Indian J Med Res 146, August 2017, pp 255-259
DOI: 10.4103/ijmr.IJMR_156_14

Quick Response Code:

Neuroprotective effect of Cubebin: A dibenzylbutyrolactone lignan on scopolamine-induced amnesia in mice

Gauresh S. Somani[1], Mruniya S. Nahire[1], Aakash D. Parikh[1], Mandar B. Mulik[2], Priya J. Ghumatkar[1], Kirti S. Laddha[2] & Sadhana Sathaye[1]

[1]Pharmacology Research Laboratory-II, [2]Medicinal Natural Products Research Laboratory, Department of Pharmaceutical Sciences & Technology, Institute of Chemical Technology[*], Mumbai, India

Received February 1, 2014

***Background & objectives*: Acetylcholinesterase (AChE) inhibitors represent a major class of drugs which provide symptomatic relief and improvement in cognitive function in Alzheimer's disease (AD). In this study, cubebin, a dibenzylbutyrolactone lignan, was isolated from *Piper cubeba* and investigated for its AChE inhibitory activity in an attempt to explore its potential for memory-enhancing activities in mice.**

***Methods*: Molecular docking of cubebin was carried out followed by *in vitro* AChE activity. Mice were treated with cubebin (25 & 50 mg/kg; i.p.), for three days and memory impairment was induced by scopolamine (3 mg/kg; i.p.). Memory function was evaluated by Morris water maze (MWM) test. Biochemical parameters of oxidative stress and cholinergic function were estimated in brain.**

***Results*: Molecular docking study revealed that cubebin was well bound within the binding site of the AChE enzyme showing interactions such as π-π stacking and hydrogen bonding with residues present therein. Cubebin inhibited AChE enzyme in an *in vitro* assay with IC_{50} value of 992 µM. Scopolamine administration caused a significant impairment of learning and memory in mice, as indicated by a marked decrease in MWM performance. Scopolamine administration also produced a significant enhancement of brain AChE activity and oxidative stress in mice brain. Pre-treatment of cubebin (25 and 50 mg/kg; i.p.) significantly prevented scopolamine-induced learning and memory deficits along with attenuation of scopolamine-induced rise in brain AChE activity and oxidative stress level.**

***Interpretation & conclusions*: Cubebin showed promising protective activity in scopolamine-induced spatial memory impairment in mice. This could be attributed to its brain AChE inhibition and antioxidant activity.**

Key words Acetylcholinesterase inhibitor - cubebin - molecular docking - scopolamine

Alzheimer's disease (AD) is the leading neurodegenerative disease causing memory loss and dementia, which mostly affects the elderly population worldwide. The pathophysiology of AD is complex, including defective beta-amyloid (Aβ) protein metabolism, tau protein hyperphosphorylation, neurotransmission

[*]University Under Section 3 of UGC Act-1956, Elite Status & Centre of Excellence-Government of Maharashtra, TEQIP Phase II Funded

deficiency, neuroinflammation, neuronal death, free radical formation and cerebrovascular dysfunction[1]. Although most of the currently used therapeutic strategies slow down the progression of dementia, but associated side effects limit their use. Since acetylcholinesterase (AChE) inhibitors represent a major class of drugs providing symptomatic therapy with resultant improvement in cognitive function, the research exploring new drugs acting on this enzyme is vital[2]. Developing new drugs from plants has emerged as an alternative for prophylactic or therapeutic intervention of AD.

Cubebin, a dibenzylbutyrolactone lignan, has been isolated from several species of plants in various families, such as *Aristolochiaceae*, *Myristicaceae*, *Rutaceae* and *Piperaceae*[3]. It exhibits various pharmacological activities such as trypanocidal[4], antimycobacterial[5], analgesic, anti-inflammatory[6] and vasodilatory[7]. Lignans have been shown to exhibit various biological activities. However, its antiamnesic potential is yet to be explored. This study was focused on screening of an identified phytoconstituent cubebin for its AChE inhibitory effect and probable bioactivity in the treatment of cognitive disorders.

Material & Methods

This study was carried out in the department of Pharmaceutical Science and Technology, Pharmacology Laboratory, Institute of Chemical Technology (ICT), in 2012.

Scopolamine, sodium hydrogen phosphate, sodium dihydrogen phosphate and dimethyl sulphoxide (DMSO) were purchased from SD Fine Chemicals Ltd., Mumbai. DTNB [5, 5'-dithiobis-(2-nitrobenzoic acid)] reagent was purchased from Sigma, India. Acetylthiocholine iodide (ATCI) was purchased from Hi-Media, Mumbai. Adult male Wistar rats weighing 180-250 g and Swiss albino mice weighing 25-30 g were used. The experimental protocol was approved by the Institutional Animal Ethics Committee of ICT, Mumbai. The animals were maintained in standard laboratory conditions with food and water *ad libitum,* under a 12 h light/12 h dark cycle.

Molecular docking: The procedure of molecular docking of cubebin was followed as described elsewhere[8]. Cubebin structure was constructed using 'build option' within Maestro using standard geometries and standard bond lengths as described earlier[8].

Isolation of cubebin from Piper cubeba (Kababchini) fruits: A 500 g powdered dried fruits of *P. cubeba* was extracted with about two litres of methanol, filtered and concentrated to obtain oily extract. Approximately 100 ml extract obtained was treated with 2g of sodium hydroxide (0.5 M NaOH) to saponify the oily matter. The dried soap obtained after saponification reaction was further extracted with 100 ml acetone 3-4 times. Acetone fraction was concentrated and dried, and subjected to silica gel column chromatography. Petroleum ether-ethyl acetate (85:15 v/v) was used as mobile phase. Of the 40 eluted fractions of 200 ml each, fraction number 19 - 30 showed presence of cubebin (2.278 g of cubebin from 500 g of crude powdered drug) which was monitored by thin layered chromatography (mobile phase- toluene: ethyl acetate at 70:30 v/v). Structure was confirmed by mass spectrometry, nuclear magnetic resonance spectrometry (done at Sophisticated Analytical Instrument Facility, IIT, Mumbai) and infra-red spectrometry (done at Dept. of Pharmaceutical Technology, ICT, Mumbai). The percentage yield of cubebin was found to be 0.23 per cent.

Experimental design: AChE enzyme was isolated from rat brain homogenate as reported earlier[8,9]. This crude enzyme was used in the study to observe inhibition by different concentrations of cubebin. The AChE activity was measured using the colorimetric method of Ellman *et al*[10]*,* with slight modifications utilizing acetylthiocholine iodide (ATCI) as a substrate and crude AChE from rat brain. Dose range of cubebin was selected on the basis of reported LD_{50} value (500 mg/kg; i.p.)[11]; $1/10^{th}$ dose of cubebin was used for investigation. Animals were randomly divided into five groups containing six animals in each group. Group I: normal control, Group II: scopolamine (SCP) 3 mg/kg, i.p.; Group III: SCP 3 mg/kg + donepezil 1 mg/kg, Group IV: SCP 3 mg/kg + cubebin 25 mg/kg, i.p. and Group V: SCP 3 mg/kg + cubebin 50 mg/kg, i.p.

Scopolamine-induced amnesia in Morris water maze (MWW) test: This test is widely accepted for the evaluation of spatial memory and learning[12]. The maze consisted of a dark circular water pool (120 cm diameter×45 cm height), containing water (20°C±1°C) to a depth of 30 cm and surrounded by extra maze distal visual cues of different shapes. A circular hidden platform, 15 cm diameter, was fixed constantly in one of the quadrants and was placed 1 cm below the water surface so that the mice could escape from swimming. After two weeks of training period, the day 0 reading was recorded in which the mice were placed into the

maze 180° from the platform always. The time taken by the mice to reach the platform was recorded which is called escape latency time (ELT). The actual trial was conducted 30 min after scopolamine administration. ELT was recorded with the cut-off time of 60 sec. The mice were sacrificed immediately after the water maze test and brains were isolated in ice-cold saline. The isolated brains were further homogenized in phosphate buffer at pH 7.4. The homogenates obtained were centrifuged at 850 g at 4°C for 15 min. The supernatant was used for the estimation of AChE[13,14], lipid peroxidation (MDA)[15] and total protein[16].

Statistical analysis: All results were expressed as the mean±standard deviation (SD). Statistical evaluation of the data was performed using ANOVA followed by Tukey's multiple comparisons test for significant differences using GraphPad prism software version 5; 2007 (GraphPad Software, San Diego, USA).

Results

Molecular docking of cubebin: To understand the interaction of cubebin with the catalytic site of the target enzyme, molecular docking study was undertaken. Cubebin molecule was found to bind well within the catalytic site of the enzyme showing π-π stacking with Histidine (His) 440, hydrogen bonding with Glycine (Gly)119, Gly118 and Tyrosine (Tyr) 121. This ligand also showed hydrophobic interactions with Tryptophan (Trp) 84, Phenylalanine (Phe) 330 at the choline binding site, Tyr 121 at a peripheral binding site and Phe 290 at the choline binding site. Glide score of cubebin (−10.51) indicated the stability of the cubebin enzyme complex (Fig. 1).

Isolation of cubebin: The isolated compound showed colourless needle of cubebin having melting point in the range of 130-132°C. TLC indicated a single band corresponding to cubebin under ultraviolet light, which was formed with concentrated H_2SO_4 reagent as a red-violet zone at R_f 0.56. Based on chemical and spectral analysis, the isolated compound was confirmed to be cubebin.

Inhibitory effect of cubebin on AChE: Cubebin strongly inhibited activity at 1122 µM (over 56%) and at other concentrations in a dose-dependent manner. The IC_{50} value of cubebin was 992 µM (Fig. 2).

Effect of cubebin on scopolamine-induced impairment of learning and memory using Morris water maze (MWM) test: Effect of cubebin was evaluated on spatial

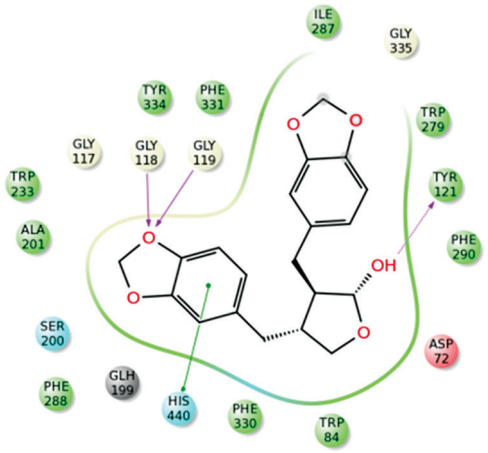

Fig. 1. Ligand interaction architecture of acetylcholinesterase target protein (PDB accession no. 1EVE) with the superimposed docked molecule of cubebin. ILE, Isoleucine; GLY, Glycine; TRP, Tryptophan; TYR, Tyrosine; PHE, Phenylalanine; ASP, Aspartate; HIS, Histidine; SER, Serine; ALA, Alanine.

memory and learning in scopolamine-induced amnesia in mice. The ELT is the time taken by the mice to locate the hidden platform in the water maze which was used as a parameter to evaluate the performance of the treated mice. The scopolamine-treated mice showed significant increase in the ELT. Moreover, administration of cubebin (25 and 50 mg/kg; i.p.) was found to decrease the ELT significantly and was comparable with standard donepezil (1 mg/kg) as shown in the Table.

Effect of cubebin on scopolamine-induced changes in AChE activity of the mice brain: Scopolamine (3 mg/kg, i.p.) significantly (P<0.05) increased the brain AChE activity when compared to control mice. The brain AChE activity was found to be significantly (P<0.05) decreased in cubebin (25 and 50 mg/kg) and donepezil-treated groups in comparison with the scopolamine-treated group and was comparable with the control group.

Effects of cubebin on scopolamine-induced changes of the mice brain: Scopolamine (3 mg/kg, i.p.) significantly increased the brain MDA levels compared to the control group of animals, reflecting enhanced oxidative stress. The elevated enzyme activity of brain MDA level was found to be significantly restored in the cubebin-treated group (25 and 50 mg/kg; i.p.) as compared to the scopolamine-treated group (Table).

Discussion

In this study cubebin was evaluated for inhibition of AChE activity, the latter being one of the major

Table. Effect of cubebin on scopolamine (SCP)-induced changes in escape latency (sec) using Morris water maze and oxidative stress level and acetylcholinesterase (AChE) activity of mice brain

Parameter/groups	Normal control	SCP	SCP + donepezil (1 mg/kg)	SCP + cubebin (25 mg/kg)	SCP + cubebin (50 mg/kg)
Escape latency (sec)	29.2±10.89	59.4±1.34$	12.5±4.92*	28.4±15*	22.8±12*
Total protein	1.97±0.52	0.81±0.23	2.88±0.38	2.04±0.64	1.98±0.40
MDA (nmoles/mg of protein)	8.66±1.98	16.83±5.42$	5.39±0.88*	9.14±2.53*	9.67±1.93*
AChE activity (U/mg protein)	12.68±4.31	22.45±4.70$	8.26±0.92*	12.07±3.85*	12.19±3.30*

Values are mean±SD (n=6). $ $P<0.05$ compared to normal control group; * $P<0.05$ compared to scopolamine-induced amnesia group. SD, standard deviation; MDA, malondialdehyde

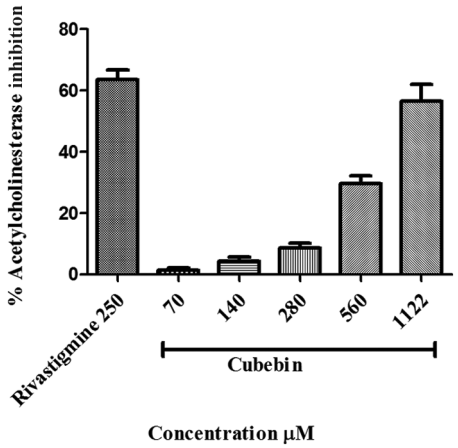

Fig. 2. Anticholinesterase activity of rivastigmine and cubebin (IC_{50}=992 µM) *in vitro*. Values are mean±standard deviation, n=3.

targets in the treatment of AD. The investigation was started by evaluating the possibility of cubebin-AChE binding through molecular modelling techniques. Cubebin was docked into the binding pocket of AChE[17]. The preliminary docking study showed minimum binding energies of cubebin with AChE binding pocket suggesting cubebin as a promising molecule for further exploration. Cubebin was isolated from *P. cubeba* and the structure was confirmed by chemical and spectral analysis. *In vitro* studies showed that cubebin at a concentration of 1122 µM exhibited approximately 56 per cent inhibitory activity with IC_{50} value of 992 µM.

Based on the results obtained from initial docking studies and *in vitro* inhibitory assay, cubebin was further assessed in scopolamine-induced amnesia model in mice. Scopolamine, a central nervous system muscarinic antagonist, causes cognitive dysfunction leading to memory impairment which can be seen as a significant increase in the ELT in MWM test. The results suggested that cubebin decreased long-term and spatial memory impairment caused by scopolamine.

The mechanism of action of cubebin was evaluated by its effect on the levels of brain AChE *ex vivo* in mice with scopolamine-induced amnesia. Cubebin pre-treatment (25 and 50 mg/kg) significantly inhibited brain AChE activity. In AD patients, AChE accelerates formation of fibrils from Aβ peptides and co-localizes with Aβ in extracellular plaques. Aβ protein also regulates AChE expression in AD patient's brain[18]. The above results showed inhibition of AChE by cubebin and thus increased cholinergic neurotransmission.

It has been reported that scopolamine impairs short memory and acquisition of new knowledge and increases AChE activity and oxidative stress in the brain[19]. The drugs that reversed the scopolamine-induced amnesia might have some effects on cholinergic system, mainly on the levels of acetylcholine in brain. Oxidative stress can be due to either an increase in reactive oxygen species production or a decrease in the activity of the antioxidant enzymes such as superoxide dismutase, catalase and non-enzymatic antioxidants. These further result in increased level of MDA which indicates peroxidative damage leading to cellular degeneration. Scopolamine significantly increased the levels of MDA, a marker of cellular degeneration. Cubebin significantly restored the MDA levels similar to those observed in the normal control group. This indicates that antioxidant property of cubebin may be responsible for its neuroprotective effect against the oxidative stress induced by scopolamine, possibly by restoring the elevated enzyme activity. This model can be used to screen the molecules which specifically act through cholinergic neurotransmission since the exact link between scopolamine in the production of brain oxidative stress and cholinergic dysfunction is yet to be explored[20]. Although cubebin has shown weaker activity as compared to donepezil, the results obtained from this study might serve basis for lead identification and further modifications. Thus, neuroprotective

role of cubebin should be further evaluated with different doses of cubebin in chronic models such as intracerebroventricular injection of streptozotocin and Aβ-induced AD in rodents[21].There are a few reports which suggest that cubebin possesses dose-related clastogenic and genotoxic effects at higher doses[11,22]. In the present study, the doses selected for cubebin were much lower than the genotoxic dose.

In conclusion, the brain AChE inhibition and antioxidant activity shown by cubebin in scopolamine-induced spatial memory impairment model in mice suggested that this phytoconstituent should be further investigated in the chronic models of AD.

Acknowledgment

The authors acknowledge financial support from University Grant Commission, New Delhi, India, to carry out the research work.

***Conflicts of Interest*:** None.

References

1. Mohandas E, Rajmohan V, Raghunath B. Neurobiology of Alzheimer's disease. *Indian J Psychiatry* 2009; *51* : 55-61.
2. Dall Acqua S. Plant-derived acetylcholinesterase inhibitory alkaloids for the treatment of Alzheimer's disease. *Botanics Targets Ther* 2013; *3* : 19-28.
3. de Pascoli IC, Nascimento IR, Lopes LM. Configurational analysis of cubebins and bicubebin from *Aristolochia lagesiana* and *Aristolochia pubescens*. *Phytochemistry* 2006; *67* : 735-42.
4. de Souza VA, da Silva R, Pereira AC, Royo Vde A, Montanheiro M, Saraiva J, *et al.* Trypanocidal activity of (−)-cubebin derivatives against free amastigote forms of *Trypanosoma cruzi*. *Bioorg Med Chem Lett* 2005; *15* : 303-7.
5. Silva ML, Coímbra HS, Pereira AC, Almeida VA, Lima TC, Costa ES, *et al.* Evaluation of *Piper cubeba* extract, (−)-cubebin and its semi-synthetic derivatives against oral pathogens. *Phytother Res* 2007; *21* : 420-2.
6. Souza GH, da Silva Filho AA, de Souza VA, Pereira AC, Royo Vde A, E Silva ML, *et al.* Analgesic and anti-inflammatory activities evaluation of (−)-O-acetyl, (−)-O-methyl, (−)-O-dimethylethylamine cubebin and their preparation from (−)-cubebin. *Farmaco* 2004; *59* : 55-61.
7. Albuquerque S, Jairo KB, inventors. Cubebin, Dibenzylbutyrolactone Lignan, Semi-Synthetic and Synthetic Derivatives thereof, and Other Lignans and Neolignans as Vasodilating Agents in the Therapy of Erectile Dysfunction. European Patent Application 20090852410, filed December 21, 2009.
8. Somani G, Kulkarni C, Shinde P, Shelke R, Laddha K, Sathaye S. *In vitro* acetylcholenestrase inhibition by psoralen using molecular docking and enzymatic studies. *J Pharm Bioallied Sci* 2015; *7* : 32-6.
9. Fadaeinasab M, Hadi AH, Kia Y, Basiri A, Murugaiyah V. Cholinesterase enzymes inhibitors from the leaves of *Rauvolfia reflexa* and their molecular docking study. *Molecules* 2013; *18* : 3779-88.
10. Ellman GL, Courtney KD, Andres V Jr., Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 1961; *7* : 88-95.
11. Maistro EL, Natel AV, de Souza GH, Perazzo FF. Genotoxic effects of (–)-cubebin in somatic cells of mice. *J Appl Toxicol* 2011; *31* : 185-9.
12. Marklund S, Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur J Biochem* 1974; *47* : 469-74.
13. Aebi H. Catalase. In: Bergmeyer HU, editor. *Methods of enzymatic analysis*. New York: Academic Press; 1974. p. 673-84.
14. Sedlak J, Lindsay RHL. Estimation of total and non-protein sulfhydral groups in tissues with Ellman's reagent. *Anal Biochem* 1979; *95* : 351-8.
15. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979; *95* : 351-8.
16. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; *72* : 248-54.
17. Shen LL, Liu GX, Tang Y. Molecular docking and 3D-QSAR studies of 2-substituted 1-indanone derivatives as acetylcholinesterase inhibitors. *Acta Pharmacol Sin* 2007; *28* : 2053-63.
18. Kwon SH, Lee HK, Kim JA, Hong SI, Kim HC, Jo TH, *et al.* Neuroprotective effects of chlorogenic acid on scopolamine-induced amnesia via anti-acetylcholinesterase and anti-oxidative activities in mice. *Eur J Pharmacol* 2010; *649* : 210-7.
19. Malik J, Kumar M, Deshmukh R, Kumar P. Ameliorating effect of lyophilized extract of *Butea frondosa* leaves on scopolamine-induced amnesia in rats. *Pharm Biol* 2013; *51* : 233-9.
20. El-Sherbiny DA, Khalifa AE, Attia AS, Eldenshary Eel-D. *Hypericum perforatum* extract demonstrates antioxidant properties against elevated rat brain oxidative status induced by amnestic dose of scopolamine. *Pharmacol Biochem Behav* 2003; *76* : 525-33.
21. Sharma M, Gupta YK. Effect of chronic treatment of melatonin on learning, memory and oxidative deficiencies induced by intracerebroventricular streptozotocin in rats. *Pharmacol Biochem Behav* 2001; *70* : 325-31.
22. de Rezende AA, E Silva ML, Tavares DC, Cunha WR, Rezende KC, Bastos JK, *et al.* The effect of the dibenzylbutyrolactolic lignan (–)-cubebin on doxorubicin mutagenicity and recombinogenicity in wing somatic cells of *Drosophila melanogaster*. *Food Chem Toxicol* 2011; *49* : 1235-41.

Reprint requests: Dr Sadhana Sathaye, Department of Pharmaceutical Sciences & Technology, Pharmacology Research Laboratories-II, Institute of Chemical Technology, Mumbai 400 019, Maharashtra, India
e-mail: sadhanasathaye@hotmail.com