



Original Article

Cytotoxic activities of *Helichrysum arenarium* on ECV 304 and Ishikawa cells

Asena Aricioglu¹, Rumeysa Cebecioglu^{1,2}, Dilan Akagunduz¹, Aykut Kul², Tunc Catal^{1,4}✉

¹Uskudar University, Istanbul Protein Research and Innovation Center, Istanbul, Türkiye

²Istanbul Kent University, Department of Medical Laboratory Techniques, Istanbul, Türkiye

³Istanbul University, Department of Analytical Chemistry, Faculty of Pharmacy, Istanbul, Türkiye

⁴Uskudar University, Department of Molecular Biology and Genetics, Istanbul, Türkiye

✉ Corresponding Author: Tunc Catal (E-mail: tunc.catal@uskudar.edu.tr)

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Abstract

Introduction: In this study, the cytotoxic activity of *Helichrysum arenarium* plant on various human cells was examined.

Methods: ECV 304 (human endothelial cell line) and Ishikawa cells (human endometrial adenocarcinoma cells) were used in order to find out the cytotoxicity results. Dimethyl sulfoxide (DMSO) extract of *Helichrysum arenarium* showed the most significant cytotoxicity effect on these cells and also lipid peroxidation (MDA) assay was performed. Also, the essential oils of immortelle extracts were analyzed using gas chromatography-mass spectrometry (GC-MS).

Results: Cell viability and MDA levels were decreased by the extract of *Helichrysum arenarium* both in Ishikawa cells and ECV304 cells at 500 µg/mL. 2-Palmitoylglycerol and palmitic acids were found as the major essential oils in DMSO extracts of *Helichrysum arenarium*.

Conclusion: *Helichrysum arenarium* may show an antioxidant activity *in vitro*.

Keywords: *Helichrysum arenarium*, cytotoxicity assay, MDA assay, GC-MS, Ishikawa cells

1. Introduction

Helichrysum arenarium (L.) Moench is an herbaceous perennial plant which belongs to the Asteraceae family (1,2). *Helichrysum arenarium* (L.) Moench has been used in folk and modern medicine since antiquity (3). Due to its diuretic, anti-infective, hepatoprotective, detoxifying, cholagogue, and choleric effects it has been used in various treatments in folk medicine. These treatments include regulating gallbladder

disorders, relieving stomach pain, improving rashes, relieving coughs, treating erythema, and diabetes mellitus (4-6). Today, scientific studies confirm the characteristics of *Helichrysum arenarium* since its antioxidant, antimicrobial, antifungal, and anti-inflammatory properties. Some of the most popular utilizations are treating digestive problems and infections, ,

improving respiratory conditions supporting heart health, and the nervous system. It has been traditionally used in European folkloric medicine for many years as well as in modern times.

Several studies have been carried out over the past years to highlight some of the traditional uses of *Helichrysum arenarium* extract and other potential applications. Many studies aimed to determine how gold grass behaves as an antimicrobial, anti-inflammatory, and natural antioxidant agent (4,7-9). *Helichrysum arenarium* helps improve heart health due to its anti-inflammatory properties and also shows positive cardiovascular effects on heart health (10). Previous studies have showed that *Helichrysum arenarium* contains special flavonoid antioxidant compounds that inhibit cancer growth and oxidative stress. Also, *Helichrysum arenarium* has anti-inflammatory properties and helps intestinal healing (11). Studies have shown that *Helichrysum arenarium* extract is effective against radiation-induced DNA damage, cell death, and mutation, as well as cancerous tumor growth. It also helps to stimulate the stomach secretions (12). In clinical trials, has been shown to contain flavonoids and fluoroglycinols, which can kill harmful bacteria, fungi, and viruses, and has been strong enough to reduce the risk of HIV contraction (13). On the other hand, *Helichrysum arenarium* is a diuretic and supports digestion through natural routes (14). Additionally, it is also used in the production of some creams (15).

In this study, the cytotoxic activities of the *Helichrysum arenarium* plant on ECV 304 and Ishikawa cells were examined using MTT assay. Malondialdehyde levels were also measured using TBARS assay. Gas chromatography mass spectrometry (GC-MS) was used for the analysis of the chemical composition of the *Helichrysum arenarium* extracts.

2. Methods

2.1. Preparation of plant extracts

Helichrysum arenarium were pulverized to weigh 10 mg. It was then extracted in DMSO (VWR, Cat No: 16I054006, France) at concentrations of 10 mg/mL.

2.2. Cell culture

In this study, ECV 304 and Ishikawa cell lines were studied. The cells were grown in a 37 °C incubator balanced with 5% CO₂. The cells were diluted to 10⁵ cell/mL with DMEM (1x) medium (GIBCO) containing 10% of FBS, L-glutamine, and penicillin-streptomycin (16, 17). Both ECV and Ishikawa cells were in cell culture petri dishes for 24 h before treatment with the extracts. Various concentrations of the plant extract were applied into the cells for 24 h.

2.2.1. Cytotoxicity assay

To determine the cytotoxic effect of plant extracts on the cells, MTT (3-(4,5-dimethyl thiazole-2-yl)-2,5-diphenyl tetrazolium bromide) assay was used (18). Here 10 mg/mL of DMSO extracts was prepared as the stock solution, and diluted with serum medium (5 µg/mL, 10 µg/mL, 50 µg/mL, 500 µg/mL). The cells were diluted to 10⁵ cells/mL. Then, 90 µL of cells were placed in each well of 96 well-plate. The cells were incubated for 24 hours in a 5% CO₂ incubator at 37 °C. After 24 h, 10 microliters of plant extract were applied. Then, the MTT solution with a concentration of 5 mg/mL dissolved in sterile PBS was added. After 3 h incubation, 100 µL of 50% DMSO-50% isopropanol mixture was added and incubated for 45 minutes at room temperature. Finally, optical density was measured with 570 nm with a multiwell spectrophotometer (Thermo Scientific, USA). Cytotoxicity index (CI) was calculated to following formula;

$$CI \% (\text{Cytotoxicity index}) = 1 - \frac{\text{OD treated wells}}{\text{OD control wells}} \times 100.$$

2.3. Lipid peroxidation (LPO)

Oxidizing agents can alter lipid structure forming lipid peroxides that result in the formation of malondialdehyde (MDA), which can be measured as Thiobarbituric Acid Reactive Substances (TBARS). 150 μ L of standards and samples were added to each well, and 75 μ L of TBA Reagent was added to each well. The optical density of each well was measured using a microplate reader at 532 nm. Then microplate was incubated for 2-3 hours at 45-50 $^{\circ}$ C. The optical density of each well using a microplate reader was measured at 532 nm.

2.4. GC-MS analysis

1 mL of the extract sample was transferred into the test tube. 10 mL of hexane was added to the test tube and the mixture was mixed using a vortex for 1 minute. Then, 0.5 mL methanolic 2 N KOH was added and the mixture was mixed using a vortex for 1 minute and kept in the dark for 30 minutes. Then, 0.5 mL 0.1 N aqueous HCl was added and kept in the room temperature for 5 minutes for phase separation. Clear phase separation was injected into the GC system. Essential oils were analyzed by using a Shimadzu GC-2010 plus gas chromatography (Shimadzu Scientific Instruments, Columbia, MA, USA), equipped with an Rtx®-5MS column (30 m \times 0.25 mm ID, 0.10 μ m film thickness) (Restek, USA) where helium was used as carrier gas (average flow rate, 1.50 mL/min). The oven temperature program increased from 140 $^{\circ}$ C (5 min) to 240 $^{\circ}$ C at 4 $^{\circ}$ C/min and kept isothermal at 240 $^{\circ}$ C for 15 min. Injector temperatures were 250 $^{\circ}$ C and, mass spectrometer parameters were as follows: source and interface temperatures, 250 $^{\circ}$ C and 275 $^{\circ}$ C, respectively; electron Impact ionization mode, at 70 eV; acquisition mass range, 40–400 m/z with a scan speed of 2000 amu/s and a scan-interval of 0.20 s. Data handling was supported by the software GC-MS solution, ver. 2.51 (Shimadzu).

The oil content of algal extracts was identified using the National Institute Standard and Technology (NIST) library. Components' relative percentages were calculated based on GC peak areas without using correction factors.

2.5. Statistical analysis

GraphPad Prism software was used to perform statistical analysis according to Dunnett's test.

3. Results and Discussion

The cell viability of the *Helichrysum arenarium* plant on ECV 304 was calculated as 81.8% at concentrations of 5 μ g/mL and 56.35% at 500 μ g/mL respectively. However, the cell viability levels in Ishikawa cell line were found as 83.06% at 5 μ g/mL and 39.24% at 500 μ g/mL. These results indicate that *Helichrysum arenarium* may show cytotoxic effect on Ishikawa cells. Mao et al. (19) reported that flavonoids obtained from *Helichrysum arenarium* may have potential for preventing AS formation (Fig 1).

In the study of Les et al. (20) which was done with the *Helichrysum stoechas* (L.) Moench plant, the basic phytochemical components of the plant were determined and evaluated in terms of antioxidant, antidiabetic, and neuroprotective activities of this plant. As a result of the phytochemical analysis of the plant extract prepared with methanol, ten different components were identified, some of which include two heterodimeric phloroglucinol, oryzanol, one homodimeric α -pyron, three phenolic acids, p-hydroxybenzoic, 5,7-dihydroxy-3,6,8-trimethoxyflavone, isoquerythrin. Bioassays have shown significant antioxidant and antiproliferative effects. The antiproliferative effects in HeLa cell line (line of human cells derived from cervical cancer) were assessed using MTT assay (mitochondrial viability test). It was found that at doses of 60 μ g/ml and above applied to the cancer cell line HeLa cells, cell viability decreased approximately by 25%. In addition, no significant difference was observed at low concentration doses. In our study, Ishikawa cells were used as a cancer cell line, and cell proliferation was decreased gradually with increased concentrations of the extract.

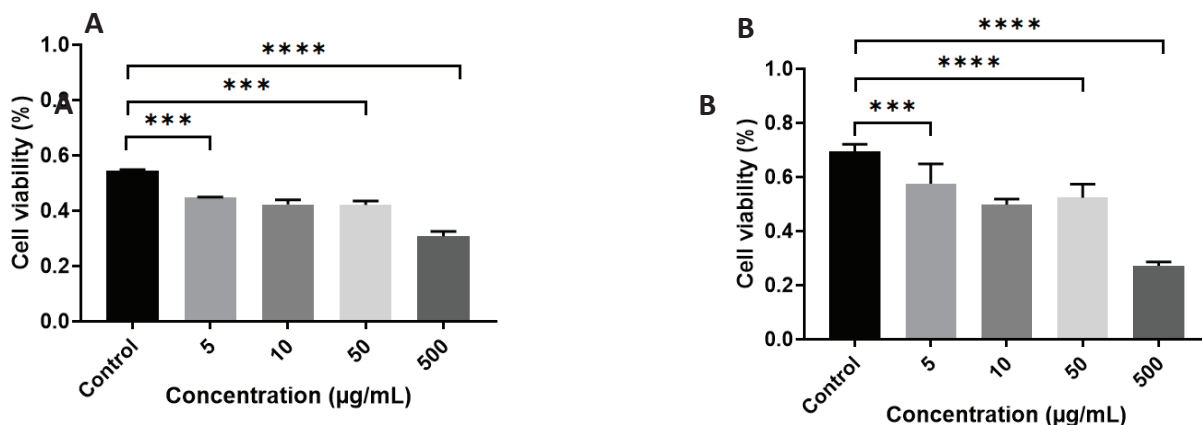


Figure 1. Cell viability results of ECV304 (A) and Ishikawa cells (B) in the presence of *Helichrysum arenarium* DMSO extracts (**** $p < 0.0001$).

TBARS assay was used to analyze the biological antioxidant level of *Helichrysum arenarium* (Fig 2). In the experiment, *Helichrysum arenarium* was applied to ECV304 cells (Fig. 2A) and Ishikawa cells (Fig.2B). According to the results, 50-250-500-2500 $\mu\text{g/mL}$ doses of *Helichrysum arenarium* applied to cancer cell line caused a significant inhibition in MDA levels. At the dose of 5000 $\mu\text{g/mL}$, a similar result was obtained with the MDA level of the control group.

Elaguel et al. (21) applied the antioxidant *Lawsonia inermis*(henna) essential oil to the human lymphatic cancer cell line (Raji) and analyzed the biological antioxidant levels with the TBARS assay method. They reported that henna essential oil inhibited the MDA level significantly (80%).

There have been found very few studies in the literature related with the effects of antioxidant substances on MDA levels in human cell lines, However, there are some studies showing that plants with antioxidant properties can inhibit MDA levels other cancerous animal tissues (22, 23). Elaguel et al. (21) reported that when 50-5000 $\mu\text{g/mL}$ doses of *Helichrysum arenarium* applied to ECV304 cells, MDA levels significantly decreased. In our opinion, the decrease in MDA level of the study above may be due to the inhibition of peroxidase enzyme activity and the decrease in lipid peroxidation.

In our study, 23 essential oil compositions were determined in the extract of *Helichrysum arenarium*. The most abundant compounds were 2-Palmitoylglycerol and palmitic acids. The detailed composition of the identified compounds is given in Table 1.

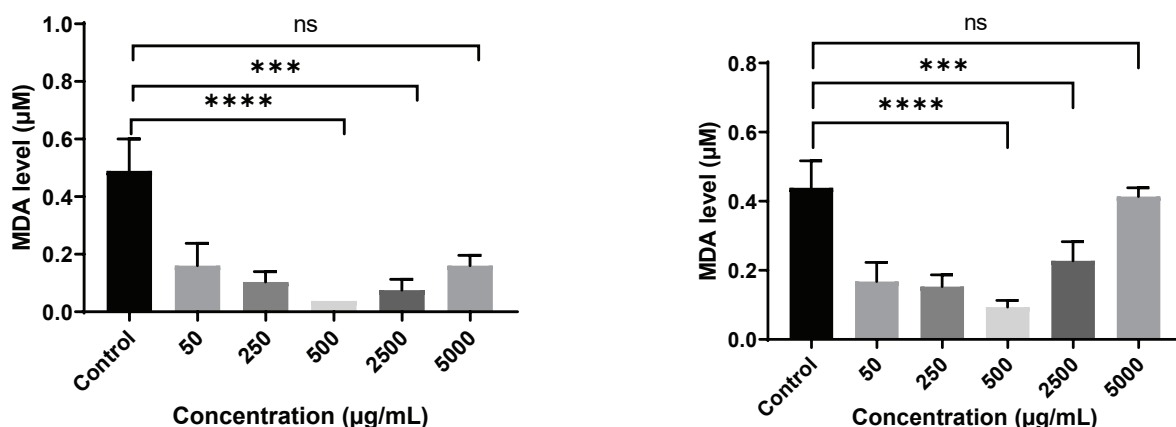


Figure 2. Changes of malondialdehyde levels in the presence of *Helichrysum arenarium* DMSO extracts in ECV304 cells (A) and Ishikawa (B) (**** $p < 0.0001$).

Table 1. Chemical composition analysis of *Helichrysum arenarium* extract detected by GC-MS.

	Compound	<i>Helichrysum arenarium</i> (%)
1	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	18,79
2	Hexadecanoic acid (Palmitic acid)	16,75
3	Phenol	14,59
4	9-Octadecenoic acid (Oleic acid)	9,49
5	9,12-Octadecadienoic acid (Linoleic acid)	7,70
6	Octadecanoic acid (Stearic acid)	7,36
7	N,N-Dimethylpalmitamide	4,94
8	Nonadecanoic acid, 18-oxo-	3,63
9	Tridecanal	2,55
10	Borane, diethylmethyl-	2,32
11	Tetradecanoic acid (Myristic acid)	2,29
12	Palmitic acid, 2-(tetradecyloxy)ethyl ester	1,81
13	1-Octanol, 2-butyl-	1,22
14	Tetratetracontane	1,05
15	Triacontane, 1-Bromo-	0,94
16	Dodecane	0,81
17	Tetratriacontane	0,81
18	1H-Purin-6-amine, [(2-fluorophenyl)methyl]-	0,73
19	Nonadecane	0,57
20	Hexadecane	0,54
21	Pentadecane	0,54
22	Docosane	0,28
23	Octadecane, 1-chloro-	0,27

Essential oils were reported very diverse in some studies (24, 25). It was reported that the major essential oil constituents were: decanoic acid (9.8%), dodecanoic acid (11.9%) and ester methyl palmitate (28.5%) (24), capric acid (19.8%) and methyl palmitate (28.5%) (26), limonene (11.4%), cyclosativene (11.9%), α -ylangene (13.9%), and diepi- α -cedrene (17.9%) (27), β -caryophyllene (9.0–25.6%), heneicosane (3.0–32.1%) and α -copaene (1.5–7.2%) (28), α -humulene (15%), 1,8-cineole (16%) and α -pinene (32%) (29) and 1,8-cineole (8.9%), δ -cadinene (9.0%) and β -caryophyllene (5.8–36.2%) (27).

The differences in essential oil constituents could be explained by diversities in their origin and the climate. Also, esterification was used to increase the volatility of fatty acids in the sample preparation of our study.

Previously, Judzentiene et al showed that essential oils of *Helichrysum arenarium* contains palmitic, myristic and lauric acids, *n*-nonanal, and trans- β -caryophyllene ($\leq 6.5\%$) (30). In various studies it has been shown that phenolic compounds of *Helichrysum arenarium* had anti-atherosclerotic and antimicrobial activities (19,31). Additionally, Kucukoglu et al. suggested that *Helichrysum* extracts can inhibit

mammalian DNA topoisomerase I enzyme and these extracts may be used to prepare anticancer medications in the future (32). Since the potential functions of substances found in herbal ingredients can be investigated by using computational methods (33), extracts of the *Helichrysum arenarium* can be found out by using computational methods and possible signaling mechanisms.

4. Conclusion

This study investigated the cytotoxic activities of *Helichrysum arenarium* on several types of human cells including ECV304 and Ishikawa cells. The results of our study have established the most significant extract dose. Furthermore, immortelle extracts' essential oils were examined by using gas chromatography-mass spectrometry. The *Helichrysum arenarium* extract reduced both cell viability and MDA levels in Ishikawa and ECV304 cells at 500 µg/mL. We have found that the main essential oils in the DMSO extracts of *Helichrysum arenarium* were palmitic acids and palmitoylglycerol. In conclusion, *Helichrysum arenarium* has exhibited antioxidant activity.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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