Original Article

Antitumor activity of an *Artemisia annua* herbal preparation and identification of active ingredients

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**ABSTRACT**

**Background:** *Artemisia annua* L. has gained increasing attention for its anticancer activity. However, beside artemisinin, little is known about the possible bioactive ingredients of *Artemisia annua* and respective herbal preparations. We hypothesized that, in addition to artemisinin, *Artemisia annua* preparations might contain multiple ingredients with potential anticancer activity.

**Methods:** MDA-MB-231 triple negative human breast cancer (TNBC) cells along with other treatment resistant, metastatic cancer cell lines were used to investigate in vitro and in vivo the anticancer efficacy of an *Artemisia annua* extract marketed as a herbal preparation, which contained no detectable artemisinin (limit of detection = 0.2 ng/mg). The extract was characterized by HPLC-DAD and the most abundant compounds were identified by 1H- and 13C NMR spectroscopy and quantified by UHPLC-MS/MS. Cell viability and various apoptotic parameters were quantified by flow cytometry. In vitro data were validated in two in vivo cancer models, the chick chorioallantoic membrane (CAM) assay and in orthotopic breast cancer xenografts in nude mice.

**Results:** The *Artemisia annua* extract, the activity of which could be enhanced by acetonitrile maceration, inhibited the viability of breast (MDA-MB-231 and MCF-7), pancreas (MIA PaCa-2), prostate (PC-3), non-small-cell lung cancer (A459) cells, whereas normal mammary epithelial cells, lymphocytes, and PBMC were relatively resistant to extract treatment. Likewise, the extract’s most abundant ingredients, chrysosplenol D, arteannuin B, and casticin, but not arteannuic acid or 6,7-dimethoxycoumarin, inhibited the viability of MDA-MB-231 breast cancer cells. The extract induced accumulation of multinucleated cancer cells within 24 h of treatment, increased the number of cells in the S and G2/M phases of the cell cycle, followed by loss of mitochondrial membrane potential, caspase 3 activation, and formation of an apoptotic hypodiploid cell population. Further, the extract inhibited cancer cell proliferation, decreased tumor growth, and induced apoptosis in vivo in TNBC MDA-MB-231 xenografts grown on CAM as well as in nude mice.

**Conclusion:** An extract of an artemisinin-deficient *Artemisia annua* herbal preparation exhibits potent anticancer activity against triple negative human breast cancer. New active ingredients of *Artemisia annua* extract with potential anticancer activity have been identified.

**Abbreviations:** ACN, acetonitrile; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ATCC, American Type Culture Collection; CAM, chick chorioallantoic membrane; ΔVm, mitochondrial membrane potential; CFSE, carboxyfluorescein diacetate succinimidyl ester; DAD, diode-array detector; DMEM, Dulbecco’s Modified Eagle Medium; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; ESI, electrospray ionization; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HE, hematoxylin and eosin staining; HP–β–CD, (2-hydroxypropyl)-β-cyclodextrin; HPLC-MS, high-performance liquid chromatography-mass spectrometry; IC₅₀, half maximal inhibitory concentration; LOD, limit of detection; NMR, nuclear magnetic resonance; NSCLC, non-small cell lung cancer; PBS, phosphate buffered saline; PBMC, peripheral blood mononuclear cells; ROS, reactive oxygen species; RT, room temperature; TNBC, triple negative human breast cancer; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; UHPLC-MS/MS, ultra high performance liquid chromatography - tandem mass spectrometry

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Introduction

The medicinal plant *Artemisia annua* L. is traditionally used for the treatment of malaria throughout Asia and Africa in form of tea or press juice. The sesquiterpene lactone artemisinin was identified as the active principle of *Artemisia annua* extracts (Slezakova and Ruda-Kucerova, 2017). In recent years, *Artemisia annua* preparations gained increasing attention for their anticancer properties. Due to poor bioavailability of artemisinin, mostly, semisynthetic derivatives of artemisinin were investigated for their efficacy towards cancer and underlying molecular modes of action. Indeed, some of them are presently being tested in clinical studies (Efferth, 2017). However, available evidence suggests that artemisinin might not be the most active anticancer ingredient of this medicinal plant (Efferth et al., 2011; Ferreira et al., 2010). The plant contains a multiplicity of other biologically active substances (Ferreira et al., 2010; Wang et al., 2009) suggesting that *Artemisia annua* might be a source of new herbal anticancer therapeutics. In general, less is known about *Artemisia annua* extracts even though there are some positive case reports about the application of *Artemisia annua* extracts marketed as herbal preparations in people and pets suffering from malignant diseases (Breuer and Efferth, 2014; Efferth, 2017).

![Diagram of extract preparation and analysis](image-url)

**Fig. 1.** Fractionation and analytical characterization of Momundo *Artemisia annua* extracts. (A) Schematic presentation of extract preparation. ACN, acetonitrile. (B) HPLC-DAD fingerprint of Momundo and Momundo-ACN extracts at 210 nm and chemical structures of identified compounds. (C) Quantification of isolated compounds by UHPLC-MS/MS, mean ± SD, n = 3. (D) Momundo extracts do not contain artemisinin as analyzed by HPLC-MS/MS.
Natural products have been and are still considered as a very important source for the identification of novel molecular structures with potential pharmacotherapeutic value (Atanassov et al., 2015; Koehn and Carter, 2005; Nosrati et al., 2018). Hence, we have chemically characterized and fractionated an Artemisia annua extract marketed as herbal preparation. Moreover, we have identified the most abundant Carter, 2005; Nosrati et al., 2018). Hence, we have chemically characterized and fractionated an Artemisia annua extract marketed as herbal preparation. Moreover, we have identified the most abundant components of the extract and demonstrate verifiably that it contains no artemisinin. We have further analyzed the Artemisia annua extract for its activity against the highly metastatic triple negative breast cancer (TNBC) cells with the intent to prove its therapeutic efficacy and identify active agents different from artemisinin.

Materials and methods

General experimental procedures

Momundo Artemisia annua extract (PZN 5466281) was obtained from MoMundo GmbH (Bad Ems, Germany). Artemannin B and artemannic acid were from Carbosynth (Berkshire, UK), casticin and 6,7-dimethoxycoumarin from Extrasynthese (Genay cedex, France), and chrysosplenol D from ChemFaves (Wuhan, Hubei, China). Stock solutions were prepared in dimethyl sulfoxide (DMSO) and further diluted with medium. The final DMSO concentration in the medium was 0.5% in all experiments. Propidium iodide, DNase-free RNase A, (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD), paclitaxel, and doxorubicin hydrochloride were from Sigma (St. Louis, MO). XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) cell proliferation assay was purchased from Roche Diagnostics (Filderstadt, Germany). Caspase 3/7 substrate (Z-DEVD-R110) was obtained from Bachem (Bubendorf, Switzerland) and the mitochondrial potential sensor JC-1 was from Molecular Probes (San Diego, CA).

For xenograft treatment in mice, HP-β-CD complexes with Moments extract were formed. For this, the extract was dissolved in ethanol/water (1:1, v/v) and HP-β-CD was dissolved in ethanol. Both solutions were mixed in a mass ratio of 1:11 for 2.5 h and were vacuum concentrated followed by lyophilization. The complexes were used for in vivo application of Moments extract in 0.9% NaCl.

Extracts

Momundo extract obtained as commercial Moments capsules was directly dissolved in DMSO. Moments-ACN extract was prepared by maceration of 80 mg/ml capsule content in acetonitrile for 1 h at RT during continuously stirring. After centrifugation, the supernatant was transferred into a new tube and the solvent was evaporated under a stream of nitrogen (Fig. 1A).

Fractionation and analytical characterization of Moments extracts

Fingerprint characterization and fractionation of Artemisia annua extracts were done by semipreparative HPLC. The HPLC system consisted of a low gradient LC-9A Shimadzu pump (Kyoto, Japan), an automatic sample injector Aspec XL (Abimed, Langenfeld, Germany), a column oven IWN CH100 (Junedis, Gröbenzell, Germany), a photodiode array detector UVD 340 U (Dionex, Idstein, Germany), and a fraction collector (Gilton, Limburg-Offheim, Germany) connected to a computer running Chromelon Software version 6.6 (Dionex). For separation and fractionation, a precolumn (Phenomenex, SecurityGuard, C18, 4 × 3 mm) and a semi-preparative column (Phenomenex, Synergi Hydro-RP, 4 µm, 80 Å, 250 × 10 mm) were used. 100 mg/ml Moments-ACN extract was dissolved in DMSO and filtered through a 0.45 µm membrane filter before injection. The flow rate was 5000 µl/min and the injection volume was 200 µl. The photodiode-array detector was set at 210 nm for absorbance tracing. The elution gradient consisted of eluent A (deionized, ultrapure water + 0.05% formic acid) and eluent B (acetonitrile + 0.05% formic acid). The details of the procedure are described in the Data in Brief article.

The fractions corresponding to the major peaks, a, b, c, d, e (Fig. 1B), were collected and the solvent was removed. Structures were identified by HPLC-MS/MS by comparison of retention times, mass spectra, and fragmentation mass spectra with reference standards. Peak b (chrysosplenol D) was subjected additionally to 1H- and 13C NMR spectroscopy with a bruker DRX 500 NMR spectrometer. The HPLC-MS/MS analysis were performed on an Agilent 1260 Infinity system (Agilent, Santa Clara, CA) coupled with an AB API 2000 (Applied Biosystems, Foster City, CA) triquadrupole mass spectrometer through an electrospray ionization (ESI) source. The data collected were processed with Analyst 1.6.1 software (Ab Sciex, Framingham, MA).

Quantification of the major ingredients was performed using an analytical UHPLC column (Dr. Maisch ReproSil-Pur Basic-C18, 1.9 µm, 75 × 2 mm) with a precolumn (Phenomenex, SecurityGuard, C18, 4 × 2 mm). The flow rate was 350 µl/min and the injection volume was 2 µl. The mobile phase consisted of eluent A (ultrapure water + 0.05% formic acid) and eluent B (acetonitrile + 0.05% formic acid). Initial conditions were 70% eluent A and 30% eluent B, followed by a linear gradient to 95% eluent B over 6.9 min, then 95% eluent B until 91.1 min. Thereafter, a linear gradient to initial conditions until 9.5 min and re-equilibration until 12.5 min were applied. In order to stabilize the chromatographic system, the column was kept at a temperature of 28 °C. MS/MS analysis was performed in the positive atmospheric pressure ESI mode and multiple-reaction monitoring (MRM) detection mode. The precursor ion at m/z 207.1 and the product ion of highest intensity at m/z 151.1 were selected for 6,7-dimethoxycoumarin. Similarly, the ions at m/z 361.3 and 327.8 were used for chrysosplenol D, m/z 375.4 and 342.0 were used for casticin and m/z 249.3 and 142.9 were used for artannuin B. Artemannic acid was analyzed in the negative ionization mode and in the selected ion monitoring (SIM) detection mode at m/z 233.2. The quantification of the compounds was performed using an external calibration method. Details of characterization and validation data are described in the Data in Brief article.

Cell culture

MDA-MB-231 cells stably expressing firefly luciferase from Cell Biosets, Inc. (San Diego, CA) were cultured in Dulbecco’s Modified Eagle Medium (DMEM, 4.5 g/l glucose, GlutaMax; Life Technologies, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.1 mM MEM non-essential amino acids, 2 mM L-glutamine, 100 µM penicillin, and 100 mg/ml streptomycin. Normal mammary epithelial cell lines hTERT-HME1 from American Type Culture Collection (ATCC, Rockville, MD) were cultured in MEGM medium (Lonza, Basel, Switzerland). The additionally used cell lines MCF-7, MIA PaCa-2, PC-3, and A549 were obtained from ATCC and cultured according to the supplier’s recommendations. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood of healthy donors by density gradient centrifugation using Biocool (Biochrom GmbH, Berlin, Germany) and cultured in RPMI 1640 medium, 2 mM L-glutamine (Life Technologies, Carlsbad, CA) supplemented with 10% FCS and penicillin/streptomycin (Li et al., 2012). For analysis of lymphocyte proliferation, CFSE-stained cells were treated with the extract, stimulated with anti-CD3/CD28 antibodies for 72 h, and analyzed by flow cytometry as described previously (Steinborn et al., 2018). Experiments using human blood cells have been approved by the Institutional Ethics Committee.

Analysis of cell viability

Cell viability after treatment with extracts, pure compounds, and doxorubicin was analyzed by XTT assay according to the manufacturer’s instructions (Roche). Absorbance was measured using an Infinite M1000 PRO Tecan plate reader at 450 nm with a 630 nm reference.
Cell cycle analysis

MDA-MB-231 cells treated with Momundo, Momundo-ACN, paclitaxel, or 0.5% DMSO were harvested and fixed with 70% ice-cold ethanol overnight. DNA was stained with propidium iodide in a buffer containing DNase-free RNase A (Morad et al., 2011) and cells were analyzed by flow cytometry using a FACSVerse cytometer (Becton Dickinson, Heidelberg, Germany) and FlowJo software (FlowJo LLC, Ashland, OR). Cells treated as above, but for 24 h, were stained with Alexa Fluor® 488 α-tubulin antibody (Cell Signaling Technology, Danvers, MA).

Fig. 2. Momundo Artemisia annua extract inhibits cell cycle progression and induces cytokinesis failure in breast cancer cells. (A) MDA-MB-231 triple negative breast cancer cells were treated with Momundo (100 µg/ml), Momundo-ACN (30 µg/ml), or paclitaxel (100 nM) for 24 h, stained with anti-tubulin antibody and Hoechst 33342, and analyzed microscopically. Graph shows percentage of multinucleated cells. (B) Cells were treated with 100 µg/ml of either extract or paclitaxel (100 nM) for 48 h, stained with propidium iodide, and analyzed by flow cytometry. (C) Momundo-ACN induces formation of polyploid (≥ 8 N) cells. Cells were treated as in (A) and were analyzed by flow cytometry. Data are mean ± SEM, n = 3, *p < 0.05, **p < 0.01, ***p < 0.001.
Danvers, MA) and Hoechst 33342 (El Gaafary et al., 2017) and imaged with a Ti-E inverse fluorescence microscope (Nikon, Düsseldorf, Germany) using x40 objective.

Analysis of apoptosis

Loss of mitochondrial potential ($\Delta \Psi _{m}$) was analyzed flow cytometrically using the lipophilic cationic JC-1 dye, which accumulates in mitochondria in a potential-dependent manner (El Gaafary et al., 2015). Cells treated with Momundo extract or paclitaxel for 48 and 72 h were incubated with 10 $\mu$g/ml JC-1 dye in DMEM (4.5 g/l glucose) for 20 min at 37 °C. $\Delta \Psi _{m}$ loss was analyzed by FlowJo software as a decrease in the red/green fluorescence intensity ratio. For the analysis of caspase 3/7 activation, cells were incubated for 1 h at 37 °C in the presence of 100 $\mu$M of fluorogenic caspase 3/7 substrate Z-DEVD-R110. The fluorescent caspase 3/7 product was detected by flow cytometry.

DNA fragmentation in vitro was analyzed by flow cytometry in MDA-MB-231 cells treated for 48 h, stained with propidium iodide using the protocol for cell cycle analysis.

Tumor xenografts

Animal experiments have been approved and permitted by the respective authorities and were conducted according to international and national guidelines. $1 \times 10^6$ MDA-MB-231 cells were grafted onto the chick chorioallantoic membrane (CAM) in medium/matrigel (1:1, v/v) 7 days after fertilization. The next 3 days, the xenografts were treated topically with 20 $\mu$l of the extracts or 0.5% DMSO. Tumor bioluminescence was measured 5 min after application of α-luciferin (0.75 mg/ml, 10 $\mu$l/egg) using an IVIS in vivo Imaging System (PerkinElmer, Waltham, MA) on day 4 of treatment. Afterwards, tumors were collected, fixed, and embedded in paraffin. For immunohistochemical analysis, 5 $\mu$m-sections were counterstained with hematoxylin, eosin and further analyzed using antibodies against proliferation marker Ki-67 (M7240, Dako, Glostrup, Denmark). For analysis of apoptosis, in vivo DNA strand breaks were visualized by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) according to the manufacturer’s protocol (Roche). Images were recorded with an Axio Lab.A1 microscope (Carl Zeiss, Göttingen, Germany) and a Zeiss 2/3″ CMOS camera using Progres Gryphax software (Carl Zeiss). Tumor volume ($mm^3$) was assessed using the formula: length (mm) x width² (mm) x $\pi/6$.

For xenotransplantation in mice, $0.5 \times 10^6$ MDA-MB-231 cells suspended in phosphate buffered saline were orthotopically implanted into the mammary gland of 8–9 weeks old female NMRI-Foxn1nu/nu mice (Janvier, Le Genest-St.-Isle, France). Starting from day 8 after xenotransplantation, mice (8 mice/group) were treated i.p. with 100 mg kg$^{-1}$ day$^{-1}$ Momundo-HP-β-CD, 2 mg kg$^{-1}$ week$^{-1}$ doxorubicin, or 1000 mg kg$^{-1}$ day$^{-1}$ solvent (HP-β-CD) for 3 weeks. Tumor volumes were calculated according to the formula

$$V = \frac{l \times w^2}{2}$$

Fig. 3. Momundo Artemisia annua extracts selectively inhibit viability of various cancer cells. (A) Momundo extracts inhibit viability of breast cancer cells MDA-MB-231, doxorubicin – positive control. (B) Momundo-ACN extract is cytotoxic to various treatment resistant cancer cell lines as analyzed by XTT. NSCLC, non-small cell lung cancer. (C) Normal mammmary epithelial cells and PBMC are relatively resistant to Momundo-ACN (48 h). (D) Lymphocyte proliferation is not affected by Momundo extract. Cells were stained with CFSE, treated with the extract, activated by CD3/CD28 antibodies for 72 h, and analyzed by flow cytometry. Cyclosporine A (5 $\mu$g/ml) served as positive control. All data are mean ± SEM, n = 3 - 5.
Blood collected by cardiac puncture of anesthetized animals at the end of experiment was analyzed by the clinical chemistry laboratory of the hospital for the presence of liver enzymes using standard methods.

### Statistical analysis

Results are expressed as mean ± SEM of at least three independent experiments. In case of two-group comparison, results were analyzed with the two-tailed Student’s *t*-test. Multi-group analysis was performed using the one-way analysis of variance, followed by Newman-Keuls post hoc test using SigmaPlot software. Significance levels were set as *p < 0.05, **p < 0.01, ***p < 0.001.

### Results

**Fractionation and chemical characterization of Momundo Artemisia annua extract**

Two *Artemisia annua* extracts, Momundo and acetonitrile-soluble fraction of Momundo (Momundo-ACN) were characterized by HPLC (Fig. 1B). The extracts contain the major ingredients (a) 6,7-dimethoxy-coumarin, (b) chrysosplenol D, (c) casticin, (d) arteannuin B, and (e) arteannuic acid (Fig. 1B). Fig. 1C shows the amounts of the identified compounds in the extracts, which were considerably higher in Momundo-ACN compared to Momundo. Artemisinin could not be detected in Momundo extracts (Fig. 1D).

**Momundo Artemisia annua extracts induce cell cycle arrest in breast cancer cells**

Morphological analysis by fluorescence microscopy demonstrates that already within 24 h of breast cancer cell treatment, *Artemisia annua* extracts, in particular Momundo-ACN, induces, similar to paclitaxel, formation of multinucleated, mostly 4N cells (Fig. 2A). At this time point, the Momundo extract did not exhibit toxicity to cancer cells with only a slight increase in the apoptotic cell number (Fig. 2A, C and Data in Brief article). Cell cycle analysis confirmed that similar to paclitaxel, treatment with *Artemisia annua* extract increased the populations of tetraploid (4N) MDA-MB-231 cells and those in the S-phase of the cell cycle (Fig. 2B) as well as the number of polyplod (≥ 8N) cells, the number of which was even more increased after 48 h (Fig. 2C). The increase in the S-phase cell population might be a result of apoptosis and loss of DNA content by 4N cells.

**Momundo Artemisia annua extracts selectively decrease the viability of treatment-resistant cancer cell lines**

*Artemisia annua* extracts inhibit concentration- and time-dependently the viability of MDA-MB-231 cells. Momundo-ACN extract exhibited considerably higher cytotoxicity towards MDA-MB-231 cells and inhibited their viability with an IC₅₀ of 18 µg/ml compared to Momundo extract IC₅₀ > 100 µg/ml after 48 h (Fig. 3A). Hence, acetonitrile extraction resulted in enrichment of lipophilic cytotoxic compounds. The Momundo-ACN extract was similarly cytotoxic towards a variety of other treatment-resistant cancer cell lines indicating no specificity for a certain tumor tissue and no dependency on expression of

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![Fig. 4. Mom undo Artemisia annua extracts induce apoptosis in vitro](image-url) (A) MDA-MB-231 cells were treated for 48 h, stained with propidium iodide, and analyzed by flow cytometry. Percentage of apoptotic hypodiploid cells is shown. (B) Cells were treated for 48 or 72 h as in (A), stained with JC-1, and analyzed by flow cytometry. Cells with depolarized mitochondrial membrane (ΔΨₘ) are shown. (C) Cells were treated for 48 h, incubated with fluorogenic caspase-3 substrate Z-DEVD-R110, and analyzed by flow cytometry. Data are mean ± SEM, n = 3–4, *p < 0.05, **p < 0.01, ***p < 0.001.
hormone receptors as the viability of both breast cancer cell lines, the TNBC MDA-MB-231 as well as MCF-7, was affected. Human NSCLC A549 cells were the most sensitive to Momundo-ACN with an IC$_{50}$ of 8 µg/ml, whereas the androgen-independent PC-3 prostate cancer cells were the most resistant ones with an IC$_{50}$ of 56 µg/ml (Fig. 3B). Of note, the Artemisia annua extracts show selectivity towards cancer cells, because Momundo-ACN exhibited no toxicity at ≤ 30 µg/ml towards normal mammary epithelial cells and PBMC, whereas at 30 µg/ml it inhibited the viability of MDA-MB-231 by about 72% (Fig. 3C). Moreover, lymphocyte proliferation remained unaffected by treatment with Momundo extract for 72 h (Fig. 3D).

Fig. 5. Artemisia annua extracts inhibit growth and induce apoptosis of breast cancer xenografts in vivo. MDA-MB-231 xenografts grown on chorioallantoic chick membranes (CAM) were treated for 3 consecutive days with 20 µl of either extract. On day 4, xenografts were analyzed by life imaging and immunohistochemically. Doxorubicin (1 µM) – positive control. (A) Hematoxylin and eosin staining (1st row), tumor images after extraction (2nd row), bioluminescence imaging of tumors in ovo after addition of luciferin (3rd row), proliferation marker Ki-67 (4th row, red-brown nuclear stain, original magnification 200x). (B) Graphs show mean tumor volumes. (C) Quantification of proliferating Ki67$^+$ cells. (D) Momundo extract induces apoptosis in breast cancer xenografts. Representative pictures and quantification of TUNEL$^+$ cells. All data are mean ± SEM of n ≥ 5 tumors/group, Kruskal-Wallis test and Dunn’s post-hoc multigroup test, or Mann-Whitney rank sum test for 2 groups, $^*p<0.05$, $^{**}p<0.01$. 

Momundo Artemisia annua extract induces apoptosis in breast cancer cells in vitro

To determine whether the cytotoxicity of the Momundo Artemisia annua extracts is due to apoptosis induction, several apoptotic parameters were analyzed (Okada and Mak, 2004). Indeed, the number of cells with hypodiploid DNA-contents treated for 48 h with Momundo or Momundo-ACN extract was significantly increased. A similar increase in the hypodiploid apoptotic cell population was observed in cells treated with paclitaxel (Fig. 4A).

Mitochondria promote apoptosis by releasing pro-apoptotic proteins into the cytoplasm activating caspase 3 (Okada and Mak, 2004). To demonstrate involvement of the intrinsic mitochondrial pathway in Artemisia annua-induced apoptosis, the mitochondrial membrane potential (ΔΨm) was analyzed. Treatment of breast cancer cells with Momundo extract or paclitaxel significantly increased the percentage of cells with reduced mitochondrial membrane potential from 4.0% to 18.1% and 43.4%, respectively, confirming mitochondrial membrane permeabilization (Fig. 4B). In agreement with these data, Momundo extract also induced caspase 3 activation confirming induction of intrinsic apoptosis by the Artemisia annua extract (Fig. 4C). However, activation of the extrinsic pathway cannot be fully excluded, as pretreatment of the cancer cells with IETD, a caspase 8 inhibitor, attenuated the extract toxicity, but only at the highest concentration of the Momundo-ACN extract (50 µg/ml) (Data in Brief article). It is worth mentioning that caspase 3 can cleave IETD as well, although with much lower efficacy compared to caspase 8 (Pop and Salvesen, 2009) and, thus, could also be inhibited by IETD.

Artemisia annua extracts inhibit growth of breast cancer xenografts grown on the CAM

MDA-MB-231 breast cancer xenografts grown on the CAM (Syrovets et al., 2005) were used to verify the antitumor activity of Momundo extracts in vivo. Treatment of the xenografts with Momundo and Momundo-ACN dose-dependently reduced the tumor volume (Fig. 5A). Immunohistochemical analysis of tumors revealed inhibition of cancer cell proliferation by Artemisia annua extract. Thus, treatment with either Momundo or Momundo-ACN extracts reduced significantly the expression of the proliferation marker Ki-67 (Fig. 5B,C). Besides, Momundo Artemisia annua extract effectively increased the number of apoptotic TUNEL-positive cells indicating induction of DNA strand breaks and apoptosis in vivo (Fig. 5D). Notably, no overt systemic toxicity on the chicken embryo was observed.

Treatment with Momundo extract inhibits growth of human breast cancer xenografts in mice

In addition, the anticancer activity of Momundo extract was analyzed in an orthotopic breast cancer model in mice. Daily treatment of the animals with Momundo extract for 3 weeks retarded tumor growth of the breast cancer xenografts to a similar extent as by the standard chemotherapeutic doxorubicin (Fig. 6A). Because of its high toxicity, differently to the Momundo extract, doxorubicin was administered once weekly. Accordingly, mice treated with doxorubicin exhibited reduced body weight, whereas animals treated with the Artemisia annua extract showed even a slight increase in body weight (Fig. 6B). In addition, doxorubicin exhibited hepatic toxicity as evidenced by significantly increased AST and ALT liver enzyme plasma levels. These parameters were considerably lower in mice treated with Momundo extract suggesting lower systemic toxicity of Momundo Artemisia annua extract (Fig. 6C).

Ingredients of Momundo Artemisia annua extract are cytotoxic to breast cancer cells

The main ingredients from the Artemisia annua extract were identified to be arteannuin B, casticin, chrysosplenol D, arteannuic acid, and 6,7-dimethoxycoumarin (Fig. 1A). While no reduction of cancer cell viability was induced by arteannuic acid and 6,7-dimethoxycoumarin, arteannuin B, casticin, and chrysosplenol D strongly inhibited MDA-MB-231 cell viability already within 24 h (Fig. 7). These results indicate that, although Momundo Artemisia annua extract contains no artemisinin, it contains additional natural compounds with potent cytotoxic...
activity against TNBC cells.

Discussion

Artemisia annua extracts marketed as herbal preparations are often taken by people suffering from malignant diseases, although their pharmaceutical contents and therapeutic efficacy had been insufficiently studied (Alsanad et al., 2016; Michaelsen et al., 2015). In this study, we have 1) characterized and fractionated an Artemisia annua extract marketed as a herbal preparation, 2) demonstrated that the extract exhibits anticancer activity in vitro and in vivo, and 3) identified active compounds of Artemisia annua beside artemisinin, which might be worth of further investigation as potential anticancer agents.

For the analytical characterization of the Artemisia annua extract, a highly sensitive HPLC-MS/MS method was developed, which allowed us to detect artemisinin with a detection limit of 0.2 ng/mg extract. Interestingly, despite of the high analytical sensitivity we were unable to detect artemisinin in the Momundo preparation. Nevertheless, the Momundo extract demonstrated anticancer efficacy indicating that the extract contains other biologically active compounds worth of investigation. When the content of a Momundo capsule was extracted with ACN, the yielded dry extract (Momundo-ACN) exhibited higher cytotoxicity towards breast cancer cells compared to the Momundo capsule content directly dissolved in DMSO. This pointed to the existence of lipophilic cytotoxic ingredients in the capsule content.

Sustained proliferative signaling and dysregulated cell cycle control are common features of most neoplasias (Hanahan and Weinberg, 2011). Momundo extract treatment induced concentration-dependent cell cycle perturbations and cytokinesis failure (Lens and Medema, 2019) in breast cancer cells. Similar effects have been described for flavonoids like quercetin, which interacts directly with DNA (Srivastava et al., 2016). Quercetin is a structurally related compound of chrysosplenol D and casticin, which we identified in the extract in rather high concentrations. Other ingredients of Momundo Artemisia annua extract might also, similar to paclitaxel, affect microtubuli dynamics leading to aberrant mitosis and accumulation of multinucleated cells (de Leeuw et al., 2015; Morris and Fornier, 2008).

Drugs leading to apoptosis are of particular interest for cancer treatment as apoptotic cells are efficiently removed by immune cells in vivo (Wong, 2011). The present data reveal that treatment of breast cancer cells with Momundo Artemisia annua extract induces typical signs of apoptosis with loss of the mitochondrial membrane potential, caspase 3 activation, and DNA-fragmentation. Furthermore, treatment of the xenograft models with the Momundo extract demonstrated induction of apoptosis in vivo. These efficacies clearly indicate that Artemisia annua contains active ingredients with therapeutic potential besides the well-known artemisinin.

Fractionation of the Momundo-ACN extract enabled the identification of five main constituents. Three of them, arteannuin B, casticin, and chrysosplenol D, could synergistically contribute to the inhibitory efficacy of the extract on tumor growth. The cytotoxicity of the flavonoids chrysosplenol D and casticin against MDA-MB-231 cells was even higher than that reported for the structurally related flavonoid quercetin (Chien et al., 2009). The reason could be the methoxylated flavonol structure leading to a more lipophilic molecule and possible better ability to permeate cell membranes.

Conclusion

This study provides evidence for an anticancer activity of an Artemisia annua extract marketed as a herbal preparation. Together, the results reveal new insights of Artemisia annua-derived compounds, their potential efficacy in anticancer therapy, and uncover compounds beside activity against highly metastatic triple negative human breast cancer cells that are different from artemisinin. In addition, the study provides evidence for therapeutically active compounds in a herbal preparation. These findings justify further exploration of these compounds for therapeutic purposes.
Compliance with ethical standards

The experiments with nude mice had been approved by the Agency for Animal Welfare and Protection of the State of Baden-Württemberg (reference number 1408). The collection of human blood and the analysis of peripheral blood mononuclear cells were approved by the University’s Ethics Committee (reference number 177/18). The volunteers provided written informed consent to participate in the study.

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Conflict of interest

The authors have stated that there is no conflict of interest associated with the publication and no financial support, which could have influenced the outcome.

Supplementary materials


References


