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Wound Healing Effects of Various Fractions of Olive Leaf Extract (OLE) on Mouse Fibroblasts[&]

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[&] *This manuscript has been written from some findings reported in the Ph.D Thesis of Dr. İpek Erdogan*

Abstract
Olive (Olea europaea) leaf has been introduced as a potential therapeutic in wound healing owing to combined antioxidant and antimicrobial activity. Comparison of crude extract and its fractions in terms of antioxidant capacity, antimicrobial and cytotoxic activity to gain insight about cell migration rate under exogenous stress of H_2O_2, as a hallmark of wound healing constituted the objective of this study. Oleuropein-containing fraction exerted the highest cell migration rate among other fractions that contains hydroxytyrosol, verbascoside and luteolin, whilst treatment with high concentrations (50µg/ml) of this fraction simultaneously with H_2O_2 caused a dramatic decline in cell migration, resulting in the loss of cell adherence. Results overall indicated that active compounds caused an imbalance in redox signaling beyond a critical concentration. Comparison of fractions and crude extract also revealed that crude extract promoted cell migration by 20%, which may be attributed to synergistic effect of undefined phenolics.

Keywords: *Olea europaea* L., oleuropein, fibroblast, antioxidant, wound healing.

1. Introduction
Impairment of skin integrity due to external factors as physical or thermal damage, as well as medical or physiological conditions can be termed as wound. Wound Healing Society defines the wound as the result of disruption of normal anatomic structure and function [1]. Wound healing process is initiated by activation of signaling pathways which proceeds with protein synthesis for proliferation and migration [2].
Wounds can be classified as acute or chronic as former commonly occurs by external factors, whilst repeated tissue insults due to medical issues such as diabetes, malignancies and persistent infections cause chronic wounds [3].
Absence of reepitelization is dominant in chronic wounds [4], caused by population of microbial colonies termes as bioburden [5]. Leukocytes migrate to wound area and generate a high-protease, high-oxidant environment as a defense action [3]. Oxidant-producing enzymes are essential to discard microbial burden from wound area to accelerate wound healing but their activity is limited in case of ischemia and resulting hypoxia, which is a characteristic of chronic wounds due to poor blood flow and high oxygen demand. High protease activity, resulting from leukocyte attack, causes degradation of extracellular matrix. Degraded matrix provides nutrient for the microorganisms and arises the microbial count while wound closure

is hindered by inhibition of cell migration. *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus* species, *Proteus* species, *Escherichia coli*, *Klebsiella* and *Citrobacter* species are the most common isolates in chronic wounds [6].
Recent studies focus on natural compounds that reduce the effects of microbial flora and high amount of free radicals in wound healing process. Especially, plant-derived components known as secondary metabolites, are commonly used due to their antioxidative, antimicrobial and anti-inflammatory properties [7, 8]. Challenge today is to isolate these bioactive components without activity loss [9].
Olive leaf is one of the prominent sources for therapeutic active compounds. Compositional analyses in various studies conclude that crude olive lef extract (OLE) is composed of oleuropeosides, flavones, flavonols, flavan-3-ols and substituted phenols [9, 10]. This polyphenolic structure makes the extract one of the most effective natural compound to scavenge free radicals. Studies indicate that it can be a potential therapeutic in oxidative-stress related medical issues such as coronary and neurodegenerative diseases and cancer [11, 12]. Oleuropein, being the most abundant phenolic compound in the extract also has protective effect against plant pathogens indicating its antimicrobial activity. The extract was shown to exhibit antimicrobial activity against especially resistant species such as *Klebsiella* and *Pseudomonas* and *Escherichia coli* [13, 14] which may be used in wound healing against opportunistic infections due to long-term antibiotic usage. This study aims to exhibit effect of OLE in crude form and as individual fractions on cell migration which is a hallmark of *in vitro* wound healing model under exogenous stress.

2. Materials and Methods

Cell lines, microorganisms and chemicals

Analytical grade ethanol, acetic acid, sodium carbonate, Folin-ciocalteu reagent and gallic acid standard (purity ≥98%) were purchased from Merck. HPLC grade acetonitrile, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl) tetrazolium bromide), MitoRed and HPLC standards of oleuropein (purity ≥98%), luteolin (purity ≥97%), hydroxytyrosol (purity ≥98%), verbascoside (purity ≥99%) and rutin (purity ≥94%), were obtained from Sigma. ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid), potassium peroxodisulfate ($K_2S_2O_8$) and Trolox standard (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (purity ≥98%), used in antioxidant analysis, were purchased from Fluka. Nutrient and potato broth were purchased from Merck and BD respectively. Bacteriological agar and peptone were obtained from Oxoid. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), penicillin-streptomycin antibiotic solution were purchased from Gibco. *Candida albicans* (ATCC 64548), *Escherichia coli* (NRRL B-3008) and *Staphylococcus epidermidis* (ATCC 12228) were purchased from ATCC as lyophilized. NIH-3T3 (mouse fibroblast) cell line was purchased from ATCC (CRL-1658).

Plant material and Preparation of OLE

Leaves of *Olea europaea* L. were collected in February, in the pruning season before flowering period, from Aquaculture Central Research Institute in İzmir.
Olive leaves were dried at room temperature and ground in a coffee grinder. Extraction was performed in 70% aqueous ethanol solution with a solid-liquid ratio of 1:20 at 180 rpm at room temperature for 2 hours. After filtration, extract was evaporated at 40 ºC to remove ethanol. Aqueous phase of extract was centrifuged at 4000 rpm for 5 minutes to remove solid residues. The liquid extracts were lyophilized for three days.

HPLC Analysis and Fractionation of OLE

Composition of crude OLE was determined by HPLC with a diode array detector at 280 nm. Zorbax C18 semi-preparative column was used as stationary phase. The flow rate was 4.5 mL/min. Mobile phases were (A) acetic acid: water (2.5:97.5) and (B) acetonitrile. Gradient was as follows 0-20 min- 95% A; 20-40 min- 75% A; 40-50 min- 50% A; 50-60 min- 20% A. Oleuropein, hydroxytyrosol, verbascoside and luteolin in OLE were identified by comparison of their retention times with the corresponding standards. Obtained crude OLE (50 mg/ml) was fractionated according to relative polarity by fraction collector at 2 minute time intervals. The mobile phase in the fractions was removed by rotary evaporator. Resultant residual fractions dissolved in deionized water, were subjected to HPLC analysis to determine the amount of active compounds.

Total Phenol Content (TPC) and Trolox Equivalent Antioxidant Capacity (TEAC) Assays

TPC of crude extract and its fractions were determined by Folin-Ciocalteu method. After incubation with Folin-ciocalteu reagent diluted by 1:10 (v/v) and sodium carbonate (7.5%), absorbances measured at 725 nm. Results were expressed as milligrams of gallic acid equivalents (GAE) per gram dry weight (DW) by using gallic acid calibration curve. The antioxidant analysis method was based on the ability of olive leaf antioxidants to scavenge the ABTS radical cation compared with Trolox. Aqueous ABTS solution of 7 mM was mixed with 2.45 mM potassium peroxodisulfate solution to form ABTS cation followed by incubation in dark at ambient temperature for 16 h. The ABTS solution was diluted with ethanol to an absorbance of 0.7 (±0.03) at 734 nm prior to use. Inhibition percentage was calculated by the formula given below and expressed as milimole TEAC per gram dried extract.

% inhibition = 1- (Absorbance final /Absorbance initial) x 100

Minimum Inhibition Concentration (MIC) Assay

Microorganisms were grown on agar overnight at 37°C followed by determining colony number by McFarland units. Crude extract and fractions dissolved in sterile deionized water, were subjected to MIC assay between concentrations of 1 to 3000 µg/ml in 96 well plate. Growth kinetics for each strain was measured at 600 nm for 24 h at 37°C.

Cytotoxicity and Wound Scratch Assays

For cytotoxicity assay, NIH-3T3 cells were grown in DMEM supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS), 1% penicillin–streptomycin, in an atmosphere of 5% carbondioxide (CO_2) at 37ºC. Crude extract and fractions were subjected to cytotoxicity assay using MTT. Cells were seeded on 96-well plates a day prior to assay at 10^4 cells/well. After removal of mobile phase, fractions were dissolved in DMEM containing 10% FBS, incubated with cells up to 72 hours followed by measurement at 545 nm. Cell viability was determined by the formula; Cell viability (%) = (Absorbance value of sample / Absorbance value of control) x100

For wound scratch assay, NIH-3T3 cells were seeded on 24-well plates at a concentration of $2x10^5$ cells/well and incubated in an atmosphere of 37°C and 5% CO_2. After overnight incubation, cells were scratched by a pre-sterilized, polyoxymethylene originated, circular cell scratcher of 5 mm diameter, followed by discarding suspended cells. Adhered cells were exposed to crude OLE and fractions, in DMEM with 10% FBS. Assay was also performed under exogenous stress created by hydrogen peroxide (H_2O_2) at the concentrations of 1, 10,

50 and 100 µM for 1 hour. Following incubation, media containing H_2O_2 was replaced with the media containing crude OLE and fractions at the concentrations of 1, 10 and 50 µg/ml. Measurement of the wound diameters and observation of cell migration were performed by camera mounted on a phase contrast inverted microscope. Media were refreshed every 48 hours to prevent nutrition deficiency. Wound closure percentage was calculated as follows;

Wound closure percentage (%) = $((D_0-D_x)/D_0)*100$

Where D_0 denoted diameter of wound on the first day of assay and D_x denoted diameter of wound on determined time points. X denoted 1^{st}, 3^{rd} and 5^{th} day of the assay in the calculations. For an additional confocal microscopy observation, cells were seeded on 35 mm dishes at the concentration of $4x10^5$ cells/ml. After overnight incubation, sterile pipette tip was used for scratching. After discarding suspended cells, adhered cells were exposed to 100 µM hydrogen peroxide for 1 hour, followed by treatment with OLE and fractions at the concentrations of 1 and 50 µg/ml. Cells were subjected to confocal microscopy (Andor Revolution) after treatment with red flourescent mitochondrial dye, MitoRed (Excitation/Emission: 622/648 nm).

3. Results and discussion

Fractions were analyzed by HPLC to confirm the separation by comparison of retention times. Purity of fractions was depicted in chromatograms in Figure 1. Ensuring that fractionation was successfully achieved, further HPLC analysis was performed to determine the retention time of each active compound and to obtain their calibration curve. Retention time of hydroxytyrosol, verbascoside, oleuropein and luteolin were found as 5.9, 17.2, 21.7 and 26.2 min, respectively. Standard addition method was also employed to confirm the presence of each active compound in individual fractions. Confirmation was provided by the shifts in the absorbances values, caused by presence of relevant active compound.

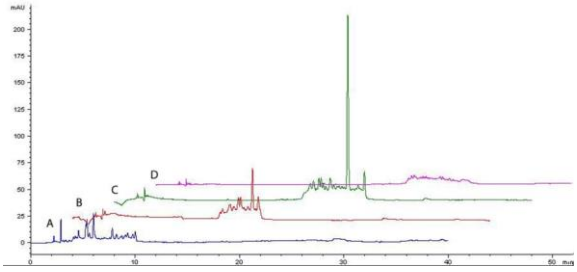

Figure 1. Fractionation of olive leaf extract (OLE) A. Hydroxytyrosol-containing fraction 2 (fr2), B. Verbascoside- containing fraction 4 (fr4), C. Oleuropein- containing fraction 5 (fr5), D. Luteolin- containing fraction 6 (fr6).

Followed by HPLC analysis of OLE (Figure 2), concentration and abundance of active compounds in crude extract and fractions were calculated based on calibration curves, prior to further analysis (Table 1). Oleuropein was found as the most abundant active compound by 22% (w/w) in crude extract as figured out by Altıok et al. [9]. Percentages of hydroxytyrosol, verbascoside and luteolin were found as 0.6%, 1.4% and 0.6%, respectively [10]. The chemical composition of olive leaves varies according to origin, proportion of branches present in the extract, storage conditions, weather conditions, moisture content and degree of soil contamination [15]. The most abundant compound in olive leaves was found as oleuropein, followed by verbascoside and hydroxytyrosol. Hydroxytyrosol is also precursor of oleuropein and verbascoside is a conjugated glucoside of hydroxytyrosol and caffeic acid

[10]. The results determined with HPLC analyses are in accordance with the findings reported in the literature earlier.

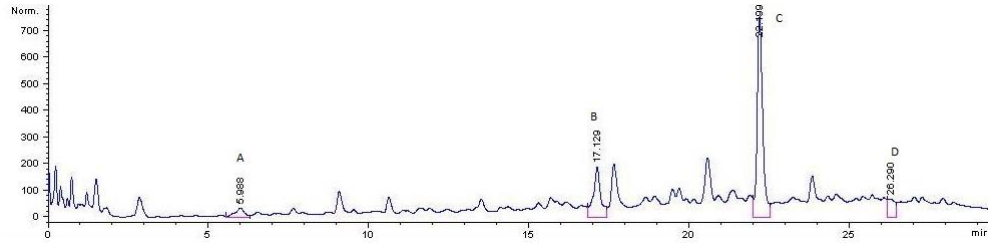

Figure 2. Chromatogram of crude olive leaf extract. Letters represent peaks of selected active compounds. A. Hydroxytyrosol, B. Verbascoside, C. Oleuropein, D. Luteolin.

Table 1. Concentration and abundance percentage of active compounds in crude extract and fractions

Active compound	Concentration in crude extract (mg/ml)	Abundance in crude extract (%)	Concentration of the active compound (no. of related fraction) (mg/ml)
Hydroxytyrosol	0.3	0.6	0.03 (Fr2)
Verbascoside	1.75	3.5	0.23 (Fr4)
Luteolin	0.33	0.65	0.02 (Fr5)
Oleuropein	2.262	22.62	0.34 (Fr6)

TPC and TEAC of crude extract were found 184±25 mg GAE/g DW and 1.34±0.18 mmole TEAC/g DW, respectively. Kontogianni et al. [16] determined total phenol content range of OLE between 70-483 mg GAE/g DW whilst Hayes et al. [17] also found TPC for OLE as 160.8 mg GAE/g DW, complying with our findings. TEAC range was also found between 1.5-7.52 mmole TEAC/g DW [10, 14] which was compatible with our results. TPC of the fractions ranged between 0.038-0.1 mg GAE/ml fraction, among which oleuropein-containing fraction 5 (fr 5) had the highest amount of phenolics. High amount of phenolics resulted in higher antioxidant capacity as oleuropein exhibited 0.36±0.06 mmole TEAC/ml fraction. The antioxidant activity of the olive leaf was attributed to its major phenolic compounds such as oleuropein, hydroxytyrosol, and luteolin-7-O-glucoside acid and the presence of a functional group in their structure, catechol [12, 17].

MIC assay was performed with *E. coli*, *S. epidermidis* and *C. albicans* with crude OLE and fractions based on concentrations which exhibited cytotoxic activity. Our results indicated that OLE exhibited bacteriostatic effect on *E. coli* and *S. Epidermidis*, which was better than antifungal effect since growth of *C. albicans* was not affected. Fractions of OLE were also subjected to MIC assay with *S. epidermidis* and *E. coli* at the concentrations of 500, 1500 and 3000 µg/ml. The results revealed that 3000 µg/ml of Fr6 exhibited better antimicrobial property in both strains, whereas 3000 µg/ml of Fr5 also inhibited the growth of *S. epidermidis*. But neither of the fractions completely inhibited the growth of the microorganisms better than crude OLE which can be attributed to its polyphenolic nature.

Crude OLE and fractions were subjected to cytotoxicity assay by MTT up to 72 hours. NIH-3T3 cells were treated with OLE and standard oleuropein at concentration range between 1-3000 µg/ml. As seen in Figure 3A, crude OLE concentrations higher than 100 µg/ml

exhibited cytotoxicity. Cytotoxic profile of oleuropein, as in Figure 3B, also exhibited similarity with crude OLE. Concentration range of fractions for cytotoxicity assay was determined based on percent abundance of each active compound in related fraction. Fractions did not exhibit cytotoxic activity up to 1500 µg/ml equivalent, contrary to crude OLE which exhibited cytotoxic activity at lower concentrations as 250 µg/ml (Figure 4) which may be attributed to synergistic effect of active compounds in crude extract.

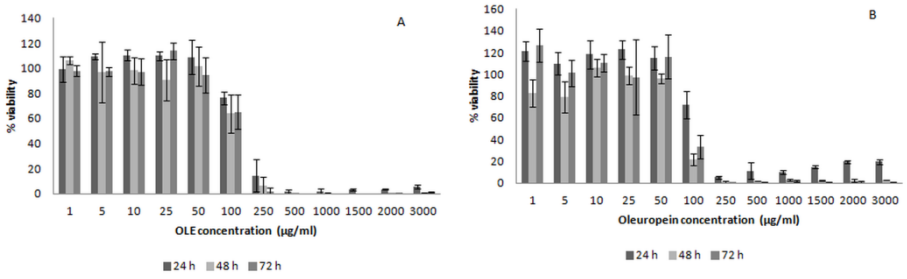

Figure 3. Cytotoxic profile of A. Crude OLE, B. Oleuropein Standard

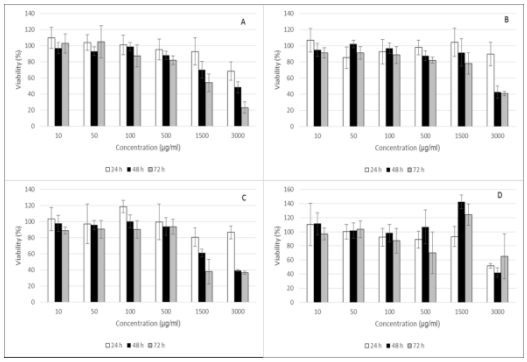

Figure 4. Cytotoxic profiles of fractions. A. Hydroxytyrosol- containing Fr2, B. Verbascoside- containing Fr4, C. Oleuropein- containing Fr5, D. Luteolin- containing Fr6.

NIH-3T3 cells were also subjected to wound scratch assay in order to determine the effect of crude OLE and its fractions on cell migration and wound closure. Assay was considered to be applied in two parts, under stress-free conditions and under stress conditions via addition of exogenous H_2O_2. Among reactive oxygen species (ROS), H_2O_2 is the most stable oxidant, and it can diffuse across cellular membranes through water channels and causes oxidative protein modifications [18], which makes it a model for oxidation studies. To determine the effects of different concentrations of H_2O_2, MTT assay was employed in migration assays. NIH-3T3 cells were treated with H_2O_2 between concentrations of 1–1000 µM. Figure 5 indicates that H_2O_2 concentrations up to 50 µM had no adverse effect on cell viability as Alexandrova et al. [19] reported in a study on rat fibroblast cells that 50 µM H_2O_2 could enhance cell adhesion and cell motility. Haendeler et al. [20] also indicated that H_2O_2 concentrations between the range of 10–50 µM H_2O_2 was protective for endothelial cells, whereas 100 µM H_2O_2 exerted cytotoxicity. Our findings show consistency with the literature.

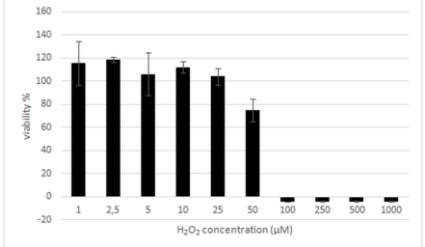

Figure 5. Cytotoxic profile of varying H_2O_2 concentrations.

NIH-3T3 cells were treated with crude OLE and H_2O_2 as controls in wound scratch assay. Figure 6 indicates the percentage of wound closure upon exposure to a range of H_2O_2 and OLE concentrations, respectively. H_2O_2 concentration of 100 µM did not provide wound closure during 5 days and wound diameter on 3rd and 5th days could not be measured due to loss of cell adherence. Hence percentage of wound closure of those samples were noted as zero. Results indicated that lower concentrations (between 1-50 µM) of H_2O_2 promoted cell migration. OLE treatment on fibroblast cells prior to wound scratch assay also revealed that non-cytotoxic concentrations of OLE also exhibited wound healing by promoting cell migration, indicated as accelerated wound closure. Concentrations of 1 and 10 µg/ml OLE provided wound closure completely while 250 µg/ml OLE was not sufficient to perform wound healing effect due to its cytotoxic activity.

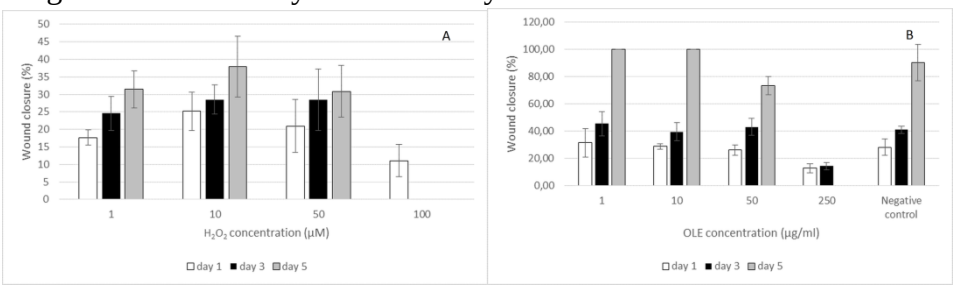

Figure 6. Wound closure percentage of fibroblast cells upon exposure to A. H_2O_2, B. OLE.

Wound scratch assay performed under exogenous H_2O_2, combined with crude OLE showed that combination of low concentrations of H_2O_2 and crude OLE (1 µg/ml) may promote cell migration and wound closure (Figure 7). When compared with results depicted in Figure 6, addition of OLE after H_2O_2 exposure performed a significant acceleration towards cell migration in a positive manner, which may be attributed to scavenging effect.

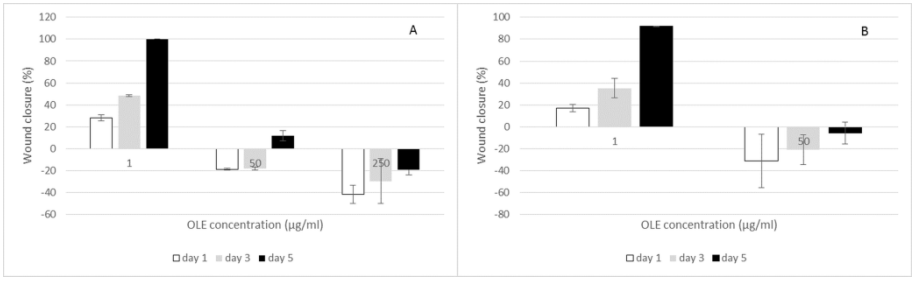

Figure 7. Wound closure percentage of fibroblast cells upon exposure to crude OLE under exogenous H_2O_2 of A. 1 µM and B. 50 µM.

As the last part, cells pretreated with H_2O_2 (1-100 µM) for 1 hour were treated with OLE fractions. Table 2 denotes the wound closure percentages of the 5th day of the experiment. As an overview it can be concluded that combination of higher H_2O_2 concentrations (50 and 100 µM) with higher fraction concentrations (50 µg/ml) led to failure in wound closure, due to combination of high antioxidant level of fractions and high level of oxidative stress caused by H_2O_2. Scavenging ability of fractions were not sufficient to promote cell proliferation and migration for wound healing. Wound scratch assay performed with crude extract under exogenous H_2O_2 stress also indicated that combination of lower concentrations of extract H_2O_2 induced cell migration and wound closure, as depicted in Figure 8 and 9. In this case, cell migration promoted by H_2O_2 and proliferative effect of OLE and fractions may result in

acceleration in wound closure. With respect to effect of fractions on wound healing, oleuropein-containing fraction 5 can be considered as having the highest potential to close the wound area by promoting cell migration.

Table 2. Wound closure percentages of fibroblast cells on the 5th day after exposure to OLE fractions of various concentrations. Cells were pretreated with a range of H_2O_2 concentrations.

H_2O_2 concentration (µM)	1	10	50	100
fr2 concentration (µg/ml)				
1	47.26±5.53	44.95±4.92	49.91±13.07	47.29±6.65
10	100.00	74.04±8.73	24.23±4.47	35.61±6.12
50	28.52±1.07	53.15±3.81	100.00	15.77±2.47
fr4 concentration (µg/ml)				
1	65.45±9.3	31.85±3.56	66.69±5.85	35.50±8.86
10	100.00	100.00	31.60±7.81	60.39±1.08
50	78.30±6.23	20.54±4.55	26.71±6.76	23.39±5.4
fr5 concentration (µg/ml)				
1	89.87±9.56	100.00	25.12±5.22	N/A*
10	51.72±0.71	81.75±4.13	58.14±0.74	N/A*
50	35.55±1.34	30.82±6.18	20.15±1.21	N/A*
fr6 concentration (µg/ml)				
1	41.07±9.97	88.90±6.71	100.00	51.46±3.33
10	56.21±4.51	34.72±3.54	49.19±1.51	N/A*
50	38.78±5.51	11.65±5.36	N/A*	N/A*

* Denotes that diameter measurement could not be achieved due to loss of cell adherence

Confocal micrographs, depicted in Figure 10, also complied with wound scratch assay results, since membrane depolarization was observed as fraction concentrations increased. Flourescence of MitoRed was observed brighter in samples with lower OLE concentrations which indicated live cells with mitochondrial membrane integrity. Lower concentrations of OLE fractions also exhibited scavenging activity sufficient to maintain membrane integrity whilst higher concentrations contributed to membrane depolarization effect by altering redox potential, which in turn affected cell viability negatively.

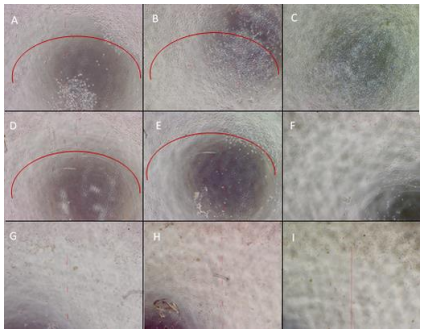

Figure 8. Micrographs of wound scratch assay. Cells were exposed to 1 µM H_2O_2 Micrographs were taken on 1st, 3rd and 5th days, denoted as A-C, D-F and G-I, respectively. Cells were treated with 1 µg/ml OLE: A-C, 50 µg/ml OLE: D-F and 250 µg/ml OLE: G-I. Red line indicates the border of wound area.

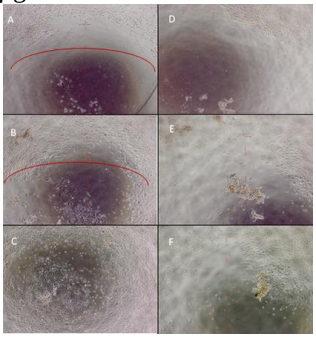

Figure 9. Micrographs of wound scratch assay. Cells were exposed to 50 µM H_2O_2 followed by OLE treatment at concentration of, A-C: 1 µg/ml and D-F: 50 µg/ml. A and D denote 1st day, B and E denote the 2nd day and C and F denote the 3rd day of the experiment. Red line indicates the wound area.

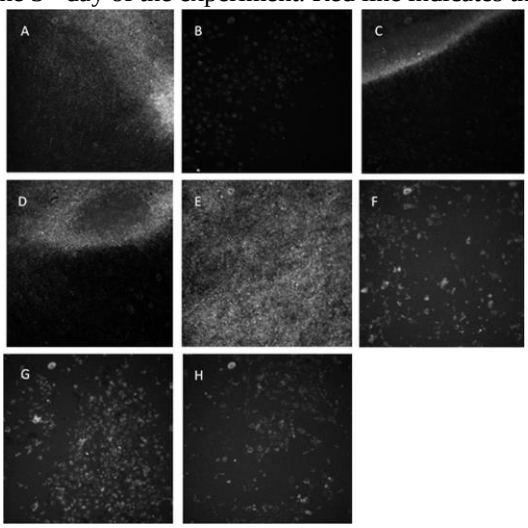

Figure 10. Confocal microscopy micrographs of wound scratch assay. Cells were exposed to 100 µM H_2O_2. Micrographs were taken on 3rd day of the assay. Cells were treated with 1 µg/ml OLE: A, C, E and G, and 50 µg/ml OLE: B, D, F and H.

This study focused on characterization of OLE in terms of active compound content, TEAC, TPC and correlation with cytotoxic activity and cell migration, within wound healing concept. Fractionation was also employed to obtain active compound-containing parts of crude extract in order to understand whether the effect exerted by the extract was based on an individual compound or synergistic effect of all compounds. Oleuropein exhibited its dominancy among four fractions, exerting highest total phenolic content and antioxidant activity which is related to the hydroxytyrosol moiety in its structure. Catechol structure produces stable resonance structures by scavenging peroxyl radicals and breaking peroxidative chain reactions [21, 22]. Cytotoxicity profile of standard oleuropein was also found to be comparable with crude OLE which indicates the abundance of oleuropein.

Free radicals are generated due to oxygen consumption in cell growth and collectively known as ROS. Pan et al. [23] found that cells treated with H_2O_2 at 20 µM showed no adverse effect on cell viability but actually stimulated adhesion and migration in cultured cells and enhanced pig eye cornea wound healing *ex vivo* as well as mouse cornea wound healing *in vivo*. These data show correlation with our findings in the aspect of tendency of cells to migrate under lower concentrations (between 1-50 µM) of H_2O_2. ROS can directly activate tyrosine kinase Src by oxidizing its two conserved Cys moieties during cell spreading in fibroblasts [24, 25] as well as regulating integrin-extracellular matrix engagement by activating epidermal growth factor receptor, EGFR [23] to enhance cell adhesion and spreading.

The interaction of generated oxygen free radicals with lipid molecules results in formation of superoxide, hydroxyl and lipoid peroxides which may exhibit cytotoxic effect on biological systems [26]. Scavenging ability of OLE prevents the cytotoxic activity of free radicals up to a critical concentration. Cells require a redox-balanced environment to maintain a healthy status. In cells treated with high amount of extract, basal level of ROS can be removed due to high amount of phenolics, resulting in a state hypo-oxidative condition because of redox imbalance [23]. In this condition excess ROS attacks and damages virtually all biomolecules

in the cells, leading to cell death and serious chronic diseases [27]. Tsuraya et al. [28] induced the proanthocyanidin-pretreated fibroblast cells with H_2O_2 and observed that generated oxidative stress was suppressed. Another study performed by Nadour et al. [29] showed that phenolic compounds extracted from green olive fruits not only exhibited antioxidant capacity significantly comparable to vitamin C but also provided membrane integrity of erythrocytes pre-exposed to H_2O_2. Quercetin and apigenin were utilized as scavenging compounds which prevented mitochondrial membrane depolarization in 3T3-L1 adipocyte cells treated with tunicamycin [30]. Pan et al. [23] also showed that toxicity of N-acetylcysteine treatment [31] was inhibited when introduced with H_2O_2 which promoted cell adhesion and migration, which in turn facilitated wound healing. In another study [32] fibroblast cells treated with 0.01% citrus polyphenols exhibited accelerated wound healing in contrast to 1% polyphenol treatment due to increased cytotoxic activity, indicating that the higher is not the better for polyphenol amount in wound healing.

In our study, cytotoxic concentration of fractions were also found higher than crude OLE, which may be attributed to synergistic effect of all compounds. Furthermore, this proposed synergistic effect of crude OLE affected antimicrobial activity and cell migration results. Results of wound scratch assay indicated that rate of cell migration and wound closure was higher when crude OLE was used, even under stress conditions generated by exogenous H_2O_2. Wound area was populated by fibroblast cells earlier when exposed to crude extract, compared to same concentration of active compound-containing fraction. These findings may indicate that efficiency of crude OLE is better than individual components, in terms of antioxidant capacity, antimicrobial activity and cell migration rate.

4. Conclusion

Oleuropein, having the hydroxytyrosol moiety in catechol structure, exhibited its dominancy among four fractions, exerting highest TPC and TEAC as well as promoting cell migration better than other fractions. It was also observed that wound closure was observed in a shorter period under exogenous stress of H_2O_2 unless high concentrations of OLE and H_2O_2 were combined.

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