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Full Length Research Paper

In vitro enzyme inhibition activities of *Myrtus communis* L.

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The crude methanolic extract and chloroform, ethyl acetate and *n*-butanol fractions of *Myrtus communis* L. were examined as inhibitors of acetylcholinesterase, butyrylcholinesterase and lipoxygenase. A significant enzyme inhibition activity (81 to 91%) was shown by the crude methanolic extract and its fractions against acetylcholinesterase, while high to outstanding enzyme inhibitory activity (72.5 to 99%) was shown against butyrylcholinesterase. The crude methanolic extract and its various fractions also demonstrated significant activity (79 to 94.5%) against lipoxygenase.

Key words: *Myrtus communis* L., acetylcholinesterase, butyrylcholinesterase, lipoxygenase, inhibition.

INTRODUCTION

Myrtus communis L. (Myrtaceae) commonly called Myrtle (English) is an evergreen shrub and is widely distributed in Mediterranean region. Myrtle has been used as a folk medicine in several remedies (Feibt et al., 2005; Sepici et al., 2004; Watt, 1972; Mulas et al., 2000; Winter and Willeke, 1951). Several studies have revealed the strong antibacterial, anti-inflammatory, anti-hyperglycemic and antioxidant activities in the various extracts of this plant (Feibt et al., 2005; Hayder et al., 2004; Romani et al., 2004; Bonjar, 2004; Rosa et al., 2003). Alpha-glucosidase (α-glucosidase) inhibition activity of aqueous extract of Myrtle has also been reported (Onal et al., 2005) and the characteristic constituents of this plant include monoterpenoids, flavonoids, triterpenoids (Diaz and Abeger, 1997) and phloroglucinol-type compounds (Rotstein et al., 1974; Kashman et al., 1974; Appendino et al., 2002).

Acetylcholinesterase (AChE) hydrolyses acetylcholine (ACh) which when released from synaptic vesicles briefly depolarizes the postsynaptic cell membrane. ACh is then hydrolyzed by AChE to choline and acetate. Thus, AChE regulates nerve impulse transmission across cholinergic synapses (Siegfried and Scott, 1990). The enzyme AChE has long been an attractive target for the rational drug design and discovery of mechanism-based inhibitors, because of its role in the hydrolysis of the neurotransmitter ACh (Taylor, 1998). AChE inhibitors, that can increase the cholinergic transmission by blocking the degradation of ACh, are therefore used for alleviating the symptoms of patients with Alzheimer's disease (AD) (Lawrence and Sahakian, 1988; Rocca et al., 1986). Inhibition of AChE is considered to be a promising approach for the treatment of AD and for possible therapeutic applications in the treatment of Parkinson's disease, aging and myasthenia gravis (Quinn, 1987; Nochi et al., 1995).

Butyrylcholinesterase (BChE) is an ester-hydrolyzing enzyme without an assigned function, taking part in a variety of pathways like detoxification, neural transmission, proliferation and lipid metabolism (Brown et al., 1981; Layer and Willbold, 1995; Kutty, 1980). It is

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differentiated from the true cholinesterase, AChE, by its ability to hydrolyze a wide range of choline esters and ester containing compounds (Tunek et al., 1988; Gorelick, 1997; Lockridge and Masson, 2000). These enzymes are named ‘‘bioscavenger enzymes’’ due to their toxicological and pharmacological importance (Ashani et al., 1991). The role of BChE in the normal aging and diseased brain is still unknown. However, recently, it has been found that BChE exists in significantly higher quantities in Alzheimer’s plaques than in plaques of age related nondemented brains. Moreover, the inclusion of cymserine which is a very potent selective BChE inhibitor in the clinical trials for AD treatment proved that BChE inhibition could be an important target for the treatment of AD and related dementias (Yu et al., 1999; Choudhary et al., 2002).

Arachidonate 5-lipoxygenase (5-LO) is the key enzyme in leukotriene (LT) biosynthesis and it catalyzes the initial steps in the conversion of arachidonic acid to biologically active leukotrienes (LTs). LTs are considered as potent mediators of inflammatory and allergic reactions, which are released by leukocytes and other 5-LO expressing cells. Arachidonic acid metabolism through lipoxygenase pathway generates various biologically active lipids that play an important role in inflammation (Steinhilber, 1999). Angiogenesis, the formation of new capillary vessels from preexisting ones, underpins a number of physiological processes and participates in the development of several pathological conditions, such as arthritis and cancer (Nie and Honn, 2002). Lipoxygenases are therefore potential targets for the rational drug design and discovery of mechanism-based inhibitors for the treatment of a variety of disorders including bronchial asthma, inflammation, cancer and autoimmune diseases. Thus, search for new LO inhibitor seems to be a promising approach for the development of new drugs.

The current study was undertaken to screen the indigenous medicinal plant of Northern areas of Pakistan *M communis* L. for the enzyme inhibition activity to provide a base for the development of rational drug design against AD, dementia, Parkinson’s disease, inflammation, allergy, asthma, cancer, etc.

MATERIALS AND METHODS

Plant

The aerial parts (8 kg dry weight (wt)) of *M. communis* L. were collected from village Kabal of Swat district, Khyber Pukhtoonkhwa, Pakistan, at an elevation of 1800 m in May to June, 2003 and was identified by Mr. Mahboob-ur-Rahman (Assistant Professor), Department of Botany, Government Jahanzeb Post Graduate College, Saidu Sharif, Swat, Khyber Pukhtoonkhwa, Pakistan). A voucher specimen (CM-03) was deposited in the herbarium of the Department Botany.

Extraction

The freshly collected air-dried plant material (leaves) was chopped into small pieces and pulverized into a fine powder. Plant material (8 kg) was soaked and extracted by maceration in 80% methanol for 10 days (3 × 50 L), the combined methanol soluble material was filtered off. The filtrate was concentrated under vacuum at low temperature (35°C) using a rotary evaporator. A blackish crude extract (600 g) was obtained.

Fractionation

The crude methanolic extract (600 g) was suspended in distilled water (1000 ml) and sequentially partitioned with n-hexane (3 × 1000 ml), chloroform (3 × 1000 ml), ethyl acetate (3 × 1000 ml) and n-butanol (2 × 1000 ml) to yield n-hexane (41 g), chloroform (55 g), ethyl acetate (80 g), n-butanol (170 g) and aqueous (210 g) fractions, respectively.

In vitro cholinesterase inhibition assay

AChE and BChE inhibitory activities were measured spectrophotometrically by modifying the method of Ellman (Ellman, 1958; Ellman et al., 1961). For this purpose, an enzyme kit (DACE-100-QuantiChrom™ Acetylcholinesterase Assay Kit) based on the Ellman method was employed. Electric-eel AChE (type VI-S, Sigma) and Horse serum BChE (Sigma) were used, while acetylthiocholine iodide and butyrylthiocholine chloride (Sigma) were used as substrates of the reaction. 5,5-dithiobis[2-nitrobenzoic acid] (DTNB, Sigma) was used for the measurement of cholinesterase activity. 140 ml of the 100 mM sodium phosphate buffer (pH 8.0), 10 ml of DTNB (1 mmol/L), 20 ml of the crude extract solution and 20 ml of AChE/BChE (0.05 mg/ml of AChE and 0.2 mg/ml of BChE) solution were mixed and incubated for 15 min at 25°C. The reaction was then initiated by the addition of 10 ml of acetylthiocholine/butyrylthiocholine (0.71 mM of acetylthiocholine and 0.2 mM of butyrylthiocholine). The hydrolysis of acetylthiocholine/butyrylthiocholine were monitored by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine or butyrylthiocholine at a wavelength of 412 nm using a spectrophotometer (Beckman DU-600 spectrophotometer, USA) in the quartz cuvettes (Starna, U.K., no: 1 OG 5391). The extracts and the control were dissolved in EtOH (5%). The rate of enzymatic reaction was finally determined by Ellman equation:

$$\text{Rate (mols / L / min)} = \frac{\text{Change in absorbance / min}}{13,600}$$

All the inhibition studies were conducted in 96-well micro-titer plates using Spectra Max 340 (Molecular Devices, CA, USA).

In vitro lipoxygenase inhibition assay

Lipoxygenase inhibitory activity was undertaken by slightly modifying the spectrophotometric method developed by Tappel (Tappel, 1962). Lipoxygenase (type I-B) and linoleic acid were purchased from sigma (St. Louis, MO). All other chemicals were of analytical grade. 160 ml of 100 mM sodium phosphate buffer (pH 8.0), 10 ml of crude extract solution and 20 ml of lipoxygenase solution (100 pmol) were mixed and incubated for 10 min at 25°C. The reaction was then initiated by the addition of 10 ml linoleic acid (0.5 mmol) solution (substrate), with the formation of (9Z,11E)-(13S)-13-hydroperoxyoctadeca-9,11-dienoate, the change in the reaction was followed for 10 min. Crude extract and the control were dissolved in MeOH (5%). All the reactions were performed in triplicate in 96 well plates.

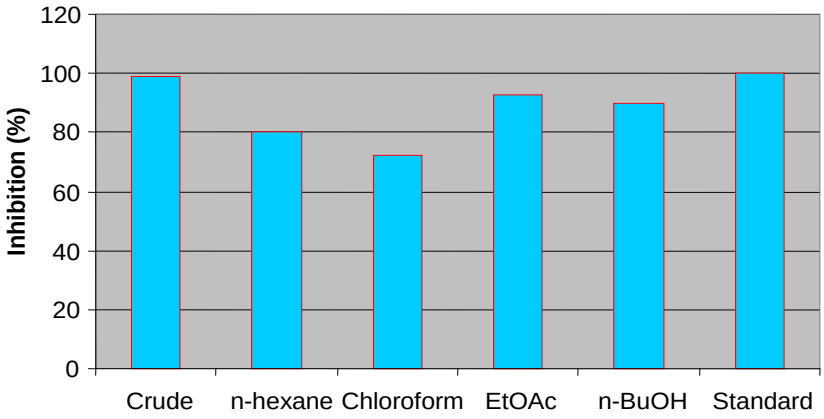

Figure 1. Butyrylcholinesterase inhibition by the crude extract and fraction of *M. communis* L., 40 µg/200 µl.

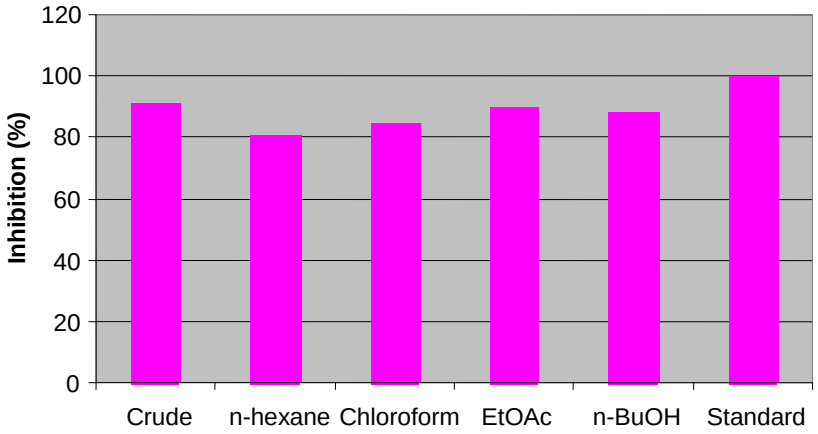

Figure 2. Acetylcholinesterase inhibition by the crude extract and fraction of *M. communis* L., 40 µg/200 µl.

RESULTS AND DISCUSSION

Pakistan has a valuable heritage of herbal remedies, and like most developing countries, its rural population still relies on the indigenous system of medicine to a great extent. Herbal medicine has the advantage of being economical and readily available. The local practitioners also claim that these remedies frequently have fewer side effects (Khattak et al., 1985). It was therefore considered to be of interest to evaluate and determine the efficacy of some medicinal plants commonly used by traditional practitioners for their effects. The current study was undertaken to screen medicinal plant *M. communis* L., for the *in vitro* inhibition of AChE, BChE and LO as drug design lends against diseases like Alzheimer, dementia, Parkinsonism, aging, inflammation, allergy, asthma, cancer, etc.

The results obtained with the crude extract of *M. communis* L., exhibits outstanding enzyme inhibitory activity against AChE (91.2%), BChE (99.1%) and LO (94.5%). The chloroform fraction exhibited good enzyme inhibition activity (72.5%) against acetylcholinesterase and significant activity against butyrylcholinesterase (85%) and lipoxygenase (89.2%). The ethylacetate fraction showed significant inhibitory activity against AChE (90%), BChE (92.5%) and good activity against LO (79%). The n-butanol fraction also showed significant activity against AChE (88%), an outstanding activity against BChE (90.1%) and significant activity against LO (83.5 %). As shown in Figures 1 to 3, it can be seen that the crude extract and subsequent fractions of *M. communis* L., are strong potential inhibitors of AChE, BChE and LO. The outstanding results obtained with the crude extract and its fractions of *M. communis* L., indicate the need for further work on the isolation, purification and investigation of the active principles responsible for the extracts inhibitory activity. The mechanism of action is still to be ascertained. Long-term toxicity study will also

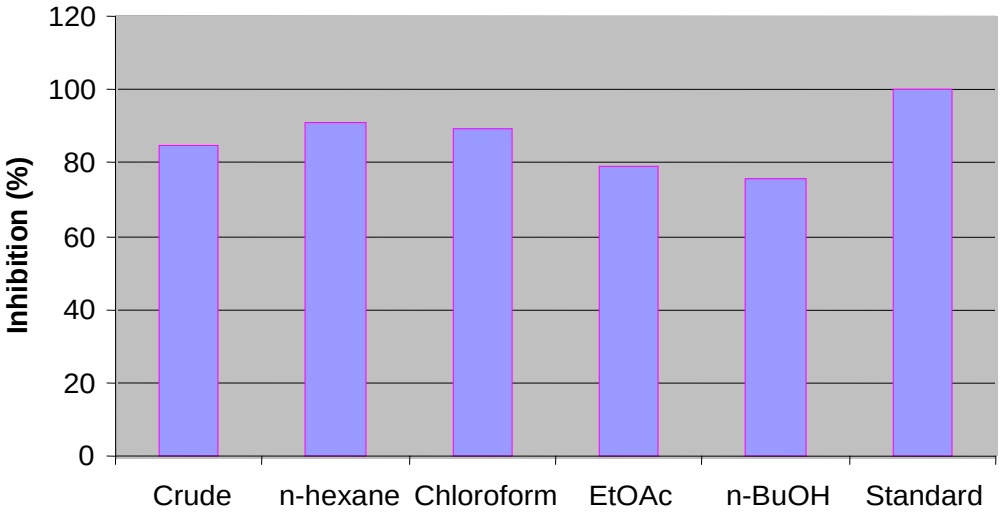

Figure 3. Lipoxygenase inhibition by the crude extract and fraction of *M. communis* L., 40 µg/200 µl.

be needed to document any cumulative adverse effects.

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