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Research Paper

Enzyme inhibitory and antioxidant activities of *Nerium oleander* L. flower extracts and activity guided isolation of the active components

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ABSTRACT

Oleander (*Nerium oleander* L., fam: Apocynaceae) is an evergreen shrub. Although it is known to be poisonous to humans, a large number of utilizations in folk medicine have been reported against diabetes, rheumatic pain and skin diseases. The present study aimed to investigate the cholinesterase inhibitory activities of oleander flower extracts and to isolate the active components responsible for the activity. The antidiabetic and skin care effects were also determined on some key enzymes (α-glucosidase, α-amylase, tyrosinase). The flower extracts obtained with aqueous, polar and apolar organic solvents were evaluated for their phenolic contents and antioxidant capacities. The enzyme inhibitory activities (cholinesterase, α-glucosidase, α-amylase and tyrosinase) were examined by microtiter plate assays. Antioxidant properties were evaluated by DPPH, FRAP and CUPRAC assays. Total phenolic and flavonoid contents were determined using the Folin-Ciocalteu reagent and aluminum chloride, respectively. The structures of the isolates were elucidated by NMR and MS experiments.In the present study, cholinesterase inhibitory activity of the EtOH extract of the olaender flowers was investigated and β-sitosterol and oleanolic acid were isolated as the active components. The less polar extracts (*n*-hexane) exerted better cholinesterase and α-amylase inhibitory activities than those of the more polar extracts (R-H_2O, EtOAc) which had better α-glucosidase and tyrosinase activities. The highest antioxidant capacity values were obtained from EtOAc and EtOH extracts. The EtOH extract was found to contain the highest levels of total phenolic and flavonoid contents. The results suggest that the flowers of oleander could be a potential source for high value phytochemicals for developing novel drug leads.

1. Introduction

Enzyme inhibitory approches are recognised as one of the most effective strategies for critical health problems including Alzheimer's disease (AD) which affects a great part of the world population (ADI, 2015; Anand and Singh, 2013; WHO, 2016). It is estimated that 46.8 million people are living with dementia and AD affects 20 million individuals worldwide as the main form of dementia (ADI, 2015; Anand and Singh, 2013).

AD arises from the deficiency of the cholinergic system and deposition of *β*-amyloid and amyloid plaques in temporoparietal cortex. Acetylcholine (ACh) hydrolysis is catalyzed by acetylcholinesterase (AChE) in the synaptic gap. In this sense, cholinesterase inhibitors enhance cholinergic transmission by inhibiting AChE. Moreover, AChE and butrylcholinesterase (BChE) play an important role in *β*-amyloid-aggregation (Anand and Singh, 2013; Chopra et al., 2011). For that reason, AChE and BChE inhibitions are known to be important targets in drug discovery challenges for AD (Anand and Singh, 2013).

On the other hand, Diabetes Mellitus (DM) is characterised by chronic hyperglycemia. An estimated 422 million adults were reported to live with DM in 2014 (WHO, 2016). This may be caused by the defects in insulin secretion or action. The enzymes responsible for carbohydrate digestion by mammals are α-glucosidase and α-amylase. Inhibition of these enzymes results in reduced rates of glucose release

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from foods and absorption in the small intestine (Tundis et al., 2010).

Tyrosinase play an essential role in the synthesis of melanin. Melanin pigmentation prevents skin against UV damage. Nevertheles, overproduction of melanin causes pigment variations such as melasma and age spots. In the treatment of skin disorders (SD), tyrosinase inhibitors (*e.g.,* kojic acid, arbutin) are used (Kim and Uyama, 2005).

Medicinal plants and their active components have been shown to exert cholinesterase inhibitory and antidiabetic activities in numerous studies (Ríos et al., 2015; Xiao and Tundis, 2013). Among them, galanthamine, as a natural alkaloid of *Galanthus* spp., was approved for the treatment of AD in many countries. On the other hand, acarbose is widely used as a digestive enzyme inhibitor for the treatment of DM which acts on α-glucosidase and α-amylase (Ríos et al., 2015; Xiao and Tundis, 2013). Although there are a number of enzyme inhibitors for the treatment of these diseases, side effects such as gastrointestinal system disturbances are very common (Chopra et al., 2011; van de Laar, 2008).

Free radical ($O_2\cdot^-$, superoxide radicals; OH· hydroxyl radical; $HO_2\cdot$, perhydroxy radical and RO·, alkoxy radicals) and non-radical (molecular) forms (H_2O_2, hydrogen peroxide and $^1O_2$, singlet oxygen) constitues reactive oxygen species (ROS). They are continuously generated as a consequence of metabolic activities in living organisms. The balance between their production and antioxidant mechanisms of the body is essential because an imbalance leads to oxidative stress. It is known that oxidative stress contributes to the pathogenesis of various debilitating diseases including cancer, diabetes and inflammatory diseases (Geronikaki and Gavalas, 2006). From this point, antioxidants from plant sources are of great importance to combat oxidative stress associated diseases. Therefore, an increasing global interest has recently been observed towards natural antioxidants (Embuscado, 2015).

Nerium oleander L. (oleander, fam: Apocynaceae) is an evergreen small shrub. It is well known for its is poisonous effect to humans which limits its usage in traditional medicine (Kiran and Prasad, 2014). However, a number of folk medicinal utilizations or preparations were reported against diabetes, rheumatic and skin diseaeses (Jamila and Mostafa, 2014; Yesilada, 2002). Moreover, some folk medicinal preparations of oleander flowers were shown to exert various activities such as cytotoxic, anti-inflammatory, analgesic, antioxidant, cardioprotective, hepatoprotective and neuroprotective activities in previous studies (Erdemoğlu et al., 2003; Gayathri et al., 2011; Singhal and Gupta, 2012a,b; Yu et al., 2004). Cardenolides, triterpenes, steroids, pregnanes and flavonoids were reported to be the chemical classes of the secondary metabolites from different organs of *N. oleander* (Abe and Yamauchi, 1992; Hanada et al., 1992; Siddiqui et al., 2012; Zia et al., 1995).

There is an increasing evidence on the effectiveness of oleander for the treatment of AD. Previously, the methanol (MeOH) extract of the flowers of the plant was reported to reverse copper and aluminium induced dementia in rats and the polysaccharides from the flowers exerted neuroprotective effects in primary rat cortical neuronal cultures against serum-deprivation and *β*-amyloid peptide toxicity (Singhal and Gupta, 2012b; Yu et al., 2004). Moreover, a method has recently been developed to treat neurological conditions including AD, Huntington's disease or stroke with an extract prepared from some oleander species and patented (Addington and Newman, 2015). However, the methanol extract of the leaves was reported to be ineffective against AChE at 1000 μg/mL concentration (Ali et al., 2013). Therefore, the present study was aimed to investigate the cholinesterase enzyme inhibitory activites of oleander flower extracts and to isolate some active components responsible for the activity. The antidiabetic and skin care effects were also determined on some key enzymes (α-glucosidase, α-amylase and tyrosinase). Furthermore, total phenolic compounds and flavonoid contents in the flower extracts were evaluated and correlated in conjunction with antioxidant capacity values.

2. Materials and methods

2.1. General

In order to record ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectra, a Varian Mercury-Mx spectrometer was used (USA) with CD_3OD as solvents. For column chromatography Kieselgel 60 (0.063–0.200 mm; Merck, Darmstadt, Germany) was used. In the thin layer chromatography (TLC) experiments, precoated Kieselgel 60 F254 (Merck, Darmstad, Germany) plates were used.

2.2. Plant material

The flowers of oleander (*N. oleander* L.) were collected from Kayışdağı-İstanbul (Turkey) in June 2010. The specimen was identified by Prof. Dr. Erdem Yesilada (one of the authors). A voucher specimen is deposited at the Herbarium of Yeditepe University (YEF 10009), İstanbul (Turkey).

2.3. Extraction and solvent fractionation

The flower extract of oleander was prepared according to Yesilada (2002). Accordingly, the fresh flowers (2.5 kg) of oleander were removed from flowers debrices and artifacts (calyx, insects, etc.) and first macerated with absolute ethanol (EtOH, 700 mL × 2) at room temperature for 50 days following the recipe described in Turkish folk medicine (Yesilada, 2002). The extract was filtered through filter paper. The organic phase was evaporated to dryness under reduced pressure at 40 °C and a crude EtOH extract was obtained (50 g, yield: 6.8%). Then the EtOH extract was redissolved in 100 mL of 90% MeOH and extracted with *n*-hexane (4 × 200 mL). The *n*-hexane subextract was obtained by evaporating the organic phase under reduced pressure (*n*-Hexane subextract; 3 g, yield: 6%). After removing the MeOH from the remaining extract and adjusting the volume to 100 mL with distilled water, partition went on with dichloromethane (CH_2Cl_2; 4 × 200 mL) and ethylacetate (EtOAc; 4 × 200 mL), respectively to yield CH_2Cl_2 subextract (2 g, yield: 4%), EtOAc subextract (1.1 g, yield: 2.2%) and remaining water (R-H_2O) subextract (30.5 g, yield: 60.1%).

2.4. Isolation of active constituents from the n-hexane subextract

The *n*-hexane subextract (2.5 g) was applied onto a SiO_2 (200 g) column eluting with *n*-hexane (100 mL) and a stepwise gradient of EtOAc in *n*-hexane (0–34% in ratios of 2%, each 100 mL) to yield four fractions (fr.), from A to D. The fr. A (35 mg) was loaded to a SiO_2 (6 g) column and eluted with *n*-hexane (100 mL) and a gradient of EtOAc in *n*-hexane (0–6% in steps of 1%, each 50 mL) and oleanolic acid (10 mg) was obtained. The Fr. C (35 mg) was applied to a SiO_2 (6 g) column eluted with *n*-hexane (100 mL) and a gradient of EtOAc in *n*-hexane (0–6% in steps of 1%, each 50 mL) to yield *β*-sitosterol (6 mg).

2.5. Structure elucidation of the isolates

The structures of the isolates were identified as β-sitosterol and oleanolic acid by interpreting NMR (^{1}H- and ^{13}C NMR) and HR-MS analysis and by comparison of their spectroscopic data with those published earlier (Kojima et al., 1990; Seebacher et al., 2003). The chemical structures of the compounds were given in Fig. 1.

2.6. Determination of total phenolic compounds (TPC) and flavonoid (TF) contents

The TPC content of extracts/subextracts was determined by the Folin-Ciocalteu (FC) method (Slinkard and Singleton, 1977). Briefly, extracts/subextracts were dissolved in 500 μL DMSO (Dimethyl sulfoxide). The extracts (500 μL) and the same amount of deionized water