Abstract. Background: Oral squamous cell carcinoma (OSCC) is a challenging disease with a high mortality rate. Natural products represent a valuable source for the development of novel anticancer drugs. We investigated the cytotoxic potential of essential oil from the leaves of a medicinal plant, Levisticum officinale (lovage) on head and neck squamous carcinoma cells (HNSCC). Materials and Methods: Cytotoxicity of lovage essential oil was investigated on the HNSCC cell line, UMSCC1. Additionally, we performed pharmacogenomics analyses. Results: Lovage essential oil extract had an IC\textsubscript{50} value of 292.6 μg/ml. Genes involved in apoptosis, cancer, cellular growth and cell cycle regulation were the most prominently affected in microarray analyses. The three pathways to be most significantly regulated were extracellular signal-regulated kinase 5 (ERK5) signaling, integrin-linked kinase (ILK) signaling, virus entry via endocytic pathways and p53 signaling. Conclusion: Levisticum officinale essential oil inhibits human HNSCC cell growth.

Oral squamous cell carcinoma (OSCC) is among the top ten most commonly occurring carcinomas worldwide with a high mortality rate. It was estimated that 35,720 people (25,240 men and 10,480 women) were diagnosed with cancer of the oral cavity and pharynx in the United States of America in 2009 and that 7,600 would die of it (1). In spite of advances in therapy, the 5-year survival rate for oral cancer patients has remained at 50% over the past five decades (2). Various multimodal therapy strategies including surgery, radiation and chemotherapy determine the standard treatments for patients with OSCC. However, the treatment of advanced-stage OSCC is associated with morbidity and poor patient outcomes (3). Therefore, alternative therapeutic strategies are called for.

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). During the past few decades, research has focused on the health effects of phytochemicals and plant-derived extracts. Plants of the genus Levisticum have been attributed with anticancer activity (5, 6). Levisticum officinale W. D. J. Koch (lovage) belongs to the family of Apiaceae. The name lovage originates from the Latin word ligusticus (meaning from Liguria, as the herb used to grow in the Liguria region of northwest Italy).

Apiaceae is a large plant family with about 3,000 species. This family includes many species with medicinal properties which are frequently used in traditional medicine. A common characteristic of this family is the presence of bioactive secondary metabolites in all plant parts: essential oils, polyphenols (flavonoids, phenolic acids), coumarins (furano- and pyranocoumarins), saponins, alkaloids and polyacetylenes (7).

L. officinale has been used as a medicinal plant for centuries due to its carminative, spasmylytic and diuretic effect (8, 9). Clinically it is a potent diuretic (9). It is approved by the German Commission E for use in lower urinary tract infections and urinary gravel (10). The antimycobacterial activity of L. officinale is rooted in its polyacetylenes (11).
There are no published data concerning the clinical application of *L. officinale* in otorhinolaryngology or experimental data of cytotoxic activity in head and neck squamous cell carcinoma (HNSCC). For this reason, the objective of the present study was to investigate the cytotoxicity of *L. officinale* leaf essential oil towards the human HNSCC cell line, UMSCC1. To gain insight into the molecular mode of action of *Levisticum officinale* leaf essential oil towards the cancer cells, microarray-based mRNA expression profiling was applied. Differentially expressed genes were subjected to signaling pathway analysis.

**Materials and Methods**

**Plant material.** *L. officinale* was cultivated in Ross-on-Wye, Herefordshire, UK. Lovage leaf oil was obtained from the leaves by steam distillation (T=100˚C). The procedure was carried out by Norfolk Essential Oils (Norfolk, UK).

The *L. officinale* leaf essential oil used in this study was provided by REHAU AG + Co. (Rehau, Germany).

**Cell culture.** UMSCC1 cells were 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 (100x), 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% antibiotic-antimycotic (100×), liquid (containing 10,000 units of penicillin G, 100 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 culture flasks at 37˚C in a humidified atmosphere containing 5% CO2.

**Gas chromatography.** The oil extract was analyzed by gas chromatography using an Agilent Technologies 6890N GC instrument and a HP-5 capillary column (0.32 mm x 30 m; 0.25 μm film thickness; Santa Clara, CA, USA). Gas chromatography was performed by Dr. Otto GmbH (Wittenberge, Germany). The oil was diluted with hexane (1:10).

**XTT cytotoxicity assay.** Cytotoxicity was assessed using the standard 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carbo-xanilide inner salt (XTT) assay kit (Roche, Indianapolis, IN, USA), which measures the metabolic activity of viable cells (13). The toxicity of compounds was compared to untreated cells (13). The cytotoxic effect of the treatment was determined as percentage of viability and compared to untreated cells (13). The toxicity of compounds was determined by means of the formula:

\[
\text{Cell viability} \% = \frac{\text{Absorbance of sample cells}}{\text{Absorbance of untreated cells}} \times 100
\]

The simple ligand-binding module of Sigma plot software (version 10.0; Systat, San Jose, CA, USA) was used for the analysis.

**RNA isolation.** The total RNA of the UMSCC1 cells was extracted from the test samples using the RNaseasy® Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer’s instructions to obtain highly pure RNA. The isolated total RNA was re-suspended in sample buffer provided by the manufacturer. The concentration and quality of the total RNA were verified by spectrophoresis using the total RNA Nanochip assay on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only the samples with RNA index values greater than 8.5 were selected for expression profiling. RNA concentrations were determined using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). All of the RNA samples were stored at −80˚C until used for microarray analyses.

**Probe labeling and Illumina Sentrix BeadChip array hybridization.** Biotin-labeled cRNA samples for hybridization on Illumina Mouse Sentrix-8 BeadChip arrays (Illumina Inc., San Diego, CA, USA) were prepared according to Illumina’s recommended 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 synthesize biotin-labeled cRNA according to the MessageAmpII aRNA Amplification kit (Ambion, Inc., Austin, TX, USA). Biotin-16-UTP was purchased from Roche Applied Science, Penzberg, Germany. The cRNA was column purified according to the TotalPrep RNA Amplification Kit, and eluted in 60 μl of water. The quality of cRNA was controlled using the RNA NanoChip Assay on an Agilent 2100 Bioanalyzer and spectrophotometrically quantified (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 biotinylated 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) blocking 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.

**Scanning and data analysis.** For microarray scanning, a bead station array scanner (Illumina, Inc.) was adjusted to a scaling factor of 1 and the photomultiplier set to 430. Data extraction was carried out for all the beads individually, and outliers are removed when >2.5 MAD (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 cells was subjected to microarray analysis at least twice. The normalized data obtained from the duplicated hybridizations were averaged to obtain a final dataset. 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 678 elements by eliminating genes with a differential expression under a single 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 allocation of p-values.

**Data analysis** was carried out with the 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. 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 Chipster were analyzed using the Ingenuity Pathways Analysis software (version 6.5) from Ingenuity Systems (Redwood City, CA, USA). This software utilizes the raw image from Chipster in order to align the image and determine expression values for all of the elements.

**Statistical analysis.** For statistical evaluations, the SPSS 10.0 for Windows software package (SPSS Inc., Chicago, USA) was used. The results were expressed as means±S.E.M. (standard error of means) of five independent experiments. In the Student’s t-test, p-values <0.05 were considered to be significant.

**Results**

**Essential oil composition.** The chemical composition of the oil as assessed by gas chromatography is shown in Table I. *L. officinale* leaf essential oil was characterized by 9 constituents (73.74 % of the total oil). Monoterpenes were the major fraction, of which α-terpinyl acetate was the most abundant compound (48.15 %).

**Cytotoxicity.** The cytotoxicity of *L. officinale* leaf essential oil towards UMSCC1 cells as determined by the XTT assay is shown in Figure 1. The dose–response curve showed a steady rise in viability to 272.1% compared to the untreated controls at 0.18 mg/ml and a subsequent rapid decrease in viability to 4.7% of control at 0.54 mg/ml. The IC_{50} value calculated from this dose–response curve was IC_{50}=292.6 μg/ml.

**Differential gene expression.** As determined by microarray hybridization, a total of 678 genes were differentially expressed after treatment with the IC_{50} concentration of lovage leaf oil (292.6 μg/ml). The positive fold changes after log 2-transformation varied in a range of 1.63 to 1.27 and

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**Table I. Chemical composition of Levisticum officinale leaf essential oil as analyzed by gas chromatography.**

<table>
<thead>
<tr>
<th>Component</th>
<th>Proportion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Terpinyl acetate</td>
<td>48.15</td>
</tr>
<tr>
<td>β-Phellandrene</td>
<td>13.16</td>
</tr>
<tr>
<td>β-Myrcene</td>
<td>5.07</td>
</tr>
<tr>
<td>Ligustilide</td>
<td>3.86</td>
</tr>
<tr>
<td>α-Pinene</td>
<td>1.52</td>
</tr>
<tr>
<td>α-Phellandrene</td>
<td>0.83</td>
</tr>
<tr>
<td>Camphene</td>
<td>0.50</td>
</tr>
<tr>
<td>β-Pinene</td>
<td>0.41</td>
</tr>
<tr>
<td>Butylidenphthalide (ligusticumlabon)</td>
<td>0.24</td>
</tr>
<tr>
<td>Falcarnol</td>
<td>Not detected</td>
</tr>
</tbody>
</table>

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**Figure 1. Cytotoxicity of Levisticum officinale leaf essential oil towards UMSCC1 cells as determined by the XTT assay. Mean dose–response curve of three independent experiments are shown (error bars showing standard error of means).**
the negative fold changes varied in a range of 0.53 to 0.78. The seven highest positive (up-regulated genes) and seven lowest negative (down-regulated genes) fold changes are shown in Table II.

**Table II. Genes down- or up-regulated after treatment of UMSCC1 cells with Levisticum officinale leaf essential oil.**

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Symbol</th>
<th>Description</th>
<th>Fold change [Log2 ratio]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Down-regulated genes</td>
<td></td>
</tr>
<tr>
<td>4120039</td>
<td>FTHL2</td>
<td>Ferritin, heavy polypeptide-like 2</td>
<td>-0.89</td>
</tr>
<tr>
<td>1230192</td>
<td>CSNK1G2</td>
<td>Casein kinase 1, gamma 2</td>
<td>-0.845</td>
</tr>
<tr>
<td>1300072</td>
<td>SRP14P1</td>
<td>Signal recognition particle 14 kDa (homologous Alu RNA binding protein) pseudogene 1</td>
<td>-0.8</td>
</tr>
<tr>
<td>670181</td>
<td>LOC585390</td>
<td>RNA, small nucleolar</td>
<td>-0.715</td>
</tr>
<tr>
<td>1470358</td>
<td>C1orf43</td>
<td>Chromosome 19 open reading frame 43</td>
<td>-0.71</td>
</tr>
<tr>
<td>2370601</td>
<td>CCDC94</td>
<td>Coiled-coil domain containing 94</td>
<td>-0.705</td>
</tr>
<tr>
<td>4490010</td>
<td>DAAM1</td>
<td>Dishevelled-associated activator of morphogenesis 1</td>
<td>-0.69</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Up-regulated genes</td>
<td></td>
</tr>
<tr>
<td>4060358</td>
<td>ABCA1</td>
<td>ATP-binding cassette, sub-family A (ABC1), member 1</td>
<td>0.705</td>
</tr>
<tr>
<td>2070520</td>
<td>CDCA7</td>
<td>Cell division cycle associated 7</td>
<td>0.705</td>
</tr>
<tr>
<td>7510309</td>
<td>FRMD6</td>
<td>FERM domain containing 6</td>
<td>0.69</td>
</tr>
<tr>
<td>2970730</td>
<td>MYADM</td>
<td>Myeloid-associated differentiation marker</td>
<td>0.69</td>
</tr>
<tr>
<td>2940110</td>
<td>UHRF1</td>
<td>Ubiquitin-like with PHD and ring finger domains 1</td>
<td>0.69</td>
</tr>
<tr>
<td>5810685</td>
<td>THBS1</td>
<td>Thrombospondin 1</td>
<td>0.65</td>
</tr>
<tr>
<td>6580039</td>
<td>PKM2</td>
<td>Pyruvate kinase, muscle</td>
<td>0.64</td>
</tr>
</tbody>
</table>

**Discussion**

*L. officinale* ethanol extracts have shown apoptosis-inducing activity in leukemia cell lines (15), but no other cytotoxic activities have been reported. The principle constituents of *L. officinale* have been shown to be polyacetylenes, i.e. 3(R)-falcarinol and 3(R)-8(S)-falcarindiol (11). Polyacetylenes of the falcarinol-type have demonstrated many interesting bioactivities including anti-inflammatory (16), antimycobacterial activity (11), antiplatelet-aggregatory (7), cytotoxic (17-19) and antitumor activities (20). 3(R)-8(S)-Falcarindiol was cytotoxic against murine L1210 leukemia cells, only (21). However, in the lovage leaf essential oil, falcarinol was not detected by gas chromatography in the present study. Therefore, we assume that constituents other than falcarniol must have been responsible for the cytotoxic activity in the present investigation. Although α-terpinyl acetate was the major constituent identified, no explicit data concerning its anticancer activity could be found in the literature, but it is reasonable to propose that the cytotoxic activity of *L. officinale* leaf essential oil towards the UMSCC1 cells was mainly due to this constituent. Nevertheless, other constituents of the essential oil may additionally contribute to cytotoxicity.

A surprising and unexpected, but repeatedly observed effect was that subtoxic concentrations of lovage essential oil stimulated proliferation and viability. At higher concentrations, dose-dependent cytotoxic effects were found. Comparable effects have previously been reported for standard anticancer agents such as doxorubicin (22, 23). 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 cytotoxic effects. The possibility that cytotoxic compounds such as doxorubicin or lovage leaf essential oil may exert tumor-promoting activity at low concentrations should also be considered.

The differentially regulated genes after lovage leaf essential oil treatment might help explain the effects on cancer cell growth. *CDCA7* acts as a c-Myc (a transcription factor and major proto-oncogene) responsive gene, and behaves as a direct c-Myc target gene. Overexpression of this gene has been found to enhance the transformation of lymphoblastoid cells, and
contributes to c-Myc-mediated tumorigenesis (24). In the present case, the up-regulated gene CDCA7 might explain the increase in viable cancer cells at low concentrations of lovage leaf essential oil. 

ABCA1 was also an up-regulated gene, encoding a protein belonging to the ABC transporters. They are involved in the active transport of phospholipids, ions, peptides, steroids, polysaccharides, amino acids, bile acids, pharmaceutical drugs and other xenobiotic compounds (25). The protein encoded by ABCA1 is a lipid transporter that plays an important role in cholesterol efflux and thereby prevents toxicity associated with cholesterol overload (24). Gene silencing of ABCA1, in resistant M14 melanoma sensitized M14 cells to the apoptotic effect of curcumin. Moreover, ABCA1 silencing alone also induced apoptosis and reduced p65 expression (26). Thus, it is plausible that up-regulation of ABCA1 might also explain the increase in viable cancer cells at low concentrations of lovage leaf essential oil. Furthermore, overexpression of ABCA1 might activate mechanisms of drug resistance and this might contribute to the more aggressive growth of multiple drug-resistant carcinomas.

UHRF1 is another up-regulated gene that might assist the cytotoxicity induced by lovage leaf essential oil treatment. UHRF1 encodes an ubiquitin ligase that plays a major role in the G1/S transition and functions in the p53-dependent DNA damage checkpoint, expression of this gene is induced in response to mitogenic stimulation (24, 27).

THBS1 was also an up-regulated gene. THBS1 encodes adhesive glycoproteins that mediate cell-to-cell and cell-to-matrix interactions. It is a potent inhibitor of tumor growth and angiogenesis (24). These data indicated that the effect of lovage leaf essential oil treatment on the UMSCC1 cells was regulated by multiple genes and that a complex regulatory network contributed to the biological activity. The down-regulated genes did not reveal any association with anticancer activity.

As yet, no data on possible mechanisms of lovage anticancer activity have been reported. Within the present signaling pathway analyses, ERK5 signaling, ILK signaling, virus entry via endocytic pathways and p53 signaling were the four most significantly regulated pathways by L. officinale essential oil treatment. ERK5, belonging to the 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 (28). The influence of L. officinale in ERK5 signaling might offer a novel target for anticancer therapy as an anti-angiogenic agent.

The oncogenic protein kinase, ILK functions as a tumor suppressor protein in vitro and in vivo in rhabdomyosarcoma (29). It is therefore conceivable that involvement of L. officinale might suppress tumorigenesis. No evidence of a link to cancer was found for the virus entry via endocytic pathway.

Interestingly, the L. officinale essential oil treatment significantly regulated one of the most important tumor suppressor pathways, namely p53 signaling in the UMSCC1 cells. The tumor suppressor protein p53 is activated, e.g. upon DNA damage, nucleotide depletion or hypoxia and initiates transcription of pro-apoptotic and cell cycle arrest-inducing target genes (30). Lovage might regulate the activity of p53 by post-translational modifications.

In conclusion, L. officinale leaf essential oil has the ability to inhibit tumor cell growth of the HNSCC cell line, UMSCC1, at high concentrations, whereas it promotes proliferation at subtoxic doses. Genes involved in ERK5

Figure 2. A: Functional groups of genes and B: Signaling pathways regulated upon Levisticum officinale leaf essential oil treatment in UMSCC1 cells. The evaluation of differentially expressed genes was performed using the Ingenuity Pathway Analysis software.
signaling, ILK signaling, virus entry via endocytic pathways and p53 signaling are associated with the cytotoxicity of *L. officinate* leaf essential oil.

**Conflict of Interest**

The Authors declare that there are no conflicts of interest.

**Acknowledgements**

Thanks are due to Jens-Uwe Bergmann, REHAU AG & Co., for the gift of lovage leaf oil extract. The UMSCC1 cells were generously donated by Dr. Christian Simon, Director of the Division of Head and Neck Surgical Oncology in the Department of Otorhinolaryngology, Head and Neck Surgery at the University of Heidelberg, Heidelberg, Germany.
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Received September 9, 2010
Revised December 6, 2010
Accepted December 7, 2010