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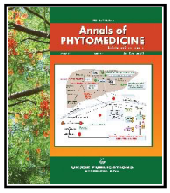

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Herbs that heal: Purification and characterization of bacoside A from *Bacopa monnieri* (L.) Pennell for its acetylcholinesterase inhibition activity implicated in neurodegenerative diseases

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Abstract

The *Bacopa monnieri* (L.) Pennell (Brahmi) is a medicinal plant used for alleviating neurodegenerative disorders. The neuroprotective effect of *B. monnieri* is primarily attributed to the presence of bacoside A, a major phytoconstituent known to inhibit the aggregation of β-amyloid protein in the case of Alzheimer's disease patients. Since bacoside A possesses other less explored bioactivities as well, the current study aimed to investigate its acetylcholinesterase inhibitory activity. An efficient high-yielding process has been developed to obtain purified bacoside A, based on solvent polarity-gradient extraction method and silica-gel column chromatography. The bacoside A content was quantified by HPTLC, and a yield of 34.6 mg bacoside A per gram of crude Brahmi extract was obtained with 93% purity. The purified bacoside A was characterized by FT-IR and HR-ESI-MS. The purified bacoside A reduced the aggregation of $A\beta_{42}$ protein by 78%. This also exhibited high acetylcholinesterase inhibitory activity as well, with an IC_{50} value of 9.91µg/ml. The compound also showed strong DPPH radical scavenging activity, with an IC_{50} value of 29.22 µg/ml compared to 70.16 µg/ml in crude extract and 259.68 µg/ml in standard. It showed no toxicity in the *in silico* test. Thus, purified bacoside A as pharmaceutical ingredient has a dual potential in ameliorating the onset and progress of Alzheimer's disease by inhibiting acetylcholinesterase and $A\beta_{42}$ aggregation.

1. Introduction

The *Bacopa monnieri* (L.) Pennell (Brahmi) has been used for centuries as an anti-inflammatory, cognitive-enhancing, and anti-epileptic agent for treating Alzheimer's disease (AD), epilepsy, anxiety, and stress (Chaudhari *et al*., 2017; Murthy, 2022). Number of reasons have been attributed to cause AD. Major among these are degradation of the neurotransmitter acetylcholine by the acetylcholinesterase (AChE) enzyme (Mateev *et al*., 2023), and aggregation of β-amyloid protein in the brain (Srivastava *et al*., 2021), produced by beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) (Bhatia *et al*., 2023). Studies so far have established that the neuroprotective effect of Brahmi is mainly caused by inhibiting aggregation of β-amyloid protein. The transgenic mice with PSEN-1 and APP mutation exhibited reduced levels of insoluble $A\beta_{40}$ and $A\beta_{42}$ proteins in their brains on the administration of Brahmi extract (Holcomb *et al*., 2006). A recent study by Deolankar *et al*. (2023) further showed enhanced cell viability of the $A\beta_{42}$-cells treated with Brahmi extract. Many *in vitro* and *in vivo* studies have confirmed significant inhibition of β-amyloid protein in presence of Brahmi extract (Fatima *et al*., 2022; Paidi *et al*., 2022; Witter *et al*., 2020).

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Within the extract, bacosides have been identified as the major bioactive compounds responsible for above effects. In a recent study, bacoside A3 was reported to downregulate the inflammatory response of Aβ aggregates on neuronal cells. Thus, bacosides are well established as important nutraceutical against neurodegenerative diseases. However, their purified form is desirable for understanding their selective neuroprotective effect, their precise mechanism of against AD, and if their action is synergistic. More specifically whether they affect other molecular targets, *viz*., AChE, BACE1, *etc*.

The bacoside A has been previously purified from the Brahmi extract by Deepak *et al*. (2005) and Sivaramakrishna *et al*. (2005) by column chromatography. However, the yield has been low. Therefore, the present work reports a high-yielding process for purifying bacoside A, with extensive characterization by HPTLC and HR-MS.

The present work further investigated the effect of purified bacoside A on the β-amyloid aggregation, AChE inhibition, and its radical scavenging activity. So far only a molecular docking study is available to indicate AChE inhibition by bacoside A (Jamal *et al*., 2020). Current study probably is the first to the best of our knowledge to confirm the AChE inhibition by bacoside A.

2. Material and Methods

2.1 Chemicals

All the solvents used were HPLC grade. The BHT, DPPH, silica gel (mesh size 100-200 mm), and ThT were procured from Sisco Research Laboratories, India. The Brahmi were procured from Himalaya™ (Batch No.:93901046). $A\beta_{42}$ protein (from *Mus Musculus*),

acetylcholinesterase enzyme (from *Electrophorus Electricus*), acetylthiocholine iodide, and standard bacoside A were sourced from Sigma-Aldrich Inc. (St. Loius, MO, USA). Pre-coated silica gel 60 F_{254} TLC aluminum plates (10 × 10 cm and 20 × 10 cm, 0.2 mm thick) were purchased from Merck (Darmstadt, Germany). All the reagents used were of analytical grade.

2.2 Extraction of bacosides

The Brahmi tablets were grounded into powdered form by mortar and pestle. The extraction was carried out by the sequential polarity gradient solvent extraction method as described by Kahol *et al*. (2004). Briefly, 100 g of powder was mixed with 500 ml of hexane, acetone, and methanol sequentially. The mixtures were refluxed at 37ºC for 6 h. The extraction was carried out twice by the same procedure. The soluble fraction obtained in each case was dried in a rotary evaporator (IKA RV 8, Karnataka, India) and stored at 4ºC until further use.

2.3 Isolation of bacoside A

Bacoside A was purified from the above crude extract on a column packed with 100-200 mesh-size silica gel. The silica slurry was prepared in ethyl acetate. 1 g of the bacoside-rich extract (BRE) was mixed with 10 g silica gel by dissolving in a minimum amount of methanol, followed by drying using a rotary evaporator (IKA RV 8, Karnataka, India). The extract was poured slowly on the top of the silica gel column (60 cm × 0.24 cm). Elution was carried by a gradient of methanol in ethyl acetate (1-30%). The fractions of 10 ml each were collected and analyzed for bacoside A.

2.4 High-performance thin layer chromatography (HPTLC)

All the fractions obtained during the extraction and isolation process were dried, and 1 mg/ml of the extract was reconstituted in methanol and analyzed by high-performance thin layer chromatography (HPTLC) (CAMAG- Anchrom Laboratory, Switzerland, with Vision CATS system manager) for the presence of bacoside A. The fractions were loaded on the pre-coated TLC plate (silica gel 60 F_{254}) by the Linomat applicator. The plates were allowed to develop in a pre-saturated chamber containing ethyl acetate, methanol, and water in the ratio of 7:2:1 as a mobile phase (Mallick *et al*., 2017). The developed plate was air-dried and derivatized by 20% (v/v) methanolic-sulfuric acid (Owolabi *et al*., 2022). These were visualized under UV light at 254 and 366 nm. The fractions showing a band corresponding to the standard bacoside A were pooled together and concentrated using a rotary evaporator to obtain dried bacoside A (Nagpal *et al*., 2022). Bacoside A content in purified fractions was quantified using a regression equation from the calibration curve of standard bacoside A.

2.5 Characterization of purified bacoside A

The signature functional groups present in the purified compound were identified by fourier transform infrared (FT-IR) spectroscopy (Agilent Technologies Cary 600, USA) and compared with the standard bacoside A (Sigma-Aldrich). Transmittance was recorded over a wavelength spectrum of 400-4000 cm^{-1}. High-resolution electron spray ionization mass spectroscopy (ESI-Q-TOF-MS) (Waters Xevo G2-XS system, Milford, MA (Q-TOF)) was conducted in positive ion mode to determine the molecular weight of the compound and identify the compound based on the online databases, including ChemSpider, MassBank, mzCloud, and PubChem.

2.6 Thioflavin T binding (ThT) assay

The anti-aggregation activity of the purified bacoside A was determined by performing ThT assay (Malishev *et al*., 2017). The reaction mixture consisted of 250 µl of $A\beta_{42}$ protein (1 µM, prepared in 50 mM phosphate buffer), 500 µl of purified bacoside A (10 µM, prepared in 10% DMSO), and 250 µl ThT (10 µM, prepared in 50 mM glycine buffer). The mixture was incubated at 37ºC, with constant shaking at 200 rpm. And the fluorescence spectra were recorded every 3 h on a fluorescence spectrometer (PerkinElmer, Model LS55, MA, USA) at excitation wavelength of 440 nm and emission wavelength of 485 nm. Fluorescent signals were recorded using the FL WinLab software.

2.7 Acetyl-cholinesterase (AChE) assay

The purified bacoside A and other reference drugs were reconstituted in 10% methanol. The AChE inhibition activity of the purified bacoside A was ascertained by conducting Ellman's assay (Ellman *et al*., 1961). Commercial drugs, Donepezil®, and Galanthamine®, which are known AChE inhibitors, were used as a reference to compare the enzyme inhibition activity of the purified bacoside A. In a 96-well plate, 100 µl of 50 mM 5,5'-dithiol-bis-(2-nitrobenzoic acid) (DTNB), 20 µl of 0.6 U/ml AChE enzyme solution (prepared in 0.1 M phosphate buffer, pH 8), and 60 µl of bacoside A/commercial drug in varying concentrations were added. The reaction mixture was incubated for 15 min at 37°C, followed by the addition of 20 µl of the 15 mM substrate, acetylthiocholine iodide. After incubating the reaction mixture at 37°C for 30 min, TNB produced by the conversion of DTNB was measured at 412 nm by UV-Vis plate reader (Thermo Scientific Multiscan Go, Vantaa, Finland). AChE inhibition was calculated using the Eq 1 and 2, where Ao represents the absorbance in the absence of an inhibitor, and Ax represents absorbance in the presence of x µg/ml inhibitor:

$$\text{AChE Activity (\%)} = \left(\frac{A_0 - A_x}{A_0}\right) \times 100 \qquad \text{Eq 1}$$

$$\text{AChE Inhibition (\%)} = 100 - \text{AChE Activity (\%)} \qquad \text{Eq 2}$$

2.8 Antioxidant activity assay

The antioxidant activity was estimated by DPPH assay method as described by Blois (1958). The radical-scavenging activity of the crude Brahmi extract, purified bacoside A, Donepezil® drug, and BHT were determined and compared. Antioxidant activity was calculated using Eq 3, where Ao represents the absorbance of the control, and Ax represents absorbance in the presence of x µg/mL of the compound:

$$\text{Antioxidant Activity (\%)} = \left(\frac{A_0 - A_x}{A_0}\right) \times 100 \qquad \text{Eq 3}$$

2.9 *In silico* toxicity prediction

The ADMET profiling tool, admetSAR, was used to predict the toxicity parameters of bacoside A and other phytochemicals that are naturally present along with it. The carcinogenicity, genotoxicity, hepatotoxicity, oral toxicity, and HERG inhibition of the compounds were ascertained as per the procedure described by Cheng *et al*. (2012).

3. Results and Discussion

3.1 Purification of bacoside A from *B. monnieri* extract

B. monnieri is known to contain bacosides as major saponins responsible for Brahmi's bioactivity. Bacoside A and B are the major bacosides. Amongst these bacosides, type A is responsible for the anti-aggregation of the β-amyloid protein in the brain, which is responsible for neurodegenerative diseases, especially the Alzheimer's disease. The concentration of bacoside A in Brahmi is quite low, about 0.5-2.5 % (w/w) of the total dry weight of the plant (Deepak *et al*., 2005).This low concentration of bacoside A makes it difficult to isolate and purify this important pharmaceutical ingredient from the Brahmi extract.

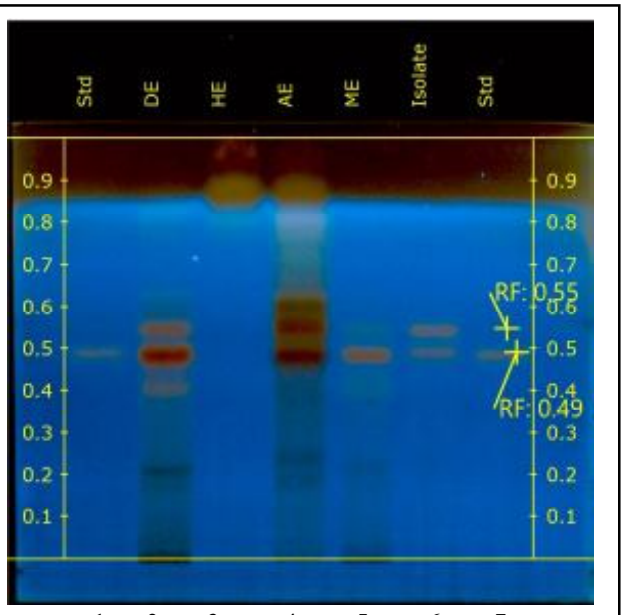

Figure 1: Derivatized TLC plate as observed at λ_{366} nm (Std: Standard bacoside A, DE: Direct crude Brahmi extract, HE: Hexane extract, AE: Acetone extract, ME: Methanol extract, Isolate: Fraction obtained after column chromatography)

In the present study, the purification was carried out by developing a procedure based on the sequential polarity-gradient solvent extraction method, followed by silicagel column chromatography. The TLC plate in Figure 1, lane 1 and 7 shows standard bacoside A (Rf values 0.49 and 0.55). Lane 2 depicts the direct extraction of Brahmi in methanol (DE, logP – 0.90). The study developed stepwise purification of the non-polar compounds by extracting Brahmi sample first with non-polar hexane (step 1, lane 3, HE, logP 4.66). This extract was further extracted by less polar solvent acetone (step 2, lane 4, AE, logP – 0.24). This extract was further extracted with polar solvent methanol (step 3, lane 5, ME, logP – 0.90). The final bacoside-rich extract was subjected to silica-gel column chromatography of bacoside-rich extract. Lane 6 shows, bacoside A was purified, as two bands corresponding to standard bacoside A (lane 1 and 7).

3.2 Quantification of bacoside A in the purified extract

The purified bacoside A was quantified by HPTLC. Table 1 shows the stepwise yield of pure bacoside A. The crude extract was found to contain 56.39 µg bacoside A per mg of the extract. The bacoside rich extract obtained after stepwise solvent gradient elution, contained 226.19 µg bacoside A per mg of the extract. The purified extract after column chromatography contained 935.95 µg bacoside A per mg of the extract. The crude extract, bacoside rich extract and the purified bacoside A demonstrated 5.64%, 22.62%, and 93.60% (w/w) purity, respectively, in terms of their bacoside A content.

The purification procedure developed provides 16.60 folds increase in the bacoside A content of the extract. We obtained 34.60 µg of the purified bacoside A per mg of the crude Brahmi extract. We were able to increase the yield of bacoside A purification by 4.94 folds compared to previously developed procedures by Deepak *et al*. (2005) and Sivaramakrishna *et al*. (2005). Previously, bacoside A has been purified by Sivaramakrishna *et al*. (2005) by subjecting the hydroalcoholic extract of Brahmi to silicagel chromatography using a gradient of chloroform, methanol and water and by Deepak *et al*. (2005) by subjecting methanolic extract of Brahmi to chloroform and methanol gradient on silicagel column. In both cases, the yield was very low in the range of 1.11 µg and 0.7 µg per mg of the extract, respectively. This yield is the highest reported so far for purified bacoside A from Brahmi.

Table 1 : Bacoside A content present in the crude and the purified extract

Sample extract	Bacoside A (µg/mg of extract)	Purity (%)
Himalaya tablet (extracted with methanol alone)	56.39	05.64
Bacoside rich extract (sequential polarity gradient solvent extraction method)	226.185	22.62
Purified bacoside A (silica-gel column chromatography)	935.95	93.60

3.3 Characterization of the bacoside A

The purified bacoside A was characterized by FTIR and HR-MS (Figure 2). The standard bacoside A was also taken for reference.

The FTIR spectra of purified and standard bacoside A (Figure 2A) exhibited the peaks at 704.33 cm^{-1}, Suggesting the presence of benzene derivative; 1054.58 cm^{-1} suggesting C-O stretch depicting the presence of aliphatic and diphenyl ether; 1383.71 cm^{-1} suggesting O-H bending representing phenol; 1456.15 cm^{-1} suggesting C-H bending representing alkane; 1644.92 cm^{-1} suggesting C=O stretching depicting ketone, and C=C stretching depicting alkene; 2846.2, 2863.85, 2928.2, 2950.28, 3420.13 cm^{-1} suggesting O-H stretching depicting alcohol. The FTIR pattern confirms the known bacoside A structure, as shown in the inset.

The HR-ESI MS chromatogram further confirms the molecular formula for the compounds in the purified bacoside A extract. It displayed $[M+Na]^+$ peak at m/z value 951.4915 and 921.4802 establishing the presence of bacopaside II ($C_{47}H_{76}O_{18}$) and bacopaside X ($C_{46}H_{74}O_{17}$) in the extract (Figure 2B).

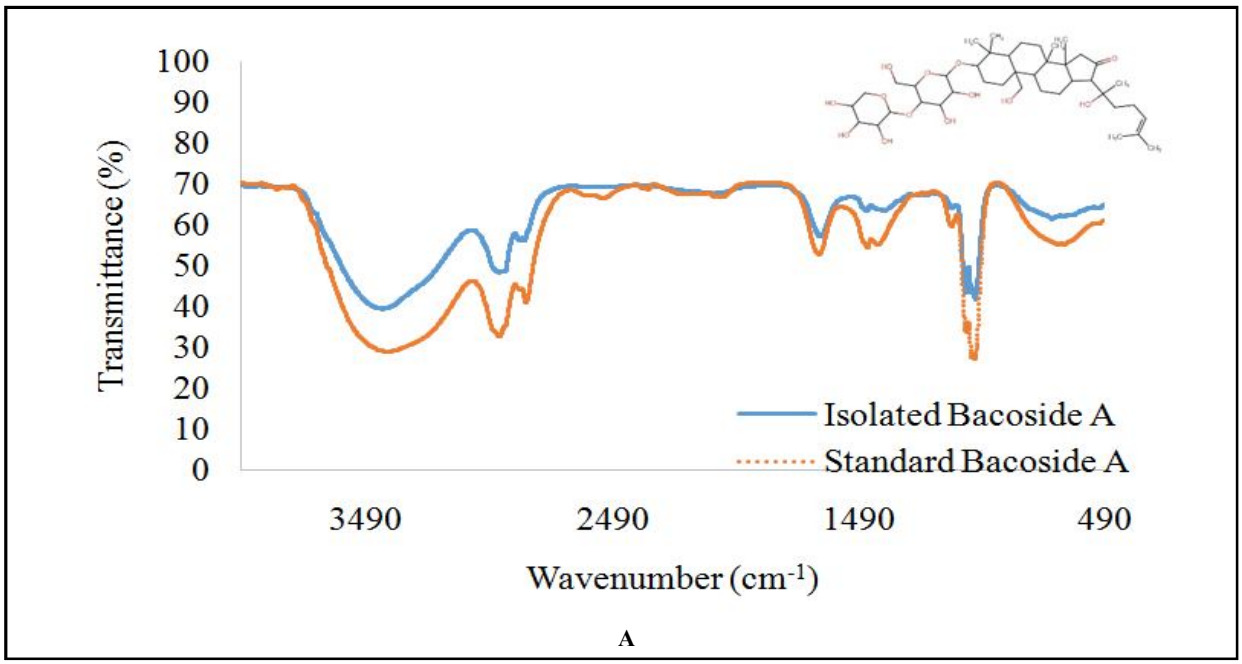

Figure 2: FTIR graph of purified and standard bacoside A (A), and HRMS chromatogram of the purified bacoside A(B).

3.4 Effect of purified bacoside A on β-amyloid aggregation

In a non-AD brain, transmembrane protein, amyloid precursor protein (APP) is cleaved by α-secretase and γ-secretase to produce soluble β-amyloid protein. However, in AD brain, APP is subjected to β-secretase and γ-secretase, producing insoluble 42-residue β-amyloid protein (Srivastava *et al*., 2021). The insoluble $A\beta_{42}$ protein, aggregates and accumulates in the brain synapses hindering the nerve impulse transmission.

The aggregation of the β-amyloid protein is measured by thioflavin T (ThT) fluorescence assay. ThT binds with the β-sheet of the aggregated protein and fluoresces (Kulichikhin *et al*., 2021). This fluorescence intensity increases as a function of protein aggregation. The native protein (non-aggregate) will have no increase in intensity. The fluorescence intensity of the native, as well as the bacoside A treated $A\beta_{42}$ protein, is shown in Figure 3.

As depicted in Figure 3, in the presence of bacoside A, the aggregation of β-amyloid protein was inhibited by 77.89 %. Despite all the conditions favorable for aggregation, bacoside A inhibited the aggregation of β-amyloid protein. There have been studies earlier on the role of bacoside A on inhibiting protein aggregation and downregulating the β-amyloid induced inflammatory responses (Bai and Zhao, 2022; Fatima *et al*., 2022; Malishev *et al*., 2017). Our results validate the previous studies.

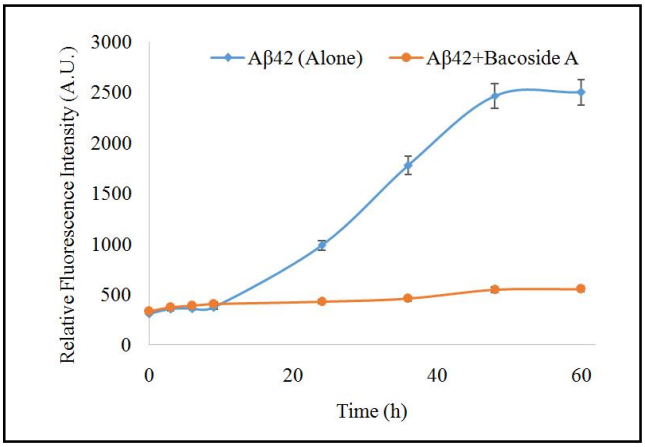

Figure 3: The inhibitory effect of bacoside A on the aggregation of $A\beta_{42}$ protein (n=3).

3.5 Effect of bacoside A on acetyl-cholinesterase inhibition

Acetylcholinesterase (AChE) is a serine protease present at post synaptic cleft in brain, and catalyzes the degradation of neurotransmitter, acetylcholine. This leads to loss of information, cognitive dysfunction, and motor function failure in Alzheimer patients. Thus, inhibiting AChE can be a potential target for treating AD (Mateev *et al*., 2023).

Inhibition of AChE enzyme in the presence of purified bacoside A as well as the commercially available AChE inhibitors, Donepezil® (synthetically produced), and Galanthamine® (isolated from *Galanthus nivalis*), as determined by Ellman assay is shown in Figure 4.

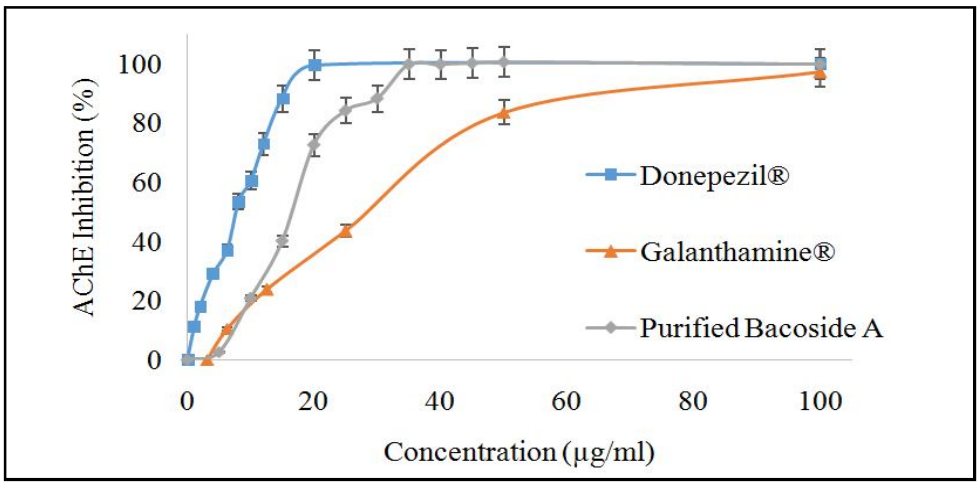

Figure 4: Effect of purified bacoside A and other reference drugs on AChE inhibition (n=3).

The IC_{50} value was determined for all the compounds, to compare the concentration of the compounds at which they inhibit 50% of the enzyme. Donepezil® exhibited the lowest IC_{50} value (7.96 µg/ml), followed by purified bacoside A (9.91 µg/ml), and Galanthamine® (29.81 µg/ml). The maximum inhibitory concentration of Donepezil®, purified bacoside A, and Galanthamine® was 20, 40, and 100 µg/ml, respectively (Figure 4). The results demonstrated that the inhibitory activity of bacoside A is comparable to that of Donepezil®. However, Donepezil® is chemically derived and can adversely affect the body. There have been cases of Donepezil® associated mania in patients with mood disorders, although the real reason is unknown (Hategan and Bourgeois, 2016). Moreover, the drug targets Alzheimer's by only inhibiting the AChE enzyme, whereas bacoside A also inhibits the aggregation of the $A\beta_{42}$ protein, as an additional activity to control the progression of AD.

3.6 Antioxidant activity

Antioxidants are the molecules with high reducing power, that are free radical scavengers, and prevent them from initiating long chain reactions (Ahmad *et al*., 2021). Generally, synthetic antioxidants are used as food additives. However, natural plant based antioxidants are preferred and thus, antioxidant activity of the purified bacoside A, and crude Brahmi extract was examined by conducting DPPH radical scavenging assay (Figure 5). BHT was used as the reference owing to its high antioxidant activity and its usage in food products.

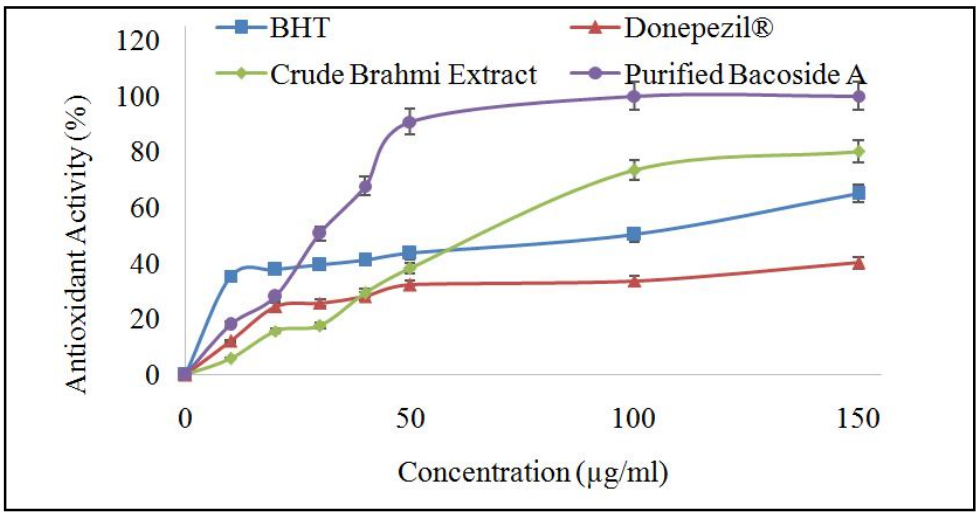

Figure 5: Antioxidant activity of the compounds in terms of their DPPH radical scavenging potential (n=3).

The purified bacoside A (IC_{50} 29.22 µg/ml) exhibited the highest antioxidant potential, followed by crude Brahmi extract (IC_{50} 70.16 µg/ml), BHT (IC_{50} 259.68 µg/ml) and Donepezil (IC_{50} 73.00 µg/ml) as seen in Figure 5. The purified bacoside A exhibited an 8.89-fold reduction in the IC_{50} value compared to BHT. Thus, it could substitute the synthetic antioxidants consumed. Synthetically derived antioxidants, when added to food products, interact with peroxide produced in the food. These compounds are cytotoxic and act as carcinogens beyond a specific concentration (Xu *et al*., 2021). Therefore, their usage should be limited, and alternative plant-derived compounds should be preferred.

3.7 Toxicity prediction of the compounds

The four saponins, comprising bacoside A3, bacopaside II, bacopaside X, and bacopasaponin C, that constitute the bacoside A. These were tested for their toxicity using the online tool admetSAR.

Table 2: Toxicity prediction of bacoside A phytoconstituents by admetSAR

	Donepezil®	Bacoside A3	Bacopaside II	Bacopaside X	Bacopasaponin C
Hepatotoxicity	- 0.78	- 0.68	- 0.70	- 0.68	- 0.70
HERG inhibition	+ 0.95	+ 0.84	+ 0.85	+ 0.80	+ 0.81
Acute oral toxicity	+ 0.56	+ 0.69	+ 0.47	+ 0.67	+ 0.67
Ames mutagenesis	- 0.59	- 0.76	- 0.75	- 0.64	- 0.68
Carcinogenesis	- 0.99	- 0.99	- 0.99	- 0.99	- 0.99
Micronucleus assay	- 0.54	- 0.88	- 0.89	- 0.88	- 0.88

In Table 2, HERG inhibition and micronucleus assay of the compounds corresponds to their cardiotoxicity and genotoxicity determination, respectively. Ames mutagenesis is a test for the identification of carcinogens using mutagenicity in bacteria. As evident all the compounds are predicted to be non-toxic with no carcinogenic or mutagenic effect on the body. This was done to rule out any possibility of cellular or organ toxicity inside the human body.

4. Conclusion

Brahmi is known for its neuroprotective property, and bacoside A is the major triterpenoid saponin present in Brahmi. Bacoside A improvescognitive skills by reducing the aggregation of the intrinsically disordered proteins in the brain that contribute to most neurodegenerative disorders like Alzheimer's, Parkinson's, and amyotrophic lateral sclerosis. Brahmi whole plant extract has low bacoside A content. Thus, purifying it would enhance the pharmaceutical efficiency of the Brahmi. A high-yielding protocol for bacoside A purification is developed leading to 4.94-fold higher yield than reported so far. The purified extract exhibited high AChE inhibition, as good as Donepezil and high antioxidant activity, besides ameliorating β-amyloid protein aggregation *in vitro*.With no toxicity, bacoside A can be safelyadministeredas pharmaceutical ingredient to patientssuffering from or at risk of developing neurodegenerative disorder.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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