The Ethyl Acetate Extract of Leaves of *Ugni molinae* Turcz. Improves Neuropathological Hallmarks of Alzheimer’s Disease in Female APPswe/PS1dE9 Mice Fed with a High Fat Diet

Daniela Jara-Moreno a,c,e,1, Rubén D. Castro-Torres a,c,d,f,1, Miren Ettcheto a,b,c,f, Carme Auladell d,f, Marcelo J. Kogan e, Jaume Folch b,c, Ester Verdaguer d,f, Amanda Cano c,g, Oriol Busquets a,b,c,f, Carla Delporte e,2 and Antoni Camins a,c,f,2,*

a Departament de Farmacologia, Toxicologia i Química Terapèutica, Facultat de Farmàcia i Ciències de l’Alimentació, Universitat de Barcelona, Barcelona, Spain
b Departament de Bioquímica i Biotecnologia, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Reus, Spain
c Biomedical Research Networking Centre in Neurodegenerative Diseases (CIBERNED), Madrid, Spain
d Departament de Biologia Cellular, Fisiologia i Immunologia; Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain
e Departamento de Química Farmacológica y Toxicológica, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Santiago, Chile
f Institut de Neurociències, Universitat Barcelona, Barcelona, Spain
1 Departament de Farmàcia, Tecnologia Farmacèutica i Físico-química, Facultat de Farmàcia i Ciències de l’Alimentació, Universitat de Barcelona, Barcelona, Spain

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**Abstract.** The most common type of dementia is Alzheimer’s disease (AD), a progressive neurodegenerative disease characterized by impairment in cognitive performance in aged individuals. Currently, there is no effective pharmacological treatment that cures the disease due to the lack of knowledge on the actual mechanisms involved in its pathogenesis. In the last decades, the amyloidogenic hypothesis has been the most studied theory trying to explain the origin of AD, yet it does not address all the concerns relating to its development. In the present study, a possible new preclinical treatment of AD was evaluated using the ethyl acetate extract (EAE) of leaves of *Ugni molinae* Turcz. (synonym *Myrtus ugni* Molina Family Myrtaceae). The effects were assessed on female transgenic mice from a preclinical model of familial AD (APPswe/PS1dE9) combined with a high fat diet. This preclinical model was selected due to the already available experimental and observational data proving the relationship between obesity, gender, metabolic stress, and cognitive dysfunction; related to characteristics of sporadic AD. According to chemical analyses, EAE would contain polyphenols such as tannins, flavonoid derivatives, and...
INTRODUCTION

Alzheimer’s disease (AD) is an irreversible, chronic, and relentless neurodegenerative disease, characterized by memory loss, psychoses, and affective disorders and behavioral alterations [1–5]. Multiple epidemiological studies have estimated that the incidence of AD is expected to increase to 66 million diagnosed cases in the world by 2030 and 131 million by 2050. These values are the result of increase in life expectancy and continuous aging of developed societies. Nowadays, there are no curative therapies for AD [1, 3] and the drugs being administered are only able to slow the progression of the disease for a reduced period of time [1]. Also, they present variable effectiveness depending on the individual characteristics of the patient and their efficacy decreases as the disease progresses [6–8].

The well-known neuropathological features observed in AD brains include the presence of extracellular amyloid-β (Aβ) plaques/deposits intracellular neurofibrillary tangles (NFTs) and synaptic dysfunction [9–14]. In addition, many reports evidence a role of activated glia and its pro-inflammatory mediators in the etiology of AD [15–18]. Although the precise role of inflammatory processes in AD pathophysiology is controversial, it is believed that microglia and astrocytes become activated, promoting the secretion of cytokines and neurotoxic mediators, including tumor necrosis factor alpha (TNF-α), superoxide (O2−·), and nitric oxide (NO), which exacerbate neuronal damage [15]. Thus, several authors have hypothesized that AD could be described as a chronic brain inflammatory disease [18]. Nonetheless, other elements have been suggested as risk factors for the development of late onset AD. Among them, the most considered are age and vascular and metabolic disorders such as hypertension, type II diabetes mellitus (T2DM), dyslipidemia, and metabolic syndrome [19–23].

U. molinae is a native and wild shrub of the Myrtaceae family. It grows in central-south Chile (between the VII and X region), especially in the Coast Mountains and in part of the pre-Andean mountains. In Chilean folkloric medicine, the aerial parts and its infusions have been used to treat urinary infections, diabetes, inflammatory states and different types of pain. Also, analgesic and anti-inflammatory properties have been demonstrated by in vivo assays using the extracts of U. molinae leaves [24–28]. In these studies, it was determined that these effects are due to the presence of phenolic compounds: genins and heterosides of quercetin, kaempferol, myricetin, epicatechin/catechin; gallic acid, quinic acid, tannins and others similar compounds and pentacyclic triterpenoids from ursane, oleanane and lupane cores (Fig. 1A, B) [24–28].

Given the previous assumption on the important role of inflammation in the development of AD-like pathologies, in this study a familial model of the disease was used to study the efficacy of the extract from U. molinae as a novel disease-modifying treatment. Likewise, since experimental preclinical reported studies strongly suggest that obesity and T2DM conditions might aggravate AD pathologies, the variable of a high-fat diet (HFD) was included [29–34].

MATERIALS AND METHODS

Plant material

U. molinae leaves were collected and botanically identified by Dr. Carla Delporte in April 2010, in south-central Chile (35°45′S, 72°33′W). A voucher specimen was deposited in the herbarium of the School of Chemical and Pharmaceutical Sciences, University of Chile (SQF: 22462). Dried and grinded leaves (2.0 kg) were successively treated for extraction by maceration at room temperature with hexane, dichloromethane, ethyl acetate, and ethanol to obtain, after the concentration, the corresponding dry extracts with a w/w yield of 1.4, 5.9, 3.7, and 22.2%, respectively. The selected ethyl acetate extract (EAE) was dissolved in dimethyl sulfoxide and later it was diluted in saline solution (NaCl 0.9% p/p) to inject at a dosage of 30 mg/kg of mice.
Fig. 1. Chemical structure of several compounds present in EAE of *U. molinae* leaves. A) Polyphenolic compounds. B) Pentacyclic triterpenoids. C) Experimental design for this study. Female WT and APP/PS1 mice were used in this study. All animals were exposed to HFD right after their weaning and, from the 4.5 months of age they were administered either the vehicle or EAE extract at 30 mg/kg daily. At 6 months of age all animals underwent the NORT and were sacrificed in order to obtain samples for IF assays.

**Chemical characterization of EAE**

**Total phenolic content**

The total phenolic content (TPC) was determined by Folin Ciocalteau assay according to Peña-Cerda et al. [25]. Briefly, EAE was dissolved in methanol: water (2:8) and 30 μL of sample was mixed with 30 μL Folin Ciocalteau reagent (1:10 in water). Then 240 μL of sodium carbonate (5% v/v) was added. Finally, the detection was made at 40°C in a 96-well plate spectrophotometer (Multiskan GO, Thermo Scientific, USA) and the absorbance was measured at 765 nm after 20 min of reaction. A calibration curve was made with gallic acid (\(y = 0.062x + 0.053\), \(R^2 = 0.998\) and \(F_{calc} = 0.30 < F_{tab} = 3.71\)) as standard compound. The TPC was expressed as mg gallic acid per g dry extract.

**Total flavonoid content**

The total flavonoid content (TFC) was calculated by an AlCl₃ complexation method according to Peña-Cerda et al. [25]. Briefly, EAE was dissolved in methanol and 30 μL of sample was mixed with 10 μL of sodium acetate (1 M) and 240 μL of distilled water. The method was performed at room temperature in a 96-well plate spectrophotometer under a 415 nm wavelength, 30 min after the beginning of the reaction. A calibration curve was made with quercetin as standard compound (\(y = 0.049x + 0.003\), \(R^2 = 0.999\) and \(F_{calc} = 0.06 < F_{tab} = 3.49\)) and TFC was expressed as mg quercetin per g dry extract.

**Identification of phenolic compounds by mass spectrometry**

Methanolic solutions of EAE (5000 ppm) were prepared and HPLC-UV-ESI-MS analyses were performed to obtain the polyphenolic fingerprint of this extract. The HPLC system was an Agilent 1100 (Agilent Technologies Inc., CA-USA) coupled with an electrospray ion trap mass spectrometer Esquire 4000 ESI-IT (Bruker Daltonics GmBH, DE). Analyses and UV detection were made at 270 and 360 nm.
correspondingly. HPLC separation was carried out on a reverse phase Purospher® STAR RP-18 column (endcapped 5 μm, Hibar® RT 250–4; Merck, Darmstadt, Germany). Methanolic solution of EAE was separated by HPLC gradient elution which was established by mixing two mobile phases: phase A (H2O/HCOOH : 98 : 2) and phase B (H2O/CH3CN/HCOOH : 18 : 80 : 2), according to methodology used by Michodjehoun-Mestres et al. [35]. The elution’s run time was 90 min and the gradient elution was: 0–8 min 90% A, 8–45 min 84% A, 45–55 min 65% A, 55–72 min 20% A, 72–75 min 0% A, 75–78 min 95% A, 80–90 min 90% A at a flow rate of 0.2 mL/min. The ionization (nebulization) was performed by an electrospray ionizer and an ion trap was used as mass analyzer. Negative polarity was employed in ESI. The following parameters were considered for mass spectrometry: spray ionization voltage was 4000 V; nitrogen (N2) was used as nebulizer gas; temperature was 365°C; pressure 45 psi; and flow rate 10 L/min. Data was analyzed using Data Analysis Version 3.2 software (Bruker Daltonik GmbH, Germany). Compound identification was done using Mass Bank database or by comparison with scientific literature.

**Animals**

Female APPswe/PS1dE9 (APP/PS1) and C57BL/6J wild-type (WT) mice were used in this study. APP/PS1 animals co-express a Swedish (K594M/N595L) mutation of a chimeric mouse/human amyloid precursor protein (APP), together with the human exon-9-deleted variant of presenilin 1 (PS1). Both mutations are associated with AD and represent a preclinical model to study some of the features of familial AD. The animals were originally purchased from Jackson Lab (https://www.jax.org/strain/004462). The original background is (C57BL/6 x C3 H)-F2. Thus, living colony in our facilities are currently maintained as hemizygote against C57BL/6J for at least 8 generations.

The mice were fed for 6 months with either control (CT) or HFD (D08061110; Research Diets Inc., New Brunswick, USA). This diet is enriched with hydrogenated coconut oil, increasing fat content up to 45% (Table 1). Mice body weight was recorded weekly from the beginning of treatment (4.5 months old) until their sacrifice at 6 months of age. Animals that underwent the treatment with EAE were injected intraperitoneally (i.p.) daily from the 4.5 months of age until their sacrifice at 6 months of age. These time periods were chosen in order to present the treatment as a prevention and possible reversal method of the negative neuropathophysiology of AD. EAE was administered at a dosage of 30 mg/kg given the maximum possible dissolution rate between dimethyl sulfoxide and saline solution. The control mice were injected with analogue proportions of dimethyl sulfoxide diluted with saline solution.

Twenty-five animals were used in total, divided into four groups: WT+HFD, APP/PS1 + HFD, WT+HFD+EAE, and APP/PS1 + HFD+EAE. Groups WT+HFD and APP/PS1 + HFD were administrated with the vehicle where EAE was dissolved. The experimental design is represented in Fig. 1. All the animals were kept under controlled temperature, humidity, and 12h light/dark conditions with food and water provided ad libitum. In all situations, mice were treated in accordance with the European Community Council Directive 86/609/EEC and the procedures established by the Department d’Agricultura, Ramaderia i Pesca of the Generalitat de Catalunya. Every effort was made to minimize animal suffering and to reduce the number of animals used. Following in vivo testing, the animals were sacrificed and all mice were used for immunohistochemistry (IHC) microscopy analysis.

### Glucose Tolerance Test (GTT) - Insulin Tolerance Test (ITT)

Before starting the treatment with EAE, the animals underwent a GTT and ITT test to determine whether their metabolic state has acquired a diabetogenic/obesogenic profile. Animals were fasted for 6 h previous to the intraperitoneal (i.p.) administration of either substance. In GTT mice were injected a glucose dosage of 1 g/kg, whereas in ITT, insulin was administered at a dosage of 0.75 ui/kg ratio. Next, blood samples were analyzed from the tail vein in consecutive time periods. For GTT samples were extracted at 5, 15, 30, 60, 120, and 180 min after the administration; in ITT measurements were made 15, 30, 45, 60, and 90 min after. Animals were continuously observed and monitored and, in those cases in which

| Table 1 | Composition of High Fat Diet |
|---------|-----------------------------|
| Proteins | 16.4 |
| Carbohydrates | 38.6 |
| Fats (coconut oil) | 45.0 |
| Total | 100.0 |
glucose dropped under a concentration of 20 mg/dL, animals were administered a glucose dosage of 1 g/kg and kept under observation until glucose blood levels stabilized and normal behavior was observed.

**Novel Object Recognition Test (NORT)**

The Novel Object Recognition Test was used to evaluate hippocampal-dependent recognition memory of mice [35]. The task procedure consisted of three phases: habituation, familiarization and probe. In the habituation phase, mice explored individually a circular open-field arena of 40 cm of diameter for three consecutive days, 10 min for each session. On the fourth day (familiarization), each mouse was placed in the arena containing two identical objects (A + A) in the middle of the field for 10 min. To perform the test phase, mice were returned 24 h later to the open-field with two objects, one was identical to the day before and the other was a novel object (A + B) for 10 min. Light intensity was kept constant in all phases and the arena and objects were cleaned with 96° ethanol between each animal to eliminate olfactory cues. Exploration activity was defined as the orientation of snout of the animals toward the object, sniffing or touching. The exploratory analysis was expressed as discrimination index (DI). DI= (novel object exploration time/total exploration time) – (familiar object exploration time/total exploration time), measured in seconds.

**Immunohistochemistry**

Mice used for IHC studies were anesthetized by i.p. injection of sodium pentobarbital (80 mg/kg) and intracardially perfused with 4% paraformaldehyde (PFA) diluted in 0.1 M phosphate buffer (PB). Brains were removed and stored in the same solution overnight (O/N) at 4°C and then, they were cryoprotected in 30% sucrose-PFA-PB solution. Samples were frozen at –80°C and coronal sections of 20 μm of thickness were obtained by a cryostat (Leica Microsystems, Wetzlar, Germany) and kept in cryoprotectant at –20°C until use.

Free-floating sections were first washed three times with 0.1 mol/L PBS pH 7.35 and, after, five times with PBS-T (PBS 0.1 M, 0.2% Triton X-100). Then, they were incubated in a blocking solution containing 10% fetal bovine serum (FBS), 1% Triton X-100 and PBS 0.1 M- 0.2% gelatin for 2 h at room temperature. After that, slices were washed with PBS-T (PBS 0.1 M, 0.5% Triton X-100) five times for 5 min each and incubated with polyclonal rabbit anti-Glial Fibrillary Acidic Protein (GFAP; 1:2000; Dako, Glostrup, Denmark), rabbit anti-Ionized calcium-binding adapter molecule (IBA1; 1:1000; Wako Chemical USA), and mouse anti-Aβ1-42 (12F4; 1:1250; Covance, USA) primary antibodies at 4°C O/N. Sequentially, sections were washed with PBS-T (PBS 0.1 M, 0.5% Triton X-100) 5 times for 5 min and incubated with Alexa Fluor 594 goat anti-rabbit and Alexa Fluor 488 donkey anti-rabbit secondary antibodies (1:500; Invitrogen, Eugene, OR, USA) for 2 h at room temperature in the dark.

The staining for plaques was performed using Thioflavin S (ThS 0.002%, Sigma-Aldrich) in a 0.033% dilution rate. Slices were incubated for 8 min in the dark, washed with ethanol 50% twice for 1 min and rinsed with PBS 0.1 M. Later, sections were co-stained with 0.1 μg/mL Hoechst 33258 (Sigma-Aldrich, St Louis, MO, USA) for 15 min in the dark, and washed with PBS 0.1M. Finally, the slices were mounted using Fluoromount G (EMS) and the image acquisition was performed with an epifluorescence microscope (BX41 Laboratory Microscope, Melville, NY-Olympus America Inc.).

**Image analysis and quantification**

We analyzed the cortex in brain coronal sections obtained from Bregma –1.28 to –2.12 mm, in accordance with the Paxinos and Franklin atlas [36]. The analysis and quantification of IHCs and ThS staining were performed using images obtained with the 20x objective. 4–8 sections/animal from 4–6 animals/group were used in all quantifications, except for particle analysis (3 animals/group).

**Evaluation of astrocyte and microglia reaction**

The quantification of astrocyte and microglia reaction was performed with GFAP and IBA1 markers. The immunopositive reactivity of the tissue were done through the percentage area occupied by

| Table 2 |
| --- |
| Content of pentacyclic triterpenoid in EAE (30 mg/Kg) |
| Pentacyclic triterpenoid | % w/w |
| Madecassic acid | 0.33 ± 0.09 |
| Asiatic acid | 2.43 ± 0.09 |
| Alpha-tocopherol acid | 2.82 ± 0.09 |
| Corosolic acid | 8.28 ± 0.21 |
| Malic acid | 2.25 ± 0.12 |
| Betulinic acid | 0.54 ± 0.21 |
| Oleanolic/ursolic acid | 6.27 ± 0.06 |
fluorescent signal as previously described by de Lemos et al. [37]. Briefly, images were captured and calibrated using a scale. Then, were transformed to 8-bits gray, inverted and highlighted the area occupied by fluorescent signal (IBA1 or GFAP cells) under fixed threshold using the NIH ImageJ software.

**Evaluation of cortical Aβ load**

To evaluate the number of Aβ plaques and their size (μm²) **Cell counter** and **particle quantification** Image J functions were used. Aβ1–42 density of aggregates were evaluated using both fluorescent IHC (12F4) and ThS staining. Intensity was determined as follows: Area of fluorescent signal (μm²) / Total analyzed area. It was expressed as % area.

**Gliosis surrounding amyloid plaque**

To quantify the astrocytes surrounding Aβ plaques, different plaques per tissue section using superimposed grid of 200×200 μm² were used. Aβ plaques were put in the center of the grid and then channels were spliced using Image J. The size of GFAP positive cells was determined using **particle quantification** of Image J. Particles with GFAP immunoreaction minor than 5 μm² were considered artefacts and therefore were excluded from the analysis. In order to identify if there was gliosis around the plaques the ratio between GFAP area surrounding the plaque and the area of this plaque was calculated.

Microglial reactivity was evaluated by analyzing microglial areas around Aβ1–42 aggregates. To do that, double IHC against IBA1 and Aβ1–42 peptides (12F4) were used. From cortical sections, the area

**Table 3**

EAE (270 nm). Identification of phenolic compounds by HPLC-UV-ESI-MS² (ESI-IT)

| Compound | Tr (min) | MW (g/MOL) | [M-H]⁻ (m/z) | MS² (m/z) | Reference |
|----------|----------|------------|-------------|-----------|-----------|
| 1. Stricnin/isostricnin isomer (galloyl-HHDP-hexose) | 3.7 | 634 | 633 | 300 274 420 | a |
| 2. Pedunculagin I/casuariin isomer (bis-HHDP-hexose) | 3.7 | 784 | 783 | 300 481 | a |
| 3. Digalloyl glucose isomer (digalloyl hexose) | 3.7 | 484 | 483 | 331 169 | a |
| 4. Gallic acid | 4.0 | 170 | 169 | 125 | b |
| 5. Stricnin/isostricnin isomer (galloyl-HHDP-hexose) | 6.4 | 634 | 633 | 300 274 420 | a |
| 6. Digalloyl glucose isomer (digalloyl hexose) | 7.0 | 484 | 483 | 331 169 | a |
| 7. Pedunculagin I/casuariin isomer (bis-HHDP-hexose) | 10.1 | 784 | 783 | 481 301 | a |
| 8. Digalloylquinic acid | 10.5 | 496 | 495 | 343 325 169 | a |
| 9. Pedunculagin I/casuariin isomer (bis-HHDP-hexose) | 11.4 | 784 | 783 | 481 300 | a |
| 10. Digalloyl glucose isomer (digalloyl hexose) | 11.4 | 484 | 483 | 331 169 | a |
| 11. Pedunculagin I/casuariin isomer (bis-HHDP-hexose) | 11.4 | 784 | 783 | 300 481 | a |
| 12. Digalloyl glucose isomer (digalloyl hexose) | 12.8 | 484 | 483 | 331 169 | a |
| 13. Digalloyl glucose isomer (digalloyl hexose) | 14.3 | 484 | 483 | 331 169 | a |
| 14. Digalloyl glucose isomer (digalloyl hexose) | 16.0 | 484 | 483 | 169 331 | a |
| 15. Epicatechin/catechin | 15.6 | 290 | 289 | 244 204 178 | b |
| 16. Trigalloyl hexose isomer | 17.0 | 636 | 635 | 464 482 313 | a |
| 17. Myricetin galloyl hexoside | 18.3 | 632 | 631 | 478 316 | a |
| 18. Trigalloyquinic acid | 18.1 | 648 | 647 | 476 325 | a |
| 19. Flavogalonic dilactone acid isomer | 18.7 | 470 | 468 | 450 425 300 | a |
| 20. Flavogalonic dilactone acid isomer | 20.2 | 470 | 468 | 425 300 450 | a |
| 21. Myricetin galloyl hexoside | 20.4 | 632 | 631 | 479 316 | a |
| 22. Digalloyl pentose | 20.6 | 454 | 453 | 313 168 326 | a |
| 23. Myricetin galloyl hexoside | 23.9 | 632 | 631 | 479 316 | a |
| 24. Myricetin deoxihexoside | 26.7 | 464 | 463 | 317 463 | a |
| 25. Myricetin hexoside | 28.0 | 480 | 479 | 316 179 271 | b |
| 26. Myricetin deoxihexoside | 28.5 | 464 | 463 | 463 317 179 | a |
| 27. Quercetin galloyl hexoside (galloylquercetin) | 31.6 | 616 | 615 | 463 317 | a |
| 28. Quercetin pentoside | 33.0 | 433 | 433 | 301 150 | a |
| 29. Myricetin pentoside | 34.3 | 450 | 449 | 318 179 152 | b |
| 30. Ellagic acid | 35.5 | 302 | 301 | 300 228 185 | a |
| 31. Ellagic acid | 36.2 | 302 | 301 | 300 228 185 | a |
| 32. Kaempferol galloyl hexoside | 38.4 | 600 | 599 | 447 312 284 | a |
| 33. Quercetin pentoside | 45.0 | 434 | 433 | 300 179 | a |
| 34. Quercetin deoxihexoside | 48.8 | 448 | 447 | 447 301 | a |
| 35. Myricetin galloyl deoxihexoside | 55.6 | 616 | 615 | 616 316 462 | a |
| 36. Quercetin | 63.7 | 302 | 301 | 178 150 300 | b |

a, scientific article; b, database; Tr, retention time; MW, molecular weight; EAE, ethyl acetate extract.
Table 4
EAE (360 nm). Identification of phenolic compounds by HPLC-UV-ESI-MS² (ESI-IT)

| Compound                        | T_r (min) | MW (g/mol) | [M-H]⁻ (m/z) | MS² (m/z) | Reference |
|--------------------------------|-----------|------------|--------------|-----------|-----------|
| 1. Digalloyl hexose isomer      | 14.4      | 484        | 483          | 313 331 169 | a         |
| 2. Epicatechin/catechin         | 15.6      | 290        | 289          | 205 245 137 | a, b      |
| 3. Digalloyl pentose            | 15.6      | 454        | 453          | 313 169   | a         |
| 4. Trigalloyquinic acid         | 17.7      | 648        | 647          | 465 477   | a         |
| 5. Trigalloyl hexose isomer     | 17.9      | 636        | 635          | 465 432   | a         |
| 6. Trigalloyquinic acid         | 18.6      | 648        | 647          | 465 477   | a         |
| 7. Trigalloyl hexose isomer     | 19.2      | 636        | 635          | 465 432   | a         |
| 8. Flavogalonic dilactone acid isomer | 20.1 | 470        | 468          | 425 450   | a         |
| 9. Digalloyl pentose            | 20.5      | 454        | 453          | 313 169   | a         |
| 10. Myricetin galloyl hexoside  | 23.5      | 632        | 631          | 479 316   | a         |
| 11. Quercetin galloyl hexoside  | 31.5      | 616        | 615          | 615 463 301 | a         |
| 12. Quercetin galloyl hexoside  | 33.2      | 616        | 615          | 615 463 301 | a         |
| 13. Ellagic acid                | 35.3      | 302        | 301          | 301 229 185 | a         |
| 14. Ellagic acid                | 36.2      | 302        | 301          | 301 229 185 | a         |
| 15. Kaempferol galloyl hexoside | 38.2      | 600        | 599          | 447 285 313 | a         |
| 16. Quercetin pentoside         | 44.7      | 434        | 433          | 300 179 150 | b         |
| 17. Quercetin pentoside         | 45.7      | 434        | 433          | 300 179 150 | b         |
| 18. Quercetin hexoside          | 48.6      | 648        | 647          | 300 179 284 271 | a         |
| 19. Myricetin galloyl deoxihexoside | 53.8 | 616        | 615          | 615 316 463 | a         |
| 20. Myricetin                    | 53.6      | 318        | 317          | 300 178 150 | b         |
| 21. Myricetin galloyl deoxihexoside | 55.4 | 616        | 615          | 615 463 317 | a         |
| 22. Myricetin galloyl deoxihexoside | 55.8 | 616        | 615          | 615 316 463 | a         |
| 23. Quercetin                    | 63.6      | 302        | 301          | 178 150 301 | b         |

a, scientific article; b, database; T_r, retention time; MW, molecular weight; EAE, ethyl acetate extract.

Fig. 2. Peripheral metabolic parameters in HFD-fed WT and APP/PS1 mice. Weekly bodyweight variation (A), GTT (B), and ITT (C) in 6-month-old mice (n = 5–12 independent samples per group). For the ITT and the GTT, AUC data were calculated from the time point 0 till the end of the experiment. The results were presented as MEAN ± SEM. Statistical analysis was performed by two-way ANOVA test, with Tukey’s post hoc test where *p < 0.05 and **p < 0.01.
occupied by the fluorescent signal from their correspond-opponent channel (green = IBA1; red = Aβ1–42) and the ratio of IBA1/Aβ1–42 were calculated.

Data analysis

All data is presented as mean alone or mean ± SEM. Level of significance was fixed at α=0.05. Thus, calculated p-values<0.05 were considered significant. Differences between samples/animals were evaluated using either two-way ANOVA with Tukey’s and unpaired Student-t as required. Both statistical analyses and graphs presented here were created with the Graph Pad InStat software V5.0 (Graph Pad Software Inc., San Diego, CA, USA).

RESULTS

Chemical characterization of EAE

EAE is composed of pentacyclic triterpenoids. They were quantified according to Goity et al. [28] (Table 2): corosolic and oleanolic/ursolic mixtures (8.28 ± 0.21% and 6.27 ± 0.06, respectively) were the most abundant triterpenoids in this extract. Also, the polyphenolic content of EAE was 75.05 ± 5.73 mg gallic acid equivalents/g dry extract (2.25 ± 0.17 mg gallic acid equivalents/g dry extract in the administrated dose, 30 mg/Kg). Additionally, the flavonoid content of EAE was 27.7 ± 5.3 mg quercetin equivalents/g dry extract. Moreover, additional polyphenolic derivatives using mass spectrometry were identified: tannins as gallotannins (di- and tri-galloyl derivatives), ellagitannins (hexahydroxydiphenoyl (HHDP) and hexose derivatives), and quinic acid derivatives. Also, flavonoids as myricetin, quercetin, kaempferol, epicatechin/catechin, its heterosides, and phenolic acids as gallic and caffeic acids derivatives were identified (Tables 3 and 4).

Treatment with EAE avoids weight gain due to HFD and improves cognitive function in APP/PS1 + HFD mice.

Our data showed that EAE treatment decreases body weight significantly in both genotypes, WT and APP/PS1 (F(1, 34)=17.48; p = 0.0002) (Fig. 2). As expected, HFD intake induced significant increases in glucose plasma levels in the GTT and ITT tests, analyzed by two-way ANOVA (F(1, 42)=17.81; p = 0.001, F(1, 24)=23.49; p < 0.0001), respectively. Furthermore, EAE treatment significantly improved cognitive performance when evaluating using the behavioral NORT (F (1, 34)=17.48; p = 0.002) (Fig. 3). It can be observed that in APP/PS1 + HFD transgenic mice that there was a significantly reduction versus WT+HFD.
Reduction in cellular glial reactivity in the brain of APP/PS1 + HFD mice treated with EAE

To evaluate the effects of EAE on the modulation of inflammatory responses, specifically, astroglia and microglial reactivity, different brain areas of WT and APP/PS1 mice fed with HFD and treated with EAE were analyzed through IHC assays. The detection for GFAP, the intermediate filament system of adult astrocytes, which is used as an astrogliosis marker, revealed a noticeable increase in the percentage of reactive tissue area in the cortex of APP/PS1 + HFD (Fig. 4 C, c) of 14.5% versus 4.5% of WT+HFD mice ($p<0.001$, Fig. 4 in Graph I) and a reduction of astrogliosis of 14.5% in APP/PS1 + HFD (Fig. 4 C, c) versus 7.5% APP/PS1 + HFD+EAE (Fig. 4D, d, $p<0.01$, Fig. 4 in Graph I). Two-way ANOVA, genotype and treatment with EAE as factor: $F(1, 12)=22.46$, $p=0.0005$ for genotype; $F(1, 12)=6.753$, $p=0.02$ for treatment with EAE; Interaction $F(1,12)=5.820$, $p=0.0328$.

Meanwhile, IHC against IBA1, a protein upregulated in activated microglia, revealed an increase in APP/PS1 + HFD of 17.5% (Fig. 4 G, g) in comparison to WT+HFD with 13.6% (Fig. 3E, e) essentially in cortical areas ($p<0.05$, Fig. 4K). In both genotypes fed with HFD (WT and APP/PS1), the treatment with EAE induced a reduction on microglial reactivity: 9.2% (WT+HFD+EAE) versus 13.3% (WT+HFD) ($p<0.05$, Fig. 4F, f versus 4E, e) and 12.4% (APP+HFD+EAE versus 17% APP+HFD+EAE) ($p<0.05$, Fig. 4 H, h versus 4G, g). Two-way ANOVA, genotype and treatment with EAE as factor: $F(1, 12)=22.46$, $p=0.0005$ for genotype; $F(1, 12)=6.753$, $p=0.02$ for treatment with EAE; Interaction $F(1,12)=5.820$, $p=0.0328$. 

Fig. 4. Fluorescent IHC against GFAP (A, a, B, b, C and c) and IBA1 (E, e, F, f, G and g) in the cortex of the mice untreated and treated with EAE. Low astrocytic reactivity in WT+HFD (A, a) and WT+HFD+EAE (B, b) mice (* indicate astrocytes). As is observed in E there is a microglial reactivity in WT+HFD (*, E, e) and a softly reduction of the reactivity due to EAE in WT+HFD+EAE (*, F, f). Meanwhile, transgenic APP/PS1 + HFD mice show a reactive morphology of astrocytes (arrowheads, C, c) and microglia (arrowheads, G, g) surrounding plaques. A reduction in astrocyte (D, c) and microglial (F, f) reactivity is observed in the APP/PS1 + HFD mice treated with EAE (arrowheads). Quantification of GFAP (J) and IBA1 (K) positive cells is shown in the graph bars. Graphs represent the mean ± SEM. ($p<0.05$, **$p<0.01$, ***$p<0.001$) Abbreviations: CTx, cortex; CA1, cornu ammonis; DG, dentate gyrus. Scale bar for A, B, C, D, E, F, G, and H = 400 μm. Scale bar for a, b, c, d, e, f, g, and h = 100 μm. Statistical analysis was performed with the two-way ANOVA, with a Tukey’s post hoc test.
Fig. 5. Staining of Aβ plaques by ThS in the cortex and hippocampus. It was performed in APP/PS1+HFD and APP/PS1+HFD+EAE (A, B) and APP+HFD+EAE groups (B, C) and allowed the visualization of cored Aβ plaques (arrowhead; a and b). The analysis of the effect of EAE on Aβ plaques revealed that the number of the plaques were reduced in APP/PS1 + HFD treated with EAE mice as is observed in cortex (A, a versus C, c; Graph in E, *p < 0.05). Also, evaluation of size plaque area (Graph in F, *p < 0.05) and ThS-positive plaque density (Graph in G, *p < 0.05) showed how EAE treatment reduces Aβ load. Graphs E and G represent the mean ± SEM Graph F represent mean with individual quantification values of n = 3/group. The statistical analysis was determined from Student t-test. Abbreviations: CTX, cortex; CA1, cornu ammonis 1; CA3, cornu ammonis 3; DG, Dentate gyrus. Scale bar for A, B, C and D = 400 μm, Scale bar a, b, c and d = 100 μm.

Attenuation of Aβ pathology in the APP/PS1 mouse brain treated with EAE

The ThS stain revealed Aβ deposits in cerebral cortex and hippocampus of APP/PS1 + HFD mice (Fig. 5A). The number of these deposits was reduced from an average of 67 plaques (APP+HFD) versus 37 plaques (Fig. 5E) (p < 0.05, Fig. 5E) and furthermore there is a reduction in the plaque size from a mean of 550 μm² (APP/PS1 + HFD) to 228 μm² (APP/PS1 + HFD+EAE) (p < 0.05, Fig. 5F). This effectiveness was confirmed by measuring Aβ in ThS staining and IHC against Aβ1–42 peptide load as the percentage of the area of plaques versus total area. We observed that there was a reduction of Aβ load using ThS staining from 2.4% (APP/PS1 + HFD) to 1.27% (APP/PS1 + HFD+EAE) (p < 0.05, Fig. 5 G). Moreover, IHC quantification for Aβ1–42 peptides revealed reduction of cortical Aβ load from 23% (APP/PS1 + HFD) to 18% (APP/PS1 + HFD+EAE) (p < 0.05, Fig. 6 C).

In addition, GFAP positive cells surrounding Aβ plaques were evaluated. A reduction of size glia surrounding Aβ plaques from 147 μm²/cell (APP/PS1 + HFD) to 87 μm²/cell (APP/PS1 + HFD+EAE) (p < 0.05, Fig. 7 G) was observed. The ratio of GFAP-area/Aβ-plaque- obtained was 3.6 APP/PS1 + HFD versus 7.0 APP/PS1 + HFD+EAE. However, statistical differences were not found (p = 0.2380, Fig. 7 H).

In addition, the ratio of IBA1-area/Aβ-diffuse plaque-area reflects the microglial reaction. The mean-ratio was reduced from 0.78 (APP/PS1 + HFD) to 0.29 (APP/PS1 + HFD+EAE) (p < 0.01, Fig. 8 G).
Fig. 6. Detection of Aβ₁₋₄₂ peptide in the cortex of APP/PS1+HFD transgenic mice untreated and treated with EAE through fluorescent IHC assay using 12F4 antibody. Images A, a and Aa correspond to the APP/PS1 + HFD experimental group; B, b and Bb correspond to the APP/PS1 + HFD+EAE. C) Aβ₁₋₄₂ peptide depositions was evaluated as % of immunoreactive area density. EAE treatment proved effective in a decrease of % value of Aβ₁₋₄₂ (*p < 0.05). Results were presented as mean ± SEM. Statistical analysis was performed through a Student t-test. Scale bar 100 μm.

DISCUSSION

EAE is composed of pentacyclic triterpenoids and different polyphenol derivatives, which have been demonstrated to have anti-inflammatory properties. The quinic acid and HHDP derivatives and similar polyphenols are a new contribution to the chemical composition of this plant [24–28].

Regarding the physiopathological conditions of AD, the polyphenols and triterpenoids found in the leaves of *U. molinae*, have indeed proved to be anti-inflammatory, anti-oxidant, and neuroprotective [38–42]. Specifically, in the present study, the beneficial effects of EAE on the amelioration of cognitive performance and improvement of some neuropathological AD biomarkers in female APP/PS1 mice fed with HFD have been demonstrated. Furthermore, previous studies reported higher abnormal Aβ production and deposition of amyloid plaques in the brain of females compared with male mice [32, 33]. Also, learning and memory loss has been described to be higher in female than males [32] and, the effects of obesogenic diet cause for an exacerbation of preclinical AD neuropathology. Thus, the positive results in the evaluation of the effects of EAE, at doses of 30 mg/kg, evidenced clearly its effectiveness as a potential new natural strategy to prevent AD [43].

The amelioration of cognitive performance and AD-related pathological features, such as neuroinflammation and Aβ deposition observed in APP/PS1 + HFD mice treated with EAE, supported that their chemical content may have therapeutic properties or preventive potential in a mixed model of preclinical T2DM and AD [44–48]. Its effects on microglial cells could explain part of the beneficial effects. Specifically, the content of EAE extracts in pentacyclic triterpenoids, such as corosolic, oleanolic/ursolic and its other triterpenoids (Table 2) which have been recognized to have anti-oxidant and anti-inflammatory properties [24–28, 39]. The identified phenolic acids, such as gallic acid, tannins, gallotannins (di-and tri-galloyl derivatives), ellagitannins (hexahydroxydiphenoyl HHDP and hexose derivatives), and for the first time identified, quinic acid
Fig. 7. Co-localization study of astrocytes surrounding Aβ depositions in the cortex of APP/PS1 + HFD (A, C, E) and APP/PS1 + HFD+EAE (B, D, F). Quantification of plaque-localized astrocyte size was performed in a 200 μm² field using 8-bit inverted and split channels. A and B = immunochemistry merged images for GFAP and ThS Staining. C and D = 8-bit image for GFAP and ThS and E and G = 8-bit image for Aβ depositions. Particle quantification was run on ImageJ software. It was observed that treatment with EAE reduces the astrocyte and plaque size (*p < 0.05, H, GFAP area (μm²)/cell), but the relationship of GFAP against of Aβ surface depositions remains unchanged (H). Results were represented as a scatter individual quantification values (Graph G) and mean ± SEM of the analysis of n = 3/group. Statistical analysis was performed with Student t-test. Scale bar = 50 μm.

derivatives (Tables 3 and 4) have also proven to be anti-inflammatory and anti-oxidant in models of neurodegenerative disease [49–51]. Although, the effect on astrocytic reaction is less patent due to the treatment does not change the area of GFAP covering a plaque and it seems that only Aβ load is decreased, our results clearly indicate that EAE has an inhibitory effect on the microglial cells reaction in the brain of APP/PS1 mice as general reactivity on the tissue as related to quantify of soluble or diffuse aggregates of Aβ₁₋₄₂ peptides. However, one of the limitations of our work has been not to evaluate the levels of
proinflammatory cytokines or enzymes involved in the inflammatory process such as cyclooxygenase-2 activity.

Moreover, the action of polyphenols has been associated with the prevention and treatment of dementia; supporting the results obtained in our study with the NORT that demonstrated the improvement in cognitive capabilities in WT+HFD and APP/PS1 + HFD mice treated with EAE. Other compounds present in EAE are flavonoids, such as quercetin, myricetin,
kaempferol, epicatechin/catechin (Tables 3 and 4) which showed beneficial effects against Aβ neurotoxicity [43]. The resulting data is in accordance with previous reported research that linked chronic treatment with anti-inflammatory drugs and improvement of AD models [52–55].

Nowadays, the exact etiology of AD is not fully clear, however, the amyloid hypothesis states that overproduction of soluble Aβ and aggregations of this peptide in the brain are primary responsible causes for the progression of the disease [10–14]. Thus, targeting Aβ reduction mechanisms and its aggregation is believed to be a possibly suitable strategy to improve the pathology [52–57]. It has been reported that the main component of diffuse plaques or pre-plaques are Aβ42 peptides probably the most pathologic in AD [10–14, 56–68]. In this way, EAE compounds have shown to modulate Aβ aggregation [56, 63–68]. Specifically, several phenols as digalloyl hexose, gallic acid, epicatechin/catechin, quercetin pentoside and ellagic acid, have been shown to both modulate amyloid oligomers aggregation and to protect from the neurotoxic consequences of their release [64–79]. The reduction of Aβ deposits with EAE administration supported this hypothesis [64]. On this point, reduction of microglia reactivity by EAE could be the result of a decrease in Aβ plaques.

In conclusion, the findings of this study demonstrate that the chemical composition of EAE has neuroprotective effects, observed in a mixed model of preclinical T2DM and AD (APP/PS1 + HFD) model. Obtained data suggested that improvement in cognitive functions is due to a microglial and astrocytic reactivity reduction, together with a decrease in Aβ plaque formation. Also, note that EAE improved peripheral parameters related to the process of obesity. Considering the reported relationship between obesity and cognitive loss, the effect of EAE on metabolism could also contribute to the beneficial effect of the extract in this preclinical model of AD. Posterior studies must be conducted to establish the mechanisms of how the compounds found in the EAE from U. molinae leaves are able to recover cognition and pathological parameters of AD, since it would be a novel potential therapeutic agent to prevent this neurodegenerative disease.

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