Original article

In vitro biological activity of *Salvia fruticosa* Mill. infusion against amyloid β-peptide-induced toxicity and inhibition of GSK-3β, CK-1δ, and BACE-1 enzymes relevant to Alzheimer's disease

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ABSTRACT

Salvia species have been traditionally used to improve cognition and have been proved to be a potential natural treatment for Alzheimer's disease. *Salvia fruticosa* Mill. (Turkish sage or Greek sage) demonstrated to have anticholinergic effects *in vitro*.

The aim of this study was to understand the mechanism underlying the neuroprotective effects of *S. fruticosa* infusion and its representative compound rosmarinic acid, which was detected by LC-DAD-ESI-MS/MS. The protective effects of the *S. fruticosa* infusion (SFINF) and its major substance rosmarinic acid (RA) on amyloid beta 1–42 -induced cytotoxicity on SH-SY5Y cells together with p-GSK-3β activation were investigated. Their *in vitro* inhibitory effects against glycogen synthase kinase 3β, β-secretase, and casein kinase 1δ enzymes were also evaluated.

The results showed that treatment with the all tested concentrations, SFINF significantly decreased Aβ 1–42-induced cytotoxicity and exhibited promising *in vitro* glycogen synthase kinase 3β inhibitory activity below 10 µg/mL (IC$_{50}$ 6.52 ± 1.14 µg/mL), in addition to β-secretase inhibition (IC$_{50}$ 86 ± 2.9 µg/mL) and casein kinase 1δ inhibition (IC$_{50}$ 121.57 ± 4.00). The SFINF (100 µg/mL and 250 µg/mL) also activated the expression of p-GSK-3β in amyloid beta 1–42 treated SH-SY5Y cells.

The outcomes of this study demonstrated that the *S. fruticosa* infusion possessed activity to prevent amyloid beta 1–42 -induced neurotoxicity and provided proof that its mechanism may involve regulation of p-GSK-3β protein.

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1. Introduction

Alzheimer's disease (AD) is a progressive and irreversible neurodegenerative disease with an increasing prevalence in aging populations. Amyloid beta (Aβ) aggregates and hyper-phosphorylated neurofibrillary tangles of tau represent the two main pathological hallmarks of AD (Atri, 2019). Although many pathological mechanisms have been proposed for AD, Aβ is thought to be one of the most important key molecules in the onset and progression of the disease. According to the amyloid cascade hypothesis, the increase in the Aβ fragment (1–42) (Aβ 1–42) causes oligomerization, deposition, and accumulation of Aβ. Then accumulated Aβ 1–42 results in oxidative stress and neuronal cell loss, one of the main causes of dementia with an inflammatory response by disturbing the homeostasis of the central nervous system (Busche and Hyman, 2020). The increase in β-secretase (BACE-1) levels leads to the generation and concentration of toxic forms of Aβ in the

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brain, resulting in neurodegeneration (Hu et al., 2010). The well-known kinase family members glycogen synthase kinase-3β (GSK-3β) and casein kinase-1δ (CK-1δ) have been recently shown in a variety of studies involving apoptotic neuronal cell death, Aβ 1–42 toxicity, and pathological accumulation of tau (Bjørklund et al., 2019; Eldar-Finkelman and Martinez, 2011). Since the key roles of these enzymes in the initiation and persistence of AD are understood, the search for compounds that act as BACE-1, GSK-3β, and CK-1δ inhibitors has become even more important.

Traditional and Complementary Medicine, especially treatment with medicinal plants has been an important healthcare component among some people who have limited facility with having difficulties in accessing modern medicine or who believe the effectiveness of the traditional medicine superior to modern medicine for many years (Hill et al., 2019; Jafari et al., 2020).

Numerous medicinal plants and their extracts containing several active constituents have been used to treat central nervous system (CNS)-related disorders. Among these, *Salvia* species are renowned for their reported favorable effects on memory failure, depression, and cerebral ischemia (Perry et al., 2003).

The genus *Salvia* (Lamiaceae) is represented by more than 900 species worldwide and 100 species in the Flora of Turkey (Celep et al., 2015). A comprehensive review about *Salvia* species gathered recent data on the biological activities, focusing on their anticholinesterase, anti-HIV, cytotoxic, antioxidant, antimicrobial, and antileishmanial activities (Wu et al., 2012). The main phytochemical groups, including caffeic acid monomers and dimers and other salvianolic acids; apigenin, luteolin, and their 6-hydroxylated derivatives; ursane-, oleanane-, and lupane-type triterpenes; and abietane- and neoclerodane-type diterpenes, of *Salvia* species are reported in broad and detailed reviews (Lu and Yeap Foo, 2002; Topçu, 2006).

Various *in vitro* and *in vivo* pharmacological studies, i.e., on anticholinesterase, anti-neuroinflammatory, antioxidative, Aβ degradation and Aβ producing secretase modulation activities, apoptotic mechanisms, and heavy metal binding properties, were performed to test the role of *Salvia* species in the treatment of AD (Howes and Houghton, 2003; Howes et al., 2003; Perry et al., 1999). The milestones of these studies were the proof of the effect of two *Salvia* species, *S. officinalis* L. (sage) and *S. lavandula* Alain (Spanish sage), on the improvement in cognitive functions when clinically tested in different types of AD (Akhondzadeh et al., 2003; Kennedy et al., 2011; Perry et al., 2018; Perry et al., 2003; Tildesley et al., 2003). Additionally, over the past 20 years, almost all of the *Salvia* species that grow in Turkey have been studied in terms of anticholinesterase activities, and it is demonstrated that the activities of these plants are due to their high content of phenolic compounds, especially phenolic acids along with some volatile constituents (Demirezer et al., 2015; Kolak Ufuk et al., 2009; Orhan et al., 2007; Orhan et al., 2012; Şenol et al., 2010). Among the species tested, *S. fruticosa* has been shown to be remarkably effective in acetylcholinesterase and butyrylcholinesterase inhibition (Şenol et al., 2011).

This background on *Salvia* species prompted us to investigate the neuroprotective effects of *S. fruticosa*, which is one of the most important herbal teas traditionally consumed throughout Mediterranean countries.

In the present study, to understand the neuroprotective effects of *S. fruticosa* infusion (SFINF) on the SHSY5Y cell line, different cell-based assays were performed in addition to examination of its *in vitro* enzyme (GSK-3β, CK-1δ, and BACE-1) inhibitory properties related to AD. Molecular docking studies of the predominant compounds, including rosmarinic acid (RA), were carried out in the active sites of GSK-3β to predict the most appropriate binding modes and support the experimental data

2. Materials and methods

2.1. Plant material, extract preparation, and determination of phenolic compounds

Aerial parts of *S. fruticosa* were collected from Antalya; Kemer, Küçükburun region, *Pinus brutia* forest, at 50 m, May 2013. Assoc. Prof. Dr. Mehmet Yavuz Paksoy identified the plants, and a voucher specimen (PAKSOY 1470) has been deposited in the Herbarium of Fırat University, Elazığ, Turkey. The dried and crude powdered aerial parts of *S. fruticosa* were immersed in 100 mL of boiling water for 5 min three times (each 10 g). After filtration, the infusions were combined and lyophilized until used for further assays (SFINF-yield 12%).

All the equipment and the chromatographic separation, elution, detection, and data collection methods used were the same as those described previously by Gürbüz et al. (2019).

2.2. Biological activity and molecular docking simulations

2.2.1. In vitro GSK-3β, CK-1δ, and BACE-1 inhibition

The method described by Baki et al. (2007) was used for the inhibition of GSK-3β. A Kinase-Glo Kit was used to measure the activity of the SFINF and RA versus CK-1δ and GSK-3β. The inhibitory activity was calculated on the basis of maximal activities measured in the absence of inhibitor. The BACE-1 assay, which was based on FRET methodology, was performed according to the manufacturer protocol available from Invitrogen (L0724). The IC_{50} was calculated as the concentration of each compound that reduces the enzymatic activity 50% with respect to that without inhibitors. The results were represented as the mean ± standard deviation from three independent experiments ($n = 3$).

2.2.2. Molecular docking

The chemical formulas of the three predominant compounds (RA, luteolin 7-*O*-glucuronide, and caffeic acid) were drawn in ChemBiodraw Ultra 12.0. The published crystal structure of GSK-3β complexed with the co-crystallized ligand AR-A014418 (N-(4-methoxybenzyl)-N′-(5-nitro-1,3-thiazol-2-yl)urea) was obtained from the Protein Data Bank (PDB code: 1Q5K) (Bhat et al., 2003). The compounds were docked into the catalytic site of the enzyme with Autodock 4.2 (Morris et al., 2009), integrated into LigandScout 4.2, using default parameters. All results obtained were ranked according to their binding affinities (kcal/mol) and the best docking pose of each compound was selected for further visual evaluation. The molecular docking figures were generated using Maestro (Schrödinger, 2019).

2.2.3. Neuroprotection

2.2.3.1. General.
For the neuroprotection assays and Western blot analysis, the SH-SY5Y (neuroblastoma) cell line was purchased from the American Type Culture Collection (ATCC, CRL 2266) and cultivated in DMEM F-12, containing FBS 10%, 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were seeded on a 96-well plate, 96-well E-plate, and 6-well plate for the Sulforhodamine B assay, neuroprotection assay, and Western blot analysis, respectively. The amyloid beta peptide (1–42) was purchased from Anaspec (Cat: AS-20276), Sulforhodamine B was purchased from Santa Cruz (3520-42-1), and SB216763 was purchased from Sigma-Aldrich as a positive control for the biological assays.

2.2.3.2. Sulforhodamine B (SRB) cell viability assay.
The SRB colorimetric method was used (Vichai and Kirtikara, 2006) after 24 h incubation of the SH-SY5Y cells with different concentrations of the SFINF (500, 250, 100, 50, 10 µg/mL) and RA (500, 250, 100,

50, 10 μM) to determine the nontoxic dose. Final absorbance was measured at 492 nm using a multifunctional microplate reader (BioTek Gen 5, USA). All the measurements were obtained at least in triplicate and IC_{50} values were calculated to assign nontoxic concentrations of the SFINF and RA (data not shown).

2.2.3.3. Real-time cell electronic sensing system. The neuroprotective effects of the SFINF on Aβ 1–42 treated and untreated SH-SY5Y cells were monitored with a real-time cell analyzer (RTCA; Roche Applied Science, Mannheim, Germany) as described by Hou et al. (2014) with slight modifications. A well-known GSK-3β inhibitor, SB216763, was used as a positive control. The RTCA monitors the impedance changes elicited by the cell index (CI) in a real-time manner, which enables concentration–activity curves to be maintained, dependent on the time point or period of time chosen. To adjust the ideal cell number in wells, 6250, 12500, 25000, and 50,000 cells/well were seeded. After half an hour of incubation at room temperature, E-plates were placed into the RTCA station. The electrical impedance was measured every 15 min by the RTCA associated software of the xCELLigence system as a dimensionless parameter termed CI. Based on the growth curve and CI values of the SH-SY5Y cells, 12,500 cells/well were selected for further analysis.

2.2.3.4. Preparation of Aβ 1–42. Briefly, the Aβ 1–42 peptide was dissolved in 1 mM in hexafluoroisopropanol (HPIF, Sigma-Aldrich) and aliquoted in sterile microcentrifuge tubes. The HPIF was then withdrawn under vacuum and stored at −20 °C. Aβ 1–42 peptide fibril formation was achieved based on the protocol explained by Dahlgren et al. (Dahlgren et al., 2002).

2.2.3.5. Cytotoxicity test of Aβ 1–42. First 12,500 cells/well were seeded in the 96-well E-plate and then 24 h post-seeding when the cells were in the log growth phase the cells were treated with different concentration of Aβ 1–42 (22, 11, 5, 2.5 μM) to determine the optimal Aβ 1–42 concentration for the assay and 22 μM Aβ 1–42 was selected for further studies (data not shown).

2.2.3.6. Effects of the SFINF and RA against Aβ 1–42 damage in SH-SY5Y cells. SH-SY5Y cells (12500 cells/well) were seeded in a 96-well E-plate. After complete adhesion for 24 h, the medium was replaced with fresh medium. The cells were treated with 22 μM Aβ 1–42 and nontoxic concentrations of the SFINF (250, 100, 50, and 10 μg/mL) and RA (50 μM and 100 μM) subsequently. Data points were collected in at least triplicate every 15 min throughout the duration of the experiments.

2.2.3.7. Western blot. Western blot analysis was carried out using crude lysates of SH-SY5Y human neuroblastoma cells. After being treated with the SFINF in 250 and 100 μg/mL concentrations for 24 h the cells were lysed in commercial RIPA lysis buffer (Santa Cruz sc-24948). The lysate was centrifuged at 4 °C for 30 min at 12000 rpm. The clear supernatant was collected and the total protein amount was determined by the Lowry method (Hartree, 1972). Next 30 μg of protein lysates were resolved on 10% sodium dodecyl sulfate–polyacrylamide (SDS-PAGE) gels and then electro-transferred onto polyvinylidene difluoride (PVDF) membrane. After being blocked with 5% nonfat milk in Tris-buffered saline (TBS, 0.1 M, pH 7.4) the membranes were incubated with primary antibodies, namely anti-GSK-3β (1:1000 dilution, Cell Signaling Technology (CST), 9315S), anti-Phospho-GSK-3β (Ser9) (1:1000 dilution, CST, 9336S), and anti-β actin (1:1000 dilution, CST, 4967S). After overnight incubation of the primary antibodies the membranes were washed three times for 15 min and were incubated with a secondary antibody, HRP-conjugated goat anti-rabbit secondary antibody (1:10.000 dilutions, Jackson Immunore-

search 111–035–144), for 2 h at room temperature. Protein bands were detected by enhanced chemiluminescence (ECL, Santa Cruz Biotechnology, CA, USA) on XO-MAT film. SB216763 was used as a positive control.

3. Results

3.1. Phenolic compounds of the SFINF

The chemical profile of the SFINF, which was determined by LC-DAD-ESI-MS/MS, is summarized in Table 1. The phytochemical examination demonstrated that the constituents attributed to the SFINF are in agreement with the published literature for *S. fruticosa* and for the genus *Salvia* (Koutsoulas et al., 2019; Vergine et al., 2019). Flavone and flavonol derivatives together with caffeic acid derivatives were detected as the main groups for the SFINF. The phytochemicals were determined from their retention times and observed $[M−H]^-$ ions with supportive UV spectra and MS/MS interpretation in comparison with the relevant literature. When considering the UV chromatogram of the SFINF, it is apparent that RA (**1**) is the major element of the SFINF. Thus, RA was selected as a major and representative molecule for the rest of the activity assays. The other predominant compounds are luteolin 7-*O*-glucuronide (**2**) and caffeic acid (**3**) (Fig. 1).

3.2. In vitro GSK-3β, BACE-1, and CK-1δ inhibitory activities

To understand the *in vitro* inhibitory effects of the SFINF, GSK-3β, BACE-1, and CK-1δ were selected as the key enzymes related to AD. In particular, the SFINF possessed promising GSK-3β inhibitory activity below 10 μg/mL (IC_{50} 6.52 ± 1.14 μg/mL), followed by BACE-1 inhibition (IC_{50} 86 ± 2.9 μg/mL) and CK-1δ inhibition (IC_{50} 121.57 ± 4.00). Consequently, the SFINF had overall higher inhibitory activity on GSK-3β compared to the other two enzymes.

3.3. Molecular docking studies

The SFINF was tested for its inhibitor activity on three different enzymes (GSK-3β, BACE-1, and CK-1δ) that play roles in the progression of AD and was found to be most effective as an inhibitor of GSK-3β. Therefore, molecular docking studies were performed to gain insights as to whether one of the predominant compounds in the SFINF is mainly responsible for GSK-3β inhibition. With this aim, three predominant compounds (RA, luteolin 7-*O*-glucuronide, and caffeic acid) were docked into the active pocket of the enzyme. We additionally re-docked the co-crystallized ligand (AR-A014418) present in the crystal structure of GSK-3β and compared the result with its original pose by using the root-mean-square deviation (RMSD) value. It was found to be 1.06 and in this way we also checked the reliability of our docking parameters.

The obtained docking results of the three compounds were ranked according to their estimated binding energies (kcal/mol) and each ligand with the best docking score (the lowest binding energy) was selected for further visual evaluation.

According to the docking poses provided in Fig. 2, caffeic acid partially occupied the binding pocket of the enzyme, as it is a less bulky compound compared to the others. It is not surprising that its binding energy was higher (-11.43 kcal/mol) than the values calculated for RA (-14.62 kcal/mol) and luteolin 7-*O*-glucuronide (-13.98 kcal/mol).

As RA has the best docking score with the lowest binding energy among those three compounds, we examined its binding mode to GSK-3β compared to the original ligand. We observed that RA has a similar orientation to AR-A014418 in the active site of GSK-3β (Fig. 3).

Table 1
Phenolic compounds tentatively identified in the SFINF.

Tentative identification	Rt (min)	[M−H]⁻	MS² (m/z)	Ref.
Apigenin di-C-hexoside	16.9	593	473, 383, 353	(Dias et al., 2013)
Caffeic acid	**20.2**	**179**	**135**	**std**
Luteolin 7-O-glucuronide	**21.1**	**461**	**285, 175**	(Cvetkovikj et al., 2013)
Salvianolic acid E/B	21.7	717	555, 519, 475, 359, 313, 295	(Barros et al., 2013)
Isorhamnetin glucuronide	21.9	491	315, 299	(Spínola et al., 2015)
Caffeic acid derivative	22.2	487	323, 179, 161, 133	(Helmja et al., 2008)
Apigenin glucuronide	22.6	445	269, 175, 113	(Guimarães et al., 2013)
Isorhamnetin glucoside	22.9	477	315, 300, 284, 175, 113	(Kontogianni et al., 2013)
Isosalvianolic acid	23.0	493	359, 313, 295, 197, 179, 161	(Barros et al., 2013)
Rosmarinic acid	**23.06**	**359**	**197, 161, 135**	(Lu and Yeap Foo, 2002)
Luteolin	26.9	285	133	(Lu and Yeap Foo, 2002)
Isorhamnetin	27.4	315	300, 297, 137	(Lu and Yeap Foo, 2002)
Salvianolic acid F	28.16	313	161, 133	(Lu and Yeap Foo, 2002)
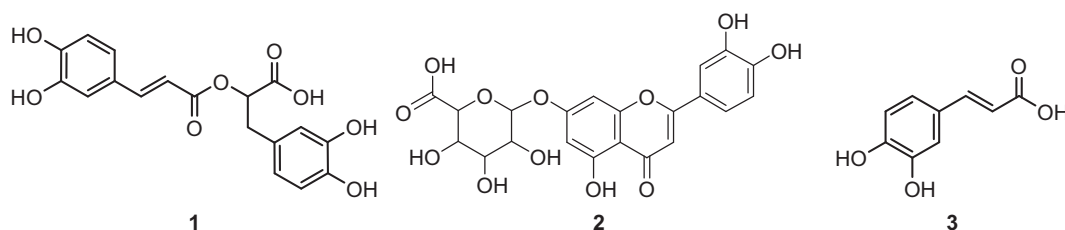
Fig. 1. The major compounds in the SFINF.
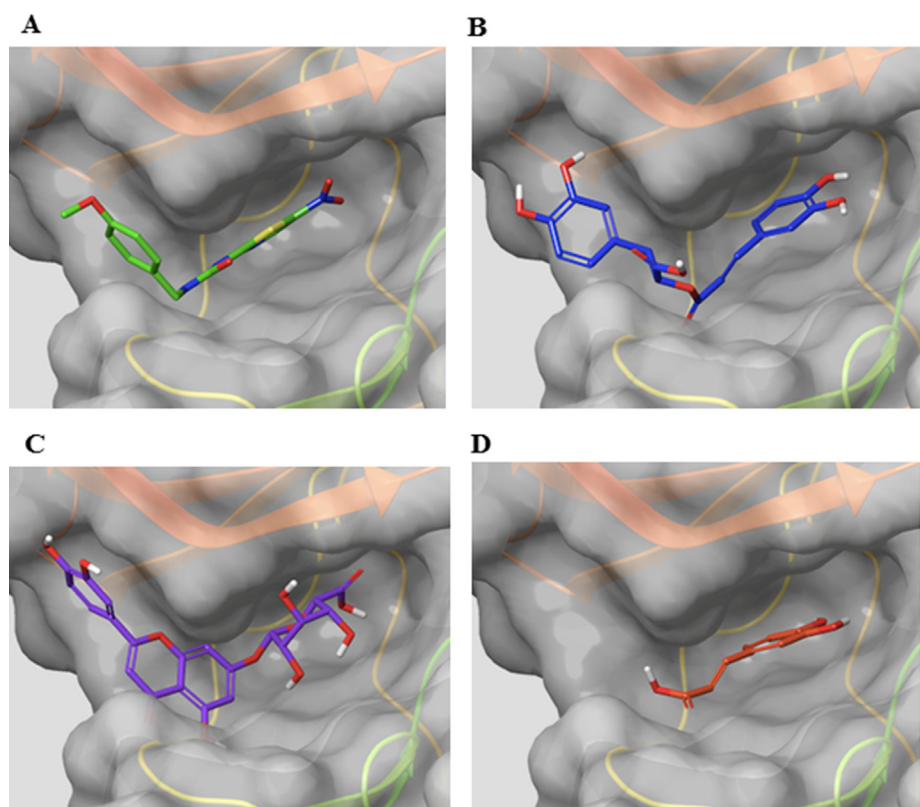
Fig. 2. Binding modes of the co-crystallized inhibitor AR-A014418 (A), RA (B), luteolin 7-O-glucuronide (C), and caffeic acid (D) in the binding pocket of GSK-3β. Ligands are shown as green, blue, purple, and orange sticks for AR-A014418, RA, luteolin 7-O-glucuronide, and caffeic acid, respectively.

Based on these findings, we decided to determine the GSK-3β inhibitory activity of RA as the major component of SFINF. However, RA did not inhibit GSK-3β effectively with IC$_{50}$ > 100 μM. This result suggested that RA as the representative compound of the SFINF was not primarily responsible for the GSK-3β inhibition. We surmise that the synergistic effect of the compounds present in the SFINF can lead to this effect in the treatment of AD through GSK-3β inhibition.
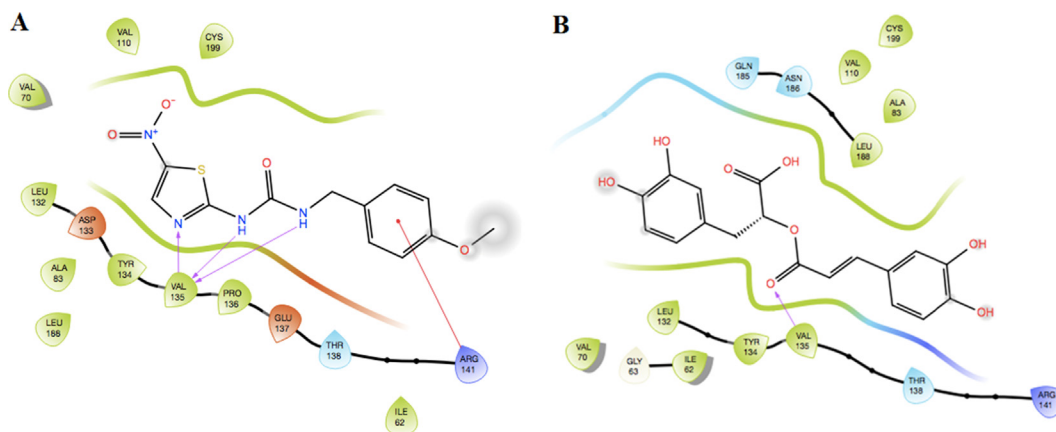
Fig. 3. 2D interactions of AR-A014418 (A) and RA (B) in the active site of GSK-3β.

3.4. Neuroprotective effect of SFINF extract on Aβ 1–42 neurotoxicity

To determine the neuroprotective effects of different concentrations prepared from the SFINF and RA in the presence/absence of Aβ 1–42 on SH-SY5Y cells, an impedance-based cell profile assay was carried out. The results are shown in Fig. 4. Each colored trace shown in the figure represents the impedance of cells calculated from the RTCA. Fig. 4 shows that the treatment of SH-SY5Y cells with Aβ 1–42 for 36 h significantly suppressed cell proliferation as determined by the RTCA at a concentration of 22 μM (**A**). In the same figure all concentrations of the SFINF, 100 μM of RA, and the positive control stimulated cell proliferation in a nondose-dependent manner. The cells were shown to grow steadily within the dose range of 10–250 μg/mL. A dose of 50 μM RA

exerted a slightly inhibitory effect on cell proliferation (B). All of the concentrations of the SFINF completely reversed the suppressive effect of Aβ 1–42 on cell proliferation and enhanced cell proliferation from 35% to 70% after incubating for 10 h. No significant differences were observed between 50 μM RA and 5 μM SB216763 neuroprotection when compared to the Aβ 1–42 treated control (**C**).

3.5. p-GSK-3β Activation

To investigate whether SFINF and Aβ 1–42 affect p-GSK-3β and GSK-3β expression in SH-SY5Y cells, Western blot analysis was performed. To evaluate activation, the p-GSK-3β to GSK-3β ratio was determined. Total protein lysate was separated by SDS-PAGE
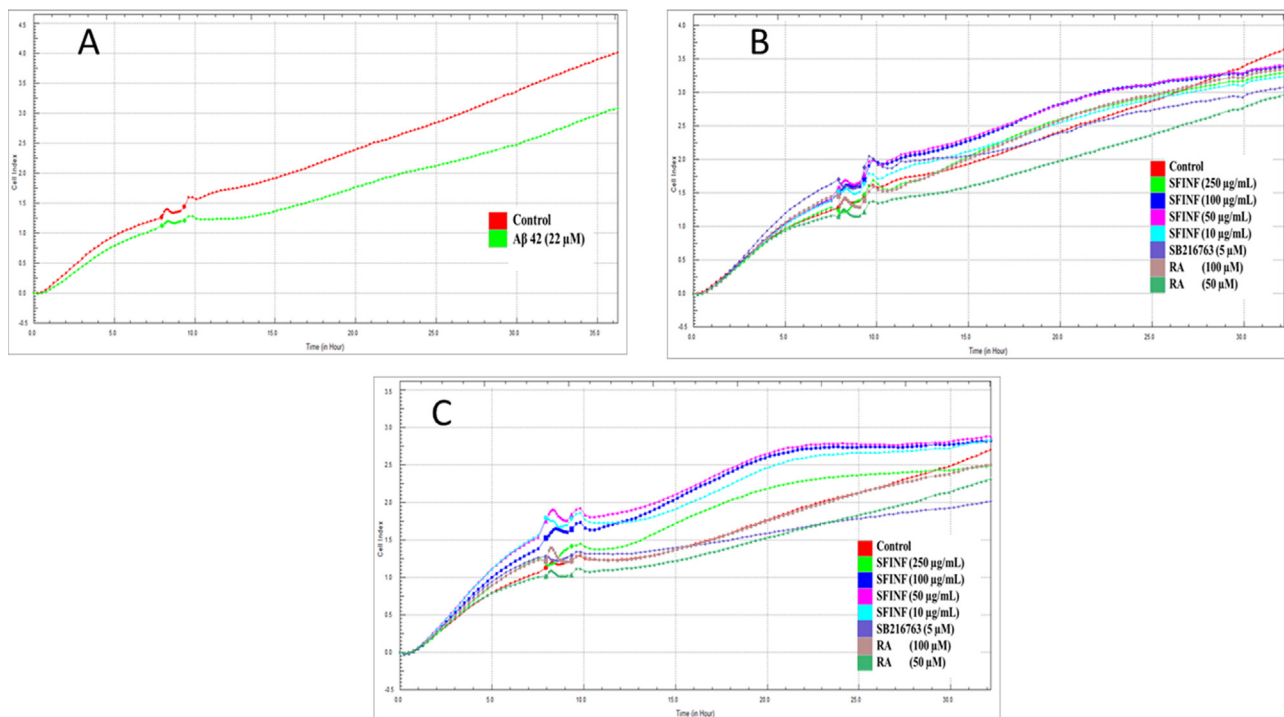

Fig. 4. Dynamic monitoring of cytotoxic responses of Aβ 1–42 to cell proliferation. SH-SY5Y cells were treated with Aβ 1–42 at a concentration of 22 μM. Aβ 1–42 reduced the proliferation of SH-SY5Y cells to approximately 30% 24 h after the treatment (**A**). Dynamic monitoring of cytotoxic responses of samples to cell proliferation. SH-SY5Y cells were treated with different concentrations of the SFINF, RA, and SB216763. None of the tested samples reduced the cell proliferation of the cells prominently 24 h after treatment. SFINF (250 μg/mL) and RA (100 μM) caused a slight increment in the cell index after treatment (**B**). Dynamic monitoring of protective responses of samples to SH-SY5Y cells in the presence of 22 μM Aβ 1–42. SH-SY5Y cells were treated with Aβ 1–42 and different concentrations of the SFINF, RA, and SB216763 (**C**).

(25 µg/lane), electro-transferred to PVDF membrane, and probed with antibody to GSK-3β and p-GSK-3β. The decrease in the p-GSK-3β/GSK-3β ratio caused by Aβ 1–42 was reversed by the SFINF in a dose-dependent manner at 100 and 250 µg/mL (Fig. 5).

4. Discussion

The aggregation of Aβ peptides from 40 to 42 amino acids is broadly accepted to be one of the main elements responsible for AD (Espargaró et al., 2017). The correlation between BACE-1, GSK-3β, and CK-1δ and the pathological accumulation of tau and formation of Aβ 1–42 represents an unconventional and promising approach for the prevention and therapy of AD. Consequently, the spotting of potential inhibitors of amyloid aggregation and related enzymes has recently attracted much interest.

S. fruticosa is widely distributed in Mediterranean countries and has a less bitter taste than *S. officinalis*. It is frequently consumed as an herbal tea prepared as an infusion, and also has economic value as a source of essential oil and as a crude drug for the local population.

The present study revealed for the first time that SFINF can protect SH-SY5Y cells against Aβ 1–42-induced toxicity. This conclusion is supported by the following evidence: 1) the SFINF prevented Aβ 1–42-mediated neurotoxicity in cultured SH-SY5Y cells; 2) the SFINF inhibited BACE-1, GSK-3β, and CK-1δ *in vitro* with a varying IC$_{50}$ value under 125 µg/mL; 3) the SFINF increased phospho-GSK-3β levels; 4) a possible synergistic effect of the compounds present in the SFINF can lead to a neuroprotective effect through GSK-3β inhibition.

Herein, all of the concentrations of the SFINF completely reversed the suppressive effect of Aβ 1–42 on SH-SY5Y cell proliferation and enhanced cell proliferation from 35% to 70%, while there was no apparent change after the treatment with RA compared to the control.

To understand the neuroprotective mechanism, the SFINF was tested for its inhibitory effect on GSK-3β, BACE-1, and CK-1δ enzymes. The SFINF possessed promising GSK-3β inhibitory activity below 10 µg/mL (IC$_{50}$ 6.52 ± 1.14 µg/mL), followed by BACE-1 inhibition (IC$_{50}$ 86 ± 2.9 µg/mL) and CK-1δ inhibition (IC$_{50}$ 121.57 ± 4.00 µg/mL). Based on these results it can be suggested that moderate CK-1δ and BACE-1 inhibitory properties of SFINF in addition to the strong GSK-3β inhibitory potential play important roles in the protection of neurons from Aβ-induced toxicity and contribute to the anti-Alzheimer effects of the SFINF together. The *in vitro* GSK-3β inhibitory potential of the SFINF was also supported by the excessive expression of p-GSK-3β with the Western blot analysis and this effect was stronger than that of the well-known GSK-3β inhibitor SB216763. The phytochemical examination led to the separation and identification of thirteen compounds in the SFINF. Based on the UV chromatogram of the SFINF, RA was detected as the main compound in the extract, followed by luteolin 7-*O*-

Fig. 5. Effect of SFINF (100 and 250 µg/mL) on Aβ 1–42-induced GSK-3β activation. Characteristic Western blot analysis of p-GSK-3β and GSK-3β in SH-SY5Y cells; treated with Aβ 1–42 (22 µM) and in the presence/absence of the SFINF and positive control (SB216763).

glucuronide and caffeic acid. Prompted by the molecular docking studies, we additionally tested RA solely for its *in vitro* GSK-3β inhibitory potential but its inhibition value suggested that, as the major component of the SFINF, RA is not responsible on its own for the strong GSK-3β inhibition effect of the infusion.

In a recent study, caffeic acid, the monomer of RA, and many other salvianolic acids were reported to decrease Aβ-induced tau phosphorylation (Sul et al., 2009). In other research, it was indicated that RA prohibits the fibrillization and decreases vibrational modes incorporated with the β sheet in tau protein linked to AD (Cornejo et al., 2017). While the complete mechanisms responsible for Aβ-mediated neurotoxicity remain unclear, various studies have shown that Aβ causes reactive oxygen species (ROS) generation and ROS induce DNA injury, enzyme oxidation, lipid peroxidation, and cell apoptosis. Thus, ROS also have been included in AD pathologies and are an important target in AD and related irregularities. Using antioxidants should be a rational and promising approach to alleviate oxidative-dependent and Aβ-caused cytotoxicity (Zhang et al., 2016). In addition to the enzyme inhibition properties of the SFINF, the phenolic pool of the SFINF with its well-known strong antioxidant effects can contribute to the neuroprotective effect.

Several cell-based studies revealed that *S. officinalis* and RA protected PC12 cells from Aβ 1–42 toxicity through different mechanisms (Iuvone et al., 2006), while radix *S. miltiorrhiza* has been proved to revise the Aβ 25–35-induced cytotoxicity in PC-12 cells (Zhou et al., 2011) *in vitro*. Shortly before it is demonstrated that different *S. fruticosa* extracts exhibited a pronounced neuroprotective effect against Aβ 25–35 toxicity in combination with their significant antioxidant potential (Ververis et al., 2020). Salvianolic acid B (SalB), one of the active antioxidant depsides from *S. miltiorrhiza*, has been shown to reduce Aβ 1–40 fibril formation and aggregation in addition to the protection of SH-SY5Y cells from Aβ 1–42-induced cell death *in vitro* (Durairajan et al., 2008; Tang and Zhang, 2001) and *in vivo* (Lee et al., 2013). Wang et al. (Wang et al., 2010) discovered that SalB exerted neuroprotective effects via anti-inflammatory properties on microglia cells. Furthermore, SalB can mediate the GABAergic neurotransmitter system to improve Aβ 25–35-induced memory impairment (Kim et al., 2011). Recently, it was demonstrated that SalB inhibits Aβ 40 and Aβ 42 generation modulating BACE-1 activity in SH-SY5Y-APPsw cells (Tang et al., 2016). In addition to salvianolic acids, many studies have demonstrated that flavonoids and their derivatives have numerous neuroprotective and neuro-inflammatory activities through various pathways related to AD (Balez et al., 2016; Rezai-Zadeh et al., 2008). More recently, some common flavonoids, i.e., kaempferol, myricetin, quercetin, and apigenin, together with phenolic compounds, i.e., curcumin and RA, were screened for Aβ 42 aggregation inhibitory potential in bacterial cells. In that study, apigenin and quercetin showed potent anti-aggregation activity, while RA displayed low and curcumin, a diarylheptanoid compound, showed moderate activity (Espargaró et al., 2017).

Supporting these related data in the literature, our study revealed that the SFINF protected SH-SY5Y cells from Aβ 1–42-induced neurotoxicity *in vitro* and, to the best of our knowledge, this is the first study to show this preventive effect in a real-time manner that can be basically linked to its GSK-3β inhibitory effect. Consequently, to interpret all the *in vitro* neurobiological activity results and the phytochemical data, it should be stated that RA not only itself but also phenolic compounds, especially flavones, and monomers and dimers of phenolic acid derivatives together may play a synergistic role in the anti-Alzheimer effects of the SFINF. The results of the present study validate the traditional use of *Salvia* species for cognitive disorders and highlight the
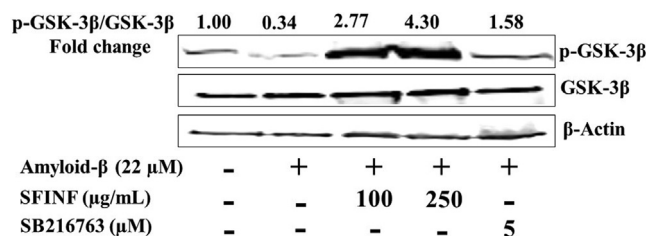
mechanisms underlying the neuroprotective effects on Aβ 1–42-induced neurotoxicity via different mechanisms for the first time.

5. Conclusion

We investigated the neuroprotective effects and potential molecular mechanisms of *Salvia fruticosa* infusion and rosmarinic acid against Aβ 1–42 toxicity in cultured SH-SY5Y neurons. The SFINF exhibits significant protection against Aβ 1–42-induced cytotoxicity, being superior to rosmarinic acid. The additional ability of inhibiting glycogen synthase kinase 3β CK-1δ and β-secretase and increasing of p-GSK-3 beta protein levels makes the *Salvia fruticosa* infusion a promising multifunctional neuroprotective agent for further studies involving the development of new anti-Alzheimer agents from natural sources.

However, one of the limitations of the present study is that the phytochemical profile could change according to different ecosystems, which requires a standardization of the major compounds for repeatable qualitative and quantitative results and for repeatable *in-vivo* studies indeed. Another limitation is that the concentrations used in the biological assays are not of physiologic relevance and future studies should be performed considering blood–brain barrier permeability and the ADME properties of *Salvia fruticosa* infusion.

Author contributions

PG designed the main study and PG and AHD performed the neurobiological assays; MBY directed the optimum *in vitro* assay conditions and concentrations in cell culture related experiments; FG carried out the LC-MS/MS experiments; MGG performed and interpreted the molecular docking studies, MYP provided the plant material; PG prepared the manuscript; CP contributed to experimental consumables of *in vitro* enzyme assays and interpreted the results; LÖD proposed the plant material; MGG, MBY, and LÖD performed the critical readings.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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