Cytotoxicity of *Thymus vulgaris* Essential Oil Towards Human Oral Cavity Squamous Cell Carcinoma

SERKAN SERTEL[1,2,3], TOLGA EICHHORN[2,3], PETER K. PLINKERT[1] and THOMAS EFFERTH[2,3]

[1]*Department of Otorhinolaryngology, Head and Neck Surgery, University of Heidelberg, Heidelberg, Germany;*
[2]*Pharmaceutical Biology (C015), German Cancer Research Center, Heidelberg, Germany;*
[3]*Department of Pharmaceutical Biology, Institute of Pharmacy and Biochemistry,*
University of Mainz, Mainz, Germany

Abstract. *Background: Oral cavity squamous cell carcinoma (OCSCC) accounts for 2% to 3% of all malignancies and has a high mortality rate. The majority of anticancer drugs are of natural origin. However, it is unknown whether the medicinal plant Thymus vulgaris L. (thyme) is cytotoxic towards head and neck squamous cell carcinoma (HNSCC). Materials and Methods: Cytotoxicity of thyme essential oil was investigated on the HNSCC cell line, UMSCC1. The IC$_{50}$ of thyme essential oil extract was 369 µg/ml. Moreover, we performed pharmacogenomics analyses. Results: Genes involved in the cell cycle, cell death and cancer were involved in the cytotoxic activity of thyme essential oil at the transcriptional level. The three most significantly regulated pathways by thyme essential oil were interferon signaling, N-glycan biosynthesis and extracellular signal-regulated kinase 5 (ERK5) signaling. Conclusion: Thyme essential oil inhibits human HNSCC cell growth. Based on pharmacogenomic approaches, novel insights into the molecular mode of anticancer activity of thyme are presented.*

Head and neck cancer is one of the ten most frequent cancers in men worldwide, comprising 500,000 newly diagnosed cases each year. The American Cancer Society estimated more than 35,000 new cases of cancer per year involving the oral cavity causing more than 7,000 deaths in the United States in 2009 (1). In Europe, the mortality rates for oral cavity squamous cell carcinoma (OCSCC) range from 29 to 40 per 100,000

Correspondence to: Professor Dr. Thomas Efferth, Department of Pharmaceutical Biology, Institute of Pharmacy and Biochemistry, University of Mainz, Staudingerweg 5, 55128 Mainz, Germany. Tel: +49 61313925751, Fax: +49 61313923752, e-mail: efferth@uni-mainz.de

inhabitants per year (2). Treatment of head and neck carcinomas comprises surgery commonly followed by concurrent chemo- and radiation therapy for advanced tumors. However, despite the improvements which have been achieved in concurrent therapies, the overall 5-year survival rate for OCSCC remains at 50% and has not significantly improved in the past 30 years (3). Novel tumor-specific therapies are required to be less toxic while maintaining a high degree of efficacy. As the majority of anticancer drugs are of natural origin, natural products represent a valuable source for the identification and development of novel treatment options for cancer (4). Over the past few decades, research has focused on the health effects of phytochemicals and plant-derived extracts.

Thymus vulgaris L. (thyme) belongs the mint family, *Lamiaceae*. 'Thymiama' is the ancient Greek expression for incense that was offered for gods in temples because of its intensive scent. This effect derives from its essential oil. Thyme essential oil and its principle compound thymol have antimicrobial, antifungal (5, 6), antioxidant (7) and anticancer activities (8, 9). In general medicine, thyme is commonly used as an expectorant in upper respiratory tract infections, *e.g.* bronchitis and pertussis (10). In odontology, thymol is used as the main active antiseptic ingredient in chemotherapeutic mouthrinses against gingivitis (11). The objective of the present study was to investigate the cytotoxicity of *T. vulgaris* essential oil towards human UMSCC1 head and neck squamous cell carcinoma (HNSCC) cells. The chemical characterization of the investigated essential oil was performed by gas chromatography. To gain insight into the molecular mode of action of *T. vulgaris* essential oil towards cancer cells, microarray-based mRNA expression profiling was applied. Differentially expressed genes were subjected to signaling pathway analysis.

Materials and Methods

Plant material. The *T. vulgaris* was cultivated in Ross-on-Wye, Herefordshire, UK. The thyme oil was obtained from a mixture of blossoms, leaves and stipes by steam distillation (T=100˚C). The

procedure was performed by Norfolk Essential Oils (Norfolk, UK). The *T. vulgaris* essential oil used in this study was provided by Rehau AG and Co (Rehau, Germany).

Cell culture. The UMSCC1 cell line was originally derived from a male patient with a T2 N0 M0 squamous cell carcinoma of the oral cavity (12). The UMSCC1 cells were cultured in McCoy's medium containing 10% fetal bovine serum (FBS) supplemented with 1% antibiotic-antimycotic (100×), liquid containing 10,000 units of penicillin (base), 10,000 μg of streptomycin (base) and 25 μg of amphotericin B/mL utilizing penicillin G (sodium salt), streptomycin sulfate and amphotericin B as Fungizone® antimycotic (in 0.85% saline) purchased from Invitrogen GmbH (Karlsruhe, Germany). The cells were maintained as monolayers in a plastic culture flask at 37°C in a humidified atmosphere containing 5% CO_2.

Gas chromatography. The oil extract was analyzed by gas chromatography using an Agilent Technologies 6890N GC instrument and a HP-5 capillary column (0.25 mm × 60 m; 0.25 μm film thickness; Santa Clara, CA, USA). The procedure was carried out by Phytolab GmbH (Vestenbergsgreuth, Germany). The oil was finally diluted with hexane (1:10).

XTT cytotoxicity assay. The cell proliferation was assessed using a standard 2,3-*bis* [2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt (XTT) assay kit (Roche, Indianapolis, IN, USA), which measures the metabolic activity of viable cells (13, 14). The cytotoxicity of *T. vulgaris* essential oil compounds was determined by means of a Cell Proliferation Kit II (Roche Diagnostics, Mannheim, Germany). This test is based on the cleavage of the yellow XTT salt by ubiquitous dehydrogenases leading to the formation of an orange formazan dye. The intensity of the dye is commensurate to the number of metabolically active cells. Fresh stock solutions of thyme essential oil was prepared in DMSO in a dilution series ranging from 0.54 μg/ml to 18 mg/ml in McCoy's medium to perform the XTT test. The cells were suspended to a final density of 1×10^5 cells/ml. One hundred microliters of the cell suspension were placed into the wells of a 96-well culture plate (Costar, Corning, NY, USA). The marginal wells were filled with 100 μl of McCoy's medium in order to minimize evaporation effects, besides, wells filled with medium were required to determine the background absorbance caused by non-metabolized XTT. A row of wells containing cells served as solvent controls. Each concentration was tested in at least two independent plates containing different batches of cells.

After incubation of 72 hours, the XTT reagent was freshly prepared and added to each well as specified by the manufacturer: XTT-labeling reagent and electron-coupling reagent was mixed in a ratio of 50:1 and 50 μl of this mixture were added to each well of the 96-well plate. The plates were incubated for 3 hours at 37°C, 5% CO_2 in a humidified atmosphere and read out after incubation. Quantification of cell cytotoxicity was performed in an ELISA plate reader (Bio-Rad, München, Germany) at 490 nm with a reference wavelength of 655 nm. The cytotoxic effect of the treatment was expressed as the percentage of viability compared to untreated cells (13). The toxicity of the compounds was determined by means of the formula:

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of sample cells}}{\text{Absorbance of untreated cells}} \times 100$$

Table I. *Chemical composition of Thymus vulgaris L. essential oil as analyzed by gas chromatography.*

Component	Proportion (%)
Thymol	33.0
ϱ-Cymol	23.8
γ-Terpinene	15.4
1-Terpinene-4-ol	6.0
Linalool	2.6
β-Myrcene	2.0

The simple ligand-binding module of SigmaPlot® software (version 10.0, Systat Software Inc., San José, California, CA, USA) was used for analysis.

RNA isolation. The total RNA of the UMSCC1 cells was extracted from the test samples using an RNeasy® Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions to obtain highly pure RNA. Isolated total RNA was resuspended in the sample buffer provided by the manufacturer. The concentration and quality of the total RNA was verified by electrophoresis using the total RNA Nanochip assay on an Agilent 2100 Bioanalyzer (Agilent Technologies GmbH, Berlin, Germany). Only the samples with an RNA index values greater than 8.5 were selected for expression profiling. The RNA concentrations were determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). All RNA samples were stored at –80°C until microarray analysis.

Probe labeling and Illumina Sentrix BeadChip array hybridization. Biotin-labeled cRNA samples for hybridization with Illumina Mouse Sentrix-8 BeadChip arrays (Illumina Inc., San Diego, CA, USA) were prepared according to Illumina's sample labeling procedure based on a previously published protocol (13). In brief, 250 ng total RNA were used for complementary DNA (cDNA) synthesis, followed by an amplification/labeling step (*in vitro* transcription) to gain biotin-labeled cRNA using the MessageAmpII aRNA Amplification kit (Ambion, Inc., Austin, TX, USA). Biotin-16-UTP was purchased from Roche Applied Science, Penzberg, Germany. The cRNA was purified in a column using the TotalPrep RNA Amplification Kit, and eluted in 60 μl of water. The quality of the cRNA was verified with the RNA NanoChip Assay and quantified on an Agilent 2100 Bioanalyzer and a spectrophotometer (NanoDrop).

Hybridization was performed at 58°C, in GEX-HCB buffer (Illumina Inc.) at a concentration of 50 ng cRNA/μl, in an unsealed wet chamber for 20 h. Spike-in controls for low, medium and highly abundant RNAs were added along with mismatch control and biotinylation control oligonucleotides. The microarrays were washed twice in E1BC buffer (Illumina Inc.) at room temperature for 5 min. After blocking for 5 min in 4 ml of 1% (w/v) blocker casein in phosphate buffered saline Hammarsten grade (Pierce Biotechnology Inc., Rockford, IL, USA), array signals were developed by 10 min incubation in 2 mL of 1 μg/ml Cy3-streptavidin (Amersham Biosciences, Buckinghamshire, UK) solution and 1% blocking solution. After a final wash in E1BC, the arrays were dried and scanned.

Table II. *Genes most down- or up-regulated in UMSCC1 cells after treatment with thyme essential oil.*

Probe ID	Symbol	Description	Fold change (Log$_2$ ratio)
Down-regulated genes			
380575	RPL23	Ribosomal protein L23	−0.99
4260368	UBE2C	Ubiquitin-conjugating enzyme E2C	−0.98
1400240	LDHB	Lactate dehydrogenase B	−0.88
5910154	NOL5A	Nucleolar protein 5A (56kDa with KKE/D repeat)	−0.805
2260066	CLIP1	CAP-GLY domain containing linker protein 1	−0.795
3290689	C14orf166	Chromosome 14 open reading frame 166	−0.79
1500010	CDC20	Cell division cycle 20 homolog (S, cerevisiae)	−0.79
Up-regulated genes			
5310044	NBPF20	Neuroblastoma breakpoint family, member 20	1.14
1510681	NBPF10	Neuroblastoma breakpoint family, member 10	0.89
6650121	C1orf71	Chromosome 1 open reading frame 71	0.815
7320561	OAS2	2-5-Oligoadenylate synthetase 2, 69/71kDa	0.775
2690224	ISG20L2	Interferon stimulated exonuclease gene 20kDa-like 2	0.7
2000148	IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	0.7
3990170	IFI27	Interferon, alpha-inducible protein 27	0.68

Scanning and data analysis. Microarray scanning was conducted using a bead station array scanner, adjusted to a scaling factor of 1 and photomultiplier set to 430. Data extraction was carried out for all the beads individually. Outliers were defined as >2.5 median absolute deviation. All the remaining data points were used for the calculation of the mean average signal for a given probe, and the standard deviation for each probe was calculated. The RNA from the UMSCC1 cell line was subjected to microarray analysis at least twice. The normalized data obtained from the duplicated hybridizations were averaged to obtain a final data set. Reproducibility of the data was assessed by calculating a percent error (standard deviation/mean ×100) for each gene element.

The data were cropped to a final set of 804 elements by eliminating genes with difference in expression exceeding standard deviation. Next, statistical significance was verified by means of empirical Bayes *t*-test and the false discovery rate was corrected with the Benjamini–Hochberg method. Ultimately, genes with *p*>0.05 were discarded after the allocation of *p*-values.

Data analysis was conducted with Chipster analysis software (http://chipster.csc.fi) for DNA microarray expression data by normalization of the signals using the cubic spline algorithm after background subtraction, and differentially regulated genes were defined by calculating the standard deviation differences of a given probe in a one-by-one comparison of samples or groups. For all the genes scored the fold change and *p*-values were determined.

Signaling pathway analysis. The data obtained from the Chipster were analyzed using pathway analysis using the Ingenuity Pathways Analysis software (version 6.5) by Ingenuity Systems (Redwood City, CA, USA). This software utilizes the raw image obtained from Chipster in order to align the image and determine expression values for all of the elements.

Statistical analysis. Statistical calculations were carried out with the SPSS 10.0 for Windows software package (SPSS Inc., Chicago, IL, USA). The results are expressed as the mean±S.E.M. of five independent experiments. Student's *t*-test was used for the statistical analyses; *p*-values <0.05 were considered statistically significant.

Results

Essential oil composition. The chemical composition of the oil is shown in Table I. *T. vulgaris* essential oil was mainly characterized by six compounds (82.8% of the total oil). The major fraction included monoterpenes, of which thymol was the most abundant (33 %).

Cytotoxicity. The cytotoxicity of *T. vulgaris* essential oil towards the human cancer cell line UMSCC1 as determined by the XTT assay is shown in Figure 1. The dose–response curve showed a steady rise in viability to 127.4% compared to the untreated control at 54 μg/ml and a subsequent rapid decrease in viability to 1.3% of the control at 540 μg/ml. The IC$_{50}$ value calculated from this dose–response curve was IC$_{50}$=369.55 μg/ml.

Differential gene expression. As determined by microarray hybridization, 804 genes were differentially expressed after treatment with the IC$_{50}$ concentration of thyme oil (369.55 μg/ml) for 72 h. The log$_2$-transformed changes of gene expression varied in a range of 1.14 to 0.68 among the up-
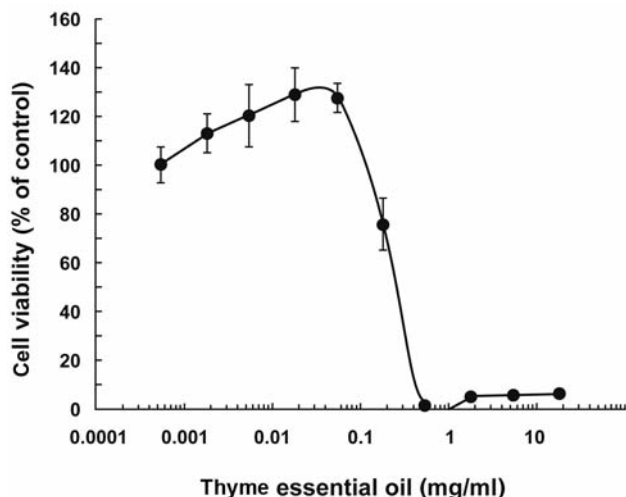
Figure 1. *Effect of Thymus vulgaris essential oil on viability of UMSCC1 cells assessed with the XTT assay after 72 h of treatment.*

Figure 2. *A: Functional groups of genes and B: signaling pathways regulated upon Thymus vulgaris essential oil treatment in UMSCC1 cells. The evaluation of differentially expressed genes was performed using the Ingenuity Pathway Analysis software.*

regulated genes and –0.79 to –0.99 in the down-regulated genes. The seven highest positively up-regulated genes and seven lowest negatively down-regulated genes are shown in Table II.

Signaling pathway profiling. Out of 67 functional groups of genes provided by the software, 54 were regulated upon thyme essential oil treatment at a significance level of $p<0.05$. The three functional groups of genes with the lowest p-values including cell cycle, cell death and cancer are shown in Figure 2A. Among the 216 signaling pathways analyzed by the pathway analysis software, the three pathways most significantly regulated by thyme essential oil, namely interferon signaling, *N*-glycan biosynthesis and ERK5 signaling ($p<0.05$; Figure 2B) were selected. The genes associated with interferon signaling, *N*-glycan biosynthesis or ERK5 signaling differentially regulated upon thyme treatment are shown in Table III.

Discussion

The aromatic compounds and oily components of thyme have shown cytotoxic activity against breast cancer (8) and ovarian adenocarcinoma IGR-OV1 cells and their counterparts resistant to chemotherapy (9). Thyme essential oil cytotoxicity might be due to its lipophilic compounds that accumulate in cancer cell membranes and increase their permeability, resulting in leakage of enzymes and metabolites (15). Interestingly, aside from its anticancer property, thyme extract has also shown immune-stimulatory effects involving leukoc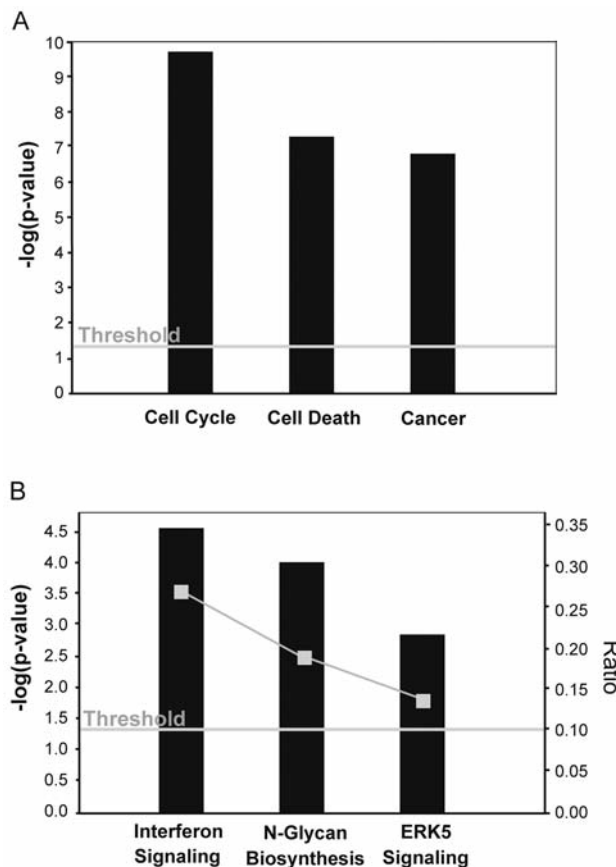yto- and thrombocytopoesis (16). This pharmacological property of thyme might be clinically beneficial as an adjuvant therapy during chemotherapy in cancer or immunocompromised patients to overcome leukopenia.

Thymol and ϱ-cymol were the most abundant constituents of the present thyme essential oil. Thymol has shown cytotoxic activity against different human tumor models (9, 17, 18) while, at the same time showing antioxidant activity with DNA-protective effects on cells (19). A Medline search revealed no data regarding cytotoxic activity of ϱ-cymol. Thus, we propose that the cytotoxic activity of *T. vulgaris* essential oil towards UMSCC1 cells was mainly due to thymol. However, the cytotoxic effect of other components present at lower percentages in the essential oil must be considered.

A surprising and unexpected, but repeatedly observed, effect was that subtoxic concentrations of the thyme essential oil stimulated proliferation and viability. At higher concentrations, dose-dependent cytotoxic effects were found.

Table III. *Signaling pathways with corresponding genes affected by treatment with Thymus vulgaris essential oil in UMSCC1 cells (according to http://www.genecards.org).*

Symbol	Description
Interferon signaling	
IFIT3	Interferon-induced protein with tetratricopeptide repeats 3
IFIT1	Interferon-induced protein with tetratricopeptide repeats 1
OAS1	2',5'-Oligoadenylate synthetase 1, 40/46kDa
JAK1	Janus kinase 1
IFITM1	Interferon induced transmembrane protein 1 (9-27)
MX1	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)
IRF9	Interferon regulatory factor 9
STAT1	Signal transducer and activator of transcription 1, 91 kDa
N-Glycan biosynthesis	
FUT8	Fucosyltransferase 8 (alpha (1,6) fucosyltransferase)
EDEM2	ER degradation enhancer, mannosidase alpha-like 2
WDFY3	WD repeat and FYVE domain containing 3
B4GALT1	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1
RPN2	Ribophorin II
ALG5	Asparagine-linked glycosylation 5, dolichyl-phosphate beta-glucosyltransferase homolog *(S. cerevisiae)*
DPM1	Dolichyl-phosphate mannosyltransferase polypeptide 1, catalytic subunit
MGAT4B	Mannosyl (alpha-1,3-)-glycoprotein beta-1,4-*N*-acetylglucosaminyltransferase, isozyme B
DDOST	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase
MGAT2	Mannosyl (alpha-1,6)-glycoprotein beta-1,2-*N*-acetylglucosaminyltransferase
ERK5 signaling	
YWHAG	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide
YWHAE	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide
CREB1	cAMP responsive element binding protein 1
CREB3	cAMP responsive element binding protein 3
RPS6KB2	Ribosomal protein S6 kinase, 70 kDa, polypeptide 2
MEF2A	Myocyte enhancer factor 2A
ATF4	Activating transcription factor 4 (tax-responsive enhancer element B67)
HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
MAP3K8	Mitogen-activated protein kinase kinase kinase 8

Comparable effects have previously been reported for standard anticancer agents such as doxorubicin (20, 21). A proliferation-stimulating effect of otherwise cytotoxic compounds can be interpreted as a rescue mechanism. At low subtoxic concentrations, cancer cells escape detrimental stimuli by the induction of proliferation, while at higher concentrations this defense mechanism is overridden by the cytotoxic effects. The possibility that cytotoxic compounds such as doxorubicin or thyme essential oil may exert tumor-promoting activity at low concentrations, should also be considered.

Up to now, there are no data concerning the molecular mode of action of *T. vulgaris* towards cancer cells. Here, for the first time microarray-based mRNA expression profiling and signaling pathway analyses are presented.

Among the down-regulated genes, *UBE2C* encodes a member of the E2 ubiquitin-conjugating enzyme family, which is required for the destruction of mitotic cyclins and for cell cycle progression (22). Consequently, down-regulation of *UBE2C* might explain one aspect of the cytotoxic activity of thyme essential oil. Another interesting down-regulated gene was *CDC20*. It acts as a regulatory protein in the cell cycle and is required for nuclear movement prior to anaphase and chromosome separation (22). Here again, thyme essential oil showed influence on cell cycle progression by down-regulating a critical mitosis regulatory protein.

Interestingly, the up-regulated gene *OAS2* contributes to this process of cell growth control. It encodes a member of the 2-5A synthetase family. These are not only essential

proteins in mediating resistance to virus infection but also in controlling cell growth, differentiation and apoptosis (22). Within our signaling pathway analyses, interferon signaling, *N*-glycan biosynthesis and extracellular-signal-regulated kinase 5 (ERK5) signaling were the three most significantly regulated pathways by *T. vulgaris* essential oil treatment. Interferons are a family of cytokines with potent antiproliferative, antiviral and immunomodulatory properties (23). The influence of thyme in interferon signaling and inhibition of tumorigenesis should be considered.

N-Glycans (oligosaccharides) play crucial roles in glycoproteins functions, *e.g.* epidermal growth factor and transforming growth factor-β receptors (24, 25). Thyme essential oil might be able to induce the growth arrest of cancer cells through targeting *N*-glycan biosynthesis.

ERK5, belonging to the mitogen-activated protein kinase (MAPK) family, is expressed in a variety of tissues and is activated by a range of growth factors, cytokines and cellular stresses. ERK5 signaling is important in endothelial cells for preventing apoptosis, regulating tumor angiogenesis and cell migration (26). The influence of *T. vulgaris* in ERK5 signaling might offer a novel target for anticancer therapy as an anti-angiogenic agent.

Despite advances in surgical techniques and adjuvant chemoradiotherapy, the prognosis for patients with OCSCC remains poor. An aggravating factor is that the toxicity of various chemoradiation protocols results in poor patient outcomes. This dilemma requires the identification of novel antitumor agents with less toxicity while maintaining a high degree of efficacy. The ability of phytochemicals such on *T. vulgaris* essential oil to inhibit human HNSCC cell growth is a small step in an auspicious new direction.

In conclusion, *T. vulgaris* essential oil has the ability to inhibit the cell growth of UMSCC1 tumor cells at high concentrations, whereas it promotes proliferation at subtoxic doses. Genes involved in interferon signaling, N-glycan biosynthesis and ERK5 signaling were identified to be associated with the cytotoxicity of *T. vulgaris* essential oil.

Conflict of Interest

The Authors have no conflict of interest to declare.

Acknowledgements

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