Antioxidant, antibacterial, and keratinocyte cell growth stimulation of herbal extracts used in traditional medicine in IRAN

Elnaz Rashidi[1], Hosein Rastegar[2], Mahmoud Aghaei[3], Atousa Salehinia[1], Hamidreza Ahmadi Ashtiani[*1]

[1]Islamic Azad University, pharmaceutical sciences branch, Tehran, Iran
[2]Food and Drug Control Laboratory and Research Center, Tehran, Iran
[3]Department of Clinical Biochemistry, School of Pharmacy & Isfahan Pharmaceutical Sciences Research Center,Isfahan University of Medical Sciences, Isfahan, Iran
Corresponding author: Hamidreza Ahmadi Ashtiani(Ph.D)
Islamic Azad University, pharmaceutical sciences branch, Tehran, Iran. P.O. Box: 19395-6466
E-mail: ahmadi@iaups.ac.ir

Abstract

Backgrounds and aims: Recently, herbal extracts attracted attention in various medical fields, including treatment of problematic wounds and dermatology. Based on these lines, this study was planned to investigate the antibacterial, antioxidant and epidermal keratinocyte growth stimulation of herbal extracts in the HEK cell lines.

Methods: MTT viability test and BrdU Cell Proliferation Assay were used to study the cell proliferation effect of herbal extracts in Human epidermal keratinocyte cell line (HEK-001). 1,1-diphenyl-2-picryhydrazyl (DPPH) radical scavenger and Reactive Oxygen Spices (ROS) assays were used as the antioxidant activity of herbal extracts.

Results: MTT and BrdU assays showed a dose-dependent increased in the cell proliferation of the epidermal keratinocyte cell line, HEK-001, following exposure to herbal extracts. The present study shows that herbal extracts decrease DPPH radical scavenging activity. Moreover, the results indicated that herbal extracts significantly decreased ROS production in HEK-001in a dose-dependent manner.The herbal extracts showed antibacterial activity against both Gram-

positive bacteria (Bacillus subtilis and Staphylococcus aureus) and Gram-negative bacteria (Escherichia coli and Pseudomonas aeruginosa).

Conclusion: These results suggest that herbal extract may produce positive effects on the epidermal keratinocyte growth promotion partly through the regulation of free radical-scavenging and ROS activity in the HEK-001 cells**.**

Key words: epidermal keratinocyte, herbal extract, ROS

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Introduction

The skin is constantly exposed to various types of stress, including oxygen in the air [1]. Among the various types of human cells, epidermal keratinocytes are in the outermost layer of the skin, and are continuously exposed to external stimuli. Therefore, these cells have self-protective functions against environmental attacks including oxidative stress. Although few comparative studies have been performed with cells from other organs, it is feasible to consider epidermal keratinocytes as a type of cell that can compete with ROS. A variety of drugs, which are well known potent oxidants, are often used for long periods of time. In fact, these drugs have a

chemical structure that produces ROS [2]. However, recent studies have demonstrated that ROS mediate the action of other drugs, such as anticancer drugs or certain specific antibiotics [3-5]. There is still a high probability that ROS are involved somewhere in the underlying mechanisms of a number of compounds. The redox state that occurs in response to external stimuli is highly organized by the endogenous redox regulation system in keratinocytes. Antioxidants modulate signaling pathways through changes of in vivo redox conditions. In addition, antioxidants modulate signaling pathways by scavenging ROS or by the direct inhibition of signal transduction through a factor like an enzyme. GSH, which is an important water-soluble endogenous antioxidant, plays a significant role in cellular redox regulation as the reduced thiol (GSH) and oxidized disulfide (GSSG) couple [6]. Thus antioxidants, which increase cellular GSH levels, have become a clue to reveal the molecular actions mediated by ROS.

Recently, the traditional use of plants for skin diseases such as wound healing has received attention by the scientific community [7, 8]. In this respect, alternative medicine has attracted interest. Although it has not yet been incorporated into mainstream of medical care, due to limited scientific evidence and incomplete knowledge of the mechanisms involved, alternative medicine has become an increasingly attractive approach worldwide [9]. The search for treatment results into few drugs of synthetic origin, but side effects associated with them cannot be neglected.

Taking into account what said above, we searched for potential therapeutics among those plant extracts that have been used traditionally in oriental medicine for treating skin diseases such as wound healing in iran. Based on these lines, this study was planned to investigate the antibacterial, antioxidant and epidermal keratinocyte growth stimulation of herbal extracts in the HEK cell lines.

Material and Methods:

Plant Material

The fruits of Persea Americana, flowers of Althaea officinalis, Chamaemelum Nobile, Thymus vulgaris, leaves of Rosmarinus officinalis, Urtica dioica were procured from local market and identified by comparing with standard herbarium specimens available in 4Food and Drug Control Laboratory and Research Center, Tehran, Iran. The various parts of plant drugs are crushed in mixer and passed through the sieve number 80. The various powder drugs were subjected to pharmacognostic studies for confirmation.

Preparation of Herbal Hair Formulation

The herbs used in the present study for making herbal extracts were dried, crushed and passed through 80 mesh stainless steel sieves and water was used as base. The hair extracts was prepared utilizing three different methodologies:

First is the direct binge method in which the crude drugs were powdered, weighed and directly bed in olive with continuous stirring and heating until the drug had completely extracted in the base. 1, 2, 3% of drugs containing s were prepared.

Secondly, paste method was used where fresh fruit or pulp or the desired part of the plants were converted into paste with very little amount of water and kept overnight. After this the wetted drug was mixed and bed with continuous stirring at a constant temperature, until the water droplets stop knocking and the drug has completely extracted. The extract was then filtered through a muslin cloth. Three different concentrations 4%, 5%, 6% were prepared containing 4, 5, 6 g of drug per 100 ml of water respectively.

Last method is cloth method, in which the dried drug was weighed and tied in a muslin cloth.

This cloth was then hanged in water base, with continuous being, stirring and finally filtered. Four different concentrations 7%, 8%, 9%, 10% were prepared containing 7, 8, 9, 10 g of drug per 100 ml of water respectively.

Preparation of combined drug herbal hair formulation of different concentration

After selection of method for preparation, multi ingredient s of effective concentrations based on the preliminary physical and biological screening was prepared. The method selected was direct binge method and three different formulations having concentrations 1% - 4.5% of drugs were prepared for maximum activity (Table1).

Table 1: Selection of concentration of herbal extracts

Amounts of drugs/100 ml							
%	Thymus vulgaris	Urtica dioica	Aloe vera	Rosmarinus officinalis	Persea Americana	Althaea officinalis	Chamaemelum nobile
1 %	1	1	1	1	1	1	1
3%	3	3	3	3	3	3	3
4.5%	4.5	4.5	4.5	4.5	4.5	4.5	4.5

Cell culture

Human epidermal keratinocyte cell line (HEK-001) was obtained from ATCC. The cell line were grown adherently in Keratinocyte-Serum Free medium with 5 ng/ml human recombinant EGF and 2mM L-glutamine (without bovine pituitary extract and without serum) according to the manufacturer's instructions (GIBCO-BRL 17005-042) at 37°C in 5% CO_2/ 95% air.

MTT viability assay

Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as previously described [10]. The HEK-001 cells were seeded at 5×10^3 cells/well

in 5% CO2 at 37°C in medium in 96-well plates. After cells were grown to 60-80% confluency, were treated with various concentrations of herbal extracts (1-4.5%). After 48 hr indicated times, 20µl of MTT (5 mg/ml in PBS) was added to each well and the cells were incubated for another 4 h at 37°C. The supernatants were then aspirated carefully and 200µl of dimethyl sulfoxide (DMSO) was added to each well. The plates were shaken for an additional 10 min and the absorbance values were read by the microplate reader (Bio-Rad, Hercules, CA, USA) at 570 nm. Cell viability was calculated as a percentage using the formula: (mean OD of treated cells /mean OD of control cells) ×100. The results expressed as percent of control cells which were not treated.

BrdU Cell Proliferation Assay

BrdU Cell proliferation was measured by colorimetric immunoassay based on bromodeoxyuridine (BrdU) incorporation by BrdU kit (Roche Diagnostics GmbH, 68298 Mannheim, Germany) according to the manufacturer's protocol as previously described [11]. In brief, the cells (5000 cells/well) were seeded in 96-well plates. After 24 hr, the cells were incubated with various concentration of herbal extracts (1–4.5%), for 48 hr. Subsequently, 20 µl of BrdU-labeling solution was added to each well and the cells were reincubated for 4 hr. During this labeling period, the pyrimidine analogue BrdU is incorporated in place of thymidine into the DNA of proliferating cells. After removal of the BrdU-labeling solution, cells were fixed and denatured with the kit's FixDenat solution for 30 min at room temperature. Denaturation of the DNA is necessary to improve the accessibility of the incorporated BrdU for detection by the following antibody. Samples were incubated for 90 min with peroxidase-conjugated anti-BrdU antibody (anti-BrdU-POD) which binds to BrdU incorporated into newly synthesized cellular DNA. After washing off the unbound anti-BrdU-POD, the color reaction was developed for 3–5

min with the substrate solution and stopped by adding 25 µl 1M sulfuric acid, and optical densities of the samples were determined using a microplate reader at 450 nm (reference value 690 nm).

Antibacterial assay

The bacteria used for the tests were obtained from the Iranian Biological Resource Center (IBRC), and included both Gram-positive and Gram-negative bacteria. The Gram-positive bacteria used were Bacillus subtilis (IBRC-M10210), Staphylococcus aureus (IBRC-M10212. Gram-negative bacteria used were Escherichia coli (IBRC-M10208) and Pseudomonas aeruginosa (IBRC-M10205). Inoculate of the microorganisms were prepared from the 24 h Mueller–Hinton broth (Sigma) cultures and suspensions were adjusted to 10^5 CFU/ml. Aqueous extracts were reconstituted in distilled water. Minimal inhibition concentration (MIC) values of the extracts were determined based on a micro-well dilution method [12]. The 96-well sterile plates were prepared by dispensing 190 µl of the inoculated broth plus a 10 µl aliquot of the plant extracts (1- 4.5%) made up in broth or 10 µl broths in the case of negative control in each well. Tetracycline (Sigma) was included as positive control. Plates were covered and incubated for 24 h at 37◦ C. Bacterial growth was determined after addition of 50 µl p-iodonitrotetrazolium violet (0.2 mg/ml, Sigma).

DPPH radical scavenging activity

DPPH radical scavenging activity was performed by the method of Cheung et al [13]. An aliquot (1 ml) of 0.2 mM DPPH radical in methanol was mixed with 200 µl of various concentration of herbal extracts (1- 4.5%) or ascorbic acid standard solution. The mixture was incubated for

30 min in the dark at room temperature, after which the absorbance was measured at 517 nm using a spectrophotometer. Butylated hydroxytoluene (BHT) was used as positive control.

Measurement of Reactive Oxygen Species (ROS)

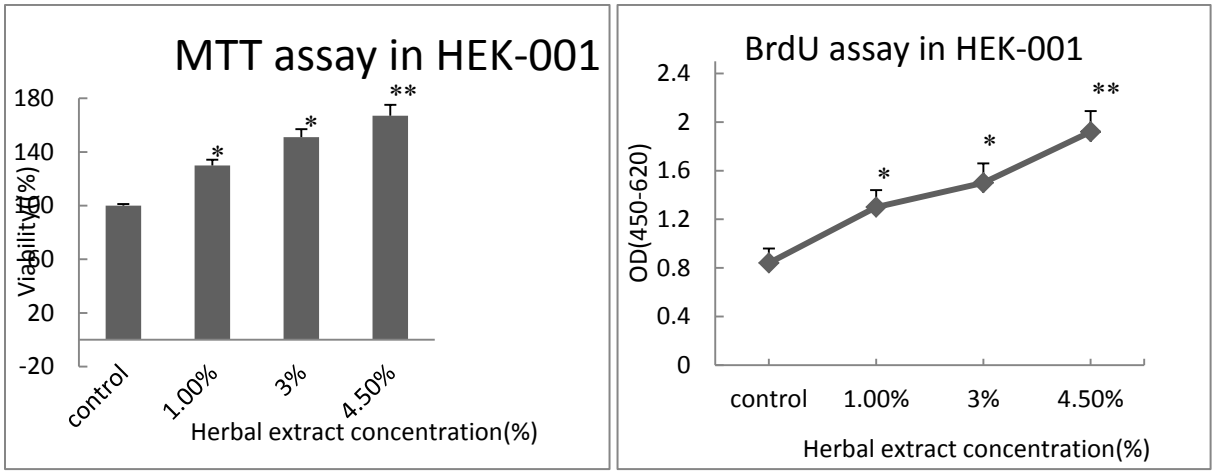
Formation of reactive oxygen species (ROS) was evaluated using the Marker Gene TM Live Cell Fluorescent ROS Detection Kit according to the manufacturer's instructions as previously described [14]. Briefly, cells plated to a density of 25×10^3 per well in 96-well plate and incubated with different concentration of herbal extracts for 48 hr. after drug treatment, cells were loaded with 2',7'-Dichlorofluorescin diacetate (20 µM) in HBSS at 37 ◦C for 30 min in the dark. The cells were then washed with HBSS and Fluorescence caused by DCF in each well was measured and recorded at 485 nm (excitation) and 528 nm (emission) by using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT).

Results

Herbal extracts induced the proliferation of human epidermal keratinocyte cell line (HEK-001)

The effects of herbal extracts on the human HEK-001 proliferation were examined. HEK-001 cells were treated with various concentrations of herbal extracts (1%- 4.5%) for 48 hours and cell proliferation was measured by MTT and BrdU methods. MTT assays showed a dose-dependent increased in mitochondrial succinate dehydrogenase activity in the HEK-001 following exposure to herbal extracts. As it is shown in Fig. 1A, growth promoting effects of herbal extracts in HEK-

001 cells starting at 1 % and increased up to 4.5% (from 130% ± 3.8% for 1% to 167% ± 7.5% for 4.5% vs. control 100%, respectively; P < 0. 01, one way ANOVA).The results of MTT assay was confirmed using BrdU incorporation assay (Fig. 1B). Proliferation of cells in response to herbal extracts was significantly increased in a dose-dependent fashion in HEK-001 (P < 0.01).



(A) (B)

Figure1: The effect of herbal extracts in promotion of cell proliferation on the human epidermal keratinocyte cells, HEK-001. Cells were treated with different concentrations of herbal extracts for 48 hr, and proliferation was assessed by MTT (A) and BrdU (B). Herbal extracts increased cell proliferation in HEK-001 in a dose dependent manner.

Antibacterial activity

As summarized in Table 2, herbal extracts showed antibacterial activity against both Gram-positive bacteria (Bacillus subtilis and Staphylococcus aureus) and Gram-negative bacteria (Escherichia coli and Pseudomonas aeruginosa). Escherichia coli were the most susceptible with MIC of 1% of herbal extract (Table 2). Therefore, herbal extracts possessed antibacterial activity.

Table 2. Antibacterial activity of herbal extracts expressed as minimum inhibitory concentrations (MICs)

Herbal extracts (%)	MIC		
	1%	3%	4.5%
Bacillus subtilis	0.5	0.35	0.25
Staphylococcus aureus	0.4	0.3	0.15
Escherichia coli	0.35	0.2	0.1
Pseudomonas aeruginosa	0.43	0.35	0.2

The MIC values are presented as percentage.

Anti-oxidative activity

Fig. 2 illustrates a significant ($p < 0.05$) decrease in the concentration of DPPH radical due to the scavenging ability of the herbal extracts. BHT, as a positive compound, presented the highest activity at all concentrations. The IC_{50} values (the concentration with scavenging activity of 50%) of scavenging activities on DPPH radical were found to be 1.5% and 0.5% for herbal extracts and BHT, respectively.

Role of Reactive Oxygen Species (ROS) in herbal extract-induced cell proliferation

To examine whether herbal extracts exert their proliferation effects in HEK-001 cells by inhibition of oxidative stress, we evaluated the levels of ROS after 48 hr treatment with various concentration of herbal extracts. The results indicated that herbal extracts significantly decreased ROS production in this cell line in a dose-dependent manner ($p < 0.05$) (Fig. 3).

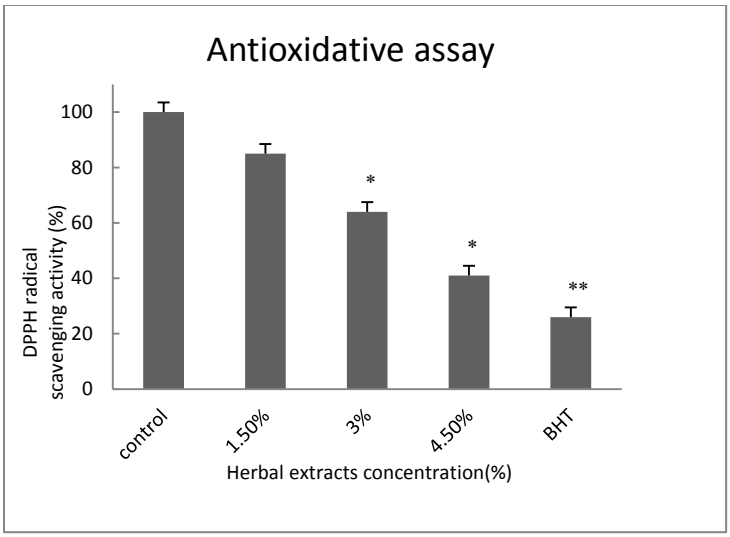


Figure2: DPPH radical-scavenging activities of BHT and the extracts herbal extracts. Values are means ± SD of three determinations. Results (mean ± SD) were calculated as percent of corresponding control values. *P <0.05, ** P <0.01, are significant. Statistical analysis was performed by ANOVA. Each point represents 4 repeats, each triplicate.

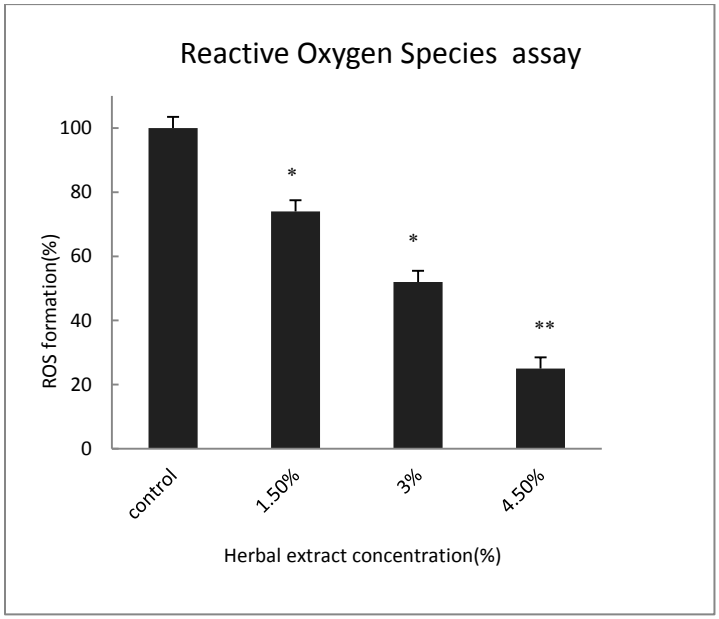


Figure 3: **Effects of herbal extracts on reactive oxygen species (ROS) generation on the human epidermal keratinocyte cells, HEK-001.** After treatment with different concentration (0.1-4.5% µM) of herbal extracts for 48hr, cells were loaded with 2',7'-Dichlorofluorescin diacetate and Fluorescence was measured by Microplate Reader. Results (mean ± SD) were calculated as percent of corresponding control values. *P <0.05, ** P <0.01, are significant. Statistical analysis was performed by ANOVA. Each point represents 4 repeats, each triplicate.

Discussion

Among the various types of human cells, epidermal keratinocytes are in the outermost layer of the skin, and are continuously exposed to external stimuli. Therefore, these cells have self-protective functions against environmental attacks including oxidative stress. Although few comparative studies have been performed with cells from other organs, it is feasible to consider epidermal keratinocytes as a type of cell that can compete with ROS. The redox state that occurs in response to external stimuli is highly organized by the endogenous redox regulation system in keratinocytes [15].

In this study, herbal extracts was found to induce significant proliferation of HEK-001human epidermal keratinocyte cell line at concentrations ranging from 1 % to 4.5%. Free radicals are known to be a major factor in biological damages, and DPPH has been used to evaluate the free radical-scavenging activity of natural antioxidants [16, 17]. DPPH, which is a radical itself with a purple color, changes into a stable compound with a yellow color by reacting with an antioxidant and the extent of the reaction, depends on the hydrogen donating ability of the antioxidant [18]. Fig. 2 shows the DPPH free radical scavenging activity of herbal extract at different concentrations.

The present study also documents that herbal extracts reduce ROS activity in cultured human HEK-001 cell line.Because ROS can alter signal transduction and gene expression, they may influence the release of cytokines and more ROS production from keratinocytes, which are critical for some inflammatory skin diseases [19, 20]. The present finding that herbal extracts partially suppressed levels of intracellular ROS, suggests that it can scavenge ROS in keratinocytes.

In recent years, *in vitro* studies demonstrated that natural extracts could regulate differentiation and proliferation of epidermal keratinocytes [21], inhibit proinflammatory responses of neutrophils [22], and suppress tumor necrosis factor-α-induced vascular cell adhesion molecule-1 expression in endothelial cells [23]. The current results suggest that the use of herbal extracts may modulate various cellular effects by modulating signaling pathways mediated by ROS.

Conclusion

These results suggest that herbal extract may produce positive effects on the epidermal keratinocyte growth promotion partly through the regulation of free radical-scavenging and ROS activity in the HEK-001 cells. Overall, we have demonstrated the potent epidermal keratinocyte growth promoting effect of herbal extracts, suggesting that herbal extracts may be a good candidate for helping keratinocyte growth promotion and support the future development of this extracts as a potential drug treatment for various skin diseases with oxidative stress.

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