

*Full Length Research Paper*

# Apoptotic and necrotic effects of plant extracts belonging to the genus *Alchemilla* L. species on HeLa cells *in vitro*

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Apoptotic and necrotic effects of plant extracts belonging to the genus *Alchemilla* on HeLa cells were investigated. The ratio of viable cells was determined by cell counter. Double staining method for the determination of apoptotic and necrotic index was carried out. The lowest apoptotic effect was found in *Alchemilla oriturcica* with the ratio of around 14% and the highest apoptotic effect found in *Alchemilla trabzonica* was 24%. Apoptotic effect was additionally determined by caspase-3 immunostaining. Increasing the concentrations of the extracts, especially in the level of 150 to 200 µg/mL caused the increase of necrotic effect on cancer cells. Particularly, the effects of *A. oriturcica* and *A. trabzonica* extracts with the density of 200 µg/mL were very high compared to the other species. The flavonoid components were detected in extracts of the species used for this study. The flavonoids identified are orientin (luteolin-8-C-glucoside), hyperoside (quercetin-3-O-galactoside) and isoquercetin (quercetin-3-glucoside) in *Alchemilla erythropoda*; rutin (quercetin-3-rutinoside), orientin, vitexin (apigenin-8-C-glucoside), hyperoside in *Alchemilla ikizdereensis*; rutin, hyperoside, isoquercetin (quercetin-3-glucoside) in *A. oriturcica*, and hyperoside, isoquercetin and quercitrin (quercetin-3-O-rhamnoside) in *A. trabzonica*.

**Key words:** Alchemilla, extract, HeLa cell, cytotoxicity, apoptosis, necrosis.

## INTRODUCTION

Cancer is a major cause of death or morbidity in human population (Miller, 1981). The human body is made up of different cells. Cells divide and multiply for the body system needs them. When these cells continue to divide when the body does not need them, the result is growth, known as tumor (Klein, 2008). Tumor is classified as benign or malignant. Benign is considered non-cancerous and malignant is cancerous. Benign tumors are rarely life threatening and do not spread to other parts of the body. Malignant tumors, however, often invade nearby tissue and other regions of the body, spreading the tumor cells (Bacac and Stamenkovic, 2008). There are four methods of treatment for cancer: surgery, chemotherapy, radiation therapy, immunotherapy and biologic therapy. Especially, chemotherapy is used in cancer treatments. But this method causes unwanted side effects which often include

nausea, loss of taste, lethargy, loss of hair, loss of libido etc. (Picozzi et al., 2001). Clinical trials may be an option for some cancer treatments that meet certain study criteria. Others may choose alternative cancer treatments (Ihde, 1992). The compositions of plant extract may include more active agents, such as vitamins, minerals, alkylating agents, anti-metabolites, antineoplastic agents, immune stimulators and anti-oxidants. In cancer cells, the extract of plant induce one or more effects of induction of cell differentiation, inhibition of cell proliferation, induction of apoptosis and inhibition of cell cycle. In spite of novel discovery of produced drugs, the potential of fitochemicals or plant extracts to bring new products for systemic diseases and cancer treatment and prevention is still enormous (Kwiecinski et al., 2008). The anticancer investigation has the greatest impact of plant derived agents, where chemotherapeutic like vinblastine, vincristine, taxol, and camptothecin have improved the chemotherapy of some tumors (Newman, 2003). The search for novel antitumor compounds in fitotherapy is a

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**Table 1.** Voucher specimens.

Species	Collection number	Herbarium
<i>A. erythropoda</i>	Yusuf 1460	ADO
<i>A. ikizdereensis</i>	Yusuf 1461	ADO
<i>A. oriturcica</i>	Yusuf 1467	ADO
<i>A. trabzonica</i>	Yusuf 1478	ADO

promising strategy for prevention of cancers (Yan-Wei et al., 2009). Numerous groups with anticancer properties are plant extracted natural products including alkaloids, phenylpropanoids, flavonoids and terpenoids (Kintzios, 2006; Park et al., 2008). *Alchemilla* is a genus of the family Rosaceae. The genus can easily be distinguished from the closely related genera by its leaves that radiate from a common point or with a fan shape; and it has small flowers without petals and appear in clusters (Pawlowski and Walters, 1972). However, *Alchemilla* species are very similar to each other and in many cases without microscopic identification they are indistinguishable. The genus *Alchemilla* is represented by nearly 1000 and 80 species all around the world and Turkey, respectively (Fröhner, 1995; Brummit, 1992; Izmailow, 1981; Sepp and Paal, 1998; Hayirlioglu-Ayaz and Inceer, 2009). Many species of the genus in Turkey are endemic to Turkey and many of them are only known from the Eastern Black Sea Region. The genus is locally known with the names aslanpençesi, aslanayağı, findikotu, yeditepe and dokuztepe in Turkey (Baytop, 1997). The plant is reported to have a medicinal use for treatment of wounds and woman's problem (Phillips and Foy, 1990). Baytop (1997) reported that its leaves are diuretic and laxative. The genus is best known in the Eastern Black Sea Region in Turkey and reported by some local people to be used externally in treating wounds. In the advertisements and some health programs, *Alchemilla* species are announced as the plants, the main active ingredient of the phytotherapy medicines used internally for treatment of woman's menstrual problems.

In this study, the effect of plant extracts belonging to the genus *Alchemilla*, *Alchemilla erythropoda*, *Alchemilla ikizdereensis*, *Alchemilla oriturcica* and *Alchemilla trabzonica* on HeLa (cancer) cells was investigated, using four different methods. These species are endemic to Turkey and have not been previously studied for their effect on HeLa (cancer) cells.

## MATERIALS AND METHODS

### Collection of plant material

*Alchemilla* species were collected from the field near Trabzon Province (Turkey). The specimens were identified and kept in Kirikkale crushed and their extractions were done according to Zhao et al. (2005). University, Anadolu Herbarium (ADO), the voucher specimens are

shown in Table 1. Leaf materials used in the study were air dried,

### Extraction and identification of flavonoid compounds

Dried and powdered leaf material (10 g) was successively extracted with 50% aqueous ethanol in a flask. The extract was subjected to maceration process in incubator for four hours at 150 rpm, at 50°C. It was left to macerate for 20 h. Then, extract was filtered using a blue band filter paper and Buchner funnel. The hydroalcohol solution was evaporated to dryness under reduced pressure. Hydrolic extract was treated with petroleum ether (40° to 60°) in separation funnel. The aqueous phase at the bottom of separation funnel was transferred to another separation funnel. After ethyl acetate was added and the funnel was mixed gently, the part with ethyl acetate was evaporated under reduced pressure and dried. Identification of flavonoids was carried out by using thin layer chromatography (TLC) and high performance liquid chromatography.

### Cytotoxicity

For cytotoxicity, HeLa cells ( $50 \times 10^3$  cells per well) were placed in DMEM by using 24-well plates. Extracts at varying concentrations (about 0 to 200  $\mu\text{g.mL}^{-1}$  in phenol red free medium) were put into wells containing cells, respectively. HeLa cells were harvested with trypsin-EDTA, and then were stained with trypan blue dye. The number of living and dead cells was counted with a cell counter (invitrogen, USA).

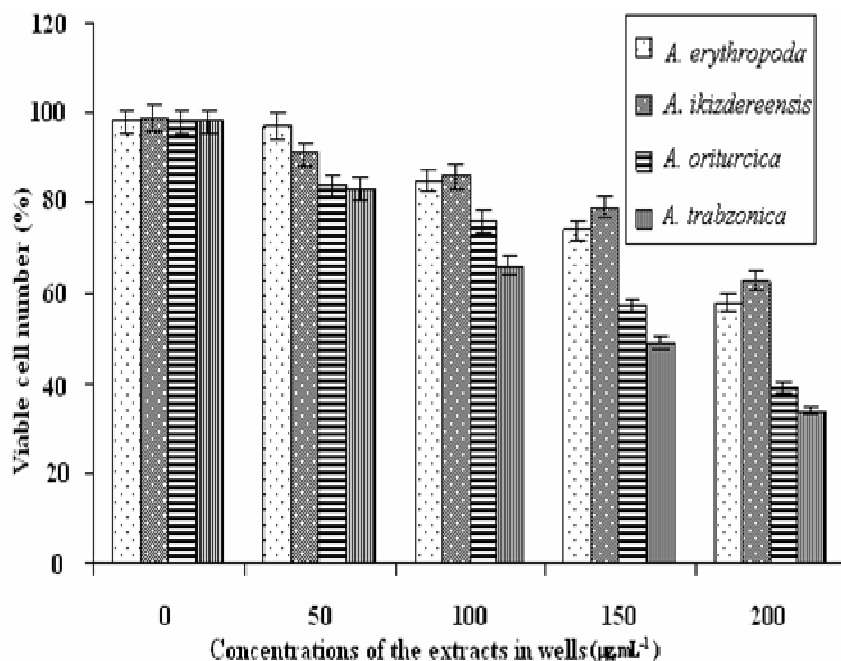
### Hematoxylen/eosin staining

For hematoxylen/eosin staining, HeLa cells ( $20 \times 10^3$  cells per well) were placed in DMEM by using 24-well plates. After treating with the extracts at different concentrations (about 0 to 200  $\mu\text{g.mL}^{-1}$ ) for 24 h period, the medium was removed, the cells washed with distilled water and fixed in ethanol, and stained with hematoxylen/eosin. After staining, the cells were observed by light inverted microscope (Leica, Germany) (Türk et al., 2010). By this way, cellular and nuclear morphology is shown in cultured cells stained with hematoxylen/eosin.

### Determination of apoptotic and necrotic indexes

Analyses of apoptotic and necrotic cells with double staining were performed to quantify the number of apoptotic cells in culture on the basis of scoring of apoptotic cell nuclei. HeLa cells ( $20 \times 10^3$  cells per well) were placed in DMEM by using 24-well plates and treating with different concentrations of functional oligomers (about 0 to 200  $\mu\text{g.mL}^{-1}$  in phenol red free medium) for 24 h period. Both attached and detached cells were collected, then stained with Hoechst dye 33342 ( $2 \mu\text{g.mL}^{-1}$ ), propidium iodide (PI) ( $1 \mu\text{g.mL}^{-1}$ ) and DNase free-RNase ( $100 \mu\text{g.mL}^{-1}$ ) for 15 min at room temperature (Ulukaya et al., 2001; McPartland, 2005).

Necrotic cells were stained red by PI. Necrotic cells lacking plasma membrane integrity and PI dye cross cell membrane, but PI dye does not cross non necrotic cell membrane. The number of apoptotic and necrotic cells were determined with DAPI and FITC filters of Fluorescence Inverted Microscope (Leica, Germany).



**Figure 1.** *In vitro* cytotoxicity of plant extracts with different concentrations at 24 h incubation. Number of viable HeLa cells in wells (%). Results are presented as means  $\pm$  SEM.

The second method, used as apoptotic detection of HeLa cells, was immunocytochemistry for caspase-3. Caspase-3 activity was determined as described previously with minor modifications (Bressenot et al., 2009). In brief, for caspase-3 immunocytochemistry, collected HeLa cells were treated in 3% H<sub>2</sub>O<sub>2</sub> for 5 min and rinsed with PBS for 15 min. The cells were blocked with blocking solution in PBS (Phosphate buffer solution) and then incubated (45 min, room temperature) with primer antibody caspase-3 (Lab Vision, 1:300 dilution) in PBS. The cells then were incubated with biotin-conjugated secondary antibody (1:300, 1 h, room temperature), avidin-biotin-peroxidase complex (Santa Cruz Biotechnology, Inc., rabbit peroxidase kit; 1 h) and 3,3-diaminobenzidine (DAB) solution. Sections were counterstained with hematoxylin. For negative controls, primary antibody was omitted in one of the slides.

## RESULTS

Identification of flavonoid compounds showed that all plant extracts used in this study included flavonoid compounds. The flavonoids identified are orientin (luteolin-8-C-glucoside), hyperoside (quercetin-3-O-galactoside) and isoquercetin (quercetin-3-glucoside) in *A. erythropoda*; rutin (quercetin-3-rutinoside), orientin, vitexin (apigenin-8-C-glucoside), hyperoside in *A. ikizdereensis*; rutin, hyperoside, isoquercetin (quercetin-3-glucoside) in *A. oriturcica*, and hyperoside, isoquercetin and quercitrin (quercetin-3-O-rhamnoside) in *A. trabzonica*.

### Cytotoxicity

Trypan blue was used to stain cells for observing the ratio

of viable and dead cells. It was observed that increasing the concentration of plant extracts led to an increase in toxicity for cells (Figure 1). Low concentrations of the plant extracts (50 to 100 µg/mL) produced low toxic effect on cells whereas at higher concentrations (above 100 µg/mL) there was higher effect in toxicity. Viability of cells was obtained as 76% in the extracts of *A. oriturcica*, 66% in *A. trabzonica* and above 80% in *A. erythropoda* and *A. ikizdereensis*. Concentration of 200 µg/mL plant extract crucially increased the toxicity effect, especially *A. oriturcica* and *A. trabzonica* caused very low viability of HeLa cells, with 39 and 34% respectively (Figure 1). It can easily be seen from Figure 1 that the highest toxicity effect was seen in the extract of *A. trabzonica*, and the lowest in that of *A. ikizdereensis*.

### Hematoxylin-Eosin staining

In this study, the effect of plant extracts on HeLa cells were investigated by observing morphological change. 1 mg/mL stock solution of each plant extract belonging to different species were prepared and dissolved to concentration of 50 to 200 mg/mL which was then applied into the cultured cancer and normal cell cultures to see the interaction for 24 h period. Control group included only medium without plant extract. According to the results, general application of extract with the concentration of 50 to 100 µg/mL for 24 h period resulted in almost similar results. When compared to control

**Table 2.** Apoptotic and necrotic effect of plant extracts (*A. erythropoda* (I), *A. ikizdereensis* (II), *A. oriturcica* (III) and *A. trabzonica* (IV)) on HeLa cell *in vitro*. Results are presented as means  $\pm$  SEM.

Extract $\mu\text{g mL}^{-1}$	Apoptotic indexes (%)				Necrotic indexes (%)			
	I	II	III	IV	I	II	III	IV
0	1 $\pm$ 1	1 $\pm$ 1	1 $\pm$ 1	1 $\pm$ 1	1 $\pm$ 1	1 $\pm$ 1	1 $\pm$ 1	2 $\pm$ 1
50	2 $\pm$ 1	3 $\pm$ 1	5 $\pm$ 1	5 $\pm$ 1	2 $\pm$ 1	2 $\pm$ 1	3 $\pm$ 1	4 $\pm$ 1
100	4 $\pm$ 1	6 $\pm$ 1	7 $\pm$ 1	10 $\pm$ 1	3 $\pm$ 1	5 $\pm$ 1	5 $\pm$ 1	9 $\pm$ 1
150	10 $\pm$ 1	8 $\pm$ 1	12 $\pm$ 1	16 $\pm$ 2	4 $\pm$ 1	8 $\pm$ 1	12 $\pm$ 1	17 $\pm$ 2
200	12 $\pm$ 1	9 $\pm$ 1	14 $\pm$ 2	24 $\pm$ 2	6 $\pm$ 2	10 $\pm$ 3	18 $\pm$ 2	25 $\pm$ 2

group, no significant difference was seen between. At the concentration of 200  $\mu\text{g/mL}$ , it was observed that when the extracts, especially of *A. oriturcica* and *A. trabzonica* were applied, some of the cells disappeared from the surface of culture dish, the others, stayed on the surface and became round, and some cell membranes were broken. It was determined that amongst the extracts of species, that of *A. trabzonica* is the most effective for causing morphological changes.

### Caspase-3 and double staining

Apoptosis rate was obtained in two ways, immunocytochemical staining of caspase-3 and double staining methods. Apoptotic index ratio based on the findings of both methods and necrotic index, based on that of only double staining method are given in Table 2. The findings showed that application of lower concentrations of extracts caused lower effect in apoptosis.

Cancer (HeLa) cells interacting with the plant extracts are shown in Figure 2. After the treatment of caspase-3, the non-apoptotic cells stained blue (Figure 2a), whereas cytoplasm of the apoptotic cells were brown in colour (Figure 2b). After double staining, nuclei of both apoptotic and healthy cells were stained blue (Figure 2c), but they appear as smooth bordered, not dispersed and medium blue in healthy cells, and as not smooth bordered, dispersed and bright blue in apoptotic cells (Figure 2d).

It was seen that apoptotic effect reduced as in the toxicity, when low concentration of the extracts applied to cells, and increasing density of solutions led to increase in apoptosis. The lowest apoptotic effect was found in *A. oriturcica* with the ratio of around 14% and the highest apoptotic effect found in *A. trabzonica* was 24%. Viability of cancer cells interacting with extracts was determined using propidium iodide fluorescent dye. This dye does not enter living cells, but freely passes through the membranes of dead cells and stains their nuclei which appear red under fluorescent light; whereas the nuclei of apoptotic and healthy cells are seen as blue in colour.

### Conclusion

Detection of necrotic index was carried out under

fluorescent light at a wavelength of 480 to 520 nm in the present study; nuclei of necrotic cells appeared red and those of apoptotic and healthy cells green in colour (Figure 2d and 2e).

However, green nuclei were blue at 358/461 nm (excitation/ emission) fluorescent filter. The findings of staining are given in Table 2. In general, necrotic effect of the extracts on cancer cells is depended on the concentration of the solution; in low concentrations (50 to 100  $\mu\text{g/mL}$ ), the effect becomes low and in higher levels (150 to 200  $\mu\text{g/mL}$ ) it gets higher. Especially, effects of *A. oriturcica* and *A. trabzonica* extracts with the concentration of 200  $\mu\text{g/mL}$  are very high with compared to the other species (Table 2).

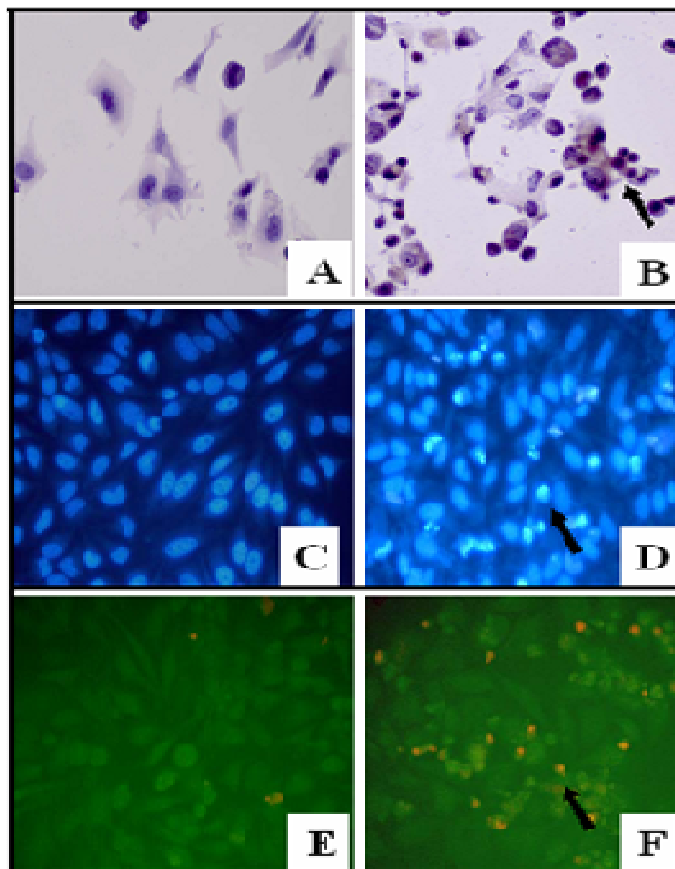
In conclusion, this study showed that the extracts obtained from *Alchemilla* species on HeLa (cancer) cells have toxic, apoptotic and necrotic effects. Especially in *A. trabzonica* extracts, toxic, apoptotic and necrotic effects were remarkable. The result in this study is very promising for the future; therefore we believe that the extracts of these plants are worth investigating for the purpose of therapeutic treatment of cancer.

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**Figure 2.** Light microscope images of (A) non-apoptotic HeLa cells as a control group (stained with caspas-3 immunostaining kit), and (B)  $200 \mu\text{g.mL}^{-1}$  concentration of *A. trabzonica* /HeLa cells conjugate (stained with caspas-3 immunostaining kit), where brown cytoplasm of cells image indicates the formation of apoptotic cells; (C) Fluorescent microscope image of nucleus of untreated HeLa cells as a control, where formation of lifeless spots demonstrates nucleus of non-apoptotic cells; (D)  $200 \mu\text{g.mL}^{-1}$  concentration of *A. trabzonica* /HeLa cells conjugate (stained with Hoechst 33342), where bright spots indicates nucleus of apoptotic cells; (E) Fluorescent microscope image of nucleus of untreated HeLa cells as a control, where formation of green spots demonstrates nucleus of non-necrotic cells; (F)  $200 \mu\text{g.mL}^{-1}$  concentration of *A. trabzonica* /HeLa cells conjugate (stained with PI dye), where red spots indicates nuclei of necrotic cells and green spots indicates nucleus of non-necrotic cells(stained with Hoechst 33342). All images were recorded with  $\times 400$  magnification. Scale bar is  $20 \mu\text{m}$ .

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