Green Chemistry-Based Investigation of Passiflora Extracts: Antioxidant and Anticholinesterase Activities Targeting Alzheimer's Disease Mechanisms

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In this study, it is aimed to determine some biological activities of the plant Passiflora incarnata L., which is primarily utilized for its sedative effects. In order to, the dry plant material obtained from the herba into a powder using a grinder, and extracts were prepared using ethanol, water, and ethanol-water (50:50; v/v). The total phenolic and flavonoid contents of these extracts were determined using pyrocatechol and quercetin as standards, respectively. The enzyme inhibitions of acetylcholinesterase and butyrylcholinesterase for the three different extracts obtained were investigated in vitro using the Ellman method to determine their activities. Notably, this study represents the first time that water bath extraction at 60°C has been applied to P. incarnata with these three solvents, combining total phenolic and flavonoid analysis with an environmental sustainability evaluation. The sustainability of the extraction methods was assessed using the AGREE: Analytical Greenness Calculator, which allowed us to evaluate their environmental impact. This unique combination of biological and green chemistry analyses emphasizes both the therapeutic potential and the environmental sustainability of P. incarnata.

Keywords: passiflora, anticholinesterase activity, LC-HR/MS, phenolic, flavonoid content

Introduction

Nervous system diseases are one of the diseases that seriously affect the health of the body. The World Health Organization (WHO) estimates that more than one billion people worldwide are affected by central and peripheral nervous system (CNS and PNS) disorders. These diseases include such as Parkinson's disease, epilepsy, schizophrenia, Alzheimer's disease and other dementias, neuroinfections, brain tumors, traumatic disorders, as well as cerebrovascular diseases like stroke and migraine [1]. Alzheimer's disease (AD) is a neurodegenerative disease and the most prevalent of dementia. It is characterized by severe memory loss and cognitive dysfunction [2]. AD is a neurodegenerative disease that leads to cognitive decline and neuronal damage, primarily affecting the elderly [3]. By 2030, it is estimated that more than 65.7 million people worldwide will have AD. This irreversible, progressive brain disease is associated with aging and is common among individuals aged 65 and older. People in the later stages of the disease are unable to perform cognitive functions, therefore they require constant care, and ultimately, AD is fatal [2]. From a clinical perspective, it is characterized by an insidious onset and a progressive deterioration of cognitive ability [4]. Aging, genetic predisposition, brain injury, infections, cardiovascular diseases, lifestyle diseases (obesity, diabetes, hypertension), and exposure to certain environmental agents (such as heavy metals) are factors associated with various causes and risks of the disease [5].

The pathology of the disease is defined by extracellular beta-amyloid (Aβ) plaque buildup and intracellular tau tangles. These abnormalities are often linked to neuroinflammation, mitochondrial dysfunction, synaptic degeneration, and neuronal necrosis. Current therapeutic approaches primarily rely on cholinesterase inhibitors and glutamate receptor antagonists. These interventions can alleviate symptoms but are ineffective in delaying or reversing disease progression. Consequently, identifying innovative treatment strategies for AD is of critical importance [4]. Most pharmacotherapeutic strategies in use today are designed to offer only symptomatic relief [3]. Furthermore, medication-related side effects can complicate care and aggravate the deterioration of patients' quality of life. For this reason, efforts in past decade have been directed towards discovering alternatives with improved safety profiles. Among these, plant-based functional foods with extensive therapeutic potential

and favorable safety characterstics have become a focal point of interest [3]. Due to their natural origin, plants have been used in the treatment of many diseases since ancient times. Secondary metabolites such as alkaloids, saponins, flavonoids and terpenes are known for their therapeutic properties. Phenolic compounds, particularly flavonoids, are bioactive substances sourced from plants and animals, with extensive evidence supporting their numerous health benefits [3].

As natural sources of antioxidants, plant extracts and their constituents have their activity primarily to secondary metabolites, including phenolic compounds like flavonoids, tannins and alkaloids. The potent antioxidant capacity of flavonoids is considered a major contributor to their physiological benefits. Numerous studies have highlighted the neuroprotective potential of flavonoids. Clinical studies suggest that various cases associated with AD contribute to increased free radical production and a decline in antioxidant levels. Flavonoids, a group of natural compounds with different phenolic structures, are abundant in vegetables, fruits, grains, flowers, tea, and wine. They lighten reactive oxygen species (ROS)-induced damage through several mechanisms, including direct scavenging of free radicals. When flavonoids are oxidized by radicals, they form more stable and less reactive species. Some flavonoids can directly scavenge superoxides, while others can scavenge highly reactive oxygen-derived radical peroxynitrite. Flavonoids demonstrate their antioxidant effects by neutralizing all types of oxidative radicals, including superoxide and hydroxyl radicals. *Passiflora incarnata* L., commonly used in Ayurveda for treating various CNS disorders, is a rich source of flavonoids [3]. *Passiflora* has been widely recognized as a medicinal plant since the 16th century in South America and Europe. It has sedative, anxiolytic, and anticonvulsant effects on the CNS[6-10].

Passiflora incarnata is a member of the Passifloraceae family, which encompasses approximately 27 genera and 694 species associated with the genus *Passiflora* L. In Turkish, *P. incarnata* is commonly known as ‘Çarkıfelek’ or ‘Saatgülü’. In English, it is known by various names, including ‘Passionflower’, ‘Maypop’, ‘Passion vine’, Granadilla’, and ‘Apricot vine’[11]. (Fig. S1).

The native habitat of this plant is America, and it is also distributed in Europe, Asia, Australia, and tropical Africa. Due to absence of *P. incarnata* in its natural form in the Türkiye, there are no records of traditional uses among the local population. However, it is known that cultivation of this species occurs in the temperate regions of Turkiye. Traditionally, *P. incarnata* has been utilized for issues like nervousness and insomnia in both America and Europe. Research findings indicate that the herb is primarily used internally to alleviate conditions such as restlessness, anxiety, insomnia, and psychological discomfort and tension related to menstruation and menopause [12].

The *Passiflora* phytocomplex contains many major flavonoids as secondary compounds, such as chrysin, vitexin, isovitexin, orientin, isoorientin, apigenin, and kaempferol. Several studies have reported that vitexin has the ability to induce sleep, is anti-diabetic, anti-inflammatory, and effective in improving sleep[6, 13-15]. They measured behavioral, physiological, and metabolic changes following *Passiflora* application. Through *Passiflora* application, serotonin and melatonin serum levels in the animals’ serum were found to be significantly increased compared to those untreated with *Passiflora* [6]. It is also known to help with sleep disorders in Alzheimer’s patients, and animal studies have shown beneficial effects in reducing Aβ levels during sleep [6, 10, 16-17].

Through various experimetal studies, it has been established that the plant exhibits significant antioxidant activity, attributed to its elevated levels of phenolic and flavonoid content. Consequently, it has been inferred that it may possess neuroprotective properties. According to these findings, it is hypothesized that it could provide neuroprotection in neurodegenerative disorders, such as AD, that are linked to antioxidant potential.

In this work, we presented a new extraction method for the herba of *P. incarnata*, highlighting its significance as a pioneering approach in the literature. The method utilized water bath extraction at 60°C with three different solvents: ethanol, water, and a 1:1 mixture of ethanol and water. The antioxidant capacities of these extracts were comprehensively evaluated by quantifying their total phenolic and flavonoid content, along with their inhibitory effects on acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes, employing the Ellman method [18] under *in vitro* conditions. This study aimed to not only assess the antioxidant, AChE, and BChE activities demonstrated in these *in vitro* experiments but also to investigate the plant’s potential as a promising phytopharmaceutical candidate for the management or alleviation of AD symptoms. Furthermore, in line with the principles of green chemistry, the sustainability of the extraction methods was evaluated using the AGREE: Analytical Greenness Calculator software. AGREE provides a comprehensive evaluation of the greenness of analytical methods based on 12 metrics, such as waste minimization, analyst safety, energy consumption, and the use of renewable materials, yielding a score between 0 and 1. The application of AGREE allowed us to assess and compare the environmental impacts of the three different solvent systems used in this study. By integrating green chemistry principles into the extraction and analysis process, this research not only emphasizes the therapeutic potential of *P. incarnata* but also highlights the importance of sustainable and environmentally friendly methodologies in the development of phytopharmaceuticals.

Experimental part

Chemicals and reagents

The reagents employed in this study were obtained from the following sources: Quercetin, Pyrocatechol, DTNB (5,5-dithiobis-(2-nitrobenzoic acid)), Acetylcholinesterase, Butyrylcholinesterase, Chloroform, Dichloromethane, Methanol, Ethanol, Aluminium nitrate, Potassium acetate, Folin-Ciocalteau reagent (FCR), Ammonium acetate, Sodium hydrogen phosphate, and Sodium dihydrogen phosphate (all from Merck, Germany). Additionally, Galantamine hydrobromide and Acetylthiocholine iodide (both from Sigma Aldrich, Germany) and Butyrylthiocholine iodide (from Fluka, Germany) were utilized.

Instrumentation and analytical conditions

The Thermo LC system utilized for the analysis consisted of a Thermo Orbitrap Q-Exactive HRMS, which was equipped with an electrospray ionization (ESI) source (Thermo, USA). Data acqusition was carried out using TRACE FINDER software. Chromatographic separations were performed on a Fortis C18 analytical column (3 µm*150 mm*3.00 mm). The mobile phases were formulated to include 0.1% formic acid in water (A) and methanol (B). The flow rate for the analyis was set at 0.35 mL/min, and the elution gradient is detailed Table S1.

The compounds from each extract were ionized using ESI in both positive and negative modes. The mass spectrometry parameters were configured as follows: sheath gas flow rate at 45 L/min, auxillary gas flow rate at 10 L/min, spray voltage at 3.8 kV, capillary temperature at 320°C [19, 20].

Plant material

Passiflora incarnata, a cultivated plant from Muğla, was collected on 26.05.2020 and identified by Çağla Kızılarslan Hançer. The dried specimen was deposited in the herbarium of the Faculty of Pharmacy of Istanbul University (ISTE) with the herbarium number: 117.298.

Extraction method

Dried plant samples (Fig. S2) was divided into three groups (Fig. S3), paying attention to the presence of both fruits and flowers in each group, and was subsequently weighed. A portion was extracted from the flower section, resulting in a total weight of 115.27 g. In order to simplify the grinding process. The plant was chopped into smaller parts using scissors. Each group of plants was afterward processed into a fine powder using separate grinders. The powdered samples were then transferred into 500 mL volumetric flasks.

Preparation of ethanolic extracts: 28.38 g of the plant material was mixed with 300 mL of ethanol and shaken thoroughly. The mixture was then placed in a water bath at 60°C under the reflux condenser (Fig. S4) for a duration of 2 hours. Once this time had elapsed, the extract was filtered through filter paper. (Fig. S5) More ethanol was added to the leftover plant material in the flask, and the same procedure was carried out for 40 minutes. The filtrates of the ethanolic extract were combined and dried using a rotary evaporator to prepare them for analysis.

Preparation of aqueous extracts: 29.34 g of plant material was combined with 300 mL of water and gently shaken. The mixture was then kept under a reflux in a water bath at 60°C for 2 hours (Fig. S4), after which it was filtered. (Fig. S5) Extra water was added to the remaining plant material in the flask, and it was left under the same conditions for 40 minutes before the filtrates were combined. The extract, which had been stored at -85°C overnight and passed through liquid nitrogen, was subjected to lyophilization to remove the solvent.

Preparation of water-ethanol (50:50;v/v) extracts: A flask containing 28.87 g of plant material was prepared by mixing 150 mL of ethanol and 150 mL of water to obtain a 50:50 homogenous solution, and afterward adding this mixture while gently shaking. Then, it was placed in a water bath at 60°C under the reflux for 2 hours (Fig. S4). Following this period, the mixture was filtered. (Fig. S5) The extract was then subjected to evaporation using a rotary evaporatör. Once the evaporation process was complete, it was placed in a freezer at -85°C overnight to faciliate the lyophilization process.

Investigation of total phenolic content

The Folin-Ciocalteu method is based on the measurement of absorbance corresponding to the color intensity generated by the FCR. Pyrocatechol (o-benzenediol) is used as the standard phenolic compound. A high absorbance value obtained from the analysis signifies a substanial presence of phenolic compound [21].

The total phenolic content of the extracts was determined using the FCR, with results expressed as equivalents of pyrocatechol [22]. A 100 ppm solution of pyrocatechol was prepared, and from this solution, 0, 1, 2, 3, 4, 5, 6, 7, and 8 µL aliquots were taken and diluted to a final volume of 184 µL with distilled water. Samples obtained from the ethanolic, aqueous, water-ethanol (50:50; v/v) extracts of *Passiflora incarnata* L. were prepared as solutions at a concentration of 100 ppm. A solution containing 1 mg of extract was prepared, from which 4 µL was taken and brought up to 184 µL with distilled water. To the pyrcatechol solutions and samples, 4 µL of FCR was added, and after a wait of 3 minutes, 12 µL of a 2% Na_2CO_3 solution was introduced. The resulting mixture was left at room temperature for 2 hours, after which the absorbance values of the samples were measured at 760 nm. The total phenolic content of the extracts were determined using the following equation (Eq.1) derived from the calibration curve of standard pyrocatechol. There are three replicates runs for each samples ($R^2 = 0.9953$).

$$Abs. = 0.0286\ (\mu g) - 0.0733,\ R^2 = 0.9953 \quad (Eq.\ 1)$$

Investigation of total flavonoid content

Flavonoids are phenolic compounds that have a diphenylpropane structure, formed by the combination of two phenyl rings with a propan chain. Quercetin is

used as a standard flavonoid compound.

The total flavonoid contents of the obtained extracts were determined using the aluminum nitrate method, with quercetin as the reference standard [23]. A quercetin solution was prepared at a concentration of 100 ppm, from which volumes of 0, 1, 2, 3, 4, 5, 6, 7, and 8 µL were transferred and diluted to 192 µL with 80% ethanol. Subsequently, 4 µL of 1 M potassium acetate was added, and after a 1 minute incubation, 4 µL of a 10% aluminum nitrate solution was introduced. The samples were then incubated for 40 minutes, after which absorance values were measured at 415 nm. Samples of aqueous, ethanolic, and water-ethanol (50:50; v/v) extracts obtained from the *Passiflora incarnata* L. plant were prepared to achieve a concentration of 1000 ppm. The absorbance values of the solutions prepared at concentration of 1000 ppm were also measured. The total flavonoid contents of the extracts were determined using the following equation (Eq. 2) gathered from the calibration curve of standard quercetin. All the samples were measured triplicates.

Abs. = 0.0394 (µg) - 0.0471, R^2 = 0.9959 *(Eq. 2)*

Anticholinesterase activity

Ellman Method. The Ellman method is a spectrophotometric technique used to assess anti-cholinesterase activity by evaluating the inhibitory effects on the enzymes AChE and BChE [18]. This method involves the *in vitro* examination of the hydrolysis of acetylcholine by either acetylcholinesterase or butyrylcholinesterase [24].

To the Ellman method, AChE derived from electric eel and BChE obtained from horse serum were used, with acetylcholine iodide and butyric acid iodide serving as substrates. The yellow compound 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) was employed to measure the inhibitory activity of acetylcholinesterase and butyrylcholinesterase. In each well of a 96-well microplate (Fig. S6), 130 µL of a phosphate buffer at pH 8 with a concentration of 0.1 M, along with 10 µL of the sample, was added. Subsequently, 20 µL of BChE solution was added to corresponding wells containing AChE and other controls. The reaction was incubated at 25 °C for 10 minutes. Following incubation, 20 µL of DTNB solution and 20 µL of the substrate acetylcholine iodide or butyrylcholine iodide resulted in the release of thiocholine, which reacted with DTNB to produce the yellow anion 5-thio-2-nitrobenzoic acid, which was spectrophotometrically analyzed at a wavelength of 412 nm. Ethanol was used as a control, while galantamine served as a standard reference. AChE and BChE activity (% inhibition) was calculated using the following equation (Eq.3). All the samples were measured triplicates.

% Inhibition=((Control-Sample))/Control x 100 *(Eq. 3)*

Green chemistry analysis

Recent studies conducted in the chemical and pharmaceutical sectors, with the advancement of technology, have made reducing scale an important criterion for minimizing environmental harm. In the recent past, researchers have focused on preventing environmental pollution and developing eco-friendly analytical methods. The newly developed 'eco-friendly' analytical techniques aim to minimize the consumption of solvents and other chemicals to reduce their irritating, toxic, corrosive effects and potential for pollution. Additionally, energy consumption in analyses can be reduced by minimizing the number of procedural steps involved in the analysis.

One of the key points emphasized by the principles of 'green chemistry' is automation. As the use of automation systems increases, so too does the safety of the analyst while reducing their labor intensity. In summary, the principles of green chemistry prioritize the implementation of necessary automation systems, the use of recyclable materials, the minimization of polluting solvents, other chemicals, and waste, the reduction of energy consumption, and the safety of analysts.

Various analytical green calculation programs have been developed with an emphasis on the principles of green chemistry. One such program, AGREE: Analytical Greenness Calculator, created by Wojnowski et al.[25], evaluates the environmental and occupational hazards linked to spesific analytical procedures based on twelve principles of green chemistry. The resulting score from this assessment is depicted in a graph, showcasing compliance with each principle. Scores range from 0 to 1, with adherence visually represented through a color scheme of green, yellow, and red, indicating varying levels of conformity to green chemistry principles.

Results and discussion

Statistical calculations

The results of the activity are presented as the mean ± standard deviation from three independent experiments. It was determined that the results fell within the 95% confidince interval, as assessed by the Student's t-test. Measurement curves illustrating the relationship between absorbance and concentration were plotted, and the corresponding regression equations were established. A linear regression analysis was performed using the least squares method to evaluate the slope, intercept, and correlation coefficients.

Results of total phenolic and flavonoid content

The extracts' total phenolic content was measured using pyrocatechol as the standard, and the total flavonoid content was evaluated with quercetin as the standard. The results for their antioxidant activities capacities are shown in the Table 1.

Based on the results, the water extract was the highest total phenolic content, followed by the water-ethanol extract, while the ethanol extract was the lowest phenolic content. With regards to total flavonoid content, the ethanol extract was the richest,

Table 1. The results of the total phenolic and flavonoid content of the extracts.

Extracts	Total Phenolic Contents (µg pyrocatechol/mg extract)	Total Flavonoid Contents (µg quercetine)/mg extract)
PaE	105.42 ± 0.12	40.58 ± 0.11
PaES	109.79 ± 0.23	28.02 ± 0.23
PaS	106.58 ± 0.08	12.53 ± 0.26

* PaE: Ethanolic extracts, PaES: Ethanol-water extracts, PaS: Aqueous extracts of *P.incarnata*

followed by the water-ethanol extract, with the water extract showing the lowest flavonoid level. In general, the ethanol-water extract was found to be rich in both phenolic and flavonoid content.and Fig S7 and Fig S8.

Results of anticholinesterase activity

The % inhibiton values of the extracts for acetylcholinesterase and butyrylcholinesterase enzymes were determined as a shown in Table S2.

When evaluating anticholinesterase activity, the ethanol extract showed the highest inhibition rate at 36% for AChE enzyme inhibition, followed by the water extract with 31% inhibition. The aqueous-ethanol extract demonstrated an enzyme inhibition rate of approximately 16%. For BChE enzyme inhibition, the ethanol extract again led with a 28% inhibition rate, while the water extract exhibited 23% inhibition. The lowest inhibition rate, at 19%, was sighted in the aqueous-ethanol extract. Generally, the ethanol extract presented a high inhibition rate for both enzymes (Fig. S9).

Results of LC-HR/MS

In the LC-HR-MS analysis (Table 2), the water-ethanol extract, Orientin was demonstrated was the most abundant compound at 68.32 µg, followed by Luteolin-7-rutinoside 3.16 µg and Naringenin 1.74 µg, and Luteolin-7-glucoside 0.84 µg. In the ethanol extract showed the highest concentration of Orientin at 103.02 µg, followed by Luteolin-7-rutinoside 4.23 µg and Naringenin 1.62 µg and Luteolin-7-glucoside 1.6 µg. The water extract included the lowest levels of Luteolin-7-rutinoside (0.379 µg), whereas Quercitrin (0.595 µg) were found. In addition to, (-)-Epicatechin was obtained only in water extract, with a value of 1.688 µg. Calibration equation, R^2 value, relative uncertainty ratio, linear range, LOD-LOQ values, relative standard deviation, and recovery of 13 phenolic compounds in all extracts are shown (Table S3).

Results of greenness

The greenness assessment of this study was calculated using the AGREE: Analytical Greenness Calculator program. In the evaluation, all 12 criteria had equal weight in scoring. The extracts obtained through three different methods (ethanolic, water:ethanol, and aqueous) were subjected to greenness metrics, yielding scores of 0.59, 0.60, and 0.64, respectively. (Figure 1) The only criterion that significantly impacted the greenness scores was criterion 7, which pertains to the consumption of analytical waste and the amount of that waste that is recycled. According to this criterion, water is a solvent that can be recycled more and generates less harmful waste compared to other solvents. Consequently, the greenness score of the aqueous extract was found to be higher than that of the others.

Table 2. Phenolic compounds determined in the all extracts by LC-HR/MS (µg analyte/g extract).

Compounds	PaES	PaE	PaS
Chlorogenic Acid	0.14	0.08	-
Fumaric Acid	47.72	0.52	-
Orientin	68.32	103.02	-
Luteolin-7-rutinoside	3.16	4.23	0.379
Luteolin-7-glucoside	0.84	1.6	-
Rutin	0.14	0.12	-
Hyperoside	0.52	0.6	-
Quercitrin	0.14	0.24	0.595
Naringenin	1.74	1.62	-
Luteolin	0.06	0.16	-
Chrysin	0.38	0.34	-
Acacetin	0.26	0.26	-
(-)-Epicatechin	-	-	1.688

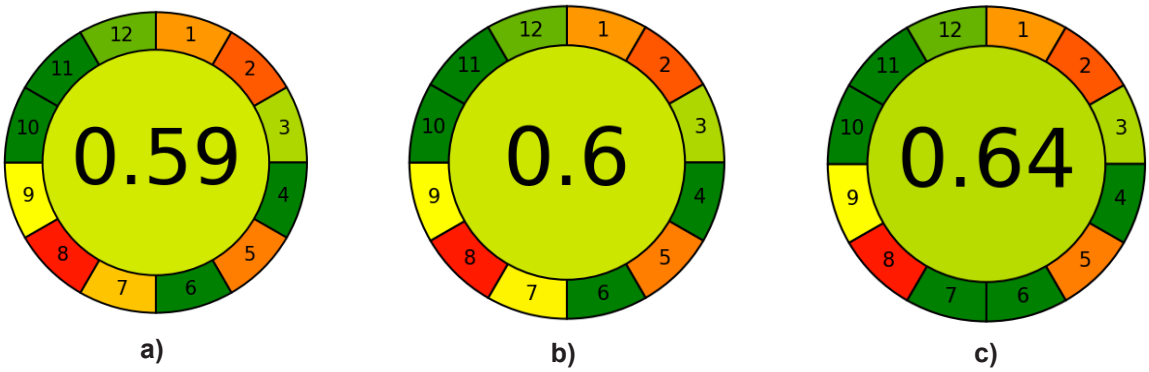

Figure 1. Greenness score of all extracts by AGREE: Analytical Greenness Calculator software (a: PaE, b: PaES, c: PE).

Conclusions

This investigation presents a novel approach for extracting *Passiflora incarnata* L. using three different solvent systems—ethanolic, ethanol-water, and aqueous—under reflux conditions at 60 °C. This method is unique in that it combines traditional extraction techniques with a sustainability assessment through the AGREE: Analytical Greenness Calculator making it the first study to explore this combination. The results provide valuable insights into the efficiency and environmental impact of each extraction method.

P. incarnata L. has been widely used to treat symptoms associated with andropause, menopause, sexual disfunctions, anxiety, hyperactivity in children, insomnia, and irritability. The plant is commonly consumed in the form of tea, tincture, or capsules derived from various extraction methods. Experimental studies have demonstrated that *P. incarnata* exhibits significant antioxidant activity, largely due to its high phenolic and flavonoid content, which may contribute to its potential neuroprotective effects [3, 26]. Given its antioxidant properties, the plant is believed to hold potential for treating neurodegenerative diseases such as Alzheimer's, offering neuroprotective benefits.

In this research, the aerial parts of *P. incarnata* were extracted using three solvent systems: water, a 50% ethanol-water mixture, and ethanol, under reflux conditions at 60 °C. The antioxidant activity and total phenolic-flavonoid content of the extracts were determined using the Folin-Ciocalteu reagent, and the anti-cholinesterase activity was evaluated through the Ellman method.

The LC-HRMS analysis revealed distinct phenolic profiles across the extracts. The ethanol extract had the highest concentration of Orientin, followed by Luteolin-7-rutinoside, Naringenin, and Luteolin-7-glucoside. The ethanol-water extract also contained a significant amount of Orientin, while the water extract exhibited lower concentrations of these compounds but contained unique compounds such as (-)-Epicatechin and Quercitrin. These compounds, particularly Orientin and (-)-Epicatechin, are known for their antioxidant and neuroprotective properties, as reported in previous studies [3, 4].

Regarding anti-cholinesterase activity, the ethanol extract demonstrated the highest inhibition rates for both AChE and BChE enzymes. The water extract showed moderate inhibition, while the ethanol-water exhibited the lowest inhibition rates. These findings indicate that flavonoids and other bioactive compounds in the ethanol extract contribute significantly to neuroprotective activities.

The sustainability of the extraction methods was assessed using the AGREE tool, revealing that the aqueous extraction method scored the highest in greenness. This result highlights the advantage of water as a solvent—non-toxic, highly recyclable, and generating less harmful waste compared to ethanol or ethanol-water mixtures. Although ethanol and ethanol-water mixtures offered higher extraction efficiencies for phenolic and flavonoid compounds, aqueous solvents represent a more sustainable option, offering an efficient balance between bioactive compound yield and environmental impact.

Ethanol-water mixtures, while efficient and safe for human consumption, also show promise in green extraction processes due to their broad polarity range and low toxicity. However, the challenges associated with pure ethanol's toxicity and waste management underline the importance of optimizing solvent systems in future studies [27]. The solvent selection is a key factor in extraction processes. Ethanol, widely used for its availability, cost-effectiveness, and biodegradability, is considered a green solvent, saf efor human consumption. Moreover, the addition of water to ethanol has been shown to enhance extraction efficiency, supporting its use in environmentally friendly extraction methods [28]. This is because these mixtures have low selectivity, both solvents offer a wide polarity range, can be mixed in any ratio anda re acceptable for human consumption [29].

These findings emphasize the importance of integrating green chemistry principles into natural product research. By combining chemical efficiency with sustainability, this work not only advances our understanding of the neuroprotective potential of

P. incarnata extracts but also sets a benchmark for the environmentally conscious development of plant-based therapeutics. Future research could focus on optimizing extraction methods to further improve greenness scores while maximizing bioactive compound yield, thus supporting both clinical and environmental goals.

Author contributions

Gizem İğdeli and Demet Dincel have made equal contributions to this work.

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Declaration of Competing Interests

The authors declare that they have no financial conflicts or personal relationships that could have impressed the work presented in this paper.

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