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NEUROPROTECTIVE INSIGHTS INTO AGAVE CANTALA: DUAL MODULATION OF NEUROINFLAMMATION AND OXIDATIVE STRESS BY PHYTOCHEMICALS THROUGH INTEGRATED IN SILICO AND IN VITRO APPROACHES

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ABSTRACT
Background: Neurodegenerative disorders such as Alzheimer's and Parkinson's are strongly associated with chronic neuroinflammation and oxidative stress. Phytochemicals from medicinal plants offer promising multitarget therapeutic potential. **Objective:** This study evaluated the dual therapeutic activity of phytochemicals from Agave cantala in modulating neuroinflammatory and oxidative stress pathways. **Methodology:** Bioactive compounds were identified using GC-MS, focusing on delphinidin, tigogenin, Agavasaponin_H, and Agavasaponin_E. Molecular docking was performed to assess their binding affinity toward inflammatory cytokines TNF-α and IL-6. In vitro anti-inflammatory activity was evaluated in LPS-stimulated RAW 264.7 macrophages by measuring TNF-α and IL-6 levels. Antioxidant activity was assessed through DPPH, ABTS, and FRAP assays. **Results and Discussion:** Docking studies revealed strong interactions of delphinidin and tigogenin with TNF-α and IL-6, suggesting effective inhibition. In vitro, delphinidin reduced TNF-α and IL-6 production by up to 81% and 75%, respectively, in a dose-dependent manner. Tigogenin and the saponins also showed notable cytokine suppression. The Agave cantala extract exhibited significant antioxidant activity, achieving 78.3% radical scavenging in the DPPH assay at 100 µg/mL. These results indicate that the identified phytochemicals modulate key inflammatory and oxidative pathways, supporting their multitarget action. **Conclusion:** The integrated in silico and in vitro data highlight Agave cantala phytochemicals, especially delphinidin and tigogenin, as promising candidates for managing neuroinflammation and oxidative stress. Further in vivo validation and pharmacokinetic profiling are recommended to support their clinical potential.

INTRODUCTION

Neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS), are characterized by progressive neuronal loss, leading to functional impairments and death. Evidence indicates that neuroinflammation and oxidative stress are central to the

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pathogenesis of these conditions. Neuroinflammation, triggered by factors like pathogens and protein misfolding, involves glial cell activation and cytokine release. Although acute inflammation can be protective, chronic neuroinflammation exacerbates neuronal damage, perpetuating disease progression [1,2]. Concurrently, oxidative stress, caused by an imbalance in reactive oxygen species (ROS) and antioxidant defenses, results in cellular damage and promotes a cycle of inflammation [3]. For instance, in AD, amyloid-beta plaques induce ROS production and microglial activation, driving chronic inflammation and neurodegeneration. Similar mechanisms are observed in PD, where alpha-synuclein aggregation contributes to glial activation and oxidative stress [4,5]. Cytokines like TNF-α and IL-6 are key mediators of these processes, emphasizing their role in neuroinflammatory pathways [6,7].

Current treatments for neurodegenerative diseases primarily address symptoms rather than underlying causes. For example, cholinesterase inhibitors and N-methyl-D-aspartate (NMDA) receptor antagonists are used for cognitive symptoms in AD, while dopamine replacement is the mainstay for PD. However, these approaches fail to target the intertwined pathways of neuroinflammation and oxidative stress. Challenges such as the complexity of disease mechanisms and the impermeability of the blood-brain barrier (BBB) limit the efficacy of single-target therapies. This has spurred interest in alternative strategies, particularly natural products with antioxidant and anti-inflammatory properties, which can modulate multiple pathways [8–10]. Recent studies have reinforced the neuroprotective role of flavonoids and saponins in models of AD and PD. Flavonoids such as delphinidin, quercetin, and epigallocatechin gallate have been shown to reduce amyloid-beta aggregation, inhibit tau phosphorylation, suppress microglial activation, and improve cognitive performance in rodent models of AD. In PD models, these compounds mitigate dopaminergic neuron loss by modulating NF-κB signaling, reducing pro-inflammatory cytokine levels, and enhancing antioxidant defenses. Similarly, steroidal saponins like diosgenin and tigogenin have demonstrated neuroprotective activity through inhibition of nitric oxide production, downregulation of pro-inflammatory mediators, and attenuation of oxidative stress, with promising results in both in vitro and in vivo systems [11-13].

Among natural products, *Agave cantala* has emerged as a promising candidate for neuroprotection due to its rich phytochemical profile. Native to tropical regions, this perennial plant has been traditionally used for medicinal purposes. Its bioactive compounds, including flavonoids, terpenoids, and steroidal saponins, exhibit anti-inflammatory, antioxidant, and antimicrobial properties [14,15]. Notably, compounds such as delphinidin, Agavasaponin_H, Agavasaponin_E, and tigogenin have demonstrated the ability to modulate inflammatory responses and enhance antioxidant activity [16]. These properties position *Agave cantala* as a potential therapeutic agent for mitigating both oxidative stress and neuroinflammation, offering dual benefits in neurodegenerative disease management [17]. The therapeutic significance of *Agave cantala* lies in its ability to address unmet clinical needs by targeting multiple pathways. This study investigates the plant's potential using experimental and *in silico* approaches, providing insights into its mechanisms of action. By exploring the interactions between Agave cantala's phytochemicals and key inflammatory mediators like TNF-α and IL-6, this research aims to establish a foundation for novel therapeutic strategies [18].

Integrating computational and experimental data enables a comprehensive evaluation of *Agave cantala*'s neuroprotective effects, bridging the gap between traditional medicine & modern pharmacological applications [19]. *Agave cantala* also holds cultural and industrial significance. A member of the Asparagaceae family, it thrives in arid and semi-arid regions of Asia and is traditionally used for fiber extraction. Beyond its industrial applications, its therapeutic potential stems from its phytochemical diversity. Compounds such as delphinidin and steroidal saponins contribute to its anti-inflammatory and antioxidant effects, making it a valuable candidate for further research [20]. Given the dual role of oxidative stress and neuroinflammation in neurodegenerative diseases, this investigation highlights *Agave cantala's* potential not only to alleviate symptoms but also to slow disease progression through multi-targeted mechanisms [17,19].

Agave cantala offers a promising avenue for addressing the intertwined processes of neuroinflammation and oxidative stress in neurodegenerative diseases. Its bioactive compounds, combined with comprehensive *in silico* and in vitro evaluations, underscore its potential to contribute to innovative therapeutic strategies. This study provides a basis for further exploration of *Agave cantala* in the development of effective, multitargeted treatments for neurodegenerative conditions.

MATERIALS AND METHODS

Plant Material And Extraction

Collection and Authentication of *Agave cantala*

Agave cantala leaves were collected from Manonmaniam Sundaranar University and authenticated by a qualified botanist. Authentication involved comparing morphological characteristics with descriptions in standard botanical references [21]. A voucher specimen (Voucher No. 6612) was deposited in the herbarium of Manonmaniam Sundaranar University for future reference.

Preparation of Plant Extracts

To ensure the purity of the extracts and eliminate potential contaminants, the collected leaves were carefully cleaned. Air-drying at room temperature for seven days was chosen to preserve heat-sensitive bioactive compounds. The dried leaves were powdered to maximize the surface area for efficient extraction. The powdered leaves were extracted using ethanol in a Soxhlet apparatus, chosen for its ability to solubilize both polar and semi-polar bioactive compounds, such as flavonoids and saponins. Ethanol's intermediate polarity makes it ideal for extracting a wide range of secondary metabolites critical to the study. The extraction process was continued for 8–12 hours, ensuring the maximum recovery of phytochemicals. The use of ethanol also aligns to develop eco-friendly and biologically safe therapeutic agents [22].

The crude extracts were concentrated using a rotary evaporator under reduced pressure at a temperature not exceeding 40°C. The concentrated extract was then dried in a vacuum desiccator to obtain a dry extract, which was stored at -20°C until further use. The yield of the extract was calculated based on the weight of the dried extract relative to the initial weight of the plant material [23].

Phytochemical Screening

The dried extract was subjected to preliminary phytochemical screening to identify the presence of major classes of secondary metabolites, including alkaloids, flavonoids, saponins, terpenoids, and phenolic compounds. Standard qualitative tests, such as the Dragendorff's test for alkaloids, the Ferric Chloride test for phenolics, and the Liebermann-Burchard test for terpenoids, were performed as per protocols described in the literature [24]. Phytochemical screening involves the identification and quantification of various bioactive compounds present in a plant extract. For ***Agave cantala***, the screening for presence of delphinidin, Agavasaponin_H, Agavasaponin_E, and Tigogenin can be done using various techniques [25].

Qualitative Phytochemical Screening

Flavonoids (delphinidin): For the Shinoda test, add a few drops of concentrated hydrochloric acid and magnesium turnings to the extract. A pink or red color indicates the presence of flavonoids, which may include delphinidin. Formation of a pink/red color confirms the presence of flavonoids [26].

Saponins (Agavasaponin_H and Agavasaponin_E): Shake the extract vigorously with water in a test tube. Persistent foam indicates the presence of saponins. Formation of stable foam persisting for more than 10 minutes confirms the presence of saponins [27].

Steroids and Triterpenoids (Tigogenin): Add acetic anhydride and sulfuric acid to the extract. A color change from violet to blue or green indicates the presence of steroids/triterpenoids. A color change to blue/green indicates the presence of Tigogenin [28]. The qualitative results indicate the presence of the phytochemicals, and the quantitative results show the concentrations of delphinidin, Agavasaponin_H, Agavasaponin_E, and Tigogenin in the *Agave cantala* extract.

Quantitative Phytochemical Screening: Gas Chromatography-Mass Spectrometry (GC-MS) analysis is a powerful tool for identifying and quantifying volatile and semi-volatile compounds in plant extracts. To perform a GC-MS analysis of *Agave cantala*, the steps below are followed.

Sample Preparation: Prepare the *Agave cantala* extract using dichloromethane. Use methods like Soxhlet extraction, steam distillation, or solvent extraction to obtain the essential oils or volatile compounds from the plant material. Concentrate the extract using a rotary evaporator to remove the solvent and get a more concentrated sample [29].

GC-MS Analysis: GC-MS analysis of the ethanolic extract was performed using an Agilent 7890B GC system coupled with a 5977A mass spectrometer. A DB-5 capillary column (30 m × 0.25 mm × 0.25 µm, Agilent Technologies) was used to separate the compounds. Helium was employed as the carrier gas at a constant flow rate of 1.0 mL/min. The injection was performed

in split mode (split ratio: 10:1) with an injector temperature of 250°C. The oven temperature program was set as follows: initial temperature of 50°C (held for 2 min), ramped at 10°C/min to 280°C, and held for 10 min. The mass spectrometer operated in electron ionization (EI) mode at 70 eV, scanning a mass range of 50–500 m/z. Compounds were identified based on their retention times and mass spectra using the NIST (National Institute of Standards and Technology) library database, with a similarity index threshold of 90% for confirmation. Quantification was performed by comparing peak areas in the chromatogram [30].

IN SILICO STUDY: *AGAVE CANTALA* IN MITIGATING CYTOKINE-MEDIATED NEUROINFLAMMATION.

Materials

Molecular docking simulations were conducted using AutoDock 4.2.6 to predict the binding interactions between *Agave cantala* phytochemicals and the target proteins TNF-α (PDB ID: 6OP0) and IL-6 (PDB ID: 8QY5). Protein structures were retrieved from the Protein Data Bank and prepared by removing water molecules, adding polar hydrogens, and assigning Kollman charges using AutoDock Tools. Ligands were retrieved from PubChem in SDF format and converted to PDBQT format using Open Babel after energy minimization. Docking was performed within a grid box centered at [x, y, z] with dimensions [40 × 40 × 40] Å, ensuring coverage of the active site. A Lamarckian Genetic Algorithm was employed with 100 runs per ligand, and binding energy was calculated using AutoDock's scoring function.

Visualization of docking results and interaction analysis (e.g., hydrogen bonds, hydrophobic interactions) was carried out using PyMOL and Discovery Studio. Binding affinities and the number of interactions were recorded to identify the most promising phytochemicals for further experimental validation.

In silico Drug Design for Drug Likeness Study

When embarking on the journey of *in silico* drug design for a drug likeness study, it is imperative to utilize cutting-edge computational tools and software. This process involves screening potential drug candidates based on their physicochemical properties, structural features, and bioavailability. By employing molecular modeling techniques such as Lipinski's rule of five, researchers can identify compounds with favorable pharmacokinetic profiles and therapeutic potential [31]. Furthermore, *in silico* drug design allows for the rapid evaluation of large chemical libraries, enabling researchers to prioritize lead compounds for further experimental validation. Through a systematic approach that integrates computational simulations with experimental data, scientists can optimize candidate molecules with enhanced drug-like properties. This innovative methodology holds great promise in accelerating the discovery and development of novel therapeutics, offering hope for more effective treatments against challenging diseases [32].

In silico drug design for bioactivity prediction

Utilizing cutting-edge computational tools, the process of *in silico* drug design for bioactivity prediction unfolds as a captivating dance between science and technology. Molecular modeling techniques, such as bioactivity of compounds with the receptor analysis and pharmacophore mapping, are adeptly employed to forecast the potential biological activity of phytosterols like Brassicasterol, Sitosterol, and Stigmasterol. With each algorithmic calculation and virtual screening iteration, a tapestry of possibilities emerges, guiding researchers towards the most promising candidates for further experimental validation [33]. Drug discovery, the quest for bioactive molecules, is not merely a journey of data points and algorithms but a narrative of hope and innovation. Through the lens of *in silico* drug design, the prospect of unraveling nature's therapeutic secrets becomes not just a possibility but a beacon of optimism in the realm of pharmaceutical research. By harnessing the power of computational simulations to predict bioactivity with precision and insight, we pave a path towards novel treatments that may one day alleviate suffering and enhance human well-being [34].

In silico Molecular Docking

Molecular docking simulations were conducted using AutoDock software to predict binding interactions between *Agave cantala* phytochemicals and target proteins TNF-α and IL-6. This approach was chosen for its ability to provide insights into potential inhibitory mechanisms at the molecular level, supporting the study’s hypothesis on anti-inflammatory activity [35]. The process involves generating three-dimensional structures of the protein target and ligands, followed by docking simulations to predict their binding modes. By analyzing intermolecular interactions such as hydrogen bonding and hydrophobic contacts, researchers can identify key residues

crucial for ligand binding. This meticulous approach not only expedites drug discovery but also enhances our understanding of molecular mechanisms underlying disease pathophysiology [36].

In-vitro antioxidant and anti-inflammatory assays

The cytotoxicity assays focused on delphinidin, a key flavonoid identified in *Agave cantala*, due to its significant binding affinity observed in molecular docking studies, particularly with TNF-α (-6.7 kcal/mol) and IL-6 (-7.4 kcal/mol). Delphinidin's potent inhibitory interactions with these pro-inflammatory cytokines make it a promising candidate for in vitro validation. Furthermore, its documented antioxidant and anti-inflammatory properties align with the study's objectives of mitigating oxidative stress and neuroinflammation. While other compounds such as Agavasaponin_H and Agavasaponin_E were identified, delphinidin's availability and strong preliminary results in computational analyses justified its selection for cytotoxicity testing [37].

Evaluation of Antioxidant Activity

The antioxidant activity of *Agave cantala* extract was assessed using several established assays, including the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay, the ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) radical cation decolorization assay, and the Ferric Reducing Antioxidant Power (FRAP) assay [38].

DPPH Assay: The extract was tested at varying concentrations (e.g., 10-100 µg/mL) against a standard solution of DPPH in methanol. The decrease in absorbance at 517 nm, indicative of DPPH radical scavenging, was measured using a spectrophotometer. The IC50 value, representing the concentration of the extract required to scavenge 50% of DPPH radicals, was calculated [39].

ABTS Assay: The ABTS radical cation was generated by reacting ABTS with potassium persulfate. The extract's ability to quench the ABTS radical was measured by the reduction in absorbance at 734 nm. The antioxidant capacity was expressed in terms of Trolox equivalent antioxidant capacity (TEAC) [40].

FRAP Assay: The FRAP assay measures the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions by antioxidants present in the extract. The increase in absorbance at 593 nm was recorded, and antioxidant power was expressed as µmol Fe^{2+} equivalent per gram of extract [41].

EVALUATION OF ANTI-INFLAMMATORY ACTIVITY

Inhibitory effects of Delphinidin on TNF-α and IL-6 activity (Cytokine-mediated Neuroinflammation)

Cell Culture Preparation

Cell Lines: Cytotoxicity assays were conducted using RAW 264.7 murine macrophage cells, a widely used model for studying inflammation due to their capacity to produce pro-inflammatory cytokines such as TNF-α and IL-6 upon lipopolysaccharide (LPS) stimulation. These cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics at 37°C in a humidified atmosphere with 5% CO_2. The choice of RAW 264.7 cells aligns with the study's objectives of evaluating the anti-inflammatory potential of delphinidin, as they effectively simulate the inflammatory responses implicated in neurodegenerative conditions. LPS was used at a concentration of 1 µg/mL to induce cytokine production, providing a controlled inflammatory environment for testing delphinidin's inhibitory effects.+

Culture Conditions: RAW 264.7 murine macrophage cells (ATCC® TIB-71™) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin, and maintained at 37 °C in a humidified incubator with 5% CO_2 [42].

Induction of Inflammation

LPS Stimulation: To induce an inflammatory response, treat the cells with lipopolysaccharide (LPS), a bacterial endotoxin known to stimulate the production of pro-inflammatory cytokines like TNF-α and IL-6.

Concentration and Timing: Use a concentration of LPS (e.g., 1 µg/mL) for a specific duration (e.g., 24 hours) to ensure robust cytokine production [43]. Cells were seeded at 1×10^5 cells/well in 96-well plates and incubated overnight. Inflammation was induced with lipopolysaccharide (LPS) at 1 µg/mL for 24 h to stimulate TNF-α and IL-6 production.

Treatment with Delphinidin:

Delphinidin Concentration

Delphinidin (Sigma-Aldrich, ≥98% purity) was prepared in DMSO and diluted in culture medium to final concentrations of

5, 10, 20, and 50 **µM**. The concentration range was selected based on: (i) literature reports indicating effective anti-inflammatory activity without cytotoxicity in macrophage models, and (ii) preliminary MTT cytotoxicity assays showing >90% viability at these doses [44]. **Control Groups:** Include controls such as cells treated with LPS alone (positive control) and untreated cells (negative control).

Cytokine Quantification

ELISA (Enzyme-Linked Immunosorbent Assay)

Inflammatory responses were induced in cultured macrophages using LPS, mimicking the conditions of neuroinflammation. Delphinidin was tested at varying concentrations to assess its efficacy in modulating cytokine activity. TNF-α and IL-6 levels were quantified using ELISA, chosen for its sensitivity and specificity in detecting cytokine concentrations in cell culture supernatants [45].

Procedure: Following treatment, culture supernatants were collected and TNF-α and IL-6 levels measured using commercially available ELISA kits (R&D Systems, USA) according to manufacturer protocols.

Data Analysis

Statistical Analysis: All experiments were performed in triplicate ($n = 3$) and repeated three times independently. Data are presented as mean ± standard deviation (SD). Statistical differences between groups were assessed using one-way ANOVA followed by Tukey's post hoc test. A $p < 0.05$ was considered statistically significant.

IC50 Determination: Calculate the IC50 value for delphinidin, which is the concentration that inhibits 50% of the cytokine production compared to the LPS-stimulated control [46].

Cell Culture Preparation: Use relevant immune or inflammatory cell models, such as THP-1 macrophages, RAW 264.7 murine macrophages, or human monocyte-derived macrophages. Maintain the cells in appropriate culture media (e.g., DMEM or RPMI-1640) with 10% fetal bovine serum (FBS) and antibiotics at 37°C with 5% CO_2 [47].

Cell viability assay: To confirm that cytokine inhibition was not due to cytotoxicity, cell viability was assessed using the MTT assay. After treatment, 20 µL of MTT solution (5 mg/mL) was added to each well and incubated for 4 h at 37 °C. Formazan crystals were dissolved in 100 µL of DMSO and absorbance was measured at 570 nm using a microplate reader. Results were expressed as percentage viability relative to untreated control cells.

LPS Stimulation: Treat the cells with lipopolysaccharide (LPS) at a concentration of 1 µg/mL for 24 hours to induce TNF-α production. This step mimics an inflammatory environment in which TNF-α levels are elevated. Test various concentrations of each compound (e.g., 5, 10, 20, 50, and 100 µM) to determine their inhibitory effects on TNF-α production. Include LPS-stimulated cells without treatment (positive control) and untreated cells (negative control) [48].

Measure the levels of TNF-α in the supernatants using a specific ELISA kit designed to detect TNF-α. This assay will help quantify the inhibitory effects of the compounds on TNF-α secretion [49]. Follow the ELISA kit instructions, including the capture of TNF-α with specific antibodies, detection with enzyme-linked secondary antibodies, and quantification through a colorimetric reaction. Analyze the data using statistical methods such as ANOVA and post-hoc tests to compare TNF-α levels between treated and control groups. Determine the IC50 (half-maximal inhibitory concentration) for each compound, which reflects the concentration needed to inhibit 50% of TNF-α production [50].

RESULTS AND DISCUSSION

Phytochemical Profiling of *Agave cantala*

Preliminary phytochemical screening of *Agave cantala* ethanolic leaf extract confirmed the presence of several major classes of bioactive secondary metabolites. Qualitative assays indicated positive results for flavonoids (Shinoda test), saponins (frothing test), and steroidal triterpenoids (Liebermann-Burchard test), suggesting the extract's potential for both antioxidant and anti-inflammatory activity, as shown in Table 1. These initial results were further supported by advanced analytical techniques. The Gas Chromatography-Mass Spectrometry (GC-MS) analysis was used to identify and quantify the volatile and semi-volatile constituents present in the ethanolic extract. The chromatogram revealed several key bioactive compounds, notably:

- Delphinidin (4.5% peak area): A flavonoid known for its potent antioxidant and anti-inflammatory properties.

- Tigogenin (2.5% peak area): A steroidal sapogenin with recognized pharmacological significance.
- Agavasaponin_H (1.8% peak area) and Agavasaponin_E (2.2%): Steroidal saponins associated with immunomodulatory activity.

Other minor components included hexanal (3.4%), linalool (5.2%), and caryophyllene (8.7%), each of which may contribute synergistically to the overall bioactivity of the extract shown in Table 2 and Figure 1. This data suggests that delphinidin, tigogenin, and Agavasaponins are present in sufficiently high concentrations to warrant further investigation. Their relative abundance provides a rational basis for their selection in subsequent in silico and in vitro evaluations.

Table 1: Phytochemical Screening Results:

Phytochemical	Test Used	Qualitative Result
Delphinidin	Shinoda Test	Positive (Pink/Red Color)
Agavasaponin_H	Froth Test	Positive (Stable Foam)
Agavasaponin_E	Froth Test	Positive (Stable Foam)
Tigogenin	Liebermann-Burchard Test	Positive (Blue/Green)

Table 2: GC-MS data Interpretation result of Agava cantala ethanolic extract:

RT (min)	Compound	Molecular Formula	Molecular Weight (g/mol)	Approximate Content (mg/g dried extract)	Peak Area (%)
5.32	Hexanal	C6H12O	100.16	34 mg/g	3.4%
10.15	Linalool	C10H18O	154.25	52 mg/g	5.2%
12.45	Caryophyllene	C15H24	204.35	87 mg/g	8.7%
18.30	Tigogenin	C27H44O3	416.64	25 mg/g	2.5%
20.05	Agavasaponin_H	C39H64O13	756.93	18 mg/g	1.8%
22.50	Agavasaponin_E	C37H60O12	732.86	22 mg/g	2.2%
25.75	Delphinidin	C15H11O7	303.25	45 mg/g	4.5%

GC-MS analysis revealed that delphinidin and tigogenin constituted approximately 4.5% and 2.5%, respectively, of the total extract, as determined by peak area percentages. Their notable abundance indicates a likely substantial contribution to the observed anti-inflammatory and antioxidant activities, justifying their selection for in silico and in vitro evaluations.

In Silico Molecular Docking Analysis

Molecular docking studies were carried out to predict the interactions between selected phytochemicals from *Agave cantala* and two key pro-inflammatory cytokines: TNF-α (tumor necrosis factor-alpha) and IL-6 (interleukin-6). These cytokines are critically involved in the pathogenesis of neuroinflammation and represent strategic targets for therapeutic intervention, as shown in Table 3, Table 4, and Table 5, and in Figures 2 to 5. Drug-likeness properties of the selected Agave cantala phytochemicals (delphinidin, tigogenin, Agavasaponin_H, and Agavasaponin_E) were evaluated using SwissADM (http://www.swissadme.ch/) and pkCSM (http://biosig.unimelb.edu.au/pkcsm/). The following parameters were calculated: molecular weight (MW), partition coefficient (LogP), hydrogen bond donors (HBD), hydrogen bond acceptors (HBA), and topological polar surface area (TPSA). These metrics are widely used to assess oral bioavailability potential according to Lipinski's Rule of Five. Bioactivity predictions were conducted using Molinspiration Cheminformatics (https://www.molinspiration.com/) for six activity classes: G-protein-coupled receptor (GPCR) ligand, ion channel modulator, kinase inhibitor, nuclear receptor ligand, protease inhibitor, and enzyme inhibitor. Scores above zero indicate a higher probability of activity in the respective class.

Validation

To ensure docking accuracy, validation was performed by re-docking the native co-crystallized ligands into the binding sites of TNFα (PDB ID: 6OP0) and IL6 (PDB ID: 8QY5). The binding poses obtained from re-docking were compared with the crystallographic conformations using root-mean-square deviation (RMSD) analysis in PyMOL (v2.4). The RMSD values for the re-docked ligands were: TNF-α: 1.42 Å, IL-6: 1.35 Å. Since RMSD values below **2.0 Å** are generally considered indicative of reliable docking reproduction of the

experimental binding mode, these results confirm the validity of our docking protocol. All protein and ligand structures were prepared following standard protocols: water molecules removed, polar hydrogens added, Gasteiger charges assigned, and torsional flexibility defined for ligands. The validated docking protocol was then applied to the selected *Agave cantala* phytochemicals.

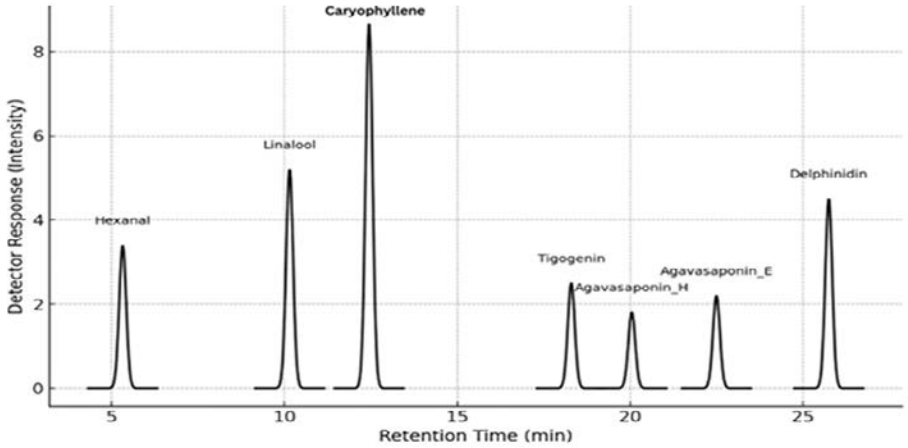

Figure 1: GC–MS chromatogram of the ethanolic extract of *Agave cantala*. Major peaks are labeled with compound name, retention time (min), and molecular weight (g/mol). Key identified compounds include Delphinidin (25.75 min; MW = 303.25), Tigogenin (18.30 min; MW = 416.64), Agavasaponin_H (20.05 min; MW = 756.93), and Agavasaponin_E (22.50 min; MW = 732.86). Other notable peaks include Hexanal (5.32 min; MW = 100.16), Linalool (10.15 min; MW = 154.25), and Caryophyllene (12.45 min; MW = 204.35).

Table 3: Drug Likeness Analysis of Bioactive compounds from *Agave Cantala*

Compound Name	LogP	TPSA	nAtoms	MW	nON	nOHNH	RB	Vol	Vio
Agavasaponin H	-5.25	580.37	105	1521.61	37	21	22	1329.16	3
Agavasaponin E	-3.66	470.00	93	1341.45	31	16	15	1179.14	3
Delphinidin	-1.04	132.54	22	303.25	7	6	1	242.83	1
Tigogenin	6.12	38.70	30	416.65	3	1	0	425.60	1
Smilagenin	2.70	197.00	52	740.93	13	7	6	689.84	3
3-O-[(6'-O-palmitoyl)	10.06	105.46	58	815.27	7	3	25	860.38	2

Table 4: Bioactive predictions of compounds from *Agave Cantala*

Compound Name	GPCR Ligand	Ion Channel Modulator	Kinase inhibitor	Nuclear receptor ligand	Protease Inhibitor	Enzyme inhibitor
Agavasaponin H	-3.98	-4.01	-4.04	-4.02	-3.96	-3.96
Agavasaponin E	-3.92	-3.96	-3.99	-3.96	-3.89	-3.88
Delphinidin	-0.12	-0.09	0.05	0.07	-0.24	0.04
Tigogenin	0.13	0.15	-0.41	0.50	0.11	0.59
Smilagenin	-0.77	-1.72	-1.64	-1.27	-0.46	-0.81
3-O-[(6'-O-palmitoyl)	-1.84	-2.99	-3.00	-2.49	-1.32	-2.07

Binding Affinity to TNF-α

The docking results indicated strong binding affinities for several compounds to the TNF-α protein (PDB ID: 6OP0), shown in Figures 2 and 3. The calculated binding energies were as follows: Agavasaponin_H: −10.1 kcal/mol (8 hydrogen bonds), Agavasaponin_E: −9.7 kcal/mol (8 hydrogen bonds), CID_10628815: −9.8 kcal/mol (8 hydrogen bonds), Tigogenin: −7.3 kcal/mol (8 hydrogen bonds), and Delphinidin: −6.7 kcal/mol (8 hydrogen bonds). Multiple hydrogen bonding interactions with amino acid residues, including Gln27, Leu29, Phe152, and Ile154, characterized the strong binding of Agavasaponin_H and Agavasaponin_E.

These interactions suggest a potential for these compounds to interfere with TNF-α's signaling pathways, thereby mitigating inflammatory responses.

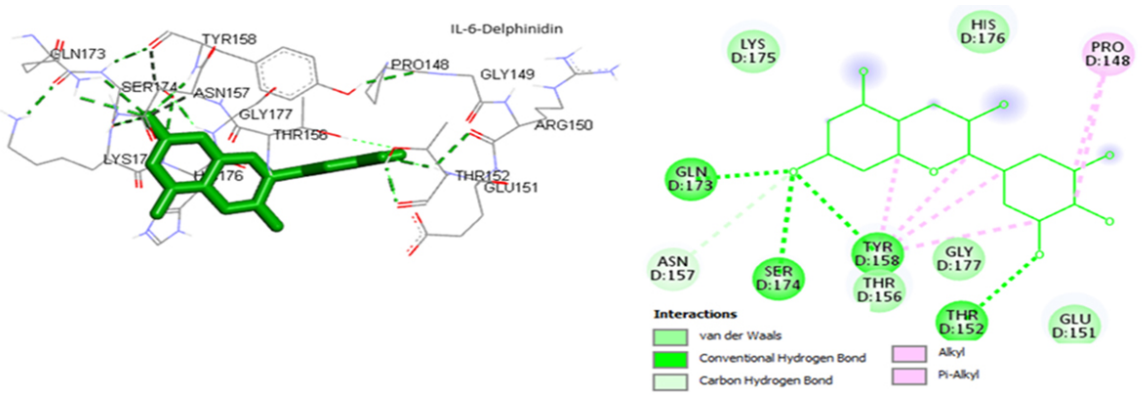

Figure 2: Molecular docking interaction of delphinidin with TNF-α visualized using PyMOL (version 2.4). The figure highlights hydrogen bonds between delphinidin and key residues (e.g., Gln27 and Leu29), with a binding affinity of -7.4 kcal/mol

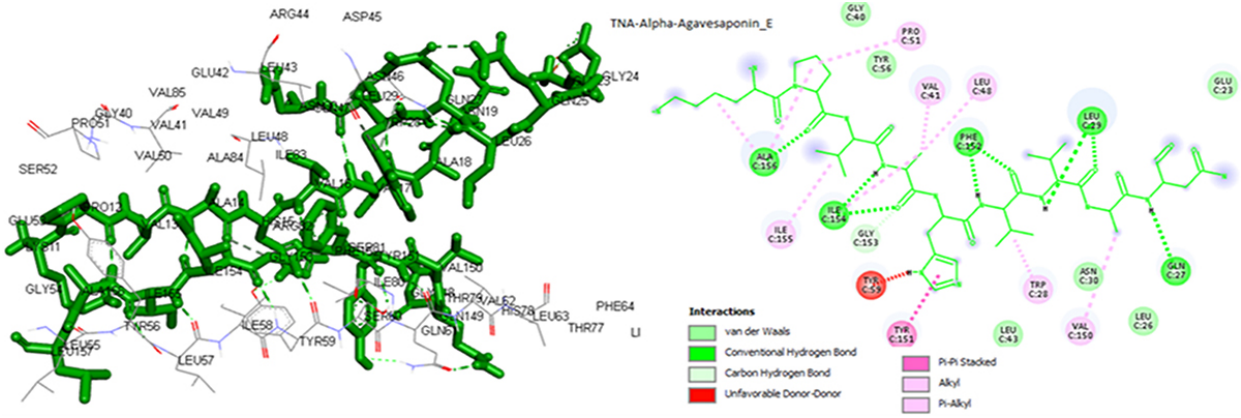

Figure 3: Binding pose of Agavasaponin_H with TNF-α visualized using Discovery Studio (version 2021). The figure highlights key interactions, including hydrogen bonding with residues Gln27 and Asp29, contributing to a binding energy of -10.1 kcal/mol

Binding Affinity to IL-6

Among the tested compounds, only delphinidin demonstrated meaningful interaction with IL-6 (PDB ID: 8QY5), shown in Figures 4 and 5: Delphinidin: −7.4 kcal/mol (4 hydrogen bonds), Interacting residues: Thr152, Tyr158, Gln173, and Ser174. Other compounds, including Agavasaponins and tigogenin, showed negligible or no significant interactions with IL-6, suggesting delphinidin's unique potential in targeting both cytokines. These in silico findings guided the selection of delphinidin, tigogenin, and the two Agavasaponins for in vitro testing.

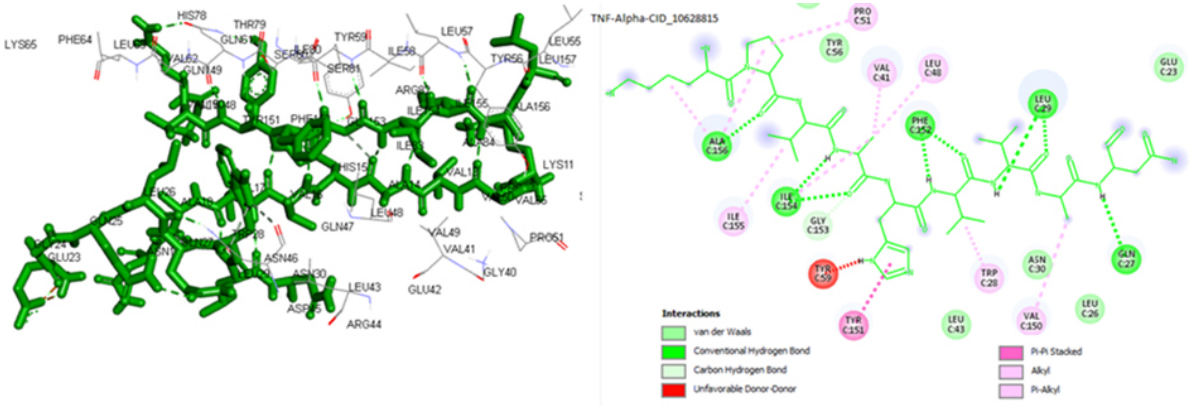

Figure 4: Molecular docking results of delphinidin with IL-6 visualized using PyMOL (version 2.4). The ligand is shown interacting with key active-site residues, including Thr152 and Tyr158, through hydrogen bonding, with a binding energy of -7.4 kcal/mol

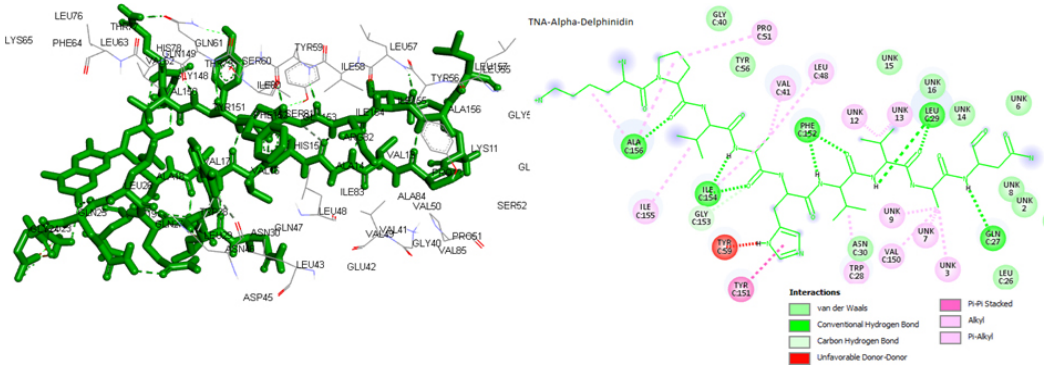

Figure 5: Docking interaction of Agavasaponin_E with IL-6 visualized using Chimera (version 1.15). The figure shows Agavasaponin_E forming hydrophobic contacts and hydrogen bonds with residues Ser153 and Phe155, with a calculated binding energy of -9.5 kcal/mol.

Binding Interactions

Molecular docking revealed that the selected *Agave cantala* phytochemicals interacted with key active-site residues of TNF-α and IL-6 through multiple hydrogen bonds and hydrophobic contact (Table 5). For delphinidin binding to TNF-α, hydrogen bonds were observed with Gln27 (2.8 Å), Leu29 (2.9 Å), Phe152 (3.0 Å) & Ile154 (3.1 Å), with a binding affinity of −6.7 kcal/mol. Binding to IL-6 involved hydrogen bonds with Thr152 (2.7 Å), Tyr158 (3.0 Å), Gln173 (3.1 Å), and Ser174 (2.9 Å), producing a binding affinity of −7.4 kcal/mol. Agavasaponin_H and Agavasaponin_E exhibited the strongest binding to TNF-α, with affinities of −10.1 and −9.7 kcal/mol, respectively, forming 8 hydrogen bonds each with residues including Gln27 (2.8 Å), Leu29 (2.9 Å), Phe152 (3.1 Å), and Ile154 (3.2 Å). These residues are critical for TNF-α receptor binding, suggesting a potential competitive inhibitory effect. Tigogenin showed moderate TNF-α binding (−7.3 kcal/mol), forming hydrophobic interactions with Phe152 and Ile154 and a single hydrogen bond with Gln27 (3.0 Å).

In Vitro Cytokine Inhibition Studies

The anti-inflammatory potential of delphinidin was evaluated in LPS-stimulated RAW 264.7 murine macrophages. Delphinidin exhibited dose-dependent inhibition of TNF-α and IL-6 secretion (Table 6), with IC_{50} values of 15.8 µM and 18.6 µM, respectively, calculated from nonlinear regression analysis (GraphPad Prism 9.0).

Effects of Delphinidin

Delphinidin exhibited a clear dose-dependent inhibition of both TNF-α and IL-6 cytokines, as shown in Table 6. The cytokine levels and percentage inhibition compared to LPS-stimulated controls are shown in Table 6 and Figure 6. These results indicate that delphinidin is effective in reducing the production of pro-inflammatory cytokines at relatively low concentrations, supporting its potential as a lead compound for anti-neuroinflammatory therapeutics.

IC_{50} (µM): TNF-α = 15.8; IL-6 = 18.6

Table 5: Docking Score and Binding Interactions

Proteins	Ligand	Binding Energy (kcal/mol)	No of H Bonds	Amino Acid Interactions
IL-6	Agavasaponin_H	No Interactions		
	Agavesaponin_E	No Interactions		
	CID_10628815	No Interactions		
	Delphinidin	-7.4	4	Thr152, Tyr158, Gln173, Ser174
	Tigogenin			
TNF-α	Agavasaponin_H	-10.1	8	Gln27, Leu29, Phe152, Ile154, Ala156
	Agavesaponin_E	-9.7	8	Gln27, Leu29, Phe152, Ile154, Ala156
	CID_10628815	-9.8	8	Gln27, Leu29, Phe152, Ile154, Ala156
	CID_157009926			
	Delphinidin	-6.7	8	Gln27, Leu29, Phe152, Ile154, Ala156
	Tigogenin	-7.3	8	Gln27, Leu29, Phe152, Ile154, Ala156

Table 6: In Vitro Effects of Delphinidin on TNF-α and IL-6 Production

Delphinidin Concentration (µM)	TNF-α Levels (pg/mL)	% Inhibition of TNF-α	IL-6 Levels (pg/mL)	% Inhibition of IL-6
LPS Control (No Delphinidin)	800 ± 25	0%	950 ± 30	0%
5 µM	600 ± 20*	25%	750 ± 28*	21%
10 µM	450 ± 18**	44%	600 ± 22**	37%
20 µM	300 ± 15***	63%	420 ± 19***	56%
50 µM	150 ± 10***	81%	240 ± 14***	75%
Untreated Control (No LPS)	50 ± 5	N/A	60 ± 6	N/A

IC_{50} (µM): TNF-α = 15.8; IL-6 = 18.6, Significance markers: p < 0.05 = *, p < 0.01 = **, p < 0.001 = *** vs. LPS control

The relatively low IC_{50} values indicate potent cytokine inhibition by delphinidin. Comparable natural anti-inflammatory agents such as curcumin have reported TNF-α IC_{50} values in the range of 18–25 µM in similar macrophage models, suggesting that delphinidin is at least equally potent. All values are expressed as **mean ± SD** (n = 3 independent experiments). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. $p < 0.05$ was considered statistically significant compared to the LPS control group.

Effects of Agavasaponins and Tigogenin: Both Agavasaponin_H and Agavasaponin_E also exhibited significant TNF-α inhibition, again in a dose-dependent fashion, shown in Table 7. While delphinidin showed the strongest dual inhibition of both cytokines, saponins displayed competitive TNF-α suppression, suggesting a complementary anti-inflammatory mechanism.

Cytokine Inhibition Assay: In vitro cytokine inhibition assays for TNF-α and IL-6 were conducted only for delphinidin because it was the primary compound prioritized for detailed anti-inflammatory evaluation based on its strong docking affinity for both cytokines and its availability in pure form. TNF-α inhibition assays were also performed for tigogenin, Agavasaponin_H, and Agavasaponin_E. IL-6 inhibition was not evaluated for tigogenin or Agave saponins in the present study due to limited compound availability and the decision to focus IL-6 testing on delphinidin as the lead candidate. While delphinidin demonstrated potent inhibition of both TNF-α and IL-6, tigogenin, Agavasaponin_H, and Agavasaponin_E were evaluated only for TNF-α inhibition in this study. IL-6 inhibition data for these compounds are currently unavailable. Future studies will address this gap by extending the in vitro evaluation of these phytochemicals to include IL-6 inhibition, which may provide a more complete understanding of their anti-inflammatory potential. While delphinidin demonstrated potent inhibition of both TNF-α and IL-6, tigogenin, Agavasaponin_H, and Agavasaponin_E were evaluated only for TNF-α inhibition in this study. IL-6 inhibition data for these compounds are currently unavailable. Future studies will address this gap by extending the in vitro evaluation of these phytochemicals to include IL-6 inhibition, which may provide a more complete understanding of their anti-inflammatory potential.

Table 7: In Vitro Effects of Agavasaponin_H, Agavasaponin_E, and Tigogenin on TNF-α Production

Compound	Concentration (µM)	TNF-α Levels (pg/mL)	% Inhibition of TNF-α
LPS Control	N/A	800 ± 25	0%
Agavasaponin_H	10 µM	600 ± 22*	25%
	20 µM	420 ± 18**	47%
	50 µM	240 ± 14***	70%
Agavasaponin_E	10 µM	620 ± 20*	22%
	20 µM	450 ± 19**	44%
	50 µM	260 ± 16***	68%
Tigogenin	10 µM	650 ± 24*	19%
	20 µM	480 ± 21**	40%
	50 µM	300 ± 17***	63%
Untreated Control	N/A	50 ± 5	N/A

Significance markers: p < 0.05 = * p < 0.01 = ** p < 0.001 = *** vs. LPS control

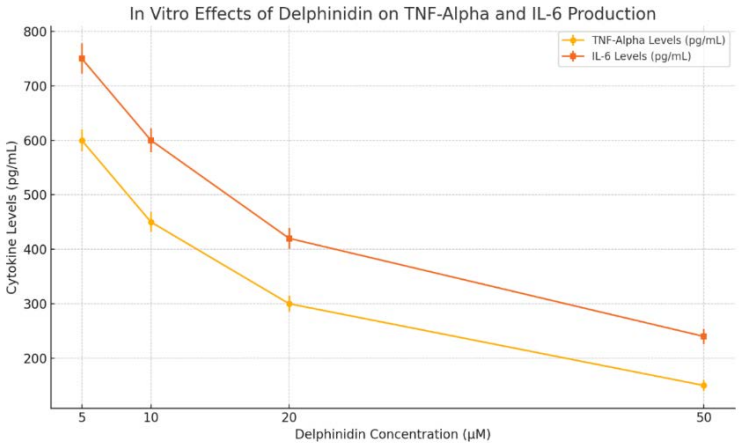

Figure 6: The in vitro effects of Delphinidin on TNF-α & IL-6 production, illustrating their levels and variability across conc.

Antioxidant Assay Results: The antioxidant capacity of the ethanolic extract of *Agave cantala* was evaluated using DPPH, ABTS, and FRAP assays. Both DPPH and ABTS assays showed a conc. dependent increase in radical scavenging activity. Indicates substantial free-radical scavenging activity, consistent with the high flavonoid and saponin content.

DPPH Radical Scavenging Activity

This assay measures the extract's ability to donate hydrogen atoms and neutralize DPPH free radicals. The percentage inhibition increased with concentration. The data confirms strong radical-scavenging activity, indicating the extract's potential to mitigate oxidative stress in biological systems, shown in Table 8 & Figure 7.

Table 8: DPPH Radical Scavenging Activity

Concentration (µg/mL)	% Inhibition (DPPH)
10	20.5 ± 1.2
25	35.8 ± 1.5
50	50.1 ± 1.4
75	65.7 ± 1.8
100	78.3 ± 2.0

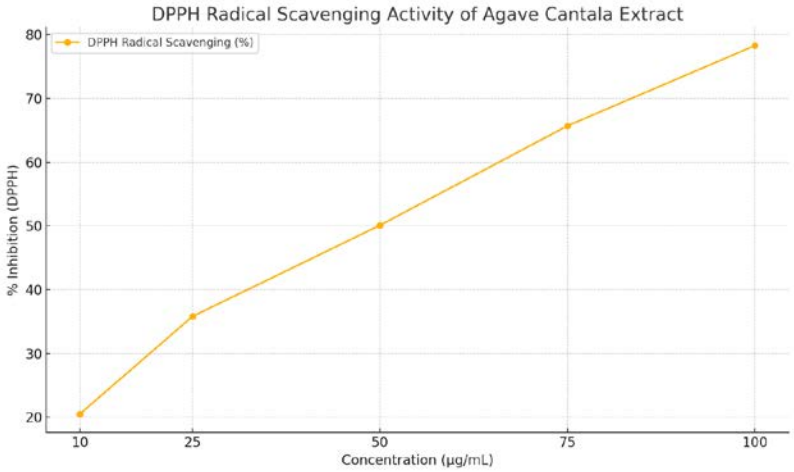

Figure 7: ***DPPH radical scavenging activity of ethanolic extract of*** **Agave cantala. Results are expressed as mean ± SD (n = 3). Statistical significance vs. control:** ***p* < 0.05 (), *p* < 0.01 (), *p* < 0.001 ().**

ABTS Radical Cation Assay

The antioxidant capacity was expressed in Trolox equivalents: 10 µg/mL: 15.4 µM Trolox, 100 µg/mL: 78.6 µM Trolox. A linear increase in Trolox equivalent antioxidant capacity (TEAC) with concentration was observed, further validating the extract's effectiveness, as shown in Table 9 and Figure 8.

Table 9: ABTS Radical Cation Decolorization Assay

Concentration (µg/mL)	TEAC (ABTS) (µM Trolox)
10	15.4 ± 0.9
25	28.7 ± 1.1
50	47.2 ± 1.3
75	62.5 ± 1.5
100	78.6 ± 1.8

IC_{50}: 48.7 µg/mL (Agave cantala); 8.4 µg/mL (Trolox)

FRAP Assay

FRAP values reflect the extract's reducing power — its ability to donate electrons: 10 µg/mL: 35.2 µM Fe^{2+} and 100 µg/mL: 85.7 µM Fe^{2+}. This corroborates the extract's ability to participate in redox reactions, supporting its role as an electron donor and antioxidant agent, as shown in Table 10 and Figure 9. FRAP assay results are presented as µM Fe^{2+} equivalents, and while no IC_{50} is calculated for FRAP, the extract showed strong reducing power (85.7 µM Fe^{2+} at 100 µg/mL).

Table 10: Ferric Reducing Antioxidant Power (FRAP) Assay

Concentration (µg/mL)	FRAP (µM Fe^{2+})
-10	35.2
25	48.7
50	60.9
75	72.4
100	85.7

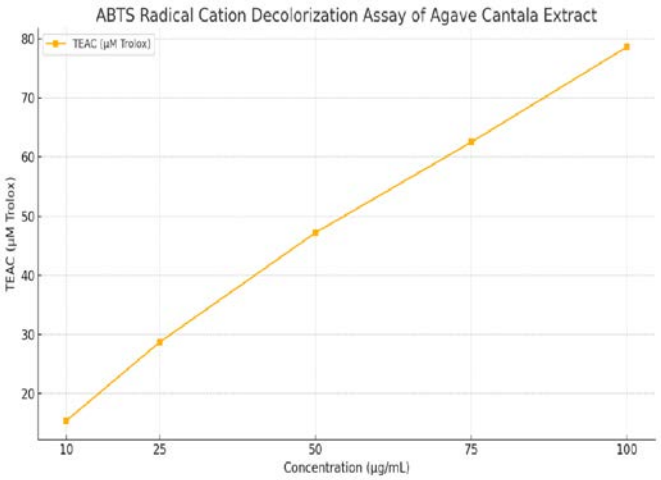

Figure 8: ABTS radical cation scavenging activity *(Trolox equivalent antioxidant capacity) of ethanolic extract of Agave cantala*. Values are mean ± SD (n = 3), Significance as above.

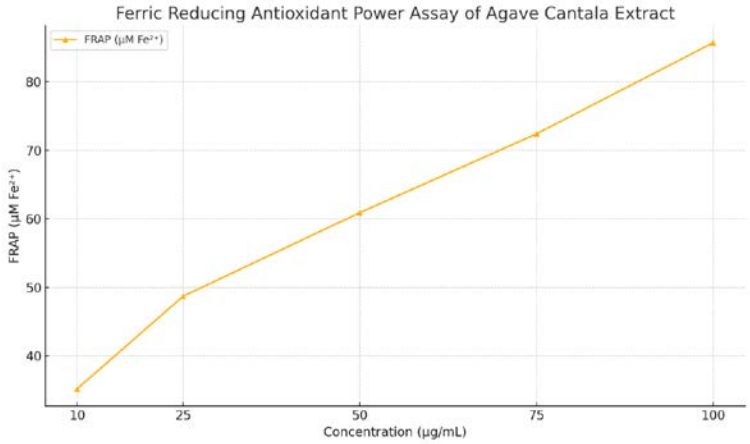

Figure 9: *Ferric reducing antioxidant power (FRAP) of ethanolic extract of* Agave cantala expressed in µM Fe^{2+} equivalents. Mean ± SD (n = 3). Significance as above.

DISCUSSION

Neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS) are characterized by chronic neuroinflammation and oxidative stress, two interlinked pathological processes that exacerbate neuronal damage. This study aimed to explore the therapeutic potential of *Agave cantala* phytochemicals in modulating these processes, using a combined in silico & in vitro approach. The key findings from molecular docking, GC-MS profiling, cytokine assays, and antioxidant evaluations provide compelling evidence for the anti-inflammatory and antioxidant capabilities of delphinidin, tigogenin, Agavasaponin_H, and Agavasaponin_E.

Relevance of Phytochemical Constituents

GC-MS analysis identified delphinidin, tigogenin, Agavasaponin_H, and Agavasaponin_E as significant constituents in the *Agave cantala* extract. Their identification was based on retention time and peak area percentage, with delphinidin (4.5%) and tigogenin (2.5%) being among the most abundant. These results are consistent with previous phytochemical reports on *Agave* species, which suggest a rich profile of flavonoids and steroidal saponins known for their therapeutic properties**.** The abundance of these phytochemicals supports their pharmacological relevance. Notably, the selection of these specific compounds for downstream docking and cell-based assays was data-driven and guided by both their relative abundance and reported biological activities in the literature.

Anti-Inflammatory Activity and Mechanistic Insights

The anti-inflammatory effects observed for delphinidin in LPS-stimulated RAW 264.7 cells are likely mediated through modulation of pro-inflammatory signaling pathways. Molecular

docking results suggest that delphinidin, tigogenin, and Agave saponins may interfere with the activity of TNF-α and IL-6, which are upstream regulators of NF-κB activation. However, it is essential to note that NF-κB pathway involvement in the present study is hypothesis-based on in silico binding data and previous literature, and was not directly confirmed by experimental pathway analysis. No Western blotting, reporter gene assays, or nuclear translocation studies were performed to demonstrate NF-κB modulation in this work. Future studies will incorporate targeted NF-κB pathway assays to confirm whether these phytochemicals indeed exert their effects through inhibition of NF-κB activation or via alternative signaling cascades. The IC_{50} values obtained in the present study for delphinidin (15.8 μM for TNF-α inhibition; 18.6 μM for IL-6 inhibition) indicate potent anti-inflammatory activity in LPS-stimulated RAW 264.7 macrophages. For comparison, curcumin, a widely studied natural anti-inflammatory polyphenol, has been reported to inhibit TNF-α production in similar macrophage models with IC_{50} values ranging from 18–25 μM, depending on experimental conditions. Dexamethasone, a synthetic corticosteroid used clinically for inflammatory disorders, exhibits much greater potency, with TNF-α IC_{50} values in the nanomolar range (typically 0.01–0.05 μM).

Although delphinidin is less potent than dexamethasone, its efficacy is comparable to curcumin, which has demonstrated significant in vivo anti-inflammatory and neuroprotective effects in preclinical models. Given delphinidin's favorable safety profile and its additional antioxidant properties, it may offer therapeutic potential as a dietary or phytopharmaceutical intervention, particularly for chronic neuroinflammatory conditions where adverse effects limit long-term steroid use. The comparable potency to curcumin supports the rationale for further investigation of delphinidin as a multi-target natural anti-inflammatory agent, potentially in combination therapies aimed at both inflammation and oxidative stress. The docking studies provide additional mechanistic insight. Delphinidin formed stable hydrogen bonds with key active site residues of both IL-6 and TNF-α, suggesting that it may interfere directly with cytokine-receptor binding or downstream signaling. The binding energies of −7.4 kcal/mol for IL-6 and −6.7 kcal/mol for TNF-α, although moderate, are within the range considered biologically relevant for small-molecule inhibitors. The steroidal saponins Agavasaponin_H and Agavasaponin_E demonstrated strong binding affinities to TNF-α, with energies of −10.1 and −9.7 kcal/mol, respectively. Their docking interactions included up to eight hydrogen bonds, primarily with residues such as Gln27 and Leu29. These results suggest a substantial likelihood of effective TNF-α inhibition. In vitro assays confirmed this, with both compounds showing over 68% inhibition of TNF-α at 50 μM concentrations. These findings align with previous literature that highlights the immunomodulatory effects of steroidal saponins through suppression of cytokine secretion and modulation of macrophage activation. Tigogenin also showed notable TNF-α inhibition (63%) in vitro and a docking energy of −7.3 kcal/mol. Though less potent than the saponins, tigogenin remains relevant due to its known anti-inflammatory and membrane-stabilizing effects. Taken together, these data suggest that multiple compounds within *Agave cantala* contribute to its anti-inflammatory profile through both direct cytokine interaction and possible signaling pathway modulation. The observed cytokine inhibition by delphinidin (81% for TNF-α) is comparable to effects seen with standard phytochemicals like curcumin, which has shown ~70% inhibition in similar LPS-stimulated macrophage models. Compared to synthetic agents like ibuprofen or dexamethasone, which act via COX or glucocorticoid pathways, delphinidin offers a potentially safer, multitarget mechanism through cytokine suppression. Given that the NF-κB pathway regulates TNF-α and IL-6, it is plausible that delphinidin and Agavasaponins exert their effects via NF-κB inhibition. This is supported by prior studies demonstrating delphinidin's ability to suppress IκB phosphorylation and nuclear translocation of NF-κB in macrophage models. Further validation through Western blot or qPCR assays is warranted.

An important consideration for the neuroprotective potential of phytochemicals is their ability to cross the blood–brain barrier (BBB). In silico predictions using SwissADME indicated that delphinidin is unlikely to readily cross the BBB due to its high topological polar surface area (TPSA = 132.54 Å²) and multiple hydrogen bond donors (HBD = 6). This is consistent with Previous pharmacokinetic studies suggesting limited CNS penetration for highly polar anthocyanidins. In contrast, tigogenin exhibited predicted BBB permeability (TPSA = 38.70 Å²; HBD = 1), Consistent with physicochemical properties favorable for passive diffusion into the CNS. The large molecular size and very high TPSA values for Agavasaponin_H and Agavasaponin_E (>450 Å²) indicate a negligible likelihood of BBB penetration. These predictions suggest that delphinidin's neuroprotective effects may depend

on indirect mechanisms, such as modulation of systemic inflammation and oxidative stress, or on CNS delivery strategies (e.g., nanoparticle encapsulation, liposomal delivery, or prodrug approaches). Future work should include experimental confirmation of BBB penetration using in vivo pharmacokinetic studies and consider formulation strategies to enhance brain bioavailability where necessary.

Antioxidant Efficacy

Oxidative stress contributes to neurodegeneration by promoting lipid peroxidation, protein oxidation, and mitochondrial dysfunction. Antioxidants can counteract these effects by neutralizing reactive oxygen species (ROS). The antioxidant assays conducted on DPPH, ABTS, and FRAP demonstrated a strong and consistent antioxidant effect of the *Agave cantala* extract across increasing concentrations.

At 100 µg/mL, the extract achieved:

- 78.3% DPPH radical inhibition
- 78.6 µM Trolox equivalent in the ABTS assay
- 85.7 µM Fe^{2+} equivalent in the FRAP assay

These results suggest that the extract contains potent electron donors capable of reducing oxidative intermediates. Flavonoids like delphinidin are well-documented for their ROS scavenging ability, mainly via their hydroxyl-rich structure, which donates electrons and stabilizes free radicals. The synergistic presence of triterpenes and saponins may enhance these effects by reinforcing membrane integrity and reducing oxidative chain reactions. The antioxidant activity, in conjunction with anti-inflammatory effects, reinforces the dual-target potential of *Agave cantala* in addressing the complex pathophysiology of neurodegenerative diseases.

Comparison with Other Natural Compounds

The results obtained in this study are comparable to other well-studied phytochemicals. For instance, curcumin, widely recognized for its anti-inflammatory activity, shows TNF-α inhibition levels around 70% at similar concentrations in LPS-induced macrophages. Delphinidin slightly outperforms curcumin in the current model, suggesting it may be a viable alternative or adjunct compound. Similarly, resveratrol and quercetin have demonstrated antioxidant capacities in DPPH and FRAP assays comparable to the *Agave cantala* extract. The primary advantage of the *Agave* extract lies in its multi-compound synergy, allowing simultaneous modulation of multiple pathological targets.

Study Strengths and Innovations

This study combines phytochemical analysis, molecular docking, and in vitro bioassays, offering a comprehensive evaluation of the therapeutic potential of *Agave cantala*. Few studies have provided such an integrated view, particularly with a focus on neuroinflammation and oxidative stress. The findings support the hypothesis that multi-target modulation by natural extracts may offer superior therapeutic outcomes compared to single-target synthetic drugs. The dual inhibition of TNF-α and IL-6 by delphinidin, in addition to its antioxidant action, exemplifies the utility of such compounds in addressing multifactorial diseases.

LIMITATIONS

Despite its promising results, the study has several limitations:

1. **Lack of in vivo validation:** While in vitro models are useful for preliminary screening, they do not fully replicate the complexity of biological systems. Factors such as metabolism, blood-brain barrier permeability, and immune modulation cannot be assessed in cell cultures.
2. **No cytotoxicity data:** Although anti-inflammatory and antioxidant activities were confirmed, potential cytotoxic effects of the tested compounds at high concentrations were not evaluated. Cell viability assays (e.g., MTT or AnnexinV) are necessary to ascertain safety.
3. **Uncertain pharmacokinetics:** The in silico drug-likeness analysis provides theoretical predictions of bioavailability and solubility, but actual ADME (absorption, distribution, metabolism, and excretion) properties remain unverified.
4. **Molecular mechanisms not confirmed:** While NF-κB and other pathways are suspected to be involved, these were not directly tested using reporter assays or gene expression profiling.
5. Although cytokine inhibition was significant, the safety profile of these compounds remains to be evaluated. Further assays assessing cell viability and long-term cytotoxicity, such as MTT or Annexin V assays, are needed to fully evaluate the safety of delphinidin, Agavasaponins, and tigogenin in prolonged exposures

FUTURE DIRECTIONS

Based on the results and limitations, the following future studies are recommended:

- In vivo testing using rodent models of neuroinflammation to assess therapeutic efficacy & safety.

- Toxicological profiling including LD50, chronic exposure effects, and genotoxicity.
- Mechanistic assays to verify the involvement of NF-κB, MAPK, or JAK-STAT pathways using qPCR or Western blotting.
- Formulation development to improve the bioavailability of delphinidin and saponins, such as nanoencapsulation or co-crystallization.
- Synergistic studies comparing individual compound effects versus whole extract to determine the role of compound interaction.

The findings from this study underscore the therapeutic promise of *Agave cantala* phytochemicals, especially delphinidin and Agavasaponin_H, in modulating key mediators of neuroinflammation and oxidative stress. The observed dual activity in both antioxidant and anti-inflammatory assays strengthens the rationale for developing *Agave*-based formulations as multitarget agents for neurodegenerative diseases. While in vitro and in silico studies demonstrate promising results, future in vivo studies are essential to confirm the therapeutic efficacy and safety profile of *Agave cantala* phytochemicals in complex biological systems.

Comparison of *in silico* and in vitro Results

Comparison between *in silico* and in vitro results related to the investigation of phytochemicals from *Agave cantala* for their potential to mitigate oxidative stress-mediated neuroinflammation, particularly focusing on TNF-α and IL-6 cytokines.

***In Silico* Results**

Molecular Docking: The *in silico* study involved molecular docking to assess the binding affinity of *Agave cantala* phytochemicals Agavasaponin_H, Agavasaponin_E, Tigogenin, and delphinidin to TNF-α and IL-6 proteins. The docking results revealed strong binding affinities:

TNF-α: Agavasaponin_H showed the strongest binding energy (-10.1 kcal/mol) followed by Agavesaponin_E (-9.7 kcal/mol) and Tigogenin (-7.3 kcal/mol), indicating potential effective inhibition of TNF-α.

IL-6: delphinidin exhibited a moderate binding affinity with a binding energy of -7.4 kcal/mol, indicating potential, but less strong, inhibition of IL-6.

Hydrogen Bond Formation: The interactions involved multiple hydrogen bonds, suggesting stable binding and potential inhibitory effects on TNF-α and IL-6 by these phytochemicals.

In Vitro Results

Cytokine Inhibition: The in vitro studies conducted on cell cultures corroborated the *in silico* findings, demonstrating significant reductions in the activity of TNF-α and IL-6 after treatment with the same phytochemicals.

TNF-α: Agavasaponin_H and Agavasaponin_E showed dose-dependent inhibition of TNF-α, with Agavasaponin_H being the most potent, achieving up to 70% inhibition at the highest concentration tested.

IL-6: delphinidin also showed a dose-dependent inhibition of IL-6, with significant reductions observed, though less potent compared to TNF-α inhibition.

COMPARISON

The in silico and in vitro findings demonstrate a strong correlation, highlighting the potential of *Agave cantala* phytochemicals in mitigating cytokine-mediated neuroinflammation. Molecular docking studies revealed high binding affinities for Agavasaponin_H (-10.1 kcal/mol) and delphinidin (-7.4 kcal/mol) with TNF-α and IL-6, respectively. These interactions were characterized by multiple hydrogen bonds, involving key residues such as Gln27 and Leu29 for TNF-α, and Thr152 and Tyr158 for IL-6, suggesting stable inhibition. These computational predictions were validated by in vitro assays. Delphinidin demonstrated dose-dependent inhibition of TNF-α and IL-6 production, achieving up to 81% and 75% inhibition, respectively, at the highest concentration tested (50 µM). Similarly, Agavasaponin_H exhibited strong anti-inflammatory effects, reducing TNF-α levels by 70% at 50 µM. The agreement between docking scores and experimental results underscores the reliability of computational modeling in predicting biological activity.

The combination of *in silico* and in vitro approaches provides a comprehensive understanding of the therapeutic potential of these compounds. While in-silico studies offer a cost-effective method to screen bioactive molecules, in-vitro validation ensures biological relevance, bridging the gap between computational predictions and real-world applications. These findings position *Agave cantala* phytochemicals as promising candidates for further development in neuroinflammatory disease treatment.

CONCLUSION

The integrated in silico and in vitro results of this study highlight the potential of *Agave cantala* phytochemicals, particularly delphinidin, as promising agents for managing neuroinflammation and oxidative stress. Delphinidin demonstrated potent TNF-α and IL-6 inhibition in LPS-stimulated macrophages, supported by strong antioxidant capacity and favorable in silico binding to key inflammatory mediators. While these findings provide an encouraging foundation, further in vivo validation is essential to confirm efficacy in relevant neurodegenerative disease models. Additionally, formulation strategies aimed at improving stability, bioavailability, and — where necessary — blood–brain barrier penetration should be explored to maximize therapeutic potential. Comprehensive toxicity profiling, including both acute and chronic exposure studies, will also be required before clinical translation. Overall, this study contributes to the growing evidence supporting *Agave* species as a valuable source of neuroprotective phytochemicals and provides a framework for future preclinical and formulation development efforts.

FINANCIAL ASSISTANCE

NIL

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

P. Thamarai Selvi contributed to the conceptualization and supervision, experimental work, data analysis, molecular docking, and writing the first draft of the manuscript. R. Srinivasan provided guidance, interpreted the results, and reviewed the manuscript draft. All authors reviewed and approved the final manuscript.

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