

## Anticancer Activity of Novel Plant Extracts from *Trailliaedoxa gracilis* (W. W. Smith & Forrest) in Human Carcinoid KRJ-I Cells

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**Abstract.** *Background:* Small intestinal (SI) neuroendocrine tumors (NETs) are rare neoplasms derived from neuroendocrine cells presenting distinct clinical symptoms according to the ability to secrete neuroamines. Nevertheless, many are asymptomatic and misdiagnosed. As response rates to chemotherapy are low, surgery remains the only effective treatment. Because many tumors have metastasized at the time of diagnosis, curative surgery is rarely achieved. Consequently, a substantial need for new therapeutic options has emerged. *Materials and Methods:* The effects of novel plant extracts from *Trailliaedoxa gracilis* (W.W. Smith & Forrest) were investigated in the SI-NET cell line KRJ-I and in KRJ-I transplanted mice. Proliferation and viability were analyzed using cell counting and WST-1 cell proliferation assay. Apoptosis was determined by DAPI staining and electron microscopy, and quantified by luminescence assays for caspases 3/7, 6, 8, 9 and 2. *Results:* Extracts of *Trailliaedoxa gracilis* showed a dose-dependent reduction of proliferation and induction of apoptosis in the KRJ-I cells. Normal fibroblasts were not impaired. Tumor growth inhibition was also observed in heterotransplanted SCID (severe combined immunodeficiency) mice. *Conclusion:* The *in vitro* and *in vivo* outcomes suggest a potential clinical effect of *Trailliaedoxa gracilis* in SI-NETs.

Small intestinal (SI) neuroendocrine tumors (NETs) are rare neoplasms originating from neuroendocrine cells that are part of the diffuse neuroendocrine system (1). These tumors are characterized by malignant potential and slow growth with an annual incidence of 4-6 per 100,000 (2, 3). Due to increasing clinical awareness and more wide-spread use of diagnostics, e.g. endoscopy, ultrasonography, CT, MRI and the measurement of biochemical markers, the incidence of SI-NETs has increased annually by 3 to 10% over the last three decades (2, 4); neuroendocrine tumors of the gut therefore are not as rare as once considered. Diagnosis is currently based on the typical clinical symptoms and imaging methods such as somatostatin receptor scintigraphy and blood levels of biochemical markers (5). Specific for the ileal carcinoid tumors are increased serum levels of serotonin, neuropeptide K and substance P (3, 4). These bioactive substances are responsible for the characteristic clinical symptoms of the carcinoid syndrome characterized by 'flushing', diarrhea and abdominal pain. However, many tumors are so-called non-functioning causing no symptoms by the secreted peptides, while functioning tumors presenting with distinct clinical symptoms are often misdiagnosed or overlooked. Therefore a delay of diagnosis (5-7 years) is typical, increasing the probability of liver metastasis (3) and consequently, more than 85% of patients show liver metastasis at the time of diagnosis. Surgery remains the only curative therapeutic option, but due to high rates of metastasis less than 15% of patients have the likelihood of a curative intervention (5). Therefore, clinical management focuses mainly on the treatment of clinical symptoms using somatostatin analogues and interferon- $\alpha$ . While biotherapy with somatostatin and interferon- $\alpha$  appears to have unquestioned tumourstatic properties, these agents do not yield significant objective

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tumor responses (5). Recently, single-agent and multi-agent chemotherapy regimens have been evaluated with response rates from 0-20% for single agent and 20-30% for multi-agent treatment (5). Due to the unchanged prognosis for SI-NETs over the past 30 years, there is a substantial need for finding new therapeutic strategies.

For centuries, plants have been used in traditional medicines for the treatment of different diseases. In recent years, oriental medicinal herbs have aroused scientific interest as complementary or alternative medicines (6). Several chemotherapeutic drugs derived from plants, such as Vinblastine, Taxol, Camptothecin and Podophyllotoxin are used in medical tumor management (7). Using modern analytical and chemical techniques, novel natural compounds from herbs can be isolated by fractionation and isolation. It has been estimated that only 5-15% of 250,000 species of higher plants have been screened systematically for natural bioactive compounds (8, 9). To study new therapeutic approaches, cell lines are used to investigate novel compounds and their effects on the tumor cells. However, few cell lines from human SI-NETs have been maintained so far, namely GOT1 (10) and CNDT2 (11), and therefore, we established four continuous tumor cell lines (KRJ-I, P-STs, L-STs, H-STs) (12, 13) from human malignant carcinoid tumors of the small intestine, KRJ-I and P-STs being derived from primary ileal carcinoids. All established cell lines were investigated morphologically and immunocytochemically and established to derive from an SI-NET (12-14). After cell lines were used as cancer models to investigate antiproliferative activities of novel compounds, findings were confirmed by *in vivo* testings in xenograft rodents (15). In recent studies, we have demonstrated a dose-dependent antitumor effect of bioactive agents derived from *Cautleya gracilis* (Smith) Dandy and extracts of *Stemona tuberosa* Lour with induction of apoptosis on medullary thyroid carcinoma cell lines established in our laboratory (16-19).

Belonging to the family of Rubiaceae, like other Chinese herbs, *Trailliaedoxa gracilis* (W.W. Smith & Forrest) was found and described by the Austrian botanist Handel-Mazzetti (20). In a previous screening, an antiproliferative effect of *T. gracilis* (W.W. Smith & Forrest) was noted in the medullary thyroid carcinoma cell lines MTC-SK (21) and SINJ (22). However, the bioactivity of *T. gracilis* is largely unclear. Thus, the aim of the present study was to investigate for the first time antiproliferative activity *in vitro* using the SI-NET cell line KRJ-I and *in vivo* in xenotransplanted mice.

## Materials and Methods

**Plant extracts and chemicals.** The plant material (whole plant) was provided by E. Stöger (Oberndorf, Austria). A voucher specimen was deposited at the Institute of Pharmacy/Pharmacognosy at the University of Innsbruck. The dried material was ground and macerated with dichloromethane (DCM), filtered and the solvent

evaporated under reduced pressure (TG-5). The extract was then re-dissolved in water/methanol and a liquid-liquid partition with *n*-hexane (TG-1), DCM (TG-2) and ethylacetate (EtOAc) (TG-3) was performed. After evaporation of the solvents under reduced pressure, all samples were re-dissolved in dimethyl sulphoxide (DMSO, Sigma-Aldrich, Vienna, Austria) at a concentration of 10 mg/ml and stored at -20°C. The fractionations of TG-5 and the subsequent subfractions were obtained by gel chromatography using a Sephadex LH-20 column and DCM:acetone (85:15) or methanol as the mobile phase. Ursolic acid was crystallized from TG-F28 and the structure elucidation was carried out by 1D and 2D NMR and MS. The concentration of ursolic acid in TG-F28 was evaluated by <sup>1</sup>H-NMR in DMSO-d<sub>6</sub>. The fifteen obtained subfractions of *T. gracilis* were tested at different concentrations on KRJ-I cells and on fibroblasts. The concentrations of each fraction and subfraction ranged from 2 µg/ml to 50 µg/ml in order to determine the IC<sub>50</sub> values.

Camptothecin (CPT) was purchased from Sigma-Aldrich (Sigma-Aldrich, Vienna, Austria), dissolved in DMSO and stored at a concentration of 1 mM at 4°C.

**Cell lines and cell culture.** A continuous cell line from a human malignant carcinoid of the small intestine, KRJ-I (12) and the normal human skin fibroblast cell line HF-SAR (23) were established in our laboratory. The KRJ-I cells had been characterized histologically and biochemically to be derived from the original tumor (12, 14). All the cell lines were *Mycoplasma*-free. The KRJ-I cells were cultured in Quantum 263 medium optimized for tumor cell growth (PAA Laboratories, Pasching, Austria) at 37°C, 5% CO<sub>2</sub> and the HF-SAR were maintained in Eagle's MEM supplemented with 10% FBS at 37°C, 5% CO<sub>2</sub>. For the *in vitro* experiments, the KRJ-I and HF-SAR cells were plated at an initial density of 2×10<sup>5</sup> cells/ml or 1×10<sup>5</sup> cells/ml, respectively.

**Cell counting.** KRJ-I cells were cultured in 24-well plates (Sarstedt, Wr. Neudorf, Austria) for 24, 48 or 72 h with extracts of *T. gracilis* dissolved in DMSO. DMSO alone was used as negative control. The samples were suspended to disperse cell clusters into single cells and then counted automatically with a CASY®-1 Cell Counter & Analyzer TTC (Schärfe System, Reutlingen, Germany). Each sample was quantified four times and the mean of the sample and standard deviations (S.D.) were calculated by the cell counter.

**WST-1 cell proliferation assay.** Cell proliferation and viability were analyzed using the WST-1 Cell Proliferation Reagent (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) (Roche Diagnostics, Vienna, Austria) according to the manufacturer's instructions. The assay principle is based on cleavage of a tetrazolium salt to a formazan by cellular enzymes, especially mitochondrial dehydrogenases (24). The number of metabolically active cells correlates directly to the amount of formazan.

The KRJ-I cells were cultured in 24-well plates for 24, 48 or 72 h with different concentrations of solvent plant extract or DMSO as negative control. After incubation, cell clusters were dispersed carefully into single cells. After 100 µl suspension of KRJ-I cells had been transferred into 96-well plates, 10 µl WST-1 cell proliferation reagent was added to each sample well followed by 2 h incubation at 37°C. Cell proliferation and viability were quantified by measuring absorbance of the formazan product spectrophotometrically using a microplate ELISA reader (Molecular Devices Corporation, Sunnyvale, CA, USA) between 420 nm to 480 nm. The HF-SAR cells were seeded directly into 96-well plates. After adherence, the

cells were treated with plant extracts for 24, 48, 72 or 96 h. The cell viability was measured as described above and the mean of six parallel samples and standard deviation were calculated.

**DAPI (4'-6-diamidino-2-phenylindole) staining.** This method of identifying typical apoptotic alterations is based on the ability of 4'-6-diamidino-2-phenylindole to form fluorescent complexes with double-stranded DNA, showing fluorescence specificity for adenosine-thymidine clusters. KRJ-I cells were cultured in 24-well plates with different concentrations of plant extract or DMSO alone as negative control for 24, 48 or 72 h. The cell suspensions were centrifuged, overlap exhausted and the cell pellet washed with DAPI methanol twice. The KRJ-I cells were then incubated with 2 ml DAPI methanol (Sigma-Aldrich) for 15 min at room temperature. After spinning down, the DAPI methanol was removed and the remaining pellet was resuspended with PBSA, mounted on a microscope slide and fixed with glycerin-aldehyde. The cells were examined using an inverted phase-contrast fluorescence microscope (Nikon eclipse TE300, Tokyo, Japan) with ultraviolet (UV) excitation at 300-500 nm. Cells with condensed chromatin or fragmented nuclei were considered as apoptotic.

**Caspase -3/7, -6, -8, -9, and -2 activity assays.** The caspase activities were quantified using Promega luminescent assay kits (Promega, Mannheim, Germany). KRJ-I cells were treated with different fractions of plant extracts at a concentration of 10 µg/ml. Camptothecin (5 µM) (25) was used as positive control. The samples were incubated at 37°C and caspase activities were measured every two or three hours for up to 36 h. Each sample was suspended to disperse cell clusters into single cells. Afterwards, 50 µl aliquots of cell suspension were transferred into a 96-well white walled plate (Nunc, Roskilde, Denmark) and Caspase-Glo Reagent was added at a ratio of 1:1. After gently mixing on a plate shaker for 30 s, the samples were incubated at room temperature (22°C) for 1 h in the dark. Finally, luminescence was measured with a luminometer (Mediators Phl; Mediators Diagnostika GmbH, Vienna, Austria) according to the manufacturer's instructions. Each sample was quantified twice and the mean value and standard deviations were calculated.

Since these caspases are characterized by similar substrate specificities to those of caspase -3 and -7, which causes cross reactions in the measurement, the specific caspase -3/7 inhibitor Ac-DEVD-CHO (Promega) (18) was added at a concentration of 10 nM, 5 nM and 60 nM for caspase -9, -6 and -2 measurements, respectively (26, 27). The effect of the specific inhibitor was tested on KRJ-I cells by evaluating caspase 3/7 activity with or without the added inhibitor. For this purpose the KRJ-I cells were seeded into a 24 well plate and treated with 25 µg/ml plant extract. One group was co-treated with specific caspase -3/7 inhibitor and compared to the control group without the specific inhibitor. The caspase -3/7 activity was measured as described above using the Promega luminescent assay kit according to manufacturer's instructions.

**Transmission electron microscopy.** KRJ-I and HF-SAR cells were cultured in a 6-well plate and treated with TG-F28 10 µg/ml for 48 h at 37°C, negative controls were treated with PBSA (control 1) or DMSO (control 2). Additionally, the specific caspase -3/7 inhibitor Ac-DEVD-CHO (60 nM) was added to half of the treated samples to inhibit apoptotic changes. Harvested cells were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, (Plano, Wetzlar, Germany) on ice for 24 h. The cells were postfixed in 1%

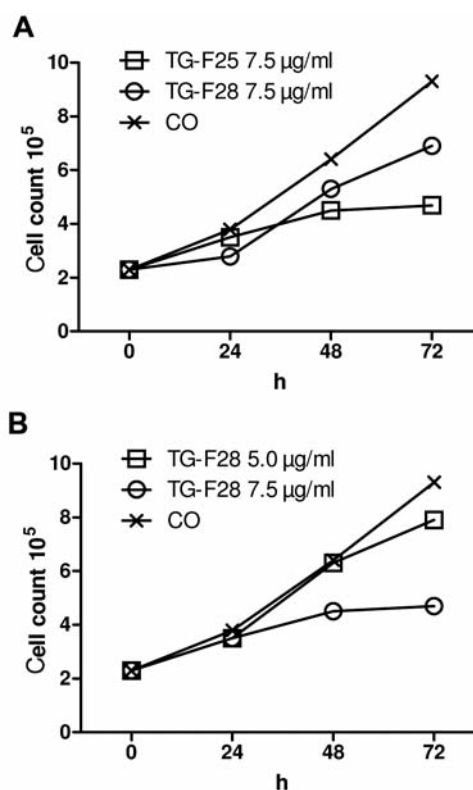


Figure 1. Antiproliferative effects of *T. gracilis* extracts on KRJ-I cells. Cell proliferation was evaluated using the CASY®-1 Cell Counter & Analyzer. A: Subfractions of *T. gracilis*, TG-F25 and TG-F28. B: Different concentrations of *T. gracilis* subfraction TG-F28; Control: DMSO alone. All data are expressed as mean±SD.

osmium tetroxide (Sigma-Aldrich) in 0.1 M cacodylate buffer and processed by modified routine methods. Samples were viewed and photographed with a FEI Technai12™ (FEI Europe, Eindhoven, the Netherlands) equipped with a Gatan CCD Camera Bioscan (Gatan, Munich, Germany).

#### Treatment of carcinoid tumor xenografts in SCID mice with TG-F28.

In order to create transplantable tumors, 14-week-old female severe combined immunodeficiency (SCID) mice (n=15) (Division of Laboratory Animal Science and Genetics, Medical University of Vienna, Himberg, Austria), were injected with  $3 \times 10^7$  KRJ-I cells/mouse suspended in 0.3 ml PBSA/mouse and transplanted subcutaneously into the flanks (12). Tumors originated at the site of injection, but no metastases were found. Fragments of these tumors (3 mm diameter) were serially transplanted. After the tumor diameters had reached approximately 10 mm, compounds were injected intratumorally: a) 9 mice were injected with 100 µg/100 µl TG28 dissolved in DMSO/PBSA per tumor, b) 5 mice were injected with DMSO/ PBSA (control). Twice a week, the diameters of the tumors were measured at three sites of each tumor by using a sliding caliper and tumor volume was calculated, respectively. The observation period was 21 days. The mice were killed at this time for ethical reasons when the tumors grew extremely rapidly and when necroses of the skin became apparent.

**Statistical evaluation.** All the statistical analyses were performed using Microsoft Excel and Prism 4 (GraphPad Software, San Diego, CA, USA). Sigmoidal dose responses were calculated to identify half-maximal inhibitory ( $IC_{50}$ ) concentration for the tested compounds.

**Results**

**Effects of *T. gracilis* on cell proliferation in KRJ-I cells.** In a preliminary screening, a DCM-extract of *T. gracilis* (TG-5) as well as three fractions obtained by liquid-liquid partition, TG-1, TG-2 and TG-3 were evaluated for their antiproliferative potential by cell counting. A strong antiproliferative effect had been noted from fraction TG-2 at a concentration of 25  $\mu\text{g/ml}$  (data not shown) and 15 subfractions of crude TG-2 were then separated for further investigation. To determine the most active fraction, each subfraction was tested for antiproliferative effects at a concentration of 10  $\mu\text{g/ml}$  and 25  $\mu\text{g/ml}$  (data not shown), and with TG-F24, TG-F25 and TG-F28 the subfractions with the most antiproliferative activity were identified and studied in more detail at different plant extract concentrations. KRJ-I cells treated with TG-F28 (7.5  $\mu\text{g/ml}$ ) and TG-F25 (7.5  $\mu\text{g/ml}$ ) reached a cell density of  $4.7 \times 10^5$  cells/ml and  $6.3 \times 10^5$  cells/ml after 72 h of incubation, while a cell density of  $9.3 \times 10^5$  cells/ml was noted in the control cells treated with DMSO alone (Figure 1A). A dose-dependent antiproliferative effect was evident in the KRJ-I cells treated with TG-F28 for 24, 48 and 72 h (Figure 1B).

To confirm the antiproliferative effects, cell viability was quantified using the WST-1 cell proliferation reagent. A dose-dependent reduction in mitochondrial activity was noted in the KRJ-I cells treated with TG-F24, TG-F25 and TG-F28 at a concentration of 7.5  $\mu\text{g/ml}$  (Figure 2A, 2B) and the  $IC_{50}$  of the most active subfractions, TG-F24 ( $IC_{50}=9.1 \times 10^{-6}$ ) and TG-F28 ( $IC_{50}=7.12 \times 10^{-6}$ ), was determined (Figure 2C).

Using light microscopy, alterations in KRJ-I cell morphology were observed after 48 h of treatment with TG-F24 (7.5  $\mu\text{g/ml}$ ) and TG-F28 (7.5  $\mu\text{g/ml}$ ). Besides a dissociation of cell aggregation, an increase in both dead cells and cell debris was noted in the treated KRJ-I cells compared to the untreated control (Figure 3 A-F).

**Induction of apoptosis in KRJ-I cells by *T. gracilis* TG-2 subfractions.** To examine whether the apoptotic pathway was involved, DAPI staining was performed in KRJ-I cells treated with all fifteen subfractions. After 48 h treatment with the plant extracts, chromatin condensation, nuclear pyknosis, increased number of nuclear body fragments and irregular edges around the nucleus were observed in treated KRJ-I cells, while round, clear edged, uniformly stained cell nuclei were noted in the untreated control (Figure 4 A-D).

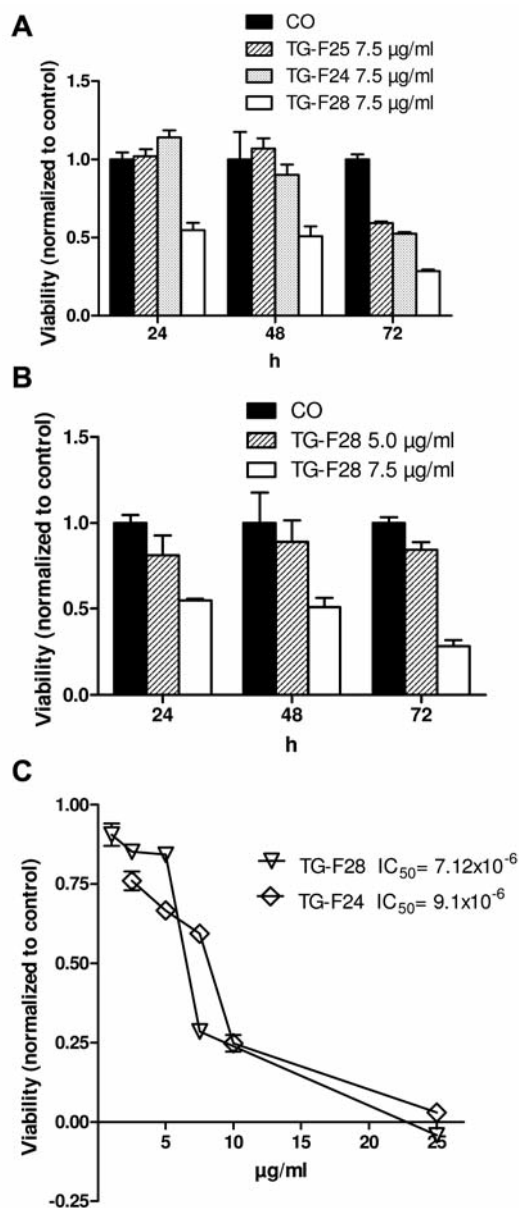


Figure 2. Effects of *T. gracilis* extracts on KRJ-I cell viability quantified using the WST-1 assay. A: *T. gracilis* subfractions TG-F24, TG-F25 and TG-F28; control: DMSO alone. B: Dose-dependent effects of TG-F28; control: DMSO alone. C:  $IC_{50}$  values of subfractions TG-F28 ( $IC_{50}=7.12 \times 10^{-6}$ ) and TG-F25 ( $IC_{50}=9.1 \times 10^{-6}$ ) quantified after 72 h of treatment. All data are expressed as mean  $\pm$  SD.

**Induction of caspase activity in KRJ-I cells by *T. gracilis* TG-2 subfractions.** To confirm the involvement of the apoptosis pathway in inhibition of KRJ-I cell proliferation, activated forms of the effector caspases 3, 6 and 7 and initiator caspases 8, 9 and 2 were analyzed.

**Caspase 3/7:** Activated forms of caspase -3/7 were evaluated in KRJ-I cells treated with the fifteen subfractions. An increase of caspase -3/7 activity was noted in the KRJ-I

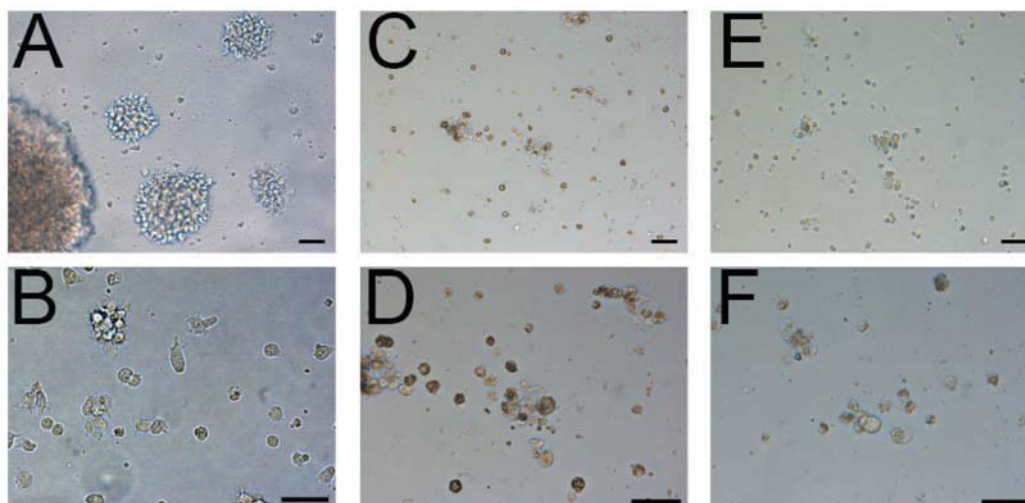


Figure 3. Effects of *T. gracilis* extracts on KRJ-I cell morphology. A-B: Control (DMSO alone): KRJ-I cells growing in clusters with only few single cells, no dead cells and little cell debris. C-F: *T. gracilis* subfractions: KRJ-I cells treated with 7.5 µg/ml TG-F28 (C, D) or 7.5 µg/ml TG-F24 (E, F) for 48 h (bar=50 µm).

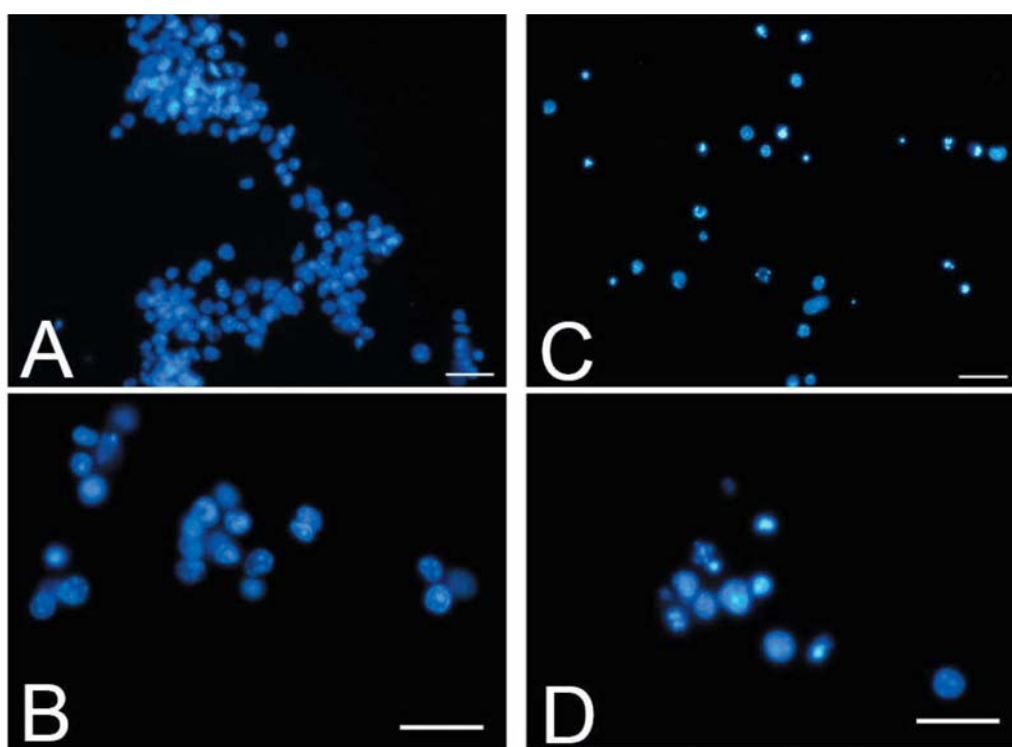


Figure 4. Effects of *T. gracilis* extract TG-F28 on apoptosis in KRJ-I cells. Nuclear staining was performed after 48 h treatment using DAPI. A, B: Control (DMSO alone): Round and clear edged cell nuclei. C, D: TG-F28 (10 µg/ml): Increased number of nuclear body fragments and irregular edges around the nucleus were noted (bar=50 µm).

cells treated with TG-F24, TG-F25 and TG-F28 (10 µg/ml) for 4, 8, 12 or 24 h compared to the untreated control (Figure 5). With TG-F28, the strongest subfraction was determined and used for studying the time response of effector as well as initiator caspases.

*Time response of effector caspases:* To evaluate the time response of effector caspases, the activity of caspase -3/7 (10 µg/ml) and -6 (10 µg/ml) was determined every three h. Activated caspase -3/7 was induced in the treated KRJ-I cells with a maximum at 19 h; similar levels were noted compared to

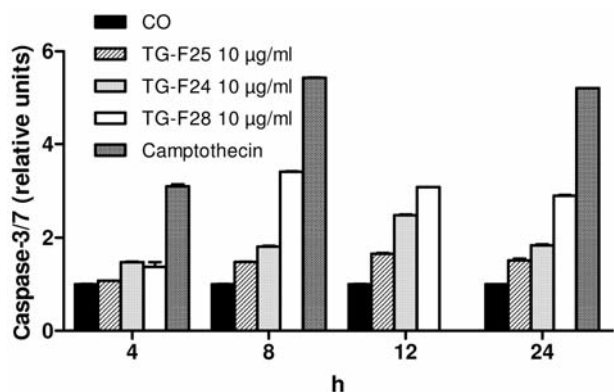


Figure 5. Effects of *T. gracilis* extracts on caspase -3/7 activity in KRJ-I cells determined by luminescence based assay. Positive control: Camptothecin (5 µM); negative control: DMSO solvent alone.

CPT (5 µM) (Figure 6A). Additionally, caspase-6 was enhanced, with a maximum increase at 25 h compared to the untreated control in the presence of Ac-DEVD-CHO (18) (Figure 6B).

*Time response of initiator caspases:* An increase of caspase-8 activity was noted in the KRJ-I cells treated with TG-F28 (10 µg/ml) for 30 h, with a maximum at 22 h (data not shown). Using the specific caspase -3/7 inhibitor Ac-DEVD-CHO, a similar increase in caspase-9 was observed, with a maximum at 15 h (Figure 7A). TG-F28 induction of caspases 8 and 9 showed similar levels compared to the positive control, CPT. Additionally, activated caspase-2 was evaluated, showing a maximum 1.7-fold increase at 24 h in the presence of Ac-DEVD-CHO (Figure 7B).

*Effects of T. gracilis subfraction TG-F28 on KRJ-I cells compared to normal human fibroblasts.* WST-1 assay and Caspase -3/7 and -6 activities were quantified in KRJ-I and HF-SAR cells, both treated with TG-F28 (10 µg/ml) for 24, 48 or 72 h. Treated HF-SAR cells showed no reduction of cell viability and no induction of caspases 3, 6 and 7 compared to untreated control, whereas a decrease of cell viability and increase in caspase activities was noted in the treated KRJ-I cells (Figure 8A-C).

*Electron microscopy.* KRJ-I cells treated with TG-F28 (10 µg/ml) showed early apoptotic alterations, including lobulated or deeply cleaved cell nuclei. Additionally, the cytoplasm contained a large number of vacuoles (Figure 9A). Treatment with TG-F28 (10 µg/ml) in combination with Ac-DEVD-CHO (60 nM) showed a lower degree of apoptosis. Fewer vacuoles were noted and the nuclei were less impaired compared to the TG-F28 treated KRJ-I cells without the inhibitor (Figure 9B). The characteristic morphology of round cells with oval or lobulated nuclei were observed in PBSA treated KRJ-I cells used as control (Figure 9C). Identical morphology was noted in KRJ-I cells treated with

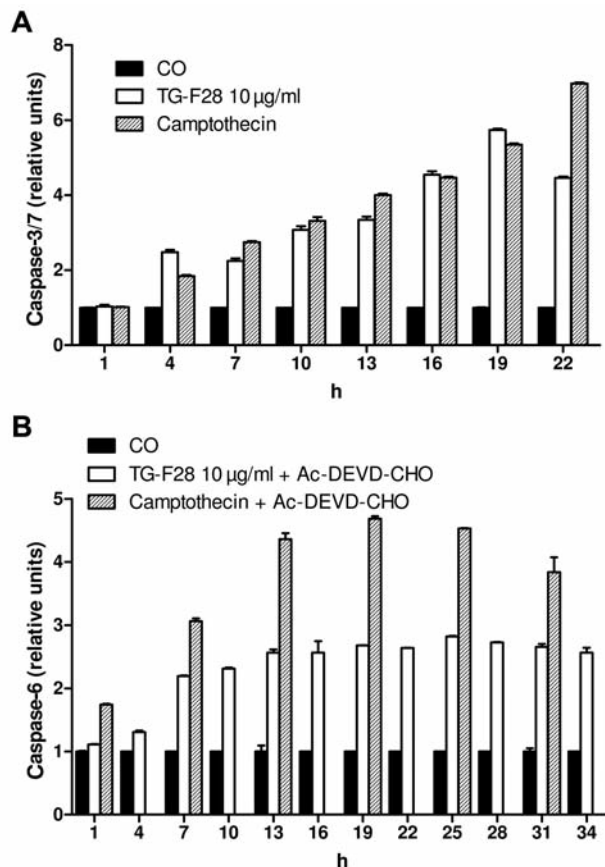


Figure 6. Time-dependent activation of effector caspases 3/7 (A) and 6 (B) in KRJ-I cells, quantified by luminescence based assay with the specific caspase -3/7 inhibitor Ac-DEVD-CHO. Positive control: Camptothecin (5 µM), negative control: DMSO alone.

DMSO (Figure 9D). No modification in ultrastructure was observed in TG-F28 (10 µg/ml) treated human fibroblast HF-SAR cells (data not shown).

*Treatment of carcinoid xenografts in SCID mice with TG-F28.* The tumor volumes were evaluated three times (day 0, 9 and 14) in TG-F28 (100 µg)-treated tumor-bearing SCID mice. A significant inhibition of tumor growth was noted after 9 days (74±22%) and 14 days (62±18%) of TG-F28 treatment compared to the untreated control (Figure 10A). Differences of the measured tumor volumes were calculated and a significant decrease in tumor progression was noted between day 0 to 9 (66±21%), day 9 to 14 (43±29%) and day 0 to 14 (55±19%) compared to the untreated control (Figure 10B).

## Discussion

The predominant aims of analyzing crude plant extracts are either to isolate bioactive agents for direct use as drugs or to identify bioactive compounds that can be used as lead

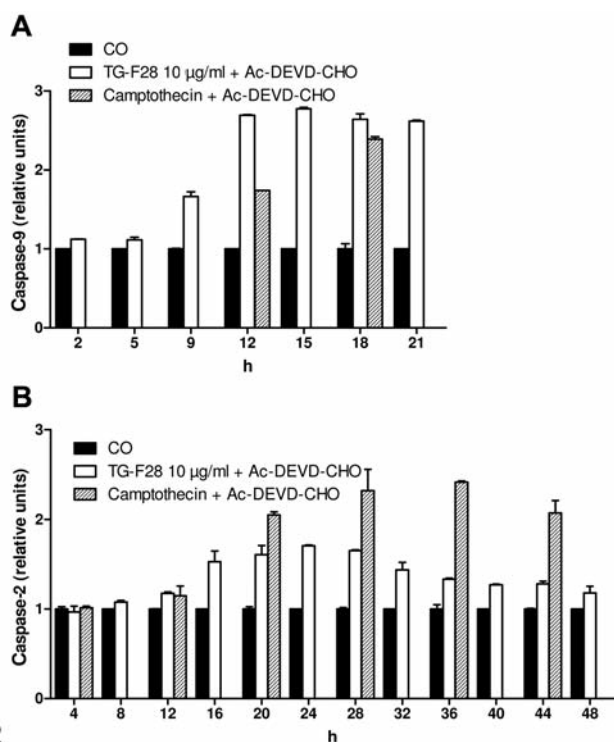


Figure 7. Time-dependent activation of initiator caspases 9 and 2 in KRJ-I cells with the specific caspase-3/7 inhibitor Ac-DEVD-CHO. Positive control: Camptothecin (5 µM), negative control: DMSO alone. A: Increase of activated caspase 9 with a maximum at 15 h (2.7-fold). B: Caspase-2 levels in treated KRJ-I cells, with a maximum at 24 h (1.7-fold), compared to untreated control.

substance in the preparation of semisynthetic drugs. In this study, we demonstrate the anticancer potential of *T. gracilis* subfractions in a well-characterized SI-NET cell line.

Tumor cell growth was inhibited by all 15 subfractions of TG-2 in a dose-dependent manner. The fractions with the strongest tumorstatic activity were the ursolic acid containing subfraction TG-F24, TG-F25 and TG-F28.

As the cell's intrinsic cell death program, apoptosis plays a key role in growth control of cells and tissue homeostasis and consequently, an imbalance or inactivation of important pathways can result in tumor formation and progression (28). Furthermore, cytotoxic therapies in anticancer treatment, e.g. chemotherapy,  $\gamma$ -irradiation, immunotherapy or suicide gene therapy, are mainly dependent on the function of cell apoptosis (29-31) and many tumors develop different escape mechanisms that subsequently result in drug resistance (32, 33). Therefore, the induction and recovery of the apoptotic response in tumor cells are relevant steps in anticancer treatment.

A complex cascade of cell signaling interactions is involved in the induction of the apoptotic pathway of a cell, while caspases – the main regulators of apoptotic cell death (34, 35) – are the most viable approach to determine the

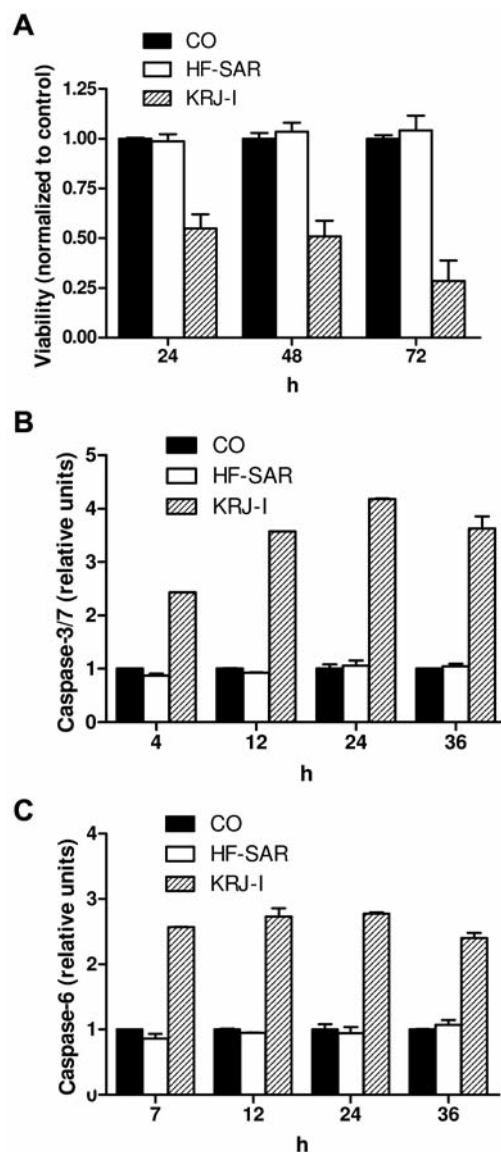


Figure 8. Comparison of *T. gracilis* effects on normal human fibroblast HF-SAR and KRJ-I cells. The cells were treated with TG-F28 (10 µg/ml) or DMSO control. Cell viability was analyzed using WST-1 assay, activated caspase-3/7 and -6 were quantified using a luminescence based assay. A: Decrease of KRJ-I cell viability after treatment with TG-F28, while HF-SAR were not impaired. B, C: Caspase-3/7 and caspase 6 activities were induced in treated KRJ-I cells with a maximum at 24 h (4.1 fold, 2.8 fold), while no increase of activated caspase-3/7 and -6 was observed in treated HF-SAR cells.

apoptotic changes (36). In the present study, caspase activation was involved in the antiproliferative effects of the *T. gracilis* extracts. The increase of the activated effector caspases 3, 6 and 7 suggested an efficient induction of the apoptotic pathway. To ensure that the apoptosis pathway was functional in the KRJ-I cells, camptothecin, a known initiator of apoptosis (25), was used as a positive control.

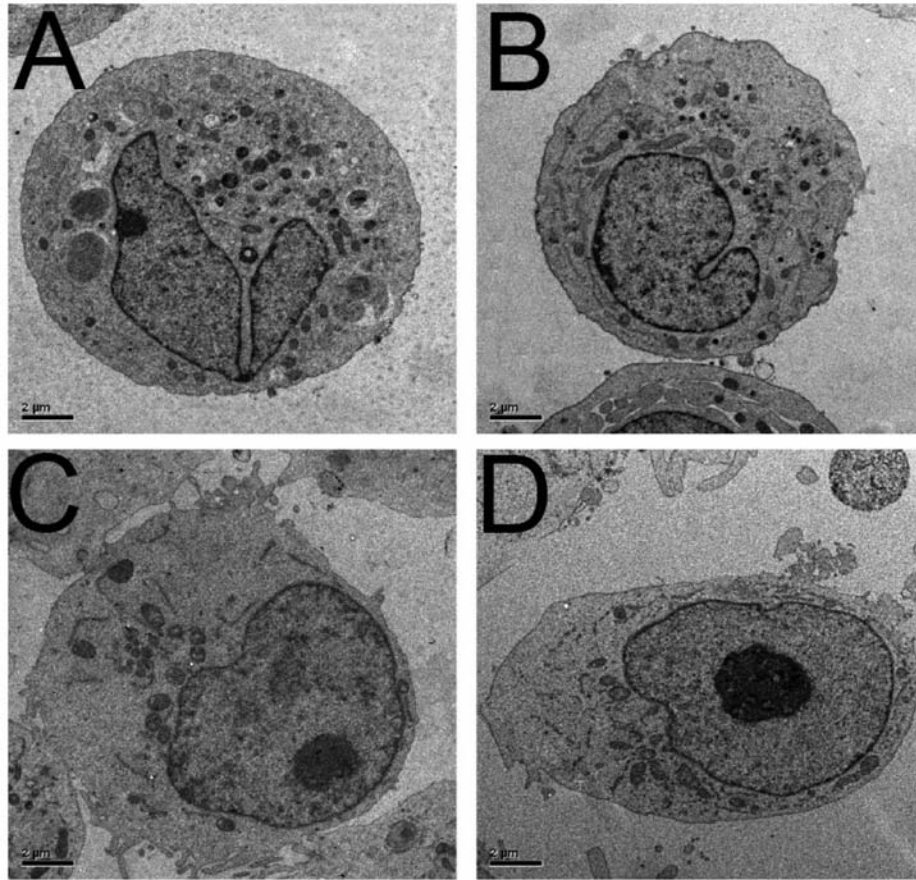


Figure 9. Ultrastructure of *T. gracilis*-treated KRJ-1 cells. A: TG-F-28 (10 µg/ml): Cleavage of the nucleus and the formation of many vacuoles, indicating an early stage of apoptosis. B: TGF-28 (10 µg/ml) and the specific caspase-3/7 inhibitor Ac-DEVD-CHO (60 nM), showing a lower number of vacuoles. C: Control treated with PBSA: Intact KRJ-1 cell, characteristic oval nucleus with a large single nucleolus. D: Control treated with DMSO: Morphology unimpaired and identical to the untreated control (C) (bar=2 µm).

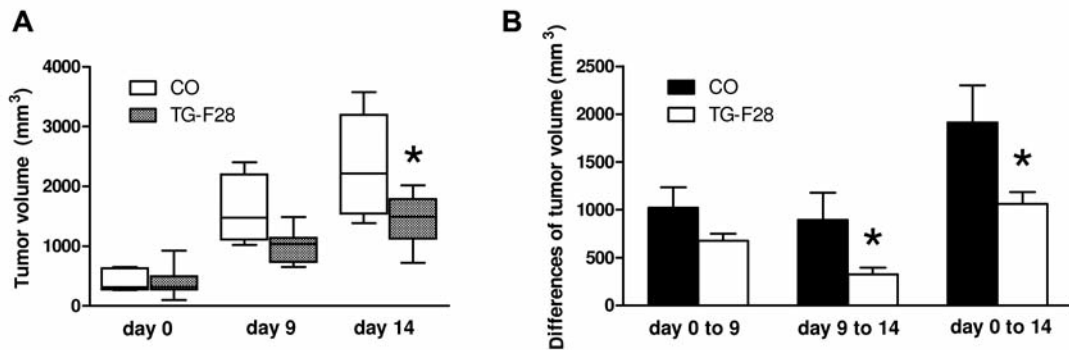


Figure 10. Effects of *T. gracilis* (TG-F28 100 µg/ml) in carcinoid xenograft SCID mice. A: A significant inhibition of tumor growth was noted in tumor bearing SCID mice after 9 and 14 d treatment with TG-F28. B: Differences of tumor growth within 9-, 5- and 14-day periods compared to untreated control. Mean±SEM; N=13, \*p<0.05.

To further define the mechanism of action of the plant extracts, levels of the initiator caspases 8, 9 and 2 were quantified. Initiator caspases are responsible for the initiation of the caspase cascade and an extrinsic cell receptor mediated

pathway can be distinguished from an intrinsic pathway triggered by the release of apoptogenic factors from intracellular compartments (37). While ligation of death receptors, e.g. CD95, TNF-related apoptosis-inducing ligand receptors



(TRAIL) and tumor necrosis factor receptors (TNF-Rs) (38, 39), is followed by activation of initiator caspase 8, the release of mitochondrial apoptogenic factor (cytochrome *c*, the bcl-2 family) (40, 41), nuclear damage (42, 43) and endoplasmatic reticulum stress (44) increase the activity of initiator caspase-9. Both pathways converge at the proteolytic activation of effector caspases 3, 6 and 7. The *T. gracilis* extracts increased activated caspase-8, -9 and -2 levels in treated KRJ-I cells, suggesting that the *T. gracilis* extracts induced the intrinsic pathway *via* apoptogenic factors and caspase-9, although activity of caspase-8 was increased. It is known that caspase-8 amplifies the apoptotic signal by cleavage of the B-cell lymphoma 2 family member *bid* (45, 46). As a result, mitochondrial cytochrome *c* is released followed by the formation of the apoptosome, a complex consisting of cytochrome *c*/ apoptotic peptidase activating factor 1/ caspase-9. Interestingly, an increase of caspase-8 was noted in tumor cells after treatment with camptothecin, which is known to induce the intrinsic apoptosis pathway *via* caspase 9 by inhibition of topoisomerase I (25). Because of a close homology of the specific caspase-8 substrate and the caspase-3/7 inhibitor Ac-DEVD-CHO (26), active caspase-8 was quantified without the caspase 3/7 inhibitor. These findings suggested an unspecific cross-reaction of effector caspases 3 and 7 in the measurement of caspase-8.

An ideal anticancer drug should specifically target the malignant cells while non-malignant cells in the body should not be impaired. While this is unfortunately rarely achieved, the present data demonstrated that a pro-apoptotic effect of the plant subfractions was only noted in the tumor cells without impairing the normal HF-SAR cells. These findings suggested a tumor-specific effect.

Ultrastructural observations confirmed the selective induction of apoptosis in the treated KRJ-I cells, while the two control groups with PBSA- or DMSO-treatment remained unimpaired. Especially, the cleavage of the nuclei and the accumulation and increase of vacuoles indicated the induction of apoptosis in the TG-F28 treated group. The apoptotic alterations were partially inhibited by the addition of the specific inhibitor Ac-DEVD-CHO. Interestingly, a complete “*restitutio ad integrum*” was not noted. This implied that another cellular pathway might be involved in the antiproliferative effect induced by the subfractions of *T. gracilis*.

The TG-F28 treatment of carcinoid xenograft SCID mice showed a significant reduction of tumor growth compared to the untreated controls. These *in vivo* data implicated a strong antiproliferative effect of *T. gracilis* subfraction TG-F28 and confirmed the *in vitro* findings in the SI-NET cell line KRJ-I.

In conclusion, inducing apoptosis by novel bioactive compounds of *T. gracilis* reduces tumor cell proliferation in a dose-dependent way without impairing normal cells. Thus, extracts of *T. gracilis* could potentially be a new therapeutic option in anticancer treatment for SI-NETs.

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