Moroccan Journal of Biology 07-2008/N 4-5

Chemical composition and anti-prolifertaive properties of the essential oil of *Tanacetum annuum* L.

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Abstract
The essential oil (EO) of *Tanacetum annuum* L. was obtained from fresh aerial parts by steam distillation. The EO was analysed by gas chromatography (GC) and gas chromatography coupled with mass spectrometry (GC/MS) by using mass spectra and retention index data. This EO contains sabinene (22.3%), camphor (13.2%), beta-pinene (10.1%), p-cymene (8.9 %) and alpha-phellandrene (7.6 %) as major constituents, and presents cytotoxic activity against a human rhabdomyosarcoma cancerous cell line with an IC50 less than 25 µl/ml. This result suggests that *T. annuum* may have potential anti-cancer activity.

Key word: *Tanacetum annuum*, cytotoxic activity, essential oil composition.

Introduction

There is increasing interest in screening medicinal plants in different regions of the world. Natural products represent an important source of compounds for human care, so intensive studies on therapies using these products have been conducted during the last decade. Most of cytotoxic agents used in chemotherapy have a natural origin (Newman *et al*., 2003) but more research into anti-cancer agents, acting on various cell targets and presenting reduced secondary effects is needed.

Several studies have shown that essential oils (EO) from some plant species present an anti-prolifertaive activity. The genus *Tanacetum*, which is an important member of the *Asteraceae* family, is widespread in Europe and western Asia and exists in about 150-200 species. These species have traditionally been used as a spicy additive for food, in cosmetic and as herbal remedies (Rohloff *et al*., 2004). *Tanacetum annuum* L. or blue camomile of Morocco is a resistant plant and grows spontaneously in the Northwest Moroccan Regions. *T. annuum* is described in traditional medicine as an anti-inflammatory, anti-flogistic, anti-pruriginic and anti-histamine plant (Crellin, 1997). It has been described as *T. parthenium* L. (feverfew), having a positive benefit when used to treat breast cancer, in combination with another anti-cancer treatment in a phase I trial (Curry *et al*., 2004). It has been reported also that the major component of this plant is parthenolide, which induces apoptosis of stem and progenitor cells (Guzman *et al*., 2005). Considering these data, this work reports the EO chemical composition of *T. annuum* from Morocco, and its ability to induce a cytotoxic effect against a human cancer cell line.

Materials and methods

Plant Material

Shoots of *T. annuum* were collected during the flowering period (September-October) from the Larache region (northwest of Morocco).

Steam Distillation

The EO was isolated from shoots by hydrodistillation (150min) through still type-apparatus (industrial extraction) and stored at -10°C as described by Greche (1999).

Quantification and identification of EO by using GC and GC/MS

The **GC**-analysis was carried out with HP 5980 and DB-5 capillary column (25m × 0.25 mm, film thickness of 0.25 µm). The analytical conditions are (i) injector and detector temperature respectively of 240 and 260°C, (ii) oven temperature programmed from 50 to 250°C with 2°C/min, (iii) isothermal at 250°C for 10 min, and (iv) N_2 is use as carrier gas with a flow of 1 ml/min. Relative concentrations were calculated using peak areas as given by HP 3396A (FID) with integrator without correction for response factors. Retention indices were obtained by injection of the homologous C_8-C_{30} hydrocarbons series in the same temperature conditions.

GC/MS

Analyses were performed with a HP 5980 Series II gas chromatograph equipped with HP5-capillary column (25 m × 0.3 mm; film thickness of 0.25 µm) and a HP 5772A mass selective detector. The analytical conditions were (i) injector and detector temperatures of 240 and 260°C in the same order, (ii) oven temperature programmed from 50 to 250°C with 2°C/min, (iii) isothermal at 250°C for 10 min, (iv) N_2 is used as carrier gas with a flow of 1 ml/min, and (v) EI is used as ionization mode (70 ev).

Identification of the EO components

Constituents of the EO were identified by the combination of retention index data (Adams 1991, Laseve 1996) and/or mass spectra data (NBS library, Adams 1989).

Bioassay

The cell line used in this study is the human RD cancer cell line (ATCC N°CCL-136) from a rhabdomyosarcoma. Cell were maintained and subcultured in 75 cm^2-cell culture flasks (Fisher Scientific, France) using 10 ml of DMEM (Dulbecco's modified eagle medium, Gibco in vitrogen), supplemented with 10 % of foetal bovine serum (FBS) (Life Technologies, Scotland), 1 % of L-glutamine (Sigma-Aldrich) and 1 % of penicillin-streptomycin from Sigma-Aldrich. The medium was adjusted to pH 7.0-7.2 and all flasks were incubated at 37°C in a humidified "5 % CO_2/95 % air" incubator.

Cytotoxicity was determined by using the the 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) assay (Mosmann 1983, Denizot & Lang 1986). Briefly, 15000 cells in a 100 µl final volume were harvested and seeded in 96-well microtiter plates. After 24h, cells were incubated for 48 h with various concentrations of *T. annuum* EO. As a positive control, cells were incubated under the same conditions with mitomycin C. EO was solubilized in DMSO and then added to the cell culture medium, with a final concentration of DMSO less than 0.2 %. After 48h incubation, 10 µl of MTT (5 mg/ml) was added to cells which were further incubated at 37°C for 4h. The formazan crystals formed were dissolved by adding 150 µl/well of 0.04 N isopropanol-HCl. The optical density was measured at 570 nm with 630 nm as the reference wavelength.

Cytotoxicity was calculated by using the following formula :

$$(1- \frac{OD_{570}\ EO}{OD_{570}\ negative\ control})\times 100$$

Results and discussion

In this study we have analysed the potential anti-cancer activity of *T. annuum* EO. This EO is a magnificent indigo blue, with a sweet and fresh flavour (Keville 1995, Fritz Weiss & Fintelmann 2000). To our knowledge, no anti-cancerous study has been conducted on this plant.

The chemical composition of EO, obtained from *T. annuum* aerial parts, is shown in Table 1. The results indicate that sabinene (22.3%), camphor (13.2%), beta-pinene (10.1%), p-cymene (8.9 %), alpha-phellandrene (7.6 %) are the major components of *T. annuum* EO. These products correspond to more than 60 % of the composition of this EO. Table 1 shows that EO from *T. annuum* contains other compounds such as alpha-pinene, myrcene, camphene, limonene, gamma terpinene, beta-caryophyllene, valencene, 3,6-dihydrochamazulene, 5,6-dihydrochamazulene, chamazulene, borneol, and terpinene-4-ol, which are present at concentration between 1 and 6%.

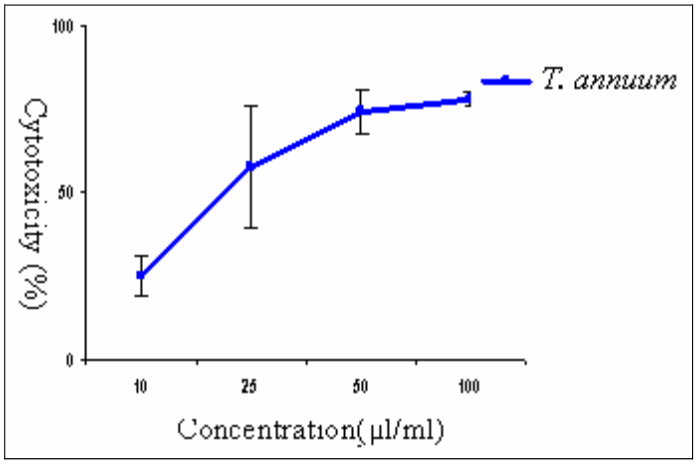
Table 1. Composition of the essential oil of *T. annuum* (%).

Compound	Percentage composition
Hydrocarbons	
alpha thujene	0.7
alpha-pinene	4.9
camphene	1.8
sabinene	**22.3**
beta-pinene	**10.1**
myrcene	**6.0**
alpha-phellandrene	**7.6**
alpha-terpinene	0.9
p-cymene	**8.9**
limonene	4.2
gamma terpinene	1.5
beta-elemene	0.2
beta-caryophyllene	1.7
(Z)-beta-farnesne	0.8
valencene	1.1
3,6-dihydrochamazulene	1.8
5,6-dihydrochamazulene	t
7,12-dehydro-5,6,7,8-tetrahydrochamazulene	t
chamazulene	2.8
9-(15,16-dihydro-15-methylene geranyl)-p-cymene	0.5
Alcohols and Ethers	
1,8-cineole	0.3
borneol	2.7
terpinene-4-ol	1.3
alpha-terpineol	t
thymol	0.8
caryophyllene oxide	t
beta-eudesmol	0.3
Ketone	
camphor	**13.2**

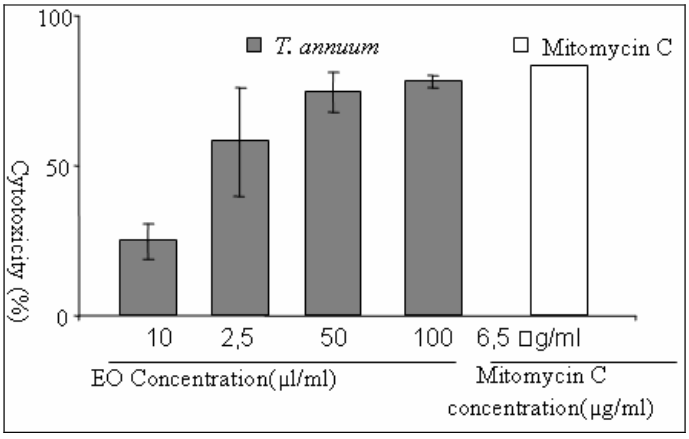
t: trace (<0.1%)

An investigation of the essential oil from the flowers of *T. annuum* from Spain (Barrero *et al.*, 1992) led to the identification of 43 components, and two of them have a homoditerpene skeleton. Five of these substances are new natural products. The major components are myrcene + alpha phelladrene (18%), chamazulène (11%), camphor (10%), beta pinene (7.5%), thymol (6.1%), sabinène (5.5%), 3,6-dihydrochamazulene (5.6%) and 5,6-dihydrochamazulene (4%).

This EO was tested for its anti-proliferative activity against a RD cell line using the MTT assay. As shown in Figure 1 (A, B), it has a strong cytotoxicity in the human cancer cell RD with an IC_{50} < 25 µl/ml. The inhibitory effect is reproducible as indicated by statistical analysis when compared to the positive control (mitomycin C) which was used as anti-mitotic control. This result is in line with recent studies showing the anti-cancer properties of essential oils from plants such as *Croton flavens*, *Comptonia peregrine,*



-A-



-B-

Figure 1. Anti-proliferative effect of *T. annuum* essential oil against a human rhabdomyosarcoma cancerous cell line (RD) (n=4).

Dose-dependant effect of *T. annuum* EO on proliferation of the RD human cancerous cell line. Cells were seeded on 96-well tissue culture plates followed by incubation with indicated concentrations of the essential oil for 48h. Cell proliferation was determined by the MTT reduction assay. Data are expressed as means ± SDs for experiments conducted in quadruplicate (n=4). As a positive control, cells were incubated under the same conditions with Mitomycin C.

Myrica gale, *Eugenia caryophyllus* and *Tanacetum parthenium* (Sylvestre *et al*. 2005, 2006, Jirovetz 2006, Wu *et al*. 2006, Sylvestre 2007). Chemical analysis of essential oils from these plants showed a high variability in the EO composition and content, indicating that various compounds may account for the anti-cancer activity of plants. In this respect, compounds such as alpha-cadinol, beta-elemene and alpha-humulene, alpha-humulene and (E)-nerolidol were identified as cytotoxic against tumor cell lines (Sylvestre, 2006).

Although our results suggest that the high cytotoxic activity may be related to the major products *i.e.* sabinene (22.3%), camphor (13.2%), beta-pinene (10.1%), p-cymene (8.9 %) and alpha-phellandrene (7.6 %) of *T. annuum* EO, however this may not exclude the possibility that the other components present at lower concentration may have an anti-tumor property. Along this line, it has been shown that essential oils with a high content of sabinene present anti-proliferative properties (Paik *et al*., 2005). In addition, it has been reported that 1,8-cineole and camphor have strong phytotoxic effects against various plant species (Koitabashi *et al*. 1997, Abrahim *et al*. 2000, Vokou *et al*. 2003, Zunino & Zygadlo 2004, Nishida *et al*. 2005, Kordali *et al*. 2005). Furthermore, it has been documented that oxygenated monoterpenes possess relatively high phytotoxic effects in comparison to monoterpene hydrocarbons (Vokou *et al*. 2003, Kordali *et al*. 2007). Thus, the herbicidal effects of *Tanacetum* oils can be attributed to their relatively high content of oxygenated monoterpenes.

With respect to cancer, a number of studies have described the anti-tumor activity of some monoterpens as well as their mechanism of action. They may inhibit cell proliferation by preventing the isoprenylation of proteins, by inhibition of DNA biosynthesis or by induction of apoptosis (Carnesecchi *et al*. 2001, Chakraborty *et al*. 2004). The active principles of the major components and their anti-tumor mechanism still need to be identified

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