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In Vitro Acetylcholinesterase Inhibitory Properties of Thymol, Carvacrol and their Derivatives Thymoquinone and Thymohydroquinone

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The aim of this study was to examine *in vitro* the inhibitory activity exerted by the main constituents of essential oil obtained from the aromatic plant *Thymus vulgaris* L. on acetylcholinesterase (AChE). The total essential oil and selected compounds, specifically linalool and thymol, carvacrol and their derivatives thymoquinone and thymohydroquinone, were tested for AChE inhibition. Thymohydroquinone exhibited the strongest AChE inhibitory effect over the range of concentrations. The AChE inhibitory potential decreased in the following order: thymohydroquinone > carvacrol > thymoquinone > essential oil > thymol > linalool. It is interesting that the AChE inhibitory effect exerted by carvacrol was 10 times stronger than that exerted by its isomer thymol, although thymol and carvacrol have a very similar structure. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords: Thymus vulgaris; acetylcholinesterase; thymol; carvacrol; thymoquinone; thymohydroquinone.

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of age-associated memory deficit. One of the therapeutic strategies developed in the AD treatment is the use of inhibitors of acetylcholinesterase (AChE), the principal enzyme involved in the hydrolysis of acetylcholine (ACh). Numerous plants and their constituents have been reputed in traditional practices of medicine to enhance the cognitive function and to alleviate other symptoms of AD, including depression (Howes *et al.*, 2003; Howes and Houghton, 2003; Perry, 1986). Besides the well known *Salvia* species as a remedy for cognitive disorders (Perry *et al.*, 2000), research on *Thymus vulgaris* essential oil also indicates its neuroprotective effects (Youdim and Deans, 2000). Due to their small molecular size and lipophilicity, volatile constituents of essential oils and liberated volatile aglycones from glycosides are likely to readily cross the blood–brain barrier (Savelev *et al.*, 2004).

This work is a part of our investigation project, dealing with the biological activities of free and glycosidically bound volatile compounds from aromatic plants (Milos *et al.*, 2000; Radonic and Milos, 2003; Jukic and Milos, 2005). Our previous investigation (Jukic and Milos, 2005) showed that the thyme essential oil, with thymol as a major compound, can be easily transformed to essential oil containing thymoquinone and thymohydroquinone among the main components. In many aromatic plants, thymoquinone and thymohydroquinone are present in the form of glycosidically bound aglycones (Stahl-Biskup *et al.*, 1993; Radonic and Milos, 2003) that become biologically active after their enzymatic hydrolysis. Since these compounds were marked as interesting for their possible use in medicine (Worthen *et al.*, 1998; Houghton *et al.*, 1995; Shoieb *et al.*, 2003), our approach to the current study was to examine their AChE inhibitory effects.

MATERIALS AND METHODS

Chemicals/reagents. All chemicals and reagents were of analytical grade and were obtained from Sigma Chemical Co. (St Louis, MQ, USA), Aldrich Chemical Co. (Steineheim, Germany), Merck (Darmstadt, Germany) and Kemika (Zagreb, Croatia). Thymoquinone and thymohydroquinone was prepared in our laboratories using the methods of Kremers *et al.* (1909) and Stolow *et al.* (1964), respectively. The purity of thymoquinone and thymohydroquinone was authenticated by NMR (Bruker AMX-400) analyses.

Plant material and essential oil. Thyme (*Thymus vulgaris* L.) was collected in central Dalmatia (Croatia). A hundred grams of dried plant material was subjected to a 3 h hydro-distillation using a Clevenger-type apparatus to produce a phenolic chemotype essential oil in the yield 1.2% (Jukic and Milos, 2005). The major compound was phenolic monoterpene thymol (35.3%).

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Other important constituents were the monoterpenic hydrocarbons p-cymene (34.7%) and terpinene (8.4%), and the oxygen-containing compounds carvacrol (5.8%), linalool (1.7%) and borneol (1.3%).

AChE inhibition assay. Inhibition of AChE from *Electrophorus electricus* (electric eel) was determined spectrophotometrically using α-naphthyl acetate as the substrate (Mastropaolo and Yourno, 1981). The method is based on an increase in absorbance at 321 nm that occurs with the hydrolysis of α-naphthyl acetate to α-naphthol. The assay was optimized regarding enzyme and substrate concentration and the reaction was linear for at least 15 min at 37 °C. A typical run consisted of 1 mL of 0.05 M Tris-hydrochloric acid buffer at pH 7.8; 10 µL of α-naphthyl acetate, at a final concentration of 0.53 mM; and 20 µL of a sample solution in 96% ethanol (EtOH). The mixture was pre-incubated at 37 °C for 5 min. The reaction was initiated by adding 20 µL of AChE, at a final assay concentration of 0.095 U/mL and measurements were taken after 15 min incubation at 37 °C. Each sample was assayed in triplicate and it also included a control in which 96% ethanol replaced the test inhibitor solution. It has been determined that an ethanol concentration of 500 mM does not affect the AChE activity which is in accordance with the Shin *et al.* (1991) study of ethanol-acetylcholinesterase interactions. A concentration of compounds that gave 50% inhibition of the enzyme was expressed as an IC_{50} value. The absorbance at 321 nm was measured on a UV-VIS (double-beam) Perkin-Elmer Lambda EZ 201 spectrophotometer.

RESULTS AND DISCUSSION

Figure 1 shows the concentration-response plots for AChE inhibition with thymol and carvacrol as well as for thymoquinone and thymohydroquinone. Their inhibitory abilities have been compared with linalool and galanthamine – a prototypical naturally occurring AchE inhibitor. The plots indicate that, after galanthamine, thymohydroquinone exhibited the strongest AChE inhibitory effect over the range of concentrations. The activity of each sample was expressed in terms of IC_{50} (the concentration required to inhibit the acetylcholinesterase level by 50%), which was calculated from log-dose curves. The AChE inhibitory potential decreased in the following order: galanthamine > thymohydroquinone > carvacrol > thymoquinone > total essential oil > thymol > linalool. Linalool inhibition of AChE was very weak (it did not achieve 50% inhibition of the enzyme within the solubility limits) which is in accordance with study done by Perry *et al.* (2000). The inhibitory effect of the total essential oil corresponded to the inhibitory effect of the main compound thymol with respect to its concentration in the total essential oil. But it is surprising that carvacrol exerted a 10 times stronger AChE inhibitory effect than its isomer thymol. This suggests that the position of the hydroxyl group in their molecular structure plays the crucial role for the AChE inhibitory effect.

In vitro study of antiinflammatory effects of thymol and different quinones (dithymoquinone, thymoquinone and thymohydroquinone) from *Nigella sativa* conducted by Marisk *et al.* (2005) suggests that these compounds participate in the general antiinflammatory activity. Research on some other plants also indicates that different quinones prevent or delay AD symptoms via their antiinflammatory activity (Mc Namara *et al.*, 2005). In our study, thymohydroquinone has been identified as a compound with the strongest AChE inhibitory effect. Generally, the strong AChE inhibitory effect was observed with the compounds that have been previously identified as strong antioxidants (Kruk *et al.*, 2000; Jukic and Milos, 2005).

In conclusion to this investigation, it is suggested that thymol and carvacrol and their derivatives thymoquinone and thymohydroquinone could be interesting for application as inhibitors of acetylcholinesterase (AChE) and that they merit further examinations for their possible application in Alzheimer's disease treatment or as remedies for cognitive disorders. It is also interesting to continue with investigations that could provide an explanation for a possible link between the antioxidant activity and the AChE inhibitory activity of the compounds mentioned (Markesbery, 1997).

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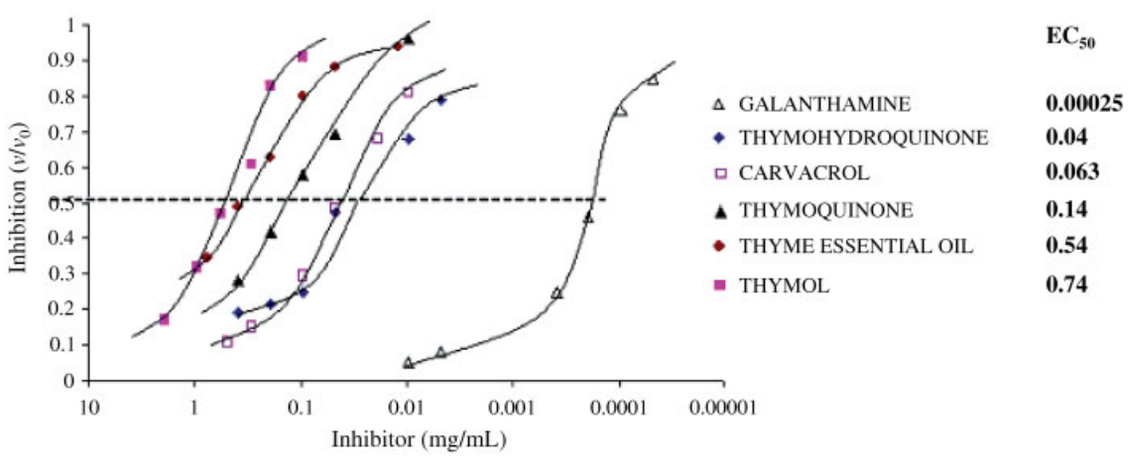

Figure 1. Dose-response plots for inhibition of acetylcholinesterase studied at a fixed concentration of substrate by thyme essential oil, its active constituents and their *in vitro* oxidation products compared with the prototypical inhibitor of AchE, galanthamine. The dashed line at v/v_0 indicates the level at which 50% inhibition of enzymatic activity is achieved.

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