

Pharmacometabolomic Study of *Pulmonaria officinalis* Polyphenol Extracts Reveals Neuroprotective Effects in LPS-Induced Alzheimer's Disease through AChE Inhibition and TLR4 Modulation

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Abstract

Background

Acetylcholinesterase (AChE) and toll-like receptor 4 (TLR4) inhibitions are used in neurodegenerative disorder treatment strategies, including Alzheimer's disease (AD), a major cause of mortality and morbidity worldwide. *Pulmonaria officinalis* (PO; lungwort) has *in vitro* antioxidant, anti-inflammatory, and AChE inhibition. Their biological property is lacking *in vivo* literature."

Methods

This study aimed to characterize the metabolomic profile and antioxidant activities of different PO fractions, specifically butanol (BuOH) and ethyl acetate (EtOAc), using colorimetric, spectrophotometric, and chromatographic methods. In addition, studying their neuroprotective effect in lipopolysaccharide (LPS)-triggered Alzheimer's in mice. The qualitative phytochemical analysis revealed that the fractions of PO contain alkaloids, glycosides, phenols, flavonoids, terpenoids, and tannins.

Results

The lungwort plant was identified 36 active compounds in the two fractions. Extractions of EtOAc and BuOH lungworts were rich in polyphenolic compounds identified by HPLC and HPLC-mass spectrometry (HPLC-MS) analysis. In regard to phenolic, tannin, and flavonoid content, PO showed that the EtOAc and BuOH fractions were more significant than the other fractions. Moreover, this fraction exhibited more potent antioxidant activity than other fractions in DPPH[•] and ABTS^{•+} assays. In the *in vivo* experiment, mice were randomly divided into four groups, each comprising eight animals. Group I was designated as the normal control, whereas Group II was administered lipopolysaccharide (LPS; 250 µg/kg, intraperitoneally). Groups III and IV were administered PO butanol and ethyl acetate fractions (400 mg/kg, orally) for seven consecutive days along with LPS treatment. PO fractions ameliorated cognitive dysfunction elevated the memory via suppressing AChE and amyloid beta, inhibited neuroinflammatory cytokines Interleukin 1 beta (IL-1β) and TLR4, and alleviated neurodegeneration.

Conclusions

P. officinalis, for the first time, was studied *in vivo* and was proved to have a neuroprotective effect against AD through the presence of catechin, methyl gallate, astragalin, nicotiflorin, quercetin 3-O-β-glucoside, cinnamic acid, catechin, daidzein, kaempferol 3-O-(6-malonyl-glucoside) and epigallocatechin. PO holds potential for future pharmacological research and is a promising candidate for therapeutic applications in treating various chronic and infectious diseases.

Background

AD is a decline in memory and cognitive and physical abilities, along with symptoms including depression, delusions, and hallucinations, which result in significant reliance and incapacity [1, 2]. Approximately 70% of cases of dementia in the elderly are caused by AD. Aging is the biggest risk factor for AD, and its prevalence is growing exponentially worldwide [3]. The pathological process of AD is multifaceted, involving β-amyloid peptide and tau protein aggregation, which results in intraneuronal neurofibrillary tangles formation [4].

Patients with AD suffer a progressive reduction in their acetylcholine levels, which is caused by cholinergic neuron loss in their hippocampal and cerebral cortex [5]. Declining plaques and neurofibrillary tangles are two more abnormalities that develop. Consequently, blocking AChE enhances the amount of acetylcholine that builds at the synapses and will focus on the cholinergic deficiency, which is a therapeutic target for AD medications. AChE inhibitors, such as glutamine, donepezil, and rivastigmine, are drugs used to treat AD. Synthetic drugs have unsatisfactory response rates and undesirable, severe adverse effects. The challenge of treating AD remains since their primary impact is on mitigating its symptoms rather than slowing down the disease's advancement [6]. Consequently, there is currently a need for safe, new and efficient medications from medicinal herbs. It has been demonstrated that medicinal plant products are an effective source of AChE inhibitors. Additionally, for AD, AChE inhibition is regarded to be a promising treatment for myasthenia gravis, Parkinson's disease, glaucoma, and other forms of dementia [7].

Toll-like receptors (TLRs), innate immune receptors, recognize different ligands related to tissue injury and pathogen-derived substances. TLR4-mediated signaling plays a role in the progression of age-related neurodegenerative disorders. It is believed that oxidative stress contributes to the neurodegeneration associated with AD because it can be triggered by the development of β -amyloid plaques. That's why it's so interesting to discover treatments that can lower oxidative stress and β -amyloid peptide synthesis and inhibit AChE [4].

There are currently only five drugs approved to treat AD, which highlights the requirement for alternative therapies that may alleviate symptoms but neither cure illness progression nor provide a remedy [8]. Prior research has indicated that oxidative damage is the primary cause of many neurological illnesses, such as AD disease, vascular dementia, stroke, and others [9]. Since scientists have searched for natural sources of antioxidants to incorporate into various cosmetic, medicinal, and food compositions, plant extracts possess important properties, one of which is their antioxidant activity. [10]. Antioxidants can be found in large amounts in the human diet, which has drawn attention as a potential treatment for AD. A number of natural substances have had encouraging outcomes in in vivo preclinical investigations, and some are currently being investigated in clinical trials. The genus *Pulmonaria*, commonly referred to as lungwort, is a member of the family Boraginaceae and comprises about eighteen species of annual flowering plants, predominantly found in Europe, Russia, and Western Asia [11]. Historically, due to the Doctrine of Signatures, PO has been used medicinally, as its appearance was thought to resemble diseased lungs [12]. Today, various commercial products containing lungwort are available, including mother tinctures, dried leaves and aerial parts (marketed as *Pulmonaria* herb), teas, cosmetics, and supplement ingredients [10].

Lungwort is used in ethnomedicine for emollient, expectorant, antitussive, antibacterial, diuretic, depurative, antilithiatic, and anti-inflammatory purposes in addition to respiratory and urinary diseases. [13]. Lungwort has been studied for its possible biological characteristics including its anti-inflammatory, anticoagulant, anti-anemic, wound-healing, skin-whitening, anticonvulsant, and anti-neurodegenerative effects. [14]. The diverse array of phytochemicals present in different plant components, such as lignans, hydroxycinnamic acid, alkaloids, anthocyanins, flavanone, flavones, flavonols, polyphenols, and polyphenolic acids, could perhaps account for the biological properties of lungwort [15]. Although ethanolic lungwort extracts exhibit a stronger inhibitory effect on AChE in vitro research compared to aqueous extracts, PO has to undergo safety and clinical trials to demonstrate the majority of its potential biological attributes. Comparably, there are insufficient in vivo investigations on their ability to treat neurodegenerative diseases or inflammation. Additional in vivo studies are required to enhance our understanding of their anticoagulant, antioxidant, and anticonvulsant characteristics [15]. A few researches have been done on PO to determine whether its medicinal qualities can be used to treat diseases or to augment current therapies. So, the aim of the current work is to characterize the phytochemical, analyze the antioxidant of the lungwort plant fractions using HPLC and HPLC-mass spectrometry (HPLC-MS) analysis and study their neuroprotective potential in LPS- triggered Alzheimer in mice.

Methods

Phytochemical section

2.1. Plant material, chemicals, and reagents

All procedures related to plant collection and experimental use complied with institutional, national, and international regulations. Dried leaves of PO(Boraginaceae) were obtained from a local herbal market in March 2023 and identified by Engineer Terese Labib, Advisor at El-Orman Botanical Garden and the National Gene Bank, Ministry of Agriculture, Egypt. To guarantee public access to the material, a voucher specimen (M251) was placed in the National Research Center's herbarium. Following authentication, the plant samples were prepared for extraction. DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), Dragendorff's reagent, ferric chloride (FeCl_3), sodium hydroxide (NaOH), hydrochloric acid (HCl), Folin–Ciocalteu reagent, tannic acid, quercetin, gallic acid, butylated hydroxytoluene (BHT), and all organic solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation and Extraction of four PO fractions.

The dried leaves (2 kg) were finely powdered and passed through a 60-mesh sieve, then extracted successively with methanol of different concentrations (100%, 80%, and 50%; 10 L each) for 48 hours at room temperature. The pooled methanolic extracts were

evaporated to dryness under reduced pressure at 45°C using a rotary evaporator (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany). The concentrated residue was suspended in 1 L of water for 24 hours and then fractionated sequentially. First, partitioning with petroleum ether produced a petroleum ether fraction and an aqueous layer. The aqueous portion was subsequently extracted with EtOAc, followed by BuOH, giving EtOAc and BuOH fractions along with the remaining aqueous fraction. Each solvent extraction step was repeated three times, yielding fractions of distinct compositions according to the polarity of the solvents used [16].

2.3. Screening for phytochemicals in a qualitative methodology

The following standard techniques were used for the phytochemical screening of the petroleum ether, EtOAc, BuOH, and water fractions of PO:

2.3.1. Alkaloids test: Each sample's 300 mg of plant fraction was mixed, and two minutes were spent with 2% H₂SO₄. The mixture was carefully filtered, followed by the addition of three drops of Dragendorff's reagent. The formation of a dark orange to reddish precipitate confirmed the presence of alkaloids [17].

2.3.2. Saponins test: approximately over 300 mg of the plant fraction's extract were placed in test tubes. After adding and 15 milliliters of distilled water for over three minutes, the froth remained apparent, indicating the presence of saponin [17].

2.3.3. Phenols & tannins test: combined with distilled water, a tiny amount of fraction. After obtaining the filtrate, a small amount of ferric chloride was mixed with it. The occurrence of a greenish-black color indicated the presence of tannin [18].

2.3.4. Flavonoids test: after dissolving 400 mg of each fraction in NaOH, HCl was added. The presence of flavonoids was confirmed when the solution turned colorless from yellow [19].

2.3.5. Terpenoids test: An amount of 300 mg from each extract fraction was combined with 2 mL of chloroform and layered with 4 mL of H₂SO₄ conc. The appearance of a reddish-brown coloration at the interface was considered a positive test for terpenoids [20].

2.3.6. Glycosides test: after adding NaOH solution to neutralize the fraction, HCl was used to hydrolyze it. Fehling solution's solutions A and B were added to it. Red precipitates have appeared as a result, indicating the presence of glycosides [21].

2.3.7. Reducing sugars test: after mixing each sample's fraction with distilled water, it was filtered. After the filtrate and Fehling's, solutions A and B were sufficiently combined. An orange-red precipitate was observed, demonstrating the reduction of the presence of sugars [22].

2.3.8. Estimation total phenolic content (TPC): The Folin–Ciocalteu method was utilized to quantify PO fractions, with gallic acid serving as a reference standard. A calibration curve was made using gallic acid after absorbance at 760 nm was measured using a spectrophotometer. The results were expressed in milligrams of gallic acid equivalents (GAE) per gram of extract [23].

2.3.9. Estimation total flavonoid content (TFC): Spectrophotometric determination of the flavonoid concentration was based on the formation of a flavonoid–aluminum complex, which exhibits maximum absorbance at 510 nm. The results were measured in milligrams of quercetin equivalents (QE) per gram of extract [24].

2.3.10. Estimation of total tannin content (TTC): Tannin levels were evaluated using tannic acid as a standard and the Folin–Ciocalteu assay, with absorbance recorded at 775 nm. Following the method described by Tempel, the results were expressed as milligrams of tannic acid equivalents (TAE) per gram of dry extract [25].

2.4. Quantitative and qualitative estimation of EtOAc/BuOH Lungworts extracts by HPLC and HPLC-MS technique.

Chromatographic and mass analyses were performed using HPLC and LC-MS/MS. HPLC was conducted on an HP 1100 system (Agilent, CA, USA) with a G1329B auto-sampler and DAD; data were processed via ChemStation [26]. Separation used a ZORBAX Eclipse XDB C-18 column (150 × 4.6 mm, 5 µm) [27]. LC-MS/MS was carried out on an Exion LCAC system coupled to a SCIEX Triple Quad 5500 with an ESI source. Chromatographic separation was achieved on an Ascentis® C18 column (150 × 4.6 mm, 3

μm) with MP: 0.1% formic acid in H₂O (A) and ACN (B). Gradient: 10% B (0–1 min) → 90% B (1–33 min), held 90% B (33–37 min), back to 10% B (37.1 min). IV: 10 μL; FR: 0.7 mL/min. MS/MS operated in negative ion mode, scanning m/z 100–1000 (MS1) and m/z 50–800 (MS2). Data acquisition/annotation was performed with MS-DIAL v4.70 [28, 29].

Pharmacology section

2.5. Estimation of the antioxidant study (DPPH[•] and ABTS^{•+})

Antioxidant activity was assessed via DPPH[•] and ABTS^{•+} assays. In the DPPH[•] assay, absorbance reduction was measured at 517 nm. DPPH[•] alone served as negative control; a standard antioxidant was the positive control. All tests were triplicated, and inhibition (%) was calculated: Inhibition (%) = [(Abs^{control} – Abs^{sample})/Abs^{control}] × 100. The IC₅₀ value (radical scavenging, 50%) was determined from the activity curve [30]. Similarly, ABTS^{•+} assay assessed extract capacity to neutralize ABTS^{•+} radicals, generated by Re et al. [31]. Absorbance was recorded at 734 nm, with Trolox as reference. Inhibition (%) was calculated by the same formula, and results expressed as IC₅₀ (μg/mL).

2.5. In vivo study

2.5.1. Chemicals and kits

Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The levels of TLR4, AChE, and IL-1β were quantified using ELISA kits supplied by SunLong Biotech Co., Ltd. (Hangzhou, China).

2.5.2. Animals

Male Swiss mice (20–35 g) were housed under controlled laboratory conditions in plastic cages with filter tops (12 h light/dark cycle, 50% relative humidity, 28 ± 2°C). Animals had free access to water and a standard pellet diet. All procedures adhered to ARRIVE guidelines and the NIH Guide for the Care and Use of Laboratory Animals (Pub. No. 8023, rev. 1978). Ethical approval was obtained from the Medical Research Ethics Committee (MREC), NRC, Cairo, Egypt (Approval No. 01410324).

2.5.3. Acute toxicity

This toxicity study was performed to assess the safety profile of *PO* leaf fractions. Sixty mice were randomly allocated into six groups. Groups I and II (controls) comprised male and female animals (n = 10 per group) administered saline orally. Groups III–VI consisted of male and female mice (n = 10 per group) that received a single oral dose (5 g/kg) of either the *n*-butanol (BuOH) or ethyl acetate (EtOAc) fractions. All animals were monitored over a 14-day period for clinical signs of toxicity, including alterations in skin and coat condition, cardiovascular functions and respiratory, autonomic, and central nervous system responses, motor activity, and general behavior [32]. Then the effective dose used in the main experiment was 1/20 of the acute toxicity dose (250 mg/kg).

2.5.4. Experimental design

Mice were divided into four groups (n = 8). Group I (control) received daily i.p. saline for seven days. Group II (LPS control) was administered lipopolysaccharide (LPS, 250 μg/kg, i.p.) for the same duration. Groups III and IV received oral doses of *PO* fractions (BuOH), and (EtOAc), respectively, at 250 mg/kg (1/10 of the acute toxicity dose), in combination with LPS treatment for seven consecutive days.

2.5.5. Measurement of Behavioral Activity with the Y-Maze

The Hidaka et al. [33] method was followed when conducting the Y-maze test. Three identical arms, designated A, B, and C, made up the apparatus. For eight minutes during the training session, each mouse was free to roam around the maze. After 24 hours, a retention trial was conducted in which the animals were again placed in the maze for 8 minutes, and their activity was recorded. Spontaneous alternation behavior was calculated as the quantity of consecutive entries in overlapping triplet sequences into three distinct arms (e.g., ABCBACA = 3 alternations), while the total arm entries were also noted (e.g., ABCBACA = 7 entries). The formula was used to determine the % of alternation [34]:

The % alternation = * 100

2.5.6. Biochemical assays

Following decapitation, brains were excised, rinsed (PBS), weighed, and homogenized (20% w/v, MPW-120, MPW Med. Inst., Warsaw, Poland). Homogenates were centrifuged (5000 × g, 5 min, 4 °C; Sigma 2k15, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) [36]. Supernatants were analyzed via ELISA kits (SunLong Biotech, Hangzhou, China) for AChE, amyloid-β, TLR4, and IL-1β [35].

2.5.7. Statistical analysis

Data expressed as mean ± SD (triplicates). TFC, TTC, and TPC contents were compared by Student's t-test. Group differences were analyzed by one-way ANOVA with Fisher's LSD or Tukey's post hoc. In vivo results are presented as mean ± SD. GraphPad Prism v5 (GraphPad Software, USA) was used. Significance set at $P < 0.05$.

2.5.8. Histopathology assays

Brains were fixed (10% formalin saline, 24 h), rinsed, and dehydrated (graded ethanol). Samples were cleared (xylene), embedded (paraffin, 56 °C, 24 h), and sectioned (4 μm, LEITZ rotary microtome). Slides were deparaffinized, H&E-stained, and examined microscopically [37].

Results

Phytochemical section

4.1. Phytochemical screening study

Utilizing phytochemical screening, the constituents of the plant extracts are evaluated, and their dominance, as well as the search for bioactive components that could be helpful in the production of pharmaceuticals [38]. As shown in Table 1, the qualitative phytochemical analysis of PO petroleum ether, EtOAc, BuOH, and water fractions is conducted in the current study. Phenols, flavonoids, tannins, and Alkaloids, were detected in all fractions. These phytochemicals may be the reason for PO therapeutic potential. Our result is supported by Chauhan et al. [15] who found that PO have been shown to contain around 90 phytochemicals from pharmacologically active phytochemical classes, which, along with micronutrients, can be regarded as one of the major contributors to the biological characteristics. Natural polyphenols, in addition to being potent antioxidants, have been shown to modulate the Aβ production pathway in AD, hence having neuroprotective benefits [39]. It is well known that flavonoids have antioxidant properties and can promote or prevent the growth and development of malignancies. Tannins possess antibacterial, antiviral, and antitumor activity [38]. Alkaloids derived from the beta-carboline group exhibit potent antibacterial, anti-parasitic, and anti-HIV properties [40]. The preliminary phytochemical screening of the EtOAc/BuOH fraction of *Lungwort* extracts is presented in Table 1.

Table 1. Phytochemical screening of PO fractions

Phytochemical compounds	PF	EF	BF	WF
Alkaloids	+	++	++	++
Saponins	-	+	+++	+++
Phenols	+	+++	+++	++
Tannins	+	+++	+++	++
Flavonoids	+	+++	+++	++
Terpenoids	+++	++	++	+
Glycosides	-	++	++	+++
Reducing sugar	-	-	-	-

(+++), (++) , (+), and (–) indicate high, moderate, low, and absent amounts, respectively.

PF= Pet. Ether fraction, EF= ethyl acetate fraction, BF= butanol fraction, WE= Water fraction.

4.2. Total phenolic, tannin and flavonoid content

Given their crucial roles in general physiological processes, the phenolic, tannin, and flavonoid contents of natural products are significant factors in the quantitative and biological evaluation of the extract [41, 42]. In order to highlight the various kinds of compounds, the POextract was separated into four fractions in this investigation using organic reagents. Spectrophotometric evaluations were used to determine the TPC, TTC, and TFC of four different PO fractions.

As shown in Table 2, the TPC was quantified as gallic acid equivalents, whereas the TTC and (TFC) were expressed in terms of tannic acid and quercetin equivalents, respectively. A significant difference of less than 0.05 was observed in the TPC, TTC, and TFC across the four fractions. The highest TPC values were found in the BF fraction at 241.86 ± 0.78 mg GAE/g extract and the EF fraction at 221.96 ± 0.90 mg GAE/g fractions. In contrast, the PF fraction exhibited the lowest TPC value of 93.57 ± 0.40 mg GAE/g fractions, indicating that the TPC in BF and EF was around two times more than PF. Similarly, the analysis of the TTC showed that BF contained the highest TTC at 199.45 ± 0.95 , followed by the EF at 186.81 ± 0.55 mg of QE/g, WF at 122.89 ± 0.84 mg of QE/g and PF at 85.53 ± 0.63 , based on these findings, the TTC of BF and EF was around two times more than PF. BF had the highest TFC content, followed by EF, WF, and PF, in line with the general pattern of TPC and TTC of the four fractions. Furthermore, EF's TFC was almost four times greater than PF's.

Table 2. Levels of total phenolics, tannins, and flavonoids in various fractions of PO.

Fractions	TPC (mg GAE/g extract)	TTC (mg TAE)/g extract)	TFC (mg QE/g extract)
PF	93.57 ± 0.40^d	85.53 ± 0.63^d	92.59 ± 0.64^d
EF	221.96 ± 0.90^b	186.81 ± 0.55^b	326.67 ± 1.11^b
BF	241.86 ± 0.78^a	199.45 ± 0.95^a	346.56 ± 0.19^a
WF	189.07 ± 0.66^c	122.89 ± 0.84^c	139.96 ± 0.68^c

The data shown here reflects the mean $\pm$ standard deviation of three separate studies. Significant differences ($p < 0.05$) are shown by means with different letters in the same column.

4.3. Quantitative polyphenolic estimation of EtOAc/BuOH Lungworts extracts by HPLC technique.

The polyphenol compounds in *PO* were identified using HPLC analysis by comparing them with standard reference substances. Nineteen polyphenol standards were used in the HPLC study, including chlorogenic acid, syringic acid, gallic acid, methyl gallate, catechin, caffeic acid, rutin, ellagic acid, vanillin, *p*-coumaric acid, naringenin, daidzein, rosmarinic acid, quercetin, cinnamic acid, ferulic acid, kaempferol, and hesperetin. The EtOAc and BuOH extracts were found to have a five-fold higher content of polyphenol compounds: chlorogenic acid, methyl gallate, caffeine, ferulic acid, and rosmarinic acid; in the ethyl acetate fraction, they were found as 4037.90, 2132.90, 2888.07, 7737.06, and 1011.41 µg/g, respectively. When compared to other butanol fractions, there are the same compounds with different concentrations plus other higher compounds as follows: Gallic acid, chlorogenic acid, caffeic acid, syringic acid, ferulic acid, and rosmarinic acid (2497.01, 6426.69, 2419.04, 1371.62, 3633.03, and 3148.22 µg/g) (Table 1 and Figure 1), illustrating that the butanol fraction was richer in polyphenolic compounds than ethyl acetate.

4.4. HPLC-MS metabolites of EtOAc/BuOH extracts.

The compounds were identified and determined based on precursor ions, fragment ions, the appropriate reference compound, literature [14, 15, 43, 44], and databases at PeakView, MS-Dial, Metlin, and PubChem. The tentatively identified compounds are listed in Table 1, with the total ion chromatogram (TIC) shown in Figure 2. Phenolic acids and polyphenols were among the many phenolic compounds of EtOAc/BuOH; altogether, 36 compounds were identified and provided with basic descriptions, forming all of the total ion chromatography. Major compounds from 6 to 11 minutes include ^{HPLC}gallic acid, ^{HPLC}methyl gallate, astragalin, nicotiflorin, quercetin 3-O-β-glucoside, ^{HPLC}cinnamic acid, monardic acid A, shimobashiric acid C, quercetin 3-O-malonylglucoside, ^{HPLC}catechin, ^{HPLC}daidzein, kaempferol 3-O-(6-malonyl-glucoside), Yunnaneic acid E, ^{HPLC}Quercetin, Rosmarinic acid methyl ester, ^{HPLC}Naringenin, ^{HPLC}Ellagic acid, ^{HPLC}hesperetin, lithospermic acid B as indicated in table (3) and figure (2). These compounds are implicated in attenuating neuroinflammation, and oxidative stress, key features of lipopolysaccharide (LPS)-induced AD. Key metabolites such as rosmarinic acid, ferulic acid, caffeic acid, gallic acid, and quercetin derivatives were abundant across both extracts. These compounds have been reported to exhibit AChE inhibitory activity, thereby enhancing cholinergic neurotransmission an essential therapeutic target in AD. For example, rosmarinic acid and its methyl ester, along with ferulic acid, showed particularly high abundance, suggesting a strong role in modulating memory and cognitive functions via AChE inhibition. Moreover, several identified compounds such as syringic acid, vanillin, *p*-coumaric acid, and rutin are also known to modulate TLR4-mediated signaling pathways, which are closely linked to microglial activation and neuroinflammation. Through TLR4 inhibition, these compounds may reduce the pro-inflammatory cascade triggered by LPS, thus offering anti-inflammatory and neuroprotective effects. The differential distribution of metabolites between EtOAc and BuOH fractions reflects their varying polarity and pharmacological contributions. While EtOAc fraction was richer in lipophilic neuroprotectants such as monardic acid A, yunnaneic acids, and pulmitric acids, the BuOH fraction contained more hydrophilic compounds like quercetin glycosides and catechin, further enhancing the spectrum of neuroprotective mechanisms.

Table 3. LC/MS metabolites of EtOAc/BuOH extracts.

*Supercritical number means HPLC conc. ug/g EtOAc/BuOH extracts

Pharmacology section

4.5. Antioxidant activity

Plant fractions can be evaluated for their antioxidant activity using a method that has been approved: scavenging free radicals using DPPH. This method is widely applied to evaluate the antioxidant activity of plant extracts due to its rapid and straightforward analysis. Owing to its ability to donate hydrogen atoms, DPPH is considered a highly effective in vitro antioxidant. The removal of these free radicals is crucial for mitigating their harmful impact on various diseases, including Alzheimer’s disease (AD) [45]. The antioxidant activity analysis of PF, EF, and PF and WF of PO are displayed in Table 3. The outcomes showed that BF of PO demonstrated higher antioxidant activity (90.61±0.60), followed by EF 87.29±0.75 at 100 µg/mL. The antioxidant activity of PO fractions demonstrated that it is dose-dependent supplied (Table 4). In comparison to PO fractions, the standard (BHT) demonstrated significantly greater DPPH radical scavenging abilities. The lowest antioxidant activity (54.64±0.36 & 64.60 ± 0.45) was shown by PF and WF, respectively, at 100 µg/mL. This study also determined the concentrations of the plant fractions

No	RT (min)	Metabolites Identified	Molecular Formula	HPLC/MS				EtOAc Fraction (EF)	BuOH Fraction (BF)
				m/z [M-H] ⁻	Mass fragments	m/z [M-H] ⁺	Mass fragments		
1	3.83	Danshensu	C ₉ H ₁₀ O ₅	197	179/135/123	199	179/154/123		*
2	3.42	HPLC Ferulic acid	C ₁₀ H ₁₀ O ₄	193	108	195	108	*7737.06	*3633.03
3	3.55	HPLC Caffeic acid	C ₉ H ₈ O	179	161/135/107	181	135/121/105	*2888.07	*2419.04
4	3.92	HPLC Rosmarinic acid	C ₁₈ H ₁₆ O ₈	359	193/165/149/107	361	163	*1011.41	*3148.22
5	4.10	HPLC p-Coumaric acid	C ₉ H ₈ O ₃	163	115/107	165	119/103	*389.28	*243.60
6	5.10	HPLC Vanillin	C ₈ H ₈ O ₃	151	108	153	107	*261.36	*479.10
7	5.26	HPLC Syringic acid	C ₉ H ₁₀ O ₅	197	151	199	154	*626.70	*1371.62
8	6.67	HPLC Gallic acid	C ₇ H ₆ O ₅	169	141/115	171	125/107	*349.06	*2497.01
9	7.44	HPLC Methyl gallate	C ₈ H ₈ O ₅	183	167/115	185	167/121	*2132.90	*9.94
10	7.63	Astragalin	C ₂₁ H ₂₀ O ₁₁	447	284/255	-	-		*
11	7.64	Nicotiflorin	C ₂₇ H ₃₀ O ₁₅	593	284/255	-	-		*
12	7.70	Quercetin 3-O-β-glucoside	C ₂₁ H ₂₀ O ₁₂	463	300/271/255	-	-		*
13	7.82	HPLC Cinnamic acid	C ₉ H ₈ O ₂	147	147/105	149	121/115	*67.19	*3.38
14	8.15	Monardic acid A	C ₂₇ H ₂₂ O ₁₂	537	491/463/300/161	-	-		*
15	8.69	Shimobashiric acid C	C ₃₆ H ₃₂ O ₁₆	719	671/359/161	-	-		*
16	8.80	Quercetin 3-O-malonylglucoside	C ₂₄ H ₂₀ O ₁₅ ⁻²	547	503/311/191/149	-	-		*
17	9.02	HPLC Catechin	C ₁₅ H ₁₄ O ₆	289	181/165	291	161/107	-	*369.12
18	9.69	HPLC Daidzein	C ₁₅ H ₁₀ O ₄	253	189/121	-	-	*363.39	*147.53
19	9.77	Kaempferol 3-O-(6-malonyl-glucoside)	C ₂₄ H ₂₂ O ₁₄	533	401/284/137	-	-		*
20	9.80	Yunnaneic acid E	C ₂₇ H ₂₄ O ₁₄	571	525/437/257	573	437/303		*
21	9.82	HPLC Quercetin	C ₁₅ H ₁₀ O ₇	301	285/151/132	303	151	*49.13	*30.08
22	10.94	Rosmarinic acid methyl ester	C ₁₉ H ₁₈ O ₈	373	285/173/147/135	375	339/151/135	*	*
23	11.25	HPLC Naringenin	C ₁₅ H ₁₂ O ₅	271	153	273	133	*260.57	*536.92
24	11.52	HPLC Ellagic acid	C ₁₄ H ₆ O ₈	301	285	303	287	*29.53	*65.66
25	11.31	HPLC Hesperetin	C ₁₆ H ₁₄ O ₆	301	285/195	303	287/195	*372.22	*271.46

26	11.77	Lithospermic acid B	C ₃₆ H ₃₀ O ₁₆	717	519/321	-	-		*
27	13.84	Pulmonarioside A	C ₄₇ H ₅₂ O ₂₄	999	501/485	-	-		*
28	13.90	HPLC Chlorogenic acid	C ₁₆ H ₁₈ O ₉	353	309/191/150	355	201/151		*
29	15.57	HPLC Kaempferol	C ₁₅ H ₁₀ O ₆	285	151/107	-	-	*431.60	*302.49
30	15.71	Cryptochlorogenic acid	C ₁₆ H ₁₈ O ₉	353	309/191/173	355	151		*
31	16.34	Yunnaneic acid E-1	C ₂₆ H ₂₂ O ₁₁	509	284/135	511	301/139		*
32	20.21	Pulmitric acid B	C ₂₇ H ₂₀ O ₁₂	535	461/443/161	-	-	*	-
33	22.86	3-O-(E)-caffeoyl-glyceric acid	C ₁₂ H ₁₂ O ₇	267	179/161/135/108	-	-		*
34	22.91	HPLC Rutin	C ₂₇ H ₃₀ O ₁₆	609	300	611	303	*135.60	*410.38
35	23.47	Actinidioionoside	C ₁₉ H ₃₄ O ₉	405	387/151	407	151		*
36	28.30	Pulmitric acid A	C ₂₈ H ₂₄ O ₁₂	551	395/275/211/133	553	287		*

required to scavenge 50% of DPPH radicals (IC₅₀). The IC₅₀ values for the BF, EF, and WF, and fractions were 38.82±0.35 µg/mL, 43.79±0.66 µg/mL, 67.50±0.50 µg/mL, and 84.60±1.18 µg/mL, respectively. In comparison, the IC₅₀ value of the standard (BHT) was 35.69±0.84 µg/mL.

Table 4. DPPH and ABTS^{•+} activity of various fractions of PO.

Sample (µg/ml)	Conc.	DPPH ⁻		ABTS ⁺	
		% Conc.	IC ₅₀	% Conc.	IC ₅₀
BHT	12.5	36.31± 0.26	35.69± 0.84 ^a	41.93±0.31	22.93± 0.31 ^a
	25	48.63±0.36		52.88±0.10	
	50	61.68±0.62		63.75±0.16	
	100	93.53± 0.26		87.89±0.21	
PF	12.5	15.12± 0.62	84.60 ± 1.18 ^e	27.12±0.62	65.84±0.20 ^e
	25	27.23±0.61		35.40±0.30	
	50	39.43±0.50		44.79±0.34	
	100	54.64±0.36		62.63±0.30	
EF	12.5	28.12± 0.27	43.79± 0.66 ^c	38.49±0.37	29.64± 0.33 ^b
	25	39.92± 0.95		49.09±0.40	
	50	52.84± 0.26		61.37±0.42	
	100	87.29± 0.75		92.04 ±0.43	
BF	12.5	31.67±0.43	38.82±0.35 ^b	34.04±0.30	37.46± 0.38 ^c
	25	42.84±0.36		44.67±0.32	
	50	55.84±0.52		55.58±0.44	
	100	90.61±0.60		89.09±0.24	
WF	12.5	20.16±0.77	67.50± 0.50 ^d	41.93±0.31	57.15± 0.31 ^d
	25	30.36±0.69		52.88± 0.10	
	50	44.39±0.52		63.75± 0.16	
	100	64.60± 0.45		87.89± 0.21	

Note: Significant differences ($p < 0.05$) exist between values in the same column that are denoted by different superscript letters. Three separate experiments' worth of data are shown as mean ± SD.

Acute toxicity

Oral dosing of both fractions at 5 g/kg did not result in mortality within 24 hours. Since this dose level was not lethal, further determination of the LD₅₀ value was deemed unnecessary [46]. Consequently, the selected experimental doses were set at 1/20 of 5 g/kg for each fraction, which corresponds to 250 mg/kg.

4.6. Effects of PO on Behavioral Activity, AChE Levels, and beta Amyloid.

LPS-induced AD is characterized by memory impairment [47] accompanied by elevated AChE release [48, 49]. In our study, LPS administration reduced Y-maze alternation performance by 55% compared with the normal control group. Treatment with the BuOH and EtOAc fractions of *PO* improved behavioral activity by 77% and 54%, respectively, relative to the LPS group. Moreover, LPS injection led to a 9-fold increase in AChE levels and a 3-fold rise in amyloid beta in the brain compared with normal mice.

Treatment with BuOH and EtOAc fractions, however, decreased AChE by 73% and 72% and reduced amyloid beta by 37% and 56%, respectively, compared with LPS-treated mice (Figure 3).

Polyphenols such as ferulic acid and rosmarinic acid are well-documented inhibitors of β -secretase (BACE1), the key enzyme involved in A β generation, and may therefore contribute to lowering A β production [50]. Additionally, their anti-inflammatory and antioxidant properties likely reduce oxidative stress and neuro-inflammation, which are known to accelerate A β aggregation and deposition [51]. Taken together, these findings highlight that PO extracts not only enhance cholinergic neurotransmission but also mitigate amyloid pathology, offering a multifaceted neuroprotective strategy against AD.

Figure 3: Effects of PO on Behavioral Activity, AChE, and beta amyloid

Data are presented as mean $\pm$ SD. Statistical comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. Values sharing the same letter are not significantly different, whereas different letters indicate significance at $p < 0.05$.

4.7. Effects of EtOAc/BuOH PO on TLR4 and IL-1 β

LPS activates glial cells through TLR4, leading to the production of pro-inflammatory cytokines such as IL-1 β [52]. In the present study, LPS administration increased TLR4 expression by 2.6-fold compared with normal mice, whereas PO butanol and ethyl acetate fractions reduced it by 63% and 52%, respectively, relative to the LPS group. Similarly, LPS elevated IL-1 β levels by 2.7-fold compared to normal mice; however, treatment with the butanol and ethyl acetate fractions decreased IL-1 β by 64% and 53%, respectively, compared with LPS-treated mice (Figure 4). This result suggests that CXB-BLs reduced gliosis and proinflammatory cytokine release mediated by LPS via inhibiting IL-1 β production in the brain. These effects may be due to its content as ferulic acid, chlorogenic acid and rosmarinic acid. Ferulic acid treat AD as it reduced decreased IL-1 β in mouse brain cortex [53]. Moreover, chlorogenic acid has neuroprotective effects through inhibition of neuroinflammation, suppression of neuronal apoptosis [54], and reduction of TLR4 in LPS-treated RAW264.7 hepatic cells [55]. Furthermore, rosmarinic acid reduced TLR4, NF- κ B, and NLRP3 inflammasome activation [56]. Clinical studies used coffee extract (Chlorogenic acid, the main component) and considered it as good therapeutic agent in AD and Parkinson's disease [57]. To date, *in vivo* experiments exploring the anti-neurodegenerative activity of *P. officinalis* are still lacking [15]. Therefore, further animal studies are warranted to investigate its antioxidant, anticonvulsant, anticoagulant, and wound-healing potentials.

Figure 4: Effects of PO on TLR4 and IL-1 β

Data are expressed as mean $\pm$ SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison tests. Values with different letters denote significant differences at $p < 0.05$, whereas identical letters indicate no significant difference.

4.8. Histopathological Observations.

Histological examination revealed no abnormalities in the control group, with normal cerebral cortex, hippocampal regions (subiculum, fascia dentata, hilus), and striatum (Figure 5a-d). In contrast, LPS-treated rats exhibited pronounced pathology, including extensive neuronal nuclear pyknosis and degeneration in the cerebral cortex and hippocampus, as well as nuclear pyknosis, neuronal degeneration, and diffuse gliosis in the striatum (Figure 5e-h). BuOH treatment partially mitigated these changes; although most cortical and striatal neurons showed nuclear pyknosis and degeneration with glial proliferation, some neurons remained intact. The hippocampal subiculum appeared normal, while the fascia dentata and hilus exhibited degenerative alterations (Figure 5i-l). Similarly, the EtOAc fraction preserved some cortical neurons but induced nuclear pyknosis and degeneration in the majority, with degenerative changes in the fascia dentata and hilus, a normal subiculum, and striatal pathology characterized by neuronal damage and glial proliferation (Figure 5m-p).

Representative histological sections of the cerebral cortex, hippocampus, and striatum: (a-d) normal control; (e-h) LPS group; (i-l) BuOH fraction; (m-p) EtOAc fraction.

Discussion

The phytochemical screening of PO revealed the presence of flavonoids, tannins, glycosides, alkaloids, triterpenoids, steroids, and saponins, whereas volatile oils were absent. These findings align with Chauhan et al. [15], who also reported saponins, tannins, alkaloids, and fatty acids among the major constituents. Such metabolites are well documented for their antioxidant, anti-inflammatory, and neuroprotective potential, thereby forming the chemical basis for the observed biological effects. This research corresponds with the investigation conducted by Ivanova et al., [58]. The content of total phenolic, tannin, and flavonoid was more in BF and EF as compared to other fractions of PO. The results showed that when it came to the extraction of phenols, tannins, and flavonoids, BF and EF were more significant than the other two fractions. Similar outcomes were observed by Shahzad et al., [59] and Jing et al., [60]. Phenolics have been demonstrated in numerous research studies to have anti-Alzheimer [61], hypolipidemic functions [62], anti-tumor [63], and hypoglycemic [64] functions, and they possess potent antioxidant properties as well, which could act as the pharmacological basis for further biological effects [65].

Polyphenols are also crucial in regulating amyloid processing. Several studies have shown that these compounds influence A β production either by stimulating α -secretase activity or suppressing BACE activity. Compounds such as methyl gallate, astragaloside, nicotiflorin, quercetin 3-O- β -glucoside, cinnamic acid, catechin, daidzein, kaempferol 3-O-(6-malonyl-glucoside), and epigallocatechin have been identified as potent inhibitors of amyloidogenic processing. *In vitro* experiments using neuronal cell lines expressing human Amyloid precursor protein (APP) revealed that epigallocatechin treatment markedly decreased A β production [66]. These results were corroborated by *in vivo* studies, where intraperitoneal administration of PO BuOH and EtOAc fractions in an Alzheimer's disease model lowered A β levels and promoted the non-amyloidogenic pathway via α -secretase, with polyphenolic compounds detected in both fractions. Furthermore, ursolic acid and rosmarinic acid enhanced α -secretase activity [67]. Both BuOH and EtOAc fractions suppressed BACE activity, with BuOH completely abolishing it *in vivo*. Co-administration of BuOH with ferulic acid, a known BACE regulator, potentiated the effect of epigallocatechin-3-gallate, further reducing BACE activity, limiting amyloidosis, and improving cognitive function. Flavones such as apigenin and nobiletin significantly reduced both soluble and insoluble A β species, as well as A β plaque deposition in AD models [67]. Chronic baicalein administration also lowered A β production, while quercetin, a widely distributed dietary flavonol, was shown to modulate soluble and insoluble A β levels in the brain. Collectively, these findings suggest that specific polyphenols regulate α -secretase and BACE activity, thereby attenuating A β production in both *in vitro* and *in vivo* models. Nonetheless, further investigations are warranted to clarify the precise mechanisms through which distinct polyphenols influence amyloidogenic and non-amyloidogenic APP processing [68].

Concerning the results in Table 4, the ABTS radical scavenging of the EF of PO has the highest ABTS radical scavenging activity at all the investigated concentrations; it records $92.04 \pm 0.43 \mu\text{g/mL}$ followed by BF 89.09 ± 0.24 at $100 \mu\text{g/mL}$. In comparison to PO fractions, the standard (Trolox) demonstrated significantly greater ABTS radical scavenging abilities. On the other hand, the PF and WF were noticed to have the lowest ABTS radical scavenging activity 62.63 ± 0.30 & 65.89 ± 0.32 , respectively. The results showed that the EF of PO had the highest with an IC_{50} of $29.64 \pm 0.33 \mu\text{g/mL}$; conversely, the PF had the lowest capacity, with an IC_{50} $65.84 \pm 0.20 \mu\text{g/mL}$. In contrast, the standard (Trolox) has an IC_{50} value of $22.93 \pm 0.31 \mu\text{g/mL}$. The antioxidant activity of the PO fractions may be attributed to phenols, tannins, and flavonoids, respectively (Tables 2 and 3). The phytochemical content of lungwort has antioxidant properties that improve cellular health overall and fight oxidative stress. Significant variations in the amounts of phenolics, tannins and flavonoids present in the various fractions of PO could account for the differences in their efficacy as antioxidants. Additionally, the extract's ability to scavenge free radicals is influenced by the solvent used during extraction. Our results are supported by Neagu et al. [69], who evaluated the antioxidant potential of ethanolic and aqueous extracts from PO by DPPH radical scavenging activity. Ivanova et al. [58] showed that PO had a high polyphenol content and stronger antioxidant activity. However, Malinowska et al., [10] demonstrated that a noteworthy antioxidant activity value was discovered during the preliminary screening of PO extracts used in the cosmetics industry that could be associated with high content of the flavonoids. Additionally, in a previous study on a number of Bulgarian medicinal plants, the antioxidant capacity of a leaf extract obtained from PO was investigated by Ivanova et al [36]. Moreover, Dyshlyuk et al. [70] found that the root culture extract of PO exhibited a rich output of flavonoids, proanthocyanidins, and other polyphenolic components.

These findings suggest that PO enhances memory and provides neuroprotective effects against AD through its AChE inhibitory activity. This effect may be attributed to its content of ferulic acid, chlorogenic acid, caffeic acid, methyl gallate, and rosmarinic acid. Notably, chlorogenic acid has demonstrated a beneficial effect on cognitive impairment by decreasing AChE activity in mouse brains [71]. In addition, ferulic acid blocked AChE activity and reduced amyloid beta plaque formation [72], and rosmarinic

acid alleviated cognition defects and promoted neurogenesis [73]. Previous research examined the inhibitory effect of POethanolic extracts' potential on AChE and reported that a higher concentration of polyphenol in the ethanolic extract may be the reason for this higher activity *in vitro* study [69]. Another study exhibited the greater ability of the phenolic compound hydroxyl groups to bind AChE, inhibiting it [74]. Amyloid beta accumulation is a hallmark of AD pathology and is strongly associated with synaptic loss, neurotoxicity, and impaired cognitive function [75]. In our study, LPS exposure significantly elevated A β levels, supporting evidence that systemic inflammation exacerbates amyloidogenic pathways [76]. Remarkably, treatment with POfractions attenuated A β burden in the brain, suggesting a dual mechanism of action both by reducing AChE activity and by interfering with amyloidogenic processes.

Overall, the results of this study reinforce the pharmacological potential of POas a multitarget agent in Alzheimer's disease. Its bioactive fractions combine antioxidant, anti-amyloidogenic, and AChE-inhibitory activities, thereby addressing multiple pathogenic mechanisms simultaneously. Future studies should aim to isolate and characterize the key active compounds, validate their mechanisms of action, and evaluate their efficacy in clinical settings. This positions POas a promising candidate for further pharmacological development in the prevention and treatment of neurodegenerative diseases.

Conclusion

The BF and EF of *PO* contain major metabolites, including catechin, methyl gallate, astragalin, nicotine, quercetin 3-O- β -glucoside, cinnamic acid, daidzein, kaempferol 3-O-(6-malonyl-glucoside), and epigallocatechin. These bioactive compounds improved memory by suppressing AChE, amyloid- β , and TLR4 activity, while inhibiting the proinflammatory cytokine IL-1 β . Collectively, *PO* demonstrated neuroprotective effects against AD, highlighting its potential as a therapeutic or supplemental candidate for clinical application in brain disorders. However, this study was limited by its reliance on an animal model and the absence of pharmacokinetic and long-term safety evaluations, which should be addressed in future investigations.

Abbreviations

ABTS

2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid

AChE

Acetylcholinesterase

AD

Alzheimer disease.

APP

Amyloid precursor protein

BACE

The enzymatic activities of β -secretase

BF

n-butanol fraction

BHT

Butylated Hydroxytoluene

BuOH

n-Butanol

DPPH

2,2-diphenylpicrylhydrazyl

EF

Ethyl acetate fraction

ESI

Electrospray ionization

EtOAc

Ethyl acetate

HPLC

Performance liquid chromatography

HPLC-MS

Performance liquid chromatography-mass spectrometry

IL-1 β

Interleukin 1 beta

MREC

Medical Research Ethics Committee

NRC

National Research centre

PBS

phosphate buffered saline

PF

petroleum ether fraction

PO

Pulmonaria officinalis

TLR 4

Toll-like receptor 4

TLRs

Toll-like receptors

WF

water fraction

Declarations

Ethical approval:

All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86 – 23, revised 1985, National Academy of Sciences). The study was approved by the IAEC and complied with the National Regulations on Animal Welfare. Ethical clearance was also obtained from the NRC Ethics Committee (Approval No. 2317/1022021).

Clinical trial number

not applicable.

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Author Contribution

Abeer Salama: Conceptualization, designing, performing pharmacological study including the neuroprotective role, and efficacy studies of the extracts, biochemical analysis, statistical analysis, writing, review and editing. 'Mona A. Mohammed: Conceptualization and Designed the plan of the plant material, phytochemical study, and were responsible for performing the

extraction of plant extract as well as identification and characterization part in vitro studies, they shared in writing and revising the phytochemical section and all manuscripts. Rasha M. M. Mohasib: Design and execution of in vitro studies, statistical analyses, manuscript writing, review, and editing. All images included in the submission were created by all authors

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Data Availability

The datasets generated during and/or analyzed in this study are available from the corresponding author upon reasonable request.

References

1. Bennett, D.A., et al., *Epigenomics of Alzheimer's disease*. Translating Epigenetics to the Clinic, 2017: p. 227–278.
2. Breijyeh, Z. and R. Karaman, *Comprehensive review on Alzheimer's disease: causes and treatment*. Molecules, 2020. 25(24): p. 5789.
3. Hampel, H., et al., *Revolution of Alzheimer precision neurology. Passageway of systems biology and neurophysiology*. Journal of Alzheimer's Disease, 2018. 64(s1): p. S47-S105.
4. Hegazi, N.M., et al., *Molecular networking-guided investigation of the secondary metabolome of four Morus species and their in vivo neuroprotective potential for the mitigation of Alzheimer's disease*. Food & Function, 2024. 15(8): p. 4354–4364.
5. Revi, M., *Alzheimer's disease therapeutic approaches*. GeNeDis 2018: Genetics and Neurodegeneration, 2020: p. 105–116.
6. Elmaleh, D.R., et al., *Developing effective Alzheimer's disease therapies: clinical experience and future directions*. Journal of Alzheimer's Disease, 2019. 71(3): p. 715–732.
7. Adetuyi, A.R., et al., *Antioxidant and acetylcholinesterase inhibitory activities, in silico analyses, and anti-Alzheimer's disease potential of leaf extracts of three Nigerian endemic medicinal plants (Spondias mombin, Carica pa paya and Kalanchoe crenata)*. Future Journal of Pharmaceutical Sciences, 2024. 10(1): p. 6.
8. Deng, M., et al., *Anti-neuroinflammatory potential of natural products in the treatment of Alzheimer's disease*. Molecules, 2023. 28(3): p. 1486.
9. Yilmaz, G., et al., *Phytochemical analysis, antioxidant, and enzyme inhibition activity of five Salvia taxa from Turkey*. South African Journal of Botany, 2023. 152: p. 212–221.
10. Malinowska, P., *Effect of flavonoids content on antioxidant activity of commercial cosmetic plant extracts*. Herba Polonica, 2013. 59(3): p. 63–75.
11. Meeus, S., et al., *Evolutionary trends in the distylous genus Pulmonaria (Boraginaceae): evidence of ancient hybridization and current interspecific gene flow*. Molecular Phylogenetics and Evolution, 2016. 98: p. 63–73.
12. Bennett, B.C., *Doctrine of signatures: an explanation of medicinal plant discovery or dissemination of knowledge?* Economic Botany, 2007. 61(3): p. 246–255.
13. Leporatti, M.L. and S. Ivancheva, *Preliminary comparative analysis of medicinal plants used in the traditional medicine of Bulgaria and Italy*. Journal of ethnopharmacology, 2003. 87(2–3): p. 123–142.
14. Krzyżanowska-Kowalczyk, J., et al., *Pulmonaria obscura and pulmonaria officinalis extracts as mitigators of peroxynitrite-induced oxidative stress and cyclooxygenase-2 inhibitors—in vitro and in silico studies*. Molecules, 2021. 26(3): p. 631.
15. Chauhan, S., et al., *Biological Activities and Phytochemicals of Lungworts (Genus Pulmonaria) Focusing on Pulmonaria officinalis*. Applied Sciences, 2022. 12(13): p. 6678.
16. Pecková, R., et al., *Effect of selected Indonesian plants on Giardia intestinalis in an experimental in vitro model*. BMC Complementary Medicine and Therapies, 2025. 25(1): p. 1–8.

17. Shah, N.A., et al., *HPLC-DAD analysis, antioxidant potential and anti-urease activity of Asparagus gracilis collected from District Islamabad*. BMC complementary and alternative medicine, 2014. 14: p. 1–12.
18. Mueller-Harvey, I., *Analysis of hydrolysable tannins*. Animal feed science and technology, 2001. 91(1–2): p. 3–20.
19. Trease, G. and W. Evans, *Pharmacognosy (13th edn)*. Bailliere Tindall, London, 1989: p. 176–180.
20. Harborne, J. and J. Harborne, *The terpenoids*. Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis, 1973: p. 89–131.
21. Lewis, D. and D. Smith, *SUGAR ALCOHOLS (POLYOLS) IN FUNGI AND GREEN PLANTS: II. METHODS OF DETECTION AND QUANTITATIVE ESTIMATION IN PLANT EXTRACTS*. New Phytologist, 1967. 66(2): p. 185–204.
22. DuBois, M., et al., *Colorimetric method for determination of sugars and related substances*. Analytical chemistry, 1956. 28(3): p. 350–356.
23. Kumbhare, M., V. Guleha, and T. Sivakumar, *Estimation of total phenolic content, cytotoxicity and in-vitro antioxidant activity of stem bark of Moringa oleifera*. Asian Pacific Journal of Tropical Disease, 2012. 2(2): p. 144–150.
24. Markham, K.R., *Isolation techniques for flavonoids*, in *The flavonoids*. 1975, Springer. p. 1–44.
25. Tempel, A.S., *Tannin-measuring techniques: A review*. Journal of chemical ecology, 1982. 8: p. 1289–1298.
26. Hassan, E.M., et al., *ANTIPROLIFERATIVE EFFECT AND CHEMICAL CONSTITUENTS OF ANNONA SPECIES*. Plant Archives, 2020. 20(1): p. 2650–2657.
27. Mohammed, M.A., et al., *Pharmacological and metabolomic profiles of Musa acuminata wastes as a new potential source of anti-ulcerative colitis agents*. Scientific Reports, 2022. 12(1): p. 10595.
28. Mohammed, M.A., et al., *Comprehensive metabolomic, lipidomic and pathological profiles of baobab (Adansonia digitata) fruit pulp extracts in diabetic rats*. Journal of Pharmaceutical and Biomedical Analysis, 2021. 201: p. 114139.
29. Mohammed, M.A., et al., *Comprehensive Tools of Alkaloid/Volatile Compounds–Metabolomics and DNA Profiles: Bioassay-Role-Guided Differentiation Process of Six Annona sp. Grown in Egypt as Anticancer Therapy*. Pharmaceuticals, 2024. 17(1): p. 103.
30. Blois, M.S., *Antioxidant determinations by the use of a stable free radical*. Nature, 1958. 181(4617): p. 1199–1200.
31. Re, R., et al., *Antioxidant activity applying an improved ABTS radical cation decolorization assay*. Free radical biology and medicine, 1999. 26(9–10): p. 1231–1237.
32. El Kady, W.M., et al., *Comparative DNA profiling, botanical identification and biological evaluation of Gazania longiscapa DC and Gazania rigens L*. Bulletin of Faculty of Pharmacy, Cairo University, 2015. 53(2): p. 129–145.
33. Hidaka, N., et al., *Inhibitory effects of valproate on impairment of Y-maze alternation behavior induced by repeated electroconvulsive seizures and c-Fos protein levels in rat brains*. Acta Medica Okayama, 2011. 65(4): p. 269–277.
34. Miwa, M., et al., *Effects of betaine on lipopolysaccharide-induced memory impairment in mice and the involvement of GABA transporter 2*. Journal of Neuroinflammation, 2011. 8(1): p. 1–13.
35. Salama, A.A., N.N. Yassen, and H.M. Mansour, *Naringin protects mice from D-galactose-induced lung aging and mitochondrial dysfunction: Implication of SIRT1 pathways*. Life Sciences, 2023. 324: p. 121471.
36. Salama, A., R. Hegazy, and A. Hassan, *Intranasal chromium induces acute brain and lung injuries in rats: assessment of different potential hazardous effects of environmental and occupational exposure to chromium and introduction of a novel pharmacological and toxicological animal model*. PloS one, 2016. 11(12): p. e0168688.
37. Bancroft, J.D., C. Layton, and S.K. Suvarna, *Bancroft's theory and practice of histological techniques*. 2013: Churchill Livingstone Elsevier.
38. Silva, A.P.S.A.d., et al., *Antimicrobial activity and phytochemical analysis of organic extracts from cleome spinosa Jacq.* Frontiers in microbiology, 2016. 7: p. 963.
39. Bukhari, S.N.A., *Dietary polyphenols as therapeutic intervention for Alzheimer's disease: a mechanistic insight*. Antioxidants, 2022. 11(3): p. 554.
40. Nugraha, S.E., S. Achmad, and E. Sitompul, *Antibacterial activity of ethyl acetate fraction of passion fruit peel (Passiflora Edulis Sims) on Staphylococcus aureus and Escherichia coli*. Indonesian Journal of Pharmaceutical and Clinical Research, 2019. 2(1): p. 07–12.

41. Owuama, C.I., *Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using a novel dilution tube method*. African journal of microbiology research, 2017. 11(23): p. 977–980.
42. Naz, R., et al., *GC-MS analysis, antimicrobial, antioxidant, antilipoxygenase and cytotoxic activities of Jacaranda mimosifolia methanol leaf extracts and fractions*. PLoS One, 2020. 15(7): p. e0236319.
43. Tawfeeq, A.A., et al., *Isolation, quantification, and identification of rosmarinic acid, gas chromatography-mass spectrometry analysis of essential oil, cytotoxic effect, and antimicrobial investigation of rosmarinus officinalis leaves*. Asian J Pharm Clin Res, 2018. 11(6): p. 126–32.
44. Krzyżanowska-Kowalczyk, J., et al., *Novel phenolic constituents of Pulmonaria officinalis L. LC-MS/MS comparison of spring and autumn metabolite profiles*. Molecules, 2018. 23(9): p. 2277.
45. Singh, S., G. Gupta, and K.R. Aran, *Emerging role of antioxidants in Alzheimer's disease: Insight to physiological, pathological mechanisms and management*. Pharmaceutical Science Advances, 2023: p. 100021.
46. Semler, D., *The rat toxicology in animal models in toxicology*. Marcel Dekke, New York, Basal, Hong Kong, 1992. 39.
47. Hegazi, N.M., et al., *Molecular networking-guided investigation of the secondary metabolome of four Morus species and their in vivo neuroprotective potential for the mitigation of Alzheimer's disease*. Food & Function, 2024.
48. Abbas, H.A., et al., *Novel Neuroprotective Potential of Bunchosia armeniaca (Cav.) DC against Lipopolysaccharide Induced Alzheimer's Disease in Mice*. Plants, 2022. 11(14): p. 1792.
49. Salama, A., R.M. Mohasib, and E.S. Shalaby, *Neuroprotective role of Origanum majorana essential oil loaded in mixed surfactants based nano emulsion against lipopolysaccharide-induced Alzheimer in mice*. Journal of Pharmaceutical Investigation, 2025: p. 1–17.
50. Saidi, A., et al., *Phytochemical analysis using cLC-DAD, nutritional importance and assessment of antioxidant, antidiabetic and anticholinesterase activities of Ruta tuberculata Forssk organic extracts*. Sustainability, 2022. 14(16): p. 10451.
51. Sharifi-Rad, J., et al., *Multi-target mechanisms of phytochemicals in Alzheimer's disease: Effects on oxidative stress, neuroinflammation and protein aggregation*. Journal of Personalized Medicine, 2022. 12(9): p. 1515.
52. Elhabak, M., A.A. Salama, and A.H. Salama, *Nose-to-brain delivery of galantamine loaded nanospray dried polyacrylic acid/taurodeoxycholate mixed matrix as a protective therapy in lipopolysaccharide-induced Alzheimer's in mice model*. International Journal of Pharmaceutics, 2023. 632: p. 122588.
53. Yan, J.-J., et al., *Protective effects of ferulic acid in amyloid precursor protein plus presenilin-1 transgenic mouse model of Alzheimer disease*. Biological and Pharmaceutical Bulletin, 2013. 36(1): p. 140–143.
54. Yao, J., et al., *Reversing ROS-mediated neurotoxicity by chlorogenic acid involves its direct antioxidant activity and activation of Nrf2-ARE signaling pathway*. BioFactors, 2019. 45(4): p. 616–626.
55. Xu, Y., et al., *Protective effects of chlorogenic acid on acute hepatotoxicity induced by lipopolysaccharide in mice*. Inflammation Research, 2010. 59: p. 871–877.
56. Wei, Y., et al., *Rosmarinic acid mitigates lipopolysaccharide-induced neuroinflammatory responses through the inhibition of TLR4 and CD14 expression and NF-κB and NLRP3 inflammasome activation*. Inflammation, 2018. 41: p. 732–740.
57. Ascherio, A., et al., *Caffeine, postmenopausal estrogen, and risk of Parkinson's disease*. Neurology, 2003. 60(5): p. 790–795.
58. Ivanova, D., et al., *Polyphenols and antioxidant capacity of Bulgarian medicinal plants*. Journal of ethnopharmacology, 2005. 96(1–2): p. 145–150.
59. Shahzad, M.N., et al., *Profiling of phytochemicals from aerial parts of Terminalia neotaliala using LC-ESI-MS2 and determination of antioxidant and enzyme inhibition activities*. PloS one, 2022. 17(3): p. e0266094.
60. Jing, L., et al., *Antioxidant potential, total phenolic and total flavonoid contents of Rhododendron anthopogonoides and its protective effect on hypoxia-induced injury in PC12 cells*. BMC complementary and alternative medicine, 2015. 15: p. 1–12.
61. Custódio, L., et al., *Phenolic composition, antioxidant potential and in vitro inhibitory activity of leaves and acorns of Quercus suber on key enzymes relevant for hyperglycemia and Alzheimer's disease*. Industrial Crops and Products, 2015. 64: p. 45–51.
62. Lee, L.-S., et al., *Hypolipidemic and antioxidant properties of phenolic compound-rich extracts from white ginseng (Panax ginseng) in cholesterol-fed rabbits*. Molecules, 2013. 18(10): p. 12548–12560.

63. Mendes, V., V. Costa, and N. Mateus, *Involvement of the modulation of cancer cell redox status in the anti-tumoral effect of phenolic compounds*. Rsc Advances, 2015. 5(1): p. 1–9.
64. Zhang, M., et al., *In vivo hypoglycemic effects of phenolics from the root bark of Morus alba*. Fitoterapia, 2009. 80(8): p. 475–477.
65. Soobrattee, M.A., et al., *Phenolics as potential antioxidant therapeutic agents: mechanism and actions*. Mutation Research/Fundamental and Molecular mechanisms of mutagenesis, 2005. 579(1–2): p. 200–213.
66. Cox, C.J., et al., *Dietary (–)-epicatechin as a potent inhibitor of $\beta\gamma$ -secretase amyloid precursor protein processing*. Neurobiology of aging, 2015. 36(1): p. 178–187.
67. Martínez-Coria, H., et al., *Anti-inflammatory effects of flavonoids in common neurological disorders associated with aging*. International journal of molecular sciences, 2023. 24(5): p. 4297.
68. Hole, K.L. and R.J. Williams, *Flavonoids as an Intervention for Alzheimer's Disease: Progress and Hurdles Towards Defining a Mechanism of Action 1*. Brain Plasticity, 2020. 6(2): p. 167–192.
69. Neagu, E., et al., *Antioxidant activity, acetylcholinesterase and tyrosinase inhibitory potential of Pulmonaria officinalis and Centarium umbellatum extracts*. Saudi journal of biological sciences, 2018. 25(3): p. 578–585.
70. Dyshlyuk, L.S., et al., *Optimization of extraction of polyphenolic compounds from medicinal lungwort (Pulmonaria officinalis L.)*. Journal of Pharmaceutical Research International, 2020. 32(24): p. 36–45.
71. Oboh, G., et al., *Comparative study on the inhibitory effect of caffeic and chlorogenic acids on key enzymes linked to Alzheimer's disease and some pro-oxidant induced oxidative stress in rats' brain-in vitro*. Neurochemical research, 2013. 38: p. 413–419.
72. Mugundhan, V., et al., *Protective Effect of Ferulic Acid on Acetylcholinesterase and Amyloid Beta Peptide Plaque Formation in Alzheimer's Disease: An In Vitro Study*. Cureus, 2024. 16(2).
73. Mirza, F. and S. Amber, Sumera; Hassan, D.; Ahmed, T.; Zahid, S. *Rosmarinic acid and ursolic acid alleviate deficits in cognition, synaptic regulation and adult hippocampal neurogenesis in an A β 1-42-induced mouse model of Alzheimer's disease*. Phytomedicine, 2021. 83: p. 153490.
74. Balkis, A., et al., *Screening flavonoids for inhibition of acetylcholinesterase identified baicalein as the most potent inhibitor*. Journal of Agricultural Science, 2015. 7(9): p. 26.
75. Sehar, U., et al., *Amyloid beta in aging and Alzheimer's disease*. International journal of molecular sciences, 2022. 23(21): p. 12924.
76. Wang, L.-M., et al., *Lipopolysaccharide endotoxemia induces amyloid- β and p-tau formation in the rat brain*. American journal of nuclear medicine and molecular imaging, 2018. 8(2): p. 86.

Figures

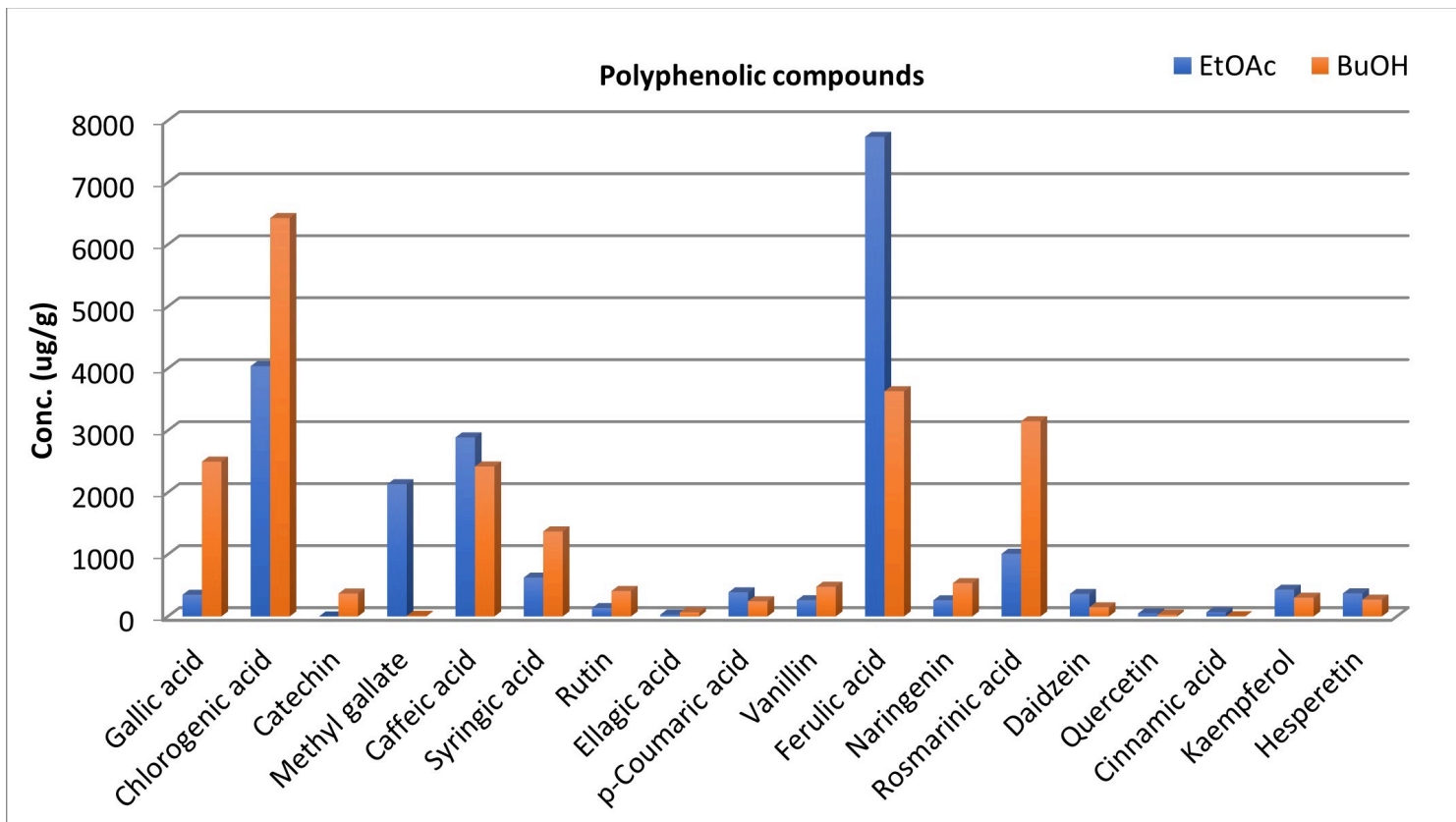


Figure 1

HPLC EtOAc/BuOH Lungworts extracts of polyphenolic metabolites.

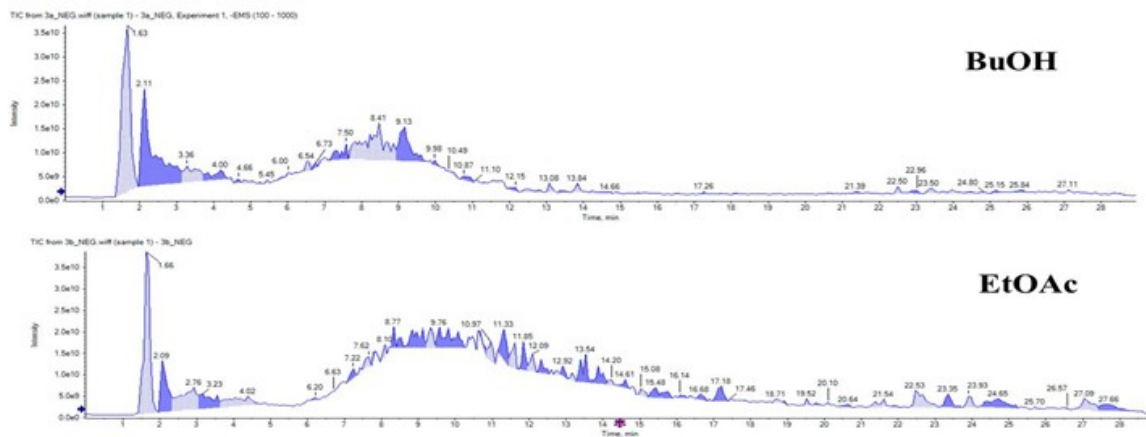


Figure 2

Total A) List of major structure metabolites and B) Ion Chromatogram of EtOAc/BuOH Lungworts extracts

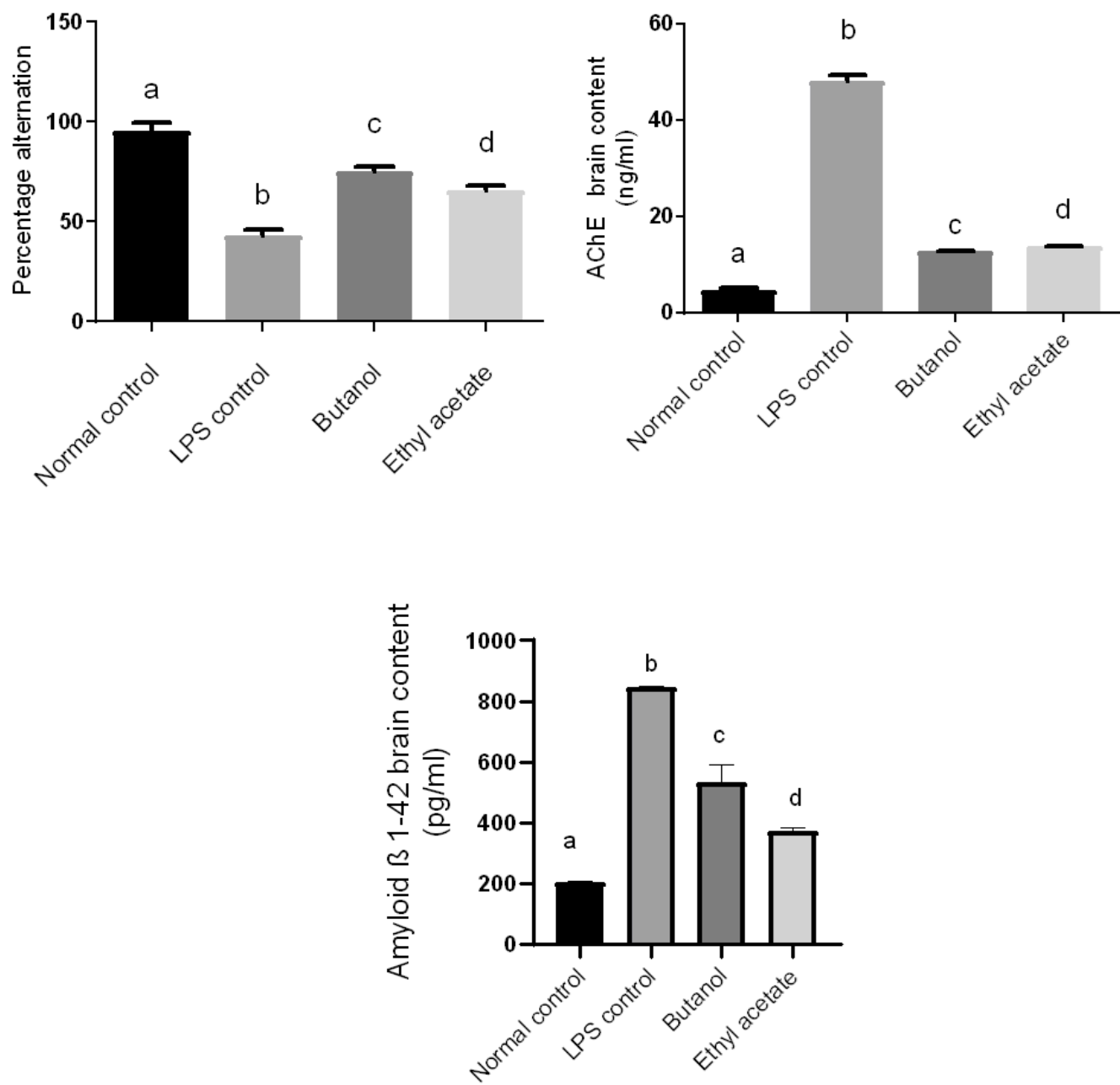


Figure 3

Effects of PO on Behavioral Activity, AChE, and beta amyloid

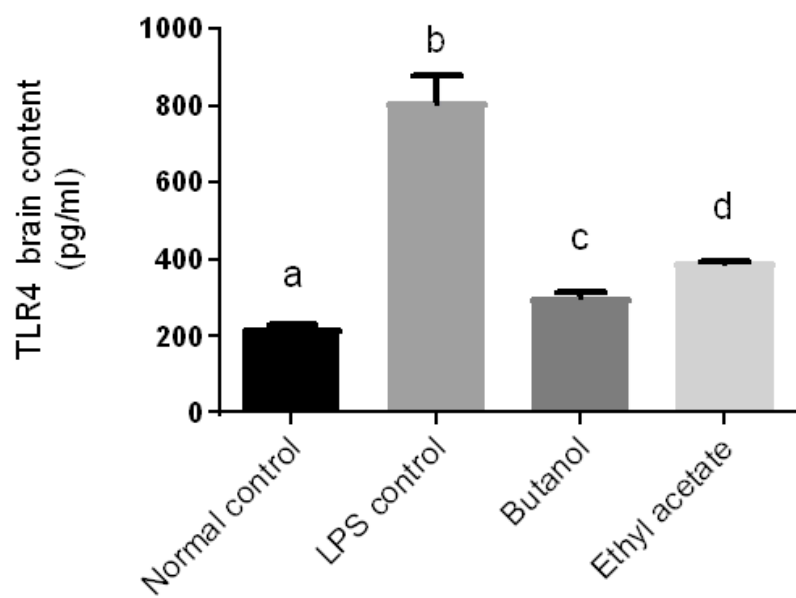


Figure 4

Effects of *PO* on TLR4 and IL-1 β

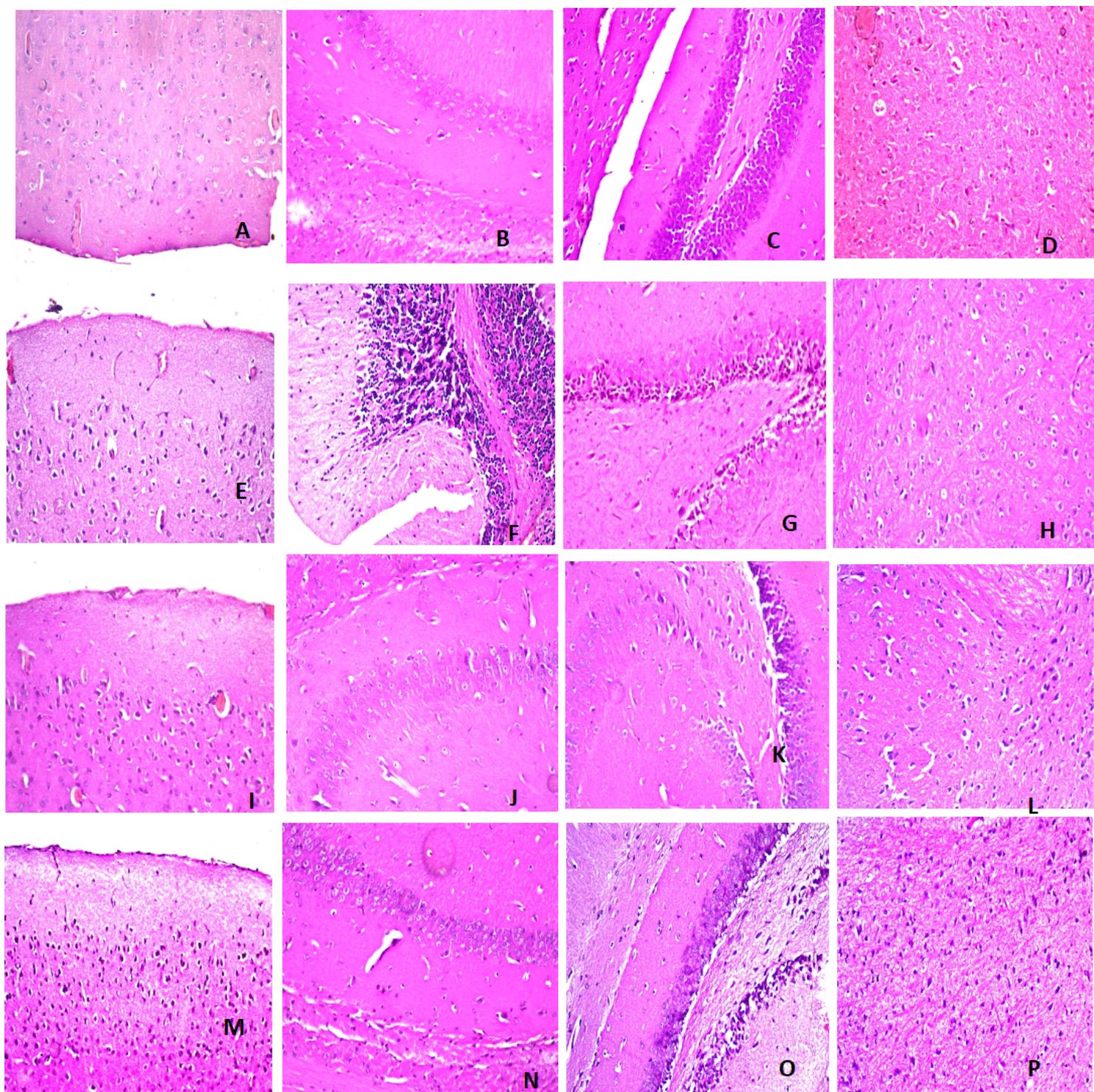


Figure 5

Effects of TP and TL on brain histopathology.