Enhanced neuroprotection against Alzheimer's disease by combining *Inula viscosa* with curcumin: an *in vitro* study

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Abstract

Introduction: Alzheimer's disease (AD), the most prevalent type of dementia among older individuals, is characterized by severe neurodegeneration with symptoms of progressive loss of cognitive capacity. We aimed to investigate the effects of curcumin (Curc) and *Inula viscosa* (IV) plant extracts on apoptosis and oxidative stress in an amyloid-β (Aβ)-induced *in vitro* AD model.
Material and methods: The human glioblastoma cell line (U87) was treated with Aβ to produce an *in vitro* AD model. Treatment groups were: Aβ + Curc, Aβ + IV, and Aβ + IV + Curc. Cell viability assay was performed to analyze cytotoxicity. Caspase-3 (CASP3) levels were measured to evaluate apoptosis. For oxidative stress, glutathione peroxidase (GPx), catalase (CAT), superoxide dismutase (SOD), total antioxidant (TAS), and total oxidant (TOS) levels were analyzed spectrophotometrically.
Results: Combinatory application of IV and Curc post-Aβ treatment increased cell viability of U87 cells. Aβ-upregulated CASP3 levels were markedly reduced upon IV and Curc co-treatment. Anti-oxidant parameters, i.e., SOD, CAT, GPx, and TAS levels, were significantly elevated in the IV + Curc-treated group compared to the Aβ group. The elevated TOS level after Aβ exposure returned to its basal level upon Curc + IV co-treatment.
Conclusions: IV + Curc co-treatment reduced apoptosis-related neurodegeneration and supported antioxidant activity in an Aβ-induced AD model of U87 cells. IV and Curc co-treatment can be a promising therapeutic strategy against AD.

Key words: neurodegeneration, cell culture, plant extract, U87 cell line.

Introduction

Alzheimer's disease (AD), the most prevalent type of dementia among older individuals, is a disease characterized by severe neurodegeneration

that manifests as symptoms of progressive loss of cognitive capacity [1]. Currently, the most prominent goal for therapeutic interventions against AD is to inhibit amyloid-β (Aβ)-induced neurotoxicity by blocking Aβ aggregation and the inflammatory response and to inhibit Aβ and amyloid precursor protein (APP) production [2]. Currently, since the drugs used for treatment are not sufficient in AD management and are only to relieve symptoms, scientists seek new effective molecules that can be used as alternative approaches. In the search for new treatment methods, plants are primarily considered due to their biologically active substances [3].

Inula viscosa (Asteraceae, abbreviated as IV) is an evergreen plant commonly found in the Mediterranean Basin [4]. As a medicinal plant, it has been used in treatments since ancient times due to its sedative, antipyretic, anti-inflammatory, and antiseptic effects [5]. Chemical elements such as azulenes, sesquiterpenes, essential oils, and flavonoids have been isolated from IV. In particular, its essential oils and sesquiterpenes (carboxy eudesmadiene) have been shown to have antifungal activity [5]. In a study by Talib *et al*. [6], the antimicrobial and antiproliferative effects of IV were evaluated. Among the flavonoids isolated from the plant, methylated quercetin was reported to exert the most anticancer and antimicrobial activities [6]. In another study, the cytotoxic activity of IV extracts was evaluated in two breast cancer cell lines. IV has been shown to have a significant cytotoxic effect on MDA-MB231 and MCF7 human breast cancer cell lines [7]. This cytotoxic activity has been attributed to the inuviscolide, tomentosin, and isocostic acid compounds in IV by the authors.

Curcumin (Curc), a turmeric biocomponent, is a pigmented substance with powerful antioxidant, anticancer, antibacterial, and anti-inflammatory actions [8]. It is used in the treatment of many disorders, namely diabetes, hypertension, open wounds, and gastrointestinal disorders [9]. In a previous study, the complex of Curc and Cu^{2+} used to prevent Curc-binding Aβ fibrils was explored in two segments of the Aβ protein, and Curc was found to act as a chelator by binding to Cu^{2+} and Aβ. Therefore, Curc was suggested to be utilized as an alternative medicine to prevent or treat AD [10].

The effects of IV extracts on AD have not been reported in the literature, and no previous studies have been conducted on this subject. For the *in vitro* model of AD, the U87 cell line was picked based on the previous literature. In brief, the U87 human glioblastoma cell line was utilized to model the cellular dynamics of AD owing to its high uptake capacity for Aβ aggregates [11, 12]. Additionally, to analyze the effect of Aβ1-42 on proliferation, cell death, ROS production, and senescence, U87 cells were used in previous studies [13, 14]. Wang *et al*. reported that Aβ-induced ROS accumulation promoted autophagic cell death in the U87 astrocytoma cell line [15].

In the present study, we investigated the synergistic effects of Curc and IV plant extracts on the Aβ-treated U87 cell line as an alternative treatment strategy against AD. For this purpose, we evaluated the prominent players involved in oxidative stress and apoptosis such as total antioxidant (TAS), total oxidant (TOS), glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT), and caspase-3 (CASP3). Additionally, we aimed to examine whether IV and Curc plant extracts have cytotoxic effects on the AD cell line model and their effects on cell proliferation using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. *In vitro* studies like ours will shed light on future studies and make an important contribution before planning an *in vivo* study with these bioactive substances.

Material and methods

Classification and extraction of *Inula viscosa* plant

IV was collected from the campus of Hatay Mustafa Kemal University during the summer season (June-August) in Antakya, Hatay, Türkiye. The IV plant was identified, classified, and recorded by Prof. Dr. İhsan Uremis (Herbarium no. H-0031). The upper part of the IV plant was dried in an oven for 24 h, and the dried plant was extracted in 80% ethyl alcohol solution (Merck SA, Darmstadt, Germany) and filtered. The alcohol of the filtered extract was evaporated and the extract remained. The obtained extract was stored at –20°C [16, 17].

Characterization of *Inula viscosa* plant extract with GC-MS analysis

The composition of IV plant extract was assessed using the gas chromatography (GC)-mass spectrometry (MS) method as described previously [18]. Briefly, we used a TG-Wax MS model column with helium gas as the carrier (ISQ Single Quadrupole, Thermo Fisher Scientific, Waltham, MA USA). MS ionization and transfer line temperatures were adjusted to 220°C and 250°C respectively. Column temperature was set to rise between 50 and 220°C (3°C/min rise, split ratio: 10 : 1, injection volume: 1 µl). For MS, scan mode was utilized (mass spectra recording: 70eV, mass range: 1.2–1200 m/z, software: Xcalibur Report program, Thermo Fisher Scientific, Waltham, MA USA). Compounds of the plant extract were compared using their retention indices (RI) and Wiley library database (Wiley Interscience, New York,

USA). The RI values of the compounds were obtained using *n*-alkane standard solution homologous series (C8-C20, C21-C40).

Cell culture

The U87 human glioblastoma cell line was obtained commercially from the American Type Culture Collection. The cells were propagated as described earlier [19]. Cell medium was prepared from Dulbecco's Eagle's Medium (DMEM, Thermo Fisher Scientific, Waltham, MA USA) and glutamine modification. Fetal Bovine Serum (FBS, Thermo Fisher Scientific, Waltham, MA USA) was added to the medium in addition to 4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid (HEPES). The complete cell medium contained 10% FBS (v/v) and 25 mM HEPES. Using this prepared medium, the cells were propagated in flasks. The confluence of the U87 cell line (ATCC number HTB 14) kept in a CO_2 incubator (Heracell 150i, Thermo Fisher Scientific, Waltham, MA USA) for 48 h was observed with light microscopy, and the cells were seeded into cell culture plates after passaging from confluence flasks for further experiments.

Experimental groups

We divided the U87 cells into 5 groups: The first group (control) received 50 µl of saline for 48 h incubation. The second group (Aβ) was treated with Aβ1-42 (Sigma Aldrich, Darmstadt, Germany) for 48 h incubation to obtain an *in vitro* AD model [13]. The third group (Aβ + Curc) was initially treated with Aβ1-42 for 48 h and then further exposed to Curc (Sigma Aldrich, Darmstadt, Germany) [20] for an additional 24 h incubation. The fourth group (Aβ + IV) received IV extract for 24 h following 48 h Aβ1-42 treatment. The fifth group (Aβ + Curc + IV) was induced with Aβ1-42 for 48 h and then was co-treated with Curc and IV extract [16] for an additional 24 h incubation. The concentrations of the reagents were as follows: 5 µM Aβ1-42 [13], 40 µM Curc [20], and 300 µg/ml IV extract [16].

Cell viability

MTT assay was utilized to assess the cell viability *in vitro* [21]. Cell counting of the U87 cell line was performed and cell viability was checked by the trypan blue exclusion test. Cell viability was determined by placing 10 µl of cells + 10 µl of trypan blue on the slide and counting non-dyed cells when examined under a light microscope. The percentage of cells that did not receive dye was calculated by counting one hundred cells in the field. Then, cells were prepared with a medium so that there were 10^4 cells per ml in the cell line. For each complex, 3 ml of cell suspension was placed in 7 wells of a separate 12-well culture plate. All plates were left to incubate for 24 h in a CO_2 incubator at 37°C in an environment containing 5% CO_2. After the incubation, the cell and medium in the first well and the solvent containing dimethyl sulfoxide (DMSO) in the second well were used as controls, in addition to the cell concentrations we added to the 12-well plates containing U87 cell lines. After incubation of U87 cells in three groups as described above, 100 µl of cell suspension/well was seeded into 96-well cell culture plates. Then, 10 µl of MTT solution was added to each well for 4 h incubation. The MTT solution was prepared by dissolving 5 mg/ml in phosphate-buffered saline (PBS) and transferring it to a vial by sterile filtration. Then, 100 µl of DMSO was added to the wells and left in a CO_2 incubator at 37°C for 10 min to dissolve the crystals formed by MTT. Each well was read at a wavelength of 570 nm with a microplate reader, and the cytotoxicity level was determined according to the absorbance value read. Cytotoxicity levels were calculated with the following formula:

$$1 - \left(\frac{\textit{Absorbance of test well} - \textit{Absorbance of control well}}{\textit{Absorbance of test well}} \right) \times 100$$

ELISA

To evaluate the antioxidant status of cells treated with IV and Curc, GPx, SOD, CAT activities, TAS, and TOS levels were measured. At the same time, CASP3 protein levels were measured by enzyme-linked immunosorbent assay (ELISA) to examine their effects on apoptosis. The experimental protocols of ELISA experiments (R&D Systems) vary for each kit.

Statistical analysis

The Gaussian distribution of the data was checked using the Shapiro-Wilk test. Depending on the normality of the data, parametric or non-parametric tests were utilized. The difference between the means of more than two independent groups was analyzed with the one-way ANOVA test for normally distributed variables. Next, Dunnett's post-hoc analysis was performed to compare two individual groups to determine the level of significance. We assumed p-values < 0.05 as statistically significant. GraphPad Prism (GraphPad Software v. 8.0.2, San Diego, USA) was used for statistical analyses. Data were expressed as mean ± standard deviation or standard error of the mean.

Results

In the present study, 5 µM Aβ-induced U87 human brain cells were used to establish an *in vitro* AD model. The cells were then exposed to a total extract of IV (300 µg/ml) and Curc (40 µM)

Table I. Composition of IV plant extract by GC-MS method

RT (min)	Compound name	SI	RSI	Area %
7.21	Toluene	963	989	0.64
14.42	3-Hexenoic acid, ethyl ester	877	935	0.42
17.03	Propanoic acid, 2-methyl	905	954	0.58
20.52	Butanoic acid, 2-methyl	817	930	0.60
20.75	Hexanoic acid	799	853	1.27
27.39	α-Terpinyl acetate	909	943	3.02
31.58	α-Toluic acid	959	982	0.60
31.94	Anethole	906	954	1.32
35.44	Nerolidol	935	949	0.90
37.27	2,5-Furandione, 3-methyl-4-propyl-	846	863	5.57
39.45	Lanceol	834	881	1.54
39.76	Pyrrolidine, 1-(1-oxo-5,8,11,14-eicosatetraenyl)	656	694	0.72
40.61	Eugenol	864	934	0.46
40.87	Nerolidol-epoxyacetate	811	832	0.52
41.07	Juniper camphor	862	869	1.46
41.84	3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one	843	960	2.17
42.31	Hexadecanoic acid, ethyl ester	942	944	4.04
43.01	Caryophyllene oxide	867	882	4.36
43.13	8-Oxo-neoisolongifolene	867	900	2.79
43.51	β-Eudesmol	921	953	0.84
43.99	Globulol	909	925	2.32
44.03	Junipene	838	864	1.86
45.25	Methyl eicosa-5,8,11,14,17-pentaenoate	798	839	6.02
46.17	Coumaran	683	889	0.67
46.68	Dihydroxanthin	812	828	0.97
47.96	Spathulenol	695	700	6.13
49.32	Oxiranecarboxylic acid, 3-methyl-, ethyl ester	659	753	0.48
49.63	Phytol	691	775	1.00
50.09	10-Heptadecen-8-ynoic acid, methyl ester	669	677	2.84
50.67	2,6-Octadien-1-ol, 2,6-dimethyl-8-[(tetrahydro-2H-pyran-2-yl)oxy]	787	812	14.82
51.25	γ-n-Heptylbutyrolactone	688	710	3.62
51.52	2H-Pyran, 2-(7-heptadecynyloxy)tetrahydro	766	769	0.69
52.13	Methyl arachidonate	791	816	0.85
52.34	Ethyl linoleate	776	792	17.69
53.26	5-Hydroxymethylfurfural	560	941	0.53
54.71	Methyl arachidonate	807	840	0.43
54.95	β-Cyclohomogeraniol	713	778	2.47
55.46	2-Pentenoic acid, 5-(decahydro-5,5,8a-trimethyl-2-methylene-1-naphthalenyl)-3-methyl-	578	609	0.53
55.93	Dihydroactinidiolide	783	875	0.68
56.17	Cholestan-3-ol, 2-methylene	656	692	1.29

RT – retention time, SI – strength index, RSI – relative strength index.

for 24 h. The control group consisted of untreated U87 cells. The effect of Curc and IV was analyzed using cell viability (MTT) and ELISA tests.

Table I summarizes the composition of the IV plant extract analyzed with the GC-MS method. The major components (>10%) of the plant extract were ethyl linoleate and 2,6-Octadien-1-ol, 2,6-dimethyl-8-[(tetrahydro-2H-pyran-2-yl)oxy].

MTT assay

Figure 1 A depicts the changes in U87 cell viability according to Aβ-treated IV and Curc. It was found that IV and Curc treatments markedly increased the reduced cell viability and proliferation in Aβ-treated U87 cells (Figure 1 A, $p = 0.0003$ and $p < 0.0001$ respectively). Additionally, the synergistic effect of Curc and IV co-treatment on cell viability was more notable compared to individual treatments (Figure 1 A, $p < 0.0001$).

ELISA and oxidative stress assays

As depicted in Figure 1 B, cleaved CASP3 levels were measured using the ELISA method to analyze the effect of IV and Curc plant extracts on caspase-dependent apoptosis in Aβ-induced AD in U87 cells. CASP3 levels significantly increased in Aβ-induced U87 cells compared to the control group (Figure 1 B, $p < 0.0001$). The individual applications of IV and Curc significantly reduced CASP3 levels compared to the Aβ group (Figure 1 B, $p < 0.0001$ and $p = 0.0003$ respectively). In addition, co-treatment of Curc and IV was more effective in reducing CASP3 levels compared to separate treatment regimens (Figure 1 B, $p < 0.0001$).

The oxidative status parameters are presented in Figure 2. SOD, CAT, GPx, and TAS levels were reduced in the cells treated with Aβ ($p < 0.05$) while Curc and IV co-administration significantly increased the levels of anti-oxidant parameters compared to the Aβ treatment group (Figures 2 A–C, E, $p < 0.05$). Aβ treatment significantly increased TOS levels ($p < 0.001$) whereas IV and Curc treatments significantly reduced TOS levels compared to the Aβ-treated group (Figures 2 D, $p < 0.05$), with a more notable synergistic effect ($p < 0.001$). In Figure 2 F, double gradient heat map analysis demonstrates the effect of Curc and IV on Aβ-induced AD cells regarding oxidative status parameters.

Discussion

AD is a prevalent neurodegenerative disease worldwide and significantly worsens the life quality of patients due to the lack of a complete treatment for the disease [1]. The current therapeutic strategies provide only palliative solutions concerning relieving the symptoms [22]. Considering the personal and socioeconomic burden of AD patients on the families and country economies, alternative treatment approaches are urgently needed [22].

Among the therapeutic options to limit AD progression, reduction of oxidative stress, prevention of Aβ toxicity, and inhibition of Tau protein hyperphosphorylation are the primary strategies. For this purpose, several plant extracts and their isolated compounds have been shown to exert anti-AD activities in *in vivo* and clinical studies [23]. In a study performed to test the effects of *G. ulmifolia*, *L. brasiliense*, *P. cupana*, *P. pluviosa*, *S. adstringens*, and *T. catigua* on an *in vitro* AD model, *S. adstringens*, *P. pluviosa*, and *L. brasiliense* ethyl-acetate fractions were reported to protect against Aβ-induced neural damage in SH-SY5Y human neuroblastoma cells [24].

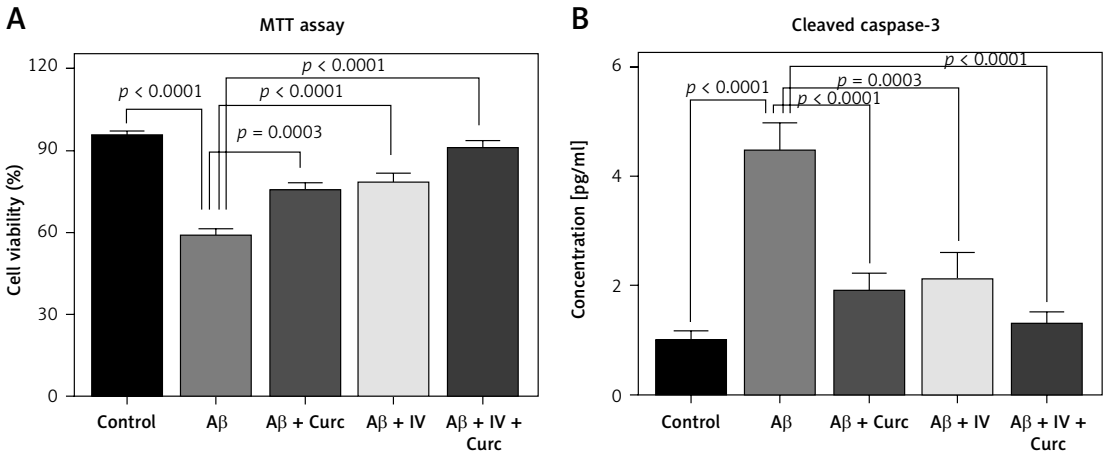

Figure 1. Effect of IV and curcumin (Curc) on the (**A**) cell viability assessed with MTT assay and (**B**) CASP3 (pg/ml) levels assayed with ELISA in U87 cell line, in the control, amyloid-β (Aβ), Aβ + Curc, Aβ + *Inula viscosa* (IV), and Aβ + IV + Curc groups. Data are presented as mean ± standard error of mean. One-way ANOVA and post-hoc Dunnett's tests were used to test for statistical significance. $P < 0.05$ was considered statistically significant

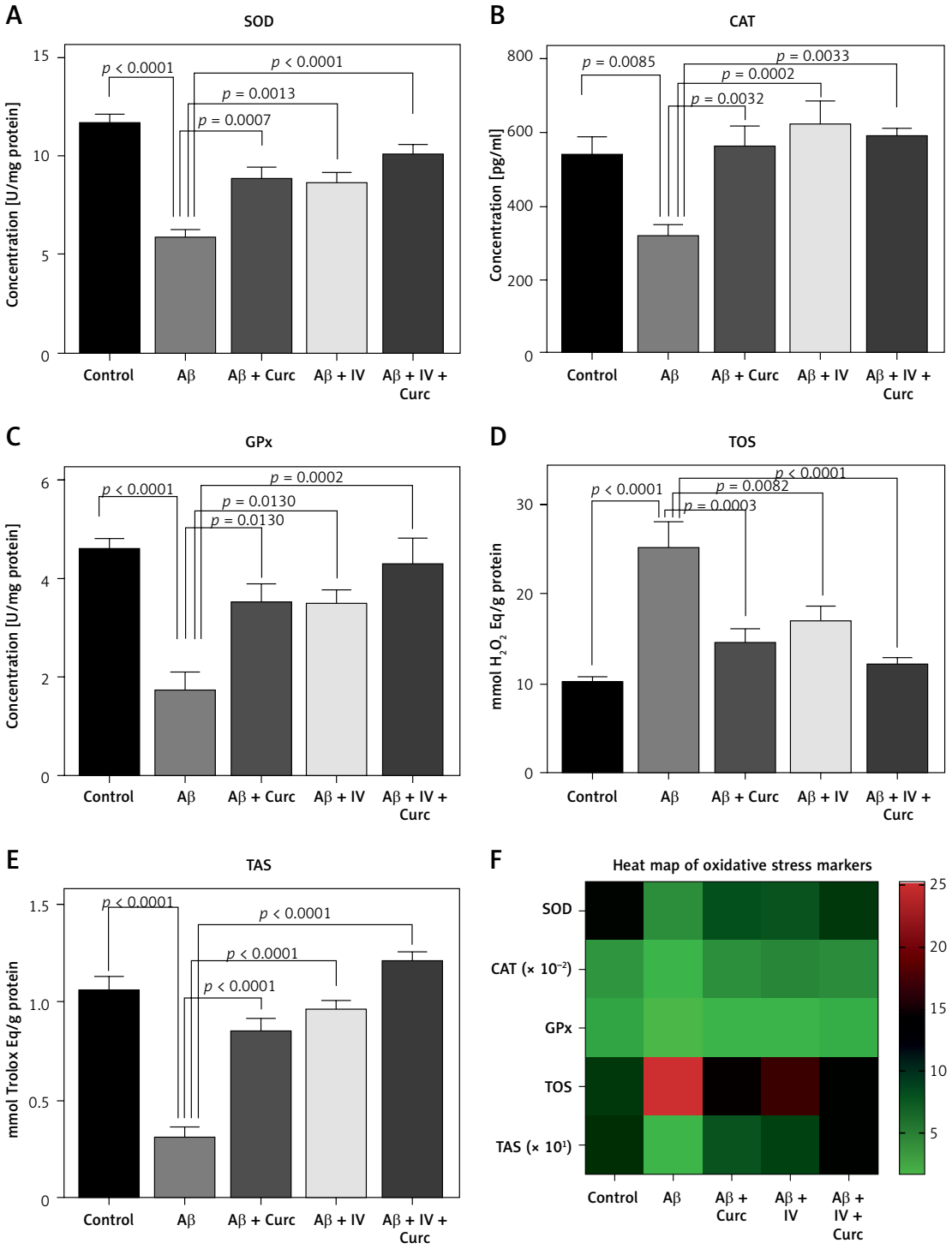

Figure 2. Effect of IV and Curc on (**A**) SOD, (**B**) CAT, (**C**) GPx, (**D**) TAS, and (**E**) TOS levels in U87 cell line in the control, Aβ, Aβ + Curc, Aβ + IV, and Aβ + IV + Curc groups. Data are presented as mean ± standard error of mean. One-way ANOVA and post-hoc Dunnett's tests were used to test for statistical significance. $P < 0.05$ was considered statistically significant. **F** – Double gradient heat map analysis of oxidative status parameters; SOD, CAT (× 10^{-2}), GPx, TOS, and TAS (× 10^{1})

SOD – superoxide dismutase, CAT – catalase, GPx – glutathione peroxidase, TOS – total oxidant, TAS – total antioxidant, Aβ – amyloid-β, Curc – curcumin, IV – Inula viscosa.

In the present study, the effects of Curc and IV plant extracts on cell viability, apoptosis, and oxidative status were investigated in the Aβ-induced AD cell line model. We found that the synergistic effect of Curc and IV co-treatment could reverse the reduced cell viability in Aβ-treated U87 cells by reducing apoptosis and restoring the distorted oxidative balance in favor of anti-oxidant capacity.

In vitro AD cell culture models with different cell lines (SH-SY5Y neuroblastoma and LN229 glioblastoma cells) demonstrated that intracellular accumulation of Aβ leads to NF-κB pathway-mediated phosphorylated Tau protein accumulation and induction of proapoptotic activities [25]. Previous studies reported that pathogenic Aβ aggregates around neurofibrillary plaques, the accumulation of hyperphosphorylated Tau protein, neuroinflammation, oxidative stress, and other prominent Aβ features have been associated with the activation of MAPK pathways [26]. In this context, researchers have focused on the effects of various drugs and active components of plant extracts on the neuroinflammatory pathways, i.e., NF-κB and MAPK, playing a role in AD pathogenesis. Rozenblat *et al*. found that some of the IV plant leaf components, e.g., sesquiterpene lactones, caused a dose-dependent decrease in expression levels of anti-apoptotic proteins as well as p65 subunit of NF-κB and survivin proteins in human melanoma cell lines [27]. In previous *in vitro* and *in vivo* cancer models, IV extract has been reported to exert anti-tumorigenic activity by triggering apoptosis via increased caspase activation in colorectal cancer cells [16] or inhibition of anti-apoptotic Bcl-2 protein family members in the Burkitt lymphoma cell line [28]. Moreover, among three flavonoids isolated from IV, sakuranetin exhibited anti-inflammatory effects that have been previously attributed to flavonoids' overall antioxidant activity [29], by directly inhibiting 5-lipoxygenase and elastase release [30]. In another *in vitro* study conducted to determine the underlying epigenetic mechanisms of anti-cancer activity, IV methanol extract was shown to induce overexpression of miR-579 and miR-524, which was implicated in the suppression of the MAPK pathway and inhibition of proliferation in human malign melanoma cells [31]. Curc has been shown to target Aβ formation at different levels in *in vitro* and *in vivo* models of AD, either via preventing plaque formation or by triggering the destruction of fibril formation [32]. Another supporting study demonstrated that several Curc derivatives could inhibit the prominent AD targets glycogen synthase kinase-3β and β-secretase and eventually prevent Aβ formation *in vitro* [33]. Curc has been shown to be implicated in cell death pathways in different disease models. Among these, Curc could upregulate the autophagic proteins (LC3-I-II, beclin1) and modulate apoptosis-related markers (Bax, CASP8, and Bcl-2) [34]. A Curc derivative called BDMC33 has been demonstrated to exert anti-inflammatory effects in IFN-γ/LPS-induced macrophages by inhibiting major pro-inflammatory mediators, i.e., NO, TNF-α, and IL-1β, via interfering with MAPK and NF-κB pathways [35]. The active components of these natural agents can modulate multiple targets synergistically through the aforementioned pathways and ameliorate cognitive deficits in AD patients by establishing a balance between antioxidant and pro-inflammatory activities [36]. The existing literature shows that Curc has neuroprotective effects in several neurodegenerative disease models. For example, Cai *et al*. observed that Curc diminished neurodegeneration by alleviating histone deacetylase 6 (HDAC6) and nucleotide-binding domain, leucine-rich repeat, and pyrin domain-containing protein 3 pathway (NLRP3)-mediated neuroinflammation in *in vitro* and *in vivo* Parkinson's disease models [37]. Parallel to these findings, Curc treatment could alleviate the epileptic phenotype in a rat model of epilepsy by blocking the NLRP3 inflammasome activity [38].

Reactive oxygen species (ROS), including hydroxyl radicals (-OH), superoxide anions, and hydrogen peroxide (H_2O_2), play a critical role in cell metabolism [39]. ROS are believed to be important in the development of many diseases, including cancer [40]. The association of elevated ROS-induced oxidative stress levels with various degenerative diseases, e.g., AD and Parkinson's disease, has been previously demonstrated [41]. ROS are produced during oxidative cellular reactions and are efficiently scavenged by cellular antioxidant defense systems, e.g., SOD, GPx, and CAT, to maintain oxidative homeostasis [42]. If the ROS level is higher than the antioxidant capacity of the cell, the excess ROS can cause irreversible oxidative modification by directly affecting and altering lipid, protein, or DNA and signaling pathways [43]. It is known that the SOD enzyme has three different forms: cytosolic (zinc-copper-dependent), mitochondrial (manganese-dependent), and extracellular (iron-dependent) forms. SOD functions by converting O_2 to H_2O_2 [44]. CAT and selenium-dependent GPx enzymes are other prominent players in the antioxidant system. These enzymes exert an antioxidant effect by converting H_2O_2 to water [44]. The studies in the literature are contradictory, with different results regarding SOD activity in patients with AD. One such study claimed a significant reduction in SOD activity in the cerebral cortex and hippocampus of patients with AD, whereas another study did not detect any difference in SOD activity in patients with AD compared to healthy controls [45, 46].

In a previous study conducted by Xue *et al*., the interactions of two different Cu(II) complexes of Curc with calf thymus DNA were investigated [47]. They showed that both complexes interacted with circulating tumor DNA. In addition, when cytotoxicity analysis was performed on MCF-7, A549, and HCT-116 tumor cell lines under similar experimental conditions, they found that Curc showed better cytotoxicity than cisplatin. The enzyme GPx is an important enzyme of the cellular defense system as an antioxidant [48]. In a previous clinical study, glutathione reductase and GPx activities were found to be higher in the amygdala, hippocampus, and piriform cortex regions of AD patients, whereas no change in GPx activity was found in the frontal, temporal, and cerebellar cortex regions of these patients [48]. Another study demonstrated the critical role of H_2O_2 in Aβ toxicity, confirming the importance of the protective action of CAT enzyme in cells. In the same study, PC12 cells resistant to Aβ toxicity were shown to exhibit high CAT and GPx activities [49].

In our study, an AD model was established with the U87 cell line, and we aimed to analyze the potential neuroprotective effects of IV extract and Curc. The MTT method was used to investigate the effects of IV extract and Curc on cell proliferation and viability and also to explore the effects on the antioxidant defense system. GPx, SOD, and CAT activities and TAS, TOS, and CASP3 protein levels were measured to determine how the ELISA method affected the apoptosis process. We observed that both Curc and IV reversed the cytotoxic effect of Aβ through the reduction of CASP3 and re-established the oxidative status in favor of TAS levels via restoring SOD, CAT, and GPx levels. More notably, the synergistic effect of Curc and IV on both cell viability and oxidative status parameters was more pronounced compared to their individual efficacy.

According to our data, when Aβ was applied to the human neuronal cell line U87, SOD, CAT, GPx, and TAS significantly decreased compared to the control group. Co-treatment of Curc and IV could reverse this decrease more substantially compared to single applications of the agents except CAT. Application of Aβ to the cells significantly increased the amount of TOS. Both IV and Curc reversed this increase, with a more marked effect when co-administered. Due to the increase in ROS levels in the cells, the antioxidant defense mechanism may have triggered and augmented cell proliferation. Accordingly, we can infer from our findings that IV and Curc increase cellular proliferation while simultaneously increasing GPx and SOD enzyme activity, thereby supporting the defense mechanism of the cell. ROS are scavenged by antioxidant defense systems, CAT, SOD, and GPx to maintain cellular redox homeostasis. However, when ROS production exceeds the detoxification capacity of the cell, the excessive amount of ROS produced causes serious damage to the proteins, DNA, and lipid barrier of the cell membrane [43]. These results suggest that ROS play a role as a second messenger in cell proliferation by activating protein kinases and transcriptional factors [50]. Previous studies have shown that Curc has beneficial effects on AD owing to its anti-inflammatory, anti-oxidant, and anti-amyloid actions [51, 52]. IV is widely used in traditional medicine as it has anti-inflammatory, antioxidant, and anticancer activities [6]. The fact that IV has antioxidant activity and the ability to scavenge ROS highlights the potential use of this plant to treat oxidative damage involving various disorders, such as age-related and neurodegenerative diseases [53].

In the present study, the synergistic effect of IV and Curc revealed a more distinct effect compared to single administrations and could reduce Aβ-induced cell death in the *in vitro* cell culture model of AD by downregulating CASP3, increasing cell viability, and reducing oxidative stress levels through elevating antioxidant enzyme activity in U87 cells.

Wang *et al*. proposed that the neuroprotective effect of Curc on spatial memory in an *in vivo* AD model might arise from the inhibitory effect on GFAP expression and astrocyte function [54]. Thus, it can contribute to the restoration of behavioral deficits caused by intracerebral Aβ injection [55]. These outcomes suggest that Curc could prevent glial cell activation and thereby attenuate the AD pathophysiology owing to its strong anti-inflammatory actions.

Inula species can be considered as a promising alternative neuroprotective agent owing to their oral bioavailability, blood-brain barrier permeability, and low toxicity features. However, further clinical studies are warranted on these aspects [56]. The effect of IV extract was analyzed *in vitro* by Qneibi *et al*., who found that IV exerted antioxidant and neuroprotective activities primarily by inhibiting the glutamate-induced excitotoxicity in the HEK293 cell line [57]. Although there are no studies in the literature analyzing the neuroprotective role of IV in a disease model, an active component of another *Inula* species has been shown to possess neuroprotective activities in *in vivo* models of cognitive impairment [58] and traumatic brain injury [59].

Our GC-MS analysis revealed that the IV extract contains various phytochemical compounds including essential oils and a high percentage (~18%) of the unsaturated fatty acid ethyl linoleate – a linoleic acid that has been reported to have anti-inflammatory activity [60]. In this regard, ethyl linoleate and other major essential oils in the composition of IV extract, namely spathulenol

(~6%), caryophyllene oxide (~4%), hexadecanoic acid, ethyl ester (~4%), α-terpinyl acetate (~3%), β-cyclohomogeraniol (~3%), globulol (~2%), lanceol (~2%), juniper camphor (~1.5%), anethole (~1.5%), phytol (1%), and nerolidol (~1%), could play a role in the prevention of Aβ-derived neurodegeneration of U87 cells.

There are major limitations of the current study. First of all, only one type of cell line was used to obtain an *in vitro* AD model, which certainly restricts the observations regarding the effect of target substances on other possible neuronal cell line-derived AD models. Although neurodegenerative culture models are common tools in novel/alternative drug discoveries, they cannot directly correspond to the complex *in vivo* pathogenesis and therefore may fail to reflect clinical cases due to lower bench-to-bedside adaptation rates. To overcome this handicap, utilizing various cell lines to model the same disease will increase the ability to mimic clinical scenarios. The U87 glioblastoma cell line has been utilized in various neurodegenerative disease models, including Alzheimer's. In this manuscript, the U87 cells were treated with Aβ to mimic the Alzheimer's disease environment, as these cells are often employed for *in vitro* studies of neurodegeneration. However, it is crucial to acknowledge that while the U87 cell line is widely used in neurodegeneration research, it is a glioblastoma model, not a neuronal model. As such, it may not fully recapitulate the pathophysiological features of Alzheimer's disease. There is literature suggesting that U87 cells, when exposed to Aβ, can be used to assess certain aspects of Alzheimer's pathology, such as oxidative stress and apoptosis, but they do not model the complex neuronal and cognitive symptoms of Alzheimer's. To improve the accuracy of the model, other cell lines such as SH-SY5Y human neuroblastoma cells or induced pluripotent stem cell (iPSC)-derived neurons are often preferred, as these more closely resemble the neurodegenerative processes in Alzheimer's disease [61, 62].

Secondly, the current study focused on observing whether the combinatory treatment of Curc and IV plant extracts has any possible effects on the cell viability, apoptosis, and oxidative stress parameters in an *in vitro* AD cell line model rather than a mechanistic and physiological approach. Further studies using an *in vivo* animal model of AD are warranted to test combinatory neuroprotective and anti-inflammatory effects of Curc and IV.

In conclusion, we examined the effects of Curc and IV plant extracts on the AD cell line model for the first time in the literature. We suggest that the synergistic effect of IV and Curc co-treatment may offer more benefits in AD treatment compared to single applications. Additionally, IV extract can be utilized as a promising compound in an alternative treatment strategy or as an adjuvant to several drugs currently used in AD therapy. As a further step, the underlying mechanism of action of IV and Curc should be analyzed concerning apoptotic pathways (intrinsic/extrinsic) and oxidative stress formation from a mechanistic perspective. In this regard, molecular docking and pathway studies can be conducted to explore the target molecules playing a role in AD pathogenesis. To elaborate, while molecular docking studies will reveal the interactions of active components of the IV and Curc with their target proteins/pathways, *in vivo* animal models of AD will provide more comprehensive data compared to *in vitro* systems in terms of cognitive and memory functions via several behavioral tests.

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Ethical approval

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

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