RESEARCH ARTICLE

Cytotoxicity and Acetylcholinesterase inhibitory and PRAP activities of the essential oils of selected Tanacetum L. species.

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Abstract

Essential oils obtained from flowers of Tanacetum mucroniferum Hub. - Mor. & Grierson, leaves of Tanacetum densum (Labill.) Heywood ssp. eginense Heywood, stems of Tanacetum densum (Labill.) Heywood ssp. sivasicum Hub. - Mor. & Grierson were investigated for their cytotoxicity, AChE inhibitory and PRAP activities. We have previously investigated chemical composition of these oils and we have also suspected that these oils also do have strong AChE inhibitory activities due to the insecticidal activities which we have reported for the other members of this genus. Highest activities were observed for pure oils: T. mucroniferum, T. densum ssp. sivasicum and T. densum ssp. eginense were 100.00±0.0%; 100.00±0.0% and 91.31±3.30% respectively (n=3). The observed activities were concentration dependent. Compared with the positive control α-tocopherol (3.735±0.063 AU; n=3) in same concentrations, all of the investigated oils showed low PRAP activity at the highest concentration (10 mg/mL). Highest activity was observed for T. mucroniferum (1.319±0.017 AU; n=3) at 10 mg/mL concentration.

Keywords: In vitro cytotoxicity, AChE Inhibition, PRAP Activity, T. mucroniferum, T. densum ssp. sivasicum, T. densum ssp. eginense.

Introduction

Previously, we have investigated essential oil composition of Tanacetum densum (Labill.) Heywood ssp. sivasicum Hub. - Mor. & Grierson, T. densum (Labill.) Heywood ssp. eginense Heywood and Tanacetum mucroniferum Hub. - Mor. & Grierson, (Polatoglu et. al., 2009; 2012a; 2012b). The essential oils of T. densum ssp. sivasicum contained 1,8 cineole (21.1% and 28.3%), camphor (19.2% and 16.4%) and bornoel (5.8% and 6.4%) main components in flower and stem oils respectively (Polatoglu et. al., 2009). The essential oils of T. densum ssp. eginense have camphor (30.9%, 25.7%, 27.7%), 1,8 cineole (12.4% flower oil), camphene (10.6%, %7.0, flower and leaf oils), bornyl acetate, (9.4%, 11.8%, stem and leaf oils), α-pinene (7.0%, %5.3, flower and leaf oils), bornoel (5.1%, 5.2%, stem and leaf oils), neodihydrocarveol (5.1%, flower oil) as well as an unidentified compound (11.5%, 27.2%, 20.5%) in flower, stem and leaf oils respectively (Polatoglu et. al., 2012a). T. mucroniferum flower essential oil contained 1,8-cineole 21.9 % and camphor 6.4 % as the main components (Polatoglu et. al., 2012b). The essential oils of T. densum were evaluated for its antimicrobial activity against selected pathogens but no significant activity was observed. The oils of this species were also evaluated for their general toxicity using Vibrio fischerii toxicity test. As a result, all of the oils inhibited the growth of this organism which suggests potential cytotoxicity (Polatoglu et. al., 2012a). The flower oil of T. mucroniferum was evaluated for its radical scavenging activity against DPPH radical but the oil produced low scavenging activity when compared with the positive control at the same concentration (Polatoglu et. al., 2012b). Pyrethrins are one of the most important commercially produced natural insecticides which are produced from Tanacetum (Pyrethrum) cineraiiifolium. The activity of pyrethrins manifests itself acting on
the nervous system of the insect species (Isman, 2006; Wandahwa et al., 1996). Unfortunately, beside *T. cinerariifolium* there are very few reports on the insecticidal activity of other *Tanacetum* species especially on the essential oils. Previously, insecticidal activity of *T. vulgare* on moths and mites (Chiasson et al., 2001; Gabel and Thiery, 1994; Palsson et al., 2008) and insecticidal activity of *T. abrotanifolium*, *T. macrophyllum* on stored product pest *Sitophilus granarius* (Polatoglu et al., 2015a, 2015b) were reported. The concerns related to food safety and environmentally friendly agricultural methods prompted acceptance of certain regulations in agricultural production. The European Pesticide Regulation (EC) No. 1107/2009 promotes the use of less harmful substances in pest management. The acceptance of this regulation by the European Union made a huge impact on pesticide industry and provided attention to the natural substances that could be used in pest management (Villaverde et al., 2014).

The purpose of this study is to evaluate the essential oils of *T. mucroniferum*, *T. densum* ssp. *eginense* and *T. densum* ssp. *sivasicum* for their acetylcholinesterase (AChE) inhibition, cytotoxic activity and PRAP (Phosphomolybdenum Reducing Antioxidant Power) activity. AChE inhibition of the monoterpenes were previously correlated with their insecticidal activity (Lopez and Pascual-Villalobos, 2010). Therefore, with this report we intend to provide initial information on their potential insecticidal activity as well as their safety.

**Materials and Methods**

**Plant Materials**

Plant materials were collected during their flowering period. *T. mucroniferum* was collected in July 2008 from Erzincan-Sakaltutan, Turkey at 2333 m altitude, voucher specimens have been deposited at the Herbarium of the Faculty of Science, Istanbul University (Voucher no. ISTE 85425 identified Prof. Dr. Neriman Özhatay). *T. densum* ssp. *sivasicum* and *T. densum* ssp. *eginense* were collected in July 2002 from Böğrüdelik Village (1800 m) and Mt. Tecer (1900 m) Sivas Turkey, voucher specimens have been deposited at the Herbarium of the Faculty of Science, Istanbul University (Voucher no. ISTE 80539 and ISTE 80538, respectively, identified by Prof. Dr. Kerim Alpinar).

**Essential oil isolation**

Plant materials (100 g each) were subjected to hydrodistillation for 4 h using a Clevenger-type apparatus to produce the oils. Essential oil yields obtained from flowers, *T. mucroniferum*, leaves of *T. densum* ssp. *eginense*, stems of *T. densum* ssp. *sivasicum* 0.1; 0.45; 0.1 (v/w) respectively. Oils were kept in amber bottles in -20°C until the biological activity assays were conducted.

**AChE inhibition assay**

The inhibitory effect of the essential oils were determined with the previously described protocol (Ellman et al., 1961). The assay solution contained 240 μL, 1.25 mM 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), 192 μL acetylthiocholine iodide (AChI), 1200 μL, 100 mM Tris–HCl buffer pH 8.0 and 20 μL essential oil. The blank solution contained 20 μL of buffer solution instead of the essential oil. Galanthamine hydrobromide (from *Lycoris* sp.), was used as a positive control in the assay. The calibration curve obtained by testing AChE inhibition of different concentrations of galanthamine was constructed the data points were fitted with logarithmic function to obtain the calibration curve $y = 24.968\ln(x) + 32.003$, that have $R^2 = 0.9934$. The calibration curve is given in Figure 1. Reactions were started by adding 0.0325 U/mL of AChE (electric eel) into the reaction mixture. The reaction was monitored for 2 min at 412 nm wavelength using a spectrophotometer (Carry 60 single beam spectrophotometer, Agilent Technologies, USA). The enzymatic
activity was calculated as the percentage of the reaction rate in accordance with the activity obtained from the blank. The data obtained from the linear section of the initial 60 s were used in the calculation of the activities. The AChE inhibition was calculated by the subtraction of the ratio of the sample activity versus blank activity from 100. The results of the experiments were given as mean ± standard deviation of three parallel experiments. The results of the AChE inhibition tests are given in Table 1.

**PRAP (phosphomolybdenum reducing antioxidant power) assay**

The antioxidant activity of the essential oils were determined with a previous spectrophotometric protocol (Falcioni et al., 2002). Stock solutions of 10% (w/v) phosphomolybdic acid, 1, 5, 10 mg/mL essential oils, positive controls: α-tocopherol and BHT were prepared. As a negative control, essential oil free methanol was used. Phosphomolybdic acid solution (200 μL) and samples (200 μL) were mixed and then incubated at 80 °C for 30 min. After all of the samples and controls were cooled to room temperature, they were measured for their absorbance at 600 nm (Agilent, Carry 60 UV-Vis. Spectrophotometer). Increased absorbance of the reaction meant increased reducing power of the compounds when compared to blank control. The results of the experiments were given as mean ± standard deviation of five parallel experiments. The results of the PRAP tests are given in Table 2.

**Cell lines and maintenance**

HeLa (human cervix adenocarcinoma), A-549 (human alveolar adenocarcinoma), MCF-7 (human breast adenocarcinoma), CaCo-2 (human colon colorectal adenocarcinoma), mPANC96 (human pancreas adenocarcinoma), PC-3 (human prostate adenocarcinoma), U87MG (human glioblastoma-astrocytoma) and as a normal cell line HEK293 (human embryonic kidney cells) were used for testing cytotoxicity. All cell lines were purchased from ATCC. The cell lines were maintained in DMEM Ham’s F12 medium supplemented with 10% FBS, 1% L-glutamine, 1% gentamycin and 1 mM HEPES in a humidified atmosphere with 5% CO₂, at 37°C. The cells were subcultured twice a week and cells in the exponential growth phase was used in the experiments.

**Cytotoxicity assay**

Cytotoxicity of the oils were determined using a modified MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide] assay (Mosmann T., 1983), which detects the activity of mitochondrial reductase of viable cells. The assay principle is based upon the cleavage of MTT that forms formazan crystals by cellular succinate-dehydrogenases in viable Adding dimethyl sulfoxide to wells helps formazan crystals to be resolved. For this purpose, all cell lines were cultivated for 24 h in 96 well microplates with an initial concentration of 1x10⁵ cells/well in a humidified atmosphere with 5% CO₂, at 37°C. Then, the cultured cells were treated with different dilutions of the extracts (0.5, 5, 50 μg/ml) followed by incubation for 48 h at 37 °C. Parthenolide was used as positive control. The optical density of the dissolved material was measured at 570 nm (reference filter, 620 nm) with UV-visible spectrophotometer (Thermo Multiskan Spectrum) (Nalbantsoy et al., 2008).

Cytotoxicity was expressed as mean percentage increase relative to the unexposed control ± standard deviation. Control values will be set at 0% cytotoxicity. Cytotoxicity data (where appropriate) will be fitted to a sigmoidal curve and a four parameters logistic model used to calculate the IC₅₀, which is the concentration of extracts causing 50% inhibition in comparison to untreated controls. The mean IC₅₀ is the concentration of agent that reduces cell growth by 50% under the experimental conditions and is the average from at least three independent measurements that will be reproducible and statistically significant. The IC₅₀ values were
reported at ±95% confidence intervals (±95% CI). This analysis was performed with Graph Pad Prism (San Diego, USA).

Results and Discussion

All of the investigated pure oils showed very high AChE inhibition. The highest inhibitions were observed for \textit{T. densum} ssp. \textit{sivasicum} (100 ± 0.00) and \textit{T. mucroniferum} (100 ± 0.00). However, when dilutions were tested, these oils produced lower inhibition. \textit{T. densum} ssp. \textit{sivasicum} oil afforded higher activity at 10 mg/mL application doses. The AChE inhibition of \textit{T. densum} ssp. \textit{sivasicum} oil at 10 mg/mL corresponds to activity of 0.529 μm galanthamine. The oil of \textit{T. densum} ssp. \textit{eginense} produced lower activity than the other oils. The \textit{T. densum} ssp. \textit{sivasicum} oil that have produced high activity contains 1,8-cineole (28.3%), camphor (16.4%) and borneol (6.4%) as major components (Polatoglu et. al., 2009). Similarly, \textit{T. mucroniferum} oil have 1,8-cineole (21.9%) and camphor (6.4%) as major component but have smaller amounts of borneol (1.7%) (Polatoglu et. al., 2012b). The oil of \textit{T. densum} ssp. \textit{eginense} contains camphor (27.7%), an unknown compound (20.5%), camphene (7.0%), \(\alpha\)-pinene (5.3%) and borneol (5.2%) (Polatoglu et. al., 2012a). The observed activity of the oils could be associated with 1,8 cineole and camphor which were reported to have high AChE inhibition (Perry et. al., 2000). None of the oils showed any cytotoxic activity against the tested cell lines. All of the oils produced very low PRAP activity, the highest activity was observed for \textit{T. mucroniferum} oil which produced 1.32 ± 0.02 AU at 10 mg/mL concentration. This activity is lower than the activity of the positive control at 1 mg/mL (1.46 ± 0.33 AU). The oils did not show considerable cytotoxic activity and PRAP activity, however they showed very high AChE inhibition. As suggested before, in most cases the insecticidal activity is related with AChE inhibition (Lopez and Pascual-Villalobos, 2010); therefore, we believe these oils do possess strong chance to have high insecticidal activity but low toxicity. The results of this study also suggest essential oils of other members of genus \textit{Tanacetum} have high potential for insecticidal activity. This is the first report on AChE inhibitory, PRAP and cytotoxic activities of the essential oils of \textit{T. densum} ssp. \textit{sivasicum}, \textit{T. densum} ssp. \textit{eginense}, \textit{T. mucroniferum}.

Table 1. AChE Activity of investigated \textit{Tanacetum} essential oils.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>\textit{T. densum} ssp. \textit{sivasicum}</th>
<th>\textit{T. densum} ssp. \textit{eginense}</th>
<th>\textit{T. mucroniferum}</th>
<th>(\alpha)-Tocopherol\textsuperscript{a}</th>
<th>Blank\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure Oil\textsuperscript{b}</td>
<td>100 ± 0.00\textsuperscript{b}</td>
<td>91.31 ± 3.30</td>
<td>100 ± 0.00</td>
<td>3.74 ± 0.06</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>10 mg/mL</td>
<td>16.09 ± 1.51</td>
<td>0.00 ± 0.00</td>
<td>13.81 ± 2.97</td>
<td>3.64 ± 0.19</td>
<td></td>
</tr>
<tr>
<td>5 mg/mL</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>5.80 ± 1.98</td>
<td>1.46 ± 0.33</td>
<td></td>
</tr>
<tr>
<td>1 mg/mL</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>1.46 ± 0.33</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}The results were given as mean ± standard deviation of three parallel experiments. \textsuperscript{b}Positive control. \textsuperscript{c}Methanol was used as the negative control.

Table 2. PRAP Activity of investigated \textit{Tanacetum} essential oils.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>\textit{T. densum} ssp. \textit{sivasicum}</th>
<th>\textit{T. densum} ssp. \textit{eginense}</th>
<th>\textit{T. mucroniferum}</th>
<th>(\alpha)-Tocopherol\textsuperscript{a}</th>
<th>Blank\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mg/mL</td>
<td>0.52 ± 0.00\textsuperscript{a}</td>
<td>0.42 ± 0.15</td>
<td>1.32 ± 0.02</td>
<td>3.74 ± 0.06</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>5 mg/mL</td>
<td>0.51 ± 0.24</td>
<td>0.22 ± 0.01</td>
<td>0.73 ± 0.07</td>
<td>3.64 ± 0.19</td>
<td></td>
</tr>
<tr>
<td>1 mg/mL</td>
<td>0.29 ± 0.04</td>
<td>0.13 ± 0.01</td>
<td>0.20 ± 0.04</td>
<td>1.46 ± 0.33</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}The results were given as mean ± standard deviation of three parallel experiments. \textsuperscript{b}Positive control. \textsuperscript{c}Methanol was used as the negative control.
Figure 1. The calibration Curve of AChE inhibition of Galanthamine.

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REFERENCES


