



Isolation and Characterization of Bacoside A from *Bacopa monnieri* for its Anti-oxidant and Acetyl-cholinesterase Inhibition Activity as Implicated in Neurodegenerative Diseases

Sukriti Srivastava, Deeksha Gopaliya, and Sunil K. Khare*

Enzyme and Microbial Biochemistry Laboratory, Department of Chemistry
Indian Institute of Technology Delhi, Hauz Khas, New Delhi-110016, India

ARTICLE INFO

Received : 12 October 2020
Revised : 21 November 2020
Accepted : 03 December 2020

Keywords:

Acetylcholinesterase,
Alzheimer's disease,
Antioxidant activity,
Bacoside A, Isolation.

ABSTRACT

Bacopa monnieri (Brahmi), is a medicinal plant used in the treatment of neurodegenerative disorders, metabolic diseases, and inflammation from ancient times. The major bioactive compound, responsible for its pharmacological properties is bacoside A, which is a mixture of four saponins: bacoside A3, bacoside II, jujubogenin isomer of bacosaponin C (bacoside X), and bacosaponin C. Along with bacoside A, other saponins, d-mannitol, Acid A, monnierin and bacoside B are also present. Owing to its pharmacological role in improving cognitive ability, we aimed to isolate bacoside A from *Bacopa monnieri* and further characterize it. A high yielding procedure for the isolation of bacoside A was developed. A bacoside-rich fraction was extracted from the Brahmi tablet (Himalaya™), by solvent polarity gradient method, and bacoside A was isolated from the crude extract employing gradient silica gel (100-200 mesh size) column chromatography. Bacoside A was obtained at a methanol concentration of 18-21% in the ethyl acetate mobile phase as confirmed by high-performance thin-layer chromatography. A yield of 92.8 mg bacoside A per gram of crude Brahmi extract was obtained. The total bacoside A content in the isolate was 83.46% as estimated by HPLC. High-performance liquid chromatography established its linearity along the concentration range 0 to 500 µg/mL and showed similar peaks in standard and isolated bacoside A. Fourier transform infrared spectra also depicted the presence of identical functional groups in both standard and the isolated bacoside A. The isolated bacoside A proved to be a potential acetylcholinesterase inhibitor, having an IC_{50} value of 9.96 µg/mL. It also proved to be a potent anti-oxidant with the IC_{50} value of 73.28 µg/mL. The admet SAR tool gave the estimation of the possible toxicity profile of the phytoconstituents of bacoside A.

© 2022 ISEES, All rights reserved

1. Introduction:

Bioactive compounds both in their innate form or their metabolites exercise beneficial impact on our well-being through ways comprising protease inhibition, chelation of metal ions, direct scavenging of free radicals, inhibition of the assembly of microtubules, and microfilaments. *Bacopa monnieri* (Brahmi), a conventional ayurvedic remedy has been used for centuries as an anti-inflammatory, cognitive-enhancing, antipyretic, analgesic, anti-epileptic, and sedative agent. Recently, studies have stated the memory-enhancing impact of various Brahmi extracts. A brightness discrimination reaction task has exhibited retention, enhanced acquisition, and prolonged extinction of newly acquired behavior. The improved cognitive ability has been ascribed to the active saponin constituent, bacosides A and B. They have exhibited regulatory effects on mental retention in avoidance response in rats and reverse amnesic effects of neurotoxin, electroshock, scopolamine, and immobilization stress. Bacoside A has been investigated for its effect in neurodegenerative disorders. Amyloid β and oxidative stress are believed to be the chief pathological and etiological elements in the induction and elevation of neurodegeneration in Alzheimer's disease. Bacoside A, is a combination

of four different saponins: bacoside II, bacoside A3, bacosaponin C and jujubogenin isomer of bacosaponin C (bacoside X) (Dowell et al., 2015).

People have been working extensively on exploring the effect of bacoside A on neurological health for a long time. Bai and Zhao studied the effect of bacoside-A3 on β -amyloid-induced reduction of U87MG cell viability, generation of oxidative radicals, and activation of NF- κ B. The pretreatment with bacoside-A3 prevented β -amyloid-mediated suppression of U87MG cell proliferation and generation of ROS in a concentration-based manner. Formation of iNOS, secretion of PGE2, over-expression of COX-2, and nuclear translocation of NF- κ B were all suppressed in pre-treated U87MG cells (Bai & Zhao, 2021). Evaluation of the neuroprotective effects of bacoside A against dichlorvos-induced toxicity in mice was established by eradication of the free radicals formed after bacoside A exposure, reduced expressions of Hsp 70, catalase, phosphorylase a and b, and increased expressions of SOD and GPx. Indicating the involvement of particular proteins responsible for cell viability and neuronal integrity (Chaudhary & Bist, 2017). Bacoside A has also been found to inhibit β -amyloid cytotoxicity, and its interaction

* Corresponding Author: skkhare@chemistry.iitd.ac.in, skhare@rocketmail.com

Table 1: List of abbreviations and acronyms used in this article

Abbreviations	Explanation
AChE	Acetylcholinesterase
ATP	Adenosine Triphosphate
Bax	BCL2 Associated X, Apoptosis Regulator
BBB	Blood-brain
BHT	Butylated hydroxytoluene
COX-2	Cyclo-oxygenase 2
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DTNB	5,5'-Dithio-bis-[2-nitrobenzoic acid]
FTIR	Fourier transform infrared
GPx	Glutathione peroxidase
HERG	Human Ether-à-go-go-Related Gene
HO-1	Hemeoxygenase 1
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
Heat shock protein 70	Heat shock protein 70
IC ₅₀	Half maximal inhibitory concentration
IL6	Interleukin 6
iNOS	Inducible Nitric Oxide Synthase
K ⁺	Potassium ion
L132 cell line	Embryonic lung tissue cancer cell line
MOA	Monoamine Oxidase
Na ⁺	Sodium ion
NF- κ B	Nuclear factor- κ B
NMDA receptor	N-methyl-D-aspartate receptor
NR1	NMDA type subunit 1
NRSF	Neuron-Restrictive Silencer Factor
PBDE-209	Polybrominated diphenyl ethers
PGE2	Prostaglandin E ₂
R ²	Regression Coefficient
REST	RE1-Silencing Transcription factor
ROS	Reactive oxygen species
SOD	Superoxide Dismutase
TLC	Thin Layer Chromatography
TNB	2-Nitro-5-Thiobenzoic Acid
TNF- α	Tumor Necrosis Factor α
UV	Ultra-Violet

with the membrane (Malishev et al., 2017; Srivastava et al., 2021). In a study on N9 microglial cells, bacoside A inhibited the *in vitro* release of TNF- α and IL-6, as well as, the release of caspase 1 and 3, and matrix metalloproteinase-3 in the cell-free assay (Nemetchek et al., 2017). In PBDE-209 exposed mice brain, PBDE-209 increased the expression of NR1 and decreased the binding of REST/NRSF to NR1 (NMDA receptor 1) promoter. In the presence of bacoside-rich extract expression of NMDAR1 (NR1) was restored and the binding of REST/NRSF to NR1 promoter was up-regulated (Verma et al., 2015). In a study conducted by Singh et al., bacoside I and bacoside A mixture inhibited the MAO-A and MAO-B enzymes. MAO is involved in removing the neurotransmitters norepinephrine, serotonin, and dopamine from the brain. MAO inhibitors prevent this from happening (Singh et al., 2014). The bacoside-rich extract was found to activate iNOS/Bax/caspase 3 mediated apoptosis in the L132 cell line (Anand et al., 2014). Administration of bacoside-rich extract in Wistar rats downregulated HO-1, iNOS, lipid peroxidation, and AChE activity (Pandareesh & Anand, 2014). Pretreatment of morphine injected rats with bacoside A has shown to possess a significant protective role against morphine-induced brain oxidative damage in the antioxidant status (total reduced glutathione, superoxide dismutase, catalase, glutathione peroxidase, and lipid peroxidation) and membrane-bound ATP-ases (Na⁺/K⁺ATPase (Sumathi et al., 2011). Administration of bacoside A in rats has been known to prevent expression of hsp70 and neuronal apoptosis during cigarette smoking (Anbarasi, et al., 2006 a). The level of antioxidants like glutathione, vitamin C, vitamin E, and vitamin A and the activities of superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase were found to be enhanced in the brain (Anbarasi, et al., 2006b). Where as, it inhibited lipid peroxidation, improved the activities of ATPases, and maintained the ionic equilibrium, indicating that bacoside

A protects the brain from cigarette smoking-induced membrane damage (Anbarasi et al., 2005). The bacoside extract, exhibited an inhibitory effect on superoxide released from polymorphonuclear cells (Pawar et al., 2001). Antioxidant activity of *Bacopa monniera* was tested in the rat frontal cortex, striatum, and hippocampus. It induced a dose-related increase in SOD, catalase, and GPX activities, in all the brain regions (Bhattacharya et al., 2000).

Pure bacoside A has been previously isolated by two research groups in India, Deepak et al., and Sivaramakrishna et al. A yield of 7.0 mg and 6.9 mg of bacoside per g of *Bacopa monniera* extract was obtained by both groups respectively (Deepak et al., 2005; Sivaramakrishna et al., 2005). We, therefore, aimed to isolate bacoside A compound from Brahmi, by developing a high yielding extraction and isolation procedure. Bacoside A is the most abundant and active pharmacological constituent of the Brahmi plant. Therefore, the same amount of this compound will have a much higher medicinal value. This would lower the amount of drug administration, reducing the cost of production and increasing patient compliance.

2 Materials and methods

2.1 Chemical reagents

All the solvents used were HPLC grade. Silica gel (mesh size 100-200 mm), DPPH, BHT were ordered from Sisco Research Laboratories, India. Brahmi tablets were bought from Himalaya™ (Batch No.: 93901046). Standard bacoside A, acetylcholinesterase enzyme, acetylthiocholine iodide were sourced from Sigma-Aldrich (US). Pre-coated silica gel 60 F₂₅₄ TLC aluminum plates (10 × 10 cm and 20 × 10 0.2 mm thick) were purchased from Merk, Germany.

2.2 Extraction of bacoside rich extract

Himalaya Brahmi tablets were grounded into powder form. 100 g of powder was extracted twice in 500 mL of hexane, acetone, and methanol respectively, following the US patent US6833143B1 with few modifications in the process. The mixtures were refluxed at 37° C for 6 hours. The soluble fraction obtained in the case of each solvent was dried in a rotary evaporator in round bottom flasks. The methanol fraction was pooled together and processed further to isolate bacoside A.

2.3 Isolation of bacoside A

Purification of bacoside A from the crude Brahmi extract was performed on a column packed with silica gel of 100-200 mesh size. The silica slurry was prepared in ethyl acetate. 1 g of the methanol extract powder was mixed with 10 g silica by dissolving in a minimum amount of methanol, followed by drying using a rotary evaporator. The obtained sample was poured slowly on the top of the column. Elution was carried on by employing a gradient of ethyl acetate and methanol solvent system. The concentration of methanol in ethyl acetate was increased from 1-30 %. And 10-10 mL fractions were collected (Deepak et al., 2005).

2.4 High-Performance Thin Layer Chromatography

All the fractions obtained during the extraction and isolation process were analyzed using HPTLC (CAMAG-Anchrom Laboratory, Switzerland using Vision CATS system manager) for the presence of bacoside A. All the fractions were loaded on the TLC plate (silica gel 60 F₂₅₄) by the Linomat applicator. The plate was allowed to develop in a pre-saturated chamber containing ethyl acetate, methanol, and water as mobile phase in the ratio of 7:2:1. The developed plate was dried and derivatized using a solution of 20% (v/v) sulfuric acid in methanol. The plates were then visualized in white light and UV_{366nm} to observe the separated bands. The fraction which showed bands corresponding to the standard bacoside A were pooled together and concentrated using a rotary evaporator to obtain dry bacoside A (Nagpal et al., 2022).

2.5 High-Performance Liquid Chromatography

The crude Brahmi extract, isolated product, and the standard bacoside A were analyzed by HPLC (Agilent Technologies, 12,000 Infinity, USA). The samples were run on a C-18 column with a mobile phase containing 31.5% acetonitrile and 68.5% anhydrous sodium sulfate [0.72% (w/v), pH 2.3, adjusted by sulfuric acid]. Injection volume was kept at 20 μ L and the flow rate 0.5 mL/minute, the running time was fixed at 30 minutes. Samples were detected in the UV-Diode Array detector at 210/ nm. The peaks were plotted by Chemstation software (Dowell et al., 2015).

2.6 Structural Analysis

Fourier Transform Infrared (FT-IR) spectroscopy was conducted to

compare the functional groups present in the isolate and the standard bacoside A. Transmittance was recorded over a wavelength spectra of 400-4000 cm^{-1} .

2.7 Acetyl-cholinesterase assay

The AChE inhibition potential of the isolated bacoside A was determined by conducting Ellman's assay (Liu et al., 2020). The enzyme was dissolved in Tris-HCl-HCl (pH 8.0), 0.6 U/mL of AChE enzyme was used to convert 15 mM of acetylthiocholine iodide into acetic acid and thiocholine. This thiocholine converts DTNB into TNB, which is measured by UV-Vis at λ_{412} nm after incubating the reaction mixture at 37 °C for 30 minutes. AChE inhibitor prevents the breakdown of the substrate, hence, lowering the conversion of DTNB to TNB. Bacoside A (1 mg/mL) was dissolved in 10 % methanol for the assay and was serially diluted until a linear range of enzyme activity w.r.t. bacoside A concentration was achieved. Commercial drugs, Donepezil® and Galanthamine®, targeting Alzheimer's disease were used as a reference to compare the potential of bacoside A.

2.8 Anti-oxidant activity assay

DPPH assay was conducted to determine and compare the radical scavenging activity of the crude extract, isolated bacoside A, Donepezil® drug, and BHT as reference. 1 mM DPPH solution was prepared in methanol and its absorbance was adjusted to 0.7 at 490 nm. Bacoside A samples were prepared with a concentration gradient of 0 to 150 $\mu\text{g/mL}$. All the samples were incubated with DPPH solution in ratio 1:1, for 30 minutes at room

2.9 In-silico toxicity prediction

ADMET profiling tool was used to predict the toxicity parameters of the phytoconstituents of the bacoside A. Bacoside A is a mixture of four saponins, bacoside A3, bacoside II, bacoside X, and bacoside C. Online tool admet SAR was used to envisage their possible toxicity. Carcinogenicity, genotoxicity, hepatotoxicity, oral toxicity, and HERG inhibition of the compounds were ascertained (Nagpal et al., 2022).

3 Results and Discussion

3.1 Identification of bacoside A in the crude extract and the isolate by High-Performance Thin Layer Chromatography

The presence of bacoside A was confirmed by performing HPTLC. The bacoside A bands appeared on the TLC plate around the R_f value of 0.4 and 0.5. The fraction that was extracted with methanol alone contained all the compounds ranging from non-polar to polar. While in the sequential polarity gradient extraction method, hexane removed the non-polar lipid content of Brahmi, and the less polar compounds were removed in the acetone extract. What was left were the highly polar compounds, obtained in methanol extract as shown in figure 1a. The methanol fractions were further pooled and run on a silica gel column (mesh size 100-200). The fractions collected at the concentration of 18-21% of methanol in ethyl acetate contained pure bacoside A (Figure 1b). These fractions were pooled and the process had an estimated yield of 92.8 mg/g of crude Brahmi extract.

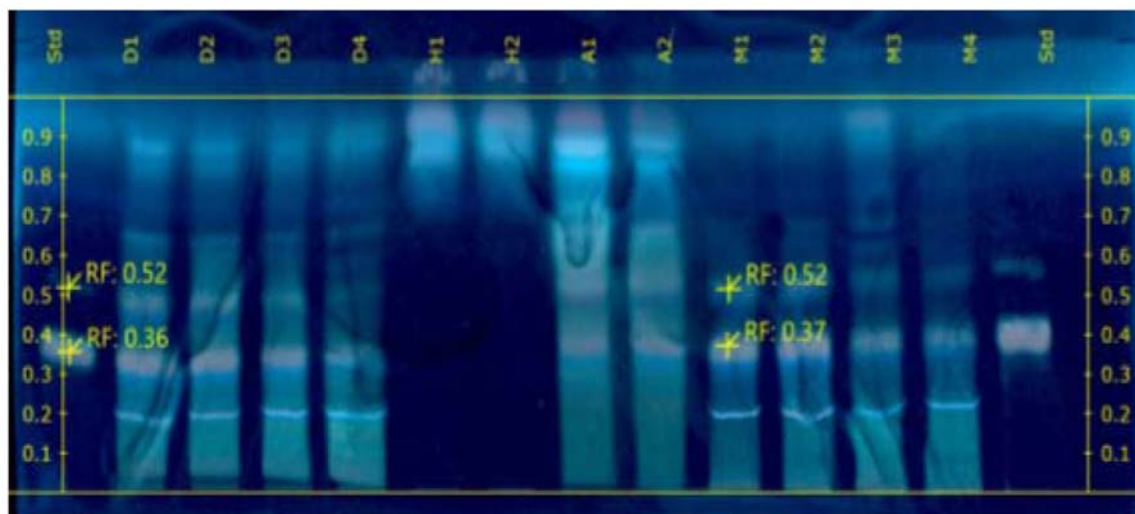


Figure 1a: Identification of bacoside A in the crude extracts obtained during sequential polarity gradient extraction method by HPTLC. Std- Standard bacoside; D1, D2, D3 and D4- Crude methanolic extract obtained at 6th, 12th, 18th, and 24th hour; H1 and H2- Hexane fraction of 6th and 12th hour; A1 and A2- Acetone fraction of 6th and 12th hour; M1, M2, M3, and M4- Methanolic fraction of 6th, 12th, 18th, and 24th hour.

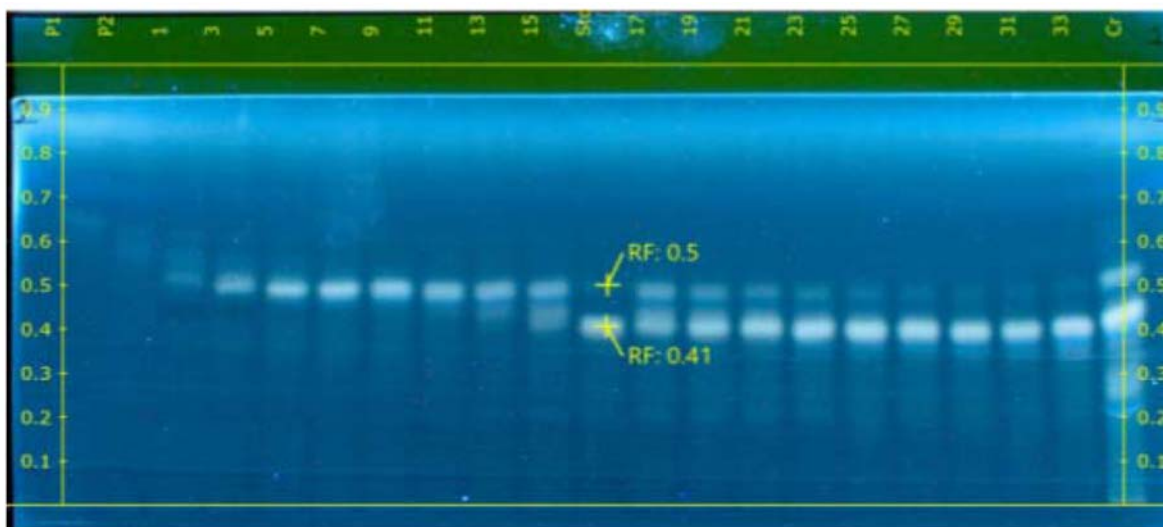


Figure 1b: Identification of bacoside A in the fractions collected after column chromatography by HPTLC

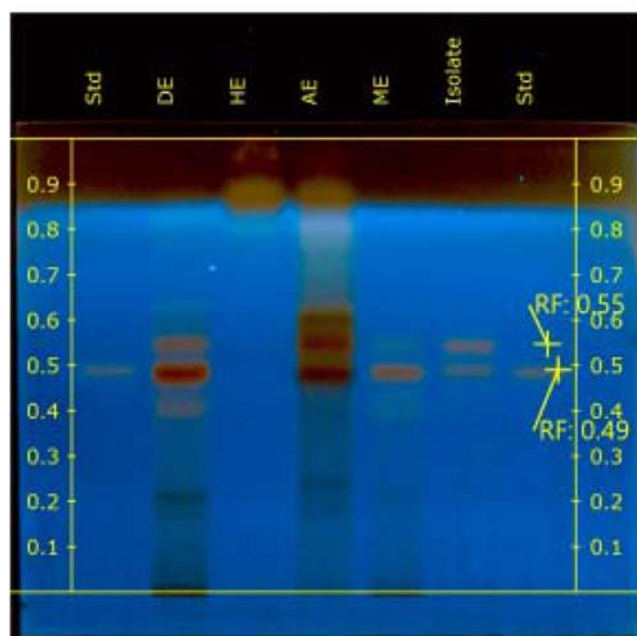


Figure 1c: Estimation of bacoside A content in the crude Brahmi extract and the isolate

3.2 Qualitative analysis by High-Performance Liquid Chromatography

To assess the linearity, a 6 point set of bacoside A, the standard solution was prepared in methanol, in the concentration range of 0 to 500 µg/mL. The standard was injected in reversed-phase C-18 HPLC column. The calibration curve was plotted employing AUC obtained corresponding to the peaks as shown in Figure 2a. The value of the regression coefficient, R^2 came out to be 0.9678. The HPLC profile of isolated bacoside A fraction recorded alongside the standard bacoside A and the crude Brahmi extract showed similar peaks. And many peaks that were present in crude extract but not in the standard were absent in the isolate as shown in Figure 2b. The purity of the isolated bacoside A as determined by the HPLC calibration curve was 83.46 %.

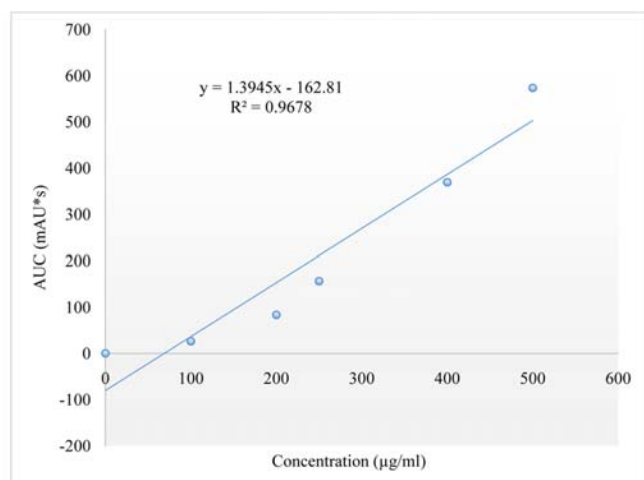


Figure 2a: Calibration curve of bacoside A plotted by HPLC

3.3 Structural analysis of the isolate

FT-IR analysis of standard and isolated bacoside A was done to compare the functional groups present. Percentage transmittance was recorded over wavelength spectra of 400-4000 cm^{-1} , graphically represented in Figure 3. Prominent IR bands and their corresponding functional groups were reported in Table 2.

3.4 Acetyl-cholinesterase inhibition

The result of Ellman's assay was reported as percentage inhibition of the enzyme at the respective concentration of the compound as observed

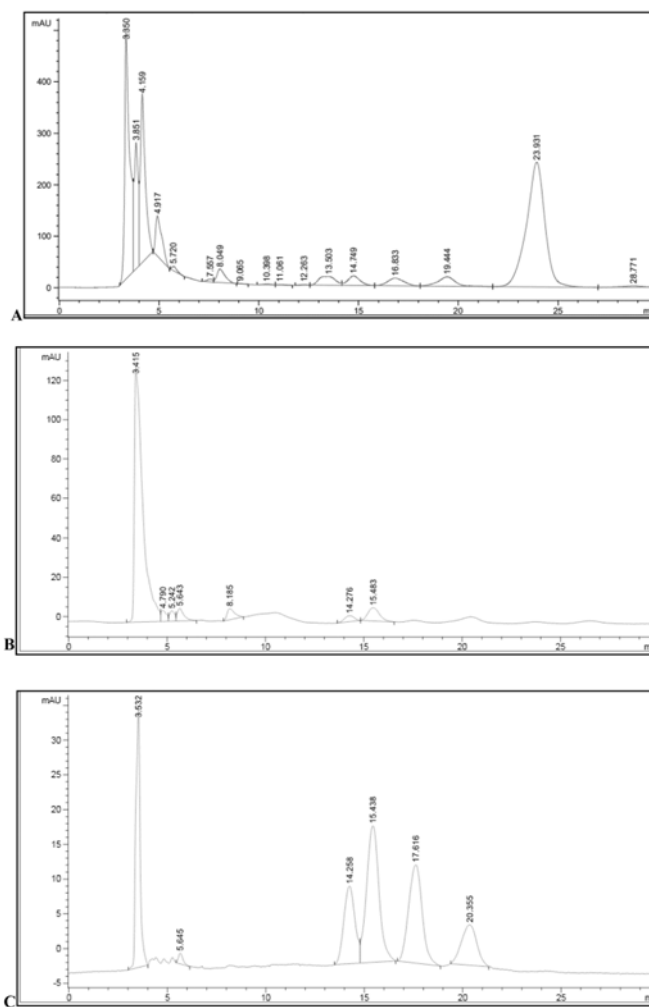


Figure 2b: HPLC of the (A) Crude Brahmi extract, (B) Isolated bacoside A and (C) Standard bacoside A

Table 2: IR peaks obtained from FT-IR spectra of standard and isolated bacoside A

Bands (cm^{-1})	Functional groups	Transmittance (%) Standard Bacoside A	Transmittance (%) Isolated Bacoside A
660	C (triple bond)	55.23	62.21
1034	CN stretch, aliphatic amines	27.23	42.27
1457	C-C stretch, presence of aromatics	54.56	63.4
1658	C=C (double bond), presence of alkenes	53.19	58.14
2845	OH stretch, presence of carboxylic acid	41.59	56.35
2954	CH stretch, presence of alkenes	32.86	48.52
3414	OH stretch, presence of alcohol, phenols	29.06	39.52

in Figure 4. The observed IC_{50} value of bacoside A was 9.96 µg/mL, which was much lower than the IC_{50} value of Galanthamine® (29.81 µg/mL), and comparable to Donepezil® (7.96 µg/mL). The AChE inhibition potential of compounds was in the following order: Donepezil® ~ Bacoside A > Galanthamine®.

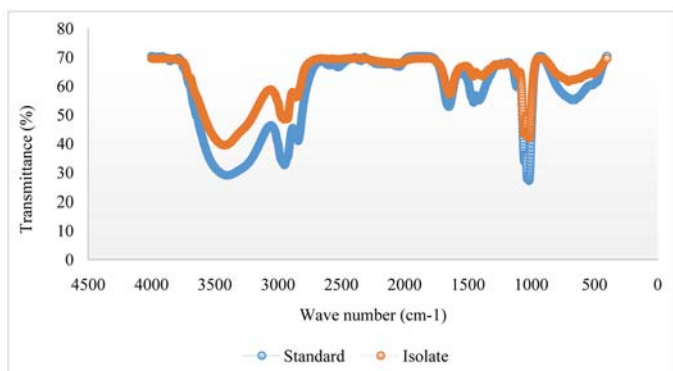


Figure 3: Structural analysis of the isolated and the standard bacoside A by FTIR

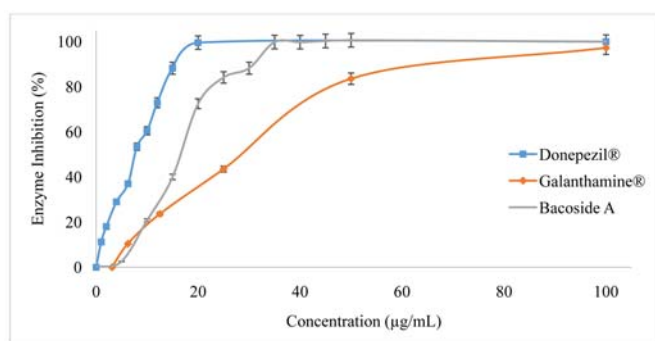


Figure 4: Acetylcholinesterase inhibition by isolated bacoside A, Galanthamine and Donepezil

3.5 Anti-oxidant activity assay

The concentration of extract at which 50% of the DPPH free radicals were reduced, was calculated as IC_{50} value. As shown in Figure 5, with an increase in concentration, the antioxidant activity of the extracts also increased. BHT was used as a reference for antioxidant activity assay, it

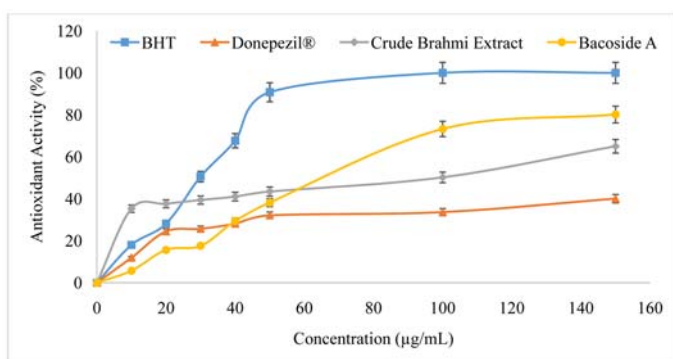


Figure 5: Antioxidant property of BHT, Donepezil, crude Brahmi extract, and isolated bacoside A

showed the IC_{50} value of 29.68 $\mu\text{g/mL}$ and 100% reduction of DPPH at 100 $\mu\text{g/mL}$. The extracts were ordered as, BHT, bacoside A (73.28 $\mu\text{g/mL}$), crude Brahmi extract (100 $\mu\text{g/mL}$), and Donepezil® (over 150 $\mu\text{g/mL}$), in decreasing order of their antioxidant activity. Table 3 depicts the IC_{50} values of the extracts and the concentration required for maximum reduction of the DPPH free radical.

Table 3: Anti-oxidant activity demonstrated by BHT, Donepezil®, bacoside A, and crude Brahmi extract

S. No.	Test compound	IC_{50} value ($\mu\text{g/mL}$)	Maximum Inhibition
1.	BHT (Reference)	29.86	100.00 %
1.	Donepezil®	150+	40.06 %
2.	Crude extract	100	65.02 %
3.	Isolated bacoside A	73.28	80.15 %

3.6 In-silico toxicity prediction

The four saponins that make up the bacoside A phytochemical were tested for their toxicity using the online tool admet SAR. This was done to rule out any possibility of cellular or organ toxicity inside the human body. As shown in Table 4, none of the compounds was carcinogenic, hepatotoxic, or genotoxic, nor they could mutate the DNA.

4 Conclusions

The procedure optimized here can be used for obtaining an enhanced yield of isolated bacoside A. The developed HPTLC and HPLC method could be used as an analytical tool for its quality control purpose. Due to its high antioxidant potential and toxicity, bacoside A can be used as a natural additive in food products instead of synthetic antioxidants like BHT. The AChE inhibitory activity of bacoside A can also be utilized to prevent and manage Alzheimer's disease. Novel methods of delivery can be established to enhance the absorption of bacoside A across the Blood-brain barrier (BBB). The BBB is a common problem faced by all the drugs used in neurological diseases.

Acknowledgment

The authors would like to acknowledge IIT Delhi for experimental facilities for conducting the research work. Financial assistance was provided by the Ministry of Human Resource and Development (MHRD), Govt. of India under the Scheme for Promotion of Academic and Research Collaboration (SPARC).

References

- Anand, T., Pandareesh, M. D., Bhat, P. V., & Venkataramana, M. (2014). Anti-apoptotic mechanism of Bacoside rich extract against reactive nitrogen species induced activation of iNOS/Bax/caspase 3 mediated apoptosis in L132 cell line. *Cytotechnology*, 66(5), 823–838. <https://doi.org/10.1007/s10616-013-9634-7>
- Anbarasi, K., Kathirvel, G., Vani, G., Jayaraman, G., & Shyamala Devi, C. S. (2006). Cigarette smoking induces heat shock protein 70 kDa expression and apoptosis in rat brain: Modulation by bacoside A. *Neuroscience*, 138(4), 1127–1135. <https://doi.org/10.1016/j.neuroscience.2005.11.029>
- Anbarasi, K., Vani, G., Balakrishna, K., & Devi, C. S. S. (2005). Effect of bacoside A on membrane-bound ATPases in the brain of rats exposed to cigarette smoke. *Journal of Biochemical and Molecular Toxicology*, 19(1), 59–65. <https://doi.org/10.1002/jbt.20050>
- Anbarasi, K., Vani, G., Balakrishna, K., & Devi, C. S. S. (2006). Effect of bacoside A on brain antioxidant status in cigarette smoke exposed rats. *Life Sciences*, 78(12), 1378–1384. <https://doi.org/10.1016/j.lfs.2005.07.030>

Table 4: Toxicity prediction of bacoside A phytoconstituents by admet SAR

	Donepezil®	Bacoside A3	Bacopaside II	Bacopaside X	Bacopasaponin C
Hepatotoxicity	- 0.775	- 0.675	- 0.7	- 0.675	- 0.7
HERG inhibition	+ 0.952	+ 0.8419	+ 0.8473	+ 0.8035	+ 0.8126
Acute oral toxicity	+ 0.5639	+ 0.6865	+ 0.468	+ 0.6698	+ 0.6698
Ames mutagenesis	- 0.59	- 0.76	- 0.7529	- 0.6429	- 0.6829
Carcinogenesis	- 0.9857	- 0.9857	- 0.9857	- 0.9857	- 0.9857
Micronucleus assay	- 0.54	- 0.88	- 0.89	- 0.88	- 0.88

5. Bai, Q.-K., & Zhao, Z.-G. (2021). Isolation and neuronal apoptosis inhibitory property of bacoside-A3 via downregulation of β -amyloid induced inflammatory response. *Biotechnology and Applied Biochemistry*. <https://doi.org/10.1002/bab.2147>
6. Bhattacharya, S. K., Bhattacharya, A., Kumar, A., & Ghosal, S. (2000). Antioxidant activity of Bacopa monnieri in rat frontal cortex, striatum and hippocampus. *Phytotherapy Research: PTR*, 14(3), 174–179. [https://doi.org/10.1002/\(sici\)1099-1573\(200005\)14:3<174::aid-ptr624>3.0.co;2-o](https://doi.org/10.1002/(sici)1099-1573(200005)14:3<174::aid-ptr624>3.0.co;2-o)
7. Chaudhary, B., & Bist, R. (2017). Protective manifestation of bacoside A and bromelain in terms of cholinesterases, gamma-amino butyric acid, serotonin level and stress proteins in the brain of dichlorvos-intoxicated mice. *Cell Stress & Chaperones*, 22(3), 371–376. <https://doi.org/10.1007/s12192-017-0773-1>
8. Deepak, M., Sangli, G. K., Arun, P. C., & Amit, A. (2005). Quantitative determination of the major saponin mixture bacoside A in Bacopa monnieri by HPLC. *Phytochemical Analysis: PCA*, 16(1), 24–29. <https://doi.org/10.1002/pca.805>
9. Dowell, A., Davidson, G., & Ghosh, D. (2015). Validation of Quantitative HPLC Method for Bacosides in KeenMind. *Evidence-Based Complementary and Alternative Medicine*, 2015, 1–8. <https://doi.org/10.1155/2015/696172>
10. Kaur, A., Nigam, K., Srivastava, S., Tyagi, A., & Dang, S. (2020). Memantine nanoemulsion: A new approach to treat Alzheimer's disease. *Journal of Microencapsulation*, 37(5), 355–365. <https://doi.org/10.1080/02652048.2020.1756971>
11. Liu, E. Y. L., Xia, Y., Kong, X., Guo, M. S. S., Yu, A. X. D., Zheng, B. Z. Y., Mak, S., Xu, M. L., & Tsim, K. W. K. (2020). Interacting with $\alpha 7$ nAChR is a new mechanism for AChE to enhance the inflammatory response in macrophages. *Acta Pharmaceutica Sinica. B*, 10(10), 1926–1942. <https://doi.org/10.1016/j.apsb.2020.05.005>
12. Malishev, R., Shaham-Niv, S., Nandi, S., Kolusheva, S., Gazit, E., & Jelinek, R. (2017). Bacoside-A, an Indian Traditional-Medicine Substance, Inhibits β -Amyloid Cytotoxicity, Fibrillation, and Membrane Interactions. *ACS Chemical Neuroscience*, 8(4), 884–891. <https://doi.org/10.1021/acscchemneuro.6b00438>
13. Nagpal, T., Alam, S., Khare, S. K., Satya, S., Chaturvedi, S., & Sahu, J. K. (2022). Effect of Psidium guajava leaves extracts on thermo-lipid oxidation and Maillard pathway born food toxicant acrylamide in Indian staple food. *Journal of Food Science and Technology*, 59(1), 86–94. <https://doi.org/10.1007/s13197-021-04984-y>
14. Nemetchek, M. D., Stierle, A. A., Stierle, D. B., & Lurie, D. I. (2017). The Ayurvedic plant Bacopa monnieri inhibits inflammatory pathways in the brain. *Journal of Ethnopharmacology*, 197, 92–100. <https://doi.org/10.1016/j.jep.2016.07.073>
15. Pandareesh, M. D., & Anand, T. (2014). Attenuation of smoke-induced neuronal and physiological changes by bacoside rich extract in Wistar rats via down regulation of HO-1 and iNOS. *Neurotoxicology*, 40, 33–42. <https://doi.org/10.1016/j.neuro.2013.11.001>
16. Pawar, R., Gopalakrishnan, C., & Bhutani, K. K. (2001). Dammarane triterpene saponin from Bacopa monnieri as the superoxide inhibitor in polymorphonuclear cells. *Planta Medica*, 67(8), 752–754. <https://doi.org/10.1055/s-2001-18351>
17. Singh, R., Ramakrishna, R., Bhateria, M., & Bhatta, R. S. (2014). In vitro evaluation of Bacopa monnieri extract and individual constituents on human recombinant monoamine oxidase enzymes. *Phytotherapy Research: PTR*, 28(9), 1419–1422. <https://doi.org/10.1002/ptr.5116>
18. Sivaramakrishna, C., Rao, C. V., Trimurtulu, G., Vanisree, M., & Subbaraju, G. V. (2005). Triterpenoid glycosides from Bacopa monnieri. *Phytochemistry*, 66(23), 2719–2728. <https://doi.org/10.1016/j.phytochem.2005.09.016>
19. Srivastava, S., Ahmad, R., & Khare, S. K. (2021). Alzheimer's disease and its treatment by different approaches: A review. *European Journal of Medicinal Chemistry*, 216, 113320. <https://doi.org/10.1016/j.ejmech.2021.113320>
20. Sumathi, T., Nathiya, V. C., & Sakthikumar, M. (2011). Protective Effect of Bacoside-A against Morphine-Induced Oxidative Stress in Rats. *Indian Journal of Pharmaceutical Sciences*, 73(4), 409–415. <https://doi.org/10.4103/0250-474X.95624>
21. Verma, P., Gupta, R. K., Gandhi, B. S., & Singh, P. (2015). CDRI-08 Attenuates REST/NRSF-Mediated Expression of NMDAR1 Gene in PBDE-209-Exposed Mice Brain. *Evidence-Based Complementary and Alternative Medicine: ECAM*, 2015, 403840. <https://doi.org/10.1155/2015/403840>