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Phytochemical analysis and evaluation of memory enhancing effects of standardised *Grewia asiatica* berry extract in scopolamine-induced amnesia rat model of Alzheimer's disease

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Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterised by memory loss and cognitive decline, with limited effective treatments. This study explores the memory-enhancing potential of a polyphenol-rich extract (PE) derived from *Grewia asiatica* L. berries. The study aims to standardise the extract and assess its potential for preventing neurodegeneration. The deseeded berry mass was extracted in the acidified methanol (0.1 % formic acid) to get PE. The PE exhibited to contain total phenolics of about 13.2±0.265 mg gallic acid equivalents (GAE) per gram and 10.71±0.688 mg quercetin equivalents (QE) per gram—total flavonoids, whereas the anthocyanin content was 2240.83±2.22 mg cyd-3-glu E/L. Two high-performance thin-layer chromatographic (HPTLC) methods were developed: one for fingerprinting anthocyanins and the other for estimating epigallocatechin gallate and gallic acid content in PE. It demonstrated significant antioxidant activity and acetylcholinesterase inhibition in *in vitro* assays. In a scopolamine-induced amnesia rat model, PE improved cognitive function, as evidenced by reduced transfer latency in the elevated plus maze and an increased discrimination index in the novel object recognition test. Thus, this study enhances scientific knowledge in quality assessment and suggests that *G. asiatica* berries could serve as a potential functional food or therapeutic adjunct for mitigating AD progression.

Keywords: Anthocyanins, *Grewia asiatica* L., High-performance thin-layer chromatographic fingerprint, Memory enhancement, Phalsa, Polyphenols

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Introduction

Alzheimer's disease (AD) is a prevalent form of dementia, marked by memory loss, declined cognition, and difficulties in performing daily tasks[1]. Multiple factors are involved in disease prognosis, such as forming beta-amyloid plaques and tau tangles, disrupting neuronal communication, and inflammation. It leads to widespread neurological death, brain atrophy, and cognitive decline. Oxidative stress, neurotransmitter deficits, genetic mutations, and vascular factors (hypertension, diabetes) also contribute to AD. The complex interplay of these factors leads to neuronal dysfunction and, ultimately, cognitive decline[2,3]. The available treatments for AD are limited, and the complex treatment regimen of medicines and non-pharmacological interventions makes it difficult for the patients to adhere to the prescribed therapies. Limited preventive options necessitate complementary therapies that can target multiple pathways. Plant-based materials often exhibit multi-target effects, making them valuable sources for combating AD[1,3,4]. *Grewia asiatica* L. berries, belonging to the family Tiliaceae, are among natural materials that contain abundant polyphenols that can target multiple AD pathways[5-10]. Traditionally, berries are known as cooling tonic and aphrodisiacs used to alleviate thirst and burning sensation, reduce biliousness, and treat inflammation, heart ailments, blood disorders, and fever. The bark acts as a demulcent, root bark has antirheumatic activity, whereas leaves are used in pustular eruptions[6,8].

A literature survey showed that different extracts of *G. asiatica* berries enhanced memory and learning abilities in scopolamine-induced amnesia animal models[11,12] and reduced anxiety-related behavioural changes and depression[9]. Brain biochemical assays and histopathology showed that berries elevated acetylcholine (ACh), decreased reactive oxygen

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species (ROS), and restored neuronal cell damage[11]. The hydro-alcoholic, dichloromethane, and methanol fractions of *G. asiatica* berries have demonstrated anti-inflammatory and anti-cancer effects in animal models and cell line assays[13].

Researchers have identified various phytochemicals in *G. asiatica*; however, the available data is limited. LC-MS and UPLC-MS analyses have revealed the presence of polyphenols, including flavonoids, phenolics, flavanols, and anthocyanins, in *G. asiatica* berries responsible for its therapeutic actions[9,10]. The LC-MS analysis revealed the presence of flavonols (44.36%), followed by anthocyanins (28.01%) and flavone (23.56%). The other classes detected include dihydroflavonols, isoflavonoids, hydroxycinnamic acid, flavanones, flavanols and other phenolics. The flavonols such as quercetin, kaempferol, myricetin, rhamnetin, and isorhamnetin and their glycosides with a significant content of quercetin-3-O-glucosyl-xyloside were detected in the fraction. The prevalent types of anthocyanins included cyanidins (79.1%), delphidins (18.3%), followed by petunidin (2.48%), with the major being cyanidin-3-O-sambubioside, followed by cyanidin-3-O-arabinoside and delphinidin-3-O-arabinosid[9]. The flavanols such as catechin, epigallocatechin and its derivatives and dihydroflavonol – dihydroquercetin were found in the fraction. The third major group, flavone include dapigenin, luteolin, hydroxyluteolin and their derivatives.

Similarly, LC-MS/MS analysis of the purified anthocyanin fraction of *G. asiatica* berries exhibited a high amount of cyanidin-3-O (6” acetyl glucoside), pelargonidin-3-O-(6” acetyl glucoside), and peonidin-3-O-glucoside, as well as the other few minor anthocyanins detected were glycosides of delphinine, pelargonidin, peonidin, and malvidin types of anthocyanidin[10]. However, there is limited information on simple and rapid analysis methods for *G. asiatica* extracts and the effect of standardised polyphenol-rich extracts on animal models of memory loss and cognition loss. We attempted to analyse *G. asiatica* berry extract to address this gap using high-performance thin-layer chromatography (HPTLC). The main objectives were to standardise the polyphenol extract (PE) of *G. asiatica* berries using HPTLC and its evaluation on a scopolamine-induced amnesia rat model. Before commencing HPTLC, we evaluated the PE for its total phenolic, flavonoids, and anthocyanin content. As a marker of the phenolic group, gallic acid was quantified, whereas epigallocatechin gallate, a representative flavonoid, was estimated in the *G. asiatica* extract. The qualitative analysis of anthocyanins was performed by developing its HPTLC fingerprinting; however, quantification was not performed as standards were unavailable. Gallic acid, epigallocatechin gallate, and anthocyanins are potent antioxidant and anti-inflammatory compounds that modulate various AD-related pathways, such as Aβ fibril formation, neuroinflammation, ACh regulation, and the neutralisation of reactive oxygen species[14-18]. The standardised PE was tested for its potential to be used as a preventive measure in case of AD and was evaluated through *in-vitro* antioxidant and enzyme inhibition activities. Then, it was tested in scopolamine-induced amnesia model in rats.

Materials and Methods

Reagents, chemicals, and consumables

Analytical-grade chemicals were used. Reagents and solvents, the reference standard of quercetin (95% purity) and the gallic acid (99% purity) standard were purchased from Loba Chemie. The reference standard of epigallocatechin gallate (98% purity) was purchased from Yucca Enterprises, Mumbai, India. TLC aluminium sheets with precoated silica gel 60 F_{254} (0.25 mm) (Merck, Darmstadt, Germany) were used.

G. asiatica berries

Fresh and ripened berries of *G. asiatica* were collected from Gandhinagar, Gujarat, India, in May 2022 and authenticated at Agarkhar Research Institute, Pune (AUTH - 21-52). The fruits were cleaned with water and wiped. The seeds were manually separated, and the deseeded berry mass was stored at -20ºC for further processing.

Experimental animals

Male Wistar rats aged 6-8 weeks, weighing approximately 180-200 g each, housed at the animal house facility at AISSMS College of Pharmacy, Pune. The animals were housed in polyacrylic cages in groups of six under laboratory conditions. They were maintained at 22-24°C and 50-60% humidity in a controlled animal facility with a 12-hour light-dark cycle. The rats were acclimatised for approximately 7 days before behavioural studies commenced. All experiments were conducted during the day between 8:00 am and 12:00 noon. The animals had access to standard food pellets and potable drinking water *ad libitum*. The study protocol was approved by the Institutional Animal Ethics Committee (Project Approval Number - CPCSEA/IAEC/PT-08/01-2K23).

Experimental

Extraction of polyphenol extract (PE)

The polyphenols were extracted using the reported method with some modifications[9]. About 5 g of deseeded berry mass was infused in 20 mL of acidified methanol (0.1% formic acid) with continuous stirring on a magnetic stirrer kept at 200 rpm for about 2 h at room temperature, protected from light in an amber-coloured vessel. The process was repeated with the same fruit material and fresh extraction solvent until it got discoloured, and after all repetitions, the collected extracts were combined. The PE was separated from marc by centrifugation, performed at 1000 rpm for 5 min. The liquid extract was concentrated in a vacuum oven (Innovative Instruments, DTC-96) for about 24 h and stored in amber-coloured bottles at -20ºC until further analysis.

Phytochemical analysis

The PE was tested for total flavonoid content (TFC) using the aluminium chloride method[19] and total phenolic content (TPC) using the Folin-Ciocalteu reagent method[19]. In contrast, anthocyanin content was measured by pH-differentiation method[19] using a UV-visible spectrophotometer (Shimadzu-1780).

HPTLC analysis

The *G. asiatica* berry is reported to contain different phytoconstituents; therefore, based on a literature survey, the presence of anthocyanins and other polyphenols such as catechins, phenolics, flavonoids[9,10] etc. was checked by HPTLC (CAMAG, Muttenz, Switzerland).

Anthocyanin analysis

About 100 mg of PE was dissolved in 10 mL methanol, and 20 µL of this was applied on a precoated silica gel plate as a band of 8 mm using a Linomat V automatic sample applicator (CAMAG, Muttenz, Switzerland) and a glass syringe (100-µL, Hamilton, Bonaduz, Switzerland). For the resolution of anthocyanins present in the PE, a mobile phase consisting of ethyl acetate: n-butanol: formic acid: trichloroacetic acid (2:7:1:1, *v/v/v/v*) with a saturation time of 30 min in a twin trough TLC chamber (20 cm × 10 cm × 4 cm; CAMAG) was used. The detection was performed at a wavelength of 530 nm, a slit dimension of 6.00 mm × 0.45 mm, with a scanning speed of 10 mm/s and the radiation source: tungsten lamp (TLC Scanner 4 connected to winCATS software V. 4.06, CAMAG).

Method development for determination of epigallocatechin gallate and gallic acid

Sample and standard preparation: The 100 mg of PE was dissolved in 10 mL methanol. The 10 mg of epigallocatechin gallate was dissolved in 10 mL methanol. Similarly, 10 mg of gallic acid was dissolved in 10 mL methanol; this served as a standard stock solution. Further, the epigallocatechin gallate of 30 µg/mL and gallic acid of 5 µg/mL were prepared from the stock and used in analysis.

Sample application and detection: About 20 µL of PE sample was applied on a TLC plate along with the standards on adjoining tracks as a band of 6 mm using a Linomat V automatic sample applicator (CAMAG, Muttenz, Switzerland) and a glass syringe (100-µL, Hamilton, Bonaduz, Switzerland). The plate was developed in an optimised mobile phase of toluene: acetone: formic acid (4.5:4.5:1 *v/v/v*) with a saturation time of 15 min and was optimised for separation. The detection was performed at a wavelength of 275 nm, a slit dimension of 6.00 mm × 0.45 mm, a scanning speed of 10 mm/s, and a deuterium lamp as a radiation source (TLC Scanner 4 connected to winCATS software V. 4.06, CAMAG). The optimised method was used to standardise the PE concerning epigallocatechin gallate and gallic acid content and validated.

Validation of high-performance thin layer chromatography method

The method was validated according to the quality guidelines (Q2 (R1)) established by the International Conference on Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH)[20].

Specificity

Both standards and PE were analysed to verify the specificity of the developed method. The presence of epigallocatechin gallate and gallic acid in PE was verified by matching the retardation factor and UV spectrum with the reference standards. The peak purity of standards was established by comparing the UV spectra of the bands at the start of peak (s), peak apex (m), and end of peak (e) in WinCATS software.

Calibration curve/linearity

Different volumes of standard (5 to 25 µL) were applied on the TLC plate to get concentrations of the spotted amount of 150-750 ng/band for epigallocatechin

gallate and 25-125 ng/band for gallic acid. A linear regression analysis was performed to correlate the amount of standards spotted with its peak area.

Accuracy

The method's accuracy was evaluated using the standard addition method at 80, 100, and 120% of standards on pre-analysed PE. The recovery percentage was calculated by comparing the measured amount with the added amount.

Precision

The inter-day and intra-day precision was assessed by analysing the six replicates of standards on different days and at different times within a day. For intraday and interday precision, the % RSD of peak areas was calculated to evaluate the method's precision. The repeatability of the sample application and peak area measurement was carried out using 6 determinants of standards in a day and represented as relative standard deviation (% RSD).

Detection limit (DL) and quantification limit (QL)

DL and QL of the developed method were calculated from the standard deviation of the response of the lowest concentration and the slope of the calibration curve as per the ICH guidelines using the equations given below:

$$DL = 3.3 \times \sigma/S \quad \text{... (1)}$$

$$QL = 10 \times \sigma/S \quad \text{... (2)}$$

where σ is the standard deviation of response, and S is the slope of the calibration curve.

Robustness

Slight variations in chamber saturation time, mobile phase composition, and detection wavelength were made to observe their impact. It was evaluated in triplicate at a concentration level of 300 ng/band of epigallocatechin gallate, and 50 ng/band of gallic acid, and % RSD of peak areas were determined.

In vitro tests

In vitro antioxidant activity

The *in vitro* free radical scavenging capacity of standardised PE was assessed by performing three antioxidant assays using a UV Spectrophotometer viz. DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay, nitric oxide assay and hydrogen peroxide assay[21].

In vitro Acetylcholinesterase (AChE) inhibition activity

The AChE inhibition capacity of the PE was assayed according to the *in-vitro* colourimetric method by Ellman using acetylthiocholine iodide as a substrate and compared to Donepezil (standard)[22-24].

In vivo testing of standardised PE on scopolamine-induced amnesia rat model

Twenty-four healthy male Wistar rats were chosen and randomly allocated into six groups of six animals each to assess their response to the exteroceptive behaviour model. The rats were administered either vehicle, PE, or donepezil daily for 14 days as per the treatment plan in Table 1. One hour after the last dose on day 14, all groups except the control group (Group I) received scopolamine 1 mg/kg by intraperitoneal group. A control group received 1 mL/kg physiological saline instead of scopolamine. Then, 45 min after the administration of scopolamine, behavioural tests were conducted to test the nootropic effects of PE[25,26].

Behavioural tests

Elevated Plus Maze (EPM)

The EPM test serves as the exteroceptive model, utilised to test learning and memory function as a nootropic activity of the treatment in laboratory animals. The test was conducted following a previously reported method[11,25,26]. The apparatus consisted of two open arms (50*10 cm) and two closed arms (50*10*40 cm) (L*W*H), extending from a central platform, elevated to a height of 75 cm from the floor. On the 14th day of study, each rat was placed on the edge of an open arm, facing away from the central platform, and the duration of time taken by the rats to move from the open arm to either of the closed arms referred to as transfer latency (TL), was recorded. This phase corresponds to the acquisition phase, indicative of learning. Subsequently, on day 15, TL was measured again, representing retention

Table 1 — Treatment Plan

Group	Day 0 to Day 14	Day 14	No of animals
I	Distilled water (1 mL, p.o)	Saline, 1 mL /kg , i.p.	6
II	Distilled water (1 mL, p.o)	Scopolamine, 1 mg/kg , i.p.	6
III	PE (400 mg/kg, p.o.)		6
IV	Donepezil (3 mg/kg, p.o.)		6

memory, corresponding to the memory retrieval index. The inflexion ratio was calculated using equation (3)[26,27] from the recorded transfer latency.

$$\text{Inflexion ratio (IR)} = (L1 - L0)/ L0 \quad \text{... (3)}$$

L1 = TL measured in seconds on day 14; L0 = TL measured in seconds on day 15 after 24 hours

Novel Object Recognition (NOR) test

The NOR test was conducted in an apparatus consisting of an open field box (40×40×40 cm) made of acrylic transparent side walls. The object recognition test was performed as per previously reported methods[27,28]. During the treatment, on day 12, each rat underwent the habituation phase in the open field. On day 13, during the training phase (T1), two identical objects (red, round shaped) were positioned in two diagonal corners of the open field, each 10 cm away from the sidewall. Each rat was placed in the centre of the open field and allowed to explore these two identical objects for 5 minutes. Afterwards, they were returned to their cages.

Subsequently, after 24 hours of T1, on day 14, the test phase (T2) was conducted. During T2, a new object (an orange, oval) was introduced, and the rats were re-exposed to both the familiar (F) and the new (N) objects for 5 minutes. The preference of each rat towards both objects was recorded via camera. The time spent by the rat exploring each object during T2 was recorded, and the discrimination index was calculated using equation (4).

$$\text{Discrimination Index (DI)} = (TN - TF)/ (TN + TF) \quad \text{... (4)}$$

TN = time spent with a novel object; TF = time spent with a familiar object

Biochemical estimation

After evaluating behavioural tests, on the 15th day, the animals were sacrificed by cervical dislocation. The whole brain was removed from the skull and weighed. Further, acetylcholinesterase activity and lipid peroxidation level were measured using the methods mentioned in the literature[29].

Histopathology

After removal, the brain samples were preserved in a 10% formalin solution. The rat's hippocampus was then stained with an eosin solution and examined under a microscope for any observable changes[29,30].

Statistical analysis

A one-way ANOVA followed by the Tukey-Kramer Multiple Comparisons Test was run using GraphPad Instat for 32-bit Windows, version 3.10. The data was represented as mean±SEM values and n = 6 per group.

Results

Estimation of TPC, TFC, and total anthocyanin content

The TPC and TFC content were 13.2±0.265 mg GAE/g and 10.71±0.688 mg QE/g of PE, respectively. The anthocyanin content, quantified using the pH differential method, was obtained to be 2240.83±2.22 mg cyd-3-glu E/L of PE.

Fingerprinting analysis by HPTLC

Two separate HPTLC methods were developed, the first to analyse anthocyanins and the second to simultaneously estimate epigallocatechin gallate and gallic acid as one of the bioactive constituents in *G. asiatica*.

HPTLC analysis of anthocyanins

The best mobile phase that could separate anthocyanins is ethyl acetate: n-butanol: trichloroacetic acid: formic acid (2:7:1:1, v/v/v/v) with a saturation time of 30 min. After plate development, two spots—pink and purple-coloured- were observed at R_f = 0.35 and R_f = 0.55; see Fig. 1(a) for the plate scanned at 530 nm. Anthocyanins were not quantified as reference standards were unavailable, but we could confirm the presence of anthocyanins by the characteristic maximum wavelength at 530 nm. The UV-visible spectra of the detected bands corresponding to the anthocyanins are represented in Fig. 1(b). This observation is consistent with the UV-visible spectra of different anthocyanins reported by Jorge Custodio-Mendoza and Marcin Kurek in the Mendeley Data[31], supporting the identification of anthocyanins in PE.

Method development and validation for estimation of epigallocatechin and gallic acid

The presence of epigallocatechin gallate and gallic acid in the PE was confirmed by comparing R_f values and UV spectra with reference standards. The optimised mobile phase that separated and resolved epigallocatechin and gallic acid from other components present in the PE was composed of toluene: acetone: formic acid (4.5:4.5:1 *v/v/v*). The epigallocatechin gallate and gallic acid were resolved with $R_{f=}$ 0.37 and $R_{f=}$ 0.54, respectively, Fig. 2.

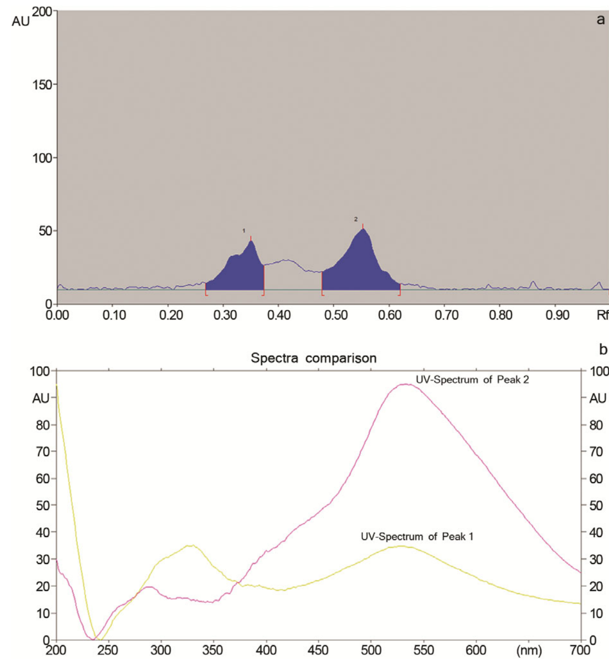

Fig. 1 — a) HPTLC Densitogram of PE representing anthocyanin peaks, and b) UV-visible spectra of peaks representing anthocyanins.

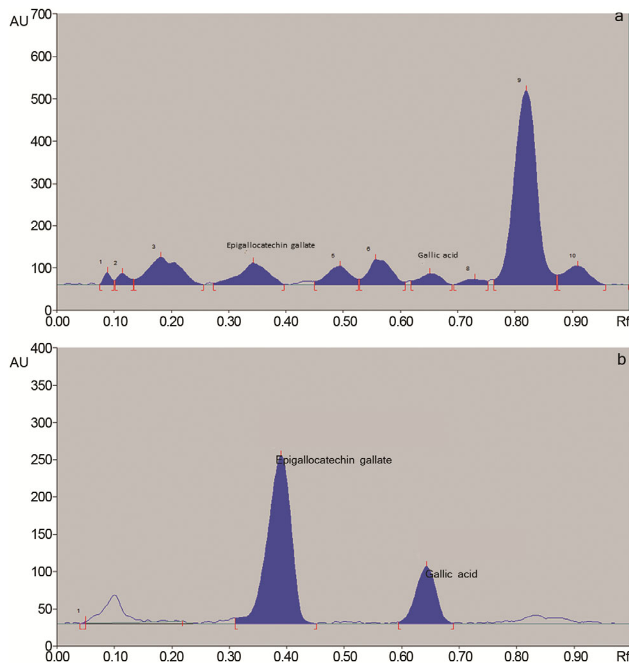

Fig. 2 — HPTLC Densitogram a) PE, and b) Standard Epigallocatechin gallate and Gallic acid.

This developed method was validated for different parameters such as specificity, accuracy, precision, DL, QL, and robustness. Specificity was verified by comparing the standard's R_f value and UV spectra with the peaks detected in the PE. The peak purity of both identified standards was defined by recording the spectra in the UV range at various positions in the peak. A satisfactory correlation was obtained between overlay spectra of the peak at peak start, peak maximum, and peak endpoints (purity > 0.99); correlation values (r) are mentioned in Table 2. The overlay spectra of standards and compounds in the extract are displayed in Fig. 3. The calibration was found to be linear within the 150-750 ng/band concentration range for epigallocatechin gallate, whereas 25-125 ng/band for gallic acid with a correlation coefficient of $r^2 = 0.99$, respectively, refer Fig. 4. The optimum recovery indicated that the developed method was accurate. DL and QL were obtained to be 11.11 and 33.66 ng/band for epigallocatechin gallate. For gallic acid, DL and QL were obtained to be 4.38 and 13.28 ng/band. The slight variations in the optimised parameters, such as mobile phase composition, saturation time, and scanning wavelength, did not impact the peak area, as % RSD was found to be < 2. % RSD of peak area of intraday and interday precision was found to be < 2. Details of validation results are given in Table 3.

Quantification of epigallocatechin gallate and gallic acid

The amount of epigallocatechin and gallic acid in the PE of *G. asiatica* berries was 13.09±0.92 and 2.13±0.02 mg/g. The previous study reports 53.5 µg/g of gallic acid in *G. asiatica* pomace extracted in aqueous methanol by LC-MS-MS[25].

***In-vitro* antioxidant and AChE inhibition activity**

The PE demonstrated significant radical scavenging potentials in the DPPH radical scavenging assay (69.45%), hydrogen peroxide assay (78.62%), and nitric oxide assay (67.58%). The IC_{50} values are mentioned in Table 4.

In addition to antioxidant capacity, the extract exhibited notable acetylcholinesterase inhibition activity (17.19 - 65.60 % over a linear concentration range), with an IC_{50} value of 41.99±0.30 µg/mL.

In-vivo evaluation of standardised PE on scopolamine-induced amnesia rat model

Scopolamine is a muscarinic antagonist that disrupts the central cholinergic system, leading to memory impairments. It induces degeneration of cortical cholinergic neurons, which is strongly linked to the cognitive deficits observed in AD. Hence, behavioural changes through different tests are evaluated in the scopolamine-induced amnesia model, which is widely used to study the anti-AD effects of therapeutics in preclinical studies[26].

Table 2 — Specificity Indicated by Peak Purity Values

Compound	r (s, m)[a)]		r (m, e)[b)]	
	Standard Track	Sample Track	Standard Track	Sample Track
Epigallocatechin gallate	0.999606	0.999609	0.999404	0.99841
Gallic acid	0.998255	0.999738	0.996016	0.999082

a) Correlation of spectrum at the start of a peak with spectrum at the centre of the peak
b) Correlation of spectrum at the centre of the peak with spectrum at the end of the peak

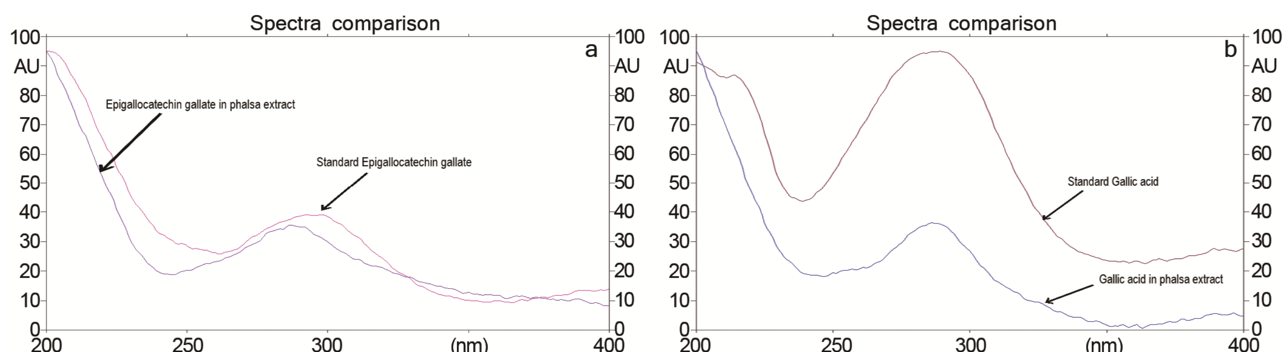

Fig. 3 — Overlay UV spectra a) standard epigallocatechin gallate and peak at same R_f from PE, and b) standard gallic acid and at same R_f from PE; of *G. asiatica berries*.

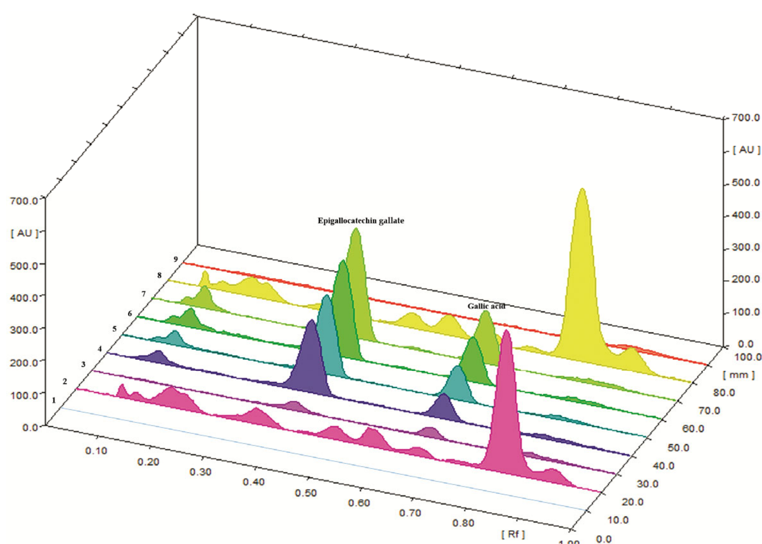

Fig. 4 — Linearity Plot - HPTLC Densitogram of Reference Standards and PE of *G. asiatica* berries. Track 1 and 9: Blank; Track 3 to 7: Standards (epigallocatechin gallate – 150 to 750 ng/band, gallic acid – 25 to 125 ng/band); Track 2 and 8: PE of *G. asiatica* berries.

Behavioural tests

Effect on transfer latency in EPM

In the disease control group, the inflexion ratio was significantly decreased, i.e., 0.056±0.0067, compared to 4.938±1.023 in the control group. We observed a significant increase in the inflexion ratio in the PE group (6.120±0.7579) compared to the disease control group (Fig. 5). This indicates improvement in scopolamine-induced memory impairments. The results reiterate that PE improved memory retention.

Effect on discrimination index in (NOR) test

The NOR test evaluated whether PE treatment could reverse the scopolamine-induced recognition impairments. We observed a drop in the discrimination index of the familiar object, which is -0.742±0.088 in the disease control group compared to the 0.6483±0.025 of the control groups. However, it improved in the PE (0.6980±0.237) and donepezil (0.6340±0.3146) groups as compared to the disease control group (Fig. 6).

Table 3 — Method Validation Data

Validation Parameter		Result	
		Epigallocatechin gallate	Gallic acid
Range		150-750 ng/band	25-125 ng/band
Linearity (Regression equation and correlation coefficient)		$y = 5.8984x - 150.89$ $R^2 = 0.9939$	$y = 15.46x - 201.82$ $R^2 = 0.996$
Accuracy (% Recovery)	80%	98.90	97.87
	100%	101.00	98.79
	120%	100.17	102.56
Precision (%RSD, n=6)	Intra-day	1.05	1.44
	Inter-day	1.60	1.22
Detection Limit (DL) (ng/band)		70.54	9.56
Quantification Limit (QD) (ng/band)		213.77	28.97
Robustness (% RSD)	Saturation time ±5 (min)	1.37	1.67
	Mobile phase composition	1.40	1.82
	Wavelength ±2 nm	1.55	1.41

Table 4 — *In vitro* activity

Sample	IC_{50} Value (µg/mL)			
PE of *G. asiatica* berries	DPPH Assay	Hydrogen Peroxide Assay	Nitric Oxide Assay	Acetylcholinesterase Assay
	165.65±3.93	52.63±2.44	195.73±5.35	41.99±0.30
Ascorbic acid (Standard)	83.59±3.93	38.02±9.52	96.08±3.26	---
Donepezil (Standard)	---	---	---	36.05±0.61

*mean ± SD (n = 3)

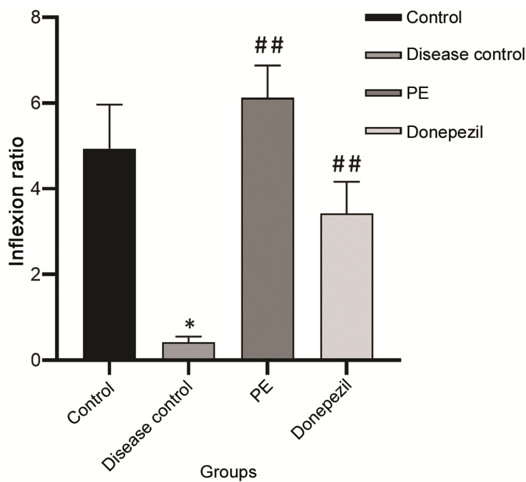

Fig. 5 — Elevated Plus Maze - inflexion ratio. [Data is represented in as mean± SEM (n=6/group)] Significant difference is denoted by *P <0.001 as compared to control group I; # #P <0.001 as compared to Disease Control group II.

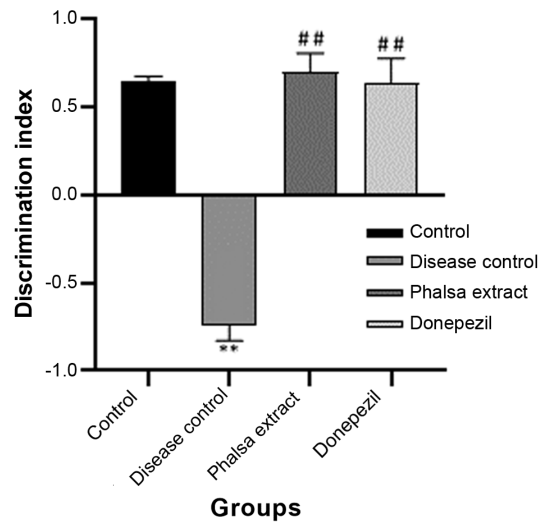

Fig. 6 — Novel Object Recognition - discrimination Index. [Data is represented in as mean±SEM (n=6/group)] Significant difference is denoted by **P <0.001 as compared to control group I; # # P <0.001, as compared to Disease Control Group II.

Biochemical estimation

The AChE activity and lipid peroxidation levels were measured in brain homogenate. The AChE activity was represented as the rate in moles of substrate hydrolysed/min/g of wet tissue. In contrast, the lipid peroxidation was expressed as nanomoles of malondialdehyde (MDA) species formed. The AChE activity decreased in Group III PE compared to the raised (0.112±0.015) in Group II, i.e., the disease control group, indicating the inhibition of AChE by PE (refer to Table 5). In the PE-treated group (Group III) reduced MDA, i.e., 0.235±0.002 nM MDA/g of wet tissue; was observed; refer to Table 5.

Histopathological studies

The control group showed normal brain hippocampus, neuronal cells, and brain parenchyma.

Table 5 — Effects of each treatment on brain MDA, AChE activity of Scopolamine-treated rats

S. No.	Group	LPO (nM MDA/g of wet tissue)	AChE activity (mole/min/g of tissue)
1	Control	4.780±0.04286	0.043±0.002417
2	Disease Control	6.195±0.03856*	0.112±0.01470*
3	PE	2.086±0.02958**	0.019±0.00217**
4	Donepezil (Standard)	1.137±0.01395**	0.056±0.003356**

Each value represents the mean ± SEM (n = 6). The level of MDA and AChE activity increased in the Disease control group (**p* <0.001 as compared to the control) and decreased in PE extract and Donepezil-treated groups (***p* <0:001 as compared to the disease control group)

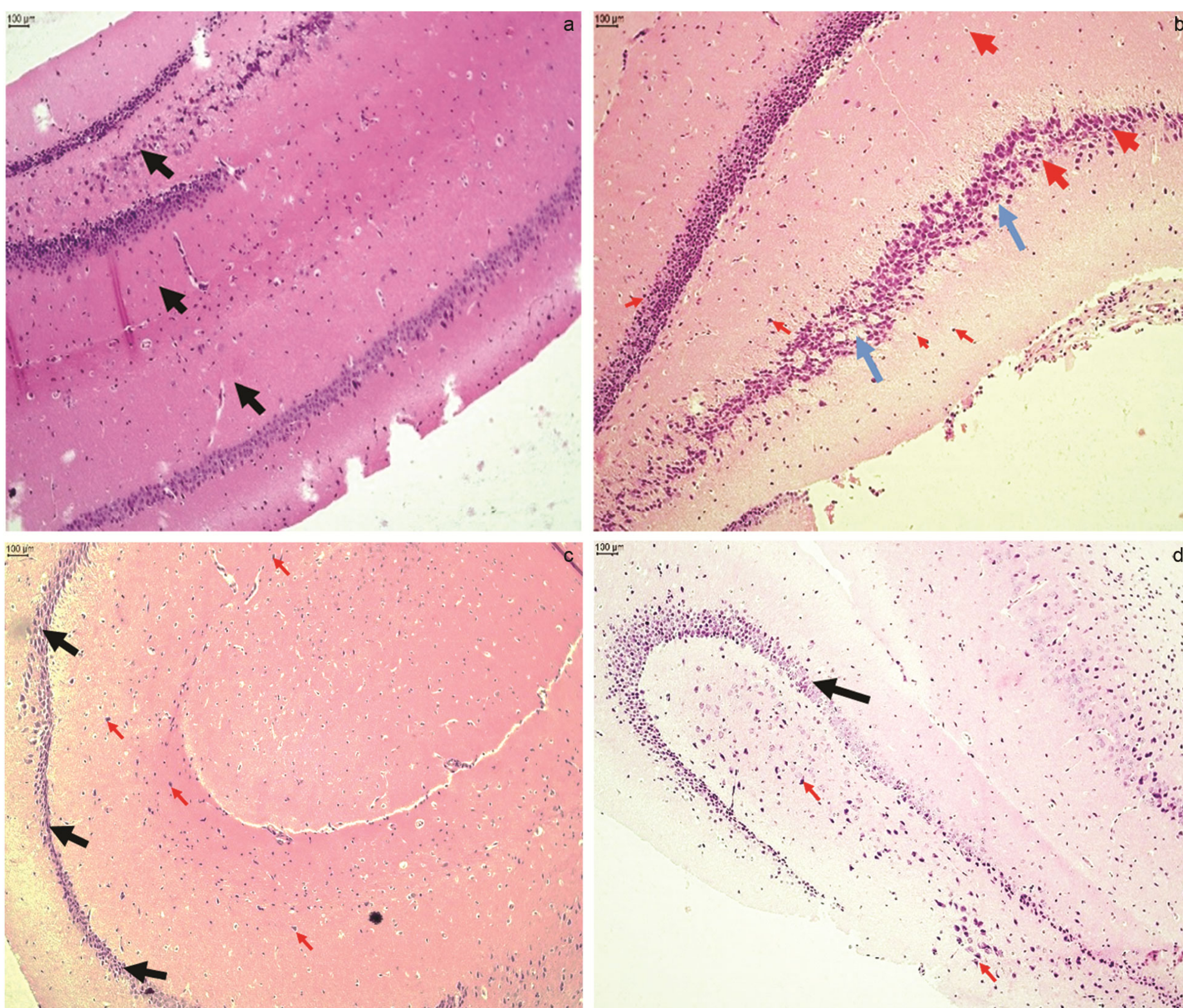

Fig. 7 — Representative photomicrographs of hippocampal sections stained with hematoxylin and eosin (HE) Histopathological changes in the hippocampal CA1 region. a) Control group, b) Disease control group, c) PE group, and d) Donepezil group. Observed Parameters - Neuronal Damage, Hippocampal Oedema, Arrangement of pyramidal cells and polymorphic cells, Pyknotic cell.

In the disease control group, histology of the hippocampus showed significant oedema, severe neurodegeneration, pyknotic nuclei, and distorted pyramidal cells[29]. The histopathological images are represented in Fig. 7. The PE and donepezil treatments showed mild oedema and mild neuronal damage, and the typical arrangement of pyramidal cells exhibited an ameliorating effect.

Discussion

AD is a complex neurodegenerative condition with numerous potential therapeutic targets. It is believed

that early intervention using scientifically validated plant materials or their regular consumption could provide substantial neuroprotective effects, potentially halting or slowing the progression of the disease. The current study aimed to explore the phytochemical profile, standardisation of PE by HPTLC, and evaluation of the memory-enhancing potential of standardised extract through *in vitro* assays and *in vivo* experiments in rats.

The phytochemical analysis of PE revealed 13.2±0.265 mg GAE/g of TPC, comparable with the previous reports on the phenolic content of the different concentrations of *G. asiatica* berry juice and dried berry extract[12]. The TPC obtained in this study is comparable to 5% (15.96 GAE/g) and less than 10, 20, and 30% *G. asiatica* berry extract[12]. The TFC that was obtained is relatively higher than previous reports where 7.92 mg QE/g of flavonoid was found in 50% hydro-methanolic extract and 0.1 to 2.5 mg QE/g in 5-30% of *G. asiatica* extract berry[12]. The anthocyanin content in the present research is higher than 8.1 mg/kg of 50 % hydro-methanolic *G. asiatica* extract[13]. Similarly, the other reported anthocyanin content of 20 and 30% *G. asiatica* extract and dried *G. asiatica* extract (3.20 and 4.12, 4.882 mg/g) are less than the obtained value in this study[12, 13].

The two peaks representing anthocyanins in the PE were detected and separated. This is the first report of anthocyanin detection by HPTLC in *G. asiatica*. The detection of two peaks in PE likely represents different anthocyanins and is consistent with the diversity of anthocyanin types reported in the literature. It is aligned with the previous findings by Koley *et al.*[9], who reported the presence of cyanidin, delphinidin, and petunidin types of anthocyanins, with the major being cyanidin-3-O-sambubioside, followed by cyanidin-3-O-arabinoside and delphinidin-3-O-arabinoside in the polyphenol fraction of *G. asiatica* berries[9]. Similarly, LC-MS/MS analysis of the purified anthocyanin fraction of *G. asiatica* berries has been reported to exhibit a high amount of cyanidin-3-O (6” acetyl glucoside), pelargonidin-3-O-(6” acetyl glucoside), and peonidin-3-O-glucoside, as well as the other few minor anthocyanins identified being glycosides of delphinine, pelargonidin, peonidin, and malvidin types of anthocyanin[10]. The discrepancies between the numbers of anthocyanins detected in this study and reported studies are directly related to a few factors in plant-based research[32]. Factors such as location, climatic conditions, extraction solvent, extraction methods, and purification methods may have impacted anthocyanin content. Therefore, chemical standardisation is essential to maintain the quality of raw herbs in the case of plant research[32].

The other two phytoconstituents, i.e. epigallocatechin and gallic acid, were estimated by HPTLC but using a mobile phase composed of toluene: acetone: formic acid (4.5:4.5:1 *v/v/v*). The method was validated according to ICH guidelines and was robust and reproducible.

This is the first report of the HPTLC method development for analysing anthocyanins and estimating epigallocatechin and gallic acid in the polyphenol extract of *G. asiatica* berries. HPTLC is a simple and cost-effective method of analysis of herbal drugs compared to other advanced and hyphenated methods such as HPLC, UPLC, NMR, HRMS, LC-MS, UPLC-MS, etc. It is a crucial technique for fingerprinting analysis and standardisation of plant-based materials and their formulations using phytochemical standards[32]. The developed method can be used to compare the quality of *G. asiatica* berries from different sources and its products concerning their anthocyanin content. The standardised extract was evaluated for its memory-enhancing activity, estimated by performing *in vitro* and *in vivo* tests.

The results of *in vitro* DPPH radical scavenging agree with the former reports, where 30% *G. asiatica* juice exhibited > 60% inhibition[12]. This can be attributed to its high content of polyphenols; these compounds are known for their strong free radical-scavenging properties, contributing to the antioxidant potential of the extract[33,34]. The notable AChE inhibition exhibited by PE in an *in vitro* test is crucial to demonstrating its neuroprotective activity, particularly in neurodegenerative disorders like AD. The high levels of phenolics and flavonoids in the PE are likely to enhance AChE inhibition[9,10]. This result is comparable to 8.33–50.66 % inhibition of AChE over a concentration range of *G. asiaica* berry juice[12].

For *in vivo* studies, standardised PE was tested in scopolamine-induced amnesia rats. The reduced transfer latency exhibited by PE in EPM and elevated discrimination index in NOR indicate its cognition and memory-enhancing effects. This finding of reduced transfer latency in EPM is in accordance with the previous study by Paul *et al.*[11], in which the petroleum ether, chloroform, and methanol extracts of phalsa berries reduced transfer latency. Recognising novelty is related to cognitive skills measuring the exploration of novel environments. This concept is

the basis of the NOR test and is thus used as a memory function in basic and preclinical research. It evaluates recognition memory and gives valuable insights to assess short-term, intermediate, and long-term memory in basic research[28]. The discrimination index in this study is comparable to the value reported for the 30% *G. asiatica* berries juice in the previous study[12].

Similarly, findings in biochemical assays indicate the beneficial effects of PE, as demonstrated by the reduced AchE activity and lipid peroxidation. These findings are aligned with the impact of methanol extract[11] and 30% juice[12] of *G. asiatica* berries in previous reports. The ACh and ROS pathways play a critical role in AD progression, leading to the formation of amyloid plaques, tau protein agglomeration, and neuronal death. Elevated AChE activity impairs cholinergic transmission through the reduction of ACh. Thus, inhibition of AChE can preserve ACh and offer neuroprotection. These effects are ascribed to the identified phytoconstituents in the PE, such as anthocyanins, epigallocatechin gallate, and gallic acid. A study by Hong and researchers[35] demonstrated that the memory enhancement potential of anthocyanin- and polyphenol-rich highbush blueberry extract is governed by cholinergic activity through AChE inhibition and enhanced antioxidant enzyme activity. Similarly, epigallocatechin gallate is a catechin with neuroprotective potential, especially in AD. It has been found to reduce oxidative damage by scavenging free radicals and activating the Nrf2 pathway, thereby upregulating cellular antioxidant defenses[36,37]. In addition to antioxidant properties, it enhances cholinergic function by inhibiting AChE, resulting in higher ACh levels in the synaptic cleft and improved cholinergic transmission[38]. This is particularly important given that the cholinergic hypothesis of AD suggests that ACh deficiency contributes to cognitive decline.

Gallic acid, another potent phenolic compound, also exerts neuroprotective effects. It acts as a powerful antioxidant by neutralising free radicals and reducing oxidative stress, essential for preventing neuronal damage in AD. It stabilises nuclear factor kappa B (NF-κB), a transcription factor involved in inflammation, thereby protecting against neuroinflammatory processes[38,39]. It has also been found to inhibit the release of pro-inflammatory cytokines, which could otherwise accelerate neurodegeneration.

Overall, the findings from this study suggest that *G. asiatica* berries could be a promising dietary intervention to enhance cognitive health and prevent age-related cognitive decline and neurodegenerative diseases like AD. However, to fully understand the therapeutic potential, phytochemical profiling to identify the active constituents and marker-based standardisation are necessary to validate efficacy and safety.

Conclusion

In the present study, the phytochemical analysis of *G. asiatica* berry exhibited the presence of an ample amount of flavonoids, phenolics, and anthocyanins. This study is the first to report the development of an HPTLC method for separating and fingerprinting the PE derived from *G. asiatica* berries. The anthocyanins were successfully detected, while epigallocatechin gallate and gallic acid were quantified using a validated HPTLC method. The developed HPTLC methods can be used to check the quality of *G. asiatica* berries. The standardised PE demonstrated significant antioxidant and AChE inhibition activities, highlighting its therapeutic potential for managing oxidative stress and cholinergic dysfunction, which are critical in the pathophysiology of neuro-degenerative diseases like AD.

In vivo studies further substantiated these findings, as the PE exhibited notable cognitive-enhancing effects in a scopolamine-induced amnesia rat model. Behavioural tests, including EPM and NOR, confirmed improved memory and learning outcomes. Biochemical assays showed reduced AChE activity and lipid peroxidation in brain tissues, while histopathological analysis revealed neuroprotective effects of *G. asiatica* berries. Overall, this study contributes to the scientific knowledge of *G. asiatica* berries, its chemical evaluation, and its benefits in improving cognition in cases of neurodegeneration. This suggests that *G. asiatica* is a valuable source of natural antioxidants, supporting its potential inclusion in functional foods and dietary supplements.

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Conflict of interest

The authors declare that they have no conflict of interest.

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