Discovery of Helminthosporin, an Anthraquinone Isolated from *Rumex abyssinicus* Jacq as a Dual Cholinesterase Inhibitor

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**ABSTRACT:** Natural products have extensively contributed toward the discovery of new leads for Alzheimer’s disease. During our search for new inhibitors of cholinesterase enzymes from natural sources, the ethyl acetate (EtOAc) extract of *Rumex abyssinicus* Jacq was identified as a dual cholinesterase inhibitor with IC$_{50}$ values of 2.7 and 11.4 μg/mL against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), respectively. The phytochemical investigation of the EtOAc extract has resulted in isolation of four anthraquinones, namely, helminthosporin, emodin, chrysophanol, and physcion, amongst which the helminthosporin has been isolated for the first time from *Rumex* sp. All isolated secondary metabolites have displayed significant inhibition of EeAChE with IC$_{50}$ values of 2.63, 15.21, 33.7, and 12.16 μM, respectively. In addition, the helminthosporin was also found to inhibit BChE with an IC$_{50}$ value of 2.99 μM. The enzyme kinetic study has indicated that helminthosporin inhibits AChE and BChE in a noncompetitive manner with $k_i$ values of 10.3 and 12.3 μM, respectively. The results of molecular modeling and propidium iodide displacement assay have revealed that helminthosporin occupies the peripheral anionic site of the active site gorge of AChE. In the PAMPA-BBB permeability assay, helminthosporin was found to possess high BBB permeability ($P_e = 6.16 \times 10^{-6}$ cm/s). In a nutshell, helminthosporin has been identified as a brain permeable dual cholinesterase inhibitor, and thus its further synthetic exploration is warranted for optimization of its potency.

**INTRODUCTION**

The genus *Rumex* (family: Polygonaceae) is widely distributed worldwide, and its many species have nutritional and medicinal properties. The *Rumex abyssinicus* Jacq. (synonyms: *Rumex schimperi* Meisn. and *Acetosa abyssinica* Jacq.) is a flowering plant (family: Polygonaceae) native to tropical Africa and is often used in African traditional medicine for various purposes including treating jaundice and related liver diseases, stomach ache, neckache, low blood pressure, pneumonia, wound healing, and in rheumatism. The plant extracts possess antimicrobial, anti-inflammatory, diuretic, analgesic, wound healing, anticancer, and antioxidant activities. However, the plant has never been investigated for its anti-Alzheimer’s potential.

Natural products (NPs) have immensely contributed in the discovery of central nervous system (CNS)-active agents. The contribution of NPs for Alzheimer’s disease (AD) is huge, and among the four currently available anti-Alzheimer’s drugs, two are NP scaffolds viz. rivastigmine and galantamine. Although numerous cholinesterase inhibitors have been reported, the search for new compounds is a continuing process. Based on the understanding of the role of butyrylcholinesterase (BChE) in late stage AD, it has now been hypothesized that dual cholinesterase inhibitors would be beneficial rather than only acetylcholinesterase (AChE) inhibitor. As a part of our efforts in this area, herein we have investigated the potential of the African medicinal plant *R. abyssinicus* Jacq. and its secondary metabolites for anticholinesterase potential. The detailed mechanistic investigation followed by molecular modeling studies were carried out to understand the mechanism and mode of cholinesterase inhibition by the most active secondary metabolite.

**RESULTS**

In Vitro Inhibition of Cholinesterase Enzymes by *R. abyssinicus* Jacq. As a part of our interest to discover new dual cholinesterase inhibitors, the ethyl acetate (EtOAc) extract of rhizomes of *R. abyssinicus* Jacq. (RAE) was screened for inhibition of EeAChE and eqBChE. Initial screening was performed at 10 and 100 μg/mL (results are shown in Figure 1). At 100 μg/mL, RAE displayed 91 and 93% inhibition of EeAChE and eqBChE enzymes. The extract RAE also displayed potent inhibition (73 and 50%) of both enzymes even at 10 μg/mL. The IC$_{50}$ values of RAE for inhibition of
these enzymes were found to be 2.7 ± 0.43 and 11.43 ± 1.23 μg/mL, respectively. The interesting activity profile of RAE against both cholinesterases prompted us to proceed for phytochemical investigation, to dig-out the secondary metabolites responsible for this activity.

**Phytochemical Investigation of R. abyssinicus Extract.**

The extract was subjected to isolation of secondary metabolites using traditional column chromatography, which resulted in isolation of four compounds RAE-1, 2, 3, and 4. The 1H NMR of RAE-1 has shown three characteristic H-bonded –OH peaks at chemical shift values of δ 11.3, 11.9, and 12.0 ppm, indicating the presence of three pairs of –OH and carbonyl groups in a pattern of 1,3-position. Further, the signals in the region δ 7.47–6.58 ppm of the 1H NMR spectrum with integration for four protons have indicated the presence of four aromatic protons. The singlet at δ 2.4 ppm confirmed the presence of aromatic –CH3 group. The 13C NMR of RAE-1 have shown two peaks at δ 190.1 and 181.8 ppm accounting for the presence of two –CO groups in the structure of RAE-1. The presence of three signals at δ 165.9, 164.9, and 161.8 indicated the presence of three OH groups on the aromatic ring. The signal at δ 22.0 ppm confirmed the presence of aromatic methyl groups. The ESI-MS (negative mode) spectrum has shown the [M − H]− peak at m/z 269.25 indicating the possible mass of RAE-1 as 270 Da. The combined information from NMR and MS data concluded the identity of RAE-1 as helminthosporin. Further, the obtained spectral data was well corroborated with the literature values.13

The NMR spectra of other three compounds RAE-2, RAE-3, and RAE-4 also showed similar characteristic peaks of the anthraquinone scaffold. Based on 1H, 13C NMR, and MS data and further their comparison with literature values, they were identified as emodin, chrysophanol, and physcion, respectively. The chemical structures of isolated secondary metabolites are shown in Figure 1.

**In Vitro Inhibition of Cholinesterase Enzymes by Isolated Secondary Metabolites.** All four anthraquinones were tested for cholinesterase inhibitory activity against both enzymes, initially at 10 and 100 μM concentrations. Results are shown in Figure 1. It was observed that helminthosporin was the most active among these four compounds, against both enzymes. Determination of IC50 values has revealed that helminthosporin inhibits EeAChE with an IC50 value of 2.7 μM, and eqBChE with an IC50 value of 2.99 μM. The IC50 of helminthosporin for inhibition of recombinant human AChE was also determined and was found to be 7.9 ± 0.14 μM. The representative dose–response curve for IC50 determination has been shown in Figure 2. Other three anthraquinones, namely, emodin, chrysophanol, and physcion were moderately active against AChE (IC50 values of 15, 34, and 12 μM, respectively) and were weakly active against BChE (Table 1).

Helminthosporin was then studied for enzyme kinetics to understand the mechanism of cholinesterase inhibition. The analysis of the Lineweaver–Burk plot (Figure 3A) has indicated that helminthosporin is a noncompetitive inhibitor of human AChE with a kᵢ value of 10.3 μM. Similarly, it showed inhibition of eqBChE in a noncompetitive mode with a kᵢ value of 12.3 μM. Further, our study has shown that the clinically used cholinesterase inhibitor donepezil is also a noncompetitive inhibitor of AChE and BChE with kᵢ values of 0.0214 (lit. 0.016)14 and 15 (lit. 1.6)14 μM, respectively. The Lineweaver–Burk plots of helminthosporin and donepezil for inhibition of AChE and BChE are shown in Figures 3 and 4, respectively. The kinetic study has shown that the Michaelis constant (Kₘ) value does not change with the change in inhibitor concentration in case of helminthosporin (Figure 3C,F) as well as donepezil (Figure 4C,F); however, the reaction rate (Vₘax) was found to be decreased with the increase in inhibitor concentration (Figures 3C,F and 4C,F). This is the typical case of noncompetitive inhibition.

Next, the in silico docking studies were conducted to explore the binding site of helminthosporin in human AChE (PDB: 4EY7) and human BChE (PDB: 6EP4) using Glide module of Schrodinger molecular modelling software. The docking investigation demonstrated that helminthosporin accommo-
dates well in the active site gorge of both enzymes, interacting mainly with the amino acids residues of peripheral anionic site (PAS) domain (Figure 5). The in silico studies are advocating that the inhibition of acetylcholinesterase is likely to be attributed to the various hydrophobic interactions of helminthosporin with the amino acid residues of PAS. As depicted in Figure 5A, the molecule stays at PAS, and does not enter deep into the active site gorge to interact with catalytic anionic site (CAS) residues. The dihydroxy aryl ring of helminthosporin (ring C) displays π−π stacking interactions with the TRP 286 and the −OH group displays H-bonding interactions with SER 293 and ARG 296 residues of AChE (Figure 5B). The carbonyl oxygen of the central quinone ring displays H-bonding interactions with PHE 295 and ARG 296. Helminthosporin also occupies the active site of the BChE (Figure 5C−D). In this case also, the molecule stays at the entrance of the cavity and further it was noticed that it orients in an angular way rather than a vertical orientation. This could be because of the wider opening of the BChE active site gorge. The hydroxyl group of ring C shows H-bonding interaction with ASN 289 of the gorge mouth. The hydroxyl of the tolyl ring (ring A) also displayed H-bonding with GLY 116 (a residue of the oxyanion hole) and the same ring also showed π−π stacking interaction with TYR 332 residue of PAS of BChE (Figure 5D). Unlike helminthosporin, donepezil was found to span across the active site gorge of both AChE and BChE because of its extra length. It shows interactions with PAS as well as CAS residues; however, its noncompetitive mode of inhibition for both enzymes must be accounted for its interactions with PAS residues. The overlay images of donepezil with helminthosporin are displayed in Figure 5E−F.

The molecular modeling studies have indicated that helminthosporin primarily interacts with PAS residues. Thereafter, the affinity of helminthosporin for the PAS binding site was assessed by displacement of propidium iodide, an AChE ligand that specifically binds at its PAS binding site and enhances its fluorescence intensity up to eight fold after binding to AChE.15 The decrease in fluorescence intensity was taken as a measure of displacement of propidium iodide by the compound and thus its affinity for PAS. Helminthosporin and donepezil were analyzed at 10 and 50 μM test concentrations. Helminthosporin displayed propidium iodide displacement by 35.9 and 69.2%, respectively, at 10 and 50 μM test concentrations. Whereas, donepezil has shown 20.8 and 29.2% displacement at 10 and 50 μM, respectively. These results supported the docking study results, that is, interactions

Table 1. Inhibitory Effect (IC_{50} Values) of RAE and Isolated Secondary Metabolites against EeAChE and eqBChE^{a}\n
| code   | test sample name            | IC_{50} in μM or μg/mL (±SD) | EeAChE | eqBChE |
|--------|-----------------------------|-------------------------------|--------|--------|
| RAE    | R. abyssinicus extract      | 2.7 ± 0.43 μg/mL              | 11.43 ± 1.23 μg/mL |
| RAE-1  | helminthosporin             | 2.63 ± 0.09 μM                | 2.99 ± 0.55 μM     |
| RAE-2  | emodin                      | 15.21 ± 3.52 μM               | nd     |
| RAE-3  | chrysophanol                | 33.7 ± 1.83 μM                | nd     |
| RAE-4  | physcion                    | 12.16 ± 0.36 μM               | nd     |
|        | positive control            | 0.049 ± 0.001 μM              | 5.52 ± 1.05 μM     |

^{a}nd: not determined. RAE: EtOAc extract of R. abyssinicus Jacq. rhizomes. IC_{50} values are average of three independent determinations.
of helminthosporin with the residues of the PAS binding site, and thus supporting the noncompetitive mode of inhibition.

**ADME Properties of Helminthosporin.** The drug-like/ADME properties of any new lead compound must be evaluated at the early discovery stage. The parameters associated with membrane permeability of the compound were assessed computationally using the QikProp module of Schrodinger molecular modeling software. The computed log P, log S, Caco-2 permeability, and MDCK permeability values were found within an acceptable range (Table 2). The BBB permeability has been considered as one of the most important criterion for CNS drugs. Therefore, BBB permeability was determined for helminthosporin, both by computational methods as well as via experimental methods. The computed log BB value was found to be within the acceptable limit. To understand whether the helminthosporin could penetrate into the brain, the parallel artificial membrane permeation assay for BBB (PAMPA-BBB) was used, which has shown its Pe value as $6.16 \times 10^{-6}$ cm/s. The computed ADME parameters, experimentally determined BBB permeability, and the summary of enzyme kinetics and molecular modeling of helminthosporin and donepezil have been shown in Table 2.

■ **DISCUSSION**

The dual inhibition of both the cholinesterase enzymes has been believed to be beneficial over inhibition of only one of the cholinesterase enzymes. The identification of a NP-based new scaffold has a special importance in the CNS domain. The activity-guided isolation of bioactive secondary metabolites has been one of the successful approaches to discover new NP inhibitors. Employing this approach, Choi and co-workers have reported the cholinesterase inhibition activity of 16 anthraquinones. Their results have shown that anthraquinones display a better activity profile against AChE in comparison to BChE. Emodin (AChE, IC_{50}: 21.8; BChE, 9.17 μM), chrysoeriol (AChE, IC_{50}: 75.8; BChE, 68.6 μM), and physcion (AChE, IC_{50}: 8.25 μM) were reported as cholinesterase inhibitors. In the present work, as a part of our campaign toward discovery of plant based new anti-AD lead candidates, the *R. abyssinicus* extract was found to show promising inhibition of AChE as well as BChE. The bioassay-guided isolation has resulted in isolation of four anthraquinones, namely, helminthosporin, emodin, chrysophanol, and physcion. Literature reports have indicated that many species of *Rumex* are reported to produce anthraquinones. Emodin, chrysophanol, and physcion were previously reported from *R. abyssinicus*; however, it is noteworthy to mention that helminthosporin is being isolated for the first time from *Rumex sp.* Previously, helminthosporin has been reported from marine fungus *Aspergillus glaucus* and from the plant *Eremurus persicus*. The anticholinesterase activity of helminthosporin is also being reported for the first time.

Majority of natural anthraquinones bear hydroxyl groups at C-1 or C-8 or at both positions. Emodin is the most widely investigated natural anthraquinone which possess −OH group at both C-1 and C-8 positions. Interestingly, all four isolated anthraquinones along with several other anthraquinones which were studied for cholinesterase inhibition bear a −OH group at 1,8-positions and a methyl group at C-3 position. Apart from 1,8-hydroxy groups, emodin bears additional-OH groups at the C-6 position. From the available cholinesterase inhibition data on anthraquinones, it was observed that the manipulation of the C6-OH group modulates the AChE inhibition activity. The replacement of C6-OH with another electron-donating group such as −OMe (e.g., physcion) does not affect the activity; however, removal of the C6-OH group has resulted in twofold decrease in activity (e.g., chrysophanol). When the
Figure 5. Molecular modeling of helminthosporin with human AChE (PDB ID: 4EY7) and BChE (PDB ID: 6EP4). (A) Surface view of AChE showing the various binding sites within the active site gorge and orientation of the molecule in the gorge; (B) interactions of helminthosporin with active site gorge residues of AChE; (C) surface view of BChE showing various binding sites within the active site gorge and orientation of the molecule inside the gorge; (D) interactions of helminthosporin with active site gorge residues of BChE; (E) overlay view of helminthosporin (grey) and donepezil (orange) in the active site gorge of AChE; and (F) overlay view of helminthosporin (grey) and donepezil (orange) in the active site gorge of BChE.

Table 2. ADME Properties and the Summary of Enzyme Inhibition for Helminthosporin and Donepezil HCl

| properties | helminthosporin | donepezil HCl |
|------------|----------------|--------------|
| ADME properties<sup>a</sup> | | |
| QP log <i>P</i><sub>o/w</sub> | 1.6 | 4.4 |
| QP log <i>S</i> | −2.7 | −4.7 |
| QPPCaco | 134 | 892 |
| QPP MDCK | 56 | 484 |
| QP log <i>BB</i> | −1.3 | 0.11 |
| PAMPA-BBB permeability [<i>P</i><sub>e</sub> × 10<sup>−6</sup> cm/s]<sup>b</sup> | 6.16 (CNS+) | 9.54 (CNS+) |
| enzyme inhibition | | |
| EeAChE: IC<sub>50</sub>, μM | 2.63 | 0.049 |
| eqBChE: IC<sub>50</sub>, μM | 2.99 | 5.52 |
| rHuAChE: IC<sub>50</sub>, μM | 7.9 | 0.017 |
| enzyme kinetics (<i>k</i>, and type of inhibition) | | |
| hAChE (μM) | 10.3 (noncompetitive) | 0.021 (noncompetitive) |
| eqBChE (μM) | 12.3 (noncompetitive) | 15 (noncompetitive) |
| molecular modeling (AChE) | | |
| binding site | PAS | both PAS and CAS |

<sup>a</sup>ADME properties were determined using the QikProp module of Schrodinger 10.2 software. QP log <i>P</i><sub>o/w</sub>, QP log <i>S</i>, QPPCaco, QPP MDCK, and QP log <i>BB</i>, indicates the octanol/water partition coefficient, aqueous solubility (mol/L), Caco-2 permeability (nm/s), MDCK permeability (nm/s), and brain/blood partition coefficient. <sup>b</sup>The permeability value (<i>P</i><sub>e</sub>) was determined experimentally using the PAMPA-BBB assay using porcine brain lipid.
C6−OH group was migrated to adjacent C5-carbon (e.g., helminthosporin) creating another (C5,C10)-keto-enol tautomer in the anthraquinone skeleton which positively modulated the activity. On addition of the fourth −OH group in the emodin skeleton (e.g., alternin), the activity was improved. The disturbance to the keto−enol tautomer (e.g., obtusin) has resulted in significant loss in activity. From these observations, it can be concluded that the presence of two or more keto−enol tautomers in the anthraquinone scaffold is important for anti-AChE activity; and the C6−OH group can be manipulated/migrated to further fine-tune the activity. The schematic view of the structure−activity relationship is depicted in Figure 6. The molecular modeling studies have shown that the additional keto−enol tautomer (C5−OH, C10−CO) present in helminthosporin display bindentage H-bonding interaction with ARG 296, and also it interacts with SER 293 and PHE 295 residues via H-bonding. These bonding interaction with ARG 296, and also it interacts with SER 293 and PHE 295 residues via H-bonding. These interactions could be accounted for stronger binding of helminthosporin with the enzyme and thus better activity over other anthraquinones.

The active site gorge of both cholinesterases is deep with about 20 Å length, wherein the catalytic site is located at the bottom of the gorge (~4 Å above the base of gorge). Within the gorge, there exists two distinct sites, the CAS and PAS. For inhibitors to competitively bind to the enzyme, it has to enter deep into the cavity to interact with the catalytic triad. The PAS is located at the entrance of the gorge and is known to allosterically modulate the enzyme activity. Donepezil which is a clinically used AChE inhibitor, spans in the entire active site gorge, there exists two distinct sites, the CAS and PAS. For inhibitors to competitively bind to the enzyme, it has to enter deep into the cavity to interact with the catalytic triad. The PAS is located at the entrance of the gorge and is known to allosterically modulate the enzyme activity. Donepezil which is a clinically used AChE inhibitor, spans in the entire active site gorge.

Figure 6. Structure−activity relationship of anthraquinones for AChE inhibition. The AChE inhibition data of obtusin and alternin has been taken from Jung et al.19

In summary, herein we report the R. abyssinicus plant extract and its isolated secondary metabolite “helminthosporin” as dual inhibitors of AChE and BChE enzymes, which are vital players responsible for cholinergic loss in Alzheimer’s patients. Helminthosporin binds to the PAS of the active site gorge of AChE in a noncompetitive mode. The anti-Alzheimer’s activity of the R. abyssinicus plant and helminthosporin has been reported for the first time, and its structure has further potential for lead optimization.

CONCLUSIONS

In summary, herein we report the R. abyssinicus plant extract and its isolated secondary metabolite “helminthosporin” as dual inhibitors of AChE and BChE enzymes, which are vital players responsible for cholinergic loss in Alzheimer’s patients. Helminthosporin binds to the PAS of the active site gorge of AChE in a noncompetitive mode. The anti-Alzheimer’s activity of the R. abyssinicus plant and helminthosporin has been reported for the first time, and its structure has further potential for lead optimization.

MATERIAL AND METHODS

General. All solvents used in the present study were obtained from CDH chemicals, Sigma-Aldrich and were used as received. 1H, 13C, and DEPT NMR spectra were recorded on Brucker-Avance DPX FT-NMR 500 and 400 MHz instruments. 13C NMR spectra were recorded at 125 MHz or 100 MHz. ESI-MS spectra were recorded on an Agilent 1100 LC-Q-TOF machine. Melting points were recorded on a digital melting point apparatus (make: Buchi M-560).

Plant Material. R. abyssinicus Jacq (family: Polygonaceae) was collected from Fongo-Tongo, West region of Cameroon and was identified by Fubert Tadjouteu of the National Herbarium of Cameroon (NHC), where the voucher specimen has been deposited (no. N°50551/NHC). The rhizomes were washed with water, sliced into small pieces, and dried in an oven at 45 °C. Thereafter, they were crushed and the obtained powder was used for extraction.

Extraction of R. abyssinicus Jacq Rhizomes. The powder of R. abyssinicus Jacq was extracted in Laboratory of Microbiology and Antimicrobial Substances (LAMAS) of Biochemistry Department, University of Dschang, Cameroon. Three hundred grams of the powder was macerated in 9 L of EtOAc for 72 h. The extract was filtered using a Whatman filter paper and the obtained filtrate was evaporated using a rotavapor to dryness to get 42 g of the EtOAc extract (RAE; extractive value, 14% w/w).

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Isolation of Secondary Metabolites. 18.6 g of the EtOAc extract was loaded on a silica gel (100–200 mesh) column and was eluted with hexane/EtOAc as a solvent system in a gradient fashion starting from 0 to 100% EtOAc/n-hexane. Fractions of 200 mL were collected and were pooled into 30 different fractions based on thin-layer chromatography analysis. Two pure compounds RAE-1 and RAE-2 were isolated in pure form (1.4 and 1.3 g, respectively) from the column when the column was eluted only with n-hexane. Two fractions containing the mixture of compounds were further subjected to column chromatography. Fraction 3 (564 mg) was subjected to a second column (100–200 mesh) which resulted in isolation of orange crystals of another pure compound RAE-3 (60 mg). Fraction 4 (654 mg) on second column chromatography (100–200 mesh) yielded the fourth pure compound as a yellow powder, which has been coded as RAE-4 (150 mg). All isolated compounds were characterized by their spectral data with literature values.27

Helminthosporin (RAE-1). It was obtained as an orange needles; mp 246–248 °C (lit. 225–226 °C).28 1H NMR (400 MHz, DMSO-d$_6$): δ 12.07 (s, 1H), 11.99 (s, 1H), 11.34 (s, 1H), 7.46 (d, J = 6.2 Hz, 1H), 7.14 (d, J = 5.4 Hz, 1H), 7.10 (d, J = 1.5 Hz, 1H), 6.58 (s, 1H), 2.40 (s, 3H); 13C NMR (100 MHz, DMSO-d$_6$): δ 190.1, 181.8, 166.0, 164.9, 161.8, 148.7, 135.5, 133.2, 124.6, 120.9, 113.7, 109.3, 109.2, 108.4, 22.0; ESI-MS: m/z 269.25 [M – H]$.^+$; HPLC purity: 94.9% ($t_R = 20.9$ min).

Emodin (RAE-2). It was obtained as orange needles; mp 258–260 °C (lit. 260–262 °C).29 1H NMR (400 MHz, DMSO-d$_6$): δ 12.11 (s, 1H), 12.04 (s, 1H), 7.51 (s, 1H), 7.19 (s, 1H), 7.15 (d, J = 2.3 Hz, 1H), 6.63 (d, J = 2.4 Hz, 1H), 2.45 (s, 3H); 13C NMR (100 MHz, DMSO-d$_6$): δ 190.1, 181.7, 166.0, 164.9, 161.9, 148.7, 135.5, 133.2, 124.5, 120.9, 113.7, 109.4, 109.3, 108.4, 22.0; ESI-MS: m/z 269.20 [M – H]$^+$; HPLC purity: 98.9% ($t_R = 12.8$ min).

Chrysophanol (RAE-3). It was obtained as light orange crystals; mp 193–195 °C (lit. 196 °C).30 1H NMR (400 MHz, CDCl$_3$): δ 12.15 (s, 1H), 12.05 (s, 1H), 7.86 (dd, J = 7.5, 1.0 Hz, 1H), 7.17–7.67 (m, 2H), 7.32 (dd, J = 8.4, 1.0 Hz, 1H), 7.14 (s, 1H), 2.50 (s, 3H); 13C NMR (126 MHz, CDCl$_3$): δ 192.6, 182.0, 162.8, 162.5, 149.4, 137.0, 133.7, 133.3, 124.3, 124.4, 121.4, 119.9, 115.9, 113.8, 22.3; ESI-MS: m/z 253.15 [M – H]$^+$; HPLC purity: 99.0% ($t_R = 17.1$ min).

Physcion (RAE-4). It was obtained as yellow powder; mp 200–202 °C (lit. 200–202 °C).31 1H NMR (400 MHz, CDCl$_3$): δ 12.33 (s, 1H), 12.13 (s, 1H), 7.65 (s, 1H), 7.39 (d, J = 2.5 Hz, 1H), 7.10 (s, 1H), 6.71 (d, J = 2.5 Hz, 1H), 3.96 (s, 3H), 2.47 (s, 3H); 13C NMR (126 MHz, CDCl$_3$): δ 190.8, 182.9, 166.6, 165.2, 162.5, 148.5, 135.2, 133.2, 124.3, 124.5, 124.5, 121.3, 113.7, 110.3, 108.3, 106.8, 52.1, 22.2; ESI-MS: m/z 283.2 [M – H]$^+$; HPLC purity: 92.7% ($t_R = 17.6$ min).

In Vitro AChE and BChE Inhibition Assay. Enzymes and Reagents. The cholinesterase enzymes viz. Electrophorus electricus AChE (EC 3.1.1.7, from electric eel, 1256 U/mg of protein), recombinant human AChE (EC 3.1.1.7, recombinant, expressed in HEK 293 cells, $\geq$1000 units/mg protein), and butyrylcholinesterase (BChE, E.C. 3.1.1.8, from equine serum, 855 U/mg protein) were purchased from Sigma-Aldrich. The reagents required in the assay viz. 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (ATChl), S-butyrylthiocholine iodide (BTChl), and reference standard donepezil hydrochloride were also purchased from Sigma-Aldrich.

Ellman Assay. The inhibitory capacity of RAE, isolated secondary metabolites (helminthosporin, emodin, chrysophanol, and physcion), and positive control donepezil hydrochloride on 25°C on 20 min. Then, ATChl solution (20 μL of 10 mM) was added and the absorbance was recorded at 340 min using a 96-well microplate reader. Each experiment was performed in triplicate. The intensity of the color (or absorbance) is directly proportional to the enzyme activity. For determination of IC$_{50}$ values, eight different concentrations of the test sample were used and the value was obtained using GraphPad Prism software.

The kinetic analysis of helminthosporin (along with donepezil) with rHuAChE and eBChE was performed using five different concentrations of the substrate (0.0625–1 mM) for different concentrations of helminthosporin/donepezil. The type of inhibition was determined from a Lineweaver–Burk double reciprocal plot, as described in our previous publications.11,12

Propidium Iodide Competition Assay. Propidium iodide is a substrate which specifically binds to PAS of AChE. The competitive displacement of propidium iodide by test compounds can be taken as a measure of binding of compounds at the PAS. 150 μL of 10 and 50 μM concentrations of test compounds were incubated with SU of EeAChE for 6 h at 25 °C. The propidium iodide (50 μL, 1 μM) was added and the assay mixture was further incubated for 10 min. The background signal was measured in control wells containing all reagents except EeAChE. The fluorometer was set on a well plate reader mode with excitation at 535 nm and emission at 595 nm, respectively, and the fluorescence intensities were measured. Each concentration was assayed in triplicate. Background signal was subtracted from all the readings. The percentage inhibition was calculated using the following formula: $100 - (IF_i/IF_0 \times 100)$, where IF$_i$ and IF$_0$ are the fluorescence intensities with and without inhibitor, respectively.15

In Vitro PAMPA-BBB Assay. The test compound was dissolved in dimethyl sulfoxide (DMSO) at 10 mM concentration, and was further diluted with phosphate-buffered saline (PBS) buffer (pH 7.4) to 100 μM. The filter membrane of the donor plate was coated with PBL in dodecane (selected empirically as 4 μL volume of 20 mg/mL PBL in dodecane). To this plate was added, 0.2 mL of test compound solution. The acceptor plate was also er

P$_V$ = C × ln $1 - \frac{[drug]_{acceptor}}{[drug]_{equilibrium}}$

where, $C = (VD \times VA)/(VD + VA) \times \text{area} \times \text{time}$.

Molecular Modelling. The crystal structure of human AChE (PDB ID: 4EY7)34 and human BChE (PDB ID: 6EP4)35 was retrieved from the protein data bank and was...
used for molecular modeling studies under default settings from Glide. The docking was performed as described earlier.1,12 The ADME properties were calculated using the QikProp module present within the Maestro program.

**ASSOCIATED CONTENT**

1. Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/10.1021/acsomega.9b03693.

- NMR, MS spectra, and HPLC chromatogram scans for all isolated compounds (PDF)

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Author Contributions

N.A. and V.K.N. equally contributed to this work as a first author. N.A. performed extraction, and isolation of compounds; V.K.N. performed biological assays; M.A. has done molecular modeling studies; S.B.B. designed and monitored the chemistry and biology experiments; Q.P.H., S.G.G., and S.B.B. guided the progress of the work; and S.B.B. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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**ABBREVIATIONS**

AChE, acetylcholinesterase; ADME, absorption distribution metabolism and excretion; ATCh, acetyl thiocholine; ATChI, acetyl thiocholine iodide; BCHE, butyrylcholinesterase; BTCh, butyryl thiocholine; BTChI, butyryl thiocholine iodide; CAS, catalytic anionic site; EeAChE, acetylcholinesterase from electric eel; eqBChE, butyrylcholinesterase from equine serum; \( K_{m} \), Michaelis constant (is the concentration of substrate which permits the enzyme to achieve half \( V_{\text{max}} \); LB plot, Lineweaver–Burk plot; PAS, peripheral anionic site; PDB, protein data bank; rHuChACE, recombinant human acetylcholinesterase; \( V_{\text{max}} \), maximum rate of reaction (the rate of reaction when the enzyme is saturated with substrate)

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