



## Research Article

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## *In vitro* assessment of genotoxic and cytotoxic effects of *Artemisia annua* L. tincture

Tamara Cetkovic, Anja Haveric, Lejla Caluk Klacar, Maida Hadzic Omanovic, Sanin Haveric

University of Sarajevo, Institute for Genetic Engineering and Biotechnology, Sarajevo, Bosnia and Herzegovina

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### Abstract

The genus *Artemisia* (fam. Asteraceae) is one of the largest and widely distributed with around 500 species, majority used as aromatic and medicinal plants. *Artemisia annua* L. is widely used as a dietary spice, herbal tea, as a supplement, and in a non-pharmaceutical form for treatment of malaria and fever. It is orally consumed as capsules, extracts and tinctures and topically applied as an essential oil diluted in lotions and ointments. Artemisinin is the main constituent of *Artemisia annua* L. extracts. Since the discovery that the artemisinin is efficient in malaria treatment, there is also a growth in consumption of *A. annua* extracts for antitumour and even recently for antiviral treatments against SARS-CoV-2 infections. This study aimed to investigate genotoxic effect in peripheral blood culture and cytotoxic effects in cancer and normal cell lines, of commercially available *A. annua* L. tincture in series of dilutions. Both comet and neutral red uptake assays revealed dose-dependent genotoxicity and cytotoxicity of *A. annua* tincture dilutions. Comet assay revealed significantly increased DNA damage in peripheral blood cells while neutral-red assays showed increase in cytotoxicity ( $p < 0.001$ ) in both normal and cancer cell cultures treated with the lowest extract dilution compared to the highest one applied. Obtained results indicate caution needed in *A. annua* L. tincture use, especially when poorly diluted.

### \*Correspondence

E-mail:  
tamara.cetkovic@ingeb.unsa.ba

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### Keywords

*comet assay, neutral red uptake assay, medicinal plants, cancer cell lines*

## Introduction

The genus *Artemisia* L. is one of the largest and widespread genera belonging to the Asteraceae family with more than 500 species (Bora and Sharma, 2010). Because of the complex chemical constitution and essential oil production, *Artemisia* species have broad ethnobotanical use for treating various diseases, including jaundice and bacterial dysentery, wounds and haemorrhoids, various viral and bacterial diseases, autoimmune diseases, malaria and related symptoms like fever and chills (Tayarani-Najaran and Emami, 2011; Pellicer et al., 2018; Nigam et al., 2019). Flavonoids and polyphenols are considered as key actors in the medicinal efficiency of Asteraceae family including *Artemisia annua* (Mesa et al., 2015). *A. annua* L. is an annual plant commonly known as “sweet wormwood”. It is traditional in Chinese medicine where it is being used for more than 2000 years (WHO, 2006, 2015; Efferth, 2017). Although with the Chinese origin, where it is known as ‘qinghao’ (green herb), it is now naturalized in Europe and America (WHO, 2006, 2015; Baraldi et al., 2008; De Ridder et al., 2008; Mesa et al., 2015). According to the Chinese and International Pharmacopoeia, the raw material is used for fever of various origins (WHO, 2006) and in Polish Pharmacopoeia for gastrointestinal problems and skin diseases. European Pharmacopoeia does not contain *A. annua* monograph (Ekiert et al., 2016; 2021).

The increased interest for *A. annua* was initiated by the discovery of artemisinin, the secondary metabolite responsible for the bitter taste of many plants. Artemisinin is a sesquiterpene trioxane lactone with a peroxide group in its chemical structure, unique to *A. annua*. The isolation of artemisinin from leaves of this plant in 1970s initiated by Chinese scientists has been one of the most successful ethnopharmacological achievements, awarded by a Nobel Prize to professor Tu YouYou in 2015 which is considered as a breakthrough in the fight against malaria and

dramatically changed treatment of malaria (Mesa et al., 2015; Su and Miller, 2015; Pellicer et al., 2018). The World Health Organization (WHO) recommends artemisinin-based combination therapy (ACT) for the non-pharmaceutical treatment of uncomplicated malaria caused by *Plasmodium falciparum*. Recommended therapy combines an artemisinin derivative such as artemether, artesunate or dihydroartemisinin with an effective antimalarial medicine (WHO, 2015; Guo, 2016; Efferth, 2017). Although developed as an antimalarial drug, artemisinin and its derivatives have been investigated for other therapeutic properties, such as antibacterial, antipyretic, antiparasitic, antiulcerogenic and anti-inflammatory (Cavar et al., 2012; Septembre-Malaterre et al., 2020). Many studies have reported anticancer effects of *A. annua* (Zhai et al., 2010; Alcantara et al., 2011; Singh et al., 2011; Xinyang, 2018; Lang et al., 2019), attributed to the presence of endoperoxide bridge (Brown, 2010; Sadiq et al., 2013). Additionally, the bioactivity of artemisinin and its semisynthetic derivative artesunate is even broader and includes the inhibition of certain viruses (Efferth et al., 2008; Lubbe et al., 2012). Results of the molecular docking methods showed that artemisinin has the potential to inhibit the main viral proteins of the SARS-CoV2 including spike protein (S), 3CLpromain protease, and PLpro proteinase, similar to hesperidin and chloroquine compounds with proven antiviral effects (Tomic et al., 2020).

Meta-analysis was performed in order to evaluate toxicity of artemisinin and its derivatives (Efferth and Kaina, 2010). Several studies were conducted to test cytotoxic, genotoxic or mutagenic effects of sesquiterpene lactones (Zhai et al., 2010), artemisinin (Cardoso et al., 2019) or its derivatives such as artesunate (Mota et al., 2011) and artemether (Alcantara et al., 2011, Cardoso et al., 2019). However, there is not as much of similar studies for commercially available products of *A. annua* (Lang et al., 2019). A lot of plant products are often commercialized as food supplements or

herbal preparations with the much more relaxed toxicity analysis requirements. Because of the growing interest and consumption of *A. annua* products for various conditions and treatments, including antiviral infections, recently for Sars-CoV2 as well, this study aimed to investigate genotoxic effect of commercially available *A. annua* tincture in peripheral blood cells, and its cytotoxicity in cancer and normal cell lines.

## Material and methods

### *Preparation of A. annua tincture dilutions*

A commercially available *A. annua* tincture (Mobis Pharm d.o.o, Mostar, Bosnia and Herzegovina) was dissolved in the dilution range of 1:10, 1:100 and 1:1000. As a solvent, the appropriate medium for each cell line was used.

### *Cell cultures and treatments*

Whole peripheral blood was used for analysis of genotoxic effects by alkaline comet assay. Peripheral blood was collected in heparinized vacutainer tubes containing lithium heparin as anticoagulant from a healthy female non-smoking donor (age 28 years) who signed informed consent form. Whole blood (350 µl) was added in 5 ml of complete PB-MAX Karyotyping Medium (Life technologies Corporation, Grand Island, NY, USA) and cultures were established under sterile conditions at 37°C in an atmosphere of 5% CO<sub>2</sub>. After 24 hours, separate whole blood cultures were treated with different *A. annua* tincture dilutions. Treatment lasted for three hours.

Four cell lines (two normal and two cancer) were used for cytotoxicity assessment. The 5637 cell line (human urinary bladder carcinoma; ATCC®HTB-9™) was cultivated in RPMI-supplemented with 10% FBS and L-glutamine. HEK 293T (human embryonic kidney; ATCC® CRL-3216™), MDBK (Madin Darby Bovine Kidney; ATCC® CCL-22™) and B16F10 cell line (murine skin melanoma; ATCC® CRL-6475™)

were cultivated in DMEM medium supplemented with 10% FBS and L-Glutamine. Both growing media contained penicillin/streptomycin. All culture reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Cells were incubated at 37°C/5% CO<sub>2</sub>. For cell detachment, medium was removed and cells were harvested by enzymatic release using 0.25% trypsin/EDTA at 37°C for 5 min, followed by inactivation with media containing serum and centrifugation at 117g for 5 min at room temperature. All cell lines were seeded at a density of 10<sup>4</sup> cells/ml in a total volume of 100 µl/well. Total cultivation period was 48h. All cultures were treated after 24h upon establishment for further 24h. 5-fluorouracil (100 ng/ml) treatment was used as positive control, and untreated cells were used as a negative control for each cell line.

### *Genotoxicity testing - alkaline comet assay*

After the treatment, cultures were centrifuged at 117g for 5 min at +4°, and alkaline comet assay as a sensitive and rapid method for measuring strand breaks in DNA (Collins et al., 2008) was performed. The resulting pellet was suspended in 0.7% low melting point agarose, applied on slides pre-coated with 1% normal melting agarose and cover-slipped. After 10 min at 4°C, the coverslips were removed, and slides for positive control were immersed in a solution of 70 µM H<sub>2</sub>O<sub>2</sub> at 4°C for 5 min and washed with dH<sub>2</sub>O according to Azqueta et al. (2011). Negative control slides remained untreated.

Slides were immersed in cold, freshly prepared lysis solution (2.5 M NaCl, 100mM Na<sub>2</sub>EDTA, 100mM Tris-HCl, 1% Triton X-100, pH 10) and left overnight at 4°C. After cell lysis, slides were placed in electrophoresis chamber and covered with freshly made cold electrophoresis solution (300 mM NaOH, 1 mM Na<sub>2</sub>EDTA, pH 13) for 20 minutes of DNA unfolding, followed by 20 min of electrophoresis at 1V/cm. After electrophoresis, slides were rinsed gently with PBS (phosphate

buffer saline) then fixed 5 min with 70% ethanol and 15 min with absolute ethanol.

Prior the fluorescent microscope analysis (U-MNU2; Olympus BX51, Tokyo, Japan), slides were rehydrated and stained with DAPI (1 µg/ml). Tail intensity - TI (% of DNA in tail of comets) was analysed in 200 comet measurements per each treatment using Comet Assay IV (Instem, UK).

#### *Cytotoxicity testing - neutral red uptake assay*

The neutral red uptake assay is a cell viability assay based on the ability of living cells to incorporate and bind neutral red, a weak cationic dye, in lysosomes (Ates et al., 2017). The dye is then extracted from the viable cells using an acidified ethanol solution and the absorbance of the dye is measured at spectrophotometer (Aslanturk, 2017). Neutral red uptake assay was used to determine lysosome membrane stability according to Repetto et al. (2008). In brief, after 24h of cell incubation with test dilutions, remaining medium was aspirated and the cells were washed with 150 µl PBS (0.01 M phosphate buffer, 0.0027 M KCl and 0.137 M NaCl) per well. In each well, 100 µl of neutral red medium (40 µg/ml) was added and incubated for 2h at 37°C. After incubation, neutral red medium was removed, and cells were washed with 150 µl PBS per well. Neutral red was extracted by adding 150 µl of dye release agent (a solution of 1% glacial acetic acid, 49% dH<sub>2</sub>O and 50% of 96% ethanol), and the plate was incubated for further 10 min on a shaker. Optical density (OD) was measured at a wavelength of 549 nm spectrophotometrically using a microplate reader (Multiskan, Thermo Fisher, Scientific, USA). The test was performed in six repeats for each treatment.

The cell survival (cell viability) was calculated as:

$$\text{Cell viability \%} = \frac{(\text{OD sample} - \text{OD blank})}{(\text{OD control} - \text{OD blank})} * 100$$

Cytotoxicity (%) was calculated as 100 – viability (%).

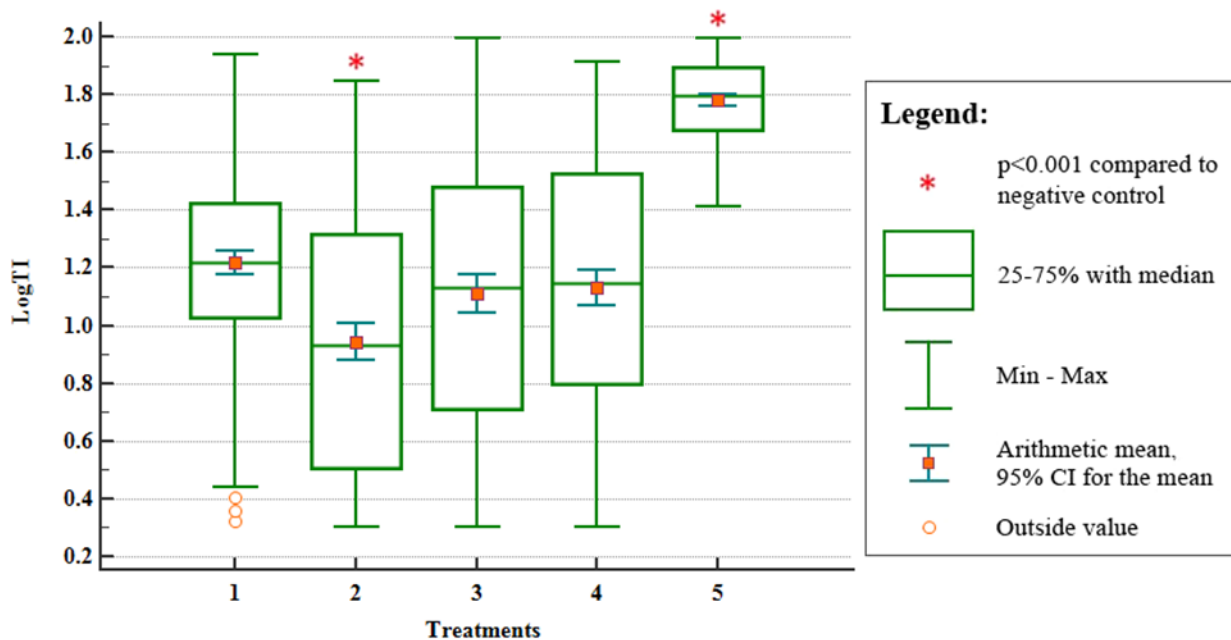
#### *Statistical analysis*

Cell viability (%) and mortality (%) were calculated from the OD values using Microsoft Excel (2010). The same software was used to log-transform tail intensity values (TI) in order to normalize distribution and equalize variances.

One-way ANOVA followed by Scheffe pair-wise comparisons in MedCalc 18.9 (MedCalc Software, Belgium) was used to test significance of differences in cytotoxicity percentage among treatments in each cell line used. Additionally, cytotoxic effects of each dilution in all of used cell lines were tested. For comet assay results in peripheral blood lymphocytes, log-transformed were compared between applied concentrations and controls. Linear regression was applied to test association between comet assay and neutral red uptake assay, and applied concentrations.

## **Results and Discussion**

Level of DNA damage in peripheral blood lymphocytes evaluated by alkaline comet assay was presented as tail intensity. The highest mean of log-transformed TI was registered for positive control (1.78) and for the 1:10 dilution among treatments (1.13). Effects of *A. annua* tincture dilutions were compared with positive (H<sub>2</sub>O<sub>2</sub>) and negative controls. A significantly lower TI (p<0.01) was detected in the highest dilution (1:1000) of the *A. annua* tincture compared to other dilutions tested and both controls. TI measured in positive control was significantly higher than in all other treatments. Dilutions 1:100 and 1:10 did not significantly differ except when compared with positive control and 1:1000 dilution (Fig 1). Simple linear regression confirmed significant positive association between applied concentration of *A. annua* tincture and TI (p<0.001).



**Figure 1:** Box-Whisker diagram of one-way ANOVA for log TI values in peripheral blood lymphocytes obtained by comet assay in different 3h treatments with *A. annua* tincture: 1 - negative control; 2 - 1:1000 dilution; 3 - 1:100 dilution; 4 - 1:10 dilution; 5 - positive control (70 $\mu$ M H<sub>2</sub>O<sub>2</sub>). \*- statistically different from negative control values

In regards to cytotoxicity, the highest cytotoxicity of *A. annua* tincture was registered for the lowest dilution of 1:10 with the values of 57.94% in 5637; 47.89% in MDBK; 24.59% in B16F10; and 65.15% in HEK cell line. Results of cytotoxicity for all cell lines used and applied treatments are presented on Figure 2.

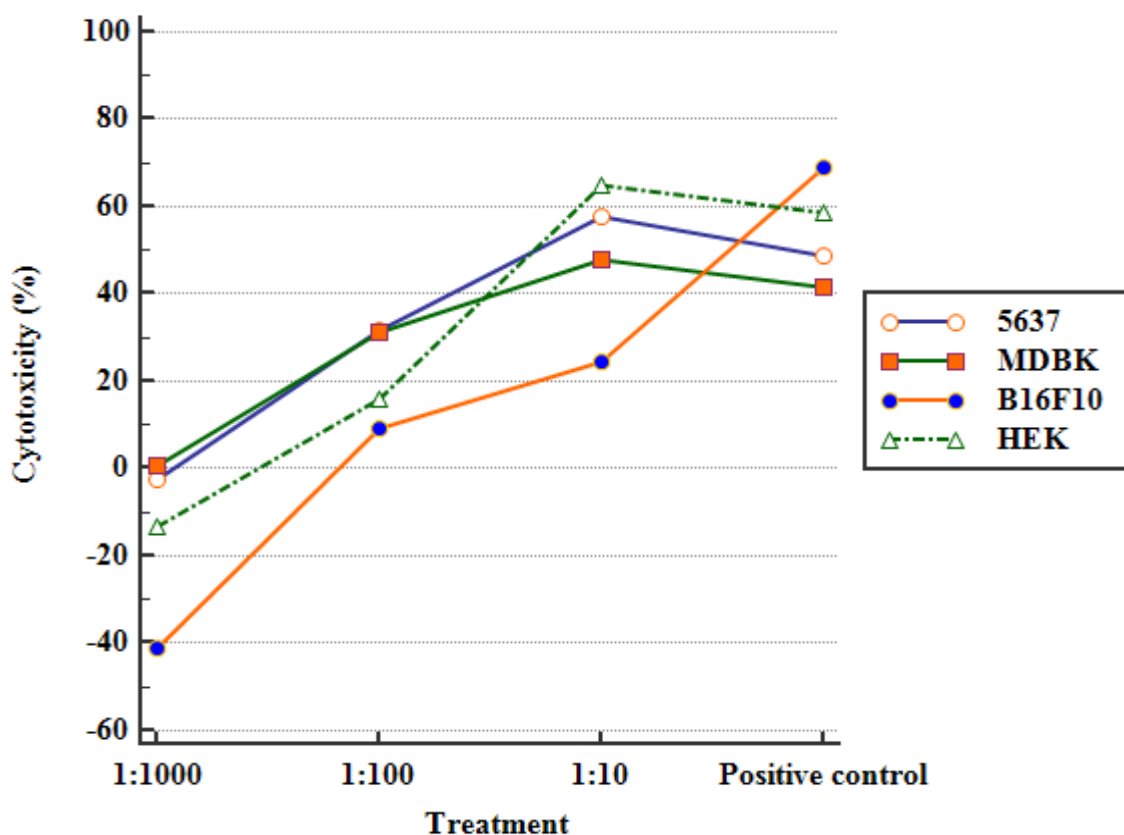
One way ANOVA followed by Scheffe post hoc multiple comparison revealed significantly lower cytotoxic effect ( $p < 0.001$ ) of 1:1000 dilution in all cell lines used, when compared with 1:100 and 1:10 dilutions, and positive control. When effects of different dilutions were compared among cell lines, significant difference was found only for 1:10 dilution that had significantly lower cytotoxicity in B16F10 cell line compared to 5637 and HEK. For 1:100 and 1:1000 dilutions, effects were not significantly different among cell lines. Simple linear regression revealed significant dose-dependent effect in all cell lines with the lowest cytotoxicity in the lowest concentration (highest dilution tested).

Even though more than 600 secondary metabolites have been identified in *A. annua* plant extracts, species from genus *Artemisia* mainly comprise of sesquiterpenoids, triterpenoids, monoterpenoids, steroids, coumarins, alkaloids, benzenoids, and flavonoids (Septembre-Malaterre et al., 2020; Koul et al., 2017). Artemisinin is one of the main components of *A. annua* extracts that has been widely used in traditional medicine for various diseases and conditions. *A. annua* extracts are also being used as antiviral agents (Efferth et al., 2008; Efferth, 2018), recently with the proven potential against SARS-CoV-2 (Tomic et al., 2020). Several studies have been conducted in order to test genotoxic or cytotoxic effects of *A. annua* extracts, artemisinin or related derivatives such as artemether, or semisynthetic compound artesunate (Mota et al., 2011; Aquino et al., 2013; Cardoso et al., 2019, Lang et al., 2019). However, there is a very few similar studies on commercially available *A. annua* preparations (tinctures or pills) (Lang et

al., 2019), although they are intensively used, even without medical prescriptions.

In our study we revealed significant genotoxic and cytotoxic effect of softly diluted *A. annua* tincture in used human and animal cells. Our results of comet assay conducted in peripheral blood lymphocytes corroborate with the study of Cardoso et al. (2019). They studied genotoxic and cytotoxic effects of artemisinin at 12.5, 25 and 50  $\mu\text{g/ml}$  and artemether at 7.46, 14.92 and 29.84  $\mu\text{g/ml}$  concentrations were tested using comet assay, micronucleus test and cytotoxicity assay for necrosis and apoptosis detection by acridine orange/ethidium bromide staining in human lymphocytes. At the highest applied concentration of artemisinin, the increase in DNA damage was significantly increased compared with negative control while for artemether significant dose-dependent DNA damage is reported. In micronucleus test, the two highest concentrations

of both artemisinin and artemether induced significant increase in micronuclei frequency. Tested concentrations of both substances did not significantly affect apoptosis induction but artemisinin had significant impact on necrosis frequencies in all applied concentrations. Artemisinin exhibits significant selective cytotoxicity toward the human hepatoma HepG2 (p53 wild-type), Huh-7 (p53 mutant), BEL-7404 (p53 mutant), and Hep3B (p53 null) cell lines, but minimal effect on non-neoplastic human liver cell line (Hou et al., 2008). Genotoxic and cytotoxic potential against human lymphocytes has also been confirmed for artesunate (Mota et al., 2011). Artemisinin and artesunate were tested for genotoxic effects using comet assay in HepG2 cell line. Both of them are genotoxic in a dose-dependent manner, however artemisinin shows more profound genotoxic potential (Aquino et al., 2013).



**Figure 2:** Cytotoxicity of *A. annua* tincture dilutions (1:1000, 1:100, 1:10) in used cell lines and in positive control treatment with 100 ng/ml 5-fluorouracil

Artemether effects were also tested *in vitro* against PG100 (gastric cancer cell line) and human lymphocytes using MTT assay, comet assay and ethidium bromide/acridine orange viability staining. Human lymphocytes showed increased sensitivity towards cytotoxic effects of artemether compared to PG100 cells (Alcantara et al., 2011). Although MTT assay is widely used for cytotoxicity assessment *in vitro*, there are reports of interference of MTT tetrazolium assay with plant extracts that may lead to false positive results (Bruggisser et al., 2002). However, when neutral red uptake assay was compared with MTT assay, neutral red did not interact with the vitamin E isomers, unlike MTT tetrazolium salt (Lim et al., 2015). Accordingly, we applied neutral red uptake assay in our study.

Anti-tumour effects of *A. annua* extracts were previously tested. Ethanolic extracts of Brazilian and Chinese *A. annua* were found to exhibit anticancer activity against Molt-4 leukemia cells (Singh et al., 2011). Methanolic extract of *A. annua* from Ethiopia was in a dose-dependent manner efficient against prostate cancer Du-145, and LNCaP, brain astrocytoma 1321N1 cells; breast cancer MCF-7 cells, and leukemia THP-1 cells (Worku et al., 2013). We have also confirmed significant inhibition of cell growth for tumour (B16F10 and 5637) but also for normal (HEK and MDBK) cell lines with the lowest dilution of used *A. annua* tincture.

The study of Singh et al. (2011) indicated a better safety of Brazilian extract. This has been confirmed in the study of Nageeb et al. (2013) who showed that the origin of the *Artemisia annua* has a significant effect on chemical composition of its extract and related bioactive potential.

In the study performed by Lang et al. (2019) *A. annua* extracts was prepared using MoMundo GmbH (Bad Emstal, Germany) capsules. However, HPLC-MS/MS confirmed absence of artemisinin in this extract. XTT assay has shown that prepared extract inhibits viability of breast

(MDA-MB-231 and MCF-7), pancreas (MIA PaCa-2), prostate (PC-3), non-small cell lung (A459) cancer cells, whereas normal mammary epithelial cells, lymphocytes, and peripheral blood mononuclear cells were relatively resistant to the treatment, confirming additional compounds, beside artemisinin, that have anti-tumour activity. Additionally, Zhai et al. (2010) have confirmed growth inhibition effect of (Z)-7-acetoxy-methyl-11-methyl-3-methylene-dodeca-1,6,10-triene (AMDT), sesquiterpene isolated from hairy roots culture of *A. annua*, on HO8910 (human high metastatic ovarian cancer), 95-D (human high metastatic lung carcinoma), QGY (human hepatoma), HeLa (human cervical carcinoma), while on normal human cell lines LO2 (human liver cell), and HF (human dermal fibroblast) cytotoxic effect has been weak. Although our study exhibited genotoxic and cytotoxic potential of *A. annua* tincture dilutions, the limitation of our study is the lack of HPLC characterization that prevents identification of extract's active compounds.

Besides numerous confirmed genotoxic and cytotoxic effects of *A. annua* extracts or its components, rapid elimination of artemisinin (Efferth and Kaina, 2010) is in the favour of the oral consumption of *A. annua* preparations.

## Conclusion

Obtained results in this study corroborate previously conducted studies and confirm caution needed in *A. annua* L. tincture use, especially when poorly diluted. Of the especial concern is the fact that the lowest dilution tested was equally toxic to normal and tumour cell lines although selective toxicity of *A. annua* extracts against tumour cells has been previously reported.

## Conflict of interest

Authors declare no conflict of interest.

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