



Attenuation of inorganic arsenic exposure-induced cognitive impairments by polyphenols-rich fraction of wild *Rubus ellipticus* berries via reshaping gut microbiota and reduction of oxidative stress-lead neuro-intestinal inflammation

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ABSTRACT

The neurotoxic effects of inorganic arsenic exposure and consequential neurobehavioral outcomes involve activation of inflammatory cascades and reactive oxygen species (ROS)-mediated neuronal damage. Functional foods with characterized bioactive(s) are gaining attention in environmental contaminants-exposure induced pathologies. Here, we investigated the use of an ultrasonication-assisted extraction (UAE) and response surface methodology (RSM)-optimized process for polyphenols-rich extract from a Himalayan berry, *Rubus ellipticus*, against inorganic arsenic [iAs(III)]-induced cognitive impairments in mice. Total phenolic content (TPC), *in-vitro* antioxidant activity and cytotoxicities, along with UHPLC and LC-MS based polyphenolic profiles were determined. iAs(III)-exposure-induced spatial navigation tasks and passive avoidance-based learning performances were assessed. Neurochemical estimations, oxido-nitrosative stress markers, histological analysis and qRT-PCR-based gene expression in brain, ileum and, colon, pro-inflammatory cytokines and LPS levels, gut permeability, short chain fatty acids (SCFAs) levels, along with nanopore-based 16s rRNA metagenomics were performed. RSM-optimized UAE methods showed high TPC and antioxidant activities in polyphenol-rich (flavanols, phenolic acids and, proanthocyanidins) extract from *R. ellipticus*. The extract showed no potential cytotoxicity and significantly prevented iAs(III)-exposure-induced cognitive impairment, especially long-term spatial learning and memory. It also prevented altered neurotransmitter turnover, neuro-/ ileal/ colonic inflammation, and ROS-induced damages, increased ileum permeability, reduced SCFA level, and gut perturbations caused by iAs(III)-exposure. These findings suggest that the polyphenol-rich extract from *Rubus ellipticus* may offer protection against environmental toxicant [iAs(III)]-induced neurotoxicity and behavioral effects, potentially through combined modulation of oxidative stress, neuroinflammation, and gut-related pathways.

Yellow Himalayan Raspberries and Cognitive Health

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1. Introduction

Environmental arsenic (As) contamination poses major health challenges to humans and animals. Though most of this environmental exposure has a natural origin from arsenic-rich rocky materials and sediments formed from these rocks, anthropogenic activities substantially add towards the bioburden of arsenic exposure. Countries in Southeast Asia, Chile, Mexico, and Europe, have shown the elevated environmental burden of arsenic contamination (Watson and Yager, 2007). The neurotoxic effects of arsenic exposure have been demonstrated in preclinical studies as well as in the epidemiological surveys. Negative effects of inorganic exposure have been observed in neurodevelopmental outcomes (Rodríguez-Barranco et al., 2013), cognitive impairments, and neurodegeneration (Qu et al., 2026; Rodrigues et al., 2016; Tian et al., 2025). The mechanistic insights on the neurotoxic effects of arsenic exposure, primarily *in-vitro* systems and in preclinical models, suggested the role of oxidative stress or impaired redox system balance, mitochondrial dysfunction/membrane instability and caloric exhaustion, epigenetic modulation, altered neurotransmitters homeostasis and synaptic transmission, activation of neuronal death pathways and structural loss of axons and myelin structure, astrocyte and microglial signaling impairments, and inflammatory cytokine burst (Garg and Bandyopadhyay, 2025; Hu et al., 2020; Kaur et al., 2024; Medda et al., 2020). Chronic arsenic exposure has been shown to trigger neuroinflammation and oxidative stress in the brain by suppressing the Nrf2 antioxidant pathway and activating the NLRP3 inflammasome as the central regulatory mechanism, where activating Nrf2 or inhibiting NLRP3 can successfully mitigate arsenic-induced neurotoxicity (Qu et al., 2025). More recently, its effect on altering gut microbiota has been shown, suggesting the role of the gut–brain axis in mediating its effect on the central nervous system (Banerjee and Chatterji, 2024; Bi et al., 2024). A recent study has provided critical insights into the impact of chronic arsenic exposure on Alzheimer’s disease-like pathologies by its ability to upregulate neuroinflammation, which is expected to be regulated through gut microbiota dysregulation, gut barrier dysfunction, and AhR/NF- κ B/NLRP3 mediated microglial pyroptosis (Qu et al., 2026).

Polyphenols have been recognized as a prominent source of antioxidants, owing to their efficient free radical scavenging, proton donor, and reactive oxygen species quenching activities. They have excellent anti-inflammatory activities and show neuroprotective properties as well (Rana et al., 2022). Positive modulation of gut microbiota and prevention of gut dysbiosis is one of the recent developments in the field of polyphenolic(s) biology (Sanz et al., 2025; Schneider et al., 2024). Arsenic, as an environmental toxicant whose exposure is unavoidable in many geographies due to its continuous presence in the drinking and irrigation water and its incorporation into the food chain (Ivy et al., 2023), polyphenols (polyphenol-rich foods) could potentially prevent or mitigate its negative health effects. Primarily, they may promote antioxidant defense and reduce oxidative stress. Targeting newly identified targets, such as gut microbiota, could provide interesting research avenues. Considering this, we narrowed down to one such potential intervention, polyphenols from an indigenous berry, *Rubus ellipticus*, which may have excellent antioxidant activities and potential for gut microbial modulation. We then tested this against inorganic arsenic [iAs(III)] induced neurotoxicity and behavioral aberration, focusing on cognitive impairments.

R. ellipticus (yellow Himalayan raspberry) is a wild edible berry native to the Indian subcontinent and other parts of South/ Southeast Asia, with a significant presence in Himalayan regions. Preliminary phytochemical screening of *R. ellipticus* berries have revealed an abundance of phenolics, flavonoids, anthocyanins, and tannins as major phytochemicals (Badhani et al., 2015; Kewlani et al., 2023; Lamichhane

et al., 2023). Furthermore, efficient extraction of polyphenols or other bioactive compounds from plant matrices is critical for enhancing their therapeutic efficacy. Ultrasonication-assisted extraction (UAE) is a green extraction technique and can provide improved yield for thermolabile polyphenols (Kewlani et al., 2022). Therefore, optimizing UAE conditions for these berries can play a pivotal role in generating potent antioxidant-rich formulations suitable for biological studies.

Interestingly, a systematic evaluation of the polyphenolics from *R. ellipticus* for their neuroprotective potential targeting brain-centered host-microbial pathways (antioxidant/anti-inflammatory, and gut microbiota modulatory effects), especially in the context of environmental neurotoxicants such as arsenic, remains unexplored. We understand that *R. ellipticus* berries are a rich but underutilized source of natural polyphenols with potential neuroprotective and gut-modulating activities. Hence, we hypothesized that the polyphenol-rich fraction of hydro-alcoholic extracts of *R. ellipticus* berries, optimized through UAE, can ameliorate iAs(III)-induced cognitive impairments in mice by modulating oxido-nitrosative stress, neurotransmitter homeostasis, neuroinflammatory signaling, and gut microbiota composition. Therefore, we optimized the UAE extraction protocols for polyphenol-rich fraction from *R. ellipticus* berries, its chemical composition analysis, and pharmacological investigations on the use of that extract against iAs (III)-exposure induced cognitive impairment.

2. Material and methods

2.1. Ultrasonication-assisted extraction, conditions optimization using response surface methodologies (RSM), and in-vitro antioxidant activities assessments of *Rubus ellipticus* extracts

Berries of *R. ellipticus* were collected in May and June of each year (2023–2025) during the project duration for the extraction. These berries were collected from the foothill region of the Himalaya in the state of Uttarakhand, India. Identification of the exact berries using local names (*Hisalu*, *Hisal*, or *Hisol*) was done through a team of locals from these regions and by one of the experimenters who is native to these areas. During the collection, storage, and transport, suitable cold chain conditions were strictly maintained until it reached the laboratory where the extraction was performed. Upon arrival, they were checked for quality by visual observation of any unwarranted damages. Once found suitable, they were stored in -80°C for further use as applicable.

Ultrasonication-assisted extraction (UAE) was performed for polyphenolic compounds from the selected berries. Briefly, 5 g of frozen berries of *R. ellipticus* were mixed with different proportions of H₂O: CH₃OH (methanol ranging from 0 to 100%) in a 100 mL volume. The samples were thoroughly homogenized (Moxcare, MT30K homogenizer, Ambala, HR, India) for 3 min and then placed in an ultrasonicator bath for a varied amount of time in temperature conditions (15–90 min; RT or 25–75 $^{\circ}\text{C}$). The sonicated mixture was filtered using a vacuum filtration assembly (using Whatman filter paper no 1 discs). The filtrate was transferred in rotatory evaporator flask, and then the evaporation was done under recirculatory chilling conditions (-60°C) in a rotatory evaporator coupled with a cold trap (Buchi R-300, BUCHI India Pvt Ltd, Mumbai, MH, India). Upon the evaporation of all organic solvent phase and most of the aqueous phase, the concentrates were lyophilized (Virtis, FM35EL-85, SP Scientific, Chennai, TN, India). The powdered extract was weighed and securely packed in airtight lab-containers for further chemical characterization, in-vitro antioxidant assays, toxicity, and in-vivo evaluations (Fig. 1a). Since the extraction was done using the frozen but not dried fruits, the extraction yield was calculated as:

$$\text{Extraction yield (\%)} = \left[\frac{\text{weight of solvent free residue (g)}}{\text{wet weight of fruits (g)}} \right] * 100$$

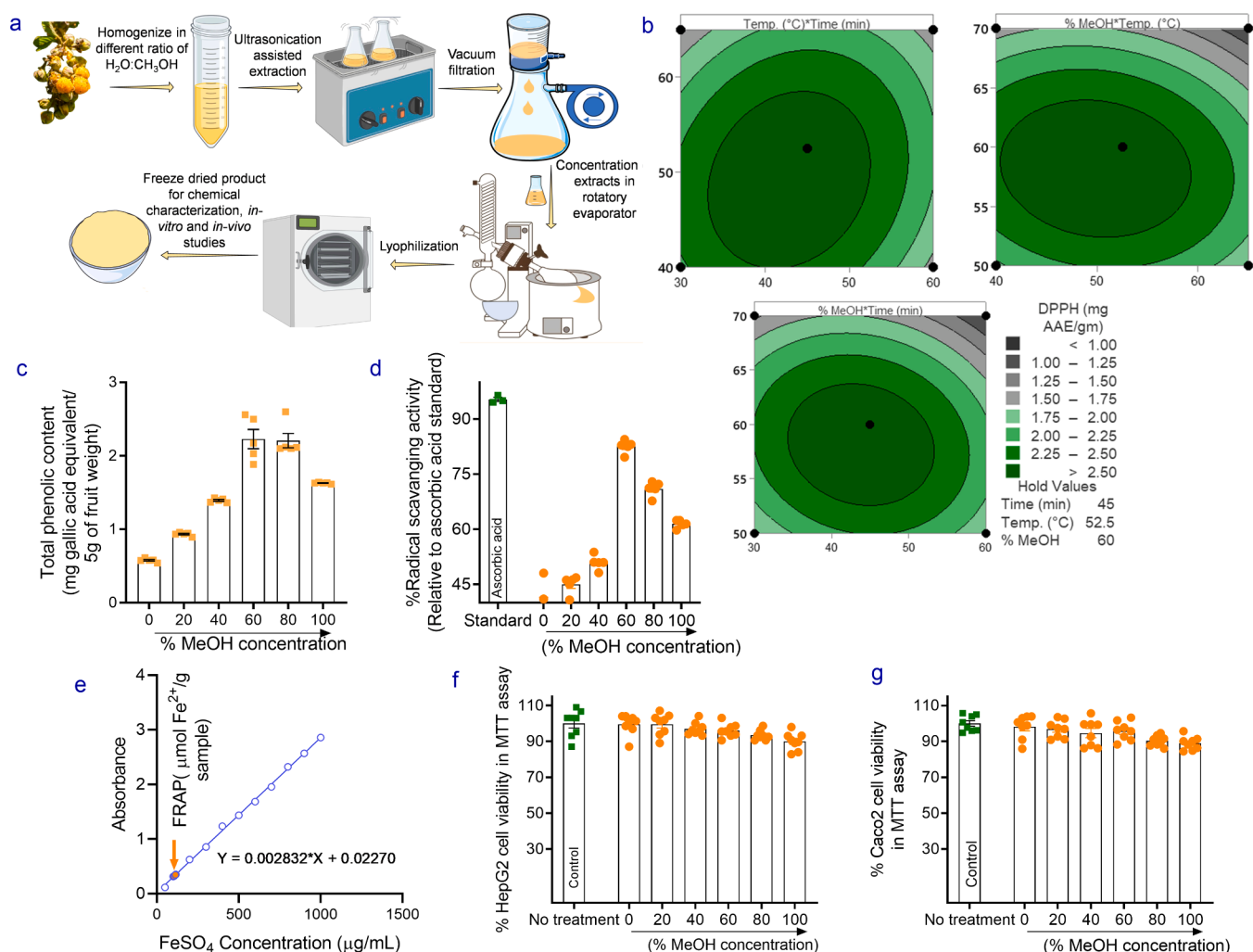


Fig. 1. Effects of extraction conditions and *in-vitro* antioxidant capacities of *R. ellipticus* berries extracts. a) General extraction procedure involving ultrasonication-assisted extraction (UAE), vacuum filtration, rotatory evaporation and lyophilization; b) response surface methodology (RSM) based extraction conditions optimization using DPPH (1,1-diphenyl-2-picrylhydrazil) radical scavenging activities; c) Total polyphenolic content (TPC) estimation of extract using Folin-Ciocalteu (FC) reagent; d) DPPH radical based % radical scavenging activity of the extract; e) Ferric Reducing Antioxidant Power (FRAP) assay based antioxidant capacity estimation of extract; MTT based colorimetric estimation of cell viability in f) HepG2 and, g) Caco2 cell lines. Data in panels c, d, e, and f are presented as mean \pm SEM. Each dot represents an individual observation. In the MTT assay, the data were compared for statistical significance using one-way ANOVA in comparison to control/untreated cells, followed by Tukey's post hoc test.

Single-factor experiments were carried out to determine the preliminary range of extraction variables, such as extraction time (A), extraction temperature (B), and solvent (MeOH) concentration (C). Then, 17 runs based on Box–Behnken Design (BBD) were carried out in random order to statistically optimize the Response Factor (R1). All the tests were performed in triplicate. Analysis of variance (ANOVA) was performed using RSM, and the test for significance was conducted at $P < 0.05$. Statistical analysis was conducted using the Design-Expert 13 software (Stat-Ease Inc., Minneapolis, MN, USA).

2.2. Total polyphenolic contents (TPC) estimations

The TPC of these extracts was determined using Folin–Ciocalteu reagent, where gallic acid was used as a reference polyphenolic antioxidant substance. Briefly, in a 96 well microwell plate, 12 μ L of extract was mixed with 60 μ L of Folin–Ciocalteu reagent. The mixture was incubated at room temperature for 5 min. Afterwards, 180 μ L of sodium bicarbonate solution (7.5%w/v) was added to neutralize the reaction. The reaction mixture was then incubated for 1 h at room temperature in the dark. Colorimetric estimation of the developed colour was measured in

the microplate reader at 765 nm (Epoch SN ELISA reader, Biotek Instruments). Total TPC in extracts was calculated against a standard curve obtained using varied concentrations of gallic acid.

2.3. Radical scavenging activities (RSA) estimations

Colorimetric estimation for free radical scavenging activity (RSA) or antioxidant activity (AA) in the berry extracts was done against the stable DPPH (1,1-diphenyl-2-picrylhydrazil) radicals. Briefly, 75 μ L of berry extract (test substance) or ascorbic acid (standard antioxidant in a concentration range of 50 μ g – 5 mg/mL) were mixed with 225 μ L of freshly prepared solution of DPPH (0.08 mg/mL in CH_3OH) and allowed to react for 30 min at room temperature in the dark. The absorbance was measured at 517 nm against methanol. The RSA or AA was calculated using the formula:

$$\text{RSA or AA (\%)} = [(A_0 - A_{\text{test}}) / A_0] \times 100$$

Where A_0 is the initial absorbance of DPPH solution, A_{test} is the absorbance of the tested sample.

2.4. Ferric reducing antioxidant power (FRAP) assay

The assay involves the reduction of ferric (Fe^{3+}) ions to ferrous (Fe^{2+}) in acidic condition forming a coloured complex. Briefly, 2.5 mL of 20 mM FeCl_3 and 25 mL of 0.3 M acetate buffer at pH 3.6 were mixed to prepare the FRAP reagent. 200 μL of extract was mixed with 1.8 mL of FRAP reagent and incubated for 10 min at room temperature. Then the absorbance of the reaction mixtures was measured using a microplate reader at 594 nm. Aqueous standard solutions of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (100–1000 μM) were used to prepare a calibration curve, and the results were expressed as the FRAP value μM of $\text{Fe}(\text{II})$ equivalent/ g of sample.

2.5. MTT assay for cell viability/cytotoxicity

The effects of direct exposure of *R. ellipticus* berry extract on their potential cytotoxic effects were tested on two cancerous cell lines from human origin, viz. human hepatocellular carcinoma (HepG2) and human colon cancer (Caco-2) cell lines (source: cell bank of National Centre for Cell Science, Pune, India). The assays were performed as per previously published and optimized methods (Bijalwan et al., 2016). Briefly, HepG2 cells were cultured in MEM media, and Caco-2 cell lines were cultured in high-glucose DMEM media. Both these media were supplemented with 10% FBS (foetal bovine serum) and antibiotics (1%, v/v, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin). Cells were grown at 37 °C in a humidified incubator having 5% CO_2 . Cells were cultured in a sterile 96-well plate at a density of 5×10^4 cells/mL (100 $\mu\text{L}/\text{well}$) for 24 h. The cells were then treated with different concentrations of extracts or left untreated (control) for 48 h. Afterwards, 10 μL of MTT (5 mg/ml) was added to each well and incubated for another 4 h. After incubation, the culture media was removed, and 100 μL of dimethyl sulphoxide (DMSO) was added in each well to dissolve the MTT-formazan, whose absorbance was colorimetrically estimated after 10 min at 490 nm using a micro-plate reader. The cell viability ratio was calculated as:

$$\% \text{cell viability} = \frac{(A_{\text{sample}} - A_{\text{blank}})}{(A_{\text{control}} - A_{\text{blank}})} \times 100$$

where 'A' denotes the average absorbance of the respective sample, control, and blank (media only well).

2.6. Procyanidins and polyphenols quantification using UHPLC

All samples were analysed on a 1290 infinity II series UHPLC-DAD system (Agilent Technologies) equipped with 1290 infinity II series pump, autosampler, column compartment and thermostat using Zorbax Eclipse Plus C-18 column (2.1 \times 100 mm, 1.7 μm) maintained at 30 °C, programmed in a gradient mode. The mobile phase consisted of an aqueous solution of 0.1% Formic acid (Solution A) and Acetonitrile with 0.1% Formic acid (Solution B). Other chromatographic parameters included: an injection volume of 3 μL , a constant flow of 270 $\mu\text{L}/\text{min}$, and a run time of 57 min, including 10 min post-run. All the samples were filtered through a 0.22 μm syringe membrane filter (Millipore) before being injected into the chromatographic system. Quantitative analysis was done using the peak areas and retention time of suitable individual analytical standards (Merck, USA) using previously published methods (Sharma et al., 2016) in the given UHPLC-DAD system.

2.7. LC-MS analysis for quantification of anthocyanins

The LC-MS analysis of samples was carried out in a UPLC system (Exion LC, Sciex) coupled to a triple quadrupole system (QTRAP6500 +; ABSciex) using an electrospray ionization. For positive ionization, the voltage was set at 5500 V. The values of gas 1 and gas 2 (70 psi), curtain gas (40 psi), collision-assisted dissociation (medium), and temperature of the source (650 °C) were used. The mass spectrometer was used in multiple reaction monitoring mode (MRM) for qualitative and

quantitative analysis using analytical standards of anthocyanins (Merck, USA). Analyst software (version 1.5.2) was used for the identification and quantitative analysis.

2.8. In-vivo evaluation of *R. ellipticus* berries extract against inorganic arsenic exposure-induced cognitive impairments

Male Swiss albino mice were acquired from the ICMR-National Animal Resource Facility for Biomedical Research (ICMR-NARFBR), Genome Valley, Hyderabad, TN, India. The animals weighed 24–26 g upon arrival. Upon arrival, mice were kept for one-week in a quarantine area and another week for acclimatization. Standard animal housing and care with close adherence to the guidelines provided by the Committee for Control and Supervision of Experiments on Animals (CCSEA) was given to the experimental animals. All animal experimentations were approved by the institute's animal ethics committee (IAEC-ICMR-NIOH/2022–23/28/03/M).

Mice were then divided into various experimental groups: (i) control ($n = 12$) received no treatment or any arsenic exposure, (ii) inorganic arsenic [iAs(III)] exposure ($n = 12$) in the form of 1.85 ppm inorganic arsenic (sourced from 3.2 ppm sodium meta-arsenite, NaAsO_2 , cat #S7400–100G, Merck KGaA, Darmstadt, Germany), (iii–v) *R. ellipticus* berries extract at dose range of 50, 100 and 200 mg/kg body weight/day was given *per orally* ($n = 12$ per group) in the arsenic exposed mice, (vi) unexposed control mice received *R. ellipticus* berries extract (200 mg/kg per day), as a *per se* group ($n = 8$), and (vii) unexposed control mice received rivastigmine (2 mg/kg, *p.o.*) as standard nootropic agent ($n = 8$) for assessing the cognitive performances. Group vi and vii received standard drinking water and were maintained and evaluated for behavioural assessment till 12 weeks (the entire duration). A detailed schematic of the experimental plan has also been presented in Fig. 3a. The 12-week exposure to iAs(III) or treatment with berry extracts was later assessed for cognitive performances in the form of spatial navigation task performances using Morris water maze or short-term cognitive preferences in a passive-avoidance test (detailed in individual experimental sections). After the behavioural assessments, the mice were euthanized, and various tissues and biospecimens were collected for further investigations. However, to make efficient use of available resources and after evaluating the behavioural data, only four groups, i.e. control, iAs(III), iAs(III) + 100 mg/kg berries extract, and iAs(III) + 200 mg/kg berries extract groups were analysed for all (until specified) biochemical, molecular, and gut microbiota assessments (detailed in the result sections).

The environmental relevance of the selected dose of iAs(III) was primarily taken from the arsenic contamination levels in various ground water sources of multiple geographies in India. The Bureau of Indian Standards (BIS) laid a desirable limit of arsenic at 0.01 mg/L and a permissible limit in case of unavailability of an alternate source, 0.05 mg/L in drinking water. However, hundreds of sites in India have shown much higher levels of arsenic contamination in groundwater in some of the most populous states of the country (ranging from 20 to 260 mg/L), covering a wide variety of climatic and geographic conditions (Marghade et al., 2023). Another dataset extracted from a recent review covering the global distribution of arsenic in groundwater, using the ranges given from many locations in India, has an estimated approximation of the median value reached up to $\sim 150 \mu\text{g}/\text{L}$ (Jha and Tripathi, 2021). This value is also ~ 3 times the permissible limits set by BIS for arsenic in drinking water. Hence, using the 0.15 mg/L median arsenic value for the exposed population, we factored it into the mice dose (12.3 times the human equivalent dose or exposure), which should be 1.85 mg/L. To achieve this, a 3.2 mg/L solution of sodium meta-arsenite has been provided to mice presented in exposure groups. This dose is still lower yet environmentally relevant than the doses used in multiple experimental studies investigating the effects of arsenic exposure on cognitive impairments (Chu et al., 2023; Mehta et al., 2021; Pandey et al., 2022). Further, the dose ranges for the berry extract (50–200

mg/kg) were empirically selected, yet significant consideration has been given to the doses selected by previously published studies using other berries (cranberry, blueberry, açai berry, and black goji berry) extracts, ranging from 50 to 500 mg/kg (Anhe et al., 2014; Cao et al., 2024; D'Amico et al., 2022; Li et al., 2022; Singh et al., 2018; Spohr et al., 2022; Tan et al., 2024). Though the pharmacological investigation in various *in-vivo* models utilizing *R. ellipticus* berry extract is limited, a summary of data suggests that it has also been used in the dose ranges of 50–500 mg/kg in various animal models for assessing its effects for antidiabetic, nephroprotective, anti-inflammatory, analgesic, and anti-proliferative activities (Kewlani et al., 2023). Additionally, as reported, the extract is well tolerated at the dose of 2000 mg/kg and did not show any neurotoxic or behavioural effects (Sharma and Kumar, 2011).

2.8.1. Morris water maze test

In the last week of the study, the spatial navigation-based learning task performances were evaluated using Morris' water maze test. After four days of training in the water maze to reach the escape platform in a cut-off time of 60 s, an acquisition trial on the 5th day of the test was performed. The time taken by the mice to reach this escape platform was noted. A probe trial on the sixth day was conducted, where the escape platform was removed and the time spent in that quadrant of the maze was recorded (Singh and Chopra, 2014b).

2.8.2. Passive avoidance test

To assess short-term learning from an aversive stimulus, utilizing a passive avoidance test, the step-through latency was recorded. Briefly, in the passive-avoidance system (Ugo Basile, Gemonio, Italy), mice were first acclimatized and trained in the light-dark shuttle box. They were kept in the light chamber. After 5 s, the door of the shuttle box was opened, and the mice were allowed to explore both compartments. Once fully inside the dark compartment, a mild foot shock (0.3 mA for 2 s) was given. After 24 h, an acquisition test was performed, where retention of memory in the form of latency to step through the dark compartment was recorded (Venable and Kelly, 1990).

2.9. Biochemical and molecular markers estimation in serum and tissues

Tissue (brain, ileum, and colon) homogenates (10%w/v in phosphate buffer saline) or serum samples were used for serotonin, 5-HIAA, dopamine, HVA, corticosterone, LPS, TNF- α , ILs levels estimations, using specific ELISA (All from Krishgen Biosystem, Mumbai, India). Dopamine and serotonin turnovers were calculated as a ratio of HVA/DA and 5HIAA/5HT, respectively. Details of ELISAs used in the study are listed in the **supplementary table 1**.

Biochemical estimations for oxidative stress markers, such as lipid peroxidation, total nitrite, reduced glutathione (GSH), and superoxide dismutase (SOD), were performed using classical biochemical methods, following the original or slightly modified protocols, previously standardized in the lab (Ellman et al., 1961; Marklund and Marklund, 1974; Singh and Chopra, 2014a; Wills, 1965).

2.10. Histology processing

Tissues (brain, ileum, and colon) were immersion-fixed in 10% normal buffered formalin and processed for paraffin embedding for sectioning. Using a rotatory microtome (MRM-ATL, Medimeas Instrument, Ambala, India), 5 μ m thick sections were obtained on a glass slide and processed for hematoxylin and eosin staining. Images (N = 10–15 images per group) were captured using an upright microscope (Leica DM750, Leica Microsystems, Wetzlar, Germany). The images were evaluated by an experimentally-blinded observer for pathological observations. In the brain, at the hippocampal level, degenerating neurons with condensed nuclei and bright eosinophilic cytoplasm were observed; in the ileum and colon, macrophage infiltration, thinning of

the epithelial layer, cryptic loss, and shortening of villus structure were also observed.

2.11. Gene expression analysis

In the brain, ileum, and colon tissues, the gene expression analyses were performed as described and previously standardized protocols (Singh et al., 2024). Trizol® reagent (Merck KGaA, Darmstadt, Germany) based total RNA extraction was done from 10 to 20 mg of tissue. Quality check on 0.8% agarose gel for its integrity, and quantitation of total RNA was done on nanodrop-OneC (ThermoFisher Scientific). Complementary DNA was prepared from 800 ng of RNA, using a QuantiTect Reverse Transcription Kit (Cat.# 205,311; Qiagen GmbH, Hilden, Germany). qRT-PCR (Biorad CFX96™ Real-Time System) based relative gene expressions for various genes were performed with SYBR® green dye and suitable primers (**supplementary table 2**) in a 10 μ L reaction. PCR conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C and 60 °C for 1 min. 2^{-ddCt} based quantification for relative fold changes to the control group was done (Livak and Schmittgen, 2001).

2.12. Short chain fatty acids (SCFAs) estimation

Cecal samples (~100 mg) were thoroughly homogenized in acidified water (0.1% formic acid v/v). After centrifugation at (20 min, 4 °C), clear supernatants were filtered through a membrane filter (0.22 μ m), and 20 μ L of the same was injected into the HPLC system coupled with a PDA detector. Chromatographic separation of acetate, propionate, and butyrate was achieved at 60 °C using an anion exchange column (300 \times 7.7 mm; 8 μ m particle size) in a 20 μ L sample volume injected. The mobile phase (0.1% formic acid in HPLC-grade water) was used in an isocratic flow rate of 0.6 mL/min at 60 °C for 30 min. Data was acquired and processed with EZchrom Elite software with the system suitability option installed using the previously optimized and published protocol (Singh et al., 2016).

2.13. Ileum permeability

To assess the ileal permeability, Evan's Blue (EB) dye elution-based assay was performed (Singh et al., 2024). Briefly, ileal sacs were prepared and filled with 200 μ L of 1.5% EB in phosphate buffer. These sacs were incubated in Krebs' solution at 37 °C for 30 min in 5% CO₂. These parts were washed with 6 mM/L acetylcysteine and dried overnight. Upon elution in 1 mL of formamide (50 °C, 24 h) the eluates were spectrophotometrically assessed (655 nm). The quantification of eluted EB was measured against a standard curve made with known concentrations of the dye.

2.14. Cecal DNA extraction, QC, and nanopore sequencing of 16S rRNA

From ~120 mg of cecal samples, total genomic DNA was extracted using QIAamp Fast DNA Stool Mini Kit (Cat #: 51604; Qiagen GmbH, Hilden, Germany). Quality check for the integrity was done on 1.0% agarose gel and further quantified using Qubit™ Flex fluorimeter with the help of Qubit 1X dsDNA kit (Invitrogen Bioservices India Pvt. Ltd, Mumbai, India). Bacterial 16S rRNA primers, S-D-Bact-0008-a-S-16/S-D-Bact-1492-a-A-16 (Klindworth et al., 2012) were used to synthesize full-length (~1.5 kb) amplicons. These amplicons were used for nanopore-based sequencing on MinION Mk1B (Oxford Nanopore Technologies, Oxford, UK), using LSK109 ligation sequencing kit, EXP-NBD196 barcoding kits (Oxford Nanopore Technologies, Oxford, UK), using the manufacturer's recommended protocol. The sequencing control suite MinKNOW v. 23.04.3 was used to control the experiment setup for a maximum of 600 megabases generation. Fast5 files were base-called and demultiplexed using Guppy (ONT) version 6.5.7. Analysis of nanopore sequencing data was performed using

epi2me-labs/wf-16 s nextflow workflow (Oxford Nanopore Technologies, UK). PCA plots were generated using CLR-transformed genus raw data abundance, with the PCA package in R (version 4.3.2).

By referring to the results from the sequencing analysis, using available phyla, family, and genus-specific primers (**supplementary table 2**), qPCR-based relative abundance analysis was done in the cecal DNA content for *Firmicutes*, *Bacteroidota*, *Eubacterium* sp., *Lactobacillus* sp., *Lachnospiraceae*, *Prevotella*, and *Roseburia* for *Rubus ellipticus* berries extract in higher dose and in *per se* groups, by following previously established methods and PCR conditions in the laboratory (Singh et al., 2018, 2023).

2.15. Statistical analysis

A priori sample size analysis of the mice before starting the experiment was done using GPower version 3.1.9.6. GraphPad Prism (version 9) was used for the majority of data analysis. Data was checked for normality using the D'Agostino-Pearson omnibus normality test for parametric data. For parametric datasets, comparison between the control group and the arsenic (III)-exposed groups and arsenic versus treatment group was done using One-Way ANOVA, followed by Tukey's post-hoc test for the determination of statistical significance, which was set at the level of $P < 0.05$. Anywhere, for datasets that violated normality assumptions, non-parametric tests such as Kruskal-Wallis test followed by Dunn's *post hoc* test for multiple group comparisons. Data is mostly presented in bar graphs (mean \pm SEM) with scattered dot bar plots, where bar heights represent the mean. In heat maps, an individual cell represents a single biological replicate, and groups were analysed using ANOVA, as mentioned before. Furthermore, Spearman correlation analysis was performed to assess the association between various factors analysed, behavioral endpoint measurements, and among various bacterial abundances and SCFAs levels.

3. Results

3.1. Ultrasonication-assisted extraction (UAE) optimization, in-vitro antioxidant activities, and polyphenols profiles of the hydro-alcoholic extracts of *R. ellipticus* berries

Based on the optimum responses from 17 different runs covering a range of duration (15–60 min), temperatures (room temperature $+10$ °C to $+52.5$ °C), and H₂O: CH₃OH concentrations (0–100%), the antioxidant activities in the form of TPC and RSA were analyzed for RSM using BBD methods. The data suggested the optimum conditions for UAE are: 60% CH₃OH as solvent, 52.5 °C temperature, and 45 min of duration (**Fig. 1b**). The average extraction yield from five different batches of berry extraction experiments was found to be $7.63 \pm 1.12\%$.

Furthermore, the extractions done with different proportions of methanol was assessed for their *in-vitro* antioxidant activities. Folin-Ciocalteu method-based TPC estimation using gallic acid as the standard suggested for maximal TPC at 60% methanol (**Fig. 1c**). DPPH assay-based estimation of RSA showed that 60% methanolic extracts showed maximum activities in comparison to ascorbic acid standard (**Fig. 1d**). Similarly, the ferric reducing potential of these extracts as assessed by the FRAP assay, showed that the 60% methanolic extracts of *R. ellipticus* berries showed a maximum ferric reduction potential (111.801 ± 4.687 $\mu\text{mol Fe}^{2+}/\text{g}$) (**Fig. 1e**). Furthermore, *in-vitro* toxicity studies were performed with the liver (HepG2) and colon carcinoma cells (Caco2), to assess the potential hepato-intestinal toxicities of the extract. The hydro-alcoholic extracts of *R. ellipticus* berries at various methanolic strengths showed no significant alterations in cellular viability in MTT assays, using these cell lines (**Fig. 1f-g**).

Chemical profiling of the polyphenolic components of these extracts was done using UHPLC. Data suggested that dihydrokaempferol, dihydromyricetin, myricetin, chlorogenic acid, rutin, t-ferulic acid, cinnamic acid, and dihydroquercetin were the major identifiable polyphenols in

the extracts, of which dihydrokaempferol was most abundant (**Fig. 2a-b**). Furthermore, four major proanthocyanidins (in their relative quantification levels) PA C1 > PA A1 > PA B2 and > PA A2 were quantified in the extracts (**Fig. 2c-d**). However, by LC-MS analysis, only delphinidin as an anthocyanidin was quantitatively determined at the levels of 0.0081 ± 0.0051 ng/mg of extract.

3.2. Effects of *R. ellipticus* berries extracts supplementation on iAs(III) exposure-induced cognitive impairments, neurotransmitter levels, oxidant-nitrosative stress markers and neuroinflammation

Twelve-week supplementation of polyphenol-rich *R. ellipticus* berries extracts significantly prevented iAs(III) exposure-induced impairment of spatial navigation task performances in Morris' water maze test. This was evident by less time taken to reach the hidden platform in the water maze by iAs(III)-exposed mice supplemented with *R. ellipticus* berries extract (100 and 200 mg/kg b.w./day) compared with the mice that did not receive the supplementation (**Fig. 3b, suppl. fig.1a**). Similarly, in the probe trials, when the hidden platform was removed from the water maze, mice supplemented with *R. ellipticus* berries extract (200 mg/kg) showed significantly higher time spent in the zone, which previously had that hidden platform (**Fig. 3c**), and with relatively less time they have taken for first such attempt as represented by latency to their first entry to this zone (**Fig. 3d**). Considering the fact that (a) *R. ellipticus* berry extract's 50 mg/kg dose was not found to be very effective against iAs(III)-induced cognitive performance, (b) no untoward effects of extract's maximum dose in control mice (200 mg/kg, *per se* group) in terms of behavioral, in-cage activity or general features for well-being and no sickness behavior reflected from normal body weights (**Suppl. fig.1b**), and (c) rivastigmine which was used as standard nootropic group for cognitive tasks, were excluded from major biochemical, and molecular investigations to suitably use the available resources. However, wherever possible, for *per se* group samples for biochemical and gene expression related experiments to have an idea about their baseline effects, efforts have been made to accommodate them as well (details available in respective figures).

For assessing the emotional learning and short-term cognitive efforts, we used a passive avoidance test. Results showed that the iAs(III) exposure did not show any improvement in initial learning (training trials) as depicted by comparable latency in training and acquisition trials in avoiding the electric shock zone of the arena as compared to control mice. Though non-significant, a slight increase in these latencies ($P = 0.280$) was shown by the mice treated with the berry extract (200 mg/kg/day) in comparison to iAs(III) exposed mice during acquisition trials (**Fig. 3e**). Moreover, iAs(III)-exposure showed a slight reduction in brain serotonin concentration which was significantly increased by *R. ellipticus* 200 mg/kg supplementation (**Fig. 3f**). Also, 5-HIAA, a primary metabolite of serotonin, was not altered significantly by iAs(III) exposure, however, berries extract (100 and 200 mg/kg) supplementation significantly reduced it (**Fig. 3g**). In turn, the overall turnover of serotonin (5-HIAA/5-HT) was reduced upon this supplementation (**Fig. 3h**). The effects on dopamine levels were quite evident as iAs(III) exposure decreased the brain dopamine levels whereas berry extract (200 mg/kg) significantly improved it. However, the supplementation did not show a significant effect on its primary metabolite, homovanillic acid. Ultimately, the turnover of dopamine (HVA/DA) was found to be increased by iAs(III) exposure, which was reduced upon berry extract supplementation (**Fig. 3i-k**). Additionally, brain corticosterone levels were found to be increased upon iAs(III) exposure, and this effect was attenuated by *R. ellipticus* extracts supplementation (**Fig. 3l**).

Histopathological investigations also revealed that the iAs(III) exposure increase the number of degenerating neurons with condensed nuclei and bright eosinophilic cytoplasm (asterisk in photomicrographs) in the perfectly rounded cytoplasmic architecture of healthier neuronal cell bodies (**Fig. 4a, suppl. fig. 2**). Furthermore, the levels of total lipopolysaccharides were also found to be increased in brain tissue

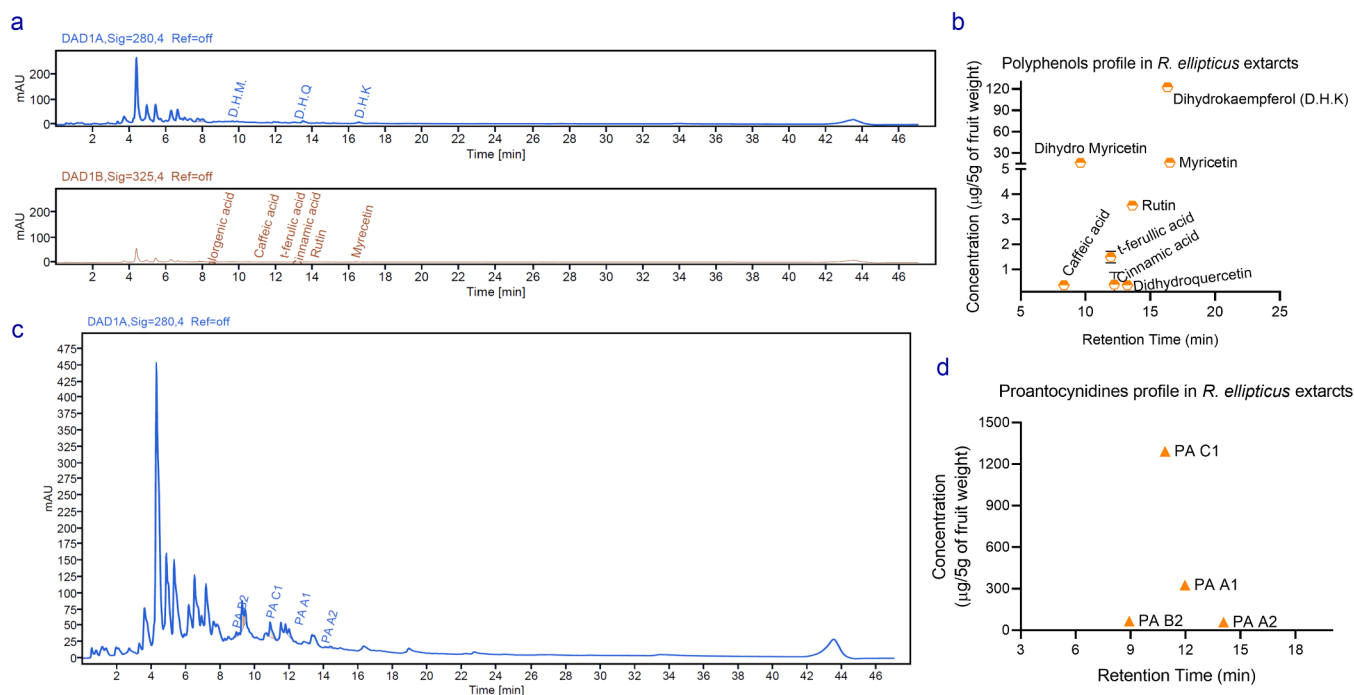


Fig. 2. Polyphenolic profiles of *R. ellipticus* berries extracts. a) Chromatograms of UHPLC-based identification of polyphenols in *R. ellipticus* extracts; b) quantification of identified polyphenols and their concentration in each gram of fruits; c) Chromatograms of UHPLC-based identification of proanthocyanidins; d) quantification data of identified proanthocyanidins in the berry extract. Data in panels b and d are presented as mean \pm SEM.

homogenate of iAs(III) exposed mice, which were significantly reduced by berry extracts (100 and 200 mg/kg) supplementation. However, no effect on brain TNF-alpha levels was observed (Fig. 4b-c). Furthermore, iAs(III) exposure affected the key oxido-nitrosative stress markers, as it increased lipid peroxidation and total nitrite levels, whereas it significantly reduced endogenous antioxidants such as reduced glutathione (GSH) and superoxide dismutase (SOD) activities. Supplementation to *R. ellipticus* extract (100 and 200 mg/kg) alleviated these alterations to an extent, with the higher dose appearing to be more effective (Fig. 4d). Furthermore, gene expression analysis also suggested that the iAs(III) exposure upregulated astrocyte and microglia activation-related genes such as *GFAP* and *AIF-1*. It also upregulated the expression of the key genes for proinflammatory response cascade upon LPS insult, such as *TLR-4* (though nonsignificant), and *NF-kB*. Along with this, it also downregulated neurotrophic factor genes such as *BDNF*, and the antioxidant defense-related genes such as *Nrf-2* and *HO-1*. Supplementation to *R. ellipticus* berries extract (200 mg/kg) efficiently alleviated these gene expression-related changes. *R. ellipticus* berry extract also promoted upregulation of *S100b*, and serotonin biosynthesis gene *TPH2*, and downregulation of *NLRP3* inflammasome in comparison to iAs(III)-exposed group (Fig. 4e).

3.3. Effects of *R. ellipticus* berries extract supplementation on circulating cytokines, gut inflammation, and permeability markers upon iAs(III) exposure

iAs(III) exposure increased circulating LPS levels ($P = 0.121$) in comparison to control, which was reduced ($P = 0.164$) upon *R. ellipticus* berries extract (200 mg/kg) supplementation (Fig. 4f). This berry extract supplementation also reduced circulating TNF-alpha and corticosterone levels in iAs(III) groups; no significant change in IL-1beta and IL-6 levels was observed in different groups (Fig. 4g-j).

Moreover, histopathological investigations in the ileum and colon showed that iAs(III) exposure prominently affected the villus structure in the ileum and reduced colonic cryptic lengths with increased inflammatory cell migration towards the mucosal barrier (Fig. 5a). In the

colon, IL-1beta levels were markedly increased upon iAs(III) exposure, which was slightly reduced ($P = 0.246$) by berries extract (200 mg/kg) (Fig. 5b). However, no effects on IL-17 levels in these tissues upon iAs (III) exposure or berries extract supplementation was observed (Fig. 5c). Furthermore, key biochemical markers for oxido-nitrosative stresses such as lipid peroxidation and total nitrite were found to be increased in ileum. Additionally, a prominent endogenous antioxidant, GSH activities were found to be reduced in ileum and colon. These effects were partially mitigated by the *R. ellipticus* berries extracts (200 mg/kg) supplementation, where in ileum and colon, it prevented the increase in MDA levels in iAs(III) exposed condition with an improvement in GSH levels (Fig. 5d). iAs(III) exposure also impacted gastrointestinal synthesis of serotonin as evident from the reduced total serotonin levels in the ileum of iAs(III) exposed mice, which was significantly reversed by the polyphenol-rich *R. ellipticus* berries extract (200 mg/kg) supplementation. No effect on the 5-HIAA levels was observed in both the investigated tissues (Fig. 5e-f). Further, Evans blue dye based ileal permeability assay also showed that iAs(III) exposure increased the translocation of dye showing an increase in permeability of this tissue, whereas supplementation to *R. ellipticus* extract (200 mg/kg) significantly prevented these effects in arsenic exposed mice (Fig. 5g). Additionally, cecal SCFAs assessment showed that the iAs(III) exposure led to or caused significant decrease in acetate and butyrate levels which were improved by the supplementation of 200 mg/kg the polyphenol rich extract of *R. ellipticus* berries (Fig. 5h).

Gene expression analysis in the ileum and colon showed some interesting signatures, where the expression of genes related to LPS-induced inflammation mediated by *TLRs* (*TLR4*, *NF-kB* in the ileum and *TLR-4*, *TLR-5*, and *NF-kB* in the colon) showed an upregulation upon iAs(III) exposure. iAs(III) exposure also reduced the expression of key free fatty acid receptor genes (*FFAR2* and *FFAR3* in the ileum and *FFAR3* in the colon) with downregulation of some of the tight junction genes, such as *Occludin* in the ileum, and *Cld-4* in the colon. It also down-regulated mucin genes *MUC-2* expression in the ileum and *MUC-2* and *MUC-4* in the colon, and interestingly, the key serotonin biosynthesis mediator gene *TPH1* in both these tissues. Supplementation with

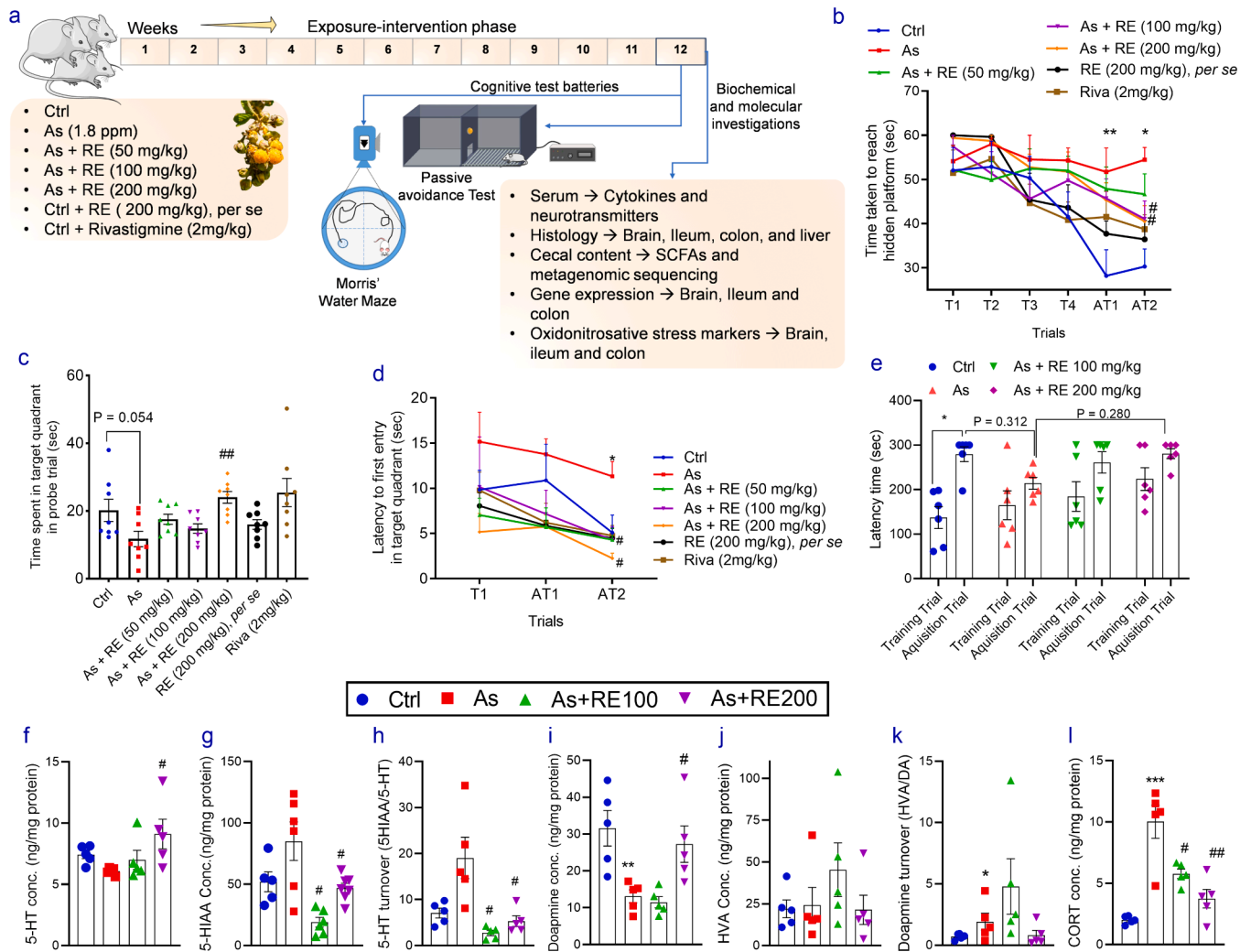


Fig. 3. Effects of *R. ellipticus* berries extract supplementation on cognitive functions and brain neurotransmitter levels or turnover in inorganic arsenic-exposed mice. a) general schematics of *in-vivo* experimental protocol involving 12-weeks exposure to inorganic arsenic and supplementation of various doses of *R. ellipticus* berries extracts and final assessment of cognitive performances in Morris water maze and Passive avoidance test; b) time taken to reach the hidden platform in the Morris water maze test; c) time spent in target quadrant, which was previously having the hidden platform in the probe test in the water maze; d) latency to first entry in the above mentioned quadrant; e) latency to entries in the shock grid zone in the passive-avoidance test; f) serotonin or 5-HT, and g) 5-hydroxyindole acetic acid (5-HIAA) concentration in brain; h) serotonin turnover in brain; i) dopamine, and j) homovanillic acid (HVA) concentration in brain; k) dopamine turnover in brain; l) corticosterone concentration in brain; Data is presented as line plots (mean \pm SEM), or scattered bar plots (mean \pm SEM, individual observation points are also presented). Statistical comparison was done using One (multigroup comparison) or Two-way ANOVA (multigroup and multi-time points observation) followed by Tukey's *post hoc* test, or paired t-test for training and acquisition trial data of same group in combination with Two way ANOVA followed by Tukey's *post hoc* test as in panel 'e' was used. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control, # $P < 0.05$, ## $P < 0.05$ vs arsenic exposed group. RE = *Rubus ellipticus*, T1, T2...T4 = training trials, AT = acquisition trials, 5-HT = serotonin, 5-HIAA = 5-hydroxy indole acetic acid, HVA = Homovanillic acid, CORT = Corticosteron.

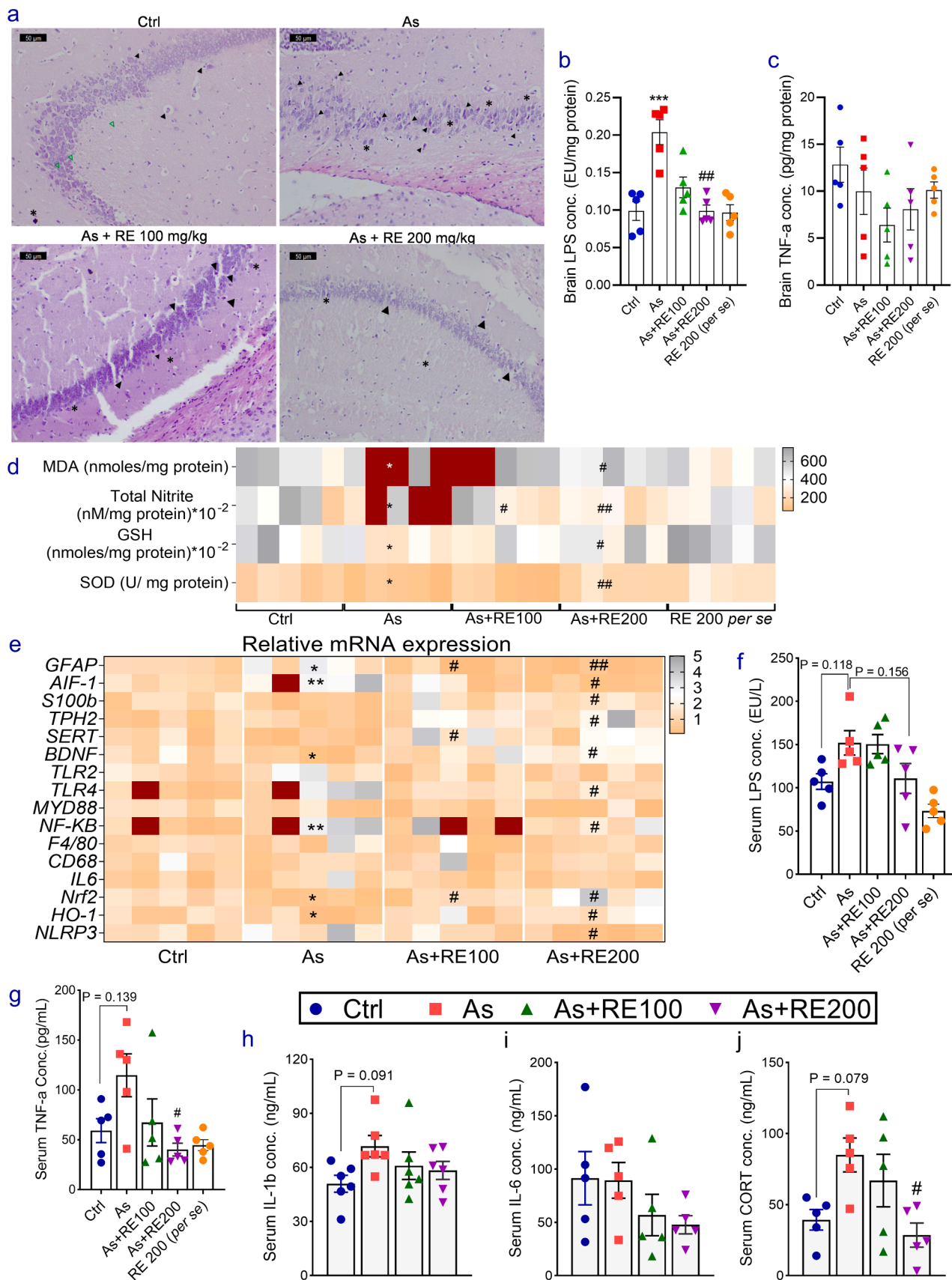
R. ellipticus berries extract showed significant reversal of these gene expression profiles in iAs(III) exposed mice (Fig. 5i-j).

3.4. Effects of *R. ellipticus* berries extract supplementation on the gut microbial profiles of mice exposed to iAs(III)

iAs(III) exposure impacted the gut microbial diversity as depicted by the alteration in alpha-diversity indices, including reduction in Chao-1 and an increase in Berger-Parker ($P = 0.190$) index. Supplementation of *R. ellipticus* berries extract (200 mg/kg) in iAs(III) exposed mice relatively prevented these alterations as it improved Chao-1 diversity ($P = 0.21$) and Pielou alpha diversity indices and reduced Berger-Parker index ($P = 0.177$) (Fig. 6a-e).

The beta diversity assessment, as presented in principal component analysis, shows clear separation of groups in various treatments where approximately 75% variance has been explained by these two axes. Taxa

contributing to the observed variance include *Marvinbryantia* and *Lachnospiraceae_uncultured*, which were positively associated with PC1, while *Lachnospiraceae_NK4A136 gp*, *Prevotellaceae*, and *Parasutterella* showed strong negative loading (Fig. 6f). Further, a Venn diagram representing common and unique taxa in the analyzed groups showed that iAs(III) exposure uniquely had some pathobionts belonging to *Acinetobacter*, *Candidatus soleiferrea*, *Escherichia-Shigella*, *Paracoccus*, and *Staphylococcus*, in comparison to control mice, which allowed harboring of *Psychrobacter*, *Stenotrophomonas*, and *Veillonella*. Interestingly, *R. ellipticus* berries extract (100, 200 mg/kg) showed the presence of *Eubacterium*, *Enterococcus*, *Clostridium cluster XIII UCG-001*, *Prevotella_9*, *Fecalibacterium*, *Lactococcus* and *Pseudomonas* (Fig. 6g). UPGMA clustering of phylum-level microbiota composition revealed group-specific clustering patterns. Here, control and iAs(III) exposed mice supplemented to *R. ellipticus* berries extract (200 mg/kg) shared highly similar profiles, suggesting comparable microbial communities.



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Fig. 4. Effects of *R. ellipticus* berries extract supplementation on neuroinflammation and circulating cytokines. a) H&E-stained section of brain at the hippocampus levels (objective 20X); b) total lipopolysaccharides, and c) total TNF-alpha levels in brain; d) Concentration of malondialdehyde (MDA) for lipid peroxidation, nitrite, reduced glutathione (GSH), and superoxide dismutase (SOD) in brain tissue; e) relative mRNA expression of selected genes in brain. Concentrations of f) total lipopolysaccharide, g) tumor necrosis factor-alpha, h) interleukin-1beta, i) interleukin-6, and j) corticosterone in serum. Data is presented as scattered bar plots (mean \pm SEM, individual observation points are also presented) or heatmaps (individual colored cells represent a single sample within a group). Statistical comparison was done using one-way (multigroup comparison) ANOVA followed by Tukey's post-hoc analysis. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control, # $P < 0.05$, ## $P < 0.05$ vs arsenic exposed group. RE = *Rubus ellipticus*, GFAP = glial fibrillary acidic protein, AIF-1 = allograft inflammatory factor-1, S100b = S100 Calcium Binding Protein B, TPH2 = tryptophan hydroxylase 2, SERT = serotonin transporter, BDNF = brain derived neurotrophic factor, TLR = toll like receptor, MyD88 = Myeloid differentiation primary response 88, NF- κ B = nuclear factor kappa beta, F4/80 = Adhesion G Protein-Coupled Receptor E1, CD68 = Macrophage Antigen CD68, IL-6 = interleukin 6, Nr2f2 = Nuclear factor erythroid 2-related factor 2, HO-1 = heme oxygenase 1, NLRP3 = NLR family pyrin domain containing 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

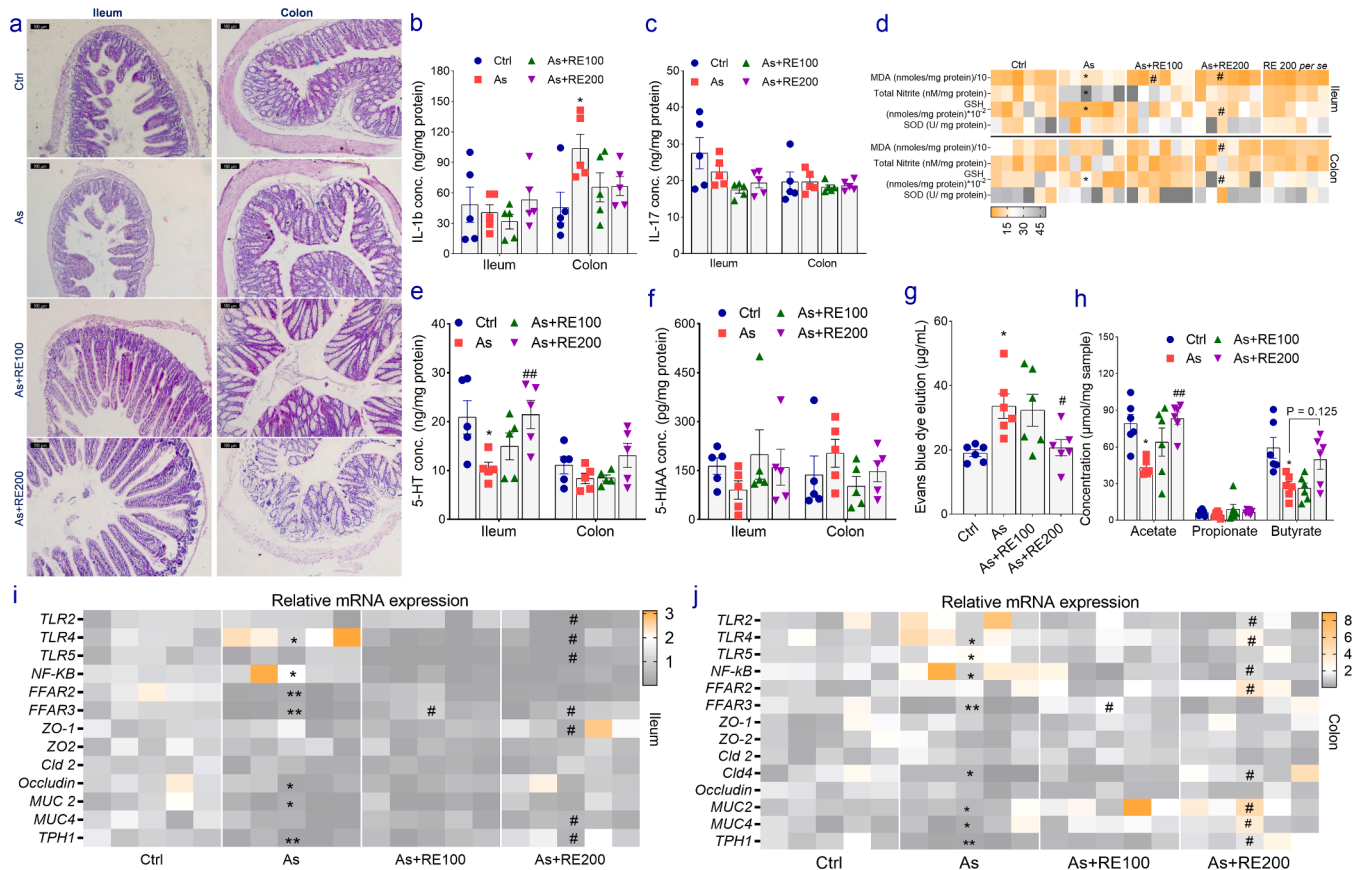
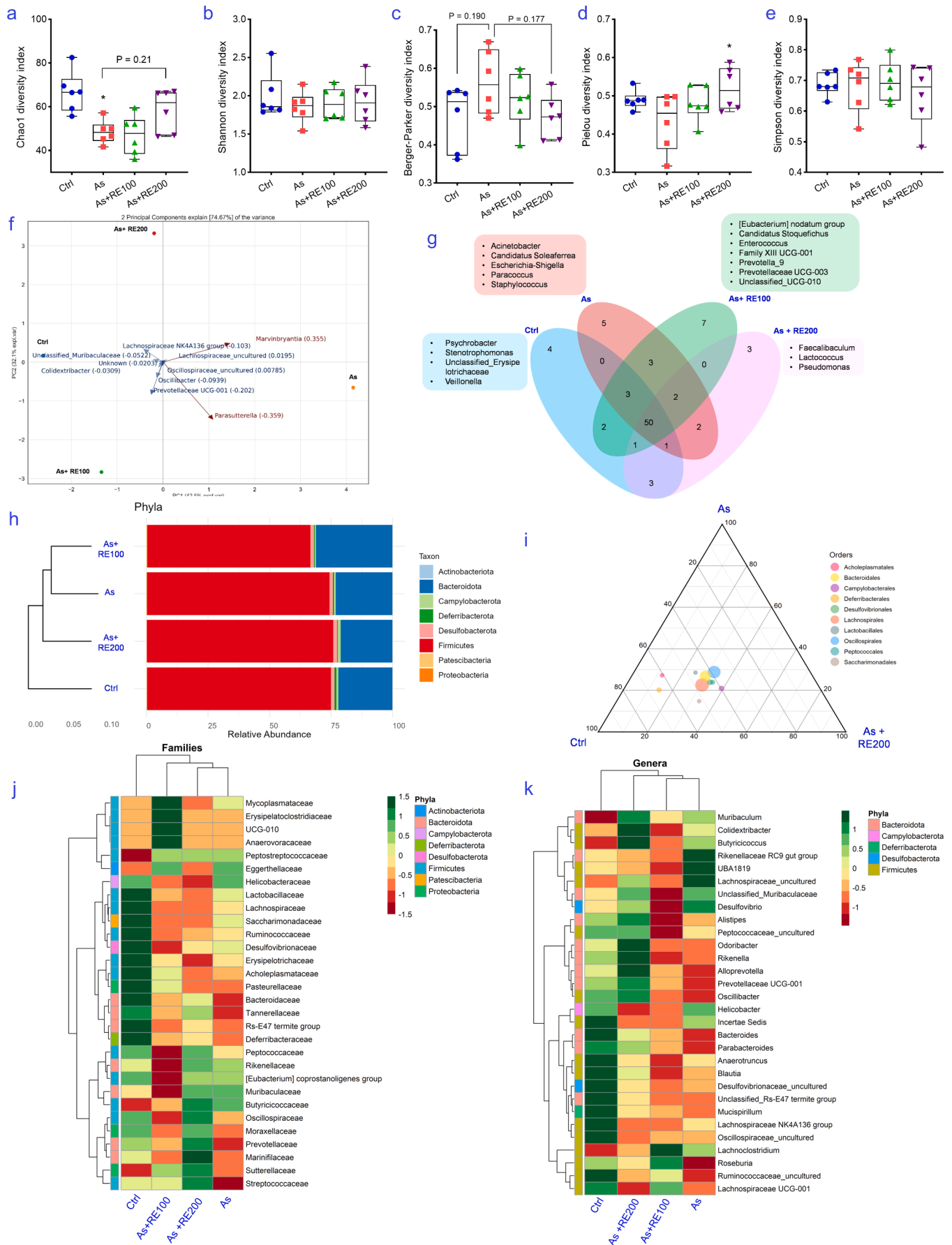


Fig. 5. Effects of *R. ellipticus* berries extracts supplementation in gut inflammatory and oxido-nitrosative stress markers, gut originated molecules and secondary metabolites, and ileum permeability in inorganic arsenic-exposed mice. a) H&E stained photomicrographs of 5 μ m thick sections of ileum and colon (objective 10X); concentrations of b) interleukin-1beta, c) interleukin-17; d) lipid peroxidation (MDA levels), nitrite, GSH, and SOD levels in ileum and colon; concentrations of, e) serotonin, and f) 5-hydroxy indole acetic acid in ileum and colon; g) Evan's blue dye permeation based ileum permeability assessment; h) cecal key short chain fatty acid concentrations; i) relative mRNA expression of selected genes in ileum; j) relative mRNA expression of selected genes in colon. Data is presented as scattered bar plots (mean \pm SEM, individual observation points are also presented) or heatmaps (individual colored cells represent a single sample within a group). Statistical comparison was done using One-way ANOVA followed by Tukey's post-hoc analysis for multigroup comparison. * $P < 0.05$, ** $P < 0.01$, versus control, # $P < 0.05$, ## $P < 0.05$ vs arsenic exposed group. RE = *Rubus ellipticus*, 5-HT = 5-hydroxy tryptamine or serotonin, 5-HIAA = 5-hydroxyindole acetic acid, MDA = malondialdehyde, GSH = reduced glutathione, SOD = superoxide dismutase, LPS = lipopolysaccharides, TPH1 = tryptophan hydroxylase 1, TLR = toll like receptor, NF- κ B = nuclear factor kappa beta, FFAR = Free fatty acid receptors, ZO = Zona occludens, Cld = Claudins, MUC = mucin gene. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In contrast, inorganic arsenic exposure induced marked divergence in this community structure. Dominance of *Firmicutes* and *Bacteroidota* was observed across all groups, while enrichment of *Actinobacteriota* and *Proteobacteria* in iAs(III) exposure group was seen (Fig. 6h). A triangulated observation in these three groups at order-level showed that *Acholeplasmatales*, and *Deferribacterales* were more abundant in control group than other, whereas most of the other orders showed equal distribution patterns in control and *R. ellipticus* berries extract supplemented mice than the iAs(III) mice alone (Fig. 6i). Similarly, family and genera level identification of these bacterial taxa showed the negative

impact of iAs(III) exposure where it distinctly separates out these bacterial taxa abundances (Fig. 6j-k). Few notable observations upon iAs (III) exposure were: reduction in the relative abundances of *Streptococcaceae*, *Marinifilaceae*, *Prevotellaceae*, *Moraxellaceae*, *Oscillosporaceae*, *Bacteroidaceae*, *Lactobacillaceae*, *Tennerellaceae*, with increased abundance of *Mycoplasmataceae*, *Butyricoccaceae*, *Muribaculaceae*, *Rikenellaceae*, *Eggerthellaceae*, and *Peptostreptococcaceae*. Many of these changes were reversed in the mice supplemented with *R. ellipticus* berries extract (200 mg/kg), interesting effects were observed with the increased abundance of *Streptococcaceae*, *Sutterellaceae*, *Marinifilaceae*,



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Fig. 6. Effects of *R. ellipticus* berries extract supplementation on gut microbial architecture in inorganic arsenic-exposed mice. Alpha diversity indices a) Chao-1 index, b) Shannon index, c) Berger-Parker index, d) Pielou index, e) Simpson index; f) principal component analysis for assessing beta diversity; g) Venn diagram showing common and unique taxa in different groups; h) UPAGMA based hierarchical clustering of phylogenetic diversity at phylum level; i) ternary plot of top 10 bacterial orders in the three distinct group; j-k) relative abundances at family and genus levels of various bacterial community members in mice gut metagenome. Data is presented as scattered bar plots (minimum to maximum with midline representing median value, individual observation points are also presented) or heatmaps (group mean values). Statistical comparison for non-parametric data as in alpha diversity indices Kruskal–Wallis test followed by Dunn's post hoc test was used for multigroup comparison. RE = *Rubus ellipticus*, PC = principal component. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Prevotellaceae, *Oscillispiraceae*, and *Peptococcaceae*, with reduction in *Mycoplasmataceae*, *Eggerthellaceae*, *Helicobacteraceae*, *Erysipelotrichaceae* (Fig. 6j). At genera level such effects were evident at increased abundance of *Lachnospirillum*, *Helicobacter*, *Desulfovibrio*, *Lachnospiraceae uncultured*, with marked reduction in some beneficial microbial taxa such as *Lachnospiraceae UCG-001*, *Ruminococcaceae*, *Roseburia*, *Bacteroides* and *Parabacteroides*, *Prevotella* and *Alloprevotella*, *Odoribacter*, and *Alistipes* upon iAs(III) exposure. A marked reversal in many of these effects was observed upon *R. ellipticus* berries extract (200 mg/kg) supplementation. Some unique observations in this supplementation group were: increased *Bacteroides*, *Parabacteroides*, *Oscillibacter*, *Prevotellaceae*, *Alloprevotella*, *Odoribacter*, *Alistipes*, and *Butyrivococcus* abundances (Fig. 6k). Further, though limited but additional validation of some of these signatures has come from classical qPCR based relative bacterial abundance studies, showing that Firmicutes and Bacteroides were highly expressed among the tested groups and a slight reduction in *Lachnospiraceae*, *Prevotella* and *Roseburia* abundances were observed upon iAs(III) exposure. These effects were effectively prevented by the *R. ellipticus* berries extract supplementations (Suppl. fig.3).

Further, correlational analysis between various behavioural, biochemical and molecular factors quantitatively determined, suggested that behavioural endpoints and performances in Morris' water maze or passive avoidance test were positively correlated to the brain or serum LPS levels, brain inflammatory signalling involving astrocyte and microglia (*GFAP* and *AIF* expression), oxidative stressors such as nitrite, gut permeability and brain antioxidant defence mechanism such as SOD levels, *Nrf2*, *HO-1* expression and intestinal serotonin production, and SCFAs (acetate, propionate, levels). Moreover, all these factors also correlated (see the correlation matrix in Fig. 7a), in such a way that drives the oxidative stress led neuro-intestinal inflammatory state. Interestingly, gut wall integrity is negatively associated with brain and gut serotonin levels and related mediators, circulating and brain lipopolysaccharides, suggesting an endotoxemia condition, and other inflammatory and oxidative stressors. SCFAs, specifically acetate levels, were positively correlated with better cognitive performances, improved dopamine levels, reduction in brain inflammation, and increased endogenous antioxidant levels. Butyrate levels were also positively correlated to passive avoidance performances, dopamine levels, and ileal serotonin levels with a reduction in brain inflammatory mediators and oxidative stressors (Fig. 7a).

Further, specific SCFAs levels were correlated with relative abundances of top bacterial hits from the metagenomic data. Results suggested that all selected SCFAs levels were positively correlated with *Anerotruncus*. Acetate was positively correlated to uncultured *Peptococcaceae*, *Desulfovibrio*, *UBA1819*, *Blautia*, *Incertae Sedis*, *Candidatus saccharimonos*, *Rikenella*, and *Biophila*. Propionate levels showed positive correlation to *Mucispirillum*, *Odoribacter*, and *Rikenella*. Butyrate showed positive correlation to *Lachnospiraceae* family bacteria, *Oscillispiraceae*, *Peptococcaceae*, *Mucispirillum*, *Desulfovibrio*, *Ruminococcaceae*, *Blautia*, *Incertae Sedis*, *Candidatus saccharimonos*, *NK4A214*, *Biophila* and *Peptococcus* (Fig. 7b).

4. Discussion

Targeting brain functions against such environmental contaminants through dietary supplementation/ potential nutraceuticals could provide an useful interventional approach. The key observations from this

study were: i) hydro-alcoholic UAE extraction of berries of *R. ellipticus* at 60%MeOH, 45 min, and 52.5 °C showed maximum antioxidant activities, which was nontoxic to the cells *in-vitro* and have multiple flavonoids, phenolic acids, and procyanidins, ii) this extract efficiently prevented iAs(III) exposure-induced impairment in cognitive performance in mice with notable control over neuroinflammation and neurotransmitters turnover disruption, iii) it also prevented iAs(III) exposure-induced increase in ileal permeability and gut inflammation, iv) lastly, it prevented iAs(III) exposure-induced gut microbial dysbiosis. This, to the best of our knowledge, is the first compelling evidence for the successful use of this polyphenol-rich fraction of *R. ellipticus* berries against iAs(III)-exposure induced cognitive impairments in mice.

Green extraction procedures, such as UAE is one of the rapid extraction strategies for bio-actives. Suitably optimized extraction conditions (time, temperature, and %CH₃OH) are optimally manipulable factors for the efficient yield of these bio-actives. Optimized RSM-based approaches have a mathematical advantage to achieve best yields (quantitative/activity-based) (Kewlani et al., 2022; Kumar et al., 2021). Other extraction methods, such as microwave-assisted extraction, pressurized-liquid extraction, supercritical fluid-based extraction, and enzymes-mediated extraction or a combination of these, could also provide an opportunity to extract polyphenols from these plant sources. However, UAE is a low-resource-intensive and rapid extraction method (Kumar et al., 2021). Considering these factors, we have utilized the UAE for polyphenols extraction from *R. ellipticus* berries using various combinations of time, temperature, and methanol concentration suggested in the RSM optimization stage. We found that 60% methanol for 45 min at 52.5 °C gives an extract with high TPC and RSA. The yield in our experiments is somewhat similar to or better than the earlier studies on these berries (Espada-Bellido et al., 2019; Kewlani et al., 2022). However, an exact comparison would not be possible, as the mentioned study used the dried berries (pre-drying for 3 days at 60 °C). This particular study found 64% methanol, for 20 min at 49 °C gave maximum yield for TPCs, which is somewhat similar to our finding, considering the wet berries as the starting sample in our study. Similarly, polyphenols profile related to caffeic acid, cinnamic acid were seen in our extract, however, we did not observe a quantifiable amount of catechins, coumaric acid, or vanillin in our extract. Interestingly, we observed the high levels of dihydrokaempferol, dihydromyricetin, myricetin, rutin, and four predominant proanthocyanidin (PA C1, PA A1, PA B2, and PA A2), and delphinidin as quantifiable anthocyanidin in these berries.

Arsenic-contaminated drinking water is still the most prevalent source of its human exposure (Kaur et al., 2024; Marghade et al., 2023). Its negative impact on human health in the form of neurological, cardiovascular, metabolic, dermatological, and immunological disorders and its potential for carcinogenic and teratogenic effects has been recognized. Oxidative stress and inflammatory signaling activation have been recognized as key underlying mechanisms for these pathologies upon arsenicals exposure (Garg and Bandyopadhyay, 2025; Vázquez Cervantes et al., 2023). Anticipated for high antioxidant activity and potential gut health-modifying activity due to polyphenols, we used *R. ellipticus* as an intervention in our study against iAs(III)-induced neurotoxicity and cognitive impairment. Neurotoxic effects of arsenic exposure precipitate in the form of behavioral aberration (Garg and Bandyopadhyay, 2025; Medda et al., 2020; Zhang et al., 2025). iAs (III)-exposure has been linked to impairment in cognitive functions

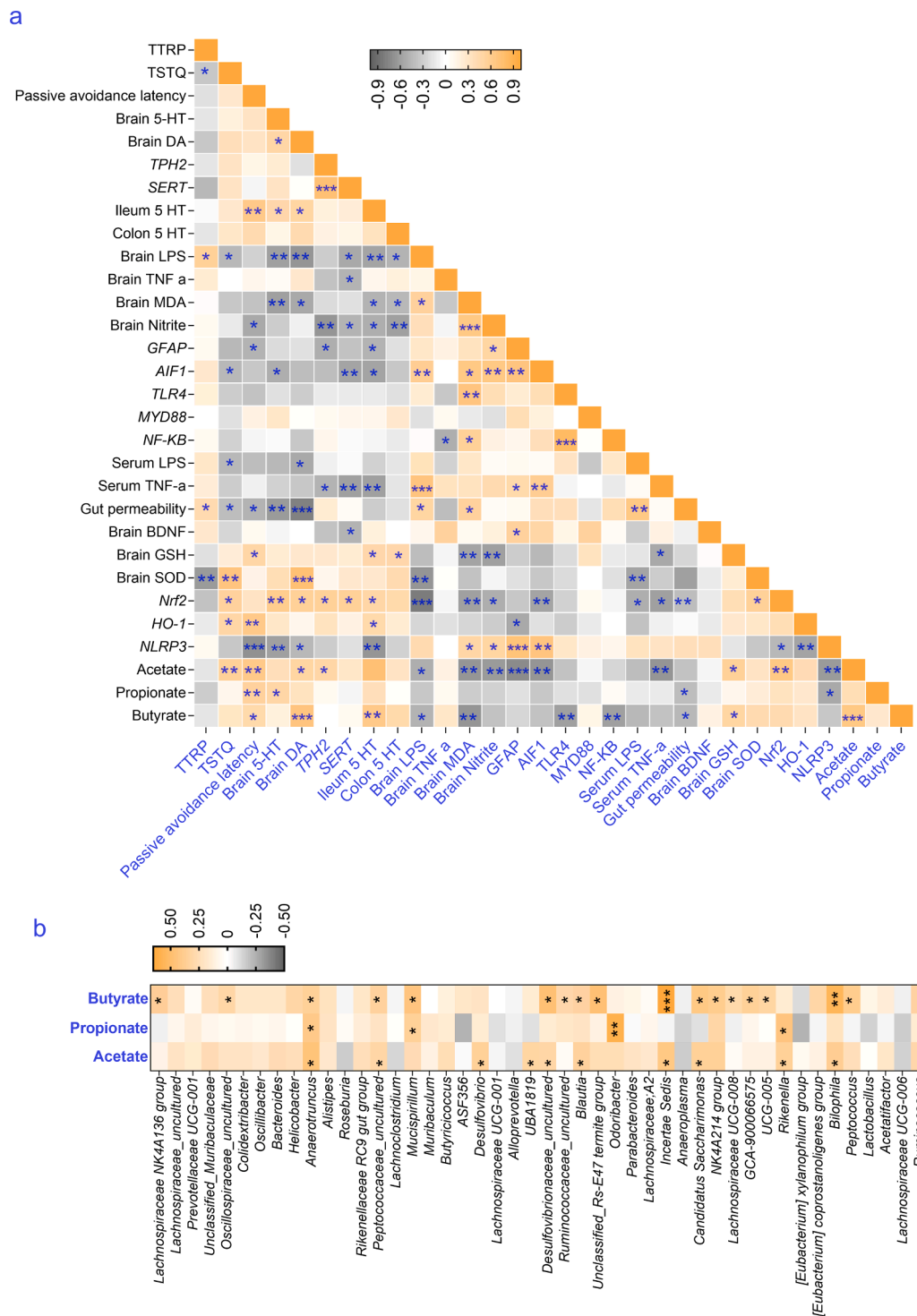


Fig. 7. Spearman correlation analysis matrix of various parameters assessed in the study. Colour gradients were given in terms of 'r' values among following: a) Time to reach the hidden platform (TTRP) and, Time spent in target quadrant (TSTQ) in Morris Water maze test, latency to reach hidden platform in passive avoidance task, brain serotonin and dopamine levels, *TPH2* and *SERT* expression, serotonin levels in ileum and colon, brain lipopolysaccharides, TNF-alpha, lipid peroxidation nitrite levels, *GFAP*, *AIF-1*, *TLR4* and *NF-kB* expression, serum LPS, TNF-alpha levels, Evans blue based gut permeability assessments, brain *BDNF* gene expression, superoxide and reduced glutathione levels in brain along with expression of *Nrf2*, *HO-1* and *NLRP3*, and short chain fatty acids such as acetate, propionate and butyrate levels. b) correlational analysis between various bacterial taxon to acetate, propionate, and butyrate. Statistical significance among different correlation coefficients is denoted as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ on individual cells of the heatmap. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

such as executive functioning (Vaidya et al., 2023), gross cognitive impairment (Karim et al., 2019; Wang et al., 2021), and impaired spatial navigational and exploratory behavior (Vázquez Cervantes et al., 2023). Furthermore, the negative impact of arsenic exposure on neuronal

health and loss of neurotransmitters has been reported (Chu et al., 2023; Medda et al., 2020). Similarly, we have also seen that chronic iAs (III)-exposure altered neurotransmitters levels, which potentially could be the aftermath of increased hippocampal neuroinflammation and

neurodegeneration. This could be led by astrocyte (*GFAP*) and microglia (*AIF-1*) markers upregulation, loss of neurotrophic factor such as *BDNF*, or upregulation of LPS mediated inflammatory cascades (tissue or circulating levels of LPS and inflammatory cytokines such as TNF α , ILs, and *TLRs*, *NF-kB* expression), or increased oxidative stressors (reduced GSH and SOD, *Nrf2* and *HO-1* genes; increased MDA and nitrite) (Chu et al., 2023; Mahalanobish et al., 2019; Singh et al., 2024; Zhang et al., 2025). Interestingly, the *R. ellipticus* berry extract's supplementation prevented these molecular events. However, studies investigating the effects of these berry extracts on neurotransmitter levels or their synthesis (upregulated *TPH2* expression in the brain), neuroinflammation, as well as molecular dissection of these events are lacking; this study is the first such report on these effects of the polyphenol-rich extract of *R. ellipticus* berries.

Further, a similar type of tissue-level response in the ileum and colon has also been observed on inflammatory mediators and oxidative stress markers upon iAs(III) exposure (Chiocchetti et al., 2018). Moreover, iAs(III)-exposure affects the intestinal permeability, as reflected by increased Evans blue dye permeation and downregulation of key tight junction genes such as *ZOs*, *Cld*, and *Occludins* in the ileum and/or colon. A direct effect of iAs on tissue inflammation and oxidative damage in the form of altered tissue histological features has been observed. LPS-mediated inflammatory pathways activation in the form of *TLRs* and *NF-kB* upregulation and impaired gut barrier may further aggravate the systemic inflammatory cascades activation, leading to cellular damages (Aburto and Cryan, 2024). Though the previous experimental data on such effects of *R. ellipticus* berries are lacking, berry polyphenols in general, owing to their high antioxidant and anti-inflammatory activities, showed beneficial effects on leaky gut phenotypes (Hidalgo-Liberona et al., 2020; Peron et al., 2021). Such disruption of epithelial barrier integrity may permit enhanced translocation of luminal antigens and endotoxins, thereby amplifying systemic inflammatory tone (Sarma et al., 2018). Elevated peripheral inflammation is known to influence microglial activation and central cytokine production, contributing to neuroinflammatory cascades and altered neurotransmitter metabolism (Aburto and Cryan, 2024; Dimeji and Ayodeji, 2025; Schneider et al., 2024). Further, multiple gut-related factors, in the present study showed iAs(III) upregulated LPS-mediated *TLRs* (*TLR-2*, *-4*, and *-5*) and could induce inflammatory cytokines production. Reduced SCFA transporter (*FFAR2* and *FFAR3*) and mucin synthesis (*MUC2* and *MUC4* expression) in iAs(III) exposed condition was prevented by *R. ellipticus* berry extract. This suggested that a disrupted gut barrier, together with the presence of pathogen-associated microbial patterns (PAMPs) like LPS, may be driving neuroinflammation and damage to hippocampal neurons, which in turn could lead to cognitive impairments.

Apart from their direct effects, these polyphenols get metabolized and activated by the activities of gut microbes (e.g., urolithin-a), which could elicit these beneficial effects (Singh et al., 2019), as these microbiota-derived metabolites affect the biological activities of dietary polyphenols in age-associated leaky gut (Hidalgo-Liberona et al., 2020). The neuroprotective effects observed in this study may also involve direct central actions of bioavailable polyphenol metabolites. Although many parent compounds identified in the *Rubus ellipticus* extract, such as flavonoids and proanthocyanidins, exhibit limited blood-brain barrier permeability and potentially undergo extensive biotransformation by host and microbial enzymes into low-molecular-weight phenolic metabolites. Among these, compounds such as ferulic acid, caffeic acid, and cinnamic acid have been reported to cross the blood-brain barrier and exert neuroprotective effects through modulation of oxidative stress and inflammatory signaling pathways (Castro et al., 2023; Elkhorsawy et al., 2024; Zhang et al., 2023). Additionally, microbial metabolism of proanthocyanidins yields phenyl- γ -valerolactones (Chen et al., 2023) and related derivatives, which could act as potential mediators of gut-brain communication by affecting oxidative stressors and inflammation. Therefore, the observed improvements in neurotransmitter

balance and neuroinflammatory status may be partly attributed to these circulating metabolites, alongside gut-mediated mechanisms. Notably, we did not measure the direct effect of such metabolites; hence, a biologically plausible mechanism based on current findings and known polyphenol metabolism could be suggested, but does not imply direct causal validation. Additionally, SCFAs such as acetate, propionate, and butyrate, are some of the major microbial metabolites from dietary carbohydrates. Catechin, anthocyanins, and proanthocyanins have shown their prebiotics effects by effectively modulating SCFAs levels in the colon (Freitas et al., 2023; Tian et al., 2024). The role of these SCFAs, especially acetate and butyrate in maintaining the gut health is crucial, both by their direct effect on inhibition of inflammation, and promotion of colonocyte growth and maintenance of mucin layer (Anhe et al., 2014; Singh et al., 2017, 2018). iAs(III) exposure significantly reduced the acetate and butyrate levels, whereas the polyphenol-rich extract of *R. ellipticus* berries effectively alleviated these alterations. Restoration of these SCFAs (especially butyrate) can be used by colonocytes as a fuel source for their growth and mucin synthesis, and reduction in inflammatory cell activation (Anhe et al., 2014; Singh et al., 2018). Also, these microbial metabolites (SCFAs, tryptophan metabolites and indole derivatives, polyphenols metabolites such as urolithin-a, bile acid metabolites, polyamines, p-cresol etc.) work systemically at various physiological sites such as gut-epithelial barrier, blood brain barrier, choroid plexus barrier to prevent the interaction of harmful substance and mostly the inflammatory mediators interaction and safeguard the cellular structure (Aburto and Cryan, 2024). Interestingly, neurotransmitter imbalance during iAs(III)-exposure may also be influenced by inflammation-led metabolic shifts. For instance, pro-inflammatory cytokines can alter tryptophan metabolism through activation of indoleamine 2,3-dioxygenase, affecting serotonergic signalling (Stone and Williams, 2024), while oxidative stress may impair dopaminergic and cholinergic homeostasis (Guo et al., 2024). As we have also seen an impaired neurotransmitter profile in the brain and in the gut, and altered expression of a key serotonin synthesis gene, upon iAs(III) exposure, which was rescued by the berry extract supplementation, could be interlinked with the complex role of gut tissue and microbial activity mediated neurotransmitter synthesis. The GI tract, which accounts for approximately 80% of total serotonin biosynthesis, participate in the gut-brain axis by the mediation of resident microbes. The role of these SCFAs particularly acetate and butyrate in neurotransmitter biosynthesis has been evident (Vincent et al., 2018; Wu et al., 2020). These microbial metabolites induced the host's enterochromaffin cells to upregulate the expression of *Tph1* gene and 5-HT biosynthesis (Yano et al., 2015). Interestingly, we have also seen that the iAs(III) exposure reduced ileal 5-HT levels and downregulated *Tph1* expression in ileum and colon. This coordinated reduction in neuroinflammation and oxidative burden following berry extract supplementation provides a plausible route through which gut-targeted modulation can direct the improved cognitive performance.

Further, it is imperative to mention that the importance of gut microbiota, the resident microbes in the gut, having the population of both beneficial and harmful microorganisms, surpasses the host genome in total size and is widely recognized as an "organ" of itself in host physiology. An alteration of this ecologically balanced microbial system is called dysbiosis. iAs exposure has recently been identified as a potential dysbiotic factor, and causes bacterial biofilm and metabolic profile derangements (Brabec et al., 2020; Coryell et al., 2019). The role of the gut-microbiota-brain axis was evident from the prevention of dysbiotic effects of iAs exposure-induced behavioural alteration upon unexposed mothers' faecal microbial transplant (Zhao et al., 2023). Previous studies have also reported that inorganic arsenic exposure in drinking water decreased Bacteroidetes, *Barnesiella*, *Lactobacillus*, *Alis-tipes*, Peptostreptococcaceae, Prevotellaceae, and increased various Proteobacteria, Bacteroidetes, Epsilonbacteraeota, *Desulfovibrionaceae*, and *Turicebacter* (Dheer et al., 2015; Guo et al., 2014; Qiu et al., 2020). Moreover, we have also seen the reduction in alpha diversity indices

such as Chao-1 upon environmentally relevant doses of iAs(III) exposure in mice. Supplementation of polyphenolic extract from the *R. ellipticus* berries improves these microbial dynamics by increasing the microbial richness. Our 16S rRNA analysis showed that iAs exposure promotes the abundances of *Acinetobacter*, *Candidatus soleiferrea*, *Escherichia-Shigella*, *Paracoccus*, *Parasutterella*, and *Staphylococcus*. These bacterial genera have shown negative outcomes to cognitive or other neurobehavioral functions in earlier published studies or where the neuronal injuries are the major pathological feature in a disease outcome (Liu et al., 2023; Xie et al., 2024; Zhou et al., 2025), whereas, unexposed mice had *Psychrobacter*, *Stenotrophomonas*, and *Veillonella*. The role of *Psychrobacter* in reduced depressive phenotypes due to its anti-inflammatory metabolites production and butyrate biosynthesis is suspected (Guo et al., 2025). Similarly, lower abundance of *Stenotrophomonas* and *Veillonella* has been linked to cognitive impairments (Hua et al., 2023; Wang et al., 2024). We believe for the first time, we are reporting the effects of polyphenolic extracts from *R. ellipticus* berries against the iAs(III) exposure induced gut microbial alteration. We saw signature key changes in *Eubacterium* sp., *Enterococcus*, *Prevotella*, *Fecalibacterium*, *Lactococcus*, and *Pseudomonas* sp. Most of these bacteria are generally considered beneficial gut microbes owing to their positive health effects in the host. *Enterococcus* sp. have shown positive effects on hippocampal neurogenesis via ERK-CREB-BDNF pathways and improved cognitive function (Takahashi et al., 2020). *Eubacterium*, *Prevotellaceae*, *Pseudomonas*, and other bacteria from *Ruminococcaceae* and *Coriobacteriaceae* families are positively correlated with fluid intelligence (Oluwagbemigun et al., 2022). Further, these polyphenols significantly increased the SCFAs-producing bacterial genus than other groups such as *Butyricoccus*, *Lachnospiraceae*, *Alloprevotella*, *Alistipes Parabacteroides*, *Peptococcaceae uncultured Odoribacter*, and *Parabacteroides* (Liu et al., 2025; Villaseñor-Aranguren et al., 2022).

Hence, the role of gut-microbiota-brain axis in iAs(III)-exposure induced cognitive impairment could be established by the involvement of gut-originated factors (gut microbes, SCFAs, neurotransmitters, LPS, local inflammatory milieu, and leaky gut phenotype) to brain function (cognitive task performance impairments, neuroinflammation, and neuronal loss). The previously minimally investigated berry (*R. ellipticus*) from Himalayan region potentially showed the phenotypically beneficial effect by targeting multiple molecular pathways in these alterations caused upon iAs(III) exposure.

The present findings support a biologically coherent interaction between intestinal homeostasis and central neurochemical regulation in the context of chronic iAs(III) exposure. While direct causality within the gut-microbiota-brain axis was not experimentally established through microbiota transplantation or depletion strategies, the convergence of behavioral, biochemical, intestinal, and microbial outcomes provides mechanistic plausibility for gut-mediated modulation of arsenic-induced neurotoxicity. Despite the fact that the findings are of associative in nature the improved behavioural performances, recovery in gut permeability, microbial balance, inflammatory tone, and neuro-/intestinal-inflammation upon berry extract supplementation, support the involvement of this intricate crosstalk in gut-microbiota and brain. Within the context of environmental toxicology, this systems-level interaction highlights the gut as a potentially critical upstream mediator of arsenic-induced neurobehavioral impairment. Future studies employing fecal microbiota transplantation, antibiotic depletion models, or metabolomic profiling of microbial-derived metabolites would be valuable to delineate causative pathways more precisely.

Limitations: Although the study presented the first functional evidence of the successful use of this polyphenolic-fraction from *Rubus ellipticus* berries against iAs(III) exposure-induced cognitive impairments, still, to pinpoint the exact mechanisms linking the gut microbiota as a key regulator to such effect could be established by experimental paradigms such as the use of germfree mice or use of antibiotics cocktail administration and fecal material transplant studies. Second, while the polyphenol-rich extract was chemically characterized, the relative

contribution of individual bioactive constituents versus synergistic interactions among flavonoids, phenolic acids, and procyanidins remains unclear. Advanced metabolomic profiling, including assessment of circulating and brain-penetrant metabolites, would help clarify bioavailability and mechanistic targets. These limitation of the present work, leaving scope for future investigation for identification of confirmatory role of gut microbiota driving these effects

5. Conclusion

The polyphenol-rich extract of *Rubus ellipticus* effectively prevented the iAs(III) exposure-mediated cognitive task performances. These effects are expected to be associated with the high TPC and antioxidant activities, and anti-inflammatory properties, prevention on neurotransmitter level alteration, and reduction in neuroinflammation. This supplementation also showed the prevention of iAs(III)-induced gut permeability alteration and structural changes, gut neurotransmitter levels, gut dysbiosis, and short chain fatty acid alterations, suggesting the plausible involvement of the microbiota-gut-brain axis. Although causality within the microbiota-gut-brain axis warrants further validation, the integrative outcomes reported here suggest the potential of *R. ellipticus* berries as a functional food source for dietary intervention against iAs(III) exposure-induced cognitive impairments. Future investigations focusing on bioavailability, metabolomic profiling of microbial-derived metabolites, dose standardization, and clinical translation will be essential to determine their practical applicability in human health contexts. Nonetheless, this study provides foundational evidence supporting the valorization of indigenous Himalayan berries as bioactive dietary resources with neuroprotective potential.

CRedit authorship contribution statement

Vandana: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Conceptualization. **Shweta Gupta:** Investigation. **Rajni Sharma:** Methodology, Investigation, Formal analysis. **Ashutosh Pandey:** Writing – review & editing, Validation, Resources, Methodology, Formal analysis. **Mahendra Bishnoi:** Writing – review & editing, Validation, Resources, Methodology, Formal analysis. **Rakesh Rawal:** Writing – review & editing, Supervision, Project administration. **Dhirendra Pratap Singh:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Data curation, Conceptualization. **Santasabuj Das:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Vandana reports financial support was provided by Department of Health Research, Ministry of Health and Family Welfare, India. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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Data availability

Data will be made available on request.

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