Enzyme inhibition, antioxidant and antibacterial potential of vasicine isolated from *Adhatoda vasica* Nees

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Abstract: Vasicine (**1**) was isolated from the ethanolic extract of *Adhatoda vasica* Nees (Acanthaceae) and the structure was confirmed using spectroscopic techniques. Acetylcholine esterase, trypsin, DPPH inhibition potential and FRAP assay were carried out using *in vitro* models. The results showed 38.4 ± 1.2% and 37.4 ± 1.1% activity in acetylcholine and trypsin inhibition assays respectively. The compound (**1**) exhibited significant DPPH inhibition activity (70.4 ± 1.3%, IC_{50} = 212.3 ± 1.9 μM). A dose dependant behavior of vasicine (**1**), was indicated in the FRAP assay. Antibacterial activity was checked according to agar well diffusion assay and results revealed that vasicine (**1**) showed moderate activity.

Keywords: Acetylcholine esterase, *Adhatoda vasica*, DPPH, FRAP, trypsin, agar well diffusion assay.

INTRODUCTION

Acetylcholine esterase inhibitors (AChEi) are the promising drugs for the treatment of Alzheimer's disease (AD). AD is a neurologic disorder, which results in progressive loss of memory and behavior. A reduction in cognitive function, associated with a cholinergic deficit is produced by this neuropathology (Plum, 1988; Bachurin, 2003; Rollinger *et al.*, 2004). Several acetylcholine esterase inhibitors such as huperzine and galanthamine are the natural products, indicating importance of medicinal plants. Among many of the known AChE inhibitors, only few are approved for AD therapy, due to their side effects (Perry *et al.*, 2003; Mukherjee *et al.*, 2007; Fang *et al.*, 2008). Therefore, more effective and less contra-indicative AChE inhibitors from plant sources are needed to be discovered (Carpinella *et al.,* 2007; Palacios *et al.*, 2007).

Proteases play a vital role in the normal physiological functions of cells. A number of diseases like cancer, pulmonary emphysema and arthritus are reported to be induced by over activity of proteases (Giovanni and David, 2005). Antioxidants are substances which extinguish free radicals or delay oxidation chain reactions through the course of different mechanisms (Velioglu *et al.,* 1998). The human body can be protected from free radicals and reactive oxygen species through antioxidants. Reactive oxygen species (ROS) is harmful not only to lipids and proteins but can also damage DNA in the cells, which results in cell death (Pryor, 1991; Kinsella *et al.,* 1993; Lai *et al.,* 2001). The oxidative damage caused by the over activity of ROS is responsible for the aging of skin and several other diseases, associated with the excessive generation of free radicals (Halliwell and Gutteridge, 1984; Halliwell and Gutteridge, 1985). *Adhatoda vasica* Nees (Family; Acanthaceae), is widely used in Ayurvedic medicine in India, Sri Lanka and Pakistan due its bronchodilator, expectorant, antiasthmatic, antiallergic activities, against dermatitis and tuberculosis (Gupta *et al.*, 1977). In China this plant has traditional use against skin disorders. Initial studies showed that the plant possesses anti-inflammatory properties (Cueller *et al.*, 1998) but its active constitute is not clearly defined. Several quinazoline alkaloids such as vasicinolone, 7-hydroxyvasicine, vasicol, 3-deoxyvasicine, vasicoline, adhatodine, vasicolinone and anisotine were detected during the phytochemical analysis of leaves and roots of *A. vasica* (Joshi *et al.*, 1994).

In continuation of the biological evaluation of natural compounds, the present study was conducted to check the enzyme inhibition, reducing ability and antibacterial activity of vasicine (**1**).

MATERIALS AND METHODS

Chemicals and instruments

Infra red spectra were measured on Perkin-Elmer 735B, ^{1}H NMR spectra was recorded at 400 MHz Bruker Avance spectrometer while ^{13}C NMR spectra were measured at 75 MHz using the same instrument. Preparation of samples for NMR spectometry was carried out in $CDCl_3$ containing tetramethylsilane as an internal standard. Mass spectrometry was carried out on a MAT 312 instrument. Silica gel 60 was used for column chromatography. Nα-benzoyl-DL-arginine-paranitroanilide-HCL, trypsin from bovine serum and DMSO were

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purchased from Fluka. Acetylthiocholine iodide, DTNB and AChI were purchased from Sigma (St. Louis, MO, USA) while, Acetylcholine esterase obtained from Mayo Hospital Lahore (Biochemistry Lab). Solvents of analytical grade were purchased from Panerac (Spain), while analytical grade reagents, agar-agar and nutrient broth were purchased from Merck (Germany).

Collection of plant material

The region of Azad Kashmir was selected to collect aerial parts of *A. vasica* (January, 2010) and the plant was identified by Dr. Zaheer Khan (Taxonomist) at Department of Botany, Govt. College University, Lahore. A reference sample was submitted at Sultan Herberium (GCU-BOT-857).

***Extraction and isolation of vasicine* (1)**

The plant material was dried under shade, pulverized and soaked in ethanol for extraction. The ethanolic extract was dissolved in 5% CH_3COOH at 60°C for fifteen min. and defatted using n-hexane via solvent extraction. The aqueous layer was basified with liq. NH_3 and extracted with $CHCl_3$ resulted crude vasicine (**1**) which was purified by recrystallization using the mixture of methanol-acetone (1:1) (136 mg, 1.1% yield).

Acetylcholine esterase assay

Acetylcholine esterase inhibitory potential of vasicine (**1**) was checked according to the method of Shahwar *et al.* (2010a; 2011a). The reaction mixture was composed of 1.5 ml of (100 mM). Tris buffer (pH 7.8), 1.0 ml of DTNB, 0.2 ml (150 μg/ml) of sample and 0.2 ml of acetyl cholinesterase solution (erythrocytes). The reaction mixture was incubated for 15 minutes at 25°C, and then added 200 μl of the substrate solution (acetylthiocholine). At 412 nm hydrolysis of acetylthiocholine was measured till 30 minutes. The assay was conducted in triplicate. The inhibitory potential was calculated as follows:

$$\%\,\text{age inhibition} = \frac{\text{E - S}}{\text{E}} x100$$

Where E is the activity of the enzyme without test sample and S is the activity of enzyme with test sample.

Protease inhibition assay

Protease inhibition activity was evaluated using the method of Jedinak *et al.,* 2006 along with some alterations (Shahwar *et al.,* 2011b,c). 12.1 g of Tris (hydroxyl-methyl)-aminomethane were dissolved in distilled water in order to prepare Tris buffer (100mM) and pH was adjusted to 7.5 with 5 M HCl. Preparation of stock solution was carried out by dissolving 2 mg of trypsin in 10 ml HCl (1.0 mM). Solution of Nα-benzoyl-DL-arginine-paranitroanilide hydrochloride (BApNA) having concentration (20 mg/ml) was prepared in DMSO. 0.3 ml enzyme and 0.1 ml of inhibitor were incubated for 15 minutes at 37°C then 0.6 mM substrate was added and the volume of the mixture was made 2.5 ml by adding Tris buffer. This reaction mixture was then incubated for 30 minutes at 37°C. The reaction was then stopped by adding 30% acetic acid (0.1 ml) and the absorbance was measured at 410 nm using UV/Vis spectrophotometer. Phenylmethanesulfonylfluoride (PMSF) was used as reference standard. Following formula was used to calculate % inhibition.

$$\%\text{ Inhibition } = \frac{\text{Absorbance (blank) - Absorbance (test)}}{\text{Absorbance (blank)}} \times 100$$

DPPH radical scavenging assay

The radical scavenging ability was measured using the method of Shahwar *et al.* (2010b,c). Methanolic solution (1.0 ml) of the sample at different concentrations (25-300 μg/ml) was added to 1.0 ml (0.2 mg/ml) methanolic solution of DPPH. Absorbance was noted at 517 nm after 30 minutes using methanol as blank.

The % inhibition was determined by the following formula;

$$\%\,\text{age inhibition of DPPH} = \frac{\text{A - B}}{\text{A}} x100$$

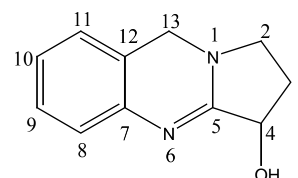
Where A is the optical density of blank and B is the optical density of sample.

Ferric reducing antioxidant power (FRAP)

Reducing potential of vasicine (**1**) was determined using FRAP assay, in the range of 25-200 μg/ml according to the method of Shahwar *et al.* (2010a). 50 μl of FRAP reagent was mixed with sample and absorbance was read at 595 nm after incubation of 15 minutes. The results were calculated as micromole equivalents to $FeSO_4.7H_2O$ using calibration curve (fig. 2).

Antimicrobial assay

Antibacterial activity was determined against four different microorganisms: *Nocardia asteroids* NRRL 174, *Micrococcus luteus* ATCC 10240, *Salmonella typhimurium* ATCC 14028 and *Proteus mirabilis* ATCC 29425 were obtained from Biochemistry Lab., Department of Chemistry, GC University, Lahore, Pakistan. The activity was carried out using the method of Shahid *et al.* (2009). Sterilized 25 ml of nutrient agar medium (Merck, pH 7) was poured in the Petri plates and seeded with the respective bacterial strain. 4 mm holes in the agar plates were filled with 50 μl of sample (5 mg/ml). Streptomycin (2 mg/ml) was the reference while dimethylsulfoxide was used as negative control. After incubation of 24 hours at 37°C, the zone of inhibition was measured. Experiments were repeated three times and data was presented as ±S.D using MS Excel 2007 software.



Vasicine (**1**)

Vasicin (1); IR (KBr, cm^{-1}) υ_{max}: 3389 (OH), 1727 (C=O), 1470 (C=C), 1070 (C-O). EIMS *m/z* (rel. int., %): 188.0 (M^+, 100), 176.1 (19.7), 148.1 (78.5), 104.0 (7.9), 76.1 (9.7), 65.0 (4.7). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 2.24 (2H, m, H-3), δ 3.71 (2H, m, H-2), δ 4.63 (2H, m, H-13), δ 4.92 (1H, d, *J* = 7.0 Hz, H-4), δ 6.95 (4H, m, H-8, H-9, H-10, H-11). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 28.9 (C-3), δ 45.6 (C-2), δ 50.9 (C-13), δ 68.6 (C-4), δ 118.9 (C-10), δ 122.1 (C-8), δ 126.8 (C-11), δ 129.3 (C-9), δ 129.9 (C-12), δ 152.1 (C-7), δ 167.3 (C-5).

RESULTS

Isolation and structure determination of vasicine (1)

The aerial parts of *A. vasica* were extracted in ethanol and the crude extract was dissolved in 5% acetic acid. The pH of the aqueous layer was adjusted to 9.0 and extracted with chloroform which on evaporation yielded vasicine (**1**) as white needles. Vasicine (**1**) showed a strong absorption band at 290 nm (Rnanjit *et al*., 2007), while a peak at 3389 cm^{-1} in the IR spectrum indicated the presence of OH group. Electron impact mass spectrometry showed M^+ at *m/z* 188.0. The proton NMR spectrum of **1** showed a doublet at δ 4.92 due to H-4. A multiplet at δ 6.95 showed the presence of four aromatic protons. The positions of the 3 upfield multiplets at δ 2.24, 3.71 and 4.63 of C-2, C-3 and C-13 respectively were confirmed using HMQC spectrum (Dhar *et al.*, 1996).

Enzyme inhibition assay

Enzyme inhibition potential of vasicine (**1**) was carried out against acetylcholine esterase (AChE) and trypsin using standard *in vitro* models. The results showed moderate inhibition activities with respective % inhibition values of 38.3 ± 1.2% against AChE (standard = 60.2 ± 0.9%) and 37.4 ± 1.1% against trypsin (standard = 87.4 ± 1.2%).

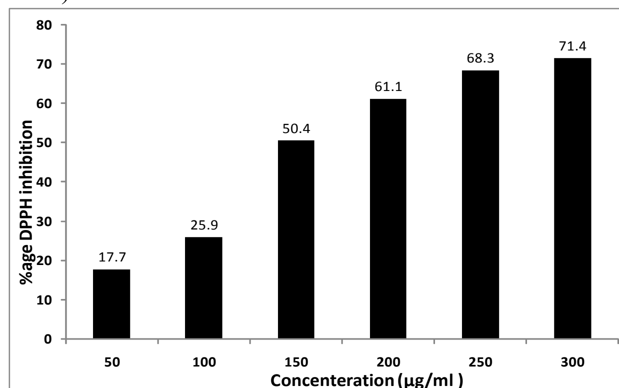


Fig. 1: % DPPH inhibition of vasicine (**1**) at various concentrations.

Antiradical activity

Free radical scavenging activity was determined using DPPH method of Shahwar *et al.* (2010b,c). Fig. 1 shows the scavenging activity of vasicine (**1**) at various concentrations. The highest activity was observed at 300 µg/ml (70.4 ± 1.3%) with IC_{50} was 212.3 ± 1.9 µM while standard had IC_{50} value of 5.6 ± 0.2 µM. Ferric reducing antioxidant power (FRAP) of vasicine (**1**) was expressed in terms of $FeSO_4.7H_2O$ equvalents (R^2 = 0.9988) (fig. 2). A correlation between different doses and ferric reducing ability of the compound was determined in the range of 25-200 µg/ml (fig. 3).

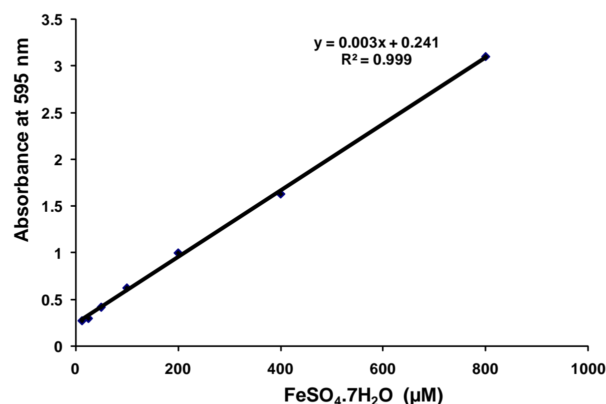


Fig. 2: Calibration curve of $FeSO_4.7H_2O$.

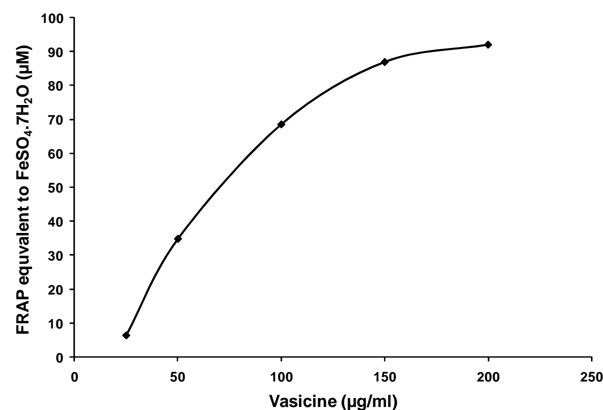


Fig. 3: Dose dependent behavior of vasicine (**1**) in FRAP assay.

Antibacterial activity

Antibacterial potential of vasicine (**1**) was checked against four bacterial strains using agar well diffusion method. The results revealed that vasicine (**1**) exhibited maximum activity against S. *typhimurium* (8 ± 1 mm) followed by *M. luteus* and showed no zone of inhibition against remaining microorganisms (fig. 4).

DISCUSSION

Alzheimer's disease (AD) can be effectively controlled by AChE inhibitors. Vasicine (**1**) showed a mild activity in the AChE inhibition assay. A possible explanation of the moderate activity is the poor tissue penetration which

results in the failuer of the approache of vasicne (**1**) at the bottom of AChE deep in the cholonergic synapses at the neuromuscular junction.

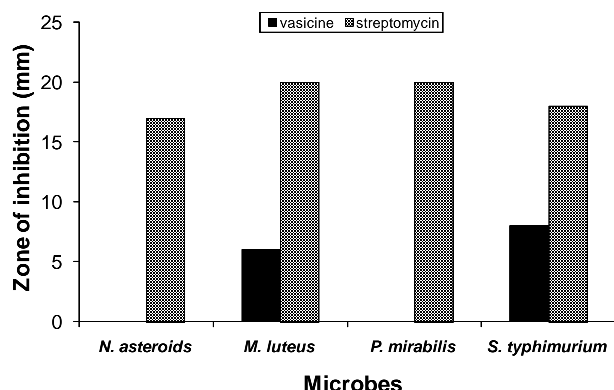
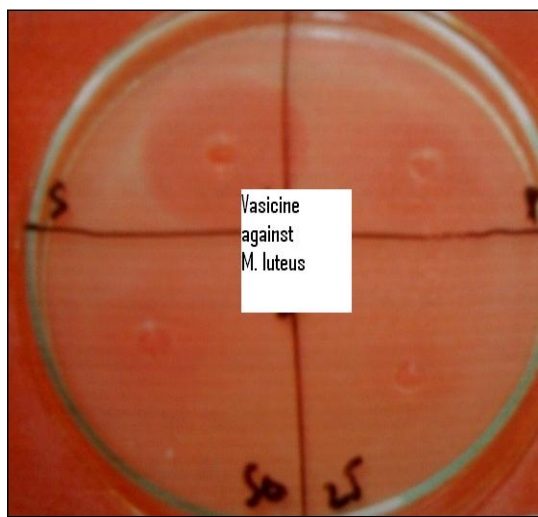
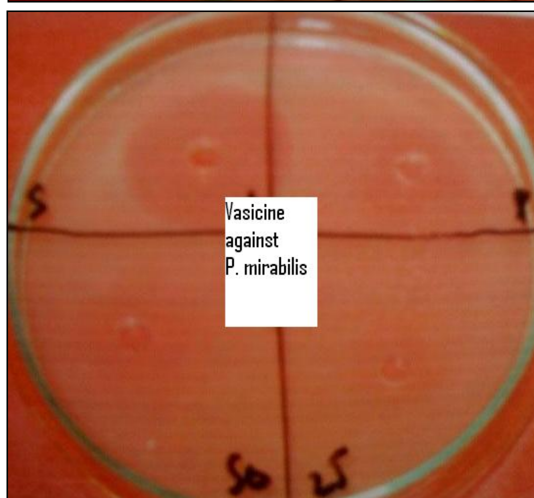


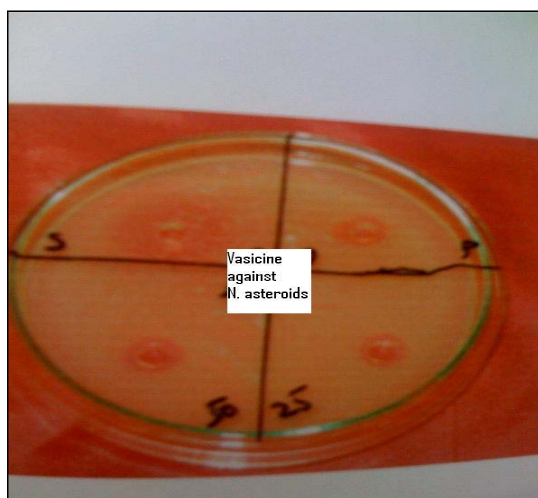
Fig. 4: Antibacterial activity of vasicine (**1**)

Many pathological disorders caused by the abnormal regulation of proteolytic enzymes resulting in tissue distruction or irregular processing of other proteins. Trypsin is a serine protease enzyme which catalyzes the hydrolysis of peptide bond of protein in small intestine. The result of trypsin inhibitory potential of vasicine (**1**) in our experiments indicated a moderate inhibition. Vasicine (**1**), a quinolizone alkaloid, characterized by the presence of heterocyclic skeleton, is famous for its bronchodialatery and anti-inflammatory activity (Srinivasarao *et al.,* 2006). Moderate activity of vasicine (**1**) colud be attributed to the presence of quinozoline skeleton. The quinazoline skeleton is equally important to chemists as well as to biologists as it is possessed by a number of medicinally important natural products. It is part of the structure of many clinically useful molecules exhibiting diverse biological activities such as antiviral, antimalarial, anticonvulsant, antibacterial, diuretic, hypnotic, anticancer, anti-inflammatory, hypoglycaemic, antitumoral and antihypertensive activities (Manjula *et al.,* 2011; David *et al.,* 2005; Vipan *et al.,* 2005; Kunes *et al.,* 2000).

The biochemical redox reactions taking place in the human body results in the generation of free radicals







which is a part of normal cell metabolism. Free radicals can be reduced easily, but oxidative stress occur when production and scanvenging of the same is imbalanced, leading to many diseases such as cancer, arthritis, antheroclerosis, etc.

In our experiments vasicine (**1**) showed significant antioxidant activity as infered by the concentration dependent increase of the reduction of Fe^{3+} in FRAP assay and significant scavenging of free radicals in DPPH assay (figs. 1 & 2). A significant increase in the serum alkaline phosphatase observed in the peripheral blood of radiation treated Swiss albino mice through oral intake of *Adhatoda vasica* leaves can be attributed to the antioxidant properties of vasicine (**1**) (Srinivasarao *et al.,* 2006). Antibacterial activity was carried using agar well diffusion method. Vasicine (**1**) remained inactive against *N. asteroids* and *P. mirabilis* and showed moderate activity against *M. luteus*, and *S. typhimurium* (fig. 4). Although the crude extract of *A. vasica* exhibited strong antibacterial activity, which may be due to some other components present in the extract (Karthikeyan *et al.,* 2009; Ilango *et al.,* 2009).

There is always a need of more selective and less contra-indicative drugs. Semi-synthetic derivatives of vasicine (**1**) are already in market in the brand name of bromohexine and ambroxol used as expectorant. Considering the high antioxidant activity and moderate protease and AChE inhibitory activity, vasicine (**1**) could be a remarkable lead compound. Some structural changes to vasicine (**1**) may yield a more effective and selective enzyme inhibitor significantly useful for clinical control of several diseases associated with the over activity of AChE and proteases.

ACKNOWLEDGMENT

We acknowledged this research work to Higher Education Commission of Pakistan for providing funds.

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