Research Article

Role of *Artemisia macrocephala* as enzymes inhibitor in dementia and nociception! A pharmacological study

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Abstract

This study was conducted for investigating potentials of methanolic extract (Am. CME) and subsequent fractions of *Artemisia macrocephala* (*A. macrocephala*) for the inhibition of acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and cyclo-oxygenase (COX-1 and COX-2). Ellman's assay was followed for the investigation of AChE and BChE inhibitory potentials of Am. CME and subsequent fractions of *A. macrocephala* while cyclo-oxygenase inhibition was investigated using a COX inhibition assay. For AChE inhibitory assay, chloroform (Am.CHF), ethyl acetate (Am.EtOAc) fractions and Am.CME showed 85.76 ± 1.49 , 77.50 ± 0.86 and 71.00 ± 1.15 % inhibition of the enzyme respectively at 1000 μ g/mL concentration. Similarly, for BChE inhibition assay, Am.CHF, Am.EtOAc and Am. CME showed 82.56 ± 0.68 , 73.56 ± 1.56 and 68.55 ± 0.81 % inhibition of the enzyme respectively at 1000 μ g/mL concentration. Am.CHF and Am.CME were found to be most active against COX-1 and COX-2. Against COX-1, Am.CHF and Am.CME showed 75.23 ± 0.98 and 73.33 ± 0.46 % inhibition respectively at 1000 μ g/mL concentration. Similarly against COX-2, they showed 81.23 ± 1.23 and 79.40 ± 0.46 % respectively at 1000 μ g/mL concentration. It can be concluded from results that *Artemisia macrocephala* can be used in the treatment of cognitive disorders, along with neurodegenerative ailments, various neuro-pharmacological ailments and certain painful conditions.

Key words: *Artemisia macrocephala*; Acetylcholinesterase; Butyrylcholinesterase; Inhibition

Introduction

Neurodegenerative disease is a general terminology used for various diseased conditions developing from persistent breakdown and corrosion of neurons in

central nervous system. Amongst several types of dementia, Alzheimer's disease (AD) has been common that affect about 20 million people in the world. Owing to its multi-factorial nature, the single therapeutic

loom is based on the cholinergic hypothesis of cognitive dysfunction [1, 2]. AD is a progressive, neurodegenerative ailment affecting mainly elders and about 50-60 % of dementia patients have age above 65 years. The main symptoms associated with the later stages of AD engross cognitive dysfunction, principally memory loss leading to sever loss in the abilities of mental level. It affects language, thought and memory control centers of the brain. It is associated with loss in neurons, abnormal tangles and plaques in neurons [3, 4].

In mammalian brain, there are present two main types of cholinesterases, namely, acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) [5]. The common notable biochemical change that AD patients are facing is diminution of acetylcholine (ACh) level in the brain. AChE is present in excitable tissues (nerves, muscles, erythrocytes and placenta), while BuChE mostly in peripheral, central nervous system, plasma and liver. Therefore, AChE and BChE inhibition, the enzymes accountable in ACh hydrolysis in cholinergic synapse, is presently the most recognized approach in treatment of AD [6].

Till date, the treatment of this ailment is based upon “cholinergic hypothesis” meaning that approved drugs for AD rehabilitation should act through counteracting the ACh deficiency, increasing its level in brain [7]. Cholinesterase inhibitors have been developed for improving the efficiency of ACh by arrest in its breakdown and raising its levels in brain or by intensifying the way nerve cells respond to it. ACh high concentration in the brain cause communication amplification among neurons and may momentarily leads to stabilization or improvement of AD symptoms. These approved drugs are effective in initial and moderate stages of AD [8, 9]. Inhibition of enzymes, AChE and BuChE is suggested desirable and thought to

be among the prime intentions for treatment of AD linked cognitive dysfunction [10, 11]. At present, frequently used drugs in AD management are AChE inhibitors such as physostigmine, tacrine, etc or inhibitors of BuChE like tetrahydro-furo-benzo-furancymserine (THFBFC). These all drugs have been proved in improving AD patient's situation to some degree [12]. Presently, Food and Drug Administration (FDA) have approved four drugs to treat A. They are galanthamine, donepezil, rivastigmine, and tacrine. All these drugs are effective in AD patients by slowing down neuro-degeneration [13]. Side effects associated are gastrointestinal disturbances; aggression, hepatotoxicity, and depression are the well-known limitations of these drugs. In addition, these drugs are costly and require regular blood monitoring [14]. Since the available drugs for AD management have certain limitations therefore, the exploration of new leading molecules from various natural product sources, which are useful in AD symptoms have gain much more attention.

Several degenerative diseases are commonly related with inflammatory processes like rheumatoid arthritis, gouty arthritis, shoulder tendonitis, cancer and asthma [15, 16]. In inflammatory responses, fatty acids mobilizations are induce by phospholipase A2 activation, particularly arachidonic acid from the membrane lipid pool. Then oxidation of arachidonic acid is carried out by constitutive cyclo-oxygenase -1 (COX-1) or inducible cyclo-oxygenase -2 (COX-2) enzymes, results in prostaglandins production [17]. Prostaglandins belong to inflammatory mediators group, involved in many pain-related disorders [18]. Because of the keen interest in the anti-inflammatory and anti-nociceptive potentials of plants by pharmaceutical firms and scientific work on innovation of new medicinal constituents, plants might be the potential precursor for

new drugs in the treatment of pain-related conditions with minimum or no side-effects. *Artemisia macrocephala* (*Artemisia griffithiana* Bioss) is a member of *Asteraceae*, which has great importance of medicinal values. *A. macrocephala*, Pashto name “Tarkha”, is 20–30 cm in height, abundantly available in Pakistan especially in northern areas [19]. Camphor, p-cymene, α -pinene, β -pinene, 1,8-cineole, limonene, camphene, borneol, enanthic acid, propionic acid, isovaleric acid and acetic acid has been isolated from *A. macrocephala*. [20]. We have previously reported *A. macrocephala* and its essential oil for phytochemical screening and antispasmodic activity [21]. We have previously testified antispasmodic potential of different fractions and essential oils of *A. macrocephala*. Antioxidant activity was reported by evaluation of its different fractions [22]. We have reported its essential oil for AChE and BuChE inhibition assays with remarkable results [23].

The purpose of recent research work was to investigate the BChE, AChE and cyclo-oxygenase inhibitory potentials of Am.CME and subsequent fractions of *A. macrocephala* in order to explore novel, effective and safest ways to treat AD, dementia, other neurological disorders and pain management.

Material and methods

Chemicals and consumables

AChE (Electric eel), BChE (equine serum), acetylthiocholine iodide, butyrylthiocholine Iodide, DTNB (5,5-dithio-bis-nitrobenzoic acid) and Galanthamine hydrobromide Lycoris Sp. Were from Sigma-Aldrich, Germany. Extra pure analytical grade K_2HPO_4 , KH_2PO_4 , and KOH were used for buffer preparation. Indomethacin was obtained from SIZA Pharmaceuticals, Pakistan.

Plant materials collection and authentication

Aerial fresh parts of *A. macrocephala* were collected in August, 2014 in hills near Badwan Chowk, Dir Lower, Pakistan. It was identified by Dr. Nasur Ullah, University of Malakand Chakdara, Dir Lower, KPK, Pakistan. Voucher sample “AM-01-2014” has been submitted in University of Malakand herbarium.

Extraction

Drying of plant material was carried out at room temperature in shade, followed by crushing and mechanical grinding to get fine powder. Soaked the powder (5.5 kg) for 22 days in commercial grade (90 %) methanol at room temperature with occasional shaking. Filtration of the materials was carried out. The materials filtered off 3 times. With the help of rotary evaporator at reduced pressure, the combined filtrate was evaporated till obtaining a dark greenish color crude Am.CME. yielding 555 gram (10.09 %).

Fractionation

Am. CME (500 g) of *A. macrocephala* suspended in distilled water (400 ml) was sequentially fractionated with n-hexane (400 mL). For thorough fractionation, process was repeated three times. The same method was followed for obtaining ethyl acetate and chloroform fractions which successively gave 40 g (8%) of n-hexane fraction (Am.NHX), 61.7 g (12.34 %) of chloroform fraction Am.CHF, 39.4 g (7.88%) of ethyl acetate Am.EtOAc and 40.7 g (8.14 %) of remaining aqueous fraction (Am.Aq).

Anticholinesterase assays

For enzyme inhibitory potentials investigation of the Am.CME and its consequent fractions AChE and BChE were used following previously reported Ellman's assay [24]. The test samples were dissolved in DMSO (few drops) and further dilution (125-1000 μ g/mL) was done in buffer (phosphate, 0.1 M). AChE (518 U/mg) and BChE (7-16 U/mg) were further diluted with 0.1 M buffer (phosphate, pH 8.0) till 0.03

U/mL (AChE) and 0.01 U/mL (BChE). Prepared DTNB (0.2273 mM), BTChI (0.5 mM) and ATChI (0.5 mM) solutions in distilled water and stored at 8 °C. Thereafter, added 5 µL enzyme solution in cuvette before the addition of test sample (205 µL) and 5 µL reagent (DTNB) in each assay. Kept this whole mixture for 15 min at 30°C in water bath and substrate solution (5 µL) was subsequently added. The absorbance was measured spectrophotometrically (Thermo electron corporation, USA) at 412 nm. Galanthamine served as reference standard. Absorbance with the time of reaction was noted at 30°C for four minutes. The experiment was performed in triplicate. Percent activity of enzyme and inhibition of enzyme by test samples and control were calculated from absorption rate with time change ($V = \Delta \text{Abs}/\Delta t$) as:

Enzyme inhibition (%) = 100 - % enzyme activity

Enzyme activity (%) = $100 \times V/V_{\text{max}}$ (V_{max} = Enzyme activity without inhibitor drug).

Cyclo-oxygenase inhibition assays

Cyclo-oxygenase enzymes (COX-1, COX-2) inhibition assays were conducted as previously described [25]. The controls contained solvent blank and background correction reaction for enzymes inactivation with HCl on ice prior to arachidonic acid (16 Ci/mol; 30 M) addition. Indomethacin was used as a reference drug (100 mg/mL). Crude extract and subsequent fractions were tested at 62.5-1000 mg/mL concentrations. COX inhibition was calculated via comparing radioactivity quantity of sample to blank using through the formula as:

COX inhibition (%) = $\{1 - (\text{DPM}_{\text{extract}} - \text{DPM}_{\text{background}}) / (\text{DPM}_{\text{solvent blank}} - \text{DPM}_{\text{background}})\} \times 100$

where DPM is disintegrations per min of extract, background and solvent blank. Results are given as means of three experiments and values are given as percentage mean $\pm$ standard error mean.

Statistical analysis

Sample concentration causing 50 % inhibition (IC_{50}) was calculated through Microsoft Excel program. Two way ANOVA followed post Bonferroni test for comparing test groups with positive reference standards was applied. P value < 0.05 was considered significant statistically. Graphs were drawn through GraphPad Prism.

Results and discussion

The acetylcholinesterase inhibitory potentials of Am.CME and subsequent fractions of *A. macrocephala* are summarized in Figure 1. Among all the tested samples, Am.CHF showed the highest percent acetylcholinesterase inhibition, causing 85.76 ± 1.49 percent inhibition at 1000 µg/mL concentration. This was followed by Am.EtOAc and Am.CME with 77.50 ± 0.86 and 71.00 ± 1.15 percent inhibition, respectively at 1000 µg/mL concentration. Galanthamine showed 94.45 ± 2.37 percent inhibition at 1000 µg/mL concentration. All samples showed good to moderate percent enzyme inhibition in a concentration dependent manner. Butyrylcholinesterase inhibition results of all the samples of *A. macrocephala* are summarized in Figure 2. Among all the samples, Am.CHF caused maximum percent butyrylcholinesterase inhibition which was 82.56 ± 0.68 % at 1000 µg/mL concentration. Similarly EtOAc, Am.CME and Am.Aq showed 73.56 ± 1.56 , 68.55 ± 0.81 and 67.57 ± 0.82 percent enzyme inhibition respectively at 1000 µg/mL concentration. All samples showed enzyme inhibition in concentration dependent manner and were from moderate to good. Standard galanthamine showed 94.45 ± 2.37 percent inhibition at 1000 µg/mL. Central cholinergic system is considered to be very important in cognitive functions regulation. Cholinergic neuronal loss is the main characteristic of AD and augmentation of

central cholinergic action by use of anticholinesterase, is currently the basis of pharmacotherapy of senile dementia of Alzheimer type [26]. AChE is among the fastest identified enzymes that catalyzes the breakage of acetylcholine after depolarization in synaptic cleft. AChE inhibitors such as galanthamine, are frequently used in AD pharmacotherapy.

The BChE, less specific, has freshly been a center of research, since its concentration remains the same, or is even up-regulated, whereas, AChE is down-regulated dramatically in AD patients [27]. Thus, regulating Ach level via AChE and BChE inhibition is considered to be a useful therapeutic approach in treatment of AD and several other types of dementia.

Figure 1. The acetylcholinesterase inhibitory potentials of Am.CME and subsequent fractions of *A. macrocephala*

The history related to drug discovery shows that plants possess active constituents which have become novel sources for investigation in pharmaceutical firms. Plant constituents may not act only synergistically with other constituents rather also work against toxic effects of constituents [28]. In traditional medicine system, several plant species are used in treatment of cognitive disorders, along with neurodegenerative ailment and several neuro-pharmacological ailments [29]. According to a scientific report, Chinese remedy of herbal nature, Yokukansan, used in treatment of several neurological conditions is very efficient

having no adverse effects [30]. An alkaloid from snowdrop, galanthamine, has been permitted by the FDA (Food and Drug Administration) of the United States in treatment of AD [31]. As AD is becoming a burden of public health, and also the normally available synthetic drugs possess unwanted adverse-effects, new treatment strategies rooted in medicinal plants have been the subject of current study. In present study; we screened *A. macrocephala* Am. CME and subsequent fractions for AChE and BChE inhibitory potentials. Among all the tested samples, Am. CHF and Am. EtOAc showed remarkable results against

both the enzymes and were quite comparable with the standard galanthamine. Am. CHF and Am. EtOAc showed 85.76 ± 1.49 and 77.50 ± 0.86 percent AChE inhibition respectively at $1000\mu\text{g/mL}$ concentration. Similarly they also caused

82.56 ± 0.68 and 73.56 ± 1.56 percent BChE inhibitions respectively at $1000\mu\text{g/mL}$ concentration. The sample Am.Aq showed 71.00 ± 1.15 and 68.55 ± 0.81 percent AChE and BChE inhibition respectively at $1000\mu\text{g/mL}$ concentration.

Figure 2. The butyrylcholinesterase inhibitory potentials of Am.CME and subsequent fractions of *A. macrocephala*

Prostanoids are bioactive constituents composed of prostaglandins (PGs), thromboxane and prostacyclin. They are originated from arachidonic acid, which come out from plasma membrane phospholipids intracellularly due to damage of tissue and inflammation. This arachidonic acid converted to the precursors of PGs. PGs, the last metabolites of arachidonic acid, are responsible for the generation of pain. Enzymes like LOX and COX are involved in the formation of these PGs [32]. Before going to test the samples for analgesic activities, their *in-vitro* screening against COX enzyme is imperative as these types study can predict the *in-vivo* fate of the test samples. Novel, clinically effective and

economically cheap anti-inflammatory and antinociceptive drugs from medicinal plants are the best alternative to the hazardous synthetic drugs. The *in vitro* cyclooxygenase assays, the crude extract and subsequent fraction of *Artemisia macrocephala* showed COX-1 and COX-2 inhibition in a dose dependent manner. Among all tested samples, Am.CHF and Am.CCME showed the enhanced inhibition activity against both COX-1 and COX-2. While other fractions showed moderate percent inhibition of the enzymes. Am.CHF and Am. CCME caused 75.23 ± 0.98 (IC_{50} 122 $\mu\text{g/mL}$) and $73.33 \pm 0.46\%$ (IC_{50} 241 $\mu\text{g/mL}$) COX-1 inhibition respectively at $1000\mu\text{g/mL}$ concentration. Similarly against

COX-2, they showed 81.23 ± 1.23 (IC_{50} $60.25 \mu\text{g/mL}$) and $79.40 \pm 0.46\%$ (IC_{50} $112.97 \mu\text{g/mL}$) respectively at $1000 \mu\text{g/mL}$ concentration as shown in Table 1. Am.Aq showed the lowest inhibitory activity for both the enzymes even the highest concentrations. The *in-vitro* enzyme inhibition activity of the plant can be attributed to the presence of bioactive

compounds like flavonoids and terpenes in the plant as reported earlier [20]. The inhibition of COX exhibited by the crude extract and subsequent fractions of *Artemisia macrocephala* make it as alternative candidate for further work in search for new anti-nociceptive and anti-inflammatory compounds.

Table 1. The COX inhibitory potentials of *A. macrocephala*

Sample	Conc ($\mu\text{g/mL}$)	COX 1 % inhibition	$IC_{50} \mu\text{g/mL}$	COX 2 % inhibition	$IC_{50} \mu\text{g/mL}$
Am.CME	1000	$73.33 \pm 0.46^{***}$	241	$79.40 \pm 0.46^{***}$	112.97
	500	$61.84 \pm 0.27^{**}$		$72.34 \pm 0.27^{**}$	
	250	$51.85 \pm 1.99^{**}$		$64.64 \pm 1.99^{**}$	
	125	46.35 ± 2.24		55.32 ± 3.21	
	62.5	31.23 ± 1.45		23.45 ± 1.15	
Am.NHX	1000	$41.13 \pm 0.98^{***}$	-	$39.13 \pm 0.90^{***}$	-
	500	$39.15 \pm 2.88^{**}$		$30.15 \pm 2.04^{**}$	
	250	$33.81 \pm 2.75^{**}$		$27.81 \pm 2.35^{**}$	
	125	$29.05 \pm 2.33^{**}$		$21.05 \pm 2.08^{**}$	
	62.5	27.30 ± 2.85		17.30 ± 2.13	
Am.CHF	1000	$75.23 \pm 0.98^{***}$	122	$81.23 \pm 1.23^{***}$	60.85
	500	$71.15 \pm 2.88^{**}$		$74.15 \pm 1.03^{**}$	
	250	$64.65 \pm 2.75^{**}$		$69.65 \pm 1.94^{**}$	
	125	$51.05 \pm 2.33^{**}$		$58.05 \pm 1.23^{**}$	
	62.5	48.35 ± 2.85		51.35 ± 1.05	
Am.EtOAc	1000	$71.87 \pm 1.50^{**}$	196	$75.87 \pm 2.06^{**}$	117.59
	500	$70.76 \pm 2.10^*$		$75.76 \pm 1.90^*$	
	250	63.65 ± 1.89		71.65 ± 2.09	
	125	49.15 ± 1.99		53.15 ± 2.90	
	62.5	39.44 ± 2.00		37.44 ± 1.90	
Am.Aq	1000	$46.88 \pm 1.50^{**}$	-	$49.88 \pm 2.09^{**}$	-
	500	$41.99 \pm 2.10^*$		$45.99 \pm 1.90^*$	
	250	39.65 ± 1.89		40.65 ± 2.09	
	125	35.15 ± 1.99		31.15 ± 2.09	
	62.5	31.44 ± 2.00		23.44 ± 1.90	
Indomethacin	100	82.56 ± 1.40	-	86.77 ± 1.64	-

Conclusion

The results of the present study show that the plant is rich in bioactive compounds which are responsible for enzymes inhibition activity. From the results of this study, it can be concluded that the plant can be used to treat cognitive disorders, including neurodegenerative diseases and different neuro-pharmacological disorders. This primary screening will further open new channels for the isolation, structural

characterization and *in-vivo* evaluation of the bioactive compounds for enzymes inhibition potentials and then ultimately will lead the molecular level investigation for enzymes inhibition responsible for AD, other forms of dementia and nociception.

Authors' contribution

Conceived and designed the experiments: I Shah, Performed the experiments: I Shah, M Shoaib, N Ali & SWA Shah, Analyzed the data: I Shah & M Shoaib, Contributed

reagents/materials/analysis tools: I Shah, N Ali & MA Nawaz, Wrote the paper: I Shah, MA Nawaz & H Rehman.

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