JBSS
Journal of Biological and Sustainability Sciences
An International Peer-Reviewed Open Access Journal
Vol. 1 • No. 1 • 2025 | DOI: 10.5281/zenodo.21530282 | jbss.biosuss.com

ORIGINAL RESEARCH ARTICLE

Solvent-Dependent Butyrylcholinesterase Inhibition and Antioxidant Activity of Cucurbitaceous Seed Extracts and *Hydrocotyle asiatica*

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PUBLICATION DETAILS

Received:
2025-06-13

Revised:
2025-07-23

Accepted:
2025-08-04

Published:
2025-08-17

Article ID:
JBSS-2025-0003

ABSTRACT

Background: Oxidative stress and impaired cholinergic neurotransmission are central features of Alzheimer's disease. Plant-derived inhibitors of butyrylcholinesterase (BChE) may provide useful leads for neuroprotective research. Objective: To compare the influence of plant species and extraction-solvent polarity on the BChE inhibitory and DPPH radical-scavenging activities of cucurbitaceous seed extracts and *Hydrocotyle asiatica*. Methods: Seeds of Cucurbita maxima, Cucumis melo, Cucumis sativus, and Citrullus lanatus, together with whole-plant material of *H. asiatica*, were sequentially extracted with petroleum ether, chloroform, ethyl acetate, and methanol. BChE inhibition and DPPH scavenging were evaluated in triplicate at 0.2, 0.5, and 1.0 mg/mL, using galantamine and ascorbic acid as reference compounds. Results: The methanolic extract of *C. lanatus* was reported to inhibit BChE by 81.19% at 1.0 mg/mL (IC_{50} = 98 µg/mL). The ethyl acetate extract of *C. maxima* produced 81.77% inhibition, whereas the chloroform extract of *H. asiatica* reached 88.88% inhibition at 1.0 mg/mL. In the DPPH assay, the chloroform extract of *C. sativus* showed the strongest seed-derived activity (63.78% at 1.0 mg/mL; IC_{50} = 642 µg/mL), followed by the ethyl acetate extract of *C. lanatus* (IC_{50} = 708 µg/mL). Conclusion: Bioactivity depended strongly on both species and solvent. The most active fractions warrant bioassay-guided isolation, mechanistic enzyme-kinetic studies, and in vivo validation; the present findings should be interpreted as preliminary in vitro evidence rather than proof of therapeutic efficacy.

KEYWORDS

Alzheimer's disease; butyrylcholinesterase; DPPH; cucurbitaceous seeds; solvent polarity; *Hydrocotyle asiatica*

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Cite this article: Senthilkumar SN, M MI, Fatima B, Nagarajan B. Solvent-Dependent Butyrylcholinesterase Inhibition and Antioxidant Activity of Cucurbitaceous Seed Extracts and *Hydrocotyle asiatica*. J Biol Sustain Sci. 2025;1(1):20-29. DOI: https://doi.org/10.5281/zenodo.21530282

1. Highlights

Sequentialextractionrevealedstrongsolvent-dependentdifferencesinBChEinhibitionandantioxidantactivity.

MethanolicCitrullus lanatus, ethyl acetateCucurbita maxima, and chloroformHydrocotyle asiaticawere among the most active BChE-inhibitory fractions.

The study identifies plant–solvent combinations for future phytochemical isolation rather than establishing clinicalefficacy against Alzheimer's disease.

2. Introduction

Alzheimer's disease (AD) is a progressive, multifactorial neurodegenerative disorder characterized by gradual deterioration of memory, cognitive function, orientation, communication, and the ability to perform daily activities. Its complex pathophysiology involves extracellular amyloid-β accumulation, intracellular tau pathology, synaptic dysfunction, mitochondrial impairment, neuroinflammation, oxidative stress, and progressive neuronal loss. These interconnected pathological mechanisms contribute to cognitive decline and present substantial challenges for effective therapeutic intervention. Although currently available pharmacological treatments provide symptomatic benefits and newer disease-modifying therapies may slow disease progression in selected patients, treatment responses remain variable, highlighting the continued need to investigate additional therapeutic targets and biologically active compounds [1,2].

Natural products represent a valuable source of structurally diverse bioactive metabolites with potential applications in neurodegenerative drug discovery. Plant-derived compounds, including alkaloids, phenolics, flavonoids, and

terpenoids, have attracted scientific interest because of their capacity to interact with enzymes, receptors, cellular membranes, and redox-related pathways. In particular, the investigation of plant extracts for cholinesterase-inhibitory and radical-scavenging activities provides a preliminary approach for identifying compounds relevant to cholinergic dysfunction and oxidative stress, two processes associated with AD pathology. However, such in vitro activities do not independently establish neuroprotective efficacy or clinical therapeutic potential. Consequently, systematic phytochemical screening, bioassay-guided isolation, mechanistic characterization, and subsequent biological validation remain essential for determining the pharmacological relevance of promising natural-product candidates [1,2].

Cholinergic dysfunction and oxidative stress are key contributors to Alzheimer's disease (AD) pathogenesis. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) regulate acetylcholine hydrolysis, with the relative contribution of BChE potentially increasing during disease progression, making it a complementary therapeutic target [3-7]. Oxidative stress further promotes neuronal damage through lipid peroxidation, protein oxidation, and mitochondrial dysfunction. Although plant-derived antioxidants may counteract oxidative processes, in vitro radical-scavenging activity does not establish neuroprotection [8]. Therefore, combined BChE-inhibitory and DPPH radical-scavenging assays provide a preliminary strategy for identifying promising botanical extracts for further phytochemical and pharmacological investigation [3-8].

Plant-derived secondary metabolites represent important sources of bioactive compounds with enzyme-inhibitory, antioxidant, and anti-inflammatory properties, offering potential leads for drug discovery. Their extraction efficiency and phytochemical composition are strongly influenced by solvent polarity. Petroleum ether predominantly extracts lipophilic constituents, chloroform recovers relatively nonpolar to moderately polar metabolites, ethyl acetate favors semipolar compounds, and methanol extracts a broad range of polar phytochemicals. Sequential solvent extraction therefore facilitates the separation of chemically diverse constituents and identification of fractions associated with specific biological activities, providing a rational basis for subsequent chromatographic characterization and bioassay-guided isolation [9-14].

Cucurbitaceous seeds are rich in nutritionally and pharmacologically relevant constituents but remain comparatively underexplored for neuropharmacological activities. The present study investigated seeds of *Cucurbita maxima* (pumpkin), *Cucumis melo* (melon), *Cucumis sativus* (cucumber), and *Citrullus lanatus* (watermelon), which contain oils, phytosterols, phenolics, flavonoid-related compounds, and other secondary metabolites. Although previous studies have reported antioxidant and related biological activities in cucurbit-derived materials, comparative investigations of sequential seed fractions for butyrylcholinesterase (BChE) inhibition remain limited. These botanical materials may therefore provide promising sources for identifying bioactive fractions and structurally diverse inhibitory compounds [15-18].

Hydrocotyle asiatica was additionally included as a medicinal comparison plant because of its reported antioxidant properties and traditional neurological applications. Its triterpenoid-rich phytochemical profile provides a relevant basis for examining solvent-dependent biological responses alongside cucurbit seed extracts [19]. Accordingly, the study comparatively evaluated BChE-inhibitory and DPPH radical-scavenging activities across sequential solvent fractions to identify promising candidates for further phytochemical and pharmacological investigation, without implying established therapeutic efficacy against Alzheimer's disease. A key research gap addressed in this study was the limited comparative evidence regarding the combined influence of plant species and extraction solvent polarity on butyrylcholinesterase (BChE) inhibition and antioxidant activity. Comparative evaluation of these biochemical responses may help distinguish enzyme-directed inhibition from chemical radical-scavenging capacity and identify plant fractions exhibiting activity in both assays. Such systematic screening provides a scientific basis for prioritizing promising extracts for bioassay-guided fractionation, phytochemical characterization, mechanistic investigation, and subsequent biological validation.

Accordingly, the present study evaluated the BChE-inhibitory and DPPH radical-scavenging activities of sequential petroleum ether, chloroform, ethyl acetate, and methanol extracts obtained from four cucurbitaceous seed species and whole-plant *H. asiatica*. Extracts were tested at concentrations of 0.2, 0.5, and 1.0 mg/mL, using galantamine and ascorbic acid as reference standards for the respective assays. The study hypothesized that differences in botanical origin and solvent polarity would produce distinct concentration-dependent biochemical activity profiles, thereby identifying promising plant–solvent combinations for further phytochemical and neuropharmacological investigation.

3. Materials and Methods

3.1 Plant materials and authentication

Seeds of *Cucurbita maxima* (pumpkin), *Cucumis sativus* (cucumber), *Cucumis melo* (melon), and *Citrullus lanatus* (watermelon), along with whole-plant material of *Hydrocotyle asiatica*, were procured from a local market in Gujrat, Punjab, Pakistan. The collected botanical materials were identified by an herbarium specialist in the Department of Botany, University of Gujrat, through morphological examination and comparison with available botanical reference materials. The voucher specimen number was not documented in the available study record and should be provided, if available, to ensure botanical traceability and facilitate future verification and reproducibility.

3.2 Sequential solvent extraction

Plant materials were shade-dried at room temperature and ground into a fine powder. Approximately 6 g of each powdered sample was subjected to sequential extraction using petroleum ether, chloroform, ethyl acetate, and methanol in order of increasing solvent polarity. Each extraction was performed in a shaking incubator at room temperature for 24 h, followed by filtration to separate the solvent extract from the residual plant material. The resulting filtrates were concentrated under reduced pressure using a rotary evaporator. Following each extraction step, the remaining plant residue was subjected to extraction with the next solvent in the sequence. The concentrated extracts were subsequently prepared at 1.0 mg/mL and diluted to 0.5 and 0.2 mg/mL for comparative evaluation of butyrylcholinesterase (BChE)-inhibitory and DPPH radical-scavenging activities.

3.3 Butyrylcholinesterase inhibitory assay

Butyrylcholinesterase (BChE)-inhibitory activity was evaluated using a modified Ellman colorimetric method [20,21], based on the spectrophotometric detection of thiocholine generated through enzymatic hydrolysis of butyrylthiocholine. The working 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) solution was prepared by mixing 3.6 mL of stock solution containing 20 mM sodium bicarbonate and 10 mM DTNB in 0.1 M phosphate buffer (pH 7.0) with 96.4 mL of 0.1 M phosphate buffer (pH 8.0). The reaction mixture comprised 1.35 mL of buffered DTNB solution, 50 µL of equine serum-derived BChE (0.4 *U*), 50 µL of plant extract (0.2, 0.5, or 1.0 mg/mL), and 50 µL of 4 mM butyrylthiocholine chloride, yielding a final reaction volume of 1.5 mL. Following incubation at room temperature for 15 min, absorbance was measured at 412 nm using a spectrophotometer equipped with a 1 cm quartz cuvette. Galantamine hydrobromide served as the positive reference inhibitor, and all measurements were performed in triplicate. Percentage BChE inhibition was calculated using the following equation: BChE inhibition (%) = [(E – S)/E] × 100, where E represents enzyme activity in the absence of extract and "S" represents enzyme activity in the presence of extract [20,21].

3.4 DPPH radical-scavenging assay

The antioxidant activity of plant extracts was evaluated using a modified 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay based on spectrophotometric measurement of DPPH radical reduction [9,10]. Briefly, 50 µL of each extract solution (0.2, 0.5, or 1.0 mg/mL) was mixed with 2 mL of freshly prepared 0.1 mM DPPH solution in ethanol. The reaction mixtures were incubated at 37 °C for 20 min, after which absorbance was measured at 517 nm using a spectrophotometer. Ethanol served as the solvent blank, while ascorbic acid was used as the reference antioxidant. All measurements were performed in triplicate. DPPH radical-scavenging activity was calculated using the following equation: DPPH radical-scavenging activity (%) = [(C – S)/C] × 100

where C represents the absorbance of the DPPH control without plant extract and S represents the absorbance of the reaction mixture containing plant extract [9,10].

3.5 Data presentation and IC_{50} estimation

Results were expressed as percentage inhibition, with error bars representing variability among triplicate measurements. Half-maximal inhibitory concentration (IC_{50}) values were obtained from concentration–response relationships reported in the original dataset. However, the specific regression method, measures of variability, and inferential statistical procedures were not documented. Consequently, the results were interpreted descriptively, and claims of statistical significance were avoided in the absence of appropriate statistical analysis. Differences among plant extracts, solvent fractions, and concentrations were therefore discussed as observed trends rather than statistically established effects. The five investigated plant materials, their respective plant parts, and the sequential extraction procedure are summarized in Table 1. Petroleum ether (PE), chloroform ($CHCl_3$), ethyl acetate (EtOAc), and methanol (MeOH) were employed sequentially, and these abbreviations are consistently used throughout the Results and Discussion sections.

Table1.Plantmaterials, plant parts, and extraction sequence. PE, petroleum ether; $CHCl_3$, chloroform; EtOAc, ethyl acetate; MeOH, methanol.

Species	Commonname	Materialtested	Sequentialsolvents
Cucurbitamaxima	Pumpkin	Seed	PE→$CHCl_3$→EtOAc →MeOH
Cucumismelo	Melon	Seed	PE→$CHCl_3$→EtOAc →MeOH
Cucumissativus	Cucumber	Seed	PE→$CHCl_3$→EtOAc →MeOH
Citrulluslanatus	Watermelon	Seed	PE→$CHCl_3$→EtOAc →MeOH
Hydrocotyleasiatica	—	Wholeplant	PE→$CHCl_3$→EtOAc →MeOH

4. Results

4.1 Solvent-dependent BChE inhibition

BChE inhibition varied markedly according to plant species and extraction solvent (Figure 1). Petroleum ether fractionsgenerallyproducedlow-to-moderateinhibition,althoughthe*C.melo*seedfractionwasreportedtoreach an IC_{50} of 565 µg/mL. Chloroform extracts from cucurbitaceous seeds were comparatively weak, whereas chloroform-extracted *H. asiatica* produced 88.88%inhibition at 1.0mg/mL anda reported IC_{50} of 161 µg/mL. Ethyl acetate fractions showed stronger activity across several seed samples. The ethyl acetate extract of *C. maxima* produced 81.77% BChE inhibition at 1.0 mg/mL. Methanolic fractions also showed consistently high activity, and the methanolic *C. lanatus* extract was reported to produce 81.19% inhibition at 1.0mg/mL with an IC_{50}of98µg/mL Galantamine produced substantially greater potency than the plant extracts (IC_{50}=3.6±0.1µg/mL).

Considered across all four solvents, the *H. asiatica* fractions shown in Figure1E were consistently active at 1.0 mg/mL: the petroleum ether, chloroform, and ethyl acetate fractions all exceeded 80% inhibition, and only the methanolic fraction fell appreciably lower, at approximately 66%. This broad, less solvent-selective response contrasts with the cucurbitaceous seeds, where high inhibition was largely confined to the ethyl acetate and methanol fractions, and suggests that the BChE-inhibitory constituents of*H. asiatica* are distributed across a wider polarity range than those of the seed matrices. The leading extracts from each species, together with the reference compound, are consolidated in Table 2 for direct comparison; the corresponding DPPH results are presented in Section 3.2.

Table2.Reportedhigh-activityextractsandreferencecompounds.

Assay	Plant/reference	Solvent	Maximumreportedrespons e	IC_{50}(µg/mL)
BChE	*Citrulluslanatus*	Methanol	81.19%at1.0mg/mL	98
BChE	*Hydrocotyle asiatica*	Chloroform	88.88%at1.0mg/mL	161
BChE	*Cucurbitamaxima*	Ethylacetate	81.77%at1.0mg/mL	Notreported
BChE	Galantamine	Reference	Positivecontrol	3.6±0.1
DPPH	*Cucumissativus*	Chloroform	63.78%at1.0mg/mL	642
DPPH	*Citrulluslanatus*	Ethylacetate	Reportedassecond-most active	708
DPPH	Ascorbicacid	Reference	Positivecontrol	4.2±0.2

4.2 DPPH radical-scavenging activity

DPPH radical-scavenging activity exhibited considerable variation among the investigated plant species and solvent fractions, demonstrating distinct concentration-dependent antioxidant responses (Figure 2). Overall, most extracts showed substantially lower radical-scavenging potency than ascorbic acid, and their antioxidant profiles did not consistently correspond to the observed butyrylcholinesterase (BChE)-inhibitory activities. Among the cucurbit seed extracts, the chloroform fraction of *C. sativus* exhibited the highest reported DPPH radical-scavenging activity, reaching 63.78% inhibition at 1.0 mg/mL, with an IC_{50} of 642 µg/mL. The ethyl acetate fraction of *C. lanatus* demonstrated the next highest potency based on its reported IC_{50} of 708 µg/mL. In comparison, the reference antioxidant, ascorbic acid, exhibited an IC_{50} of 4.2 ± 0.2 µg/mL, indicating substantially greater radical-scavenging potency than the tested seed fractions. The variation in antioxidant responses across petroleum ether, chloroform, ethyl acetate, and methanol fractions suggests that the distribution of radical-scavenging constituents differed among the investigated botanical materials. Notably, fractions demonstrating comparatively strong BChE inhibition did not necessarily exhibit correspondingly high DPPH activity. This divergence indicates that the two biochemical assays assess distinct properties of the extracts and may reflect differences in the chemical characteristics of the constituents contributing to each response. However, the present screening data do not establish which specific phytochemicals are responsible for the observed activities.

The whole-plant fractions of *H. asiatica* also displayed pronounced solvent-dependent differences in DPPH radical-scavenging activity (Figure 2E). Among the four fractions, ethyl acetate exhibited the strongest antioxidant response, achieving approximately 51% inhibition at 1.0 mg/mL, whereas the chloroform and methanol fractions showed comparatively weaker activity. Interestingly, this antioxidant profile contrasted with the BChE-inhibitory results, in which the chloroform fraction demonstrated the greatest inhibitory activity for *H. asiatica* (Section 3.1). Thus, the fraction exhibiting the strongest enzyme inhibition was not the fraction showing the highest radical-scavenging activity within the same plant species.

Collectively, these findings demonstrate that DPPH radical-scavenging activity varied according to both botanical source and extraction solvent, with chloroform *C. sativus* emerging as the leading antioxidant fraction among the investigated cucurbit seeds. Nevertheless, the substantially higher IC_{50} values of the active seed fractions relative to ascorbic acid indicate modest antioxidant potency under the reported experimental conditions. The contrasting responses observed between DPPH and BChE assays further emphasize that antioxidant and cholinesterase-

inhibitory activities should be evaluated independently. The potential phytochemical basis and biological implications of these differences are discussed further in Section 4. 4.

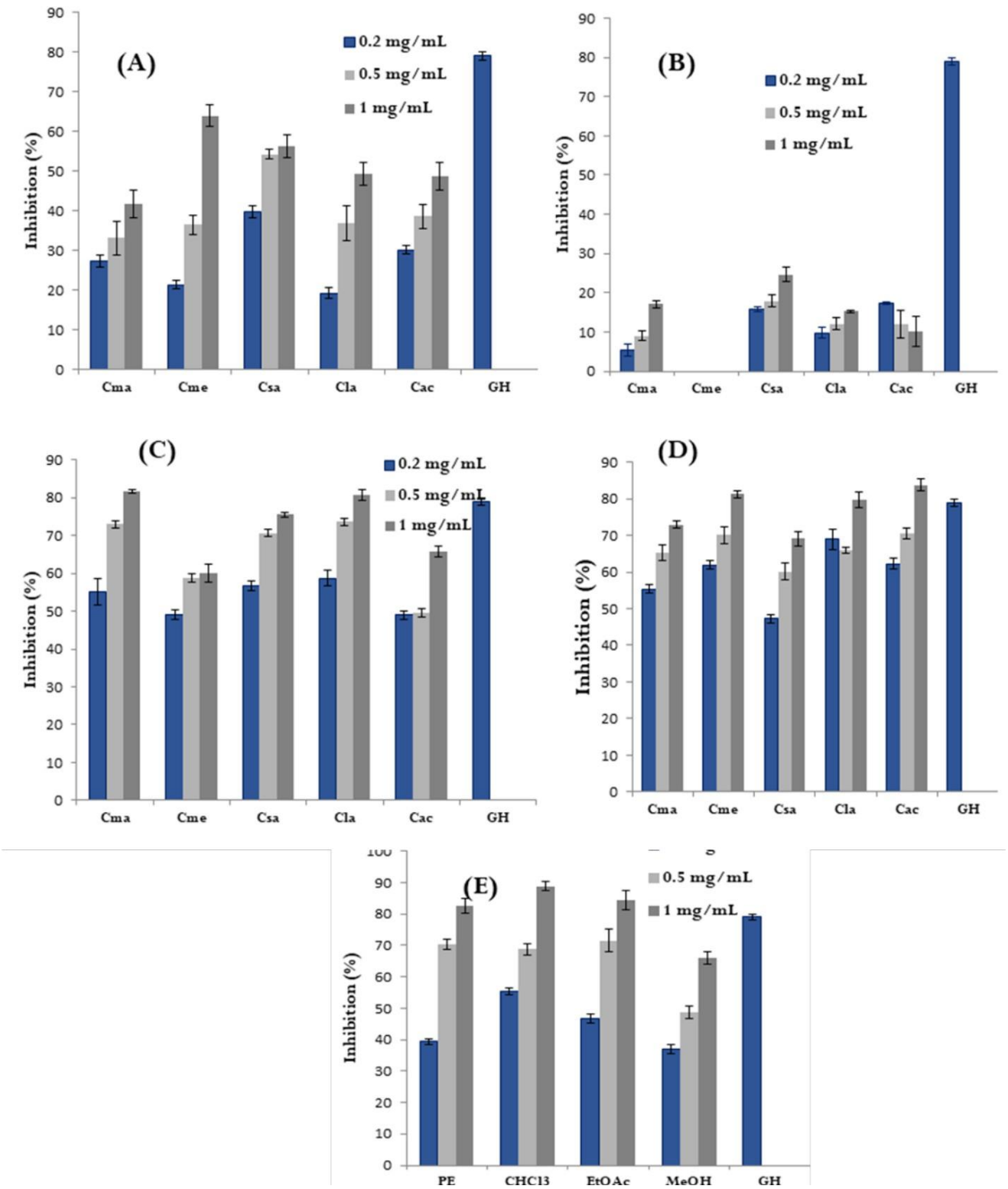


Figure 1.Butyrylcholinesterase inhibition by sequential solvent extracts. Panels A–D show petroleum ether, chloroform, ethyl acetate, and methanol seed fractions, respectively; panel E compares the four solvent fractions of *Hydrocotyle asiatica*. Cma, Cucurbita maxima; Cme, Cucumis melo; Csa, Cucumis sativus; Cla, Citrullus lanatus; Cac/Mac, combined cucurbitaceous seed extract as labeled in the source figure; GH, galantamine hydrobromide. Values are shown as mean with error bars from triplicate measurements.

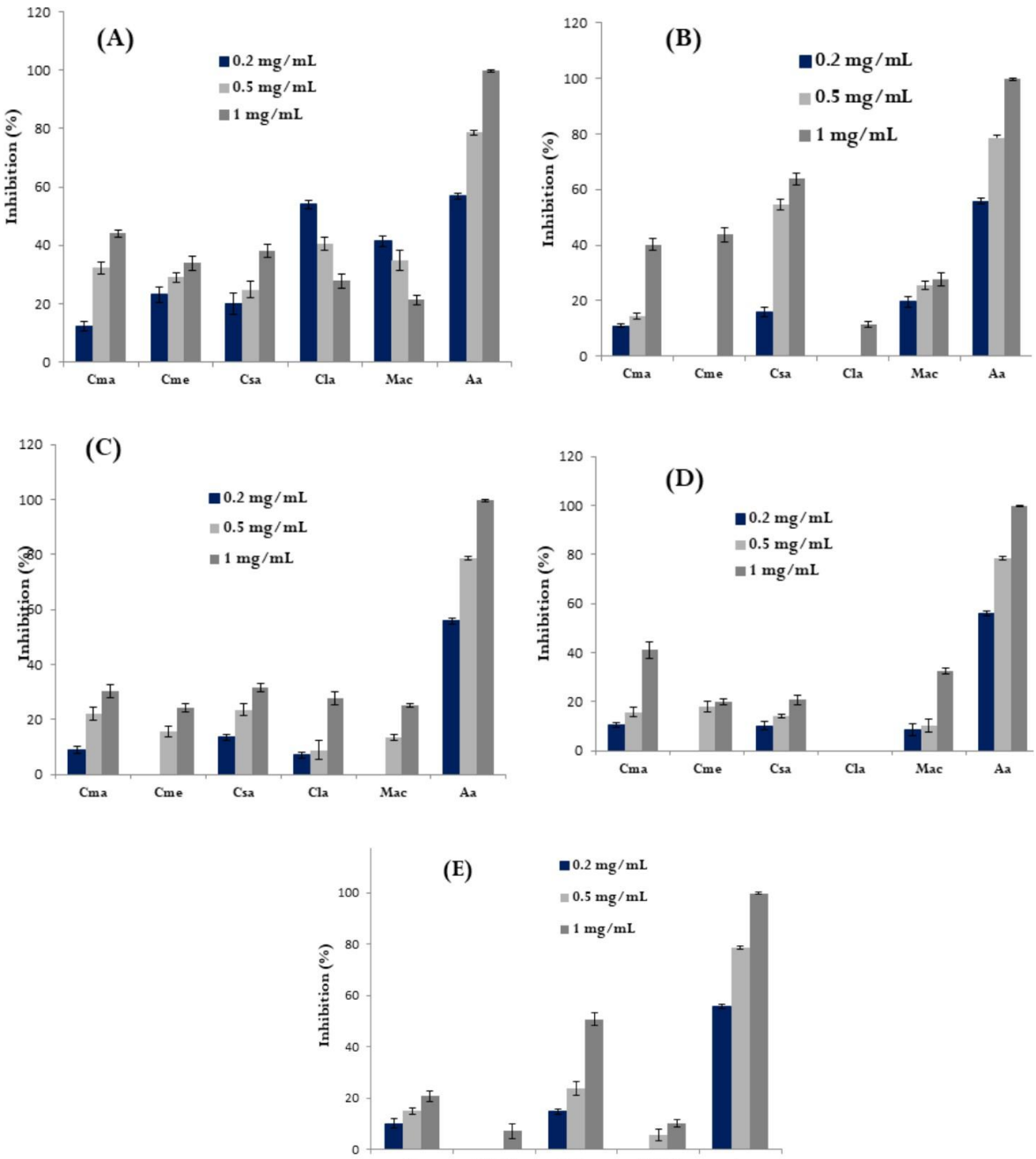


Figure 2. DPPH radical-scavenging activity of sequential solvent extracts. Panels A–D show petroleum ether, chloroform, ethyl acetate, and methanol seed fractions, respectively; panel E compares the four solvent fractions of *Hydrocotyle asiatica*. Cma, Cucurbita maxima; Cme, Cucumis melo; Csa, Cucumis sativus; Cla, Citrullus lanatus; Mac, combined cucurbitaceous seed extract as labeled in the source figure; Aa, ascorbic acid. Values are shown as mean with error bars from triplicate measurements.

5. Discussion

5.1 BChE inhibition and solvent polarity

The present findings demonstrate that BChE inhibition was strongly influenced by both solvent polarity and plant identity. Petroleum ether and chloroform seed fractions generally displayed lower inhibition than ethyl acetate and methanol fractions, whereas the chloroform fraction of *H. asiatica* was notably active. This pattern suggests that the compounds contributing to BChE inhibition were not confined to a single polarity class. Rather, the activity may reflect several chemically distinct groups distributed differently among the plant materials. Sequential extraction was therefore useful not only for recovering constituents but also for separating broad chemical classes and revealing activity that could have been diluted or masked in a single crude extract.

The comparatively strong responses of several ethyl acetate and methanol fractions are consistent with the ability of these solvents to recover semi-polar and polar metabolites, including many phenolic, flavonoid-related, nitrogen-containing, and glycosylated compounds. However, qualitative solvent behavior alone cannot identify the active constituents, and the strong chloroform response of *H. asiatica* indicates that less-polar metabolites may also contribute. Similar variability has been observed in plant cholinesterase screening studies, where activity depends on the botanical source, plant part, extraction protocol, enzyme source, substrate, and assay conditions [6,11,12]. The

present data should therefore be viewed as a comparative ranking within this experimental system rather than as an absolute potency classification across studies.

The concentration-dependent increases observed in many fractions support the presence of inhibitory activity rather than a single-point experimental effect. Nevertheless, percentage inhibition at a high extract concentration can be influenced by sample color, turbidity, nonspecific protein interactions, or interference with the chromogenic reaction. Future confirmation should include extract blanks at every concentration, full concentration–response curves, nonlinear regression with confidence intervals, and enzyme kinetic experiments to determine whether inhibition is competitive, noncompetitive, mixed, or time-dependent. Counter-screening against AChE would also clarify whether the active fractions are selective for BChE or act broadly against both cholinesterases [6,7,20,21].

5.2 Species-specific patterns and priority extracts

Among the tested seed samples, the methanolic extract of *C. lanatus* emerged as one of the most promising butyrylcholinesterases (BChE)-inhibitory fractions, whereas the ethyl acetate extract of *C. maxima* also exhibited substantial inhibition at 1.0 mg/mL. These findings suggest that different cucurbit species may contain distinct distributions of BChE-inhibitory phytochemicals across solvent fractions. The comparatively strong activity of methanolic *C. lanatus* indicates the potential contribution of polar metabolites, while the pronounced inhibition observed in ethyl acetate *C. maxima* suggests the involvement of moderately polar constituents. However, solvent-dependent activity alone cannot establish the chemical identity or relative abundance of individual inhibitory compounds. The comparatively weaker responses of several chloroform seed fractions further demonstrate that extraction efficiency and biological activity may vary according to the botanical source and phytochemical composition [13,19]. Consequently, the optimal extraction solvent for recovering biologically active compounds from one plant species cannot necessarily be generalized to another.

The whole-plant fractions of *H. asiatica* exhibited a distinctive inhibition profile, maintaining measurable BChE-inhibitory activity across all four solvent fractions, with the chloroform fraction demonstrating the greatest potency among the *H. asiatica* fractions (Figure 1E). This observation distinguishes the medicinal comparison plant from the cucurbit seed samples and suggests that less-polar metabolites may contribute to its inhibitory activity. Nevertheless, the possible involvement of multiple active constituents with overlapping polarity characteristics cannot be excluded. The strong activity of the chloroform fraction provides a rationale for further chromatographic separation and bioassay-guided fractionation [13,19]. Repeated evaluation of the resulting subfractions would help identify the constituents responsible for inhibition and determine whether the observed activity is attributable to a dominant compound or the combined effects of multiple phytochemicals. Potential additive, synergistic, or antagonistic interactions should be experimentally evaluated rather than inferred from crude-extract inhibition alone [13,19].

Despite their promising percentage inhibition, the crude extracts exhibited substantially lower BChE-inhibitory potency than galantamine when evaluated on an IC_{50} basis. The leading fraction, methanolic *C. lanatus* (IC_{50} = 98 µg/mL), was approximately 27-fold less potent than galantamine (IC_{50} = 3.6 ± 0.1 µg/mL), as summarized in Table 2. This difference is consistent with the comparison of a purified reference inhibitor against chemically heterogeneous crude extracts containing variable proportions of active and inactive constituents. Importantly, the IC_{50} of a crude extract reflects its total mass concentration rather than the concentration of an individual inhibitory compound. Consequently, the observed potency difference does not exclude the possibility that certain isolated constituents may exhibit stronger inhibitory activity. However, increased potency following purification cannot be assumed, particularly when interactions among constituents contribute to the overall inhibitory response [13,19].

The relationship between percentage inhibition and IC_{50} requires careful interpretation. High inhibition at a single extract concentration does not necessarily indicate superior potency across the complete concentration–response range. Reliable potency comparisons require standardized assay conditions, appropriate concentration–response modeling, and assessment of experimental uncertainty. Furthermore, plant extracts may contain pigments, insoluble materials, or reactive constituents capable of interfering with spectrophotometric enzyme assays. Therefore, the observed responses should be verified using concentration-matched extract blanks, appropriate reference controls, and complementary methods where feasible. Such validation would strengthen confidence that the measured activity reflects genuine BChE inhibition rather than assay-related artifacts.

From a pharmacological perspective, the present findings provide preliminary evidence of BChE-inhibitory potential rather than demonstrating therapeutic effectiveness. Although cholinesterase inhibition is relevant to cholinergic modulation and has been investigated in relation to neurodegenerative disorders, biological activity in an isolated enzyme assay cannot establish efficacy in cellular, animal, or clinical systems. Further investigations should therefore assess inhibitory selectivity against acetylcholinesterase (AChE), enzyme inhibition kinetics, cytotoxicity, chemical stability, and the pharmacological characteristics of isolated constituents. For compounds intended to act within the central nervous system, blood–brain barrier permeability and the ability to achieve effective concentrations at the intended site of action would also require evaluation.

Collectively, these findings identify methanolic *C. lanatus*, ethyl acetate *C. maxima*, and chloroform *H. asiatica* as priority fractions for further phytochemical and pharmacological investigation. Subsequent studies should first confirm their inhibitory activities using independently prepared extracts, authenticated botanical materials, validated

concentration–response assays, and appropriately replicated experiments. Bioassay-guided chromatographic fractionation, followed by characterization using high-performance liquid chromatography (HPLC), liquid chromatography–mass spectrometry (LC–MS), and nuclear magnetic resonance (NMR) spectroscopy, may facilitate the identification and structural elucidation of active constituents [13,19]. Isolated compounds should subsequently undergo standardized potency, selectivity, and mechanistic investigations to establish their inhibitory characteristics and potential pharmacological relevance. Thus, the principal contribution of the present study lies in identifying promising botanical sources for targeted BChE inhibitor discovery rather than proposing crude plant extracts as direct alternatives to established cholinesterase-inhibiting medications.

5.3 Antioxidant activity and assay interpretation

DPPH radical-scavenging activity was generally modest among the investigated plant fractions and exhibited a solvent-dependent pattern distinct from butyrylcholinesterase (BChE) inhibition. Among the cucurbit seed extracts, the chloroform fraction of *C. sativus* demonstrated the strongest antioxidant activity, while selected *C. lanatus* fractions also showed measurable responses. However, the most active seed-derived fraction, chloroform *C. sativus* (IC_{50} = 642 µg/mL), was approximately 150-fold less potent than ascorbic acid (IC_{50} = 4.2 ± 0.2 µg/mL; Table 2). This substantial difference indicates relatively weak radical-scavenging potency under the tested conditions. Variations among petroleum ether, chloroform, ethyl acetate, and methanol fractions further suggest that antioxidant activity depends on the distribution and chemical properties of extracted metabolites rather than solvent polarity alone [9,10,14]. The contrasting DPPH and BChE inhibition profiles suggest that the two activities may be mediated by different phytochemical constituents. Radical-scavenging efficiency is influenced by molecular structure, electron- or hydrogen-donating capacity, solubility, and interactions with the assay medium. Consequently, strong BChE inhibition does not necessarily correspond to high antioxidant activity, and the presence of both activities within a fraction does not establish that they arise from the same compounds. Further phytochemical characterization and bioassay-guided fractionation are therefore necessary to identify the metabolites responsible for these responses [9,10,14].

The DPPH assay evaluates the capacity of compounds to reduce a stable synthetic radical through electron-transfer and/or hydrogen-atom-transfer reactions. Although widely employed for preliminary antioxidant screening, it does not reproduce the complex mechanisms of oxidative injury in biological systems, particularly neuronal tissues, where mitochondrial dysfunction, lipid peroxidation, endogenous antioxidant defenses, metabolism, and cellular bioavailability influence oxidative stress. Furthermore, extract pigmentation, turbidity, solvent composition, and reaction conditions may interfere with absorbance measurements at approximately 517 nm. Accordingly, appropriate sample blanks, standardized reaction conditions, and validated concentration–response analyses are essential for reliable interpretation. DPPH activity should therefore be regarded as evidence of chemical radical-scavenging capacity rather than direct proof of cellular antioxidant effects or neuroprotection [9,10,14].

The comparatively higher activity of chloroform *C. sativus* is consistent with previous reports describing antioxidant constituents in cucumber-derived materials [16,18]. Nevertheless, differences in plant parts, maturity, cultivation conditions, and extraction procedures may substantially influence phytochemical composition and antioxidant responses. As the present investigation focused on seed fractions, direct comparisons with studies examining fruit or whole-plant extracts require caution. The relatively stronger response of chloroform *C. sativus* warrants further phytochemical investigation, although its high IC_{50} indicates that substantial improvements in inhibitory concentration would be required to approach the radical-scavenging potency of the reference standard [16,18]. Future investigations should prioritize bioassay-guided fractionation and chemical characterization of the most active extracts using chromatographic and spectroscopic techniques. Complementary antioxidant assays, including ABTS, ferric-reducing antioxidant power (FRAP), and lipid-peroxidation inhibition, could provide a broader assessment of antioxidant properties. Subsequent cellular oxidative-stress models, supported by cytotoxicity assessment and appropriate experimental controls, would help establish whether the observed chemical activity translates into biologically relevant protection [9,10,14]. Overall, the findings identify chloroform *C. sativus* as the leading seed-derived DPPH-active fraction, although its potency remains substantially lower than that of ascorbic acid. The distinct antioxidant and BChE inhibition profiles highlight the importance of evaluating these activities independently. Thus, the present results provide a preliminary basis for phytochemical characterization and further biological validation rather than definitive evidence of therapeutic antioxidant or neuroprotective efficacy.

5.4 Relationship between BChE inhibition and antioxidant capacity

A notable outcome of the study was the absence of a simple parallel relationship between BChE inhibition and DPPH scavenging. Some fractions were strong enzyme inhibitors but only modest radical scavengers, whereas others showed better antioxidant activity without equivalent BChE inhibition. This divergence indicates that the two assays probably respond to different constituents or different chemical features within the same fraction. It also argues against attributing BChE inhibition merely to non-specific antioxidant capacity.

From a discovery perspective, selective and dual-activity fractions are both valuable. A selective BChE-inhibitory fraction may contain molecules with direct affinity for the enzyme, while a fraction active in both assays may provide compounds with complementary biochemical properties. Future studies should calculate correlations using the

complete concentration-response dataset, test purified subfractions in both assays, and examinewhethercombinationsofisolatedcompoundsproduceadditiveorsynergisticeffects.Suchworkwould establish whether the apparent dual activity arises from a single multifunctional molecule or from different constituents coexisting in the crude extract [8,13,14].

5.5 Scientific significance, limitations, and future directions

A major strength of this study is its comparative design, employing four sequential extraction solvents across multiple cucurbitaceous seed species and *H. asiatica*, followed by evaluation using two distinct biochemical assays. This plant-by-solvent approach enables identification of active species and solvent fractions for further investigation. Concentration-dependent assessments, triplicate measurements, and established reference compounds further strengthen its exploratory value. Nevertheless, several limitations should be acknowledged. Active constituents were neither isolated nor structurally characterized, extraction yields were unavailable, and complete raw absorbance data and inferential statistical analyses were not documented. The absence of voucher specimen numbers and reliance on market-sourced materials may introduce botanical variability. Moreover, the study was restricted to BChE inhibition and DPPH assays without assessing AChE selectivity, enzyme kinetics, cytotoxicity, blood–brain barrier permeability, cellular neuroprotection, pharmacokinetics, or in vivo efficacy.

The next stage should begin with independent replication using authenticated plant material, documented voucher specimens, extraction-yieldmeasurements, and standardized sample preparation. The leading fractions should then undergo bioassay-guided chromatography, followed by LC-MS/MS, HPLC, GC-MS where appropriate, and spectroscopic structure elucidation. Confirmed constituents should be tested in full BChE and AChE concentration-response assays, with kinetic analysis and orthogonal methods to exclude color or aggregation artifacts. Molecular docking may help generate mechanistic hypotheses, but predicted binding should be verified experimentally rather than treated as proof of inhibition.

Biological validation should proceed through neuronal or glial cell models assessing oxidative stress, mitochondrial function, cytotoxicity, and inflammatory responses, followed by suitable animal studies evaluating bioavailability, brain exposure, and safety. Such investigations would establish whether the observed in vitro activities translate into meaningful neuropharmacological effects. Overall, this study identifies promising fractions from underexplored cucurbitaceous seeds and *H. asiatica*, highlighting the influence of solvent polarity on biological activity and providing a foundation for further phytochemical characterization and pharmacological evaluation of potential BChE inhibitors and antioxidant compounds.

6. Conclusions

Sequential solvent extraction revealed clear species- and solvent-dependent differences in BChE inhibition and DPPH radical-scavenging activity. Methanolic *C. lanatus*, ethyl acetate *C. maxima*, and chloro form *H. asiatica* were the most notable BChE-inhibitory fractions, whereas chloro form *C. sativus* showed the strongest seed-derived DPPH response. The different rankings obtained in the two assays indicate that enzyme inhibition and chemical antioxidant activity were not governed by a single common pattern and may arise from distinct phytochemical constituents. The results support the use of cucurbitaceous seeds as underexplored sources of bioactive metabolites and confirm the importance of solvent selection during preliminary screening. At the same time, the crude extracts were much less potent than the reference compounds, andthe assays were limited to in vitro biochemical measurements. The findings therefore identify promising research leads rather than demonstrating clinical efficacy, disease modification, or suitability for direct therapeutic use in Alzheimer's disease. Future studies should prioritize reproducible preparation and bioassay-guided fractionation of the leading extracts,followed by chemicalidentification, BChE/AChE selectivity testing, enzyme kinetics, broader antioxidant evaluation, cytotoxicity assessment, and validation in cellular and in vivo models. By narrowing a large set of plant-solvent combinations to a manageable group of priority fractions, this study provides a practical starting point for discovering and characterizing plant-derived compounds with potential relevance to cholinergic dysfunction and oxidative stress in neurodegenerative research.

Declarations

Author contributions: Sankara Narayanan Senthilkumar: Conceptualization, Methodology, Investigation, Formal analysis, Writing – Original Draft. Mohamed Isthiyak M: Investigation, Validation, Data curation, Writing – Review & Editing. Bilqees Fatima: Literature review, Visualization, Writing – Review & Editing. Balaji Nagarajan: Conceptualization, Supervision, Project administration, Resources, Writing – Review & Editing. All authors reviewed and approved the final manuscript, including the revised title, text, figures, declarations, and authorship order.

Funding: This research received no external funding.

Institutional review board statement: Not applicable. The study involved plant extracts and commercially obtained enzyme reagents and did not report experiments involving human participants or live animals.

Informed consent statement: Not applicable.

Data availability: The data supporting the findings of this study are available from the corresponding author upon reasonable request. The raw data are securely maintained by the Post Graduate Department of Biotechnology,

Microbiology & Bioinformatics, National College, Tiruchirapalli, Tamil Nadu, India, and will be made available in accordance with institutional policies.

Acknowledgements: The authors gratefully acknowledge the Post Graduate Department of Biotechnology, Microbiology & Bioinformatics, National College, Tiruchirapalli, Tamil Nadu, India, and the Department of Biochemistry, Minhaj University, Lahore, Pakistan, for providing the facilities, laboratory resources, and institutional support necessary to conduct this study.

Conflicts of interest: The authors declare no conflict of interest.

Use of artificial intelligence tools: AI-assisted editorial tools were used for language improvement, structural reorganization, and journal formatting. The authors remain responsible for the accuracy of the scientific content, data, citations, authorship, and final conclusions.

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