RESEARCH PAPER

HPTLC fingerprinting and in-vitro evaluation of Aquilaria agallocha against MPTP-induced neurotoxicity and oxidative insult in PC12 cells

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ABSTRACT

Background: Parkinson's disease (PD) are primarily associated with oxidative stress–induced neuronal damage and mitochondrial dysfunction. Aquilaria agallocha exhibits significant neuroprotective potential by combating oxidative stress and neuroinflammation.

Objectives: The present study investigated the neuroprotective and antioxidant potential of Aquilaria agallocha (AA) in an in vitro model of MPTP induced Parkinson's disease using PC12 cells.

Methodology: Phytochemical analysis revealed that Aquilaria agallocha (AA) extracts possess substantial levels of alkaloids, phenolics, and flavonoids with HPTLC as confirmed by colorimetric assays. Invitro study using PC12 neuronal cells that indicating the presence of potent bioactive constituents with strong antioxidant potential.

Result: Treatment of PC12 cells with MPTP resulted in a marked reduction in cell viability (~50%), confirming successful induction of cytotoxic stress. However, co-treatment with AA extract (10–40 µg/mL) significantly restored cell viability in a dose-dependent manner, with near-complete recovery observed at higher concentrations, suggesting robust neuroprotective efficacy. Fluorescence-based assays, including DAPI, PI, and DCFH-DA staining, revealed that AA treatment mitigated ROS generation, reduced apoptotic cell death, and preserved nuclear morphology.

Conclusion: Aquilaria agallocha exhibits strong antioxidant, cytoprotective, and neurorestorative properties, effectively counteracting MPTP-induced oxidative stress and apoptosis in neuronal cells. The study highlights the therapeutic promise of AA as a natural neuroprotective agent for the prevention or management of Parkinson's disease and related neurodegenerative conditions.

Keywords: Aquilaria agallocha, Parkinson's disease, PC12 cells, HPTLC, oxidative stress, neuroprotection, antioxidants.
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INTRODUCTION

Parkinson's disease (PD) stands as the second most prevalent neurodegenerative condition, severely impacting the quality of life due to its chronic and progressive nature. PD is fundamentally a progressive nervous system disorder primarily defined by debilitating motor symptoms, including bradykinesia, rigidity, rest tremor, and postural instability, which significantly affect patient mobility and independence [1]**.** The global burden of PD is substantial, with estimates suggesting that 6.3 million individuals worldwide currently suffer from the condition. This translates to a considerable incidence rate,

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approximated at 4.5–21 cases per 100,000 populations annually [2]. The underlying pathology of PD is characterized by the selective degeneration of dopaminergic neurons in the substantia nigra pars compacta and the presence of intracellular inclusions known as Lewy bodies, composed primarily of misfolded alpha-synuclein protein [3, 4].

Despite decades of intensive research, current therapeutic strategies, while effective at managing symptoms offer no neuroprotective or curative solutions. Traditionally, *A. agallocha* has been used in Ayurvedic and Unani medicine for treating various ailments such as inflammation, pain, asthma, rheumatism, and neurological disorders. Recent pharmacological studies have highlighted its antioxidant, anti-inflammatory, antimicrobial, and neuroprotective properties. The presence of high phenolic and flavonoid content contributes significantly to its free radical–scavenging and cytoprotective effects, making it a promising natural candidate for combating oxidative stress–related diseases, including neurodegenerative disorders like Parkinson's disease [5, 6].

Aquilaria agallocha (Thymelaeaceae) is an indigenous evergreen plant found in India, China, and Tibet. It is widely known for its resinous wood, commonly referred to as "agarwood" or "aloewood," and is considered a valuable non-timber aromatic resource. Agarwood has been used globally for many years and is well documented in Ayurveda and traditional medicinal systems.

Different parts of the plant, including the bark, roots, leaves, and heartwood, are utilized for their therapeutic and medicinal properties. Phytochemical studies have reported the presence of compounds such as agarol, aquillochin, agarospiranes (sesquiterpenes) including agarospirol, baimuxinic acid, baimuxinal, dihydroxyagarofuran, and 2-(2-phenylethyl)-4H-chromen-4-one derivatives.

Due to its increasing demand, there is a need for proper standardization of agarwood and its essential oil based on detailed chemical analysis. The major bioactive constituents identified in agarwood include alkaloids, steroids, saponins, tannins, terpenoids, flavonoids, and phenolic compounds. *Aquilaria agallocha* exhibits a wide range of pharmacological activities, including antidiabetic, antibacterial, anti-inflammatory, cardiovascular protective, and antifungal effects.

Given its rich phytochemical profile and therapeutic potential, *Aquilaria agallocha* serves as an important source for developing natural antioxidant and neuroprotective agents aimed at mitigating neuronal injury and promoting brain health [7].

MATERIAL AND METHODS

Plant Extration procedure of *Aquillaria agallocha*

The fresh leaf of AA was dried in sun light and converted into dried powder form and stored in air tight container. Standardized powdered plant materials extracted with 50% ethanol in water. Soxhlet's extraction technique to reduce solvent use and maximum extraction efficiency. After maximum extraction achieved, filter while hot and filtrate collected. Reduce the volume of filtrate in rotary vacuum evaporator. Hydro-alcoholic extract of both the plants was collected by above process [7, 8].

High-Performance Thin Layer Liquid Chromatography (HPTLC) Analysis

High-performance liquid chromatography (HPLC) was employed for the qualitative phytochemical profiling of Aquilaria agallocha extracts to to assess chemical consistency and identify major constituents. The dried hydroalcoholic extract was accurately weighed and dissolved in HPLC-grade methanol to obtain an appropriate concentration. The solution was sonicated for 10 minutes to ensure complete dissolution and then filtered through a 0.45 µm membrane filter prior to chromatographic analysis. HPTLC analysis was performed using a CAMAG system comprising a Linomat 5 applicator, TLC Scanner-4, CAMAG visualizer, and winCATS software. Samples were applied as bands on silica gel 60 F254 plates (7.0 × 10.0 cm) at an application rate of 150 nL/s. Plate development was carried out in an automatic development chamber using chloroform:ethyl acetate:formic acid (5:4:1, v/v/v) as the mobile phase.

IN-VITRO EXPERIMENTAL GROUP DESIGN

The in vitro study was performed using the PC-12 cell line to evaluate the effect of the test extract under both neurotoxic and normal conditions. The experiment was divided into two sets: with MPTP induction and without MPTP induction.

Experimental Design with MPTP Induction

To establish a Parkinsonian neurotoxic model, cells were exposed to MPTP. The cells were divided into six experimental groups.

Table 1. Experimental Design with MPTP Induction

Group	Treatment
I (Control)	Untreated PC-12 cells
II (MPTP)	MPTP
III	MPTP + Test Compound (10 µg/mL)
IV	MPTP + Test Compound (20 µg/mL)
V	MPTP + Test Compound (30 µg/mL)
VI	MPTP + Test Compound (40 µg/mL)

Experimental Design without MPTP Induction

To evaluate the direct cytotoxic or protective effects of the test compound under normal conditions, PC-12 cells were divided into six groups without MPTP exposure:

Table 2. Experimental Design without MPTP Induction

Group	Treatment
I (Control)	Untreated PC-12 cells
III	Test Compound (10 µg/mL)
IV	Test Compound (20 µg/mL)
V	Test Compound (30 µg/mL)
VI	Test Compound (40 µg/mL)

Reagents and chemicals

Anthrone reagent, sulfuric acid, Congo red, n-butyl alcohol, methanol, hematoxylin, acetic anhydride, chloroform, ammonium buffer solution, hydrochloric acid, lead (II) acetate trihydrate, ninhydrin, Fehling A and B solutions, potassium ferricyanide, trichloroacetic acid,), 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide (MTT), H2DCFDA (dichlorofluorescein diacetate).

HPTLC Fingerprinting Procedure

A high-performance thin-layer chromatographic analysis was performed to develop a distinctive fingerprint profile of the leaf extract from Aquilaria agallocha (AA). The separation was carried out using pre-coated silica gel 60 F_{254} HPTLC plates measuring 10 × 10 cm and 20 × 10 cm. The hydroalcoholic extract was dissolved in chosen volatile solvent mixtures toluene: ethyl acetate: methanol (5:4:1, v/v/v) and chloroform: methanol (9:1, v/v) which were selected following initial optimization to ensure good separation of bands. Sample volumes between 10 and 25 µL were applied as 8 mm bands using an automated applicator in a controlled nitrogen stream to ensure even application. The original application line was kept 10 mm away from the bottom edge, with uniform spacing between tracks. Chromatographic development took place in a twin-trough chamber that had been pre-saturated with the corresponding mobile phase for 20 minutes at 25 ± 2 °C, enabling the solvent front to travel 70 mm from the starting point. After development, the plates were dried in air and then analyzed using densitometric scanning at wavelengths of 254 and 366 nm. Chromatographic software was used to record and analyze Rf values, peak areas, and absorption spectra. Reproducibility was evaluated using triplicate analysis, showing consistent band patterns and little variation in Rf values, thus confirming the method's reliability for phytochemical fingerprinting and authentication.[9, 10].

Cell culture and maintenance

PC12 a was procured from National Centre for Cell Science (NCCS, Pune, India). All the cell culture products including Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), antibiotic, and antimycotic solution were purchased from Gibco™ (Thermo Fisher Scientific). Cells were maintained in a humidified environment at 37°C and 5% CO2. Cell culture media DMEM was supplemented with 10% FBS and 1% antibiotic and antimycotic solution [11–13].

Cell viability

Cell viability was assessed using the MTT assay. After each treatment, cells were washed with PBS and incubated for 3 h at 37 °C in serum-free RPMI medium containing 0.25 mg/mL MTT solution. Formed purple formazan crystals were dissolved in 200 µL isopropanol–HCl, and the absorbance was measured at 570 nm with a reference wavelength of 630 nm using a microplate reader. Cell viability was also confirmed by trypan blue exclusion. Treated cells were harvested with trypsin–EDTA, washed with PBS, and stained with 0.4% trypan blue. The percentage of cell death was calculated from the ratio of dead to total cells [12].

ROS Generation

PC12 cells were seeded in 24-well plates at a density of 50,000 cells per well. After 24 h, the old medium was replaced with fresh medium, and the cells were treated with the compound at various concentrations. Subsequently, 10 µM DCFH-DA was added to each well and incubated for 30 min at 37 °C. The cells were then imaged to assess fluorescence intensity, indicating intracellular ROS levels [14].

PI (Propidium Iodide)

PC12 cells (50,000 cells/well) were cultured in 24-well plates and treated with the test compound for 24 h. Following treatment, 10 µM propidium iodide (PI) solution (10 µL dissolved in 1 mL distilled water) was added to each well. After 15 min of incubation at room temperature, the cells were imaged to assess PI fluorescence, indicating membrane-compromised or dead cells [11].

DAPI staining Protocol

PC12 cells were seeded in 24-well plates at a density of 50,000 cells/well and treated with the desired compound concentrations for 24 h. After treatment, 20 µL of DAPI dye was added to each well and incubated for 15 min. The dye was then removed, and the cells were imaged using a FLoid fluorescence microscope to visualize nuclear staining [15], [16].

DAPI/PI dual staining

In brief after the incubation period of DAPI, the dye was removed and PI (Propidium iodide) dye was subjected to each well. Afterward, imaging was taken using the dual filter on the FLoid microscopy (Invitrogen, ThermoScientific) under the blue and red filter [17].

RESULTS

Effect of *A. agallocha* on TLC

At lower sample volumes (10–15 µL), the bands appeared faint, indicating a lower concentration of analytes. With increasing sample volume (20–25 µL), the band intensity and sharpness increased, confirming a higher presence of bioactive constituents. The alkaloid fraction showed well-defined and separated spots, suggesting the presence of multiple alkaloid components. Similarly, the flavonoid and phenolic fractions displayed several distinct bands with strong fluorescence, signifying a rich composition of antioxidant compounds as showed on figure 1,2,3.

HPTLC Fingerprinting and Phytochemical Profiling of *A. agallocha*

The HPTLC fingerprint of the leaf extract of A. agallocha showed a clear and distinct chromatographic profile, different from Phoenix sylvestris (Figure 1). Under short-wave UV light (254 nm), multiple strong dark bands appeared against a fluorescent background (Figure 1A). This indicates the presence of UV-absorbing compounds with conjugated aromatic systems. When viewed at 366 nm, several fluorescent bands were visible (Figure 1B), suggesting the presence of flavonoid and coumarin-type compounds.

Densitometric scanning at 254 nm produced sharp and symmetrical peaks with little tailing (Figure 1C). This confirms effective chromatographic separation. Clear and reproducible peaks were seen at Rf values between 0.338 and 0.351. The consistency of Rf values and peak patterns across replicate tracks shows the reliability of the method for generating fingerprints and quality control.

A comparative chromatographic study showed significant differences between A. agallocha (Figure 2), especially in the mid-Rf region. The distinct grouping of peaks in A. agallocha highlights its unique phytochemical profile and confirms the effectiveness of the HPTLC method developed. Peak purity scans showed that the major band was homogeneous (Figure 3) because of strong similarity in the spectra across the start, peak, and end positions.3.2 Probable Phytoconstituents Based on Literature Correlation

The chromatographic behavior observed matches the previously reported phytochemical components of Aquilaria agallocha, including:

(i) 2-(2-Phenylethyl) Chromones

Chromone derivatives are seen as markers for the genus Aquilaria. Many studies have noted the presence of agarotetrol, agarospirol-related chromones, and other phenylethyl chromone derivatives in various Aquilaria species. These compounds absorb UV light strongly around 250 to 280 nm due to their conjugated benzopyran structure. This aligns with the intense bands noted at 254 nm in the current study. (Naef, 2011).

(ii) Sesquiterpenes (Agarofuran-type Compounds)

Sesquiterpenoids like agarospirol and jinkohol have been frequently identified in Aquilaria species. While many sesquiterpenes show weak UV absorbance, some oxygenated derivatives can create moderate-intensity bands in the mid-Rf regions. The grouping of peaks observed in this area might correspond to these sesquiterpenoid components.

(iii) Flavonoids (Quercetin and Kaempferol Derivatives)

Flavonoids have been found in the leaves of Aquilaria agallocha. Quercetin and kaempferol glycosides show distinct fluorescence under 366 nm, which aligns with the fluorescent bands seen in this study. Their strong conjugated systems also lead to absorption at 254 nm.

(iv) Alkaloids

Early phytochemical studies of A. agallocha reported minor alkaloidal components. Alkaloids usually absorb in the UV range due to their nitrogen-containing aromatic rings, which supports the detection of UV-active bands.

(v) Phenolic Acids and Polyphenols

Phenolic compounds are known for their antioxidant and neuroprotective benefits linked to Aquilaria. These compounds showcase their characteristic UV absorption at 254 nm because of their aromatic hydroxyl groups.

Overall, the HPTLC fingerprint indicates a diverse chemical composition containing chromones, sesquiterpenoids, flavonoids, alkaloids, and phenolic compounds, in line with published research on Aquilaria agallocha.

The HPTLC densitogram of the ethanolic extract of Aquilaria agallocha developed in chloroform:ethyl acetate:formic acid (5:4:1 v/v/v) showed major peaks at Rf 0.14, 0.33, and 0.35. Polar phenolic acids or flavonoid glycosides may be responsible for the low Rf band (0.14), while 2-(2-phenylethyl) chromone derivatives and flavonoid aglycones, which are typical phytoconstituents of agarwood, are indicated by peaks at Rf 0.33–0.35 (Figure 1).

The fluorescent bands seen at Rf 0.16, 0.24, 0.45, 0.74, and 0.84 under 366 nm may be ascribed to flavonoids and 2-(2-phenylethyl) chromone derivatives, plus other conjugated aromatic components typical of Aquilaria agallocha.(Figure 2)

The characteristic peak corresponding to less polar chromone derivatives or other conjugated aromatic components pesticides typical of Aquilaria agallocha is Rf ≈ 0.72 (figure 3).

There were five prominent fluorescent bands visible in the densitogram recorded at 366 nm with Rf values at 0.16, 0.24, 0.45, 0.74 and 0.84. These major bands are indicative of flavonoid glycosides (Rf 0.16–0.24), chromone derivatives (Rf 0.45–0.74), and less polar aromatic components comprising sesquiterpene linked compounds (Rf 0.84) characteristic phytoconstituents to Aquilaria agallocha (figure 4).

3.3 Suggested Marker Compounds for Future HPLC Confirmation

For further analytical validation and quantitative profiling using HPLC or UHPLC, the mention table-1 marker compounds are recommended:

For further confirmation by HPLC analysis, detection may be performed at 254 nm for general phytochemical screening, 280 nm for phenolic compounds, and 320 to 340 nm for flavonoid compounds. It is recommended to use a photodiode array (PDA) detector to allow for peak purity assessment and spectral comparison. Additionally, LC-MS analysis can be used for structural confirmation of chromone derivatives and related secondary metabolites.

The developed HPTLC method produced a reproducible and species-specific chromatographic fingerprint for *A. agallocha*. The observed UV absorption characteristics, fluorescence response, and consistent Rf distribution strongly align with previously reported chromones, sesquiterpenoids, flavonoids, alkaloids, and phenolic compounds of the species. Overall, the method shows robustness and is suitable for verifying phytochemicals, confirming authenticity, and controlling the quality of A. agallocha leaf extracts.

Effect of *A. agallocha* on cell viability

In the control group, PC12 cells exhibited maximum cell viability (~75%), with abundant and intensely stained formazan crystals, indicating normal mitochondrial function and healthy cellular metabolism.

Exposure of PC12 cells to MPTP (neurotoxin) resulted in a marked reduction in cell viability accompanied by sparse and faint formazan crystal formation. This significant decrease confirms MPTP-induced mitochondrial dysfunction and cytotoxicity, consistent with parkinsonian neurodegeneration mechanisms. Treatment with AA extract at increasing concentrations (10–50 µg/mL) produced an improvement in cell viability. 10 µg/mL AA: Moderate increase in cell viability compared to the MPTP group, with partial restoration of formazan crystal density. 20 µg/mL AA: Further enhancement of cell survival, indicating improved mitochondrial activity. 30 µg/mL AA: Substantial increase in cell viability, with dense and uniformly distributed MTT crystals. 40–50 µg/mL AA. Near-normalization of cell viability comparable to control cells, with prominent and intense formazan crystal formation. The increase in MTT crystal deposition with higher AA concentrations clearly demonstrates restoration of mitochondrial dehydrogenase activity and enhanced cell survival. **[Figure-5,6]**

Effect of *A. agallocha* on ROS generation

Reactive oxygen species (ROS) generation in PC12 cells was assessed using DCFH-DA fluorescence staining under both normal and MPTP-induced conditions. In the normal control group, very weak green fluorescence was observed, indicating basal ROS levels and normal cellular redox homeostasis. In the absence of MPTP, treatment with the test extract at 10 and 20 µg/mL resulted in a moderate increase in green fluorescence, suggesting a slight elevation of ROS at lower concentrations. However, at 30 and 40 µg/mL, fluorescence intensity was markedly reduced and comparable to the control group, indicating effective suppression of intracellular ROS generation. At 50 µg/mL, a mild increase in fluorescence was again observed, possibly due to concentration-dependent cellular stress. In contrast, the MPTP control group exhibited intense green fluorescence, confirming excessive ROS production and pronounced oxidative stress. Treatment with the test extract significantly reduced MPTP-induced ROS generation in a dose-dependent manner. Cells treated with 10 and 20 µg/mL showed partial attenuation of fluorescence intensity compared to the MPTP control. Further reduction in ROS levels was observed at 30 µg/mL, while 40 µg/mL treatment resulted in minimal fluorescence, nearly restoring ROS levels to those of the normal control group. The results demonstrate that the test extract effectively modulates intracellular ROS levels under normal conditions and provides significant protection against MPTP-induced oxidative stress, with maximum antioxidant and neuroprotective activity observed at 30–40 µg/mL. **[Figure-7, 8].**

INVITRO FLUORESCENCE IMAGING ASSAY

Effect of DAPI staining assay

Nuclear morphology of PC12 cells following treatment with the test extract at varying concentrations was evaluated using fluorescent nuclear staining. In the control group, cells exhibited uniformly stained, round to oval nuclei with intact nuclear architecture, indicating normal cell integrity and absence of apoptotic features. Cells treated with 20 and 40 µg/mL of the extract displayed nuclear morphology comparable to the control group, with well-defined nuclei and no evident signs of chromatin condensation or fragmentation. These observations suggest that lower to moderate concentrations of the extract did not induce cytotoxic effects and maintained nuclear stability. At 40 µg/mL, early morphological alterations became evident, including mild nuclear condensation and reduced nuclear size in a limited number of cells, as indicated by arrow markings. Treatment with 40 µg/mL resulted in a more pronounced appearance of condensed and fragmented nuclei, characteristic of apoptotic changes. At the highest concentration (50 µg/mL), marked nuclear abnormalities were observed, including significant chromatin condensation, nuclear fragmentation, and reduced cell density, indicating concentration-dependent induction of apoptotic cell death. the results demonstrate that the test extract maintains nuclear integrity at lower concentrations, while higher concentrations induce distinct apoptotic nuclear changes**. [Figure-9]**

Effect of Propidium Iodide (PI)

Fluorescence microscopic analysis revealed distinct, concentration-dependent changes in red fluorescent signal intensity and cellular morphology following treatment with the test extract. In the control group, only sparse red fluorescence was detected, indicating minimal membrane damage and a predominance of viable cells. Cells treated with 20 and 50 µg/mL exhibited a mild increase in red fluorescent cells; however, the majority of cells maintained normal morphology, suggesting limited loss of

membrane integrity at lower concentrations. At 50 µg/mL, a marked increase in red fluorescence was observed along with cellular shrinkage and aggregation, indicating enhanced uptake of the dye due to compromised membrane permeability. Treatment with 80 µg/mL resulted in pronounced red fluorescence with irregularly shaped and condensed cells, reflecting a substantial increase in non-viable or late apoptotic cells. At the highest concentration (50 µg/mL), intense red fluorescence was evident with a notable reduction in cell number, confirming severe cytotoxicity and extensive loss of membrane integrity. These findings demonstrate a clear concentration-dependent increase in red fluorescent signal, indicative of progressive cell death at higher extract concentrations. **[Figure-10]**

Effect of DAPI and PI Merge

Apoptotic and necrotic cell death in PC12 cells treated with *A. agallocha* extract was assessed using dual fluorescent staining with DAPI and propidium iodide (PI). In the control group, cells predominantly exhibited uniform blue DAPI fluorescence with intact nuclear morphology and an absence of PI uptake, indicating viable cells with preserved membrane integrity. Treatment with *A. agallocha* at 20 and 40 µg/mL resulted in a slight increase in merged blue–pink fluorescence, indicating early apoptotic changes in a limited number of cells. The majority of cells retained normal nuclear morphology, suggesting minimal cytotoxicity at lower concentrations. At 50 µg/mL, a noticeable increase in PI-positive nuclei was observed, accompanied by nuclear condensation and partial fragmentation, indicative of progression toward late apoptosis. Cells treated with 50 µg/mL displayed prominent merged fluorescence with intense pink coloration and marked nuclear fragmentation, reflecting extensive membrane permeabilization and advanced apoptotic or secondary necrotic cell death. At the highest concentration (50 µg/mL), the field showed a substantial reduction in cell density with predominance of PI-stained nuclei, confirming severe cytotoxicity and loss of cell viability. These observations demonstrate a clear concentration-dependent induction of apoptosis and loss of membrane integrity in PC12 cells following *A. agallocha* treatment**. [Figure-11]**

DISCUSSION

The current investigation based on neuroprotective and antioxidant activity analysis of *A. agallocha* extract revealed a distinct phytochemical profile, indicating the presence of multiple bioactive compounds with varying polarity and concentration. The progressive increase in spot intensity with rising sample volumes (10–25 µL) suggests a concentration-dependent enrichment of phytoconstituents within the extract. The well-resolved and prominent bands observed under UV light confirm the chemical diversity of the extract, particularly highlighting the abundance of alkaloids, flavonoids, and phenolic compounds [9,13]**.**

The quantitative estimation of total alkaloids, phenolics, and flavonoids in extracts revealed a consistent increase in absorbance with increasing concentrations, suggesting a strong presence of bioactive phytochemicals. Each class of compound plays a distinct but complementary role in contributing to the plant's therapeutic potential, particularly its antioxidant and neuroprotective properties [18, 20]**.**

HPTLC profiling produced well defined and reproducible chemical fingerprints for *A. agallocha*, highlighting at 366 nm with Rf values at 0.16, 0.24, 0.45, 0.74 and 0.84. These major bands are indicative of flavonoid glycosides (Rf 0.16–0.24), chromone derivatives (Rf 0.45–0.74), these show the stability of their phytochemical composition and supporting their traditional medicinal applications. The chromatograms showed several clearly separated peaks at ultraviolet absorption wavelengths commonly linked to phenolic compounds, flavonoids, and alkaloids. These categories of secondary metabolites are well-documented for their antioxidant, anti-inflammatory, and neuroprotective effects, properties that are especially important in reducing oxidative stress and neuroinflammation associated with Parkinson's disease [22]

A. agallocha is a rich source of phytochemicals with potent antioxidant capacity, which could contribute to its neuroprotective effects observed in PC12 cell models. The synergistic action of alkaloids, phenolics, and flavonoids may underlie the extract's ability to counteract oxidative stress, prevent neuronal damage, and offer therapeutic potential against neurodegenerative diseases [5, 21].

These results align with the unique phytochemical profile of Aquilaria agallocha offers a reasonable chemical explanation for its reported neurostimulant effects and relevance in treating neurological conditions. The HPTLC fingerprint profiles support the HPTLC data and the in vitro assessments conducted with the PC12 cell model, together reinforcing the idea that the noted cytoprotective, anti-apoptotic, and reactive oxygen species (ROS)-modulating effects result from the combined action of several bioactive components, rather than from a single compound. The suggested HPTLC method showed reliability, consistency, and analytical accuracy, making it appropriate for regular quality control, phytochemical standardization, and future bioactivity-directed fractionation of these medicinal plants [23]**.**

The present findings demonstrate the neuroprotective and antioxidant potential of *A. agallocha* extract in PC12 cell models of Parkinson's disease. MPTP treatment significantly reduced cell viability, confirming its cytotoxic effect and successful induction of neuronal injury. However, co-treatment with AA extract markedly improved cell survival in a dose-dependent manner, with near-complete recovery observed at 30–40 µg/mL. This suggests that AA contains bioactive compounds capable of counteracting MPTP-induced oxidative damage and apoptosis [24]**.**

The observed (ROS)antioxidant activity of the extract may be attributed to the presence of bioactive phytoconstituents

such as flavonoids, phenolic compounds, and alkaloids, which are known to neutralize free radicals, chelate metal ions, and modulate redox-sensitive signaling pathways. By reducing intracellular ROS accumulation, the extract likely prevents mitochondrial dysfunction and inhibits downstream apoptotic cascades[12].

Collectively, these findings suggest that the test extract confers significant neuroprotection against MPTP-induced oxidative damage in PC12 cells. The ability of the extract to regulate ROS levels highlights its therapeutic potential in mitigating oxidative stress mediated neurodegeneration and supports its possible application as a neuroprotective agent for the management of Parkinson's disease.[25, 26].

Nuclear integrity is a critical indicator of cellular health, and alterations in nuclear morphology are hallmark features of apoptosis and cytotoxicity. In the present study, fluorescent nuclear staining was employed to assess the effect of the test extract on nuclear morphology of PC12 cells across a wide concentration range [27].

This dual behaviour cytoprotective at lower concentrations and cytotoxic at higher concentrations is a characteristic feature of many bioactive phytochemicals and may be attributed to dose-dependent modulation of intracellular signaling pathways. At optimal concentrations, antioxidant and neuroprotective constituents of the extract likely stabilize cellular redox balance and nuclear integrity, whereas excessive concentrations may overwhelm cellular defense mechanisms, leading to apoptosis [28].

These findings indicate that the test extract exhibits a favourable safety profile at lower concentrations, while higher concentrations induce apoptotic cell death and supports the potential use of the extract as a neuroprotective agent within a defined therapeutic window. [19].

In vitro finding from DAPI-dominant fluorescence to PI-dominant merged signals highlights the narrow therapeutic window of *A. agallocha*. While lower concentrations maintain cellular integrity and viability, higher concentrations induce significant apoptotic and necrotic cell death. This biphasic effect is characteristic of phytochemicals containing bioactive constituents that exert protective effects at optimal doses but become cytotoxic upon excessive exposure.

Collectively, these findings demonstrate that *A. agallocha* extract induces at higher dose apoptotic cell death in PC12 cells, with a clear distinction between safe and cytotoxic concentration ranges. The DAPI–PI merge analysis complements the ROS and cell viability data, strengthening the mechanistic evidence for neurotoxicity and supporting careful dose optimization for therapeutic applications [29].

Taken together, these results suggest that *A. agallocha* extract exerts potent antioxidant and neuroprotective effects by reducing ROS generation, preventing apoptosis, and enhancing cell viability in MPTP-treated PC12 cells and may serve as a promising therapeutic candidate for managing oxidative stress–related neurodegenerative conditions, particularly Parkinson's disease [30].

CONCLUSION

This investigation delivers an integrated analysis of the antioxidant and neuroproptective activities exhibited by *A. agallocha* extracts confirmed the presence of multiple phytochemical constituents, including alkaloids, flavonoids, and phenolic compounds. The intensity and clarity of the bands increased with concentration, indicating a strong presence of bioactive compounds. These findings support the phytochemical richness and antioxidant potential establishes that *A. agallocha* extract exhibits strong neuroprotective and antioxidant properties in PC12 cell models of Parkinson's disease. Quantitative phytochemical analysis confirmed the presence of bioactive alkaloids, flavonoids, and phenolic compounds contributing to its antioxidant activity. In MPTP-induced neuronal injury, *A. agallocha* treatment significantly restored cell viability and achieving near-complete recovery at 30–40 µg/mL also effectively reduced intracellular ROS levels, demonstrating its ability to alleviate oxidative stress and maintain redox balance. Fluorescence staining (DAPI and PI) confirmed decreased apoptotic cell death and preservation of normal nuclear morphology, supporting its cytoprotective action. Future work should focus to promotes cell survival, mitigates oxidative damage, and offers potential therapeutic value for the prevention and management of neurodegenerative diseases, particularly Parkinson's disease.

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TABLES AND FIGURE

Table -1: Marker Compounds for Future HPLC Confirmation

Phytochemical Class	Suggested Marker Compound	Rationale
2-(2-Phenylethyl) chromones	Agarotetrol	Chemotaxonomic marker of *Aquilaria*
Sesquiterpenes	Agarospirol	Characteristic agarwood sesquiterpene
Flavonoids	Quercetin	Common flavonoid with strong UV absorption
Flavonoids	Kaempferol	Frequently reported flavonol
Phenolic acids	Gallic acid	Representative polyphenol
Alkaloids	β-Carboline-type alkaloids (if reported)	Minor nitrogenous constituents

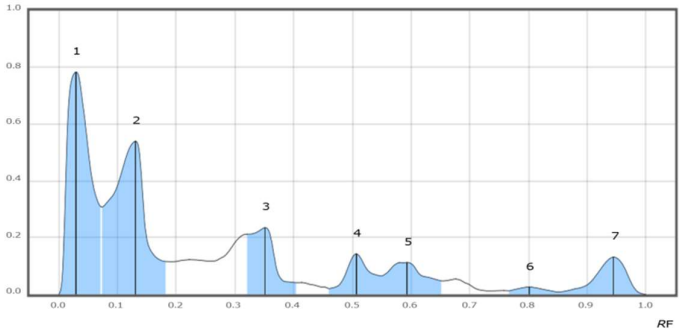

Figure 1 . HPTLC densitometric chromatogram of the ethanolic extract of Aquilaria agallocha developed in chloroform: ethyl acetate: formic acid (5:4:1, v/v/v) and scanned at 254 nm. Prominent peaks were observed at Rf ≈ 0.14, 0.33, and 0.35. The band at Rf ≈ 0.14 may correspond to polar phenolic constituents, while the peaks at Rf ≈ 0.33–0.35 are tentatively attributed to 2-(2-phenylethyl) chromone derivatives and flavonoid aglycones characteristic of agarwood.

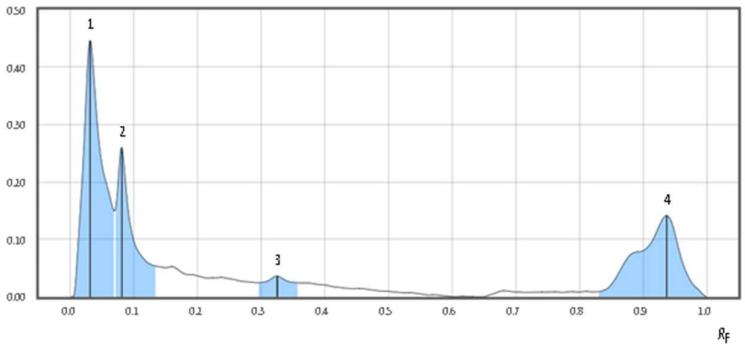

Figure 2. HPTLC densitometric chromatogram of the alkaloid fraction of *Aquilaria agallocha* developed on silica gel and scanned at 254 nm. A prominent peak observed at Rf ≈ 0.35 suggests the presence of alkaloid-like constituents, based on its chromatographic behavior and UV absorption characteristics.

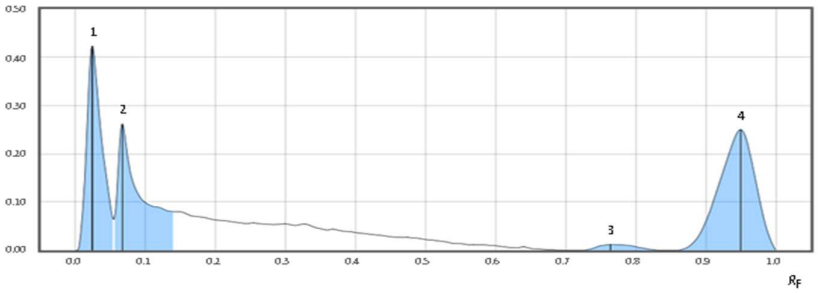

Figure 3. HPTLC densitogram of Aquilaria agallocha extract scanned at 254 nm showing a prominent peak at Rf ≈ 0.72, tentatively indicating alkaloid-like constituents.

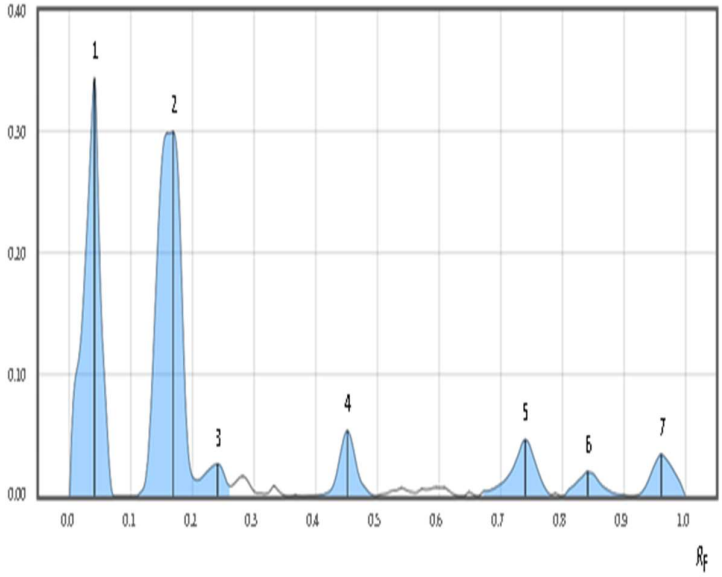

Figure 4. HPTLC densitogram of *Aquilaria agallocha* extract scanned at 366 nm showing fluorescent bands at Rf ≈ 0.16, 0.24, 0.45, 0.74, and 0.84, suggestive of flavonoids and chromone-type constituents.

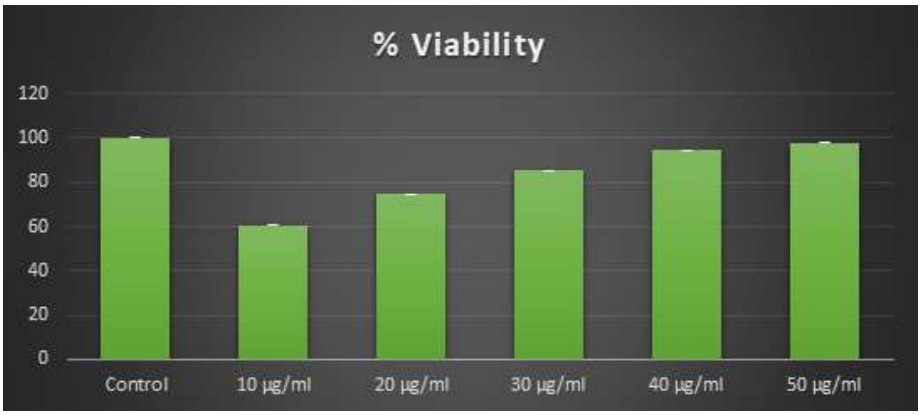

Fig: 5. Cell viability of PC12 cells treated with *Aquilaria agallocha* extract in the absence of MPTP.

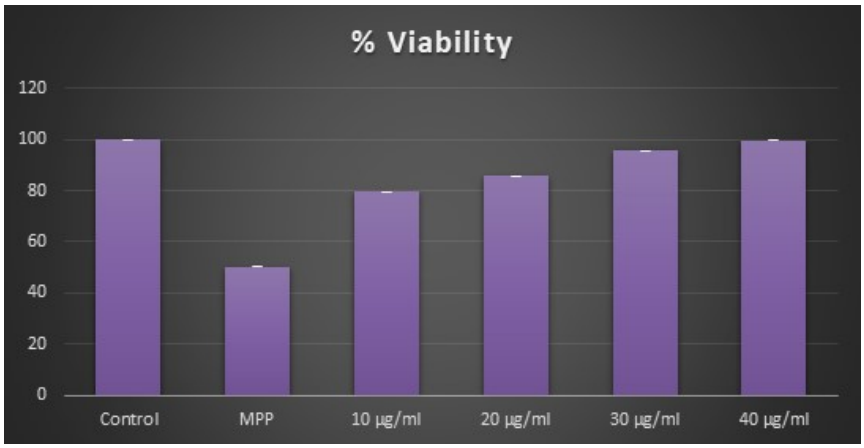

Figure: 6 Cell viability of PC12 cells treated with *Aquilaria agallocha* extract in the. With MPTP

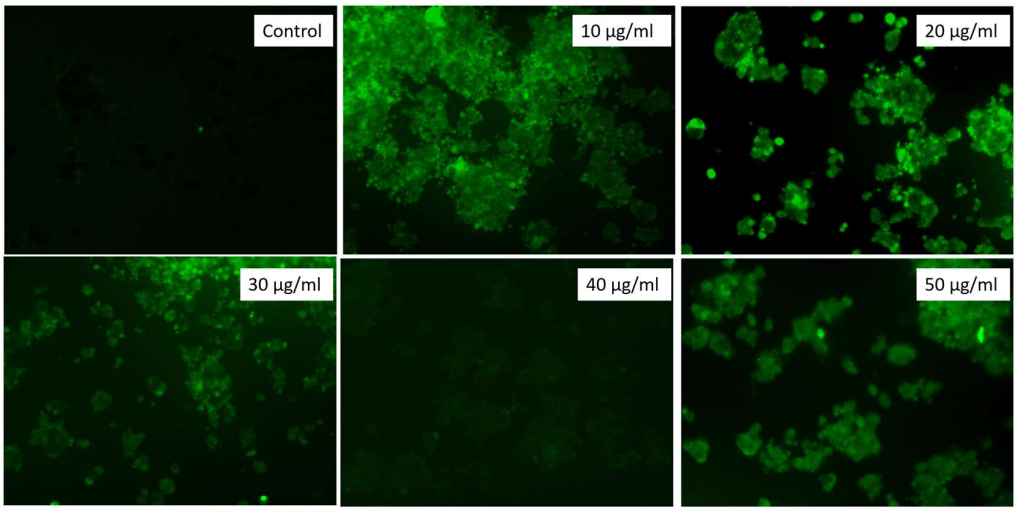

Figure- 7- Effect of test extract on intracellular ROS generation in PC12 cells under normal conditions as assessed by DCFH-DA fluorescence microscopy.

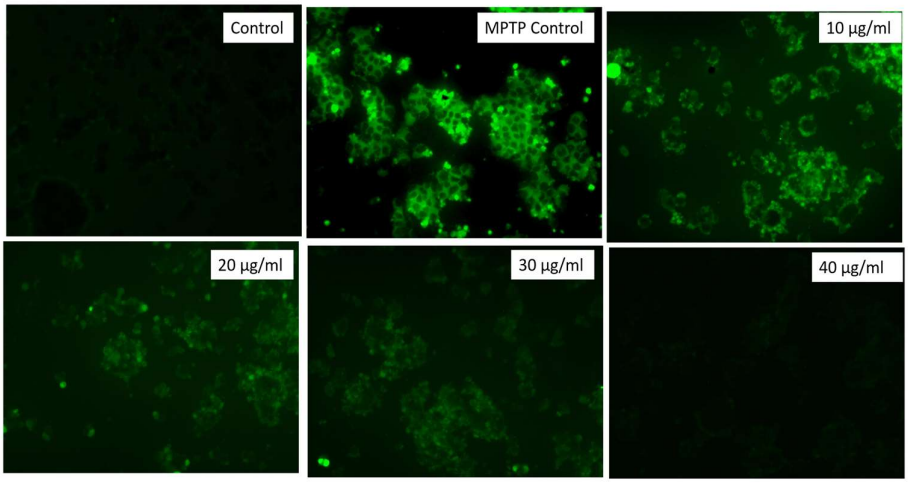

Figure -8 Effect of test extract on intracellular ROS generation in PC12 cells MPTP-induced conditions as assessed by DCFH-DA fluorescence microscopy.

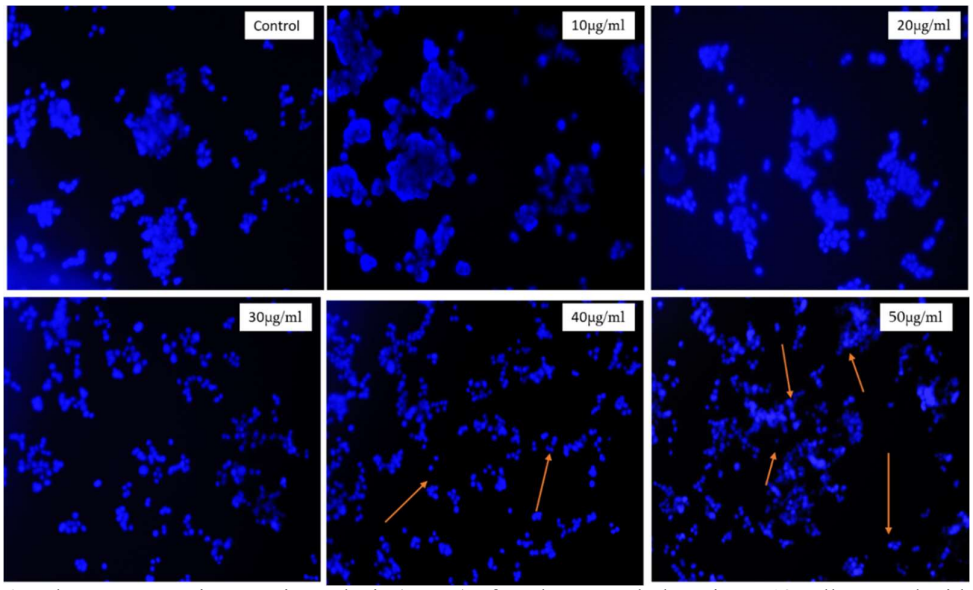

Figure – 9 - Fluorescence microscopic analysis (DAPI) of nuclear morphology in PC12 cells treated with different concentrations of the AAs extract, showing concentration-dependent apoptotic nuclear changes as evidenced by chromatin condensation and nuclear fragmentation (arrow marked).

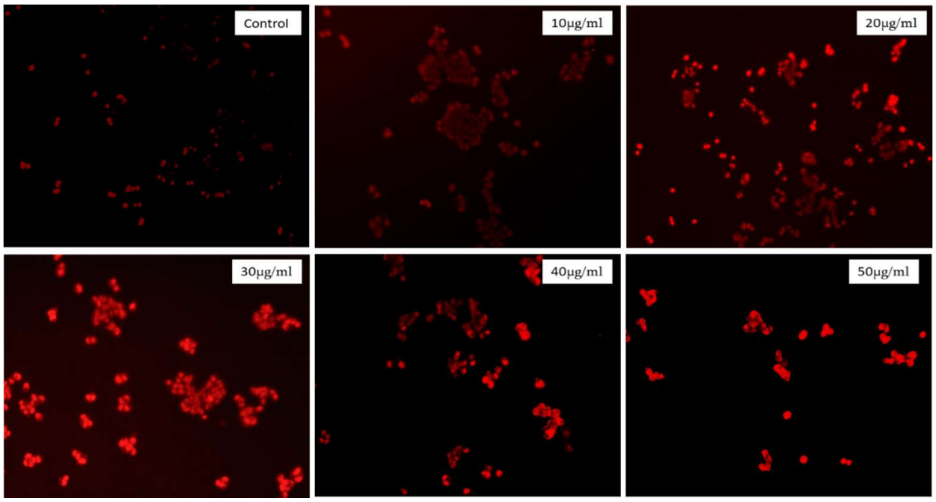

Figure –-10: Red fluorescence microscopic analysis (PI) of PC12 cells treated with increasing concentrations of the test extract, showing concentration-dependent loss of membrane integrity and increased cell death.

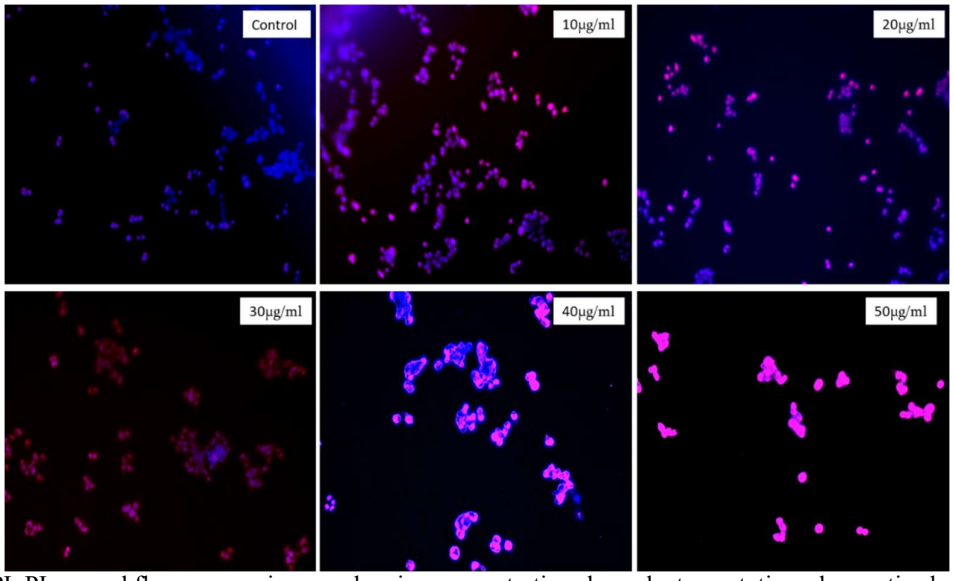

Fig -11*:* DAPI–PI merged fluorescence images showing concentration-dependent apoptotic and necrotic changes in PC12 cells treated with *Aquilaria agallocha* extract**.**