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Extraction and Purification of Ursolic Acid from the Apple Peel and *in vitro* Assessment of the Biochemical Antibacterial, Antioxidant and Wound Healing Characteristics

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Abstract

Background and Objective: Ursolic acid is a pentacyclic triterpenoid with various biological characteristics. The objective of this study was to investigate potentially biological activities of ursolic acid extracted from apple peels.

Material and Methods: Ursolic acid was extracted from apple peels and purified using column chromatography. Then, the biochemical was analyzed using ultraviolet-visible spectroscopy, high-performance thin-layer chromatography, Fourier-transform infrared spectroscopy and nuclear magnetic resonance techniques. Antimicrobial effects of the purified ursolic acid on pathogenic bacterial species of *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis* were assessed using minimum inhibitory concentration and disc diffusion methods. Furthermore, the biochemical radical scavenging ability was assessed using 1,1-diphenyl-1-picrylhydrazyl method. Wound healing characteristics of the purified ursolic acid was studied using scratch assay method.

Results and Conclusion: Minimum inhibitory concentration and disc diffusion results verified antibacterial effects of ursolic acid on Gram-positive bacterial species. Ursolic acid at concentrations higher than 625 µg ml^{-1} showed significant antioxidant activity, compared to that vitamin C did as reference antioxidant. It was shown that migration and proliferation of human umbilical vein endothelial cells can be promoted by the extracted ursolic acid, which was assessed via wound healing assays and 3(4,5- dimethylthiazol- 2- yl)- 2,5- diphenyltetrazolium bromide. Wound closure was 97%, revealed by the purified ursolic acid after 24 h. A low concentration of ursolic acid (< 20 µg ml^{-1}) stimulated proliferation of human umbilical vein endothelial cells; however, 100 µg ml^{-1} of the extracted ursolic acid decreased the number of viable cells within 24 h ($p < 0.05$). Purified ursolic acid (10 µg ml^{-1}) was able to upregulate (almost two times) FLT1 and VEGF-A gene expression in human umbilical vein endothelial cells. Results suggest that ursolic acid is an effective antioxidant and includes excellent antibiotic characteristics. In addition, it can affect endothelial cell proliferation, which is significant to enhance angiogenesis and improve wound healing processes.

Conflict of interest: The authors declare no conflict of interest.

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1. Introduction

Fruits and vegetables are within the most important food supplies in human life, which are consumed because of their nutritionally advantageous constituents and health benefiting effects. Apple is consumed frequently worldwide. This fruit includes phytochemicals and antioxidants, various vitamins, high quantities of fibers and moderate calorie contents [1,2]. Studies have reported isolation of natural biocomponents from apple peels. Examples of these biocomponents include fatty acids, polysaccharides, proteins, polyphenols [3], pectin [4], flavonoids and triterpenoids [5]. Ursolic acid (3-β-

Hydroxy-12-ursen-28-ic acid) as a well-known pentacyclic triterpenoid includes various biological characteristics, including antibiotic, anticancer [6], antiviral, anti-inflammatory and antidiabetic characteristics [7]. Anticancer effects of ursolic acid are seen because of the biochemical potentials to inhibit proliferation and metastasis as well as promoting apoptosis in cancer cells [8]. Anticancer effects of ursolic acid in human metastatic melanoma cancer cells (SK-MEL-24) are majorly due to the inhibition of mitogen-activated protein kinase (MAPK)/ERK signaling pathway, arrest of cell cycle, inhibition of cell migration and invasion, and apoptosis induction [9]. For example, ursolic acid has shown anti breast cancer characteristics in multiple breast cancer cell lines and animal samples by targeting various cellular signaling pathways involved in breast tumors [10]. Kang et al. [11] investigated antitumor effects of ursolic acid by mediating inhibition of STAT3/PD-L1 signaling in quite large-cell lung cancers.

Nowadays, bacterial resistance to antibiotics is a critical health issue worldwide. Bacteria can form biofilms that enhance the bacterial resistance by blocking antibiotic actions. Several studies have reported that ursolic acid includes antimicrobial activities against various pathogenic species. Zhou et al. [12] investigated antibacterial activities of ursolic acid against methicillin-resistant *Staphylococcus* (*S.*) *aureus* (MRSA) and indicated that the biochemical included significant activities against MRSA infections and could be used as an effective natural antibiotic. Ursolic acid at 60 μg ml^{-1} doses was shown to inhibit biofilm formation by *S. aureus* up to 71.5%.

Moreover, this compound could enhance sensitivity of bacterial strains to various antibiotics [13]. Qian et al. [14] investigated antimicrobial and antib-iofilm activities of ursolic acid against carbapenem-resistant *Klebsiella pneumoniae* and showed that ursolic acid was effective against carbapenem-resistant *Klebsiella pneumo-niae* at the MIC of 0.8 mg ml^{-1}. This acid provided excellent inhibitory effects against formation of biofilms. Do Nascimento et al. [15] reported major antibacterial activities of ursolic acid and its associated derivatives against bacterial strains. They also reported synergistic functions between these compounds and antibiotics such as aminoglycosides, neomycin, amikacin, kanamycin and gentamicin.

Oxidative stress can be induced in cells by an elevated level of reactive oxygen species, causing cellular damage by enhancing oxidative impairment of the cell components and initiating various conditions [16]. Oxidative stress highly affects pathogenesis of the chronic diseases such as cardiovascular diseases, neurological disorder, cancers, age-related optical diseases and diabetes [17]. When oxidative stress destroys body antioxidant capacity, physiological functions may be impaired; thus, human diseases can be triggered. Therefore, using exogenous sources of antioxi-dants can delay these detrimental effects [18]. Chang et al. [19] demonstrated the antioxidant activities of ursolic acid. Furthermore, antioxidant and antiproliferative potentials of Ursolic acid were shown by Srinivasan et al. [20]. Another study assessed the antioxidant characteristics of ursolic acid and its derivatives using 1,1-diphenyl-1-picrylhydrazyl (DPPH) assay and detected essential scavenger effects for these biochemical compounds [15]. Moreover, the radical scavenging ability has been verified in several previous studies. Ursolic acid includes substantially antibacterial characteristics and has demonstrated synergistic activities with the aminoglycoside antibiotics such as gentamicin, amikacin, kanamycin and neomycin; hence, the biochemical can be a radical scavenger [15,19,21]. Several studies have verified the minimum toxicity, acceptable safety and tolerability of ursolic acid at various doses, revealing its ability to be used as an appropriate therapeutic agent for the treatment of certain diseases [22,23].

Ursolic acid can include significant effects on improving wound healing processes. It is effective on increasing vital proteins for the terminal differentiation of keratinocytes, including philagrin, involucrin and loricrin [24]. In addition, ursolic acid improves intercellular lipids such as ceramide, which support the epidermal barrier recovery [25]. Moreover, ursolic acid can constrain intracellular reactive oxygen species creation and control oxidative effects of UVB radiation by inhibiting lipid peroxidation, which is responsible for skin damage [26]. In a study by Carletto et al. [27], poly(L-lactic acid),lipid-core nanocapsules containing ursolic acid were developped to accelerate healing processes of skin damage in ovariectomized rats.

Although several studies have demonstrated biological effects of ursolic acid; however, efficiency of ursolic acid extracted from apple peels for antibiotic, antioxidant, angiogenesis and wound healing purposes has not been demonstrated. Due to the importance of cost-efficient and facile extraction methods to ensure availability of ursolic acid from apple peels and considering the numerous therapeutic characteristics of ursolic acid, isolation and purification of ursolic acid from apple peels were carried out in this study. Although ursolic acid has been extracted from apple peels in previous studies, separation with two hydrophilic and hydrophobic phases is easier and faster, resulting in high purity of the produced ursolic acid. Potentially biological characteristics of the purified ursolic acid, including the antibacterial and antioxidant activities, were also assessed. Moreover, angiogenesis activity of the purified ursolic acid on human umbilical vein endothelial cells (HUVECs) from the endothelium of umbilical cord veins was investigated to show effects of purified ursolic acid on cell migration and wound healing.

2. Materials and Methods

2.1. Materials

Red Fuji apples were purchased from a local market, Tehran, Iran, December 2020. Peels were separated from the fruits, dried and ground to produce a fine powder with particle sizes of 150 µm and moisture contents of ~10%; from which, ursolic acid was extracted. Standard ursolic acid (≥ 90%) and vitamin C (99%) were purchased from Sigma-Aldrich, USA. For antimicrobial and cell assessments, *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 25922, *S. aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633 and human umbilical vein endothelial cells (HUVECs) were provided by Pasteur Institute of Iran, Iran. All reagents included analytical grades.

2.2. Ursolic acid extraction and purification from apple peels

Apple peel powder was soaked in n-hexane for 5 min to remove impurities. To extract ursolic acid from the peels, powder was soaked in ethanol (96%) at 45 ℃ for 24 h, followed by concentrating the extract using rotary evaporator. To isolate ursolic acid, pH of the extract was adjusted close to the pKa of the carboxyl group of ursolic acid (4.7). Then, Na_2CO_3 was added to the mixture and agitated overnight to create a hydrophilic phase. Chloroform was added to the mixture to form hydrophilic and lipophilic phases and direct the ursolic acid to the hydrophilic phase. For further purification, ursolic acid was passed through column chromatography (C18) and analyzed using ultraviolet-visible (UV-vis) spectrophotometer (Analytik Jena, Germany) for the availability of ursolic acid in the extracted solution using ethanol as the standard. Ursolic acid extracted from the apple peels was quantified using high-performance thin-layer chromatography (HPTLC) technique. Samples were analyzed on HPTLC plates. Then, silica gel 60 F_{254} was pre-coated on aluminum sheets (Merck, Germany) using solvent system of a mixture of ethyl acetate, toluene and formic acid (1:7:0.1, v/v/v) at 530 nm with saturation time of 10 min [28]. Characterization of the purified ursolic acid was carried out using Fourier-transform infrared spectroscopy (FTIR, Perkin Elmer Spectrum 100, Perkin Elmer, USA) and nuclear magnetic resonance (^{1}H-NMR Bruker AVANCE 400 MHz). The ^{1}H-NMR spectroscopy was carried out to analyze purity of the extracted ursolic acid. Hence, $CDCl_3$ was used as a solvent at room temperature (25 ℃) [29].

2.3. Antimicrobial activity of the purified ursolic acid

2.3.1. Assessment of minimum inhibitory concentration

Microdilution assay in 96 well-plates was used to assess minimum inhibitory concentration (MIC) of the pure ursolic acid, based on the guidelines by the Clinical and Laboratory Standards Institute [30]. Briefly, procedure began with the addition of 100 µl of sterilized Mueller-Hinton (MH) broth to the wells, except for the first column. Wells were treated with serial dilutions (2000-250 µg ml^{-1}) of ursolic acid dissolved in 5% dimethyl sulfoxide (DMSO) and mixed with the broth [31]. Bacterial strains were cultured in Luria-Bertani broth using incubator at 37 °C for 24 h. Then, 100 µl of the microbial cultures (0.5 McFarland standard) were added to each well. The MIC of samples was assessed by the addition of p-iodonitrotetrazolium chloride (0.2 mg ml^{-1}) into each well and incubated at 37 ℃ for 30 min. Apparent changes in color from yellow to pink were indicators of microbial growth. The MIC was recorded and specified visually as the minimum concentration inhibiting the bacterial growth.

2.3.2. Antimicrobial assay of ursolic acid using disc diffusion method

Antimicrobial effects of the purified ursolic acid on various pathogenic bacterial species were assessed using disc diffusion technique. Based on a method described in the literature [32], bacterial species included *P. aeruginosa*, *Escherichia coli*, *S. aureus* and *Bacillus subtilis*. Microbial suspensions were prepared in Luria-Bertani broth and their turbidities were adjusted to 0.5 McFarland standard at 620 nm [10^8 colony forming units (CFU) ml^{-1}]. Nearly 100 µl of the suspension were spread over the surface of Muller-Hinton agar plates (100 × 15 mm) and 10 µl of the ursolic acid solution at MIC were transferred onto the sterile blank discs (6-mm diameter). Petri dishes were incubated at 37 ℃ for 24 h. Then, diameters of the inhibition zones (including the sterile disc diameter) were recorded. Ciprofloxacin discs (Padtan Teb, Iran) were used as antibiotic references [33].

2.4. Assessment of the antioxidant activity of purified ursolic acid

Based on the standard approach, antioxidant effects of the purified ursolic acid were assessed by measuring reduction in free radical 1,1-diphenyl-1-picrylhydrazyl (DPPH) [15,34]. Therefore, 200 µl of various concentrations of ursolic acid (e.g., 39.06 to 2500 µg ml^{-1}) were dissolved in methanol (≥ 99.9 %), mixed with 100 µl of DPPH methanolic solution (60 mM) and added to wells of the flat-bottom microplate. Samples were incubated at ambient temperature for 30 min in dark. Then, microplate spectrophotometer (Thermo Fisher Scientific, USA) was used for reading the absorbance at 620 nm. Nearly 200 µl of methanol and 100 µl of DPPH solutions were used as controls. Vitamin C was used as positive control through an identical procedure. Equation 1 was used for calculating the inhibition proportion of the radical scavenging effect:

$$\text{Proportion of inhibition (\%)} = (Abs_{Control} - Abs_{Sample}) / Abs_{Control} \times 100 \quad \text{Eq. 1}$$

The scavenging activity was presented as the effective concentration needed to scavenge 50% of DPPH, expressed by IC_{50} value using regression curve [35].

2.5. MTT assay of ursolic acid on HUVEC cell line

Dulbecco modified eagle medium (DMEM) was used for culturing HUVECs, including 1% of streptomycin/penicillin (Biosera, UK) and 10% of fetal bovine serum (FBS) (Gibco, Germany) supplementations. The HUVECs can be an appropriate model system to study the function and pathology of endothelial cells, particularly angiogenesis, which is essential in the wound healing process [36]. Viability of the cells was assessed based on a protocol described by Kanjoormana and Kuttan [37] with some modifications. Briefly, 1% trypsin (Biosera, UK) was used for harvesting HUVECs and cells were seeded at a density of 5×10^3 cells per well using 96-well culture plates. Cells adhered to the surface of the wells and propagated for nearly 16-20 h. then, cells were washed using phosphate-buffered saline solution. Cells of the test group were treated with purified and standard ursolic acid at varying concentrations (4100 µg ml^{-1}) for 24 h, while the control wells were untreated. Cell viabilities were quantified using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After completing the treatment, 20 µl of the MTT solution were added to the wells and the incubation process was carried out for 3 h. Solution was replaced with 150 µl of dimethyl sulfoxide (DMSO). Then, ELISA plate reader (Qualigen, France) was used for measuring the absorbance at 570 nm. The cell viability proportion was described as the treated cell absorbance divided by the control cell absorbance multiplied by 100 [38].

2.6. Flow cytometric analysis

For apoptosis, HUVECs were treated with 0, 4, 6 and 20 µg of the purified ursolic acid for 24 h. Cells were harvested and stained with propidium iodide (PI) and annexin V-FITC at ambient temperature for 30 min in dark. Flow cytometry analysis was carried out for assessing the proportion of apoptotic cells. To this end, a FACS Aria flow cytometer was used (Becton, Dickinson Biosciences, Germany). Cells at early apoptosis were characterized as PI negative and annexin V-FITC positive, while cells at late apoptosis were characterized as PI double-positive and annexin V-FITC.

2.7. In vitro wound healing assay

First, the process began by seeding 12×10^4 HUVECs in a 12-well plate and propagating them in DMEM media with 10% FBS supplement. Cells were incubated for 24 h, reaching 60-70% confluence. Four horizontal wounds were created in the center of wells using 200-µl sterile pipette tips. Then, cell debris was washed out and wells were treated with DMSO (2% v v^{-1}), allantoin (positive control for healing the wounds) and 10 µg ml^{-1} of ursolic acid (purified and standard). Treatment continued for 24 h. A vertical line was created to ensure that the wound visualization was at the same point. After completing the treatment, wound closure and empty scratched sites were photographed using inverted light microscope (Model ECLIPSE Ti2, Nikon Instrument, USA) and the photographs were analyzed using Image J Software at 0, 6, 12 and 24 h after the treatment. Equation 2 was used for calculating the proportion of wound closure:

$$\text{Proportion of wound closure (\%)} = [(A_0 - A_h) / A_0] \times 100 \quad \text{Eq. 2}$$

where, A_0 and A_h were the primary and the secondary wound areas, respectively.

2.8. Expression assay

Briefly, HUVECs were cultured in 25-ml flasks using purified and standard ursolic acid at three various concentrations (0, 4 and 10 µg ml^{-1}) and DMSO (0.5%). The TRIzol reagent (Invitrogen, USA) was used to extract total RNA after 24 h. Total RNA was converted to cDNA using SOLIScript RT cDNA Synthesis Kit (Solis BioDyne, Tartu, Estonia). Furthermore, TaqMan Universal PCRMaster Mix (Applied Biosystems, USA) was used for real-time PCR. The HPRT1 served as reference. Table 1 summarizes characteristics of the probes and primers.

2.9. Data analysis

Empirical data were provided as mean ±SD (standard deviation) for a minimum of three replicates. Student t-test was used for the analysis of statistical significance and differences. Null hypothesis was rejected using Mann-Whitney U test with a probability (*p*-value) of 0.05 or less. Statistical data analysis was carried out using SPSS Software v.19 (IBM Analytics, USA). WinCATs Software was used for calculating R_f values of bands in images from the HPTLC plates.

3. Results and Discussion

3.1. Characterization of the purified ursolic acid

The HPTLC method was used for assessing purity of the extracted ursolic acid. As shown in Fig. 1, mobile phase resulted in high-resolution separation of triterpenoids and well-separated peaks for ursolic acid at the corresponding R_f values. As demonstrated in Fig. 1(a), achiviening a single peak in 3D HPTLC dendrogram was indicative of the good separation and high purity of the extracted ursolic acid. Moreover, HPTLC fingerprinting profiles of the standard and purified ursolic acids were developed as under white [Fig. 1(b)] and ultraviolet lights at 366 [Fig. 1(c)] and 254 nm [Fig. 1 (d)], they represented unique patterns and specific colors by a set of R_f values. Based on the analysis results, ursolic acid included purple color under white light and pink/red bands under ultraviolet light at $\lambda = 366$ nm.

Table 1. Nucleotide sequences of the probes and primers

Gene	Primer sequence	Probe sequence
FLT1	Forward: ACCGTCATCAGCACATTCCC Reverse: CTCTTCTGGCTAGTGAGTCTTCC	FAM-CCTACTGGCTCCTGGCAGCGGCT-TAMRA
VEGF-A	Forward: AGGAAAGACTGATACAGAACGATC Reverse: AGGTTTCTGGATTAAGGACTGTTC	FAM ACCACGCTGCCGCCACCACAC-TAMRA
HPRT1	Forward: TATATCCAACACTTCG Reverse: CTTTCCTTGGTCAGG	FAM-AAGCTTGCGACCTTGA-TAMRA

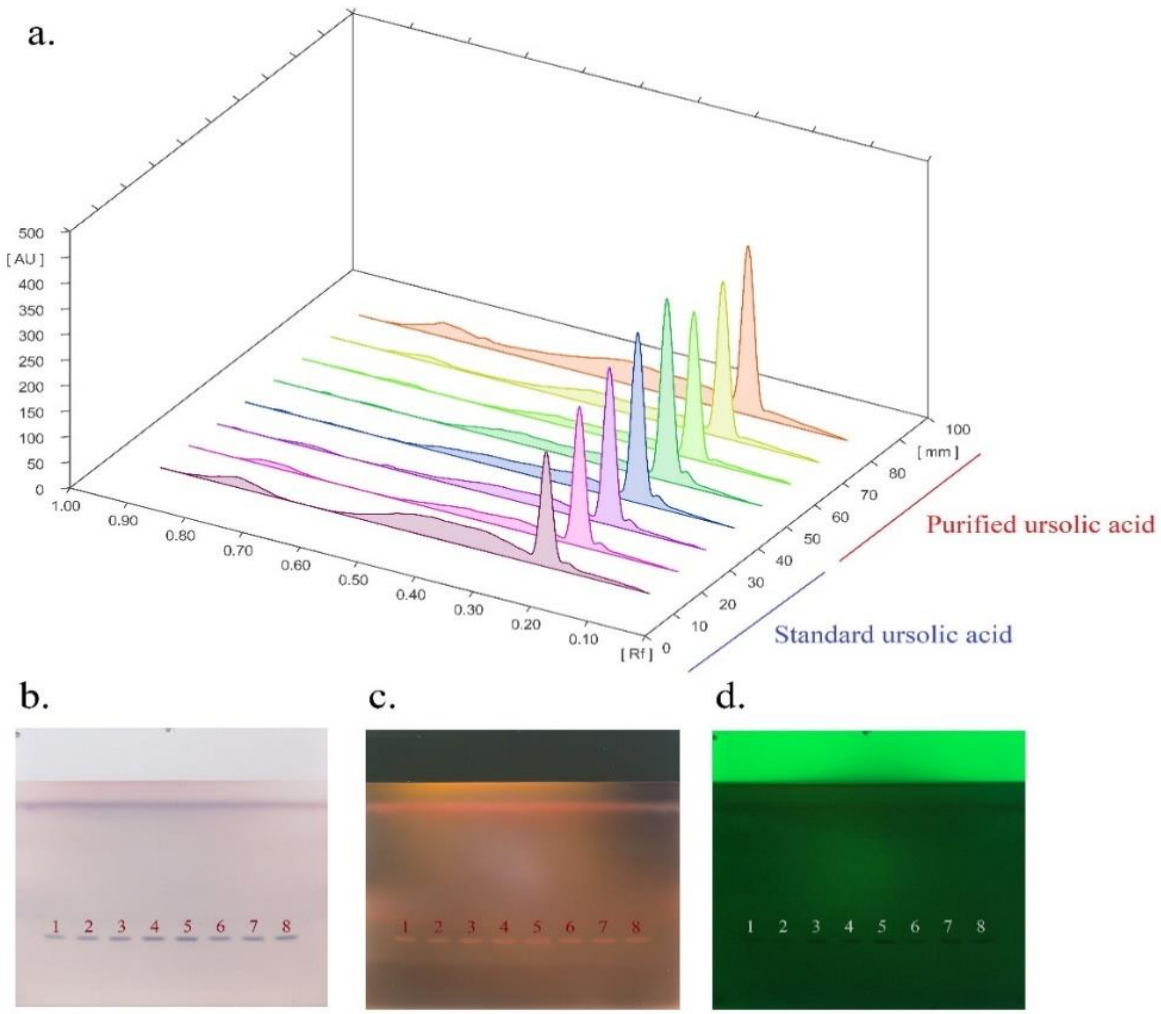

Figure 1a. The HPTLC 3D densitometric chromatograms of ursolic acid at 530 nm. Photographs of HPTLC plates: 1–5, standard and 6-8, purified ursolic acid assessed under b) white light, c) ultraviolet light at λ = 366 nm and d) ultraviolet light at λ = 254 nm

Figure 2(a) illustrates FTIR spectra of the standard and purified ursolic acids. Characteristic peaks of the standard ursolic acid were as follows: 2926 and 2368 cm^{-1} (C–H stretching vibration in CH_3 and CH_2) [39,40], 3434 cm^{-1} (stretching vibration of O–H bonds), 1749 cm^{-1} (carboxyl groups, stretching vibration of C=O bond), 1595 cm^{-1} (stretching vibration of C=C), 1350 cm^{-1} (C–H deformation in gem dimethyl), 1059 cm^{-1} (C-O bond) and 965 and 802 cm^{-1} (attributing to C=C-H group) [41]. Absorbance spectra of the standard and purified ursolic acids in the range of 400-600 nm were captured using UV-vis spectrophotometer [Fig. 2(b)]. The UV spectra of the standard and purified ursolic acids showed a characteristic peak at 550 nm. This revealed that the characteristics of the purified ursolic acid were similar to those of the standard ursolic acid.

Figure 2(c) illustrates ^{1}H-NMR spectra of ursolic acid. The peak range of 0.80-0.87 ppm could be associated with the protons of methyl groups ($–CH_3$) in the ursolic acid structure (a). Peak in the range of 0.90-1.07 ppm could be attributed to the single hydrogen attached to a carbon with one hydrogen ($-R_3$- H) in the structure (b).

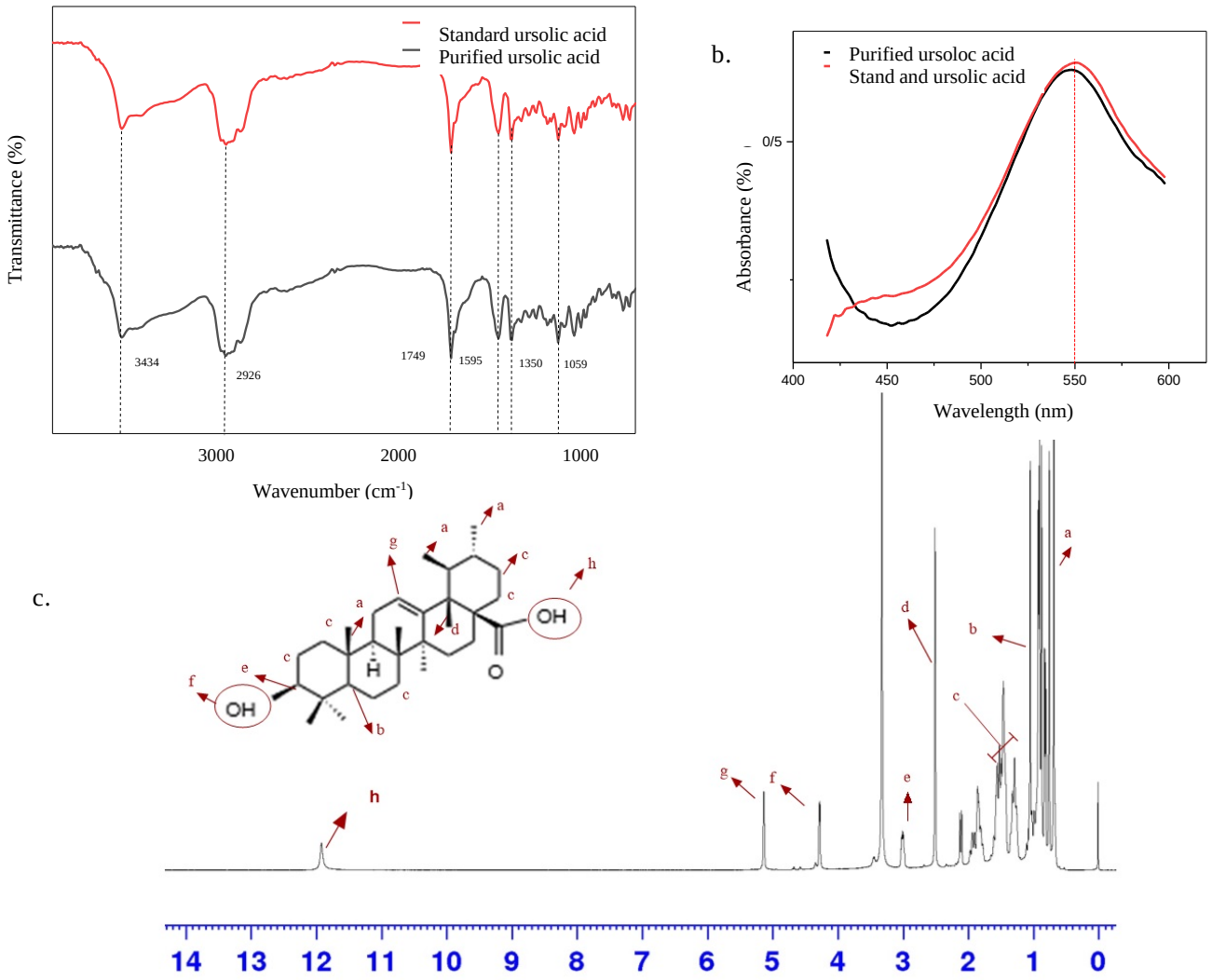

Figure 2a. The FTIR spectra of standard and purified ursolic acids, b) UV-vis spectra of the standard and purified ursolic acids and c) ^{1}H-NMR spectra of the purified ursolic acid

Peak in the range of 1.26-1.93 ppm was linked to the proton in various positions of cyclic structure (c). Peak in the range of 2.90-2.12 ppm could be due to the single hydrogen attached to the third carbon in the structure (d). Peak in the range of 3.00-3.23 ppm could be due to the hydrogen attached to a carbon that included a hydroxyl group in the structure (e) [42]. Peak in the range of 4.27-4.28 ppm could be associated to the proton in the hydroxyl group (–OH) in the structure (f). Peak at 5.13 ppm might be due to the hydrogen attached to the carbon with a double bond (–C=CH) in the structure (g). Peak at 11.92 ppm is linked to the proton of the hydroxyl (–OH) group in the form of the carboxylic acid (–COOH) in the structure (h) [43].

3.2. Antimicrobial activity of the purified ursolic acid

Antimicrobial characteristics of the extracted ursolic acid were assessed by calculating the MIC value, which was reported as the minimum concentration of ursolic acid that showed no significant bacterial growth after an overnight incubation. Based on the results, ursolic acid included significant inhibitory effects on Gram-positive bacterial growth (*Bacillus subtilis* and *S. aureus*) ($p < 0.05$). The MIC values of *Bacillus. subtilis* and *S. aureus* were 128 and 64 µg/ml, respectively. Ursolic acid slightly inhibited Gram-negative bacterial species (*P. aeruginosa* and *Escherichia coli*) ($p < 0.05$) with a similar MIC value of 625 µg ml^{-1}. These results indicated a higher sensitivity of Gram-positive bacteria to ursolic acid. Table 2 demonstrates effects of ursolic acid at various concentrations on Gram-negative and Gram-positive bacteria represented as inhibition zone diameter (mm) around the assessed microorganisms after 24 h of incubation. A plate with susceptibility of *S. aureus* to the extracted urloic acid using disc diffusion method is shown in Fig. 3.

Table 2. Antibacterial activity of ursolic acid and ciprofloxacin against Gram-negative and Gram-positive bacteria

Ursolic acid (µg ml^{-1})	Diameter of inhibition zone (mm)			
	S. aureus	*B. subtilis*	*E. coli*	*P. aeruginosa*
2000	24.6±0.4	20.0±1.0	9.2±0.5	10.2±0.5
1000	22.0±1.0	17.7±0.9	8.0±0.3	8.0±0.1
500	19.9±0.6	14.4±0.5	7.1±0.3	7.8±0.2
250	14.3±0.9	12.0±1.4	Resistant	Resistant
Ciprofloxacin (5 µg)	26.8±2.0	24.0±1.0	23.2±0.0	14.0±2.0
MIC	≥ 6.0±1.0	≥ 3.0±0.3	Resistant	Resistant

* MIC of ursolic acid against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* was 64, 128, 625 and 625-µg ml^{-1}, respectively.

These effects were compared to those of ciprofloxacin as reference antibiotic. Diameter values of the inhibition zone were categorized as resistant (for the zone of 8 mm or less), intermediate sensitive (for the zone of 8-14 mm) and highly sensitive (for the zone of 14-20 mm) [44]. Based on the results, inhibition zone of the purified ursolic acid ranged 8-16 mm (Table 2). Within the four selected Gram-negative and Gram-positive species, *S. aureus* was identified as the most susceptible species to ursolic acid ($p < 0.05$).

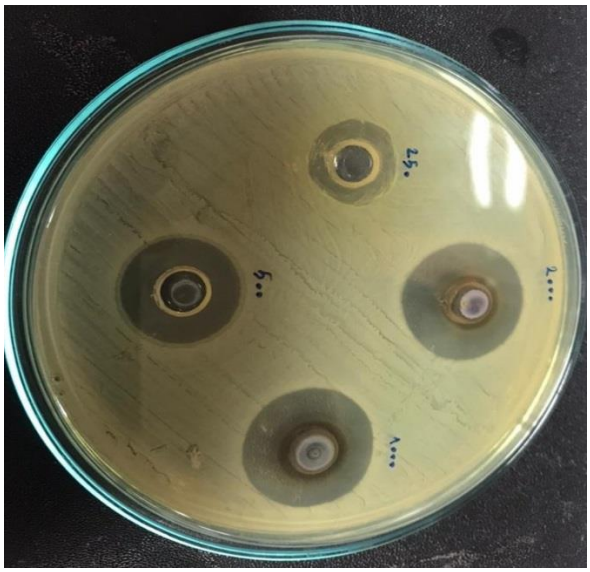

Figure 3. Assessment of the antimicrobial activities of various concentrations of the purified ursolic acid against *Staphylococcus aureus*

3.3. Assessment of antioxidant activity of the purified ursolic acid

The DPPH technique was used for assessing antioxidant effects of the purified ursolic acid for the free radical scavenging function. Antioxidant activities of various concentrations of the extracts (39-2500 µg ml^{-1}) were assessed in comparison to those of vitamin C as standard. As illustrated in Fig. 4, radical scavenging capacity of ursolic acid increased with increasing concentration of this biochemical. Ursolic acid at concentrations greater than 625 µg ml^{-1} showed significant antioxidant effects ($p < 0.05$).

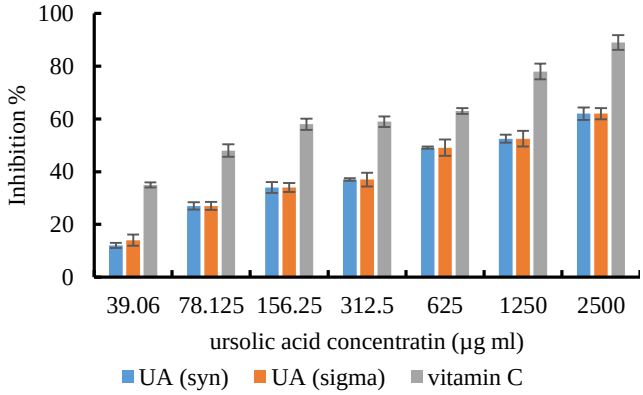

Figure 4. Antioxidant activities of various concentrations of ursolic acid in comparison with the antioxidant capacity of vitamin C (*$p < 0.05$ was considered as the significance level)

3.4. MTT assessment of ursolic acid on HUVEC cell line

Cells were treated using ursolic acid at various concentrations (0, 4, 6, 10, 20, 50 and 100 µg ml^{-1}) and analyzed using MTT assay to assess the ursolic acid effects on the proliferation of HUVECs. Based on the results, proliferation of HUVECs was stimulated by a low concentration of ursolic acid (< 20 µg ml^{-1}). The mean survival curves from the MTT assay are shown in Fig. 5(a).

Data revealed that 10 µg ml^{-1} of ursolic acid included significant effects on the viability of HUVECs. However, HUVEC treatment with 100 µg/ml of the extracted ursolic acid for 24 h caused significant decreases in the number of viable cells, compared to untreated cells in the control group ($p < 0.05$). Using standard ursolic acid as positive control, the extracted ursolic acid was shown to include similar effects on the viability of HUVECs to the effects of standard ursolic acid.

3.5. Flow cytometric analysis

To verify whether there is a relationship between the proliferative impact of ursolic acid and the apoptosis rate, the HUVECs were treated with ursolic acid at different concentrations (0, 6, 10 and 20 µg ml^{-1}). They were then stained by PI and Annexin V-FITC and flow cytometry was used for their measurement to identify the impact of ursolic acid as an indicator of early apoptosis. As shown in Fig. 5(b), the early apoptosis in Q3 was 7.91, 6.8, 5.1 and 10.2% for the concentrations of 0, 6, 10 and 20 µg ml^{-1}, respectively. According to these results, the inhibitory impact of ursolic acid on early apoptosis of HUVECs is identified, which is related to the proliferative impact stimulated by ursolic acid ($p < 0.05$) compared to the negative control.

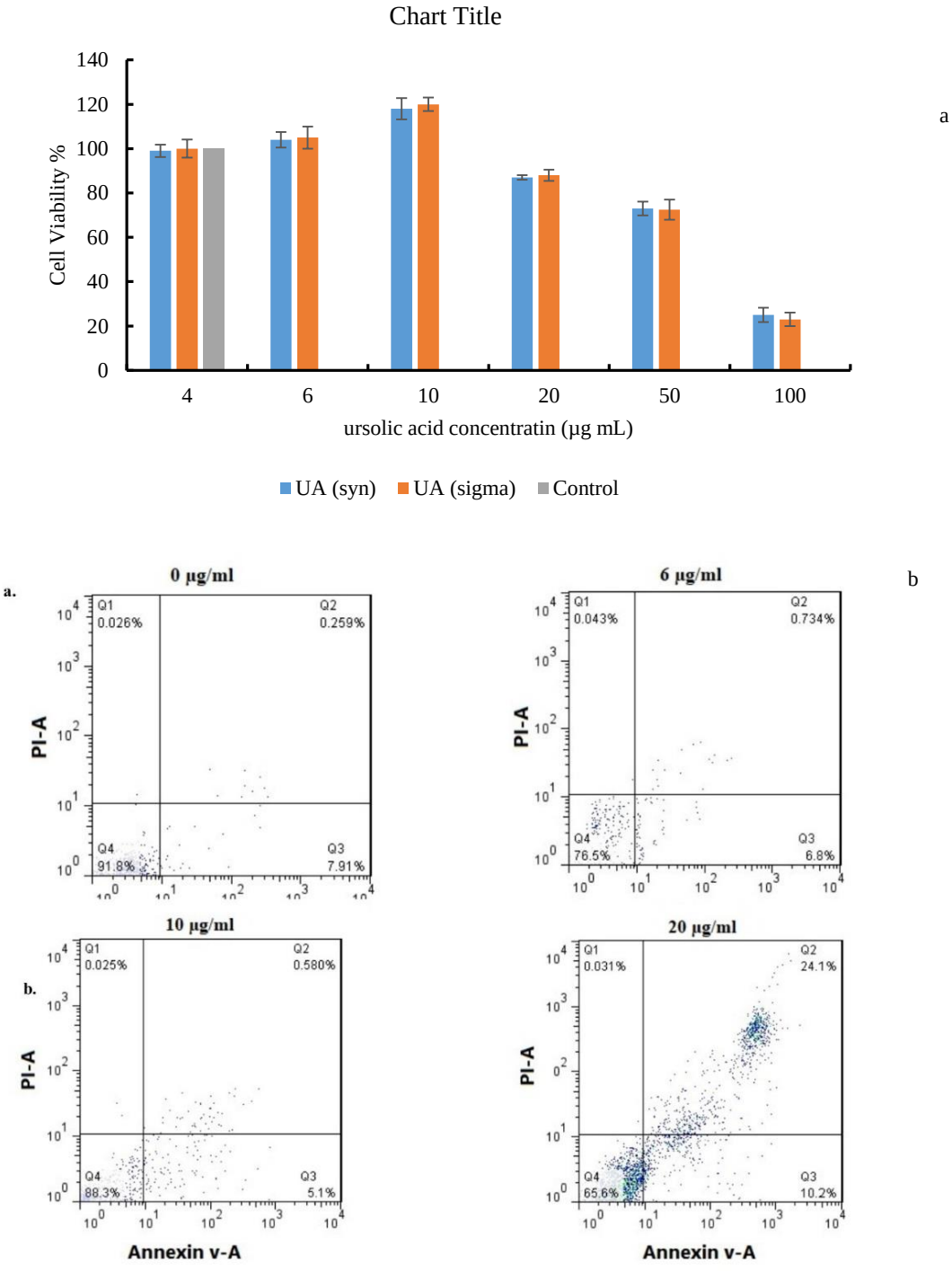

Figure 5. a) Proliferative effects of the standard and extracted ursolic acid on HUVECs and b) ursolic acid effects induced by apoptosis on HUVECs (representative scattergrams from the flow cytometry profile indicating a Annexin V-FITC staining on X-axis and PI on Y-axis)

3.6. In vitro wound healing assay

Migratory effects of ursolic acid on HUVECs were analyzed using monolayer wound scratch assay. As shown in Fig. 6(a,b), treatment with the purified and standard ursolic acid induced migration of HUVECs toward center of the scratches in a time-dependent manner, compared to DMEM and DMSO-treated control cells ($p < 0.05$). Wound closure induced by the purified and standard ursolic acid treatments was 97 and 99% after 24 h, respectively. Allantoin, as a growth factor and positive control, significantly increased migratory activity of the HUVEC cells.

3.7. Expression study

Based on Fig. 7, standard and extracted ursolic acid significantly increased VEGF-A Fig.7 (a) and FLT1 Fig.7 (b) expression levels with an increasing concentration of ursolic acid. No significant differences were seen between the results from purified and standard ursolic acid. Gene expression studies provided similar findings, improving angiogenesis-linked gene expression (FLT1 and VEGF-A). Moreover, VEGF-A gene expression increased by keratinocytes and macrophages following the wound injury. Expression receptor in the blood vessels showed that VEGF-A stimulated angiogenesis at the wound site. This finding suggested that ursolic acid could promote neovascularization.

Discussion

The major aims of the present study were to isolate and purify ursolic acid from apple peels and investigate the biochemical biological effects. After extracting and purifying ursolic acid, the biochemical was characterized using UV-vis, HPTLC, FTIR and NMR methods.

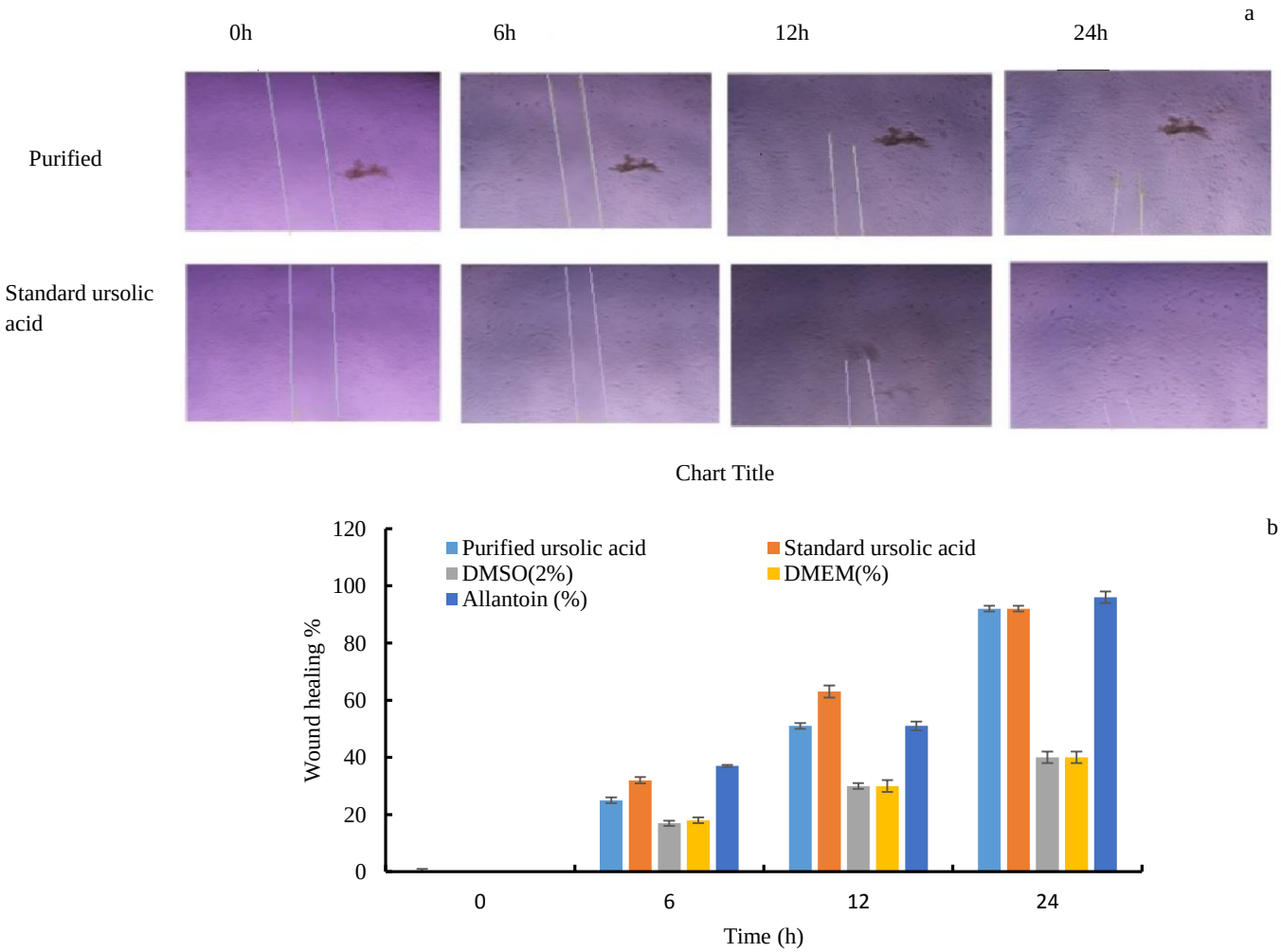

Figure 6. a) Wound-healing effects of the purified and standard ursolic acid (10 µg ml^{-1}) at 0, 6, 12 and 24 h and b) cell migration area against the control (mean ±SD)

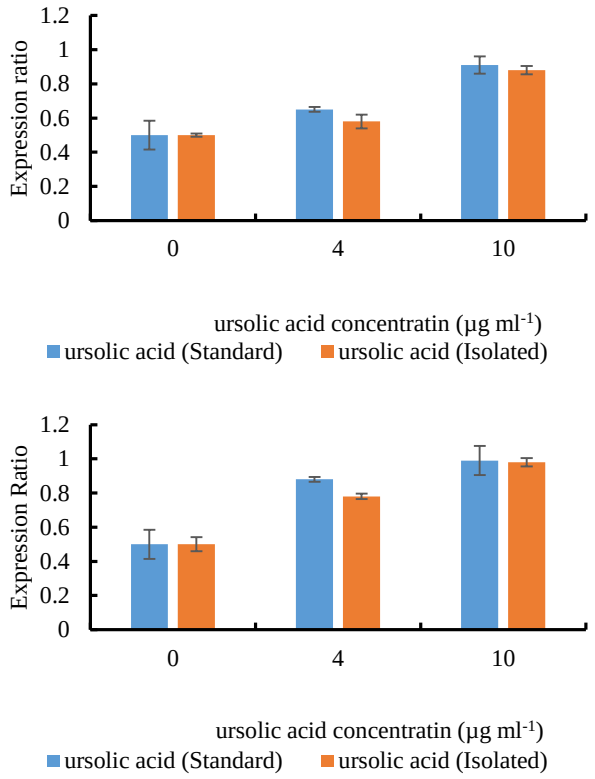

Figure 7. Upregulation of VEGF-A and FLT1 in HUVECs in response to the treatment of ursolic acid

The UV-vis spectrophotometry showed identical peaks at 550 nm with similar characteristics for the standard and purified ursolic acids, suggesting effectiveness and appropriateness of the extraction method used for the isolation of ursolic acid. The HPTLC technique was used for the identification of extracted ursolic acid from apple peels. Results from this method revealed high purity of the extracted ursolic acid. The FTIR analysis showed that the extracted ursolic acid included a high purity. The biological activities were studied in three steps for the analysis of the antibacterial, antioxidant and cellular activities of the purified ursolic acid. In general, MIC values demonstrated that ursolic acid included significant inhibitory effects on the Gram-positive bacterial growth, compared to that the biochemical did on the Gram-negative bacteria. Disc diffusion method using ciprofloxacin as reference antibiotic revealed that only *S. aureus* was significantly susceptible to ursolic acid. Data were similar to those of previous studies. Wang et al. [45] investigated the antibacterial activity of ursolic acid against *S. aureus*. They showed that this biochemical caused decreases in staphylococcal membrane integrity and could affect the bacterial translational machinery, chaperon system, oxidative stress, ribonuclease enzyme function and glycolysis. Zhao et al. [46] extracted seven ursolic acid derivatives from *Ilex hainanensis* Merr leaves and investigated their antibacterial characteristics. Result showed that three derivatives included significant antibacterial effects on Gram-positive *Streptococcus mutants* as well as minimum activities against Gram-negative *Fusobacterium nucleatum*. In contrast, Do Nascimento et al. [15] semi-synthesized two derivatives of ursolic acid from *Sambucus australis* and assessed their antioxidant and antibacterial characteristics against Gram-negative and Gram-positive bacteria using microdilution. Interestingly, they reported that within the assessed microbial species, *S. aureus* demonstrated the highest resistance against the highlighted biochemicals. Differences between the current data and those from their study might be linked to the modifications they carried out at C-3 of ursolic acid derivatives. Cunha et al. [47] extracted ursolic and oleanolic acids from the methylene dichloride extract of *Miconia ligustroides* Naudin (Melastomataceae) and found that only ursolic acid included antibacterial effects against *B. cereus*. Furthermore, they verified lack of activities for ursolic acid derivatives against the highlighted microorganisms.

In this study, DPPH assay was used for assessing radical scavenging activity of the extracted ursolic acid. Based on the results, ursolic acid showed significant antioxidant activities at concentrations higher than 625 µg ml^{-1}. Moreover, effects of the extracted ursolic acid on HUVEC proliferation were studied and compared to those of untreated control and standard ursolic acid-treated cells as positive control. Results revealed that treatment of HUVECs by the extracted and standard ursolic acid at concentration of 10 µg.ml^{-1} for 24 h significantly induced cell proliferation, compared to untreated control cells. The HUVECs were treated with 0, 6, 10 and 20 µg ml^{-1} of purified ursolic acid and the biochemical apoptosis effects were assessed using flow cytometry analysis. Results indicated inhibitory effects of ursolic acid at low concentrations against early apoptosis, promoting migration at low concentrations. Data verified that ursolic acid included promoting effects on angiogenic capacity and proliferation of HUVECs *in vitro*. However, researchers do not commonly believe ursolic acid

effects on various aspects of angiogenesis. Studies on anticancer effects of this biochemical have shown that ursolic acid plays prohibitory roles in angiogenesis. Cardenas et al. [48] demonstrated that ursolic acid was capable of blocking major angio-genesis processes *in vitro*, including endothelial cell migration, differentiation and proliferation. Moreo-ver, strong angiogenic and inhibitory effects of ursolic acid were verified by Sohn et al. [49]. They indicated that ursolic acid could effectively hinder bovine aortic endothelial cell proliferation in a dose-dependent manner; as shown by other studies. As reported by Zhu et al. [50], treatment of HUVECs with ursolic acid significantly terminated the angioge-nesis blockage induced by TGF-β1 treatment. They suggested an angiogenesis-promoting activity for ursolic acid. In another study by Kiran et al. [51], it was revealed that ursolic acid treatment of HUVECs significantly induced expression of several pro-angiogenic factors such as CD-31 and I-CAM, E-selectin, VEGF and FGF-2 and their receptors and increased the PGE2:PGD2 ratio. Endothelial cell migration is a critical process in angiogenesis, a process that is necessary for wound healing [52]. In the present study, a monolayer wound healing experiment was designed for the assessment of ursolic acid effects on HUVEC migration. Based on the findings, the currently purified ursolic acid significantly and time-dependently enhanced mig-ratory activities of the endothelial cells, similar to the standard biochemical and allantoin as positive control. This suggests that ursolic acid includes potentials to promote proliferation and migration of the endothelial cells to begin new blood vessel formation in wound repair processes. Wounds are clinical problems, especially for people with diabetes. Since ursolic acid affects decreases blood glucose levels by increasing insulin synthesis in diabetic individuals [53], the current investigation may include practical solutions for diabetic patients.

4. Conclusion

Due to the importance of cost-efficient and facile extraction process to guarantee the availability of ursolic acid from apple peels and assess therapeutic qualities of ursolic acid, isolation and purification of ursolic acid from apple peels was carried out in the present study. Bioactivity of ursolic acid extracted from apple peels was investigated in antibiotic, antioxidant, angiogenesis and wound healing processes. Based on the results, antibacterial effects of this biochemical against Gram-positive bacteria were more significant than Gram-negatives bacteria. Furthermore, radical scavenging capacity of ursolic acid was significant, suggesting its use as supplement for the treatment of oxidative stress-induced complications in various diseases. Results verified that treating endothelial cells with ursolic acid could contribute to the migration and proliferation of these cells, inducing pro-angiogenic activities that are essential for the improvement of wound healing processes.

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6. Conflict of Interest

The authors report no conflicts of interest.

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استخراج و خالص‌سازی اورسولیک اسید از پوست سیب و بررسی برون‌تن خواص ضدباکتریایی، ضداکسایشی و ترمیم زخم آن

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چکیده

سابقه و هدف: اورسولیک اسید ترپنوییدی پنج حلقه‌ای با خواص زیستی گوناگون است. هدف این مطالعه بررسی خواص زیستی بالقوه اورسولیک اسید استخراج شده از پوست سیب است.

مواد و روش ها: اورسولیک اسید از پوست سیب استخراج و با استفاده از کروماتوگرافی ستونی خالص‌سازی شد. سپس، با استفاده از طیف‌سنجی فرابنفش-مرئی[۱]، کروماتوگرافی لایه نازک با کارایی بالا [۲]، طیف بینی مادون قرمز تبدیل‌فوریه [۳]و تشدید مغناطیسی هسته‌ای[۴] مورد آنالیز قرار گرفتند. اثرات ضدمیکروبی اورسولیک اسید خالص روی سوش‌های باکتری‌های بیماری‌زای *سودوموناس آئروژینز*، *اشرشیا کلی*، *استافیلوکوکوس اورئوس*، و *باسیلوس سوبتیلیس* با استفاده از روش‌های حداقل غلظت مهاری[۵] و انتشار دیسک[۶] مورد مطالعه قرار گرفت. سپس، توانایی بیوشیمیایی آن در حذف رادیکال DPPH [۷] با روش 1,1-diphenyl-1-picrylhydrazyl بررسی شد. خواص ترمیم زخم اورسولیک اسید خالص به‌روش سنجش خراش مورد بررسی قرار گرفت.

یافته‌ها و نتیجه‌گیری: : نتایج حداقل غلظت مهاری و انتشار دیسک اثرات ضدباکتریایی اورسولیک اسید بر سوش‌های باکتری‌های گرم مثبت را تایید کرد. اورسولیک اسید در غلظت بالاتر از $^{-1}$ µg ml ۶۲۵ فعالیت ضداکسایشی قابل توجهی در مقایسه با ویتامین C، به‌عنوان آنتی‌اکسیدان مرجع داشت. سنجش ترمیم زخم و MTT [۸] نشان داد که اورسولیک اسید استخراج شده می‌تواند موجب افزایش مهاجرت و تکثیر سلول‌های اندوتلیال ورید ناف انسان[۹] شود. پس از ۲۴ ساعت، بسته‌شدن زخم توسط اورسولیک اسید خالص تا ۹۵٪ نشان داده شد. غلظت کم اورسولیک اسید ($^{-1}$ µg ml ۲۰ >) تکثیر سلول‌های اندوتلیال ورید ناف انسان را تحریک کرد، اما $^{-1}$ µg ml ۱۰۰ اورسولیک اسید استخراجی تعداد سلول‌های زنده را در مدت ۲۴ ساعت کاهش داد ($p <$ ۰/۰۵). اورسولیک اسید خالص ($^{-1}$ µg ml ۱۰) قادر بود به‌میزان تقریبا دو برابر بیان ژن FLT1 و VEGF-A در سلول‌های اندوتلیال ورید ناف انسان را تنظیم کند. براساس نتایج به‌دست آمده می‌توان اورسولیک اسید را به‌عنوان آنتی‌اکسیدانی موثر و دارای خواص آنتی‌بیوتیکی عالی پیشنهاد کرد. به علاوه، می‌تواندبر تکثیر سلول‌های اندوتلیال موثر باشد، که در رگزایی و بهبود ترمیم زخم اهمیت دارد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

تاریخچه مقاله

دریافت ۳ می ۲۰۲۱
داوری ۳۰ می ۲۰۲۱
پذیرش ۱۸ جولای ۲۰۲۱

واژگان کلیدی

- آنتی‌بیوتیک
- آنتی‌اکسیدان
- پوست سیب
- اورسولیک اسید
- ترمیم‌کننده زخم

***نویسنده مسئول**

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پست الکترونیک:

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[۱] Ultraviolet-visible spectroscopy or UV-vis
[۲] High-performance thin-layer chromatography or HPTLC
[۳] Fourier-transform infrared spectroscopy or FTIR
[۴] Nuclear magnetic resonance or NMR
[۵] Minimum inhibitory concentration or MIC
[۶] Disc diffusion
[۷] 1,1-diphenyl-1-picrylhydrazyl or DPPH
[۸] 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT
[۹] Human umbilical vein endothelial cells or HUVECs