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Original Research Article

Molecular docking study on columbin isolated from *Tinospora cordifolia* as a cholinesterase inhibitor

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Abstract

Purpose: *To investigate the acetylcholinesterase (AChE) inhibitory potential of columbin and also to assess its binding affinity against AChE protein.*
Methods: *Crystals of columbin were isolated from the ethyl acetate fraction of Tinospora cordifolia using column chromatography and its structure was determined using x-ray crystallography. Ellman colorimetric assay was used to determine the AChE inhibitory effect in vitro while molecular docking was performed using the MOE 2015.010 software. The selected protein data bank (PDB) was modeled using PDB ID: 10CE (pacific electric ray).*
Results: *The crystal and structure refinement data of columbin were: $C_{20}H_{22}O_6$, Orthorhombic, $P2_12_12_1$, a = 7.4951(2) Å (α = 90°), b = 11.6451(3) Å (β = 90°), c = 19.5882(5) Å (γ = 90°), V=1709.68(8) Å^3, Z = 4, Density (calculated) = 1.392 Mg/m^3, absorption coefficient = 0.851 mm^{-1}, goodness-of-fit on F^2=1.091, T = 100(2) K. Columbin demonstrated good AChE inhibitory effect with half-maximal inhibitory concentration (IC_{50}) of 1.2993 ± 0.17 mg/mL. Molecular docking data revealed that it exhibited hydrophobic and hydrogen bonding interactions with the surrounding residues, and this accelerated complexation between the ligands and the active site of the enzyme.*
Conclusion: *Columbin may be useful in the management of neurodegenerative conditions such as Alzheimer's disease.*

Keywords: *Tinospora cordifolia, Columbin, Acetylcholinesterase, Single crystal diffraction, Molecular docking*

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INTRODUCTION

Molecular docking, a method that helps predict or anticipate the favored orientations of drug candidates (ligands) against macromolecular targets (protein) to make stable complex, has found remarkable importance in drug design and discovery. This field of drug design and discovery using computer aided simulations has witnessed several achievements in recent years, especially towards discovery of new drug leads [1].

Neurodegenerative disease refers to conditions resulting from prolonged collapse and deterioration of the nervous system, especially the nerve cells in the brain. [2]. Alzheimer's

disease (AD) is a neurodegenerative condition that results in progressive loss of structure and function of neurons, ultimately leading to cognitive decline and deterioration [3]. Acetylcholine (ACh), which plays an important role in learning and memory processes, was the first neurotransmitter defect discovered in AD [4]. In patients with AD, there is a drastic decrease in the production and half-life of ACh due to the presence of the enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) which catalyzes the breakdown of ACh [5].

Acetylcholine is a serine protease (EC 3.1.1.7) with binding site consisting of many domains [6] (Figure 1). The anionic domain is responsible for the cation – π interactions with the protonated head of ACh while the acyl pocket dictates its selective binding capacity. The oxyanion hole, consisting of one molecule of water, facilitates the binding between enzyme and substrate through hydrogen-bond and also stabilizes the substrate tetrahedral transition state. The peripheral anionic site (PAS) moderates catalysis [7].

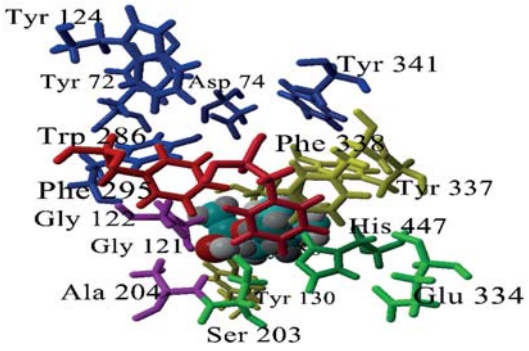

Figure 1: Domains of the binding site of human AChE: showing catalytic domain, (green) anionic domain (red), acyl pocket (purple) oxyanion hole (blue), peripheral anionic site (balls)

Tactics to improve cholinergic function in AD have included stimulation of cholinergic receptors or increasing the half-life of acetylcholine released into the neuronal synaptic cleft by use of agents which restore the level of acetylcholine through inhibition of acetylcholinesterase and butyrylcholinesterase [8]. It has been shown that the inhibition of acetylcholinesterase holds a key role not only in boosting cholinergic transmission in the brain but also in decreasing aggregation of β-amyloid plaques and formation of neurofibrillary tangles in AD [9]. Consequently, AChE inhibitors are considered effective in the treatment of AD. *Tinospora cordifolia* is a shrub that belongs to the family Menispermaceae. This plant has several pharmacological effects such as antiallergic, bactericidal, osteoprotective, antioxidant, genoprotective anti-inflammatory, uricosuric, antimalarial, antiperiodic, antispasmodic, antistress, hepatoprotective, antiviral, diuretic, febrifuge and immuno-stimulant [10-12]. The stem and root of the plant contains alkaloids like tembetarine, choline, magnoflorine, berberine, tinosporin, isocolumbin, palmetine, jatrorrhizine, aporphine, and tetrahydropalmatine which had anti-cancer, anti-diabetes, anti-viral, anti-inflammatory, anti-psychiatric and immunomodulatory actions [13]. Furthermore, the whole plant of *T. cordifolia* contains furanolactone, diterpenoid lactones, cleodrane derivatives columbin, tinosporides, tinosporin and jateorine which exerts vasorelaxant, anti-inflammatory, anti-microbial, anti-hypertensive and anti-viral effects [14]. The present study was aimed at determining the crystal data and structure refinement of columbin isolated from *T. cordifolia* and assessing its binding affinity against acetylcholinesterase protein.

EXPERIMENTAL

Plant collection, extraction and purification

The stem of *T. cordifolia* collected from Enugu State, Nigeria was authenticated at Forest Research Institute of Nigeria with voucher number FHI 112287. Oven dried (40ºC) and powdered stem bark was extracted with methanol and the extract was further partitioned into hexane, dichloromethane, ethyl acetate and methanol. The ethyl acetate fraction was purified using column chromatography on a 600 g silica gel (60 - 200 mesh size) to yield crystals of columbin (eluting solvent: dichloromethane 100%).

Single-crystal x-ray diffraction analysis

The colorless needle-shaped crystal of columbin was subjected to single-crystal X-ray diffraction analysis to determine its structure unambiguously. The solid state experiment was accomplished at 100 K using φ-ω scan mode for collection of diffraction data on Bruker D8 venture fitted with Cu Kα radiation source (λ = 1.54178 Å) and CCD detector (PHOTON 100 diffractometer). Crystal data were reduced using SANIT program [15] while the structure was solved by direct method with the aid of SHELXS-86 program [16]. All non-hydrogen atoms were refined anisotropically through full matrix least

square refinement methods on F^2 by using SHELXL-97 program during the structure elucidation [17]. The hydrogen atoms on the parent compound in the constructed structure were placed at calculated positions (0.95 - 1.0 Å) with isotropic thermal displacement parameter U_{iso} (H) = 1.2 - 1.5 through riding model. ORTEP was used to plot the diagram of molecular structure with 40% thermal ellipsoid [18]. The significant supramolecular interactions within the crystal lattice, were found with PLATON program [19], while quantitative analysis of inter-molecular interaction was done using the Crystal Explore 3.0 program [20].

Hirshfeld surface analysis

The number of interactions present in the crystal lattices was determined qualitatively and quantitatively *via* three-dimensional Hirshfeld surface analysis and two-dimensional fingerprint plots, respectively. The strength of non-covalent interactions was forecasted by using the color-coding scheme in 3D Hirshfeld surface while two-dimensional fingerprint plots was used to find out the contribution of different contacts utilized in crystal packing toward the total interactions.

Assay of anticholinesterase activity

Inhibition of AChE by columbin was assessed by the method of Ellman *et al* [21]. The AChE activity was determined in a reaction mixture in a 96-well plates containing 240 µL of buffer (50 mM Tris-HCl, pH 8.0), 20 µL of columbin (1 mg/mL), and 20 µL of AChE enzyme (0.28 U/mL). After incubation for 30 min at 37ºC, 20 µL of 10 mM 5,5-dithio-bis(2-nitrobenzoic) acid (DTNB) was added. The AChE activity was determined with a microplate reader from the absorbance changes at 412 nm over a period of 4 min at 30 s intervals. The experiments were done in triplicate with eserine - used as standard. The inhibition (H) of AChE was determined by comparison of rates of reaction of samples relative to blank sample using Eq 1.

$$H\ (\%) = \{(E - S)/E\}100 \quad \text{..... (1)}$$

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

Ferrous chelation assay

The ferrous ion-chelating (FIC) assay was carried out according to the method of Singh and Rajini, [22] with some modifications. Solutions of 2 mM $FeCl_2 \cdot 4H_2O$ and 5 mM ferrozine were diluted 20 times in distilled water. In the assay, 1 mL of columbin (1 mg/mL) was mixed with 1mL $FeCl_2 \cdot 4H_2O$. After 5 min incubation at 25ºC, the reaction was initiated by the addition 1mL of ferrozine. The mixture was shaken vigorously and after a further 10 min incubation period at 25ºC, the absorbance of the solution was measured at 562 nm. The control was prepared as above but the test drug was replaced with 1mL of methanol. Ethylenediaminetetraacetic acid (EDTA) was used as positive control. The inhibition (B) of ferrozine–Fe^{+2} complex formations was calculated as in Eq 2.

$$B\ (\%) = \{(Ac - As)/Ac\}100 \quad \text{..... (2)}$$

where Ac and As are the absorbance of control and test samples, respectively. The extract concentration providing 50 % inhibition (IC_{50}) was calculated by interpolation from linear regression analysis.

Molecular docking studies

In order to predict the binding mode of columbin as putative acetylcholinesterase inhibitor [], molecular docking studies were carried out with the available protein data bank (PDB) ID:10CE (acetylcholinesterase (E.C. 3.1.1.7) of the enzyme from *Tetronarce californica* (*Pacific electric ray)* complexed with an inhibitor MF268 (physostigmine analogue 8-(cis-2,6-dimethylmorpholino) octylcarbamoyleseroline) [23]. The builder module in MOE 2015 was used to draw the structures. The compound was energy-minimized, followed by addition of partial charges as per Merck Molecular Force Field (MMFF94). Docking was done using MOE 2015.010 after initial protein preparation. The default rigid docking protocol in MOE Suite was utilized for docking. The resulting poses of the compound was visually inspected to comprehend protein ligand interactions. The interactions were analyzed with PLIP web server (https://projects.biotec.tu-dresden.de/plip-web/plip). All the visuals were recorded using MOE 2015 Suite.

Statistical analysis

Applicable data were analyzed using GraphPad Prism 6.0, and expressed as mean ± standard deviation (SD). $P < 0.05$ was considered statistically significant.

RESULTS

Crystal packing

The packing arrangement of the molecule showed the presence of five different inter-

molecular interactions dominant in the crystal structure with no intra-molecular interaction. In crystal lattice, asymmetric unit joined with the neighboring molecules *via* O3-H1...O4, C12-H12A...O2, C13-H13...O2, C12-H12A...O4 and C18-H18...O4 inter-molecular hydrogen bonding, and form $R_2^1(7)$ $R_2^1(8)$, and $R_2^2(7)$ ring motifs. The seven-member ring motifs $R_2^1(7)$ and $R_2^2(7)$ generated by self-linkage of neighboring molecule through C12-H12A...O4, C18-H18...O4 C12-H12A...O2, C12-H12A...O4, and O3-H1...O4, interactions respectively. The ring motif $R_2^1(8)$ was assembled by C12-H12A...O2 and C13-H13...O2 inter-molecular interactions as shown in Figure 2. The non-covalent interactions O3-H1...O4, C12-H12A...O2, C13-H13...O2, C12-H12A...O4 and C18-H18...O4 aligned the molecules in three-dimensional network with donor-acceptor bond distances of 3.183(2) Å, 3.210(2) Å, 3.482(2) Å, 3.156(2) Å, and 3.315(2) Å, respectively. The crystal lattice of columbin molecules are inter connected with one another through the screw symmetry (Figure 2).

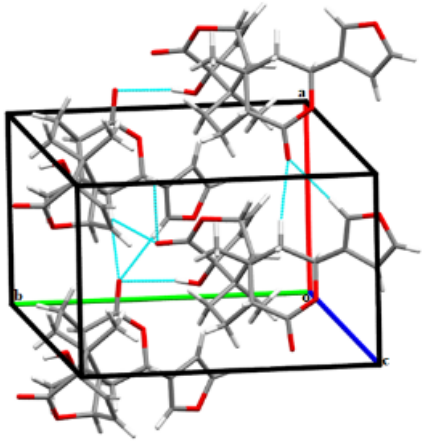

Figure 2: Unit cell diagram showing the packing arrangement of columbin molecules

Hirshfeld surface analysis result

The relative contributions of H…H, O…H, C…H, O…O and C…O interactions toward crystal stability were 51.1, 38.2%, 8.5, 0.7, and 2.2 % respectively over 100% interaction (Figures 3 and 4).

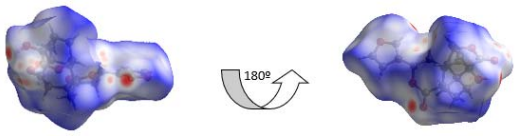

Figure 3: The d_{norm} mapped on the Hirshfeld surface for visualizing the intermolecular contacts of columbin

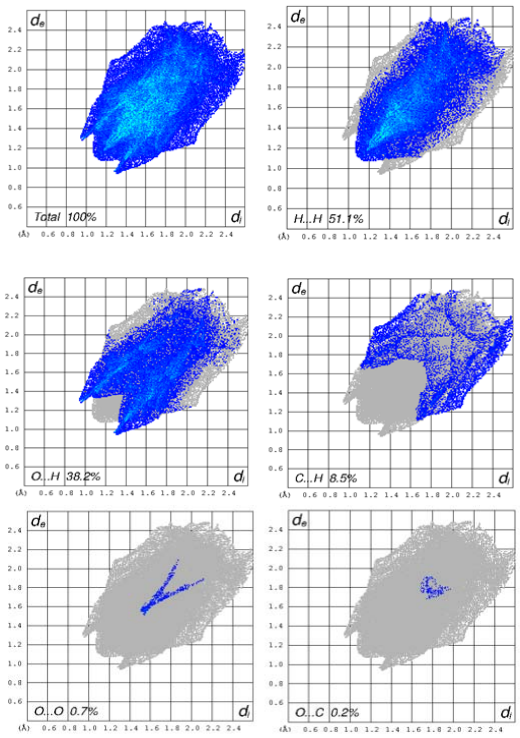

Figure 4: Two-dimensional fingerprint plots indicating the major and minor contributions of inter-molecular interactions in columbin

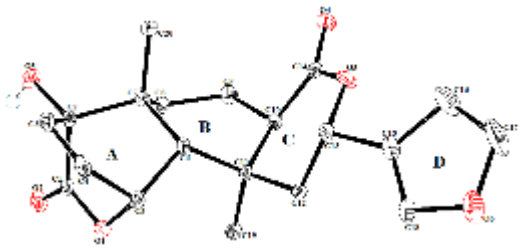

Figure 5: The molecular structure of columbin drawn at 40 % probability level

Acetylcholinesterase inhibitory and metal chelating potential of columbin

The acetyl cholinesterase inhibitory and metal chelating effects of columbin are as shown in Table 1.

Molecular docking of protein data bank (PDB ID: 10 CE) complexed with columbin

The top ranked docked pose of columbin **(**Figure 6) revealed that there were four hydrophobic interactions of columbin with ASP 72, TRP 84A, Phe 330A and a hydrogen bond with GLU199 (1.77) with an estimated free binding energy (ΔG), of -7.1371 kcal/mol.

Table 1: Acetylcholinesterase and metal chelating effect of columbin

Compound	AChE inhibition (IC_{50}±SD (mg/mL)	Fe 2+ chelating activity IC_{50}±SD (mg/mL)
Columbin	1.2993±0.17	1.8131±0.01
Eserine (standard)	0.5318±0.34	NA
EDTA (standard)	NA	0.0450±0.11

Values are presented as mean ± standard deviation (n=3). NA = not applicable

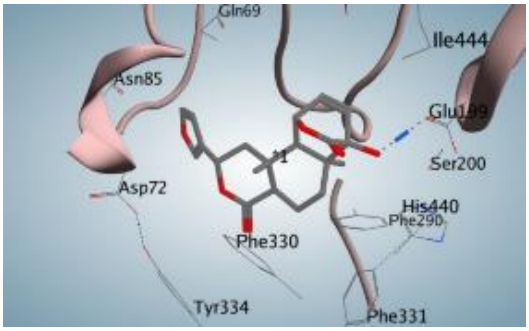

Figure 6: Simulated poses of Columbin. Hydrogen bonds are presented in blue lines. Grey sticks show the ligand while the acetylcholinesterase residues are shown as pink ribbons. The images were generated using MOE 2015.01.08

DISCUSSION

Single crystal X-ray diffraction is the main source of information on the geometrical structure of molecules and molecular solids, including bond distances (and hence bond orders), bond angles, shapes of coordination, conformations of flexible molecules, as well as intermolecular contacts. It can always distinguish between configurational isomers (e.g. cis and trans), and optical isomers (enantiomers). The single-crystal X-ray diffraction analysis of columbin describes non-centrosymmetric structure consisting of a bicycle ring A (O1/C1-C5/C2-C7) fused with six-membered rings B (C6-C7)/C8-C11) and C (C10/C11/C12/C13/O5/C14) along C6/C7 and C10/C11 respectively. The solid state chemistry showed a bicyclic ring A with a lactone moiety and an olefinic bond between C3 and C4 (1.326(3) Å). Furthermore, the hydroxyl group on the bicyclic ring was found to be equatorially oriented at C2 with O3/C2/C3/C4 torsion angle of 176.4(18)º. The bicyclic ring A (O1/C1-C5/C2-C7) having puckering parameters Q = 0.781(2) Å, ɵ = 90.55(15)º, φ = 298.90(14)º for ring O1/C1-C5, and Q = 0.823(2) Å, ɵ = 91.82(14)º, φ = 2.40(14)º for ring C2-C7 exhibited boat conformation. Ring B (C6-C7)/C8-C11) was also found to exist in boat conformation as indicated by puckering parameter Q = 0.683(2) Å, ɵ = 90.59(17)º, φ = 282.12(17)º with axially oriented methyl on junction carbon atoms C7 and C11 with respect to ring A and ring C respectively (Figure 5). However, another lactone ring C, substituted with *pseudo* equatorially oriented planer furan ring (O6/C15-C18) at C13 adopts half chair conformation. The torsion angle of bridge head carbon atoms C2-C3-C4-C6 was 0.3(2)º and it showed syn-periplanar geometry of atoms. On the whole, the geometrical parameters of the compound are as follows; crystal size: 0.210 x 0.090 x 0.070 mm^3, empirical formula $C_{20}H_{22}O_6$ and weight of 358.37. The crystal system is orthorhombic with space group of $P2_12_12_1$. The Unit cell dimensions were: a = 7.4951(2) Å (α = 90°), b = 11.6451(3) Å (β = 90°), c = 19.5882(5) Å (γ = 90°), Volume = 1709.68(8) $Å^3$, Z = 4. The density (calculated) was 1.392 Mg/m^3 with absorption coefficient value of 0.851 mm^{-1} (See supplementary data).

One of the most important approaches for treatment of AD involves the enrichment of acetylcholine level in the brain using acetylcholinesterase (AChE) inhibitors [24]. Columbin, a furanoid diterpenoid isolated from ethyl acetate fraction of *T. cordifolia* stem demonstrated good acetylcholinesterase inhibitory effect with IC_{50} value of 1.2993 ± 0.17 mg/mL, when compared to the standard eserine with IC_{50} value of 0.5318 ± 0.34 mg/mL (Table 1) which indicates that columbin has the potential to increase the half-life of acetylcholine in the brain thereby improving learning and memory. The AChE inhibitory effect of columbin is in agreement with other related diterpenes such as dihydrotanshinone and cryptotanshinone isolated from *Salvia miltiorhiza* reported to inhibit AChE in a dose-dependent manner [25]. It was reported also that compounds with aromatic hydrocarbons are more potent AChE inhibitors [26]. This study thus confirms the use of *T. cordifolia* as cognitive enhancer and columbin could serve as a potential drug lead for development of new drugs against Alzheimer's disease.

New molecular modeling tactics driven by rapidly improving computational platforms, have resulted in many success stories of the use of computer-assisted drug design in the discovery of new mechanism- or structure-based drugs. For instance, berberine, an isoquinoline alkaloid isolated from the dried rhizome of *Rhizoma coptidis* showed promising cholinesterase inhibitory potential with mostly hydrophobic interactions with the enzyme [27]. The possible interactions between geissospermine, an indole-indoline alkaloid isolated from *Geissospermum vellosii* and AChE of the Pacific electric ray have

been studied with molecular docking, in which case hydrogen bonds, hydrophobic interactions and p-p stacking were reportedly involved in the interactions [28]. Infractopicrin an indole alkaloid isolated from *Cortinarius infractus* binds preferentially to the oxyanion hole of the AChE enzyme via p-p interactions with the aromatic residues [29]. Molecular modeling studies on columbin was performed in order to investigate its binding mode against *Tetronarce californica* (*Pacific electric ray)* acetylcholinesterase. A protein data bank (PDB) was chosen and the compound was docked using the coordinates of the cognate ligand [30]. Four hydrophobic interactions of columbin, including with ASP 72, TRP 84A, Phe 330A and a hydrogen bond with GLU199 were observed. Thus, columbin has the potential to delay the neurodegeneration associated with AD due to its ability to bind to the inhibitor site with moderate energy leading to competitive inhibition of the enzyme AChE. In a study, the binding energies of the US-FDA approved drugs donepezil (Aricept), rivastigmine (Exelon), galantamine (Reminyl) and tacrine (Cognex) were found to be 3.58, -5.61, -7.86 and -6.95 kcal/mol, respectively [31]. Thus, columbin is quite comparable with existing drugs in term of binding energy.

As the demand for new and more effective drugs for AD treatment continues to grow, pharmacological strategies aimed at lowering brain metal ions and targeting A*β*/metal ion interactions has been proposed and this offers good potential to chelation therapy [32]. Metal ions have been shown to abnormally accumulate in the brain with aging as well as in the course of several neurodegenerative disorders including AD [33]. Columbin showed good metal chelating potential with IC_{50} value of 1.8131±0.01 mg/mL when compared to standard EDTA with IC_{50} value of 0.0450±0.11 mg/mL. Columbin therefore, has the ability to lower brain metal ions which has been linked to several neurodegenerative diseases such as Alzheimer's.

CONCLUSION

Columbin, a furanoid diterpenoid with an orthorhombic crystal system, has been successfully isolated from ethyl acetate fraction of *T. cordifolia*. It has the potential to improve memory and learning, as well as delay the neurodegenerative process associated with Alzheimer's disease due to its ability to competitively inhibit cholinesterase enzyme. The compound could therefore serve as potential lead for novel drug development for the treatment of Alzheimer's disease.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. TOE designed the study, supervised the study and produced the final manuscript. JOO collected, analyzed the data and wrote draft manuscript. ZA and ZU did the molecular docking work. All authors read and approved the manuscript for publication.

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