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ORIGINAL ARTICLE

# Real-Time Cell Analysis of the Cytotoxicity of *Origanum acutidens* Essential Oil on HT-29 and HeLa Cell Lines

## *Origanum acutidens* Uçucu Yağının HT-29 ve HeLa Hücre Hatları Üzerine Sitotoksisitesinin Gerçek Zamanlı Hücre Analizi

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## ABSTRACT

**Objectives:** The aim of this study was to investigate the cytotoxic and radical scavenging effects of the essential oil from *Origanum acutidens* (Hand-Mazz.) letswaart and to determine its chemical composition.

**Materials and Methods:** The essential oil was obtained by hydrodistillation and analyzed by gas chromatography/mass spectrometry (GC-MS). The cytotoxic effect of the essential oil on the growth of human colorectal adenocarcinoma (HT-29) and human cervical adenocarcinoma (HeLa) cell lines was investigated by xCELLigence Real Time Cell Analyzer instrument. Moreover, radical scavenging effect of the oil was determined by using DPPH method.

**Results:** The main constituents of the oil were found to be carvacrol (61.69%), *p*-cymene (17.32%), γ-terpinene (4.05%), and borneol (3.96%). The essential oil of *O. acutidens* exhibited significant cytotoxic effect against HeLa and HT-29 cell lines at the tested concentrations. The essential oil had moderate DPPH radical scavenging activity compared to butylated hydroxytoluene (BHT).

**Conclusion:** The data in the present study clearly demonstrated inhibitory effect of the oil on two human cancer cell lines. According to these results and other reported studies, this observed high effect may be attributed to the presence of the carvacrol component in the oil.

**Key words***: Origanum acutidens*, carvacrol, antiproliferative, HeLa, radical scavenging

## ÖZ

**Amaç:** Bu çalışmanın amacı, *Origanum acutidens* (Hand-Mazz.) letswaart uçucu yağının sitotoksik ve radikal giderim etkisinin araştırılması ve kimyasal kompozisyonunun belirlenmesidir.

**Gereç ve Yöntemler:** Uçucu yağ, hidrodistilasyon yöntemi ile elde edilmiş ve gaz kromatografisi/kütle spektrometresi (GC/MS) ile analiz edilmiştir. Uçucu yağın, insan servikal adenokarsinom (HeLa) ve insan kolorektal adenokarsinom (HT-29) hücre hatları üzerine sitotoksik etkisi xCELLigence Gerçek Zamanlı Hücre Analizör cihazı ile araştırılmıştır. Ayrıca, uçucu yağın radikal giderim aktivitesi DPPH yöntemi kullanılarak belirlenmiştir.

**Bulgular:** Uçucu yağın ana bileşenleri karvakrol (61.69%), *p*-simen (17.32%), γ-terpinen (4.05%) ve borneol (3.96%) olarak bulunmuştur. *O. acutidens* uçucu yağı HeLa ve HT-29 hücre hatlarına karşı test edilen konsantrasyonlarda önemli sitotoksik etki göstermiştir. Uçucu yağ, BHT ile karşılaştırıldığında orta derecede DPPH radikal süpürücü etkiye sahiptir.

**Sonuç:** Bu çalışmada sunulan sonuçlar, *O. acutidens* uçucu yağının iki insan kanser hücre hattı üzerine olan inhibitor etkisini açıkca göstermektedir. Bu sonuçlara ve rapor edilen diğer çalışmalara gore, gözlenen bu yüksek etki yağda karvakrol bileşiğinin bulunmasına bağlanabilir.

**Anahtar kelimeler:** *Origanum acutidens,* karvakrol, antiproliferatif, HeLa, radikal giderimi

## INTRODUCTION

Cancer has emerged as one of the most alarming diseases in the last few decades throughout the world. The steep rise in the number of cancer cases may be attributed to the changes in food habits, use of tobacco and alcohol, chronic infections, exposure to harmful radiations and chemicals, or more widely due to change in lifestyle and environmental pollution.[1] No extremely effective drug to treat most cancers is available in the market. There is a general call for new drugs that are highly effective, possess low toxicity and have minor environmental impact. Novel natural products offer opportunities for innovation in drug discover.[2] Questions concerning the safety

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of synthetic agents have increased the interest in the use of natural compounds and have encouraged more detailed studies of plant resources, which are a rich source of bionutrients or bioactive phytochemicals.[3]

Essential oils from aromatic plants have been reported to possess anticancer properties as well as antioxidant activity. Most of essential oils have been first characterized for the treatment of inflammatory and oxidative diseases. It appeared that these essential oils could also have anticancer effects as there is a relationship between the production of reactive oxygen species to the origin of oxidation and inflammation that can lead to cancer.[4,5]

*Origanum* L. (Lamiaceae) is represented by 30 taxa in Turkey, 15 of them are endemic and 5 taxa are hybrid.[6-10] *Origanum* species have traditionally been used as spices and herbal tea. They are traditionally used as a sedative, diuretic, degasifier, sweater and antiseptic, and also in the treatment of gastrointestinal diseases and constipation. This genus is rich in essential oils and bitter substances.[11] Oregano essential oil has been found to be amongst the most effective antioxidant natural agents.[12] The antioxidant effect of these essential oils is attributed to their major components, carvacrol and thymol, and it is the result of various possible mechanisms: free-radical scavenging activity, transition-metal-chelating activity, and/or singlet-oxygen quenching capacity.[13]

*Origanum acutidens* (Hand.-Mazz.) Ietswaart is an endemic, herbaceous, and perennial plant growing mainly in East Anatolia.[8] Essential oil of this plant possesses a variety of biological activities, including antioxidant activity.[14] The aim of this study was, i) to investigate the essential oil composition of *O. acutidens* by gas chromatography/mass spectrometry (GC-MS), ii) to evaluate the cytotoxic effect of the oil on the growth of human cervical carcinoma cell line (HeLa) and HT-29 cell lines by xCELLigence method, and iii) to determine its radical scavenging effect by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.

## MATERIALS AND METHODS

*Chemicals and reagents*

DPPH, butylated hydroxyanisole (BHA), 2,6-di-tert-butyl-4-methylphenol (BHT), dimethyl sulfoxide (DMSO), formic acid, Trypsin-ethylenediaminetetraacetic acid, fetal bovine serum (FBS), penicillin/streptomycin and Dulbecco's modified Eagle's medium (DMEM)-high glucose were purchased from Sigma-Aldrich GmbH (Taufkirchen, Germany).

*Plant materials*

*Origanum acuditens* materials were collected from Bingöl city to Aşağıköy village, 8-9. km, in *Quercus* L. woods (Bingöl, Turkey) in June 2011. The identification of plant materials was confirmed by taxonomist in the Department of Biology, Bingöl University, Turkey. A voucher specimen (*L. Behçet* 8046) was deposited at the Herbarium of the Biology Department, Bingöl University, Turkey.

*Essential oil isolation procedure*

Two kilograms of dried aerial parts of the plant were shredded and combined with liquid nitrogen in a clean metal container. Then, the plant materials were processed for approximately 3 h in a Clevenger hydro-distillation apparatus and the essential oil was obtained. Anhydrous sodium sulfate was added to eliminate residual water in the oil. Finally, filtration was performed using blue band filter paper. The essential oil was stored in amber glass bottles at 4˚C.

*Gas chromatography/mass spectrometry analysis*

The essential oil of *O. acutidens* were analyzed with an Agilent Technology 7890A GC system coupled to a 5975C inert MSD with Triple-Axis Detector (Agilent Technologies) on a capillary column [Agilent Technologies HP5-MS (30 m x 0.25 mm I.D. x 0.25 µm film thickness)]. GC temperature program was as follow: From 60 to 150˚C at a rate of 3˚C/min and holding there for 10 min, from 150 to 200˚C at a rate of 5˚C/min and holding there for 3 min, from 200 to 250˚C at a rate of 15˚C/min. Inlet temperature was 250˚C. Spectra were obtained for the range of 50-550 m/z. The GC temperature program was run with helium as carrier gas, at a flow rate of 1 mL/min and injections in split mode (1:50). The mass-spectrometer interface temperature was set to 250˚C. The temperature of the ion source was 230˚C, electron energy 70 eV and quadruples temperature 150˚C. The injection volume was 1 µL.

*xCELLigence real time cell analyzer assay*

The cytotoxic effect of the essential oil against the HT-29 and HeLa cell lines was determined by using the xCELLigence real-time cell analyzer-single plate (RTCA) instrument (Roche Applied Science, Basel, Switzerland) according to method of Koldas et al.[15] DMEM with 10% FBS and 2% penicillin-streptomycin was used as the cell culture medium during the assessments. First, 50 µL of medium was added to each well of E-plate 96 and the plate was left in the hood for 15 min and then in the incubator for 15 min to let both the E-plate's golden electrode well bottoms and the medium reach a thermal equilibrium. Then, the E-plate was inserted into the RTCA station in the incubator and a background measurement was performed. After ejection of the Eplate from the station, 100 µL HT-29/HeLa cell suspensions were added to the wells to obtain a $2.5x10^4$ cell/well concentration in each well except for three of the wells. The cell concentration (cells/well) was analysed by using fully automated Cedex Hires Analyzer system (Roche Diagnostic Ltd, Rotkreuz, Switzerland) based on the manual Trypan Blue Exclusion Method. Three wells were left without cells to check if there would be an increase in cell index (CI) originating from the medium. 100 µL of medium was added to these wells instead of the cell suspension. After leaving the E-plate in the hood for 30 min, it was inserted to the RTCA station and the second step measurement was initiated for 80 min. During this period, the cells adapted to the bottom of the wells and entered into a growth and division phase. After this step, the E-plate was ejected from the station and solutions of the essential oil that was prepared with DMSO (final concentration of DMSO

was less than 1% in each of the wells) and medium were added to the wells to obtain final concentrations of 250, 100, 50, and 10 µg/mL. Then final volume of the wells was completed to 200 µL by adding medium. After this step, the E-plate was inserted into the station and the main measurement period of 48 hours was initiated.

*2,2-diphenyl-1-picrylhydrazyl radical scavenging assay*

The free radical scavenging activity of the essential oil was determined by the method of, Blois[16] with some modifications.[17] The solution of DPPH in methanol (0.004%) was prepared fresh daily and 1 mL of this solution in methanol was mixed with 1 mL of sample solution of varying concentrations. Each mixture was kept in the dark for 30 min at room temperature. The absorbance was measured at 517 nm against a blank on a ultraviolet (UV) visible light spectrophotometer (Rayleigh, UV-2601). BHT and BHA were used as positive controls. The activity was calculated using the following formula: Scavenging %= $[(A_{control}-A_{sample})/A_{control}]x100$.

*Statistical analysis*

All experiments were done in triplicate. The results were expressed as means ± standard deviations. Statistical analyses were performed using the SPSS 11.5 (SPSS, Chicago, IL). Differences among means were done by ANOVA, and averages were compared using the Tukey test. The level of statistical significance was taken at $p<0.01$.

## RESULTS AND DISCUSSION

*Essential oil composition*

Hydro-distillated essential oil of *O. acutidens* was analyzed by GC/MS. Twenty eight components were identified representing 98.99% of the oil (Table 1).

According to the GC-MS analysis results, carvacrol was the most abundant component of the oil (61.69%). Other main components of the oil were found to be *p*-cymene (17.32%), γ-terpinene (4.05%), and borneol (3.96%). As seen in Table 1, our findings are in agreement with previously published studies determined carvacrol (64.58-72%) and *p*-cymene (7.5-13.99%) were the major components of the oil.[14,18,19]

*Cytotoxic effect by using real time cell analyzer*

The cytotoxic effect of the essential oil was examined on HT-29 cell line at the concentrations of 10, 50 and 100 µg/mL by using real time cell analyzer xCELLigence method. The system measures impedance differences in order to derive CI values at time points whose intervals can be set by the operator. These impedance differences and thus the CI values depend on the cell activity at the bottom of the wells.[20] CI is a dimensionless parameter derived as a relative change in measured electrical impedance to represent cell status. If it is decreasing, it shows us that the cancer cells are dying. Our data showed that the essential oil displayed significant cytotoxic effect against HT-29 cell line (Figure 1). The essential oil was most active at the concentration of 50 and 100 µg/mL.

Cytotoxic effect of the essential oil was also examined on HeLa cell line at the concentrations of 50, 100 and 250 µg/mL. The essential oil exhibited excellent cytotoxic effect against HeLa cell line at the tested concentrations [inhibitory concentration $(IC)_{50}<10$ µg/mL]. The Figure 2 belonging to the CI changing according to the cell numbers against to time was obtained.

Begnini et al.[21] reported that *Origanum vulgare* essential oil was composed mostly of 4-terpineol and induced a high cytotoxic effect in HT-29. Sivropoulou et al.[22] investigated cytotoxicity of three *Origanum* essential oils and the oils exhibited high levels of cytotoxicity against four permanent animal cell lines including HeLa. Hussain et al.[23] reported the inhibitory effect of *Origanum*

**Table 1. The essential oil composition of *Origanum acutidens***

| No | Component | Area % |
|---|---|---|
| 1 | α-Thujene | 0.32 |
| 2 | α-Pinene | 0.50 |
| 3 | Camphene | 0.69 |
| 4 | 1-Octen-3-ol | 0.26 |
| 5 | 3-Octanone | 0.67 |
| 6 | β-Pinene | 0.75 |
| 7 | α-Phellandrene | 0.12 |
| 8 | α-Terpinene | 1.01 |
| 9 | *p*-Cymene | 17.32 |
| 10 | Eucalyptol | 0.70 |
| 11 | γ-Terpinene | 4.05 |
| 12 | Terpinolene | 0.28 |
| 13 | 2-Caren-4-ol | 0.23 |
| 14 | Borneol | 3.96 |
| 15 | 4-Terpineol | 1.46 |
| 16 | *p*-Cymen-8-ol | 0.23 |
| 17 | α-Terpineol | 0.27 |
| 18 | Dihydrocarvone | 0.30 |
| 19 | Thymol | 0.76 |
| 20 | Carvacrol | 61.69 |
| 21 | 2-Ethyl-5-propylphenol | 0.41 |
| 22 | Caryophyllene | 1.52 |
| 23 | Alloaromadendrene | 0.41 |
| 24 | β-Guaiene | 0.33 |
| 25 | Spathulenol | 0.25 |
| 26 | Caryophyllene oxide | 0.50 |
| 27 | Trienbolone | 0.45 |
| 28 | 2-[4-methyl-6-(2,6,6-trimethylcy-clohex-1-enyl)hexa-1,3,5-trienyl] cyclohex-1-en-1-carboxaldehyde | 0.56 |

essential oils (*O. majorana*, *O. vulgare*) on the cell proliferation of two human cancer (MCF-7 and LNCaP) cell lines and the $IC_{50}$ values indicating that the tested *Origanum* essential oils showed prominent cytotoxicity against both the cancer cell lines.

In the present study, carvacrol (61.69%) was found to be the main component of the essential oil. It may be an important component in the cytotoxic effect of this oil. Mehdi et al.[24] demonstrated that carvacrol is an effective anticancer compound with an $IC_{50}$ of 50 mg/L at 48 h inducing growth inhibition in human cervical cells by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and lactate dehydrogenase assays. In our previous study, we reported the inhibitory effect of the essential oil and the isolated pure or mixture components from *Satureja boissieri* essential oil on HeLa. In that study, the components containing the high concentrations of p-cymene, thymol and carvacrol effectively inhibited the growth of HeLa cells.[25] Akalin and Incesu[26] demonstrated that the carvacrol has cytotoxic effect on H-ras transformed 5RP7 and *N*-ras transformed CO25 cell lines upon time- and concentrations. Therefore, in our present study, the high cytotoxic effect of the essential oil against HeLa and HT-29 cells may be attributed the presence of carvacrol.

**Table 2. Radical scavenging activity of the essential oil**

| | DPPH $IC_{50}$ (mg/mL) |
|---|---|
| Essential oil | 0.387±0.005[a] |
| BHT | 0.022±0.000[b] |
| BHA | 0.003±0.000[b] |

DPPH: 2,2-diphenyl-1-picrylhydrazyl, BHT: Buthylated hydroxytoluene, BHA: Butylated hydroxyanisole, $IC_{50}$: Inhibitory concentration, *Values in the same column with different superscripts are significantly ($p<0.01$) different

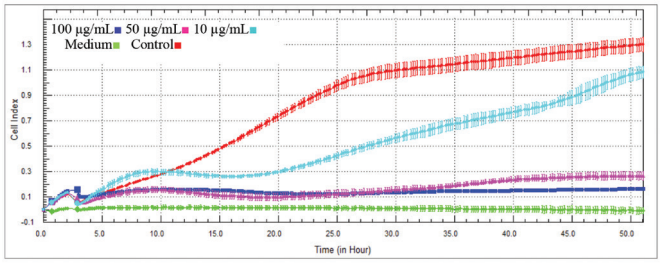



**Figure 1.** Real-time cell monitoring of the proliferation of HT-29 cells treated with the essential oil for 48 h period

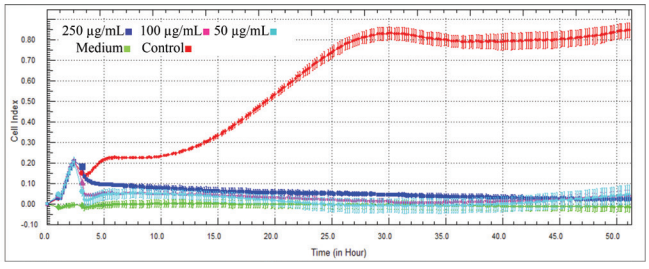



**Figure 2.** Real-time cell monitoring of the proliferation of HeLa cells treated with the essential oil for 48 h period

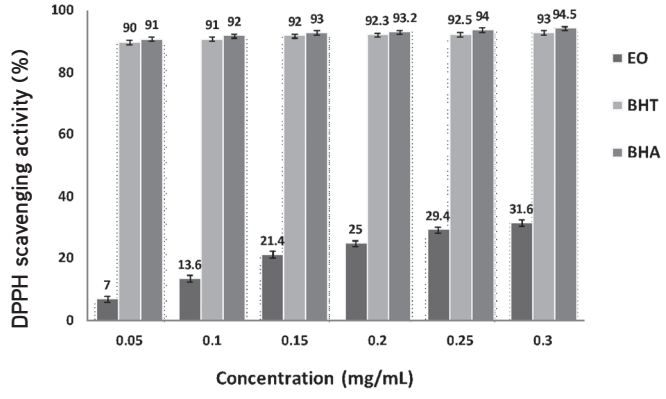



**Figure 3.** 2,2-diphenyl-1-picrylhydrazyl scavenging activity of the essential oil and synthetic antioxidants at tested concentrations

DPPH: 2,2-diphenyl-1-picrylhydrazyl, EO: The essential oil, BHT: Buthylated hydroxytoluene, BHA: Butylated hydroxyanisole

*2,2-diphenyl-1-picrylhydrazyl radical scavenging activity of the oil*

The free radical scavenging activity of essential oil from *O. acutidens* was measured by DPPH assay. DPPH radical scavenging activity assay is a widely used method to evaluate antioxidant activities in a relatively short time compared with other methods. This assay measures the ability of a sample to donate hydrogen to DPPH radical. DPPH radical scavenging activities of the essential oil, BHT and BHA are given in Table 2. Lower $IC_{50}$ values indicate higher free radical scavenging activity. The radical scavenging activity of the essential oil increased by increasing the oil concentration (Figure 3). The $IC_{50}$ value of the oil was determined as 0.387±0.005 mg/mL.

The essential oil of *O. acutidens* had moderate radical scavenging activity compared to the synthetic antioxidants. Sokmen et al.[14] was determined the radical scavenging effect of *O. acutidens* essential oil with a $IC_{50}$ value of 133.7±0.5 µg/mL and the oil exhibited lower radical scavenging activity than BHT (19.8±0.5 µg/mL). Hussain et al.[23] reported the radical scavenging activities of *Origanum* oils (*O. majorana* and *O. vulgare*) was less than that of the positive control.

Sharopov et al.[27] investigated the radical scavenging activities of the eighteen different essential oil components and carvacrol showed the one of best radical scavenging activity in the DPPH and 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid assays. Lin et al.[28] reported that thymol and carvacrol were the major components attributing the DPPH free radical scavenging activity in the thyme wild essential oil. In the study of Ali et al.[29] carvacrol exhibited 33.9% radical scavenging activity at 5 mM concentration. Therefore, the observed radical scavenging activity of the essential oil is mainly attributed to its main constituent carvacrol.

## CONCLUSION

In this study, we have identified the chemical composition of *O. acutidens* essential oil and evaluated the oil's cytotoxic effect on HT-29 and HeLa cell line. Our results clearly show that this essential oil is active against both tumor cell lines tested. These cytotoxic properties could be explained, in part, by the presence of carvacrol which is a main component of the oil. According to the

results, *O. acutidens* essential oil may be suggested as a promising natural agent for alleviating HeLa and HT-29 cell growth.

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## REFERENCES

1. Gautam N, Mantha AK, Mittal S. Essential oils and their constituents as anticancer agents: a mechanistic view. Biomed Res Int. 2014;2014:154106.
2. Cai Y, Luo Q, Sun M, Corke H. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. Life Sci. 2004;74:2157-2184.
3. Aydin S, Seker E. Effect of an aqueous distillate of *Origanum* onites L. on isolated rat fundus, duodenum and ileum: evidence for the role of oxygenated monoterpenes. Pharmazie. 2005;60:147-150.
4. Jackson AL, Loeb LA. The contribution of endogenous sources of DNA damage to the multiple mutations in cancer. Mutat Res. 2001;477:7-21.
5. Bayala B, Bassole IHN, Scifo R, Gnoula C, Morel L, Lobaccaro J-MA, Simpore J. Anticancer activity of essential oils and their chemical components - a review. Am J Cancer Res. 2014;4:591-607.
6. Ietswaart J. A taxonomic revision of the genus *Origanum* (Labiatae). Leiden University Press, Netherlands, 1980.
7. Davis P. Flora of Turkey and the East Aegean Islands. Vol. 7, pp. 297-313, Edinburgh University Press, Edinburg, 1982.
8. Davis P, Mill R, Tan K. Flora of Turkey and the East Aegean Islands. vol. 10 (supp. I). Edinburg Univ. Press, Edinburgh, 1988.
9. Guner A, Özhatay N, Ekim T, Baser K. Flora of Turkey and the east Aegean islands, Vol. 11. (Supp. II), Edinburg Univ. Press, Edinburgh, 2000.
10. Guner A, Aslan S, Ekim T, Vural M, Babac M. Türkiye bitkileri listesi (Damarlı Bitkiler), syf: 1290, NGBB ve Flora Aras. Der. Yayını, İstanbul, 2012.
11. Baytop T, Therapy with medicinal plants in Turkey (Past and Present). pp. 166-167, Istanbul University Press, Istanbul, 1999.
12. Rodriguez-Garcia I, Silva-Espinoza BA, Ortega-Ramirez LA, Leyva JM, Siddiqui MW, Cruz-Valenzuela MR, Gonzalez-Aguilar GA, Ayala-Zavala JF. Oregano Essential Oil as an Antimicrobial and Antioxidant Additive in Food Products. Crit Rev Food Sci Nutr. 2016;56:1717-1727.
13. Shan B, Cai YZ, Sun M, Corke H. Antioxidant Capacity of 26 Spice Extracts and Characterization of Their Phenolic Constituents. J Agric Food Chem. 2005;53:7749-7759.
14. Sokmen M, Serkedjieva J, Daferera D, Gulluce M, Polissiou M, Tepe B, Akpulat HA, Sahin F, Sokmen A. *In vitro* antioxidant, antimicrobial, and antiviral activities of the essential oil and various extracts from herbal parts and callus cultures of *Origanum acutidens*. J Agric Food Chem. 2004;52:3309-3312.
15. Koldas S, Demirtas I, Ozen T, Demirci MA, Behcet L. Phytochemical screening, anticancer and antioxidant activities of *Origanum vulgare* L. ssp. viride (Boiss.) Hayek, a plant of traditional usage. J Sci Food Agric. 2015;95:786-798.
16. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature. 1958;181:1199-1200.
17. Oke F, Aslim B. Protective effect of two edible mushrooms against oxidative cell damage and their phenolic composition. Food Chemistry. 2011;128:613-619.
18. Baser K, Tümen G, Duman H. Essential Oil of *Origanum acutidens* (Hand.-Mazz.) letswaart. J Essent Oil Res. 1997;9:91-92.
19. Dagdemir E, Cakmakci S, Gundogdu E. Effect of Thymus haussknechtii and *Origanum acutidens* essential oils on the stability of cow milk butter. Eur J Lipid Sci Technol. 2009;111:1118-1123.
20. Isik S, Karagoz A, Karaman S, Nergiz C. Proliferative and apoptotic effects of olive extracts on cell lines and healthy human cells. Food Chemistry. 2012;134:29-36.
21. Begnini KR, Nedel F, Lund RG, Carvalho PH, Rodrigues MR, Beira FT, Del-Pino FA. Composition and antiproliferative effect of essential oil of *Origanum vulgare* against tumor cell lines. J Med Food. 2014;17:1129-1133.
22. Sivropoulou A, Papanikolaou E, Nikolaou C, Kokkini S, Lanaras T, Arsenakis M. Antimicrobial and cytotoxic activities of *Origanum* essential oils. J Agric Food Chem. 1996;44:1202-1205.
23. Hussain AI, Anwar F, Rasheed S, Nigam PS, Janneh O, Sarker SD. Composition, antioxidant and chemotherapeutic properties of the essential oils from two *Origanum* species growing in Pakistan. Rev Bras Farmacogn. 2011;21:943-952.
24. Mehdi SJ, Ahmad A, Irshad M, Manzoor N, Rizvi MMA. Cytotoxic effect of Carvacrol on human cervical cancer cells. Biology Med. 2011;3:307-312.
25. Oke-Altuntas F, Demirtas I, Tufekci AR, Koldas S, Gul F, Behcet L, Gecibesler HI. Inhibitory Effects of the Active Components Isolated from *Satureja Boissieri* Hausskn. Ex Boiss. On Human Cervical Cancer Cell Line. J Food Biochem. 2016;40:499-506.
26. Akalin G, Incesu Z. The effects of carvacrol on apoptosis of H-ras and *N*-ras transformed cell lines. Turk J Pharm Sci. 2011;8:105-116.
27. Sharopov FS, Wink M, Setzer WN. Radical scavenging and antioxidant activities of essential oil components--an experimental and computational investigation. Nat Prod Commun. 2015;10:153-156.
28. Lin C-W, Yu C-W, Wu S-C, Yih K-H. DPPH Free-Radical Scavenging Activity, Total Phenolic Contents and Chemical Composition Analysis of Forty-Two Kinds of Essential Oils. J Food Drug Anal. 2009;17.
29. Ali HM, Abo-Shady A, Sharaf Eldeen HA, Soror HA, Shousha WG, Abdel-Barry OA, Saleh AM. Structural features, kinetics and SAR study of radical scavenging and antioxidant activities of phenolic and anilinic compounds, Chem Cent J. 2013;7:1-9.