

Christia vespertilionis plant extracts as novel antiproliferative agent against human neuroendocrine tumor cells

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Abstract. Neuroendocrine tumors respond poorly to radiation and conventional chemotherapy, hence surgical removal of the neoplastic tissue is still the most effective way of treatment. In an attempt to find new therapeutic plant extracts of *Christia vespertilionis* (CV) their antitumor potential in human medullary thyroid carcinoma (MTC) and human small intestinal neuroendocrine tumor (SI-NET) cell lines were tested. Proliferation and viability were analyzed using cell counting and WST-1 assay. Apoptosis was determined by microscopy, luminescence assays for caspases 3/7, and expression studies of apoptosis-related genes. CV extracts showed antiproliferative and proapoptotic effects in all MTC and SI-NET cell lines, whereby high growth inhibition was observed by treatment with the ethylacetate-extracts (CV-45) in tumor cell lines but not in normal human fibroblasts. Furthermore CV-45 treatment resulted in alterations of gene expression of PDCD5, MTDH and TNFRSF10b in MTC as well as in SI-NET cells. The results indicate that *Christia vespertilionis* could serve as an anticancer therapeutic for treatment of neuroendocrine tumors.

Introduction

Neuroendocrine tumors (NETs) represent rare tumors that are derived from cells of the embryonic neural crest. They account for ~0.5% of all malignancies and the incidence is ~2/100,000 (1). The ability to secrete hormones and release them into the bloodstream is one characteristic of NET cells. These tumors originate from endocrine glands, whereby their origin and behavior can vary over a wide range. MTCs are calcitonin producing tumors arising from the parafollicular C-cells and occur in ~4-10% of all thyroid tumors (2). MTCs

(75%) mainly occur sporadically, but there is also a familial form of MTCs without any associated endocrinopathies (FMTC), in which germline mutations of the 'rearranged during transfection' (RET) proto-oncogene have very often been identified. The remaining 25% of MTC cases are autosomal dominantly inherited and are combined with other endocrinopathies summarized as multiple endocrine neoplasia (MEN) type 2A or 2B (3,4). SI-NETs, formerly called carcinoids, have their origin in enterochromaffin cells throughout the intestinal tract. They have the ability to secrete and release serotonin into the bloodstream, which can result in flushing, diarrhea, bronchoconstriction and fibrosis, summarized as the carcinoid syndrome (5).

SI-NETs can occur sporadically, but also in a dominantly inherited form associated with multiple endocrine neoplasia type 1 (MEN1), which occurs due to loss-of-function germline mutations of the *MEN1* gene. Since these NETs usually have already metastasized when diagnosed, none of the treatment options reported have been found to be effective and the response rate has generally been low (6). As the only curative treatment for NETs is early removal of all neoplastic tissue (7,8) new strategies for the treatment remain crucial. Medicinal herbs are of high interest in anticancer drug research (9-11). *Christia vespertilionis*, a south-east Asian Fabaceae, is known to exhibit antiplasmodial activity and to increase cytotoxicity in HeLa cells compared to normal human lung cells (12). Triterpenes, alkaloids, fatty acids, phenols, alkanes and long chained alcohols have been identified as main constituents of this plant.

Remarkably, isoquinoline alkaloids, usually typical for Papaveraceae or closely related families, were found in *Christia* plant extracts. In a previous study, pheophorbide-a, a chlorophyll derivative in *Christia vespertilionis* plant extracts showed high antiproliferative activity in MTC cells (13). The aim of the present study was to examine the *in vitro* effects of bioactive agents extracted from *Christia vespertilionis* in chemo- and radiation-resistant NET cells.

Materials and methods

Plant material. Specimen of *Christia vespertilionis*, a south-east Asian Fabaceae, was deposited at the Institute of Pharmacy at the University of Innsbruck. The plant was originally bought

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at a market in Vietnam. Aerial parts of the plant were successively extracted in dichloromethane in a Soxhlet apparatus and four subfractions extracted by petroleum ether (CV-44), ethyl acetate (CV-45), butanol (CV-46) and water (CV-47). Two pure substances, palmitine (CV-48) and corynoxidine (CV-49), were isolated from CV-46. The identity was confirmed by 1D and 2D NMR spectroscopy and mass spectrometry. Dried extracts were redissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Vienna, Austria) at a concentration of 5 mg/ml and stored at -20°C.

Cell lines and cell culture. The human MTC cell line *MTC-SK* (14), the SI-NET cell line *KRJ-I* (15) and the normal human fibroblast cell line *HF-SAR* (Pfragner R, personal communication) were established in our laboratory. *MTC-SK* and *KRJ-I* cells were cultured in Ham's F12:M199 medium (1:1) (BioWhittaker; Lonza, Verviers, Belgium) with 10% fetal bovine serum (FBS) (PAA Laboratories, Pasching, Austria) at an initial cell number of 2×10^5 cells/ml. Human skin fibroblasts, *HF-SAR*, isolated from a 2-year-old male served as control and were cultured in DMEM (BioWhittaker; Lonza), supplemented with L-glutamine and 10% FBS (PAA Laboratories, Vienna, Austria) at an initial cell number of 1×10^5 cells/ml. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. Cell lines were *Mycoplasma*-free, as tested with MycoAlert *Mycoplasma* detection kit (Lonza, Rockland, ME, USA). For all experiments, cells were cultured without antibiotics.

Cell counting. Cells were counted with the CASY-1® Cell Counter Analyser TTC (Schärfe system; Reutlingen, Germany). *MTC-SK* as well as *KRJ-I* cells were seeded into 24-well plates at a density of 2×10^5 cells/ml and incubated for 24, 48 and 72 h, respectively, with DMSO (control) or supplemented with 10 µg/ml of CV extracts. Cell clusters were pipetted into single cells and each sample was analyzed in triplicate by cell counting.

Cell proliferation. WST-1 cell proliferation reagent (Roche Diagnostics, Vienna, Austria) was used to quantify viability and proliferation in *MTC-SK*, *KRJ-I* and *HF-SAR* after CV treatment. Mitochondrial dehydrogenases are able to convert WST-1, a tetrazolium salt, into formazan, which leads to a change of absorbance. *MTC-SK* and *KRJ-I* cell suspensions were seeded into 24-well plates and incubated with DMSO (control) or 10 µg/ml of plant extracts; then cell viability was measured. After 24, 48 and 72 h of treatment, cell aggregates were pipetted into single cells. The measurement was carried out according to the manufacturer's protocol. Adherent *HF-SAR* cells were directly seeded into 96-well plates and cell viability was measured as above. Samples were tested in 6 replicates.

DAPI staining. DAPI (4',6'-diamidino-2 phenylindole) is a fluorochrome that forms fluorescence complexes with double-stranded DNA. Treatment of cells with the fluorescent dye leads to a blue coloring of the nuclei when irradiated at an excitation wavelength of 350 nm. Apoptotic nuclei can be identified by the condensed chromatin gathering at the periphery of the nuclear membrane or a fragmented morphology of nuclear

bodies, observed by fluorescence microscopy. Cell pellets of *MTC-SK* as well as *KRJ-I* cells were resuspended in DAPI (Sigma) solution pursuant to the manufacturer's instructions. The stained cells were pipetted onto glass slides, covered with a cover slip and analyzed under a fluorescence microscope (Leica DM 4000/EL6000; Leica, Wetzlar, Germany). Nuclei that showed clearly condensed and fragmented chromatin were scored as apoptotic.

Caspase-Glo 3/7 assay. To measure the activity of apoptotic key effector caspases 3 and 7, the Caspase-Glo® 3/7 assay (Promega, Mannheim, Germany), which is based on a luminescence reaction, was used. The obtained luminescence signal is proportional to caspase 3/7 activity. In a time period of 5 or 8 h, respectively, CV extract or DMSO was added to the cells. Samples were transferred into white-walled 96-well plates (Nunc™, Thermo Fisher Scientific, Vienna, Austria). Caspase-Glo 3/7 reagent was added according to the manufacturer's instructions and luminescence of each sample measured with the GloMax®-Multi+ Microplate Multimode Reader with Instinct™ (Promega). Medium supplemented with 10% FBS was used for blank reactions. DMSO treated cells were used as control. Each treatment was measured in triplicate; arithmetic mean and SD were calculated automatically by the GloMax software.

Cell morphology. Effects of CV-45 on cell morphology of treated *MTC-SK* and *KRJ-I* cells were observed with the Nikon inverted microscope (Eclipse TE 300; Nikon, Tokyo, Japan). *MTC-SK* and *KRJ-I* cells (2×10^5 cells/ml) were treated with either 10 µg/ml of CV-45 or DMSO (control) and incubated for 24, 48 and 72 h in 24-well plates. After each incubation day, cell morphology was observed and images were taken (Nikon 12-bit CCD camera, Nikon).

RNA extraction and reverse transcription. *MTC-SK* and *KRJ-I* cells (2×10^5 cells/ml) were transferred into cell culture flasks (75 cm²) and incubated for 2 h with 10 µg/ml of CV-45, or DMSO (control). After incubation, cells were transferred into 50 ml tubes (Sarstedt), and centrifuged for 10 min at 300 x g; the cell pellets were used for RNA isolation with the TRI Reagent® (Molecular Research Centre, Cincinnati, OH, USA). The concentration of isolated RNA was determined using the Nanodrop (NanoDrop® Spectrophotometer ND-1000, Peqlab Biotechnology GmbH, Erlangen, Germany). Each preparation (1 µg) was reverse-transcribed using the High Capacity RNA to cDNA kit (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (q RT-PCR). The relative gene expression of *MTDH* (*Metadherin*, also known as *lyric*), *PDCD5* (programmed cell death 5) and *TNFRSF10b* (tumor necrosis factor receptor superfamily, member 10b) in CV treated cells in comparison to untreated cells (control) was analyzed. Primers are summarized in Table I. SYBR Green I fluorescence dye (Bio-Rad) was used for detection. IQ Sybergreen Supermix, 12.5 µl, with 5.5 µl RNase free water and 0.5 µl of each forward and reversed primer (10 µM) were mixed thoroughly and 6 µl cDNA (5 ng/µl) of CV-45 treated and untreated *MTC-SK* and *KRJ-I*

Table I. Primer sequences for quantitative RT PCR.

GenBank Acc. no.	Gene ID	Gene name	Forward primer	Reverse primer	Product size (bp)
NM_178812	MTDH	Metadherin	CAGATCAGTGCAGGTGAGGA	GGAATTGGTTCCTGGGACTT	374
NM_004708	<i>PDCD5</i>	Programmed cell death 5	AAAGCACAGGGAAGCAGAAA	GGGCACATAGTCATCCCACT	275
NM_003842	TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b	CACCAGGTGTGATTCAGGTG	CCCCACTGTGCTTTGTACCT	221
NM_001256799	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	AATGCCTTGTCGAAGTCCAG	TCTGTGTCTGCCTTTGTTGG	236

cells added to the Master mix in PCR tubes. For the calibration curve of each gene of interest, cDNA of pooled RNA (treated + untreated) was added and combined with Master mixes for each gene. The following PCR conditions were used: 95°C for 3 min, 44 cycles at 95°C/10 sec, 60°C/45 sec, 72°C/40 sec, followed by 55°C for 10 sec and the condition for the melting curve (55°C-95°C for 5 sec). Data were normalized using the expression of the housekeeping gene *GAPDH*. Relative gene expression was assessed using the $\Delta\Delta C_q$ -method (16).

Statistical analysis. Medians and SD were calculated using Microsoft Excel software (Microsoft Office, version 2011). A statistical analysis of differences between treated and untreated tumor cells was carried out with a two-tailed unpaired Student's t-test. A P-value <0.05 was considered to indicate significance; P-values <0.01 indicated 'very significant'. Experimental results are shown in the graphs as mean value \pm standard error of the mean.

Results

Cell proliferation. The effect of the petroleum ether (CV-44), ethylacetate (CV-45), butanol (CV-46) and water (CV-47) fractions and the two isolated pure substances (CV-48 and CV-49) of *Christia vespertilionis* on cell-proliferation and cell-viability was tested by cell counting in *MTC-SK* cells. At a concentration of 10 μ g/ml all subfractions showed a reduction of cell viability within 72 h (Fig. 1a). After 72 h treatment with CV-45, numbers of total viable *MTC-SK* cells were significantly lower than those treated with DMSO or other fractions. Isolated pure *Christia vespertilionis* showed very weak inhibition of cell viability; only after 72 h did CV-48 show decreased number of viable cells as compared to DMSO treated control cells. We obtained the highest inhibition of proliferation with the ethylacetate fraction CV-45 compared to other tested fractions and pure substances. Treatment of *MTC-SK* as well as *KRJ-I* cells with 10 μ g/ml of CV-45 showed significant inhibition of cell growth. As shown in Fig. 1b, the cell number obtained by cell counting of *MTC-SK* cells after CV-45 treatment compared to control cells (DMSO) was 11.3% less after 24 h, 28.9%

less after 48 h and 32.5% less after 72 h (t-test $p < 0.05$). The cell number obtained by counting *KRJ-I* cells (Fig. 1c) after CV-45 treatment was also significantly decreased as compared to control cells. Following CV-45 treatment, total viable cell counts decreased by 11.1% after 24 h, by 20.8% after 48 h and by 29.9% after 72 h (t-test $p < 0.01$).

Cell viability. The potency of CV plant extracts to reduce cell viability of *MTC-SK* cells is shown in Fig. 1d. After 48 h of exposure, we measured a decrease in cell viability with CV-44, CV-45 and CV-47, but not with CV-46 and only very weakly with the isolated pure substances CV-48 and CV-49; after 72 h of treatment CV-46 and CV-47 as well as CV-48 and CV-49 we did not observe antiproliferative effects; on the contrary, CV-44 and CV-45 showed the highest antiproliferative effects after 48 and 72 h of exposure (77% cell viability after 48 h and 69% cell viability after 72 h with CV-44 compared to control cells and 26% cell viability after 48 h and 31% cell viability after 72 h with CV-45 compared to control cells). The highest antiproliferative effect was measured for CV-45. As shown in Fig. 1e, cell viability of *MTC-SK* decreased 21.1% after 24 h, 31.8% after 48 h and 37.4% after 72 h exposure to 10 μ g/ml of CV-45 when compared to control cells (DMSO). Similarly to *MTC-SK* cells, *KRJ-I* cells were also suppressed by CV-45 fraction (Fig. 1f). After 24 h of CV-45 treatment (10 μ g/ml) cell viability decreased by ~18.3%, after 48 h, significantly by 23.5% (t-test $p < 0.05$) and after 72 h by 42.8% compared to DMSO treated cells (control). In human skin fibroblast *HF-SAR* serving as control cells, the same concentration of CV-45 did not inhibit cell proliferation (Fig. 2).

DAPI-staining. We found morphological changes in *MTC-SK* as well as in *KRJ-I* cells including chromatin condensation, cell shrinking and apoptotic bodies when cells were treated for 48 and 72 h with CV-45 (Fig. 3). No significant changes were observed in *MTC-SK* and *KRJ-I* control cells (DMSO).

Induction of apoptotic pathway. CV-45 treatment increased caspase 3/7 activity in *MTC-SK* and *KRJ-I* cells within 24 h, which correlated with a decrease in viable cells. An increase

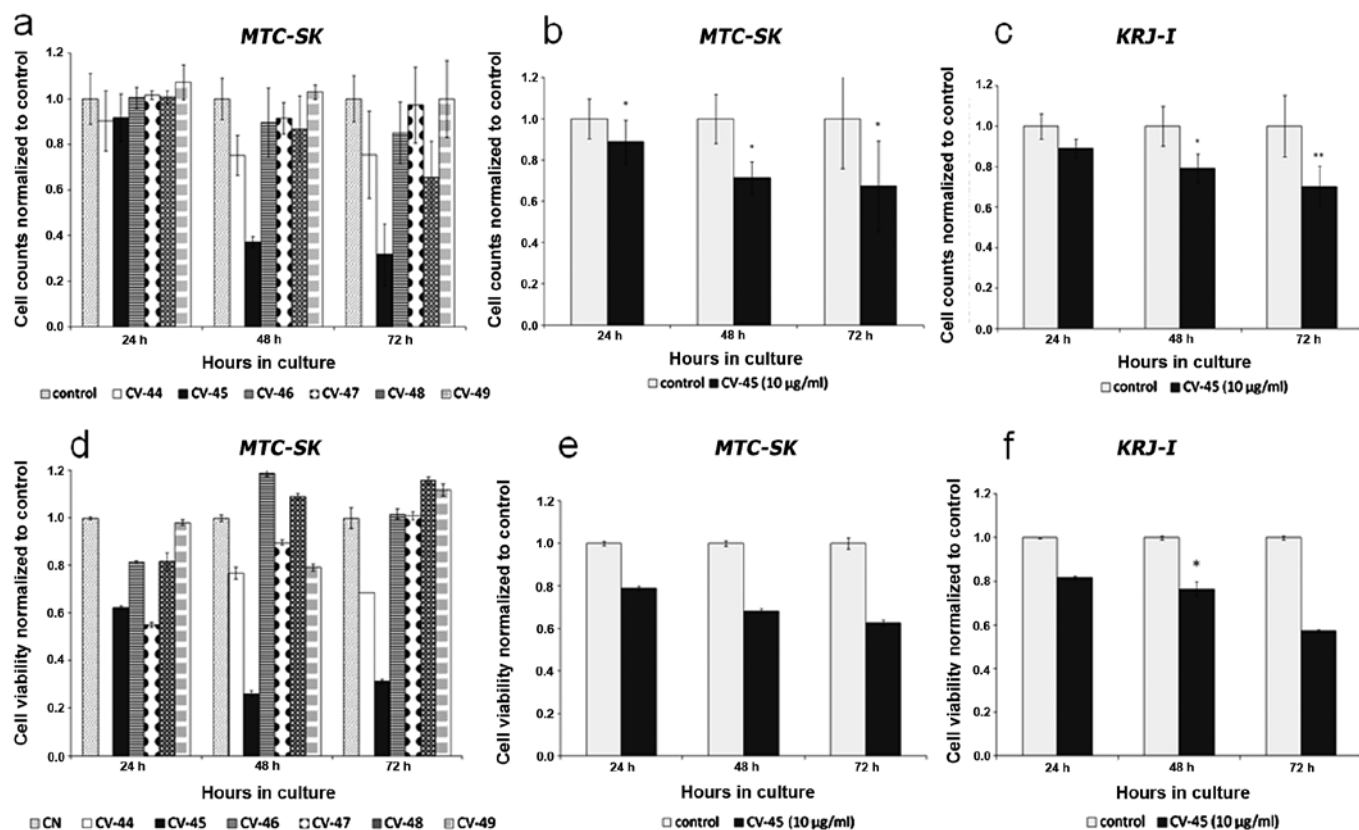


Figure 1. Cell proliferation and viability in *MTC-SK* and *KRJ-I* cells treated with 10 µg/ml *Christia vespertilionis* subfractions and pure isolated substances. Cell proliferation was analyzed by CASY-1 Cell Counter and Analyzer and viability measured by WST-1 cell proliferation assay. (a) Subfractions CV-44 - CV-47 inhibited cell growth after 48 and 72 h of incubation. Pure isolated substance CV-48 showed inhibition after 48 and 72 h. Treatment with CV-49 showed no growth inhibiting effect in *MTC-SK* cells. (b) Ethylacetate fraction CV-45 showed significant growth inhibiting effects in *MTC-SK* cells after 48 and 72 h of treatment ($p < 0.05$). (c) *KRJ-I* cells, treated with 10 µg/ml of CV-45, showed decreased cell proliferation after 24, significantly after 48 h ($p < 0.05$) and highly significantly after 72 h ($p < 0.01$). (d) Highest antiproliferative effects were shown after 48 and 72 h by treatment with CV-44 and CV-45 in *MTC-SK* cells. (e) CV-45 subfraction (10 µg/ml) exhibited antiproliferative effects in *MTC-SK* cells after 24, 48 and 72 h of treatment. (f) Cell viability in *KRJ-I* cells after treatment with 10 µg/ml of CV-45 subfraction. CV-45 exhibited antiproliferative effects in *KRJ-I* cells after 24, significantly after 48 and after 72 h of treatment, measured by WST-1 assay. * $P < 0.05$, ** $P < 0.01$.

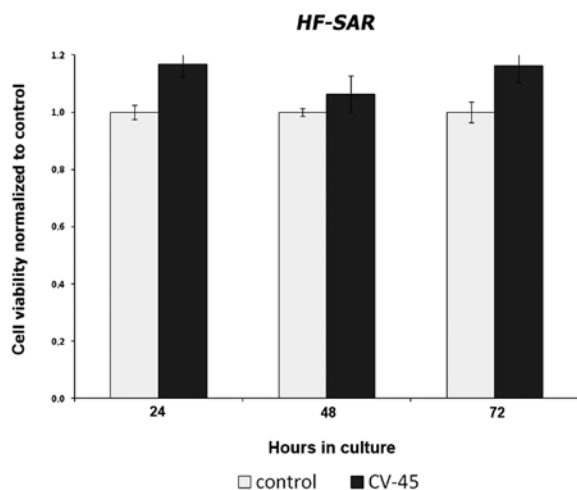


Figure 2. Relative cell viability in human *HF-SAR* cells after treatment with 10 µg/ml of CV-45 subfraction measured by WST-1 assay. CV-45 exhibited no inhibitory effects in *HF-SAR* cells after 24, 48 or 72 h of treatment.

of caspase 3/7 activity was no longer evident after 48 and 72 h of CV-45 treatment, although the amount of viable cells after CV-45 treatment still decreased after 48 and 72 h (data not

shown). After 6 h of exposure at a concentration of 10 µg/ml, the caspase 3/7 activity of CV-45 treated *MTC-SK* cells was ~21.4% higher and after 8 h caspase 3/7 activity nearly doubled (93.1%) compared to control cells (Fig. 4a). In *KRJ-I* cells, caspase 3/7 activity had increased by 13.5% after 2 h of CV-45 treatment, 17.9% after 3 h, 49.1% after 4 h and 61% after 5 h CV-45 compared to control cells (Fig. 4b).

Cell morphology in vitro. *MTC-SK* as well as *KRJ-I* cells grow in multicellular spheroids. Fig. 6 shows *MTC-SK* and *KRJ-I* cells after treatment with 10 µg/ml of CV-45 compared to control. Forty-eight hours after adding CV-45 to *MTC-SK* cells, treated cells showed multicellular aggregates of ~300 µm diameter and more single cells were visible, compared to the much larger aggregates (800-900 µm) formed by control cells (Fig. 5a and b). In *KRJ-I* cells, CV-45 treatment also led to reduction of aggregate formation. After 48 h of CV-45 treatment, multicellular clusters of *KRJ-I* were mostly dissociated into single cells. Aggregates of control cells were twice as large as those of treated cells (Fig. 5c and d).

Gene expression of PDCD5, MTDH and TNFRSF10b. The data on cycles of quantity (C_q -value) of the reference gene *GAPDH* showed stable results in *MTC-SK*, indicating that

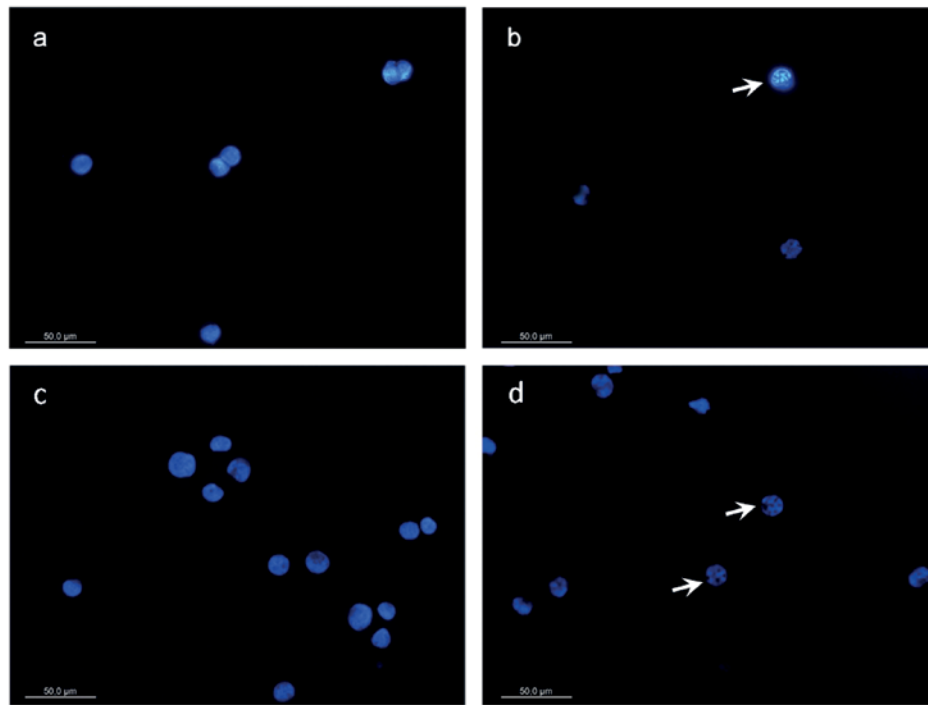


Figure 3. *Christia vespertilionis* CV-45 induces apoptosis in *MTC-SK* and *KRJ-I* cells. *MTC-SK* as well as *KRJ-I* cells were treated with 10 $\mu\text{g/ml}$ of CV-45 or DMSO and stained (DAPI) after 48 and 72 h, respectively. (a) *MTC-SK* control cells after 48 h (b) apoptotic body in *MTC-SK* after 48 h of CV-45 treatment (c) *KRJ-I* control cells after 72 h (d) apoptotic bodies in *KRJ-I* after 72 h of CV-45 treatment. Bar, 50 μm . (apoptotic bodies are marked with arrows).

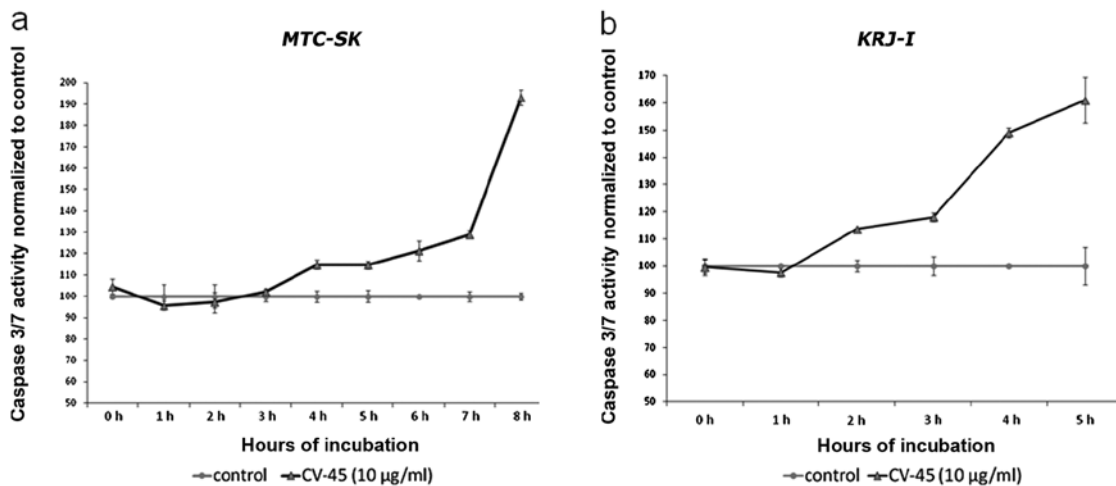


Figure 4. Caspase 3/7 activity after CV-45 treatment in *MTC-SK* and *KRJ-I* cells and normalized to control cells. (a) Caspase activity had increased 3 h after addition of CV-45 in *MTC-SK* cells and reached the highest level after 8 h. (b) In *KRJ-I* cells, caspase 3/7 activity was already increased after 2 h of CV-45 treatment.

expression of *GAPDH* was not influenced by CV-45 treatment (17.96 in CV-45 treated versus 18.01 in control, data not shown). Quantitative RT-PCR showed increased expression of *MTDH* and downregulation of *PDCD5* and *TNFRSF10b* in CV-45 treated *MTC-SK* cells (Fig. 6a). Although *GAPDH* gene expression was slightly influenced by CV-45 treatment in *KRJ-I* cells, the gene expression analysis showed that CV-45 treatment lead to downregulation of *MTDH* and upregulation of *PDCD5* gene expression in *KRJ-I* cells. The expression of *TNFRSF10b* was not significantly altered in *KRJ-I* cells when treated for 3 h with CV-45 (Fig. 6b).

Discussion

The establishment of new treatment options for chemo- and radiation-resistant NETs is essential because of the inefficacy of conventional chemotherapy. Medicinal herbs have come increasingly into the spotlight as complementary medicines. In the present study, we provide a first report of the antitumor activity of plant extracts from *Christia vespertilionis*, in which the ethylacetate fraction CV-45 showed significant antiproliferative and pro-apoptotic effects in *MTC-SK* as well as in *KRJ-I* cells.

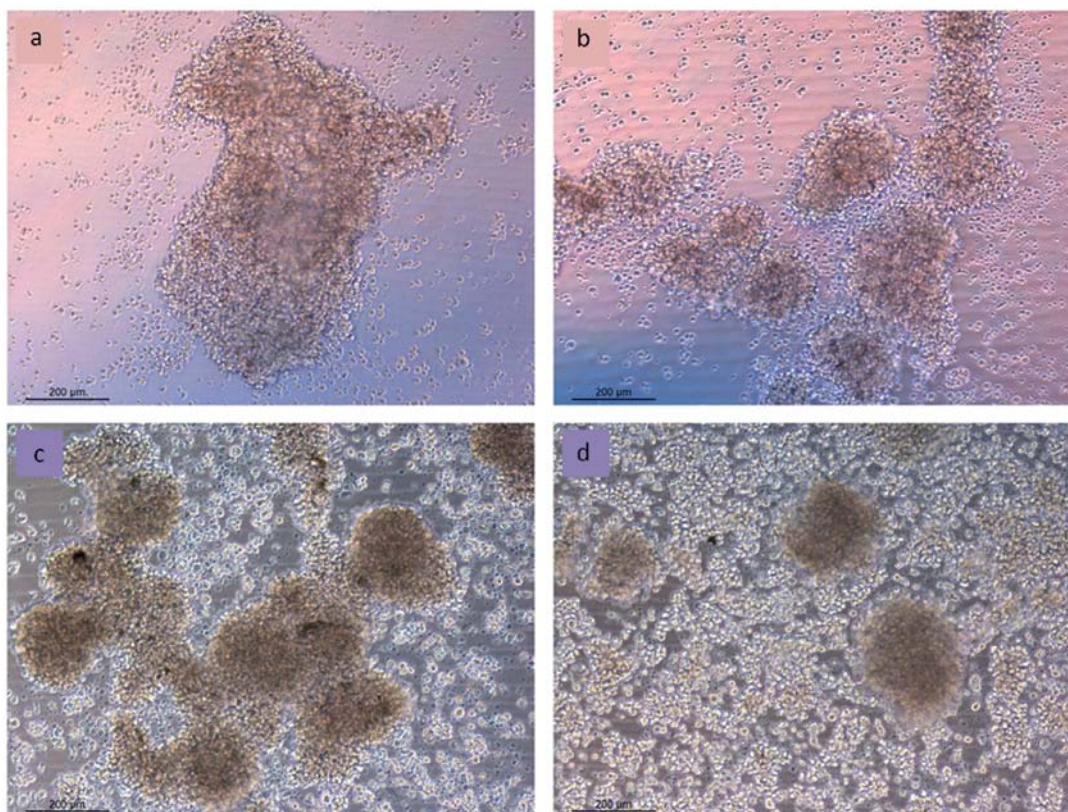


Figure 5. *MTC-SK* as well as *KRJ-I* cells grow in suspension and form multicellular aggregates. After suspending into single cells, *MTC-SK* and *KRJ-I* cells were treated with 10 µg/ml CV-45 or DMSO as control. (a) *MTC-SK* control after 48 h of incubation (b) CV-45 treated *MTC-SK* cells after 48 h (c) *KRJ-I* control cells after 48 h (d) CV-45 treated *KRJ-I* cells after 48 h. Compared to control cells, CV-45 treatment for 48 h lead to reduction of cell aggregates in *MTC-SK* as well as in *KRJ-I* cells.

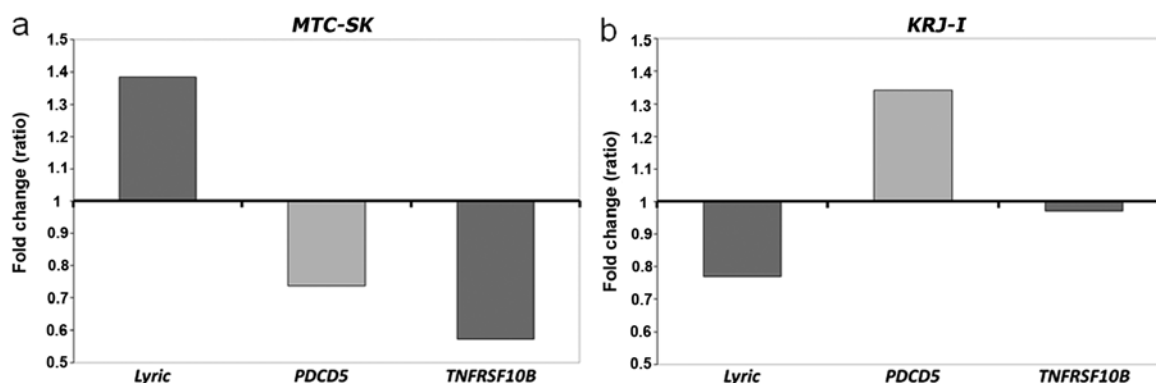


Figure 6. Relative gene expression of *MTDH*, *PDCD5* and *TNFRSF10b* in CV-45 treated *MTC-SK* and *KRJ-I* cells, compared to control cells (DMSO). (a) *MTC-SK* cells, treated for 2 h with 10 µg/ml of CV-45, showed increased relative gene expression of *MTDH* and decreased expression of *PDCD5* and *TNFRSF10b*, compared to control. (b) In CV-45 treated *KRJ-I* cells, gene expression of *MTDH* and *TNFRSF10b* was downregulated and *PDCD5* upregulated, when compared to control. *KRJ-I* cells were treated for 3 h with 10 µg/ml of CV-45 or DMSO as control.

It is known that many chemotherapeutic agents are able to induce apoptosis in cancer cells, as with sorafenib or 5-fluorouracil for human hepatoma cells (18,19). One goal in the establishment of new therapies against NETs is to define substances that have the ability to trigger anticancer effects and to induce apoptosis specifically in tumor cells, but not in normal cells.

In the human fibroblasts (*HF-SAR*) tested, the same concentration of CV-45 (10 µg/ml) as used for tumor cells

did not inhibit proliferation, suggesting that the composition of this fraction does affect tumor cells specifically. Moreover, bioactive ingredients of *Christia vespertilionis* extracts, only some of which have been identified to date, look like good candidates for further evaluation. The CV plant extracts were resolved in DMSO, which is often discussed to be cell toxic. However, Da Violante *et al* showed, DMSO can be used as solvent at even higher concentrations, up to 10%, without significant cell damage (17).

The tendency of MTC as well as SI-NETs to grow in multicellular aggregates when cultured *in vitro* may be evidence for the resistance of NETs to conventional chemo- and radiation therapies (20,21). As reported by Pfragner *et al* (15), both *MTC-SK* as well as *KRJ-I* cells grow in multicellular spheroids. To optimize treatments for MTCs and carcinoid cells, these cell clusters must be dissociated into single cells to make them more accessible to chemotherapeutics. Treatment of *MTC-SK* and *KRJ-I* cells with CV-45 showed only weak effects on disruption of cellular aggregates. This may be due to the low concentration of the plant extract in contrast to the pure substance. It should be noted that we do not yet know whether this spheroid destroying effect could also be achieved by other components.

Many chemotherapeutic drugs degrade tumor cells by activating cascades of reactions that generally result in apoptosis. The induction of apoptosis follows a very complex pathway involving many cell proteins. Caspases 3 and 7 are known to be proteases inducing and activating proteins responsible for cell fragmentation and cell shrinking. Further, an evaluation of the activity of caspase 3/7 is a meaningful method to identify induction of apoptosis. The natural compounds of CV-45 showed strong evidence of triggering apoptosis in NET cells. Within 24 h of CV-45 treatment, caspase 3/7 activity was increased in *MTC-SK* as well as in *KRJ-I* cells, but no longer after 48 and 72 h. When compared to cell viability, these results correlate within 24 h of CV-45 treatment, with a decrease in cell counts compared to an increase of caspase 3/7 activity, but no longer correlate after 48 and 72 h. We know accordingly that caspase 3 activation is an early-stage event in apoptosis; thus, this could be the reason why a significant decrease in cell viability and proliferation occurred later than caspase 3/7 activation. The apoptotic potential of CV-45 was also evident in the formation of apoptotic bodies that were observed with fluorescence microscopy after DAPI staining. Nucleic aberrations, manifested in chromatin condensation and nucleic shrinking, were determined in *MTC-SK* as well as in *KRJ-I* cells, confirming the apoptosis inducing effect of *Christia vespertilionis* plant extracts.

The object of the present study was also to examine the effects of CV-45 treatment on the relative gene expression of *MTDH*, *PDCD5* and *TNFRSF10b* in *MTC-SK* and *KRJ-I* cells. We found these genes to be expressed in our cell lines and their expression was altered after treatment. Proteins encoded by the genes *PDCD5*, *MTDH* and *TNFRSF10b* are known to be involved in programmed cell death. *PDCD5* protein is believed to participate in regulation of apoptosis. Its expression was downregulated in gastric tumor tissue as compared to normal gastric tissue. However, when treated with diallyl trisulfide, which induced apoptosis in gastric cancer, *PDCD5* expression was upregulated (22). In *KRJ-I* cells, *PDCD5* gene expression was also upregulated with 3 h of treatment with CV-45, in agreement with literature data. Unlike gene expression in *KRJ-I*, *PDCD5* gene expression was downregulated in *MTC-SK* with 2 h stimulation with apoptosis inducing CV-45. This result could possibly be related to insufficient incubation time with CV-45 in these cells. Altered expression of *PDCD5* in *MTC-SK* occurred also in *MTC-SK* treated for a longer time with CV-45. *MTDH* is known to have a multifaceted role in cancer progression,

taking part in the complex network of oncogenic signaling pathways. It is involved in the processes of proliferation, metastasis, survival, chemoresistance and invasion of tumor cells. Furthermore, it interacts with NF- κ B by binding directly to the p65 subunit of the necrosis factor (23).

The upregulation of *MTDH* in CV-45 treated *MTC-SK* cells, might lead to the apoptotic effect by the activation of NF- κ B. NF- κ B on its part is known for its anti-apoptotic activity, acting as transcription factor for cell survival genes. In contrast *MTDH* was downregulated in *KRJ-I*. A possible explanation could be that downregulation of *MTDH* leads to less NF- κ B activation and further to an inhibition of the expression of cell survival genes involved in cell death. In the present study, the apoptotic potential of CV-45 was demonstrated via caspase 3/7 activation and fluorescence staining in both *MTC-SK* and *KRJ-I* cell lines. Besides inhibition of *MTDH*, we suppose the presence of another pathway for CV-45 to activate apoptosis in *MTC-SK*. Hu *et al* (23) also described a connection between *MTDH* and tumor necrosis factor-related apoptosis-inducing ligand (*TRAIL*), which is known to activate TRAIL induced apoptosis via the following pathway: TRAIL binds to TNFRSF10b and activates further proteins, known as FADD and initiator caspase 8, which thereupon activate effector caspases 3 and 7 and finally apoptosis.

Hu *et al* explained that overexpressed *MTDH* leads to downregulation of *TRAIL*. Thus, decreased TRAIL could possibly mean less TRAIL-induced apoptosis, suggesting that this probably does not occur in CV-45 treated *MTC-SK* cells. However, knowing that many proteins, also ones yet unknown, are involved in the complex pathway of apoptosis, explanations can only be vague and speculative. The protein encoded by *TNFRSF10b* gene is a receptor that is also known as death receptor 5 (DR 5), containing a death domain (DD). Some parts of this receptor interact with TRAIL (24), whereas FADD binds with its DD at the DD of TNFRSF10b resulting in formation of the death-inducing signaling complex (DISC). Further, it recruits caspase 8 to DISC, engaging the caspase cascade to induce apoptosis (25). It seems that *TNFRSF10b* is selectively expressed in cancer cells and may induce TRAIL-induced apoptosis in cancer cells (26).

Overexpression of *TNFRSF10b* led to significant activation of NF- κ B in human embryonic kidney cells (HEK 293). Thus, *TNFRSF10b* was involved in both induction of apoptosis and activation of NF- κ B (27). The downregulation of *TNFRSF10b* in CV-45 treated *MTC-SK* cells remains to be clarified. In *KRJ-I* cells, *TNFRSF10b* gene expression was not significantly altered when compared to control cells. Chaudhary *et al* showed that overexpression of *TNFRSF10b* induced apoptosis in mammalian cells via a caspase-dependent mechanism (27). Although caspases 3 and 7 were activated after CV-45 treatment in *MTC-SK* and *KRJ-I* cells, there was no significant upregulation of *TNFRSF10b*, but quite the opposite. These results lead to the conclusion that apoptosis might not be induced by the TRAIL/TNFRSF10b mechanism.

The present *in vitro* study demonstrates the antitumor effects of novel plant derived agents in human neuroendocrine tumor cells. The ethyl acetate fraction of *Christia vespertilionis* (CV-45) had an antiproliferative and pro-apoptotic effect in MTC cells as well as in SI-NET cells. Fibroblasts were not impaired, indicating a lack of side effects. The *in vitro* effects of

the selected plant-derived compounds suggest potential clinical effects in patients with neuroendocrine tumors.

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