Cumhuriyet Üniversitesi Fen Fakültesi
Fen Bilimleri Dergisi (CFD), Cilt:36, No: 3 Özel Sayı (2015)
ISSN: 1300-1949

Cumhuriyet University Faculty of Science
Science Journal (CSJ), Vol. 36, No: 3 Special Issue (2015)
ISSN: 1300-1949

Cytotoxic effect of *Zataria multiflora* on cervical cancer cell line (HeLa) and normal fibroblast cells

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Received: 01.02.2015; Accepted: 05.05.2015

Abstract. In this study, cytotoxic effects caused by Zataria multifloraessential oil, an Iranian species of family of Laminaceae, in human cervical cancer cell line (HeLa) and human fibroblast cells was investigated. The HeLa cells were seeded in 96-well culture plates in the presence and absence of different concentrations of Z. multifloraessential oil(0, 6.25, 12.5, 25, 50, 100, 200 and 400 µg/ml)for 24, 48 and 72 hours using MTT assay. Results showed thatessential oil of Z. multiflorashowed strong inhibitory effects on HeLa cells when compared to control. The cytotoxicity effect after 72h incubation was greater than 24 and 48h and the highest cytotoxicity of Z. multifloraessential oil was related to 400 µg/ml (100.08%) . Also results showed that more effect of Z. multifloraessenceon the growth inhibitory of normal fibroblast cell was observed at 100 µg/ml after 48h (98.82%).

Keywords: zataria multiflora, cytotoxic effect, HeLa cells, fibroblast cells, MTT assay

1. INTRODUCTION

Cervical cancer (Cervical carcinoma) after breast, lung, colorectal, endometrium and ovarian cancers is sixth most common cancer in women. It caused by sexually-acquired infection with human papillomavirus (HPV), continues to be a public health problem worldwide as it imperils the lives of more than 270,000 women every year [1]. Also, this cancer is the most common gynecological cancer in Iran [2], where various methods of screening, diagnosis, and treatment have been reported [2-7].

Because of high death rate associated with cancer and because of serious side effects of chemotherapy and radiation therapy, many cancer patients and scientistsseek alternative complementary methods of treatment [8]. Over the past decade, herbal medicine has become a topic of global importance, making an impact on both world health and international trade. Among the human diseases treated with medicinal plants is cancer, which is probably the most important genetic disease. The anti-tumor activity and the possible applications of medicines from medicinal plants for cancer prevention have been recently described [9-17].

Zataria multiflora Boiss is a medicinal plant that belongs to the family Laminaceae. This plant (vernacular name of Avishan Shirazi, in Iran) geographically grows in Iran, Pakistan and Afqanistan [18, 19] and has been traditionally used as an antiseptic, anesthetic and anti-spasmodic drug [18]. Also, this plant is extensively used as a flavor ingredient in Iranian food. The essential oil of *Z.multiflora* is extracted from the flowered browses of the plant, having the compounds with important pharmaceutical, antimicrobial, and antioxidant effects [20, 21]. The main constituents of its essential oil are phenolic compounds such as carvacrol and thymol [22, 23]. It is also well known that essential oils, which are rich in carvacrol, possess strong antioxidant properties equivalent to those of ascorbic acid, butyl hydroxyl toluene (BHT) and vitamin E [24, 25]. Since many antioxidants exert anticarcinogenic effects [26- 29], and reported that essential oil of *Z.multiflora* is rich in carvacrol and thymol,it is possible that this

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Special Issue: The Second National Conference on Applied Research in Science and Technology

essence functions in a similar way and the anti-proliferative properties of carvacrol on non-small cell lung cancer cells, A549, chronic myeloid leukemia cells, K562, murine B16 melanoma cells and human metastatic breast cancer cell line, MDA-MB 231 have been shown [30-35].

Based on this hypothesis and reported data, the present study is aimed to evaluate the cytotoxic effects of *Z. multiflora* essential oil on human cervical cancer HeLa cells and human normal fibroblast cells.

2.MATERIAL AND METHODS

2-1. Plants Mateerial

The aerial parts of *Z. multiflora* were harvested in pre-flowering stage from wild grown plants in the Mountain regions of Fars province. Voucher specimen was deposited at the herbarium of medicinal and aromatic plants of Islamic Azad University, Qaemshahr branch, Mazandaran, Iran. The harvested plants were dried at room temperature (25°C) for 2 weeks, then, air-dried plants (in each habitat) were ground and powdered with mixer for essential oil extraction and other experiments.

2.2. Essential Oil Extraction

The dried aerial parts of *Z. multiflora* were subjected to hydro-distillation for 3 hours using a Clevenger-type apparatus. The essential oil obtained was separated from water and dried over anhydrous sodium sulfate. The concentrated plant essential oil were dissolved in dimethyl sulphoxide (DMSO) (SIGMA, USA) to obtain appropriate solutions of the essence.The sub-stock solutions of 6.25, 12.5, 25, 50, 100, 200 and 400 µg/ml was prepared by diluting the stock solution into serum-free culture medium, RPMI 1640 (the percentage of DMSO in the experiment should not exceed 1). The stock and sub-stock solutions were both stored at 4 ºC.

2.3. Cell Culture

Human cervical cancer cell line (HeLa) were purchased from Pasteur Institute of Iran (NCBI C115). The cells was grown and maintained in a humidified incubator at 37 ºC and in 5% CO2 atmosphere. RPMI-1640 medium (SIGMA) was supplemented with 0.01 mg/ml heat inactivated Fetal Calf Serum (FCS), 100 units/ml penicillin, and 100 µg/ml streptomycin (ALL FROM INVITROGEN GIBCO) was used for cell culture. Upon reaching confluency, the cells were passaged. After being harvested from sterile T75 culture flasks, the cells were counted using a hemocytometer and cell viability was determined by trypan blue exclusion. Three thousand cells from log phase cultures were seeded in 100 µl of RPMI medium supplemented with 10% fetal bovine serum per well of 96-well flat-bottom culture plates [36]. Cells were incubated with the *Z. multiflora*essential oil for defined tisme (24, 48 and 72 hours). Proliferative response and cell death of the *Z. multiflora*essential oil-treated cells were determined using MTT assay and cell death ELISA Cell Viability Assay, respectively.

2.4. Cytotoxicity Analysis By Mtt Assay

Growth of tumoral cells quantitated by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl- 2-thiazolyl)-2,5-diphenyl-2H-terazolium bromide (MTT) to a blue formazan product. MTT assay is an accurate method to evaluate the survival of cells.This assay is based on the cleavage of the yellow tetrazolium salt, MTT, to form a soluble blue formazan product by mitochondrial enzymes, and the amount of formazan produced is directly proportional to the number of living cells [47]. In brief, this color can be measured by spectroscopic methods. There is a linear relation between the number of viable cells and absorption for each cell. This

relationship allows providing accurate determination of any changes in the rate of cell proliferation [48]. In this study, after HeLa and fibroblast cells were incubated for 24, 48 and 72 hour with different values of essential oil, 20 µl MTT solution 0.5 mg/ml was added into the wells and incubation continued at 37°C for 3 to 5 hours. After this time the supernatant cells were removed, and instead of that 200 µl of DMSO solution was added for 15 min at room temperature. Finally, the percentage of cell viability was determined by ELISA reader in 570 nm. The percentage of cell viability was calculated using the equation: [mean optical density (OD) of treated cells/mean OD of control cells] × 100. Percent of cytotoxicity = 100 – percent of Viability [49]. The effects of essential oil were expressed by IC_{50} values (the drug concentration reducing the absorbance of treated cells by 50% with respect to untreated cells).

2.5. Statistical Analysis

The data are expressed as mean ± standard deviation (SD) for at least three independent determinations in triplicate for each experimental point. SPSS Statistics 22 Software was used to perform statistical analysis. One-way ANOVA method followed by Duncan test was used to determine the differences among the groups (P<0.05).

3. RESULTS

Data on the cytotoxic effects of *Z. multiflora*essential oil using human HeLa tumor cell line and fibroblast cells *in vitro* are showed in table 1 and 2. The cytotoxic effect of *Z. multiflora*essential oil was determined using concentrations ranging 6.25 - 400 µg/ml for 24, 48 and 72 h.

After 24 h exposure, *Z. multiflora* induced cytotoxic effects in cervical cell lines with cell growth inhibition percent of 92.05 - 99.77% and IC50 of 35.59 µg/ml in HeLa cells using MTT method.

The more effect of *Z. multiflora*essenceon the growth inhibitory of HeLa cell line was observed at 400 µg/ml, (99.77%) and this effect highly significant as a comparison with the control group (p<0.01).

After 48h incubation, HeLa cells showed growth inhibition in a dose dependent manner when treated with *Z. multiflora*essential oil at concentrations ranging from 6.25 - 200 µg/ml (table 1). The percentage of dead cells for each concentration was found to be from -5.30 to 43.14%; but in 400 µg/ml the cell gowth inhibition was reduced (2.48%). The more effect was absorved at 200 µg/ml (43.14%) and this effect had significant difference compared with control group (p<0.01). The 50% cytotoxic effect (IC50) of *Z. multiflora* was found to be 10.48 µg/ml afetr 48h.

After 72h, the percentage growth inhibition was found to be increasing with increasing concentration of test compounds, and *Z. multiflora*essential oil concentration-dependent inhibitory effect on HeLa cell line was increased from -52.68 to 100.08 µg/ml (Table 1).

Overall, the highest cytotoxicity of *Z. multiflora*essential oil was related to 400 µg/ml (inhibition % = 100.08) after 72 hours incubation, with significant difference compared with control group (p<0.05).

Table 1. Cytotoxicity activity of *Z. multiflora* essential oil against HeLa cell line at different concentrations by MTT assay. Values represent the mean of three experiments (*P< 0.05, **P< 0.01, ***P<0.001)

Concentrations of *Z. multiflora* essential oil (μg/ml)	Absorbance	Inhibition (%)	IC_{50} (μg/ml)	P-value
After 24h incubation				
Control	0.353±0.010	-		-
6.25	0.353±0.014	92.058		0.975
12.5	0.444±0.016	97.764**		0.004
25	0.457±0.023	96.430*	35.593	0.011
50	0.353±0.012	96.023		1.000
100	0.165±0.015	94.392*		0.010
200	0.049±0.001	99.728***		0.0009
400	0.044±0.002	99.772**		0.001
After 48h incubation				
Control	0.633±0.047	-		-
6.25	0.841±0.044	-5.309		0.069
12.5	0.851±0.008	-7.972*	10.484	0.031
25	0.744±0.039	3.110		0.169
50	0.680±0.055	5.360		0.525
100	0.309±0.041	42.177*		0.041
200	0.046±0.004	43.144**		0.004
400	0.049±0.005	2.488**		0.004
After 72h incubation				
Control	0.770±0.139	-		-
6.25	1.102±0.039	-52.681		0.061
12.5	1.024±0.067	-43.427		0.207
25	0.859±0.068	-19.658		0.631
50	0.768±0.040	-5.268		0.990
100	0.501±0.042	34.561		0.153
200	0.055±0.001	99.812*		0.024
400	0.053±0.001	100.08*		0.024

In addition, the effect of *Z. multiflora* essential oil on inhibition of normal fibroblast cells, in different concentrations of it include6.25, 12.5, 25, 50, 100, 200 and 400 μg/ml, was checked using the MTT assay after 24, 48 and 72 hours incubation and results are tabulated in Table 2. After 24h incubation, results showed lower effect on fibroblast cells compared with 48 and 72h. Nevertheless, the *Z. multiflora* essential oil was able to induce inhibitory of the fibroblast cells after 48 and 72h incubation, from 18.67 to 98.88 and from 41.65 to 94.34, respectively, as exhibited in table 2. The Maximum cytotoxic to fibroblast cells were found at concentrations of 100 μg/ml, After 48 hours of treatment, with more cytotoxic effects on normal cells (98.88%). The IC_{50} values (50% inhibitory concentration) of this assay after 24, 48 and 72 h were 6.04, 25.11 and 66.71 μg/ml respectively.

Table 2. Cytotoxicity activity of *Z. multiflora* essential oil against normal fibroblast cells at different concentrations by MTT assay. Values represent the mean of three experiments (*P< 0.05, **P< 0.01, ***P< 0.001).

Concentrations of *Z. multiflora* essential oil (µg/ml)	Absorbance	Inhibition (%)	IC_{50} (µg/ml)	P-value
After 24h incubation				
Control	0.175 ±0.009	-		-
6.25	0.161 ±0.018	9.674	6.041	0.454
12.5	0.158 ±0.003	11.803		0.069
25	0.155 ±0.007	13.734		0.060
50	0.142 ±0.009	22.836		0.081
100	0.129 ±0.008	31.360*		0.020
200	0.104 ±0.016	48.375**		0.003
400	0.071 ±0.029	71.402*		0.032
After 48h incubation				
Control	0.520 ±0.145	-		-
6.25	0.179 ±0.006	18.674		0.051
12.5	0.185 ±0.003	31.945		0.054
25	0.261±0.002	47.154	25.110	0.166
50	0.115 ±0.003	86.878		0.074
100	0.069 ±0.0008	98.823		0.062
200	0.072 ±0.001	97.736		0.065
400	0.082 ±0.002	95.507		0.065
After 72h incubation				
Control	0.386 ±0.042	-		-
6.25	0.127 ±0.040	41.654*		0.041
12.5	0.229 ±0.042	56.456*		0.030
25	0.292 ±0.005	61.838*		0.033
50	0.379 ±0.027	64.657	66.710	0.887
100	0.468 ±0.040	71.847		0.259
200	0.505 ±0.042	75.729		0.162
400	0.495±0.023	94.348		0.108

From figures 1 and 2 it can be inferred that inhibition percentage of human cancer and fibroblast cells in presence of various volumes of essential oil (6.25, 12.5, 25, 50, 100, 200 and 400 µg/ml) after 24, 48 and 72h incubation using MTT method has been presented. Each experimental curve represents the average of a series of three different experiments.

It was found that the percentage of cancer cell growth inhibition to be increasing with increasing concentration of test compounds, thus *Z. multiflora* essential oilat 6.25 to 200 µg/ml exhibited dose-dependent inhibitory effects on the proliferation of HeLa cells, but after 48h high cell inhibitory reduction was observed after 400 µg/ml than 200 µg/ml from 43.14 to 2.48%.

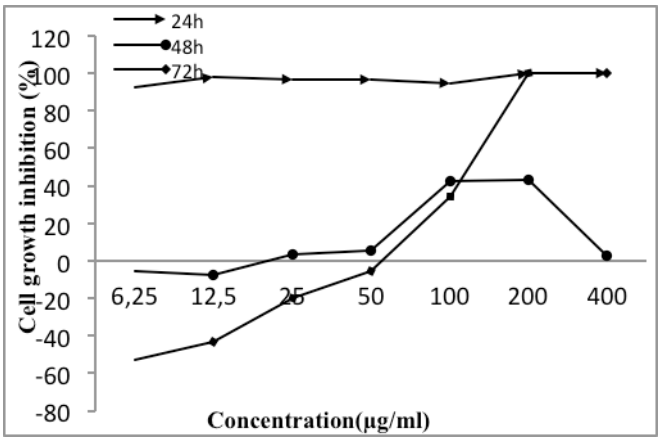

Figure 1. Concentration-cell inhibitory curves of *Z. multiflora* essential oil following 24, 48 and 72 hour exposure on HeLa cells.

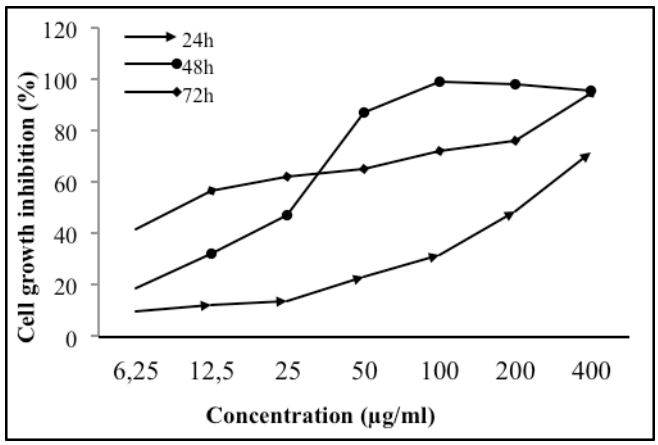

Figure 2. Concentration-cell inhibitory curves of *Z. multiflora* essential oil following 24, 48 and 72 hour exposure on human fibroblast cells.

4. DISCUSSION

In the last few decades, human cancer cell lines have aggregated an accessible, easily usable set of biological models to examine cancer biology [37]. The utility of cell lines acquired from tumor allows the investigation of tumor cells in a simplified and controlled environment [38].

MTT cytotoxicity assay used to measure the cytotoxic effect of *z. multiflora* on cervical carcinoma (HeLa) cells. In screening result, *z. multiflora* has shown broad cytotoxicity and it had most active cytotoxic activity on HeLa cells after 72h at 400 µg/ml. MTT proliferation assay was carried out to determine the growth rate of cells. In this study, essential oil of *z. multiflora* have indicated significant growth inhibition in HeLa cell line at high concentration. The IC50 of extract on cell line less than 100 g / ml is categorized as a potential cytotoxic substance [39]. *z. multiflora* treatment on HeLa cell lines showed significant decrease in growth rate compared with control. On the other hand the percentage of non-viable cells on cell lines increased with the increasing time of treatment except after 48h that severe decrease was observed at 400 µg/ml than 200 µg/ml. Several mechanisms of action were detected in HeLa cells.

The composition of the essential oil of *Z. multiflora* has been reported previously by more researchers. Alizadeh and Shaabani (2014) reported that essential oil composition in *Z. multiflora*, is rich in phenolic compounds as carvacrol and thymol and his precursors, also the extracts of *Z. multiflora* indicating good phenolic content and antioxidant activity. They showed that, Iranian *Z. multiflora* are strong radical scavengers and can be considered as a good source of natural antioxidant for traditional and medicinal uses [40]. The main constituents in *Z. multiflora* are phenolic compounds such as thymol and carvacrol and the other compounds are p-cymene, c-terpinene, b-caryophyllene and monoterpenes [22, 41, 42]. Alipour-eskandani (2011) found that the major components of *Z. multiflora* obtained from Shiraz Province of Iran was Carvacrol (71.1%) [43].

Previous studies have shown that carvacrol can exhibit strong antitumor activity. They revealed that carvacrol suppresses the growth of mouse B16 melanomas[30] and human larynx carcinoma Hep-2 cells [44] *in vitro* and was active against DMBA-induced lung tumors in rats [45]. Additional evidence for its antitumor activity was found: inhibition of DNA synthesis in mouse myoblasts bearing a human N-ras oncogene [46]. The further study indicated that carvacrol, dose-dependently, decreased the number of cancer cells, and total protein content and increased the degeneration of cell morphology of the human non-small cell lung cancer (NSCLC) cell line A549 [33]. Therefore, this essential oil may be efficient for the cancer treatment.

5. CONCLUSIONS

In summary, this is the evidence for the effect of *Z. multiflora* essential oil on cervical cancer cell line. These findings suggest that this plant essence may be a potential medicine for cancer and further studies, as a result work, should be done for evaluation of cell death mechanisms of this plant, and also it is crucial to examine other cancer cell lines; similarly, in vivo studies are prominent to decipher the exact consequence of this plant. Hence present study shows the efficacy of *Z. multiflora* for the cytotoxicity towards HeLa cells thus suggesting protection against cervical cancer.

REFERENCES

[1] Butler, M.S.(2004),"The role of natural products chemistry in drug discovery,"Journal of Natural Products 67, pp. 2141–2153.

[2] Behtash, N., Ghaemmaghami, F., Ayatollahi, H., et al (2005),"A casecontrol study to evaluate urinary tract complications inradical hysterectomy,"World J Surg Oncol, 3,pp. 12.

[3] Behtash, N., Mousavi, A., Mohit, M., et al (2003),"Simplehysterectomy in the presence of invasive cervical cancer inIran,"Int J Gynecol Cancer, 13, pp.177-81.

[4] Behtash, N., Nazari, Z., Ayatollahi, H., et al (2006), Neoadjuvantchemotherapy and radical surgery compared to radicalsurgery alone in bulky stage 11b-11A cervical cancer,"Eur JSurg Oncol, 32,pp.1226-30.

[5] Mousavi, A. and Karimi-Zarchi, M. (2007),"Isolated cervical metastasisof breast cancer: a case report and literature review,"J LowGenit Tract Dis, 11,pp. 276-8.

[6] Ghaemmaghami, F., Karimi-Zarchi, M., Mousavi, A. et al (2008), "Results of cervical cone excision biopsy in Iran,"Asian Pac J Cancer Prev, 9, pp.45-7.

[7] Mousavi, A., Karimi- Zarchi, M., Gilani, M.M., et al (2008),"Radical hysterectomy in the elderly,"World J Surg Oncol, 6, pp.38.

[8] Talib, H. W.(2011), "Chapter 6: Anticancer and antimicrobial potential of plant-derived natural products. P: 141-158. University of Applied Science, Jordan. Phytochemicals - bioactivities and impact on health,"Edited by Prof. Iraj Rasooli. Pp: 388.
[9] Chiu, H.F. and Wu, Y.C.(2002),"Cytotoxic constituents of the stem bark of Neolitsea acuminatissima," Journal of Natural Products, 65, pp.255-258.
[10] Cragg, G.M. and Newman, D.J.(1999),"Discovery and development of antineoplastic agents from natural sources," Cancer Investigation, 17, pp.153-163.
[11] Katsube, N., Iwashita, K., Tsushida, T., Yamaki, K. and Kobori, M.(2003),"Induction of apoptosis in cancer cells by Bilberry (Vaccinium myrtillus) and the anthocyanins," Journal of Agricultural and Food Chemistry, 51, pp. 68-75.
[12] Mukherjee, A.K., Basu, S., Sarkar, N. and Ghosh, A.C.(2001),"Advances in cancer therapy with plant based natural products," Current Medicinal Chemistry, 8, pp. 1467-1486.
[13] Richardson, M.A.(2001),"Biopharmacologic and herbal therapies for cancer: research update from NCCAM," Journal of Nutrition, 131, pp. 3037-3040.
[14] Tatman, D. and Mo, H.(2002),"Volatile isoprenoid constituents of fruits, vegetables and herbs cumulatively suppress the proliferation of murine B16 melanoma and human HL-60 leukemia cells," Cancer Letters, 175, pp.129-139.
[15] Wargovich, M.J., Woods, C., Hollis, D.M. and Zander, M.E.(2001),"Herbals, cancer prevention and health," Journal of Nutrition, 131, pp.3034-3036.
[16] Popov, A.M., Atopkina, L.N., Uvarova, N.I. and Elyakov, G.B.(2001),"The antimetastatic and immunomodulating activities of ginseng minor glycosides," Doklady Biochemistry and Biophysics, 380, pp.309-312.
[17] Ruffa, M.J., Ferraro, G., Wagner, M.L., Calcagno, M.L., Campos, R.H. and Cavallaro, L.(2001),"Cytotoxic effect of Argentine medicinal plant extracts on human hepatocellular carcinoma cell line," Journal of Ethnopharmacology, 79, pp. 335-339.
[18] Ali, M.S., Saleem, M., Ali, Z. and Ahmad, V.U. (2000), "Chemistry of Zataria multiflora (Lamiaceae)," Phytochem, 55, pp.933–6.
[19] Hosseinzadeh, H., Ramezani, M. and Salmani, G. (2000), "Antinociceptive, anti-inflammatory and acute toxicity effects of Zataria multiflora Boiss. extracts in mice and rats," J Ethnopharmacol, 73, pp. 379–85.
[20] Jafarian-Dehkordi, A., Emami, S.A., Saeidi, M. and Sadeghi, H. (2004), "Cytotoxicologic studies of the extracts of iranian Juniperus sabina and Platycladus orientalis on cancer cells," J Res Med Sci, 5, pp.205–9.
[21] Saeedi-Saravi, S.S. and Shokrzadeh, M. (2008), "The study of hepatic and renal disorders in mice which were administered ethyl acetate extract of plant Sambucus ebulus Intraperituenally (IP) and effect of vitamins C and E on prevention of its disorders," Toxicol Lett,180, pp.57–58.
[22] Shaffiee, A. and Javidnia, K. (1997), "Composition of essential oil of Zataria multiflora," Planta Med, 63, pp. 371–2.
[23] Saei-Dehkordi, S.S., Tajik, H., Moradi, M. and Khalighi-Sigaroodi, F. (2010),"Chemical composition of essential oils in Zataria multiflora Boiss. From different parts of Iran and their radical scavenging and antimicrobial activity," Food Chemistry and Toxicology 48, pp.1562-1567.
[24] Aeschbach, R., Loliger, J., Scott, B.C., Murcia, A., Butler, J., Halliwell, B. and Aruoma, O.I.(1994),"Antioxidant actions of thymol, carvacrol, 6-gingerol, zingerone and hydroxytyrosol," Food and Chemical Toxicology, 32, pp. 31–36.

[25] Mastelic, J., Jerkovic, I., Blazevic, I., Poljak-Blazi, M., Borovic, S., Ivancic-Bace, I., Smrecki, V., Zarkovic, N., Brcic-Kostic, K., Vikic-Topic, D. and Muller, N.(2008), "Comparative study on the antioxidant and biological activities of carvacrol, thymol, and eugenol derivatives," Journal of Agricultural and Food Chemistry, 56, pp. 3989–3996.

[26] Kallistratos, G., Evangelou, A., Agnantis, N., Fasske, E., Karkabounas, S. and Donos, A.(1994), "Enhancement of the antineoplastic effect of anticarcinogens on benzo[a]pyrene-treated Wistar rats, in relation to their number and biological activity," Cancer Letters, 82, pp. 153–165.

[27] Evangelou, A., Kalpousos, G., Karkabounas, S., Liasko, R., Nonni, A., Stefanou, D. and Kallistratos, G.(1997), "Dose-related preventive and therapeutic effects of antioxidants - anticarcinogens on experimentally induced malignant tumors in Wistar rats," Cancer Letters, 115, pp.105–111.

[28] Liasko, R., Kabanos, T., Karkabounas, S., Malamas, M., Tasiopoulos, A., Stefanou, D., Collery, P. and Evangelou, A.(1998),"Beneficial effects of a Vanadium complex with cysteine administrated at low doses of benzo[a]pyrene - induced leiomyosarcomas in Wistar rats," Anticancer Research, 18, pp. 3609–3613.

[29] Karkabounas, S., Binolis, J., Zelovitis, J., Kotsis, N., Charalabopoulos, A., Avdikos, A., Zouridakis, A., Liasko, R., Giannakopoulos, X. and Charalabopoulos, K.(2002),"Inhibition and modification of benzo[a]pyrene-induced chemical carcinogenesis by ascorbic acid alone or in combination with a-tocopherol in Wistar rats," Experimental Oncology, 24, pp. 274–278.

[30] He, L., Mo, H., Hadisusilo, S., Qureshi, A.A. and Elson, C.E. (1997), "Isoprenoids suppress the growth of murine B16 melanomas in vitro and in vivo," J Nutr 127, pp. 668–674.

[31] Horvathova, E., Turcaniova, V. and Slamenova, D.(2007),"Comparative study of DNA damaging and DNA-protective effects of selected components of essential plant oils in human leukemic cells K562," Neoplasma, 54, pp.478–483.

[32] Karkabounas, S., Kostoula, O.K., Daskalou, T., Veltsistas, P., Karamouzis, M., Zelovitis, I., Metsios, A., Lekkas, P., Evangelou, A.M., Kotsis, N. and Skoufos, I.(2006)," Anticarcinogenic and antiplatelet effects of carvacrol," Experimental Oncology, 28, pp.121–125.

[33] Koparal, A.T. and Zeytinoglu, M.(2003), Effects of carvacrol on a human non-small cell lung cancer (NSCLC) cell line A549," Cytotechnology, 43, pp. 149–154.

[34] Lampronti, I., Saab, A.M. and Gambari, R.(2006), "Antiproliferative activity of essential oils derived from plants belonging to the Magnoliophyta division," International Journal of Oncology, 29, pp. 989–995.

[35] Arunasree, K.M.(2010), "Anti-proliferative effects of carvacrol on a human metastatic breast cancer cell line, MDA-MB 231," Phytomedicine, 17, pp. 581–588.

[36] Phelan, M.C. (1998), "Basic Techniques for Mammalian to Cell Tissue Culture," Current protocols in cell biology; 7, pp. 1-10.

[37] Green, J.E. (2003), "Mouse models of human breast cancer: evolution or convolution," Breast Cancer Res. 5(1), pp.1.

[38] Arya, V., Kashyap, C.P.,Bandana, T.,Shiksha, S.,Sweta, K.,Verma,P. and Sharma, S. (2011),"Human cancer cell lines A brief communication, J. Chem. Pharm. Res. 3(6), pp.514-520

[39] Spavieri, J., Allmendinger, A., Kaiser, M., Casey, R.,Hingley-Wilson S.and Lalvani, A. (2010),"Antimycobacterial, antiprotozoan and cytotoxic potential of twenty-one brown algae (Phaeophyceae) from British and Irish waters," Phytother. Res. 24 : 1724-29.

[40] Alizadeh, A. and Shaabani, M. (2014), "Essential oil composition, total phenolic content and antioxidant activities of Iranian Zataria multiflora Boiss," International Journal of Biosciences. 4 (4), pp. 97-104.

[41] Ebrahimzadeh, H., Yamini, Y., Sefidkon, F., Chaloosi, M. and Pourmortazavi, S.M. (2003), "Chemical composition of the essential oil and supercritical CO2 extracts of Zataria multiflora Boiss," Food Chem., 83, pp. 357-361.

[42] Sharififar, F., Moshafi, M.H., Mansouri, S.H., Khodashenas, M. and Khoshnoodi, M. (2007), "In vitro evaluation of antibacterial and antioxidant activities of the essential oil and methanol extract of endemic Zataria multiflora Boiss," Food Control, 18, pp. 800-805.

[43] Alipour-eskandani, M. (2011), "Effect of Zataria multifloraBoiss essential oil on the growth of bacillus cereus in a commercial chicken soup," Book of proceedings 6, pp. 389-392.

[44] Stammati, A., Bonsi, P., Zucco, F., Moezelaar, R., Alakomi, H.L. and Von-Wright, A. (1999), "Toxicity of selected plant volatiles in microbial and mammalian short-term assays," Food Chem Toxicol 37, pp. 813–823.

[45] Zeytinoglu, M., Aydin, S., Ztrk, Y., Hsn, K. and Baser, C. (1998), "Inhibitory effects of carvacrol on DMBA induced pulmonary tumorigenesis in rats," Acta Pharmaceutica Turcica 40, pp. 93–98.

[46] Zeytinoglu, H., Incesu, Z. and Baser, K. (2003), "Inhibition of DNA synthesis by Carvacrol in mouse myoblast cells bearing a human N-RAS oncogene," Phytomedicine 10, pp. 292–299.

[47] Sylvester, P.W. (2011), "Optimization of the tetrazolium dye (MTT) colorimetric assay for cellular growth and viability," Methods Mol Biol, 716, pp.157-68.

[48] Maioli, E., Torricelli, C., Fortino, V., Carlucci, F., Tommassini, V. and Pacini, A. (2009), "Critical appraisal of the MTT assay in the presence of rottlerin and uncouplers," Biol Proced Online, 3(11), pp. 227-40.

[49] Zamanian-Azodi, M., Rezaie-Tavirani, M., Heydari- Kashal, S., kalantari, S., Dalilan, S. and Zali, H. (2012), "Proteomics analysis of MKN45 cell line before and after treatment with Lavender aqueous extract," Gastroenterol Hepatol Bed Bench, 5, pp.35-42.