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ORIGINAL ARTICLE

Cholinesterase Inhibitory and Antioxidant Activities of Seed Extracts from Cucurbitaceous Plants and *Hydrocotyle asiatica*: Potential Natural Therapeutics for Alzheimer's Disease

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Abstract

Neurodegenerative disorders such as Alzheimer's disease are closely associated with oxidative stress and cholinergic dysfunction. This study evaluated the cholinesterase inhibitory and antioxidant potential of sequential solvent extracts of seeds from *Cucurbita maxima, Cucumis melo, Cucumis sativus,* and *Citrullus lanatus*, along with *Hydrocotyle asiatica*. Butyrylcholinesterase (BChE) inhibition and radical scavenging activity were assessed in vitro at concentrations of 0.2, 0.5, and 1.0 mg·mL^{-1}. The methanolic seed extract of *C. lanatus* showed the strongest BChE inhibition with an IC_{50} of 98 µg·mL^{-1} and 81.19% inhibition at 1 mg·mL^{-1}. The ethyl acetate extract of *C. maxima* demonstrated 81.7% inhibition at the same concentration, while the chloroform extract of *H. asiatica* achieved 88.88% inhibition at 1 mg·mL^{-1}. Antioxidant assays revealed that the chloroform seed extract of *C. sativus* was the most active scavenger with an IC_{50} of 642 µg·mL^{-1}, followed by the ethyl acetate extract of *C. lanatus* (IC_{50} = 708 µg·mL^{-1}). In comparison, the positive controls galantamine and ascorbic acid exhibited IC_{50} values of 3.6 ± 0.1 µg·mL^{-1} and 4.2 ± 0.2 µg·mL^{-1}, respectively. These findings suggest that cucurbitaceous seeds and *H. asiatica* are promising sources of neuroprotective phytochemicals with dual cholinesterase inhibitory and antioxidant activities. Future studies should focus on bioassay-guided isolation and in vivo validation of the active constituents to confirm their therapeutic potential.

Keywords Alzheimer's disease, Antioxidant, Butyrylcholinesterase, DPPH, Extracts

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1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the leading cause of dementia worldwide, currently affecting over 33.9 million individuals globally, a number expected to nearly triple by 2065 due to aging populations (https://www.who.int/news-room/fact-sheets/detail/dementia). In the United States, approximately 7.2 million people aged 65 and older are living with AD, projected to rise to nearly 13 million by 2050, with women being disproportionately affected. The societal and economic burdens continue to escalate, costing over $400 billion annually in the U.S. alone (1, 2). Cholinesterases (ChEs), consisting of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), catalyze the hydrolysis of choline esters and regulate cholinergic neurotransmission in the brain (3). BChE, a serine esterase abundant in the brain and plasma, has emerged as an important target in AD therapy alongside AChE. Current treatment strategies largely focus on symptom management through ChE inhibitors and memantine, which improve cognitive function and quality of life (4). Moreover, advanced disease-modifying

therapies such as FDA-approved anti-amyloid monoclonal antibodies lecanemab and donanemab have demonstrated efficacy in slowing cognitive decline in early AD, signaling a shift towards targeting underlying pathologies (5).

Pathologically, AD is characterized by intracellular tau hyperphosphorylation and extracellular beta-amyloid deposition leading to synaptic dysfunction and neuronal loss (6). Recent advances in PET imaging and fluid biomarkers have enhanced early diagnosis and our understanding of disease progression. Research presented at the 2025 Human Amyloid Imaging conference highlights hybrid diagnostic models combining imaging and blood-based biomarkers for improved staging and patient stratification (7). In addition to pharmacologic advances, integrated, personalized treatment approaches incorporating lifestyle interventions, cognitive training, and caregiver support are increasingly recommended to manage AD's multifactorial nature effectively. Despite these strides, challenges remain in patient selection, treatment accessibility, and minimizing adverse effects, emphasizing the need for continued innovation and real-world evidence gathering (5, 7).

Cholinesterase inhibition has become one of the most-recommended treatment approaches for AD. In fact, cholinesterase inhibitors had been as well reported for their efficacy in some other diseases counting myasthenia gravies, glaucoma and Down syndrome, lately. So, these are main biological targets responsible for control of cholinergic conduction, and their inhibitors are employed for the management of Alzheimer's disease (8, 9). Studies on the relation between BChE and Alzheimer's disease had been done in terms of etiopathogenesis, predisposition and drug treatment. Changed levels of AChE and BChE were described in the cerebral cortex of the people with Alzheimer's disease (10). When the AChE Activity in individual's plasma was assessed with Alzheimer's disease, the intensity was inferior as compared to BChE; but the stage and patterns of the molecular forms were similar to those in individuals with silent BChE forms. A number of existing drugs for cure of AD target collectively AChE and BChE, but some are more discriminatory than others. Cholinesterase inhibitors contribute in the protection of the levels of the acetylcholine by inhibiting the enzymes concerned with its degradation, butyrylcholinesterase and acetylcholinesterase. This pharmacological action had an important function in several diseases, along with neurodegenerative diseases such as Alzheimer's (10, 11).

Antioxidants are the substances those inhibit the progression of oxidation, even at quite low concentrations and have diverse physiological role in the body. Many oxidative stress related diseases are as a result of accumulation of free radicals in the body. A lot of researches are going on worldwide directed towards finding natural antioxidants of plants origins. Oxidative stress is an important issue, and one that works at primary phases of Alzheimer's disease (AD) pathogenesis. The decrease of oxidative stress had been experienced as a therapy for AD. As the examination of vitamin E additives in somewhat severe AD is the most expectant so far, it was also accounted the limitations of general antioxidant therapies that essentially lower oxidative stress and then the difficulty of the redox system (10-12).

The aim of this study was to systematically evaluate the butyrylcholinesterase inhibitory and antioxidant activities of sequential solvent extracts from four cucurbitaceous seeds (*Cucurbita maxima, Cucumis melo, Cucumis sativus,* and *Citrullus lanatus*) and the medicinal herb *Hydrocotyle asiatica,* in order to identify natural sources of bioactive compounds with potential therapeutic relevance against Alzheimer's disease.

2. Materials and Methods

2.1. Plant Materials

Seeds of four plant species *Cucurbita maxima* (Pumpkin), Cucumis sativus (cucumber), *Cucumis melo* (melon), *Citrullus lanatus* (water melon) and another whole plant *Hydrocotyle asiatica* were purchased from local market of Gujrat, Punjab, Pakistan. All the samples were identified by Department of Botany, university of Gujrat by herbarium specialist using reference materials.

2.2 Preparation of Crude Extracts

All seeds and *Hydrocotyle asiatica* whole plant were dried in shade at room temperature. The dried material was grinded to fine powder and six grams of each sample sequentially extorted by petroleum ether (PE), chloroform (CHCl3) ethyl acetate (EtOAc), and methanol (MeOH) separately. All the samples were extracted by using a shaking incubator at room temperature for 24 hours. After obtaining the PE extracts, the extracts were filtered by using a filtration assembly and then organic phases were concentrated by using a rotary evaporator under vacuum. The residue of each PE extract was then extracted with CHCl3 and then with ethyl acetate and methanol respectively. The concentration of each extract was then adjusted to 1mg/ml by using concentrated samples of all extracts. This concentration was then successively adjusted to, 0.500 mg/ml and 0.200 mg/ml by successive dilutions.

2.3 Determination of Butyrylcholinesterase (BChE)

Inhibitory Activity

Butyrylcholinesterase inhibitory activity was measured by a modification Walsh (13) of Ellman method (14) with butyrylthiocholine chloride as a substrate. Briefly, in this method, a running DTNB solution was made by mixing 3.6mL of stock solution, consisting of 20mM NaHCO3 and 10 mM DTNB in 0.1 M phosphate buffer at pH 7.0, along with 96.4 mL of 0.1 M phosphate buffer at pH 8. The test was carried out by addition of 1.35 mL of buffered DTNB running solution (pH 8), 50 uL (0.4 unit) of BChE (from equine serum, SIGMA), 50 uL of sample (at different concentrations 0.200, 0.500 and 0.750 mg/ml) and 50 uL of aqueous butyrylthiocholine chloride (4 mM). The final reaction volume was 1.5 mL. The hydrolysis of butyrylthiocholine chloride was observed by the development of the yellow 5-thio-2-nitrobenzoate anion as a result of the thiocholines reaction with DTNB, catalyzed by enzyme.

After 15 min incubation at room temperature, the absorbance was measured at 412nm in 1 cm path length quartz cuvette by using spectrophotometer (think HS 3300). Inhibitory percentage of BChE was estimated by evaluation of rates of response of samples to blank sample (phosphate buffer with water, pH 8) using formula (E-S)/E × 100, where E is enzyme activity without test sample and S is the enzyme activity with test sample. All experiments were prepared in triplicates. Galantamine hydrobromide (from lycroris sp.) was procured from sigma and used as a reference.

2.4 DPPH Free Radical Scavenging Assay

Free radical scavenging activities of all the 24 extracts at different concentration in methanol were determined with 1, 1-diphenyl-2-picrylhydrazyl (DPPH) (SIGMA-ALORICH) by modification (15, 16). The 50 uL of each sample (at different concentrations; 0.200, 0.500 and 0.750 mg/ml) was mixed with 2 mL of 0.1 mM DPPH in ethanol. The reaction mixture was mixed well and incubated for 20 min at 37 Co. The absorbance was measured at 517 nm by using spectrophotometer (think HS 3300). Percentage radical scavenging activity was measured by comparing the samples with blank sample (ethanol), by using the formula:

Percentage radical scavenging activity = (C-S)/C× 100

Where C is DPPH solution without sample and S is the DPPH with sample. All the reactions were done in triplicates. Ascorbic acid was used as a reference at three different concentrations.

3. Results and Discussion

Butyrylcholinesterase has reduced hydrolyzing power against acetylcholine but act as backup stimulant for the homologous AChE. This result in playing a vital role in aggravating Alzheimer's, even at very low concentration of AChE (17). Therefore, BChE inhibition appeared to be another approach to mediate the development of AD. In our study, most of the seed extracts with different solvents exhibited low to good butyrylcholinesterase inhibition except Cucumis melo chloroform extract that did not reveal any activity at all three concentrations (Figure. 1). Overall, out of 24 extracts, the best inhibitory activity was showed by methanol extracts while the least active were with chloroform extracts. There was another study conducted on Rosmarinus officinalis L. extracts resulted in same pattern of inhibition with same solvents. The maximum inhibition was by methanol extract while there was no inhibition by chloroform extract (18). Therefore, Butyrylcholinesterase (BChE) inhibition is highly significant in Alzheimer's disease (AD) because, at the later stages of AD, acetylcholinesterase (AChE) activity can decrease by up to 85%. When this occurs, BChE becomes the dominant enzyme responsible for hydrolyzing acetylcholine in the brain. Notably, BChE concentration and activity increase during this advanced stage, thereby maintaining the hydrolysis of acetylcholine despite the loss of AChE. This compensatory role of BChE makes it a critical therapeutic target for AD, as inhibiting BChE can help preserve acetylcholine levels, potentially improving cognitive function when AChE activity is severely diminished (19, 20).

In the petroleum ether extracts, the highest butyrylcholinesterase (BChE) inhibition was observed with *Cucumis melo* seeds at 1 mg/mL, exhibiting an IC50 of 565 µg/mL. Chloroform extracts of all seeds showed low BChE inhibition across tested concentrations, with the highest activity displayed by *Cucumis sativus* at 24.56% inhibition. Ethyl acetate extracts demonstrated moderate to good BChE inhibition, with *Cucurbita maxima* seed extract exhibiting the highest inhibition of 81.77% at 1 mg/mL. Methanol extracts showed consistently good inhibitory activity, with the extract comprising a mixture of all seeds yielding the highest BChE inhibition. *Hydrocotyle asiatica* extracts exhibited moderate to good inhibitory effects against BChE with different solvents. Notably, the chloroform extract of *Hydrocotyle asiatica* presented maximum inhibition (88.88% at 1 mg/mL), contrasting with the low inhibition observed in seed chloroform extracts. Additionally, a study on *Salvia* species supports these findings, reporting maximum butyrylcholinesterase inhibition by chloroform extracts of whole plants (19, 21)

The chloroform extract exhibited an IC50 of 161 µg/mL against

butyrylcholinesterase, indicating significant inhibitory activity. In Alzheimer's disease, oxidative stress is recognized as a crucial pathogenic element and has been considered a key therapeutic target for disease modification and potential cure (22). The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was used to estimate the antioxidant potential of the extracts because it is a widely employed and reliable method to evaluate antioxidant activity. The DPPH radical is a stable free radical that undergoes a gradual weakening upon reaction with antioxidant compounds that are sufficiently potent hydrogen donors. Such antioxidants possess promising physiological relevance due to their capacity to stoichiometrically react with and neutralize DPPH radicals, leading to a measurable color change from deep violet to pale yellow (15, 23). The method is advantageous because it accommodates both hydrophilic and lipophilic antioxidants and provides reproducible, easily interpretable results without the need for radical generation (15, 23).

This assay specifically measures the hydrogen atom or electron-donating ability of extracts to the strong DPPH radical generated in solution, leading to a measurable color change from purple to pale yellow. The extent of this discoloration is proportional to the antioxidant capacity of the sample, making the DPPH assay a convenient and widely recognized method for antioxidant evaluation (15, 23).

Most of our seed extracts and *Hydrocotyle asiatica* extracts obtained with different solvents exhibited poor inhibition of DPPH radicals (Figure 2). Among seed extracts, the highest antioxidant activity was observed with petroleum ether extracts, while methanol extracts showed the least activity. The pattern of antioxidant activity for seed extracts was petroleum ether > chloroform > ethyl acetate > methanol. This finding contrasts with a study on *Salvia* species, where the antioxidant potential followed the order methanol > ethyl acetate > chloroform > petroleum ether. The substantial antioxidant activity of *Hydrocotyle asiatica* in chloroform extract is consistent with reports indicating the presence of triterpene glycosides, such as Asiatic acid, contributing to its antioxidant effects (24).

In the present study, maximum free radical scavenging activity was observed with *Cucumis sativus* chloroform extract, showing 63.78% inhibition at 1 mg/mL, while *Citrullus lanatus* exhibited the highest activity at 0.2 mg/mL with 54.06% inhibition of DPPH. These findings suggest that these extracts may have physiological relevance in living systems. Supporting this, a study on *Centella asiatica* reported that a crude methanol decoction administered at 50 mg/kg daily for two weeks significantly improved antioxidant enzyme activities *in vivo* (25, 26). The free radical scavenging activity of the fruit extract of *Cucumis sativus* at 500 µg/mL concentration was 56.15% against the DPPH radical, indicating significant antioxidant potential. This activity is largely attributed to phytochemicals such as flavonoids and tannins, which efficiently donate hydrogen atoms to neutralize free radicals. The antioxidant capacity of seed extracts was comparatively low, correlating with their reduced extract yield, whereas flesh extracts displayed superior activity. These findings align with earlier studies demonstrating the antioxidative and anti-inflammatory properties of *Cucumis sativus* fruit extracts, which contain bioactive compounds such as cucurbitacins and phenolics contributing to their efficacy (25, 27, 28). Further investigation into these phytochemicals may support development of therapeutic agents derived from this common vegetable.

This study provides a novel and systematic evaluation of cholinesterase inhibitory and antioxidant activities in seed extracts of cucurbitaceous plants; an underexplored plant part compared to leaves and peels. The use of sequential solvent extraction highlighted the role of solvent polarity in modulating bioactivity, with methanol and chloroform fractions showing the strongest effects. The inclusion of *Hydrocotyle asiatica* as a reference strengthened the pharmacological context, while comparative analysis with published data highlighted the novelty of the findings. Importantly, the study generated quantitative IC_{50} values for several extracts, providing a useful benchmark for future phytochemical and pharmacological research on neuroprotective agents.

However, the scope of the study was limited to in vitro assays, which do not fully capture the complexity of in vivo biological systems. The active phytoconstituents responsible for the observed activities were not isolated or structurally identified, and the mechanisms underlying their effects remain to be clarified. Although experiments were performed in triplicate, larger datasets and detailed dose–response curves could yield more precise potency estimates. Furthermore, while reference standards were used for comparison, advanced mechanistic approaches such as enzyme kinetics or molecular docking were beyond the present scope and are recommended for future studies.

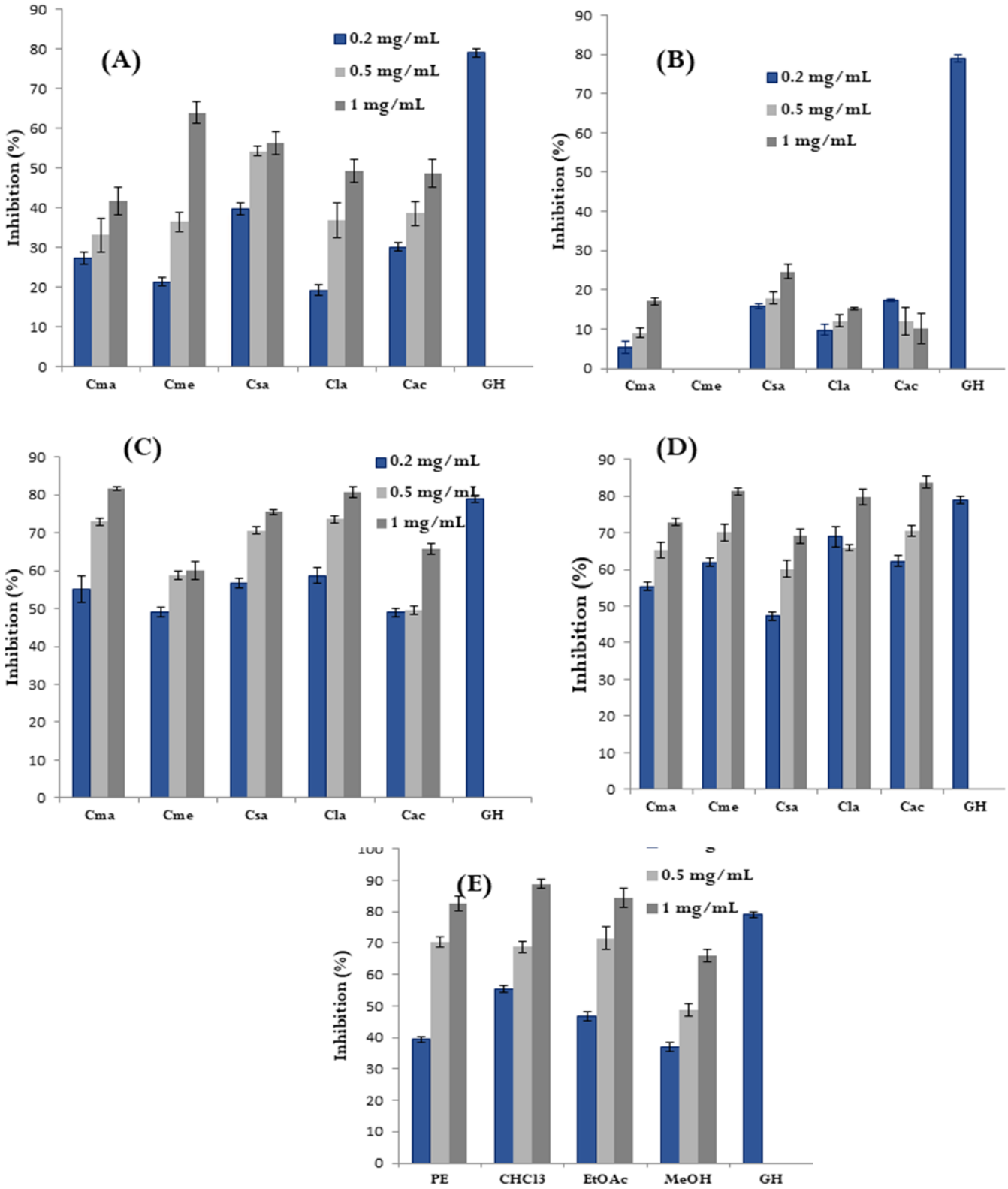

Figure 1. Anti butyrylcholinesterase activity of seeds and *Hydrocotyle Asiatica* with petroleum ether, chloroform, ethyl acetate, and methanol extracts. (Cma= *Cucurbita maxima*, Cme= *Cucumis melo*, Csa= *Cucumis sativus*, Cla=*Citrullus lanatus*, PE= Petroleum ether fig A, CHCl3=Chloroformed fig B, Ethyl acetate fig C, MeOH=methanol fig D, *Hydrocotyle Asiatica* fig E

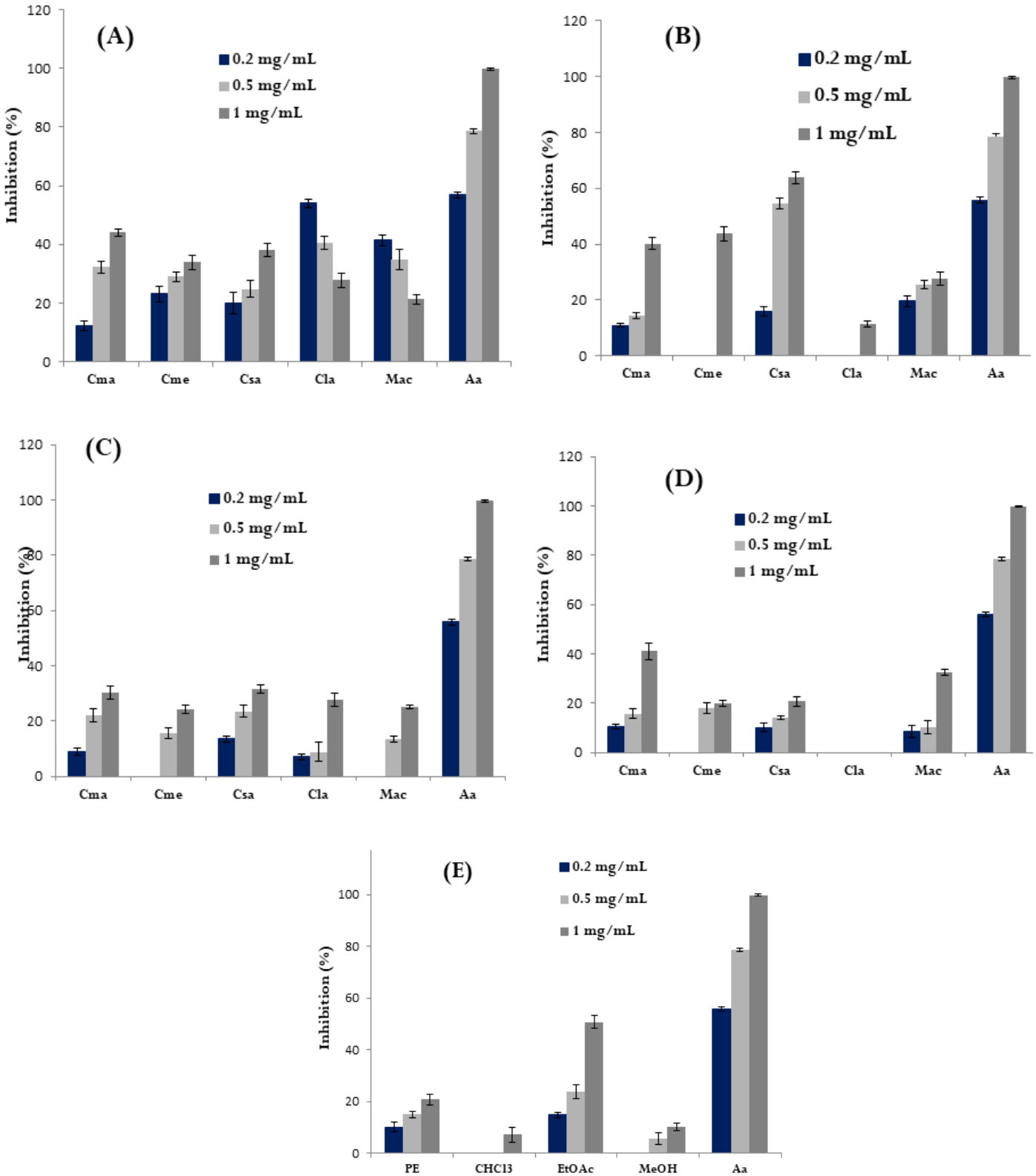

Figure 2. DPPH activity of seeds and *Hydrocotyle Asiatica* with petroleum ether, chloroform, ethyl acetate, and methanol extracts. (Cma= *Cucurbita maxima*, Cme= *Cucumis* melo, Csa= *Cucumis sativus*, Cla=*Citrullus lanatus*, PE= Petroleum ether fig A, CHCl3=Chloroformed fig B, Ethyl acetate fig C, MeOH=methanol fig D, Hydrocotyle Asiatica fig E.

4. Conclusion

This study demonstrates that cucurbitaceous seed extracts and *Hydrocotyle asiatica* possess significant cholinesterase inhibitory and antioxidant activities, with solvent polarity strongly influencing bioactivity. The methanolic seed extract of *C. lanatus* exhibited the highest BChE inhibitory effect (IC_{50} = 98 μg·mL^{-1}, 81.19% at 1 mg·mL^{-1}), while the chloroform extract of *H. asiatica* showed 88.88% inhibition at 1 mg·mL^{-1}, validating its traditional use in neurological disorders. The ethyl acetate extract of *C. maxima* also demonstrated potent inhibition (81.7% at 1 mg·mL^{-1}). In antioxidant assays, the chloroform seed extract of *C. sativus* was the most effective (IC_{50} = 642 μg·mL^{-1}), followed by the ethyl acetate extract of *C. lanatus* (IC_{50} = 708 μg·mL^{-1}). Although less potent than reference standards galantamine (IC_{50} = 3.6 μg·mL^{-1}) and ascorbic acid (IC_{50} = 4.2 μg·mL^{-1}), these extracts represent valuable natural leads. Collectively, these results highlight seeds as underexplored reservoirs of neuroprotective agents, supporting their potential role in managing Alzheimer's disease. Future investigations should include phytochemical profiling by advanced chromatographic techniques to identify bioactive compounds. Furthermore, in vivo pharmacological studies are essential to validate their safety and efficacy in neurodegenerative disease models.

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Data Availability Statement: The data supporting the findings of this study are available in the Lab manual at 1Department of Biochemistry and Molecular Biology, University of Gujrat, Gujrat, Pakistan.

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Conflicts of Interest: Authors declare there is no conflict of interest.

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