the inverted microscope (motic ae31) and photographs were taken.

### Statistical analysis

The data is expressed as means ± SEM. Biological activity was examined in three individual experiments, performed in triplicate for each dose. Statistical significance was determined using the Student's t-test. Differences were considered to be a statically significant when P < 0.05 and P < 0.01. The IC<sub>50</sub> value was obtained from MTT assay and calculated using Microsoft excel software from linear regression analysis.

## RESULTS



**Fig. 1:** (a) Control HeLa cells. (b) HeLa cells treated with *C. microphylla* flower and leaf bud extract after 72 h. Results revealed that morphological changes were typical of apoptosis (Magnification 200X)

This experiment investigated the cytotoxic activity of the ethanolic extract of flower and leaf bud of *C. microphylla* in various concentrations (0.156, 0.312, 0.625, 1.25, 2.5, 5, 7.5, 10 mg/mL) by MTT assay method in HeLa cell lines. The growth inhibition and morphological changes were compared with untreated cells. Decrease in cell viability and increase in growth inhibition was observed after 72 h of treatment. The cell growth was significantly lower ( $p < 0.05$ ,  $p < 0.01$ ) when compared to untreated control cells [Table 1]. This significant decrease in cell growth was observed for all concentrations.

The highest percentage of growth inhibition at concentrations of 1.25mg/ml was % 85.741 for HeLa cells and the Inhibitory Concentration ( $IC_{50}$ ) value was 0.871 mg/ml.

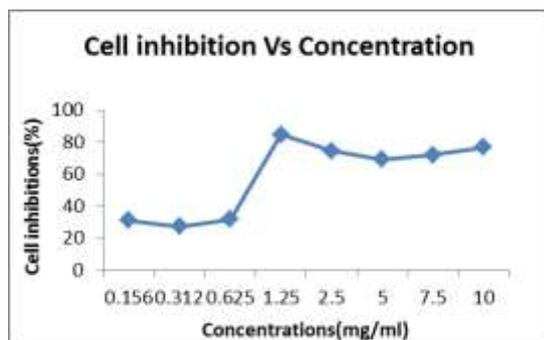
On treating the normal blood lymphocytes and monocytes, with 10mg/ml of extract of the flower and leaf bud of *C. microphylla*, there was no significant decrease in cell viability.

After 72 hours, cell population decreased compared with the control group. Certain degree of morphological changes were observed under the inverted microscope [Fig. 1(a) and (b)]. Results revealed that morphological changes were typical of apoptosis. As the concentration increased from 0.156 to 10 mg/ml, there was more number of dead cells. The apoptotic cells appeared round in shape

and had detached from the surface and intercellular disruption, vacuolation, and pigmentation were apparent at all concentrations.

**Table 1:** The cytotoxic effect of ethanolic extract of flower and leaf bud of *C. microphylla* on HeLa cells, after 72 h exposure. The effect was measured by MTT cell viability assay. The data are mean  $\pm$  SEM of three independent experiments

Concentration Crataegus microphylla mg/ml	Observation	%Cell growth inhibiting	$IC_{50}$ mg/ml
0.156	0.542 $\pm$ .0063*	31.004	
0.312	0.541 $\pm$ .012*	27.245	
0.625	0.540 $\pm$ .027*	31.579	0.0871
1.25	0.184 $\pm$ .0027**	85.741	
2.5	0.236 $\pm$ 0.014**	74.614	
5	0.279 $\pm$ 0.009**	69.16933	
7.5	0.254 $\pm$ 0.022**	72.18	
10	0.227 $\pm$ 0.009**	79.792	
Control	0.770 $\pm$ 0.0322		



**Fig. 2:** Effect of leaf and flower bud extract of *C. microphylla* on HeLa cells. The highest percentage of growth inhibition at concentrations of 1.25mg/ml was % 85.741

## DISCUSSION

Cancer is considered as the serious health problem worldwide and medicinal plants represent a vast potential resource for anticancer compounds [18]. There are various medicinal plants reported to have anti-cancer activity but they are toxic to normal cells and cause immunotoxicity [19]. Finding of new antitumor drug with low side effects on immune system have guided the investigators to consider more herbal medicine to be tested in many studies of immunopharmacology [20].

In this research, the cytotoxic effect of flower and leaf bud extract of *C. microphylla* in different concentrations (10, 7.5, 5, 2.5, 1.25, 0.625, 0.312, 0.156 mg/ml) was studied.

The results of cytotoxic assays of different concentrations of *C. microphylla* on HeLa showed significant cytotoxicity in all the concentrations [Table 1]. The highest cytotoxicity of this extract against HeLa cell was found in 1.25 mg/ml concentration with 85.741 percent of cell growth inhibition (Diagram 1). And also this extract did not have any cytotoxic effect on normal (lymphocyte and monocyte) cells. The approximate concentrations of the extracts to reduce viability of the cells to about 50% (IC<sub>50</sub>) showed for HeLa 0.871 (mg/ml).

Our study showed that ethanolic extract of flower and leaf bud of *C. microphylla* has an in vitro inhibitory effect on the proliferation of human cervical cancer cell line.

The results indicate that *C. microphylla* is considered to be a particularly valuable source of effective anti-proliferative and cytotoxic substances.

In studies of quantitative composition of secondary metabolites from *C. microphylla* has indicated that the main compounds are flavonoids, triterpene, saponins, organic acids, and amines[10]. this kind of valuable secondary metabolites can cause major inhibitory effects on the growth of cancer cells.

Previous research of *C. microphylla* in turki [21] and Iran[11] has indicated high concentration of phenolic compounds, responsible for high antioxidative and soybean lipoxygenase activity.

Our results are in agreement with the findings of other researchers. Kao and et al has shown that polyphenols derived from the fruit of *Crataegus pinnatifida* have anti-tumor activities on skin [12]. Min and et al, has shown that trieterpens derived from *Crataegus pinnatifida* exhibited potent cytotoxic activities both in murine and in human cancer cell lines [13].

Sandra and et al identified that flower bud extract of *crataegus monogyna* showed high anti proliferative activity in MCF-7, NCL-H460, HeLa, HepG2 cell line. They show that flavonoid, particularly flavonols and flavones were the main compound in flower buds. They conclude that the high bioactivity observed in flower buds might be related with its high content in phenolic compounds [14].

Flavonoid anticancer activities include inhibition of cell growth, inhibition of protein kinase activities, induction of apoptosis [22]. Based on these arguments, we can conclude that the high concentration of phenolics and flavonoids is most likely responsible for the significant cytotoxic activity of ethanolic extract of flower and leaf bud of *C. microphylla*.

Another group of active constituents of this species are Triterpenoids. Triterpenoids exert a plethora of biological activities including suppression of inflammation, reduction of oxidative stress, regulation of cell cycle, inhibition of cell proliferation, induction of apoptosis, and interaction with tumor microenvironment through modulation of multiple signal transduction pathways. Therefore, this could explain, at least in part, antineoplastic properties of flower and leaf bud extract of *C. microphylla* in HeLa but it is well known that tumor cells use multiple survival pathways to prevail over their normal counterparts [23].

These results represent the first report on cytotoxicity of this plant, therefore it is indicated to continue investigation of cytotoxicity on other cancer cell lines and *in vivo* tests on animal model.

## CONCLUSION

In conclusion, the results of *in-vitro* antitumor activity revealed that flower and leaf bud extract of *C. microphylla* exhibits good antitumor activity. The best antitumor activity by this extract was shown at 1.25 mg/ml concentration. The anticancer activity of this extract has not been reported in the literature. The IC<sub>50</sub> value was found to be 0.0871 mg/ml. Additionally, it was revealed that there is valuable bioactive compound in this extract, showing its anticancer potential and relative non-toxicity to the normal healthy lymphocytes and monocytes. Thus, further research should be carried out to isolate and identify biologically active substances from *C. microphylla*, with an anti proliferative activity.

### CONFLICT OF INTEREST

There is no conflict of interest

### ACKNOWLEDGEMENTS

None

### FINANCIAL DISCLOSURE

None

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