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Neuroprotective potential of *Viburnum orientale* Pallas through enzyme inhibition and antioxidant activity assays

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ABSTRACT

Neurobiological effects of the fruit, leaf, and branch extracts of *Viburnum orientale*, used in the traditional drink called "gilaburi" in Anatolia, were tested against acetyl- (AChE), butyrylcholinesterase (BChE), and tyrosinase (TYR). Antioxidant effect of the extracts was determined using six *in vitro* methods. The fruit and branch methanol extracts with the richest total phenolic contents exerted the highest inhibition against all enzymes. These extracts were the most active in DPPH radical scavenging, ferric- (FRAP) and phosphomolibdenum-reducing antioxidant power (PRAP) assays, which was followed by the leaf aqueous extract with the best nitric oxide (75.00 ± 1.22%), DMPD scavenging (33.70 ± 1.13%), and metal-chelating (54.66 ± 3.56%) effects at 2500 μg/mL. We previously reported that the fruits are rich in chlorogenic acid, which might be related to its marked enzyme inhibitory and antioxidant effect. Our results indicated that all methanol and leaf aqueous and branch extracts of the plant are promising for their neuroprotective potential.

1. Introduction

The genus *Viburnum* L. (Caprifoliaceae), which comprises of over 230 species in the world, (Lobstein et al., 1999), is represented by four species in the flora of Turkey as *V. opulus* L. (cranberrybush), *V. lantana* L., *V. tinus* L. and *V. orientale* Pallas, called "kartopu" among local people (Chamberlain, 1972). Several species of *Viburnum* possess edible fruits consumed either raw or in the form of jam such as *V. trilobum* consumed by native North Americans (Burns Kraft et al., 2008). On the other hand, the berry type of fruits of some species of *Viburnum* is used to prepare a kind of traditional drink, locally known as "gilaburu" in inner Anatolia. The genus *Viburnum* L. has a very rich diversity of chemical compounds such as vibsane-type of diterpenes, triterpenes, sesquiterpenes, flavonoids, lignans, iridoids, and phenolic compounds (Wang et al., 2008; Sever-Yilmaz et al., 2013; Hu et al., 2014).

Alzheimer's disease (AD) is a progressive neurodegenerative disorder with a complicated pathogenesis. One of the accepted hypotheses suggested for AD pathology is cholinergic hypothesis described as the obvious deficit of acetylcholine in the brains of AD patients, which is hydrolyzed by acetylcholinesterase (AChE, EC 3.1.1.7) (Orhan et al., 2011). Furthermore, excess amount of another enzyme, butyrylcholinesterase (BChE, EC 3.1.1.8), has been also found in the plaques in the brain (Nordberg et al., 2013).On the basis of this knowledge, inhibition of these two sister enzymes has been an important strategy for AD treatment and cholinesterase inhibitors have become one of the most prescribed drug class against this disease. Tyrosinase (TYR; polyphenol oxidase or oxygen oxidoreductase, EC 1.14.18.1) is a copper-containing enzyme that catalyzes the rate-limiting oxidation of tyrosine to melanin. TYR exerts a vital role in pigmentation of skin related to melanoma, detrimental browning of fruits and vegetables, molting process of insects and well as neuronal death in Parkinson's disease (PD) and Huntington's disease (Mendes et al., 2014).

We previously investigated *in vitro* neuroprotective effect of *V. opulus*, *V. lantana*, and *V. tinus* (Sever-Yilmaz et al., 2013) and among them, the branch-ethyl acetate and fruit-methanol extracts of *V. tinus* possessed a significant cholinesterase inhibitory effect, while the fruit-methanol extrac of the same speciest exerted the hightest inhibition against TYR. Besides, the extracts showed a remarkable antioxidant activity. Taking these results into account, we aimed to establish cholinesterase and TYR inhibitory effect of another *Viburnum* species, *e.g. V. orientale*, using ELISA microtiter assays. In addition, antioxidant potential of the extracts of the plant was investigated a number of *in vitro* methods. Total phenol and flavonoid quantities of *V. orientale* extracts were calculated spectrophotometrically adapted to ELISA microplate assay.

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2. Materials and methods

2.1. Plant material

The flowering samples of *V. orientale* were collected in 2011 from the vicinities of Artvin, Turkey. The plant was identified by Dr. Ayse Mine Ozkan from the Department of Pharmaceutical Botany, Faculty of Pharmacy, Ankara University (Turkey) and the voucher specimen is deposited in the Herbarium of Faculty of Pharmacy of Ankara University (AEF number 25988).

2.2. Preparation of the extracts

The air-dried and powdered branch, leaf, and fruit parts of *V. orientale* were extracted sequentially with ethyl acetate (EtOAc), methanol (MeOH), and distilled water (H_2O). The extracts were prepared by macerating 10 g of each plant powder in 100 mL of the corresponding solvents for 8 h, respectively. The macerates obtained with EtOAc and MeOH were evaporated until dryness using rotary evaporator, while the H_2O extract was lyophilized. The extracts amounts were obtained as follows: for the fruits - EtOAc: 0.6581 g, MeOH: 0.6310 g, H_2O: 0.4606 g; for the leaves - EtOAc: 0.3588 g, MeOH: 1.0138 g, H_2O: 1.4701 g; for the branches - EtOAc: 0.1842 g, MeOH: 0.7510 g, H_2O: 0.2289 g.

2.3. Enzyme inhibition assays

2.3.1. Cholinesterase inhibition

AChE and BChE inhibitory activity was measured by slightly modified spectrophotometric method of Ellman et al. (1961). Electric eel AChE (Type-VI-S; EC 3.1.1.7, Sigma, St. Louis, MO, USA) and horse serum BChE (EC 3.1.1.8, Sigma, St. Louis, MO, USA) were used, while acetylthiocholine iodide and butyrylthiocholine chloride (Sigma, St. Louis, MO, USA) were employed as the substrates of the reaction. 5,5′-Dithio-bis(2-nitrobenzoic)acid (DTNB; Sigma, St. Louis, MO, USA) was used for the measurement of the anticholinesterase activity. All reagents and conditions were same as described in our previous publication (Ercetin et al., 2012). Briefly, in this method, 140 μL of sodium phosphate buffer (pH 8.0), 20 μL of DTNB, 20 μL of test solution and 20 μL of AChE/BChE solution were added by multichannel automatic pipette (Gilson pipetman, Paris, France) in a 96-well microplate and incubated for 15 min at 25 °C. The reaction was then initiated with the addition of 10 μL of acetylthiocholine iodide/butyrylthiocholine chloride. Hydrolysis of acetylthiocholine iodide/butyrylthiocholine chloride was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalyzed by enzymes at 412 nm utilizing a 96-well microplate reader (VersaMax Molecular Devices, Sunnyvale, CA, USA). The measurements and calculations were evaluated by using Softmax PRO 4.3.2.LS software (Sunnyvale, CA, USA). Percentage of inhibition of AChE/BChE was determined by comparison of reaction rates of the samples relative to blank sample (ethanol in phosphate buffer pH = 8) using the formula $(E - S) / E \times 100$, where E is the activity of enzyme without test sample and S is the activity of enzyme with test sample. The experiments were done in triplicate. Galanthamine (Sigma, St. Louis, MO, USA), the anticholinesterase alkaloid-type of drug obtained from the bulbs of snowdrop (*Galanthus* sp.), was used as the reference.

2.3.2. Tyrosinase inhibition

Inhibition of TYR (EC 1.14.1.8.1; 30 U, mushroom tyrosinase, Sigma) was determined using the modified dopachrome method with *L*-DOPA as substrate (Masuda et al., 2005). The assays were conducted in 96-well microplate using ELISA microplate reader (VersaMax Molecular Devices, USA) to measure absorbance at 475 nm. An aliquot of the extracts dissolved in DMSO with 80 μL of phosphate buffer (pH 6.8), 40 μL of TYR, and 40 μL of *L*-DOPA were put in each well. Results were compared with control (DMSO). Baicalein (Sigma, St. Louis, MO, USA) was used as the reference. The percentage TYR inhibition (I%) was calculated as follows:

$$I\% = (\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}) / \text{Absorbance}_{\text{control}} \times 100$$

2.4. Antioxidant activity by radical-formation methods

2.4.1. DPPH radical scavenging activity

The stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was determined by the method of Blois (1958). The samples (30 μL) and reference dissolved in ethanol (75%) were mixed with 2700 μL of DPPH solution (1.5×10^{-4} M). Remaining DPPH amount was measured at 520 nm using a Unico 4802 UV–visible double beam spectrophotometer (Dayton, NJ, USA). Gallic acid (Sigma, St. Louis, MO, USA) was employed as the reference. Inhibition of DPPH in percent (I%) was calculated as given below:

$$I\% = \left[(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}\right] \times 100,$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test sample), and A_{sample} is the absorbance of the extracts/reference. Analyses were run in triplicate and the results were expressed as average values with S.E.M.

2.4.2. DMPD radical scavenging activity

The assay is based on reduction of the purple-colored radical DMPD + (*N,N*-dimethyl-*p*-phenylendiamine). According to the method (Schlesier et al., 2002), a reagent comprising of 100 mM DMPD, 0.1 M acetate buffer (pH = 5.25), and 0.05 M ferric chloride solution, which led to formation of DMPD radical, was freshly prepared and the reagent was equilibrated to an absorbance of 0.900 ± 0.100 at 505 nm. Then, the reagent was mixed up with 50 μL of the extract dilutions and absorbance was taken at 505 nm using a Unico 4802 UV–visible double beam spectrophotometer (Dayton, NJ, USA). Quercetin was employed as the reference and the experiments were done in triplicate. The results were calculated according to the same formula given for DPPH radical scavenging test and expressed as average values with S.E.M.

2.4.3. Nitric oxide (NO) radical scavenging activity

The scavenging activity of the extracts against NO was assessed by the method of Marcocci et al. (1994). Briefly, the extract dilutions were mixed with 5 mM sodium nitroprusside and left to incubation for 2 h at 29 °C. An aliquot of the solution was removed and diluted with Griess reagent (1% sulfanilamide in 5% H_3PO_4 and 0.1% naphthylethylenediaminedihydrochloride). The absorbance of the occurred chromophore was measured at 550 nm using a Unico 4802 UV–visible double beam spectrophotometer (USA). Inhibition of NO radical in percent (I%) was calculated as given below:

$$I\% = \left[(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}\right] \times 100,$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test sample), and A_{sample} is the absorbance of the extracts. Analyses were run in triplicates and the results were expressed as average values with S.E.M. (Standard error of the mean). Quercetin was the reference in this test.

2.5. Antioxidant activity by metal-related and other methods

2.5.1. Metal-chelating capacity

The metal-chelating capacity of the extracts through ferrous ion was estimated by the method of Chua et al. (2008). Briefly, dilutions of the extracts were incubated with 2 mM $FeCl_2$ solution. The reaction was initiated by the addition of 5 mM ferrozine into the mixture and left