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Acetylcholine esterase inhibitors and melanin synthesis inhibitors from *Salvia officinalis*

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ABSTRACT

Background: *Salvia officinalis* is a traditionally used herb with a wide range of medicinal applications. Many phytoconstituents have been isolated from *S. officinalis*, mainly phenolic diterpenes, which possess many biological activities.
Purpose: This study aimed to evaluate the ability of the phenolic diterpenes of *S. officinalis* to inhibit acetylcholine esterase (AChE) as well as their ability to inhibit melanin biosynthesis in B16 melanoma cells.
Methods: The phenolic diterpenes isolated from the aerial parts of *S. officinalis* were tested for their effect on melanin biosynthesis in B16 melanoma cell lines. They were also tested for their ability to inhibit AChE using Ellman's method. Moreover, a molecular docking experiment was used to investigate the binding affinity of the isolated phenolic diterpenes to the amino acid residues at the active sites of AChE.
Results: Seven phenolic diterpenes–sageone, 12-methylcarnosol, carnosol, 7b-methoxyrosmanol, 7a-methoxyrosmanol, isorosmanol and epirosmanol–were isolated from the methanolic extract of the aerial parts of *S. officinalis*. Isorosmanol showed a melanin-inhibiting activity as potent as that of arbutin. Compounds 7a-methoxyrosmanol and isorosmanol inhibited AChE activity by 50% and 65%, respectively, at a concentration of 500 μM.
Conclusions: The results suggest that isorosmanol is a promising natural compound for further studies on development of new medications which might be useful in ageing disorders such as the declining of cognitive functions and hyperpigmentation.

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Introduction

Alzheimer's disease (AD) is an age-related, irreversible neurodegenerative disorder characterized by progressive memory loss and impairment in cognitive function, often accompanied by behavioral disturbances. In elderly people, AD is the most frequently occurring form of dementia, especially if considered alongside concomitant cerebrovascular disease. AD is associated with loss of cholinergic synapses in the hippocampus and neocortex, resulting in deficiencies in the neurotransmitter, acetylcholine (ACh). Inhibition of acetylcholin esterase (AChE), the enzyme responsible for the hydrolysis of ACh, elevates ACh levels, and thus is considered a promising strategy for temporarily addressing AD symptoms, such as memory loss and confusion, though not for curing Alzheimer's disease or stopping it from progressing (Mesulam 2004). As two of the U.S. Food and Drug Administration (FDA)-approved acetylcholin esterase inhibitors (AChEIs)—galantamine and rivastigmine—are naturally derived, the potential for plants to yield other therapeutic agents has stimulated extensive research into the discovery of new AChEIs (Howes and Perry 2011).

Hyperpigmentation is a common, harmless skin condition in which patches of skin become darker in color than the surrounding area due to excess production of melanin. A common form of hyperpigmentation is age spots, which are dark, uneven patches of skin that appear in response to sunlight exposure. Several factors such as sun exposure, inflammation, free radicals and hormonal changes cause reductions in the number of melanocytes in elderly people. As a result, melanocytes are stimulated to overproduce melanin, and the excess melanin is unevenly distributed in the

Abbreviations: S. officinalis, *Salvia officinalis*; AChE, acetylcholine esterase; AD, Alzheimer's disease; AChEIs, acetylcholine esterase inhibitors; FDA, U.S. food and drug administration; Aβ, amyloid-β peptide; Rp HPLC, reversed phase high performance liquid chromatography; ^{1}H and ^{13}C NMR, proton and carbon-13 nuclear magnetic resonances; ACTI, acetylthiocholine iodide; MTT, thiazolyl blue tetrazolium bromide; DTNB, 5,5-dithiobis [2-nitrobenzoic acid]; NaOH, sodium hydroxide; DMSO, dimethyl sulfoxide; EMEM, Eagle's Minimum Essential Medium; FBS, fetal bovine serum; CO_2, carbon dioxide; PBS, phosphate-buffered saline.

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epidermis, causing hyperpigmentation (Matt et al. 2007; Videira et al. 2013).

The medicinal use of plants and their phytoconstituents, especially those long used in folk medicine, is becoming very popular worldwide. *S. officinalis* (common sage) is a common culinary herb native to the Mediterranean region and Europe. Known in Arabic as mairamia, *S. officinalis* has been traditionally used to treat digestive disorders, circulation disturbances, bronchitis, cough, asthma, and angina and to reduce excessive perspiration. It is also used as a mouth wash for the treatment of inflammations of the mouth and throat mucosa such as gingivitis and pharyngitis (Saad and Said 2011; Hamidpour et al. 2013). Scientific researches have reported that hydroalcoholic and methanolic extracts of *S. officinalis* and several isolated compounds showed a wide range of biological activities (Devansh 2012; Hamidpour et al. 2013), such as antibacterial, antifungal (Gracia et al. 2012; Stefanovic et al. 2012; Abdelkader et al. 2014), antioxidant (Rasmy et al. 2012; Neagu et al. 2014), anti-inflammatory (Baricevic et al. 2001), anti-angiogenic (Keshavarz et al. 2010) and anti-cancer activities (Janicsak et al. 2011), in addition to having anti-diabetic potential (Christensen et al. 2010).

S. officinalis has been reported to produce many biologically active compounds, mainly phenolic diterpenoids (Masahiro et al. 1994; Masahiro et al. 1997; Fischedick et al. 2013), triterpenoids (Topçu 2006) and polyphenolic compounds (Lu and Yeap 2000).

The alcohol extract of *S. officinalis* is known to be effective in the management of mild-to-moderate Alzheimer's disease (Akhondzadeh et al. 2003; Sandra et al., 2014) through improvement of cognition and memory with no adverse effects, even after many years of use (Perry et al. 1999). Several researches have suggested that *S. officinalis* extract improves memory and cognition through dose-dependent inhibition of AChE (Kennedy et al. 2006; Russo et al. 2013) and by exerting a neuroprotective effect against Aβ-induced toxicity (Iuvone et al. 2006).

However, there is a lack of research about the effect of the individual phenolic diterpenes comprising the major bioactive phytoconstituents of *S. officinalis* in the inhibition of AChE, and thus in the possible management of AD. In the present study, therefore, we evaluated individual phenolic diterpenes as AChEIs.

Although skin-whitening creams containing *S. officinalis* are used (Toshitsugu and Juko, 1995), there are no detailed data or investigations on the phytochemical constituents of *S. officinalis* responsible for this antimelanogenesis effect. We thus also evaluated the effect of several isolated phenolic diterpenes from the aerial parts of *S. officinalis* as melanin synthesis inhibitors, with the aim of developing effective treatments against hyperpigmentation (age spots). Together, therefore, the present experiments evaluated the ability of several isolated phenolic diterpenes from the aerial parts of *S. officinalis* as both AChE and melanin synthesis inhibitors.

Materials and methods

General experimental procedures

Rp HPLC was performed using a Cholester column (4.6 mm × 250 mm) at 210 nm and 1 ml/min ^{1}H and ^{13}C NMR spectra were recorded on a *Bruker AV 400* spectrometer (400 and 100 MHz for ^{1}H and ^{13}C, respectively).

Plant material

The dry aerial parts of *S. officinalis* (3 kg) were purchased from the local market in Cairo, Egypt in 2012. The aerial parts were compared with a reference sample kept at the Pharmacognosy Department, Faculty of Pharmacy, Mansoura University, Egypt. A voucher specimen (2012-SO/01) was placed at the Herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Mansoura University.

Extraction and isolation of compounds

The powdered plant material (3 kg) was extracted with methanol (10 l × 3), and the collected methanolic extracts were evaporated under reduced pressure, yielding a dark green viscous residue (500 g). The methanolic extract residue was fractionated using n-hexane, chloroform and ethyl acetate, respectively. The n-hexane fraction (130 g) was chromatographed on a silica gel column using a petroleum ether : ethyl acetate gradient elution, then purified on a Sephadex LH20 column using methylene chloride for elution, yielding compound **1** (3.4 mg). The chloroform fraction (15 g) was chromatographed on a silica gel column using methylene chloride : methanol (100:0–0:100), yielding 5 groups, **I-V**. Group **I**, eluted with 100% methylene chloride, yielded on evaporation compound **2** (10 mg). Group **II**, eluted with methylene chloride : methanol (99:1), was further purified on a silica gel column using methylene chloride : methanol (100:0 and 99:1), yielding compound **3** (10 mg). Group **III**, eluted with methylene chloride : methanol (97:3), was purified on the Sephadex LH20 column, yielding Group **III A** and Group **III B**. Group **III A** (20 mg) was further purified by Rp HPLC using acetonitrile : water yielding compound **4** (*Rt* 33 min) and compound **5** (*Rt* 35 min). Group **IV**, eluted with methylene chloride : methanol (95:5), was purified on a Sephadex LH20 column, yielding compound **6**. Group **V**, eluted with methylene chloride : methanol (95:5), was purified on a silica gel column using petroleum ether : ethyl acetate (100:0 to 0:100), yielding compound **7**.

Reagents for biological assays

Acetylthiocholine iodide (ACTI) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Thiazolyl blue tetrazolium bromide (MTT), galantamine hydrobromide and AChE from Electrophorus electricus (electric eel), 500 U/mg, were purchased from Sigma (St. Louis, MO, USA). 5,5-Dithiobis [2-nitrobenzoic acid] (DTNB), NaOH and DMSO were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Media for cell lines

Eagle's Minimum Essential Medium (EMEM) was purchased from Nissui Pharmaceutical (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from Gibco BRL (Tokyo, Japan). Buffer A (50 mM Tris-HCl, pH 8, 0.1% BSA) and Buffer B (50 mM TrisHCl, pH = 8, 0.1 M NaCl, 0.02 M $MgCl_2.6\ H_2O$) were used.

Cell line

A mouse B16 melanoma cell line was obtained from RIKEN Cell Bank. The cells were maintained in EMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.09 mg/ml theophylline. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2.

Melanin biosynthesis inhibitory activity assay

The assay was carried out according to Ashour et al. (2013). The mouse B16 melanoma cells were cultured in a pair of 24-well plates (one plate for melanin content determination and the other for cell viability testing), at a density of 1×10^5 cells/well. After 24 h, the medium was replaced with 998 µl of fresh medium and 2 µl of the test compound at maximum solubility (n = 3). The final concentrations of the tested compounds are shown in

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