Investigation of Antioxidant and *In Vitro* Wound Healing Activity of Fulvic Acid

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ABSTRACT: This study aimed to investigate the antioxidant and *in vitro* wound healing activities of fulvic acid (FA) purified from Hüsamlar leonardite in Muğla/Turkey. Purified FA was analyzed with FT/IR spectrophotometer and flame photometer for determining functional groups and cation impurities, respectively. 1, 5, 10, 20, 30, 50, 100 µg mL^{-1} concentrations of FA were tested for antioxidant activity (by using DPPH radical scavenging and H_2O_2 scavenging assays). Also, effect of FA on BJ human foreskin fibroblast and HaCaT spontaneously immortalized non-tumorigenic human keratinocyte cells proliferation was tested by *in vitro* MTT and WST-8 assays and on cell migration by wound healing assay (scratch assay). The results of the study show that the FA has a low DPPH radical scavenging activity, but it exhibited high H_2O_2 scavenging activity at low concentrations. The effect of FA on the proliferation of BJ and HaCaT cells varied according to the cell type, FA concentration and treatment time. However, it accelerated wound healing by increasing cell migration, especially in HaCaT cells. 1 and 10 µg mL^{-1} FA had a significant wound healing effect on BJ cells, and all concentrations of FA had a significant wound healing effect on HaCaT cells at the end of the 24 h treatments.

Keywords: Antioxidant activity, cell proliferation, fulvic acid, humic substance, wound healing

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INTRODUCTION

Skin is the largest organ in the body comprising approximately 15% of body weight and consists of epidermis and dermis. Furthermore, subcutaneous layer is present under the dermis. Epidermis is the outermost layer of the skin and it maintains a vital barrier against external trauma. The major cellular content of the epidermis is keratinocytes (approx. 95% of the epidermis) and fibroblasts are major cellular component of the dermis (Zouboulis, 2000; Braun-Falco et al., 2000). When the outer covering of the skin, the epidermis, is injured a wound healing response is initiated. Wound healing is a complex process that occurs in different stages and involves many different cell types (Martin, 1997). Wound repair requires the proliferation and migration of fibroblasts and keratinocytes. These cells re-establish the normal cellular and extracellular matrix composition of skin, and the growth of vascular endothelial cells (VECs) to form new blood vessels that supply nutrients to the skin cells (Barrientos et al., 2008). The ability of these three cell types (fibroblasts, keratinocytes and VECs) to repair skin integrity and function is jeopardized in non-healing wounds (Schafer and Warner, 2008).

Humic substances are formed from the decomposition of plants and occur naturally in water, peat, soil and brown coal. Humic substances have a complex structure and can be fractioned into humines, humic acids and fulvic acids (MacCarthy, 2001). Fulvic acid (FA), a class of compounds of humic substances, is a mixture of polyphenolic compounds formed through the degradation of organic substances such as plants, microbes and animals by chemical and biological processes (Motojima et al., 2011). FA has been reported recently to have nutraceutical properties and physiological action on the human body (Winkler and Ghosh, 2018). It is one of the naturally occurring phytochemicals with neuroprotective effect (Cornejo et al., 2011; Guzmán-Martinez et al., 2013), antimicrobial and anti-inflammatory properties (Van Rensburg et al., 2001; Sherry et al., 2013).

Although with the presence of information about neuro-protective, antioxidant, antimicrobial and anti-inflammatory properties of various FA products and derivatives, no data have been reported previously on the *in vitro* wound healing activities of FA. Therefore, the aim of our study was to investigate the antioxidant and *in vitro* wound healing activities of FA purified from Hüsamlar leonardite in Muğla/Turkey on BJ human foreskin fibroblast cells and HaCaT spontaneously immortalized non-tumorigenic human keratinocyte cells.

MATERIALS AND METHODS

Fulvic acid (FA) purification from Leonardite and FT/IR analysis

In this research, a method of purification process of fulvic acid was aimed. Thus, two different processes yielding calcium fulvate and ethyl fulvic ester were developed. FA is purified by method of extraction, coagulation, vacuum distillation, esterification, solid-liquid seperation and ion exchange respectively (Smith and March, 2001). In experimental studies, produced amounts of calcium fulvate and ethyl fulvic ester from 100 g of Muğla Milas Hüsamlar leonardite were 832 mg and 764 mg, respectively. After purification of these products, remaining amount of pure fulvic acid produced from ethyl fulvic ester and calcium fulvate were 800 mg and 739 mg, respectively. Thus, products were analyzed with FT/IR spectrophotometer in order to determine functional groups, and flame photometer was used for determining cation impurities (Fig. 1 and Fig. 2). After purification studies at laboratory scale, leonardite operating plants with a capacity of 1 tonne per hour were designed for each method. 14 kg/tonne raw material calcium fulvate was produced using

Muğla Milas Hüsamlar leonardite (Sönmez 2011). Purified FA has carboxylic acid functional groups. Carboxyl acid groups are bonded with calcium and magnesium in coagulation phase of production process, and these ions are exchanged with sodium and hydrogen respectively. Carboxyl acid salts of calcium, magnesium, sodium and potassium are
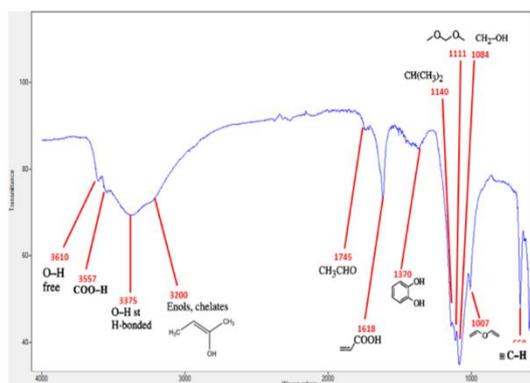
changed to hydrogen with ion exchange stages of production. After these processes, the end product is two seperate FA components: Esterificiation product FA and Calcium salt precipitation product FA. Fulvic acid purchased from International Humic Substances Society (IHHS) was used as standard agent.
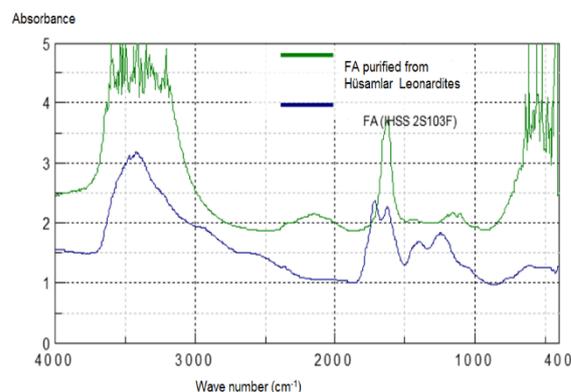

Fig.1.FT/IR analysis of ethyl fulvic ester

Fig. 2. FT/IR comparison of ethyl fulvic ester and pure fulvic acid

Determination of antioxidant activity

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay

The free radical scavenging activity of FA was tested for its ability to bleach the stable 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH) (Brand-Williams et al.,1995). One milliliter of 0.1 mM DPPH methanol solution was added to 3 ml of 1, 5, 10, 20, 30, 50, 100 µg mL^{-1} concentrations of FA. The mixture was shaken vigorously and left at room temperature. After 30 min, the absorbance of mixture was measured at λ=517. Tests were carried out in triplicate. Ascorbic acid (10µg mL^{-1}) and Rutin (10µg mL^{-1}), a citrus flavonoid glycoside, were used as standard.

H$_2$O$_2$ scavenging assay

Hydrogen peroxide scavenging activity of FA was determined by the method described by Ruch et al. (1989). A solution of H$_2$O$_2$ (40mM) was prepared in phosphate buffer (pH 7.4). Reaction mixtures contained 40mM of H$_2$O$_2$ and

different FA concentrations (1, 5, 10, 20, 30, 50, 100 µg mL$^{-1)}$, and absorbance values were measured after 10 min using wavelength of 230 nm. Ascorbic acid was used as the standard.

Cell culture and proliferation assay (MTT and WST-8 assays)

Human foreskin fibroblast cells (BJ) (ATCC CRL-2522) and a spontaneously immortalized nontumorigenic human keratinocyte cell line, HaCaT (CLS 300493 provided by Dr. Ersan KALAY, Karadeniz Techical University, Trabzon/TURKEY) were maintained in Eagle's minimal essential medium supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, Germany), penicillin (100IUmL^{-1}), and streptomycin (100µg mL^{-1}) under humidified atmosphere of 5% CO$_2$ at 37°C until confluent. The cells were trypsinized and proliferation assays were carried out in 48 well-plates. BJ and HaCaT cells were seeded into 48-well plates (5x10^3 cells per well) and incubated for 24 h, during which a partial monolayer formed. After 24 h incubation, the cells were

treated with 1, 5, 10 and 20μg mL^{-1} FA for 24h and 48h. Negative control cells received only culture medium. Crystalin (10%) was used as standard wound healing agent for BJ and HaCaT cells. 5μg mL^{-1} citric acid was also used as a solvent control (solvent of FA). At the end of the treatment times, cellular viabilities were determined by MTT assay (Mossman 1983) and WST-8 assay (Tominaga et al. 1999). The absorbance was read at 570 nm for MTT assay and at 450 nm for WST-8 assay by using a microplate reader (Thermo Labsystems Multiscan Spectrum).

Wound healing assay (Scratch assay)

The wound healing assay was used in this study, because it is a surrogate method for evaluation *in vivo* tissue healing assays and also allows the researcher to study cell migration and cell interactions (Hoang et al., 2000; Liang et al., 2007). BJ and HaCaT cells were plated in a 24-well plate (10x10^{3}cells per well) in Eagle's minimal essential medium containing 10% fetal bovine serum. After 24 h of cell culture at 37 ºC and 5% CO$_2$, the 100% cell confluence was

observed before the scratch assay was performed. A sterile yellow (100μL) pipette tip was used to make a straight scratch on the monolayer of cells, stimulating a wound. Wounded monolayers were washed twice with phosphate buffered saline (PBS) to remove cell debris. Then, cells were treated with medium containing FA at different concentrations (1, 5, 10, 20μg mL^{-1}), which were determined according to proliferation assay results. Negative control cells received only culture medium. Furthermore, wound healing agent Crystalin (10%) was used as standard wound healing agent for BJ and HaCaT cells. 5μg mL^{-1} citric acid was used also as a solvent control (solvent of FA). Wound healing by migrating cells after FA treatment was observed at 0, 12, and 24th hours by inverted light microscope (Olympus CK40) equipped with a camera. Photographs were analyzed by using Image J software (NIH, USA). The % relative migration of BJ and HaCaT cells treated with FA and controls were calculated according to the following equation:

$$\% \text{ Relative migration} = \frac{Area\ between\ cells\ 0h - Area\ between\ cells\ 12\ or\ 24\ h}{Area\ between\ cells\ 0h} \times 100$$

(1)

Statistical analysis

All the experiments were carried out triplicate, and obtained data were analyzed by One Way ANOVA (SPSS 20.00 software package program). The statistically significant difference was considered to be p<0.05. The normality of variables was evaluated using the Kolmogorov – Smirnov Z test. The statistical differences between the control and treatment groups were carried out using the non-parametric Mann Whitney Test (for independent samples). The correlations between different variables were determined using the Spearman Rank Correlation Test.

RESULTS AND DISCUSSION

DPPH radical scavenging activity

The data on the DPPH radical scavenging activity of the fulvic acid (FA) are given in Table 1. Tested concentrations of FA exhibited very low DPPH radical scavenging activity. The DPPH radical scavenging activity of the 100μg mL^{-1} FA (the highest concentration in experiment) was determined as 12.37%. DPPH radical scavenging activity of citric acid, and rutin and ascorbic acid are 24.22%, 91.75% and 90.95%, respectively.

H_2O_2 scavenging activity

The data concerning the hydrogen peroxide (H_2O_2) scavenging activity of FA are present in Table 1. According to these data 1, 5 10 and 20 µg mL^{-1} concentrations of FA exhibited high H_2O_2 scavenging activities (p <0.05). With an increase in FA concentration from 30 µg/mL, the H_2O_2 scavenging activity began to decline, and the H_2O_2 scavenging activity at the highest concentration of 100µg mL^{-1} decreased to 25.78%.

Table1. DPPH radical scavenging and H_2O_2 scavenging activities of Fulvic acid

Concentrations	DPPH scavenging activity (% ± SD)	H_2O_2 scavenging activity (% ± SD)
1 µg mL^{-1} FA	9.28±0.006	86.72±0.001*
5 µg mL^{-1} FA	9.28±0.005	71.10±0.041*
10 µg mL^{-1} FA	1.55±0.015	74.61±0.022*
20 µg mL^{-1} FA	1.00±0.007	75.00±0.011*
30 µg mL^{-1} FA	1.03±0.008	39.06±0.045*
50 µg mL^{-1} FA	5.79±0.004	35.16±0.019*
100 µg mL^{-1} FA	12.37±0.011	25.78±0.070
Citric acid (50 µg mL^{-1}) (solvent control)	24.22±0.005	80.86±0.019*
Rutin (10 µg mL^{-1}) [x]	91.75± 0.003*	---
Ascorbic acid (10 µg mL^{-1}) [xx]	90.95± 0.012*	41.73±0.047*

*p<0.05 (FA: Fulvic Acid) [x] Standard in DPPH scavenging assay; [xx] Standard in DPPH and H_2O_2 scavenging assay.

FA, a soluble class of humic substances at each pH value, is a mixture of polyphenolic acid compounds that occur as a result of chemical and biological degradation of organic materials such as dead plants, microbes and animals (Motojima et al., 2011; Guzman-Martinez et al., 2013). There are studies to investigating antioxidant activity of FA obtained from different natural sources e.g. FA isolated from dissolved sludge, FA purified from coal mines in China, and FA isolated from turf (Ueda et al., 2004; Tachibana et al., 2004; Motojima et al., 2011; Gao et al., 2018). In our study, although FA showed high H_2O_2 scavenging activity in comparison with control, it exhibited low DPPH radical scavenging activity.

DPPH can only be soluble in organic solvent and interference of absorbance from the sample compounds could be a problem for quantitative analysis (Arnao, 2000). In addition, the spectrophotometric measurements can be affected by compounds, such as carotenoids, that absorb at the wavelength of determination as well as by the turbidity of the sample (Apak et al., 2013). FA solution has a dark yellow to brown color. Although the color is very light at low concentrations, the color is getting darker at higher concentrations. As the color of the FA solution could be interfered, the measured absorbance values in DPPH and H_2O_2 assays may be high. Interference of color of FA may have led to a low rate of antioxidant activity.

Effect of fulvic acid on the proliferation and viability of BJ and HaCaT cells

Effect of FA on the proliferation and viability of BJ cells are present in Table 2. MTT test results showed that 20µg mL^{-1} FA treatment decreased cell viability and proliferation significantly in comparison with control (p <0.05). However, other FA concentrations have not significantly effect on BJ cell viability and proliferation. Citric acid and Crystalin (10%) also have not an important effect on cell viability and proliferation.

According to WST-8 test results, it was observed that the 1 and 5µg mL^{-1} FA increased the proliferation of BJ cells at 24 h, but reduced the proliferation after treatment for 48 h. The decrease in cell proliferation after 48 h treatment of 5 and 10µg mL^{-1} FA was statistically

significant (p <0.05) in comparison with control. 20μg mL^{-1} FA treatment for 24 and 48 h decreased cell viability and proliferation significantly (p <0.05). Citric acid and Crystalin

(10%) decreased cell viability and proliferation insignificantly. Results of the MTT and WST-8 assays exhibited that 20μg mL^{-1} FA significantly reduced viability and proliferation of BJ cells.

Table 2. Effect of Fulvic acid on proliferation of BJ human foreskin fibroblast cells

Groups	Concentrations	Proliferation (%±SD)			
		MTT assay		WST-8 assay	
		24 h	48 h	24 h	48 h
Negative Control	---	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
Citric acid (solvent control)	5 μg mL^{-1}	101.01±0.191	96.36±0.126	90.20±0.074	87.40±0.060
Crystalin (standard agent)	% 10	96.03±0.162	97.15±0.177	97.89±0.050	87.73±0.109
Fulvic Acid	1 μg mL^{-1}	102.40±0.168	102.64±0.119	114.15±0.290	98.85±0.049
	5 μg mL^{-1}	102.35±0.079	97.98±0.037	106.60±0.061	84.60±0.103*
	10 μg mL^{-1}	92.98±0.271	87.87±0.118	87.89±0.060	85.44±0.010*
	20 μg mL^{-1}	83.34±0.102*	84.84±0.038*	81.02±0.057*	82.93±0.009*

*p<0.05

Results about effect of FA on proliferation and viability of HaCaT cells are presented in Table 3. MTT test results showed that FA treatment decreased cell viability and proliferation concentration dependently. After 24h treatment, decrease of cell viability and proliferation was found statistically significant at all FA concentrations. 20μg mL^{-1} FA decreased cell viability and proliferation statistically significant after 24 and 48 h treatment (p <0.05).

According to WST-8 test results, it was observed that none of the FA concentrations affected HaCaT cell viability and proliferation significantly, in comparison with control groups. Citric acid and Crystalin decreased cell viability and proliferation after 24 h, but they increased cell viability and proliferation after 48 h treatment.

Table 3. Effect of Fulvic acid on proliferation of HaCaT human immortalized keratinocyte cells

Groups	Concentrations	Proliferation (%±SD)			
		MTT assay		WST-8 assay	
		24 h	48 h	24 h	48 h
Negative Control	---	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
Citric acid (solvent control)	5 μg mL^{-1}	96.10±0.159	102.66±0.104	84.38±0.182*	101.50±0.135
Crystalin (standard agent)	% 10	88.17±0.076	101.75±0.259	85.00±0.604	101.28±0.935
Fulvic Acid	1 μg mL^{-1}	83.80±0.185*	94.81±0.195	102.01±0.518	110.70±0.733
	5 μg mL^{-1}	75.19±0.296*	92.99±0.137	101.61±0.539	109.80±0.434
	10 μg mL^{-1}	67.40±0.061*	86.90±0.104	96.63±0.325	104.95±0.268
	20 μg mL^{-1}	58.15±0.181*	84.18±0.172*	92.02±0.564	100.52±0.324

*p<0.05

Wound healing effect of fulvic acid in BJ and HaCaT cells

Data for wound healing efficacy of FA on BJ cells are presented in Table 4 and Figure 3. When obtained data is compared with the control group, it is observed that crystalin has a considerably high wound healing effect at 24th h and this effect is considerably higher than that

the negative control group. However, when the effect of crystalin is compared to the citric acid, it appears that the wound healing effect of citric acid is higher than that of crystalin. 1μg mL^{-1} FA has significantly higher wound healing effect than the negative control at 12th and 24th h but, this effect is lower than that of crystalin and citric acid.
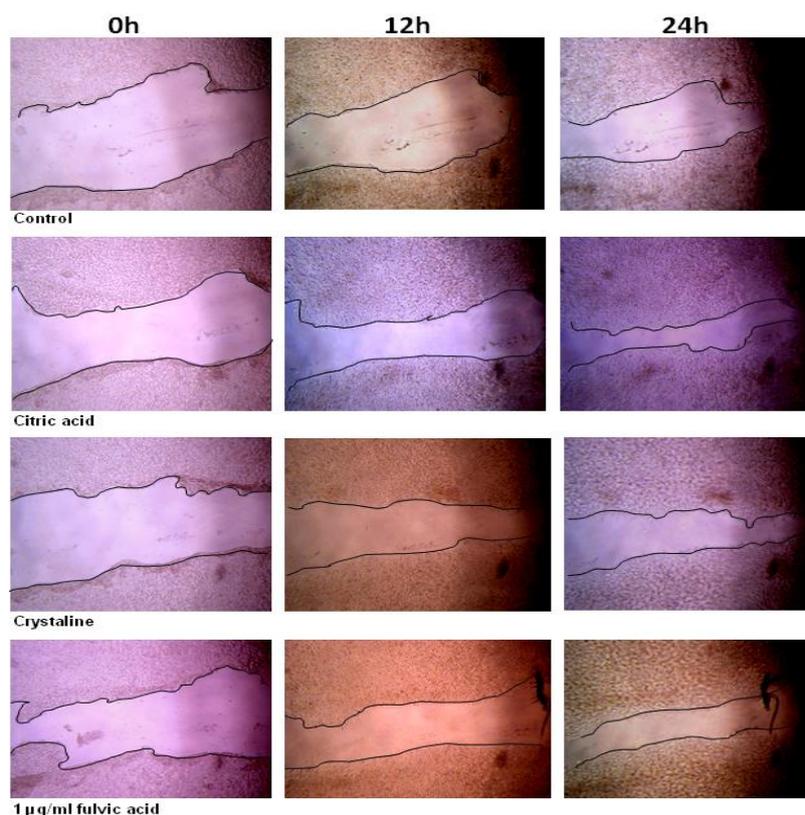
Table 4. Wound healing effect of Fulvic acid on BJ human foreskin fibroblast cell

Groups	Wound healing (%±SD)		
	0 h	12 h	24 h
Negative Control	0.00±0.00	28.42±0.220	45.63±0.630
Citric acid (Solvent control)	0.00±0.00	35.74±0.200*	62.55±0.200*
Crystalin (Standard agent)	0.00±0.00	34.14±0.140*	55.29±0.090*
1 µg mL^{-1} FA	0.00±0.00	36.69±0.190*	53.25±0.250*
5 µg mL^{-1} FA	0.00±0.00	30.77±0.100	47.53±0.200
10 µg mL^{-1} FA	0.00±0.00	17.58±0.200*	53.30±0.361*
20 µg mL^{-1} FA	0.00±0.00	28.48±0.300	45.45±0.250

*p<0.05

5 and 10µg mL^{-1} FA remained far behind the control for the first 12 h and did not show any significant wound healing effect. However, at 24 h, it surprisingly showed a significant wound-healing effect compared to the negative control. 20µg mL^{-1} FA did not show any significant wound healing effect on BJ cells. The citric acid showed considerable wound healing effect from the 12th h and reached a high value (62.55%) at 24th hour compared with negative control group, and FA and crystalin (p <0.05).

Fig.3. Micrographs showing the coverage of scratched wounds by BJ cells in the absence or presence of fulvic acid

Data for wound healing efficacy of FA on HaCaT cells are presented in Table 5 and Figure 4. According to the findings, all concentrations of FA exhibited wound healing effects at a significant level on HaCaT cells relative to the negative control group at the 12th h. The wound healing effects of the other FA concentrations except 10µg mL^{-1} were found to be higher than that of citric acid and crystalin.

At 24th h, all concentrations of FA showed significant wound healing effects in HaCaT cells in comparison with the negative control group, citric acid and crystalin (p<0.05). The most effective concentration of FA was $1\mu g\ mL^{-1}$ FA on HaCaT cells at the end of the 24-hour treatment period.

Table 5. Wound healing effect of Fulvic acid on HaCaT human immortalized keratinocyte cells

Groups	Wound healing (%±SD)		
	0 h	12 h	24 h
Negative Control	0.00±0.00	9.24±1.61	31.91±2.23
Citric acid (Solvent control)	0.00±0.00	18.12±0.20*	37.02±5.87
Crystalin (Standard agent)	0.00±0.00	18.82±2.44*	37.52±1.76
1 µg mL^{-1} FA	0.00±0.00	30.08±2.29*	63.82±6.35*
5 µg mL^{-1} FA	0.00±0.00	23.81±4.81*	49.82±4.98*
10 µg mL^{-1} FA	0.00±0.00	18.35±4.93*	59.40±4.90*
20 µg mL^{-1} FA	0.00±0.00	21.03±6.53*	53.20±3.52*

*p<0.05
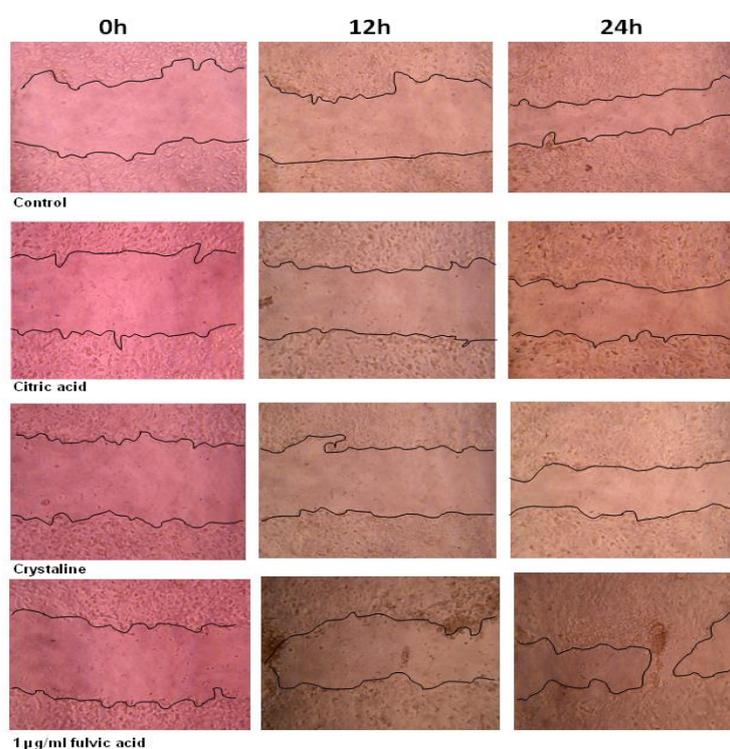
Fig.4. Micrographs showing the coverage of scratched wounds by HaCaT cells in the absence or presence of fulvic acid

Wound healing is a highly complex biochemical event that develops in the injured organism. Disruption or prolongation of any stage of wound healing causes the wound to become chronic, which may not be delayed or improved in wound healing (Guo and Di Pietro, 2006). In our study, FA increased wound healing in both BJ and HaCaT cells related to the duration of administration. The healing process requires extra oxygen, and this demand appears in the first minute after wounding due to phagocytosis, the main event in wound healing process, which is very oxygen-consumptive (Jurcsik, 1994). Humic acid (HA) can increase the production of active oxygen during the wound healing process and in anti-tumor process. Reports by Jurcsik have shown that when HA was incubated with Hep-2 cancer cell line for 24 hours, the proliferation of the HEp-2 cancer cell line was found to be decreased by 65% as compared to the control cell line (68). HA can inhibit the tumor cell multiplication by

intercalating with DNA, blocks the DNA opening and destroys the DNA by producing the reactive oxygen derivatives (Jurcsik, 1994). Fulvic acid may also increase wound healing with a mechanism similar to humic acid.

In the elderly people and diabetic patients, it is often the case that the above-mentioned stages of healing of wounds become late. Oxidative stress is also one of the factors that contribute to impaired wound healing in non-healing wounds (Schafer and Warner, 2008). Endogenous antioxidant's levels such as glutathione and vitamin E are diminished under conditions that impair wound healing including aging and diabetes (Rasik and Shukla, 2000; Ullah et al., 2016). Therefore, the wound healing potential of exogenous antioxidants has received an increasing interest.

Fibroblast proliferation and migration are important factor in wound healing. This event plays an important role in the healing process by initiating the proliferative phase of repair (Fronza et al., 2009; Ebeling et al., 2014; Ascione et al., 2016). The results of proliferation tests (MTT and WST-8) of BJ and HaCaT cells were found different from the wound healing assay results. Cell division (proliferation) is an irreversible process, and it is started by a single and rapid event, while migration is a reversible process (De Donatis and Cirri, 2009). This may be the reason for the difference between the results of cell proliferation and wound healing.

CONCLUSION

The results from our study show that fulvic acid purified from Hüsamlar Leonarditis exhibit a low DPPH radical scavenging activity, but exhibited high H_2O_2 scavenging activity at low concentrations. Fulvic acid accelerated wound healing especially in HaCaT cells by increasing cell migration, in comparison with negative control, citric acid, and wound healing agent crystalin. Therefore, fulvic acid may be a promising agent for wound healing efficacy,

especially at low concentrations. However, in order to reach a more accurate judgment in this regard, more detailed studies should be carried out using different test systems, including *in vivo* tests.

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