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***Original Article***

# Identification and characterization of a compound from *Cuminum cyminum* essential oil with antifibrilation and cytotoxic effect

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## *Abstract*

Amyloid pathology is associated with fibril aggregation of different proteins which results in the progressive damage of affected organs. It is strongly believed that specific small molecules interfere with fibrillation by interacting with the amyloidogenic proteins. We had previously reported the strong and long-term inhibition of fibrillation of hen egg white lysozyme (HEWL) by *Cuminum cyminum* oil. Herein, it was intended to rationally identify the active anti-amyloidogenic compounds of the oil. After fractionation, the highest inhibitory effect was observed in the toluene-ethyl acetate part of the oil. Gas chromatography-mass spectrometry (GC-MS) analysis of this fraction indicated that eight compounds were predominantly present in the fraction. Unexpectedly, two compounds including terpinolene and limonene, having very similar chemical structures, inhibited and induced fibrillation, respectively. PC12 cells (derived from a transplantable rat pheochromocytoma) were affected by HEWL fibrils, whereas the inhibited forms of fibrils in the presence of terpinolene led to higher levels of viability, as shown by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), lactate dehydrogenase (LDH) and flow cytometry assays. Molecular local docking analysis suggested a site of interaction for terpinolene in the flexible cleft of the protein. This interaction site is close to tryptophan -62 and -63 and two other hydrophobic residues in the hot spot regions of the protein. Seemingly, these interactions interrupt protein self-assembly and therefore, fibril formation. Despite previously reported small anti-amyloid molecules which have aromatic flat rings, terpinolene ring is not flat. This functionally durable small molecule may aid us toward developing new anti-amyloidogenic compounds with extended activity.

***Keywords:*** Anti-fibrillation compounds; Essential oil fractionation; HEWL fibrillation; Iranian *Cuminum cyminum;* Local docking

## INTRODUCTION

There are a growing number of incurable human diseases known as amyloidosis, which are associated with a specific form of protein aggregation, known as the amyloid fibrils. With the extension of life expectancy and the ageing of the general population in both developed and developing countries, the prevalence of many chronic and progressive physical and mental conditions including amyloid-related disorders are developing considerably. The World Health Organization (WHO) estimates that neurodegenerative diseases will rise to 14.7%, by 2020. While amyloid diseases have been the center of intensive research efforts, there is still no ideal treatment available for these diseases. Amyloidogenic proteins do not usually share any similar amino acid sequences, and do not have any homology in their three-dimensional structures. However, regardless of their

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origins, amyloids from various proteins have demonstrated strikingly similar structures (1). Studies from various cell and animal models suggest that the reduction of amyloid aggregation is beneficial for reducing the symptoms of amyloidosis (2,3). There is also a worldwide effort to identify the anti-aggregating substances. Accordingly, a number of molecules have so far been discovered which include antibodies, synthetic peptides, heat shock proteins and other chemically-synthesized compounds (4-6). A large number of small molecules have been found to have potential use in the treatment of amyloidosis. However, understanding the mechanisms of how such small molecules impede the fibrillation process is still at a preliminary stage.

The structures of known small molecules with inhibitory roles do not have any rational similarity, and sometimes very similar structures have opposite effects on the fibrillation process. Medicinal herbs and their essential oils contain valuable small natural molecules that can inhibit, or even reverse amyloid accumulation, and alleviate the fibril-related symptoms. For example, rosmarinic acid isolated from *salvia officinals*, or cuminaldehyde from *Cuminum cyminum,* both inhibit alpha-synuclein fibrillation. Furthermore, some herbal compounds protect cells from the toxic effects of fibrillation. The extract of *Melissa officinalis* is an example that improves memory function in mouse models with Alzheimer's disease (7-10). Many believe that natural compounds are safer and have fewer adverse impacts than chemically synthesized drugs (11,12). However, the increased global usage of medicinal herbal extracts has raised a number of concerns in relation to undesirable health effects. Generally, the presence of phenols, aldehydes and alcohols is the major reason for the cytotoxic activities of different medicinal herbal extracts (13).

This cytotoxic property is of great importance in the applications of essential oils, not only against certain human or animal pathogens, but also for the preservation of agricultural or marine products (13). Hence, herbal products contain complex mixtures of active components (phytochemicals) which makes the identification and determination of the biological activity of its individual constituents rather difficult (14-16).

Previously, we determined that *C. cyminum* can significantly inhibit hen egg white lysozyme (HEWL's) amyloid aggregation (17). However, cell culture tests indicated that it has serious cytotoxic effects. In the present study, different fractions of the oil extract were screened to find a compound with the highest inhibitory effects on the fibrillation and the lowest cytotoxic effects. The anti-amyloidogenic ability of different fractions of the Iranian *C. cyminum* species was investigated with regard to the fibrillation of HEWL as a model protein. It was also shown that the toluene-ethyl acetate fraction of *C. cyminum* inhibited HEWL's amyloid aggregation without any noticeable cytotoxic effects. It was also revealed that different kinds of compounds, like terpinoids, flavonoids, glycosides, glucoseindoates, can to be extracted from their herbal sources by using various organic solvents which have different physical and chemical properties (18). Through the application of standard methods, it was shown that the inhibitory effects of the fraction in preventing amyloid formation was strong and continued over a long period of time. Rat pheochromocytoma cells (PC12) were protected against the cytotoxic effects of amyloid fibrils. In order to identify the active compounds of the extract, GC-MS was applied. Subsequently some of the main components were incubated individually with HEWL and their inhibitory effects were then studied. Furthermore, their abilities to protect PC12 cells from incubated HEWL's-induced cytotoxicity were assessed. Moreover, in order to identify the binding model of the active compound, Autodock analysis through local docking was carried out.

## MATERIALS AND METHODS

### *Chemicals and biological reagents*

Hen egg white lysozyme (HEWL, EC 3.2.1.17), thioflavin T (ThT), congo red 1,8-cineol, p-cymene, limonene , terpinolene and beta-myrcene were obtained from Sigma

(USA). Local essential oils were purchased from Barij Essence Pharmaceutical Co. (Kashan, Iran). Rat pheochromocytoma (PC12) cells were acquired from the Pasteur Institute (Tehran, Iran). All salts and organic solvents were obtained from Merck (Darmstadt, Germany).

The lactate dehydrogenase (LDH) assay kit was acquired from Ziestchem Diagnostics (Tehran, Iran). The cell culture medium (RPMI 1640) and antibiotics (penicillin, streptomycin) were purchased from Gibco-BRL (Life Technologies, Paisley, Scotland). Fetal bovine serum (FBS) was obtained from Biosera (England). The culture plates were purchased from Orange Scientific Products (USA).

***Lysozyme fibril preparation***

Lysozyme was dissolved at 2 mg/ml in 50 mM glycine buffer (pH 2.5), and incubated at 57° C while being shaken (80 rpm) gently in a shaking water bath. To study the inhibition of fibrillation, lysozyme was incubated in the presence of the *C. cyminum* oil fractions (5% (v/v)) or the other tested small molecules (as mentioned above) for 24 to 92 h (kinetic study).

***Thioflavin T fluorescence assay***

Ten µl of HEWL solution (2 mg/ml) was added to 490 µl of 10 µM thioflavin T (ThT) solution,containing 10 mM Tris (pH 8.0), and mixed gently. Fluorescence emission spectra (450-550 nm) were taken using an excitation wavelength of 440 nm. The excitation and emission slit widths were set as 5 nm (19). A Cary Eclipse VARIAN fluorescence spectrophotometer (Mulgrave, Australia) was used for the fluorescence assays.

***Congo red absorbance assays***

Congo red was dissolved at 1 mg/ml in a buffer consisting of 150 mM sodium chloride and 5 mM potassium phosphate (pH 7.4). Then it was filtered using a center-glass N4 filter. A 10 µl sample of the well-mixed incubated mixture was added to 490 µl of the congo red solution and incubated for 5 min. Absorbance spectra were recorded (400–600 nm) using a PGT80+UV–Visible spectrometer (Leicestershire, England) (20).

***Fluorescent staining of nanofibrils***

By using the amyloid-specific fluorophore, ThT, fluorescence microscopy images of different stages of protein fibrillation can be observed (19). Briefly, 15 µl of the incubated protein was added to 15 µl of ThT (500 µM), under amyloid-inducing conditions. Samples were incubated for 5 min at room temperature, and were then spread onto a microscopic slide. Thereafter, they were studied by fluorescence microscopy, Ceti inverso TC100 microscope (Medline scientific, Oxon, UK).

***Silica gel column chromatography***

For separation of the essential oil compounds, a column (1.5 cm in diameter and 30 cm in length) was packed with 5 g of silica gel (0.2-0.5 µm size). A 3 ml sample of the essential oil was dissolved in 5 ml of n-hexane and applied on the column. Details of the procedure for this chromatography have been described elsewhere (21). Briefly, a series of solvents were used for the separation of the essential oil compounds, based on their hydrophobicity strengths, from highest to lowest hydrophobicity strength, as follows: n-hexane, 60% n-hexane + 40% toluene, toluene, 60% toluene + 40% ethyl acetate and ethyl acetate. The solvent from each fraction was evaporated until a final volume of 30 µl was reached (at 30-35ºC). The concentrated extracts were kept in dark vials at 4º C, until further use.

***Gas chromatography–mass spectrometry analysis***

Gas chromatography–mass spectrometry (GC-MS) analyses were performed on a Thermoquest 2000 GC coupled with A Quadruple (Thermoquest-Finnigan) TRACE Mass system and a CP-sil5 capillary column (60 m × 0.25 mm; 0.25 µm film thickness). Mass range was from m/z 45–460 amu. The GC oven temperature was programmed from 60 ºC to 250 ºC at a rate of 5 ºC/min, then held for 10 min at 250 ºC, at a split ratio of 10:1. The carrier gas was helium. Mass spectra were taken at 70 eV. An injection volume of 0.2 µl was used. The components of the oils were identified by comparison of their mass spectra and retention indices with those published in the literature (22) and presented in the MS computer library (NIST 05.L).

***Cell culture and treatments***

PC12 cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 U/ml of the penicillin and 100 µg/ml of streptomycin in a 5% $CO_2$ humidified atmosphere, at 37 °C. Cells were plated at a density of $3 \times 10^4$ cells/well in 96-well plates, and incubated for 24 h. The medium was subsequently changed and treated with 20 µl of incubated HEWL, with or without the *C. cyminum* fractions and other test molecules. Samples of the cultivated cells, to which the same volume of glycine buffer were added, were used as control. Cells were then incubated for another 24 h.

***Cell viability assay***

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was carried out to measure cell viability. A 10 µl sample of the 5 mg/ml MTT stock solution was added to individual wells, and incubated for 4 h at 37 °C in a 5% $CO_2$ humidified atmosphere. Then, medium was removed, and 100 ml of dimethyl sulfoxide (DMSO) was added to each well in order to solubilize formazan crystals. The absorbance of the samples was read with a 580 nm filter and a 692 nm reference filter using a multi-well assay plate reader (Expert 96, Asys Hitchech, Austria).

***Lactate dehydrogenase release assay***

To assess the plasma membrane integrity of PC12 cells following 24 h of treatment, cytosolic Lactate dehydrogenase (LDH) release was measured using the LDH kit, according to the manufacturer's instructions (Ziestchem Diagnostics, Iran). Briefly, 10 µl of medium from each well was taken and mixed with 1 ml of substrate solution. The absorbance of the resulting mixture was then measured at a wavelength of 340 nm at 37 °C, using a PGT80+UV–Visible spectrometer (Leicestershire, England).

***Flow cytometry***

Flow cytometry assay was employed to find more details about the effect of terpinolene on the cytoxicity of fibrils, the percentage death rate, and also the type of cell death. After 24 h of incubation, the cells ($10 \times 10^5$) were treated with different samples including HEWL fibrils, and also HEWL fibrils treated with terpinolene. The resulting mixtures were then incubated for another 24 h. Subsequently, the plates were detached with 0.25% trypsin and cells were collected by centrifugation at 1000 rpm for 5 min. The pellets were washed with phosphate buffer solution (PBS) and resuspended in 500 µl of binding buffer. Fluorescein isothiocyanate (FITC) conjugated Annexin V was added to samples, which were then incubated for 5 min in a dark place at ambient temperature. In the next step, *propidium iodide (PI)* was added to the samples, and the resulting solutions were incubated for another 5 min. For the analysis of the cell death rate, the samples were transferred to a BD FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Data was analyzed using a Flowing software v.2.5, so as to discriminate the early and late apoptosis/necrosis rates (23).

***Detecting the hot spot areas of the protein by Aggrescan***

In order to predict the areas of protein which are susceptible to self-aggregation, the Aggrescan program was used (http://bioinf.uab.es/aggrescan). The areas of the protein which had the highest tendencies to self-interact and aggregate were predicted by submitting the HEWL sequence input data to the Aggrescan program.

***Autodocking***

Docking procedure was carried out with the Autodock software (version 1.5.6rc3, autodock.scripps.edu). The structure of HEWL was obtained from the Protein Data Bank (PDB) with ID: 2VB1. Furthermore, the structure of terpinolene was derived from the ChemDB database, ID: 6691720.

To limit the screening (local docking), docking was analyzed in the selected areas proximate to the hot spot regions of the protein to see whether terpinolene has favorable interactions with the parts of protein that are prone to aggregation. The sizes of the grid spacing used, included 46, 16, and 18 with 0.63 A. Different binding models were obtained and the lowest energy conformation

was chosen and analyzed to find the residues that had direct contact with the target molecule.

***Statistical analysis***

All experiments were carried out in triplicate, and data was presented as means ± SD. Statistical significance between two groups was concluded by the unpaired Student's t-test. Also, one-way ANOVA was employed for the results of more than two experimental groups to state differences between groups. $P_{Value}<0.05$ was *considered as significant*.

## RESULTS

***Fractionation of Cuminum cyminum essential oil and analysis the effects of the fractions on the fibrillation of hen egg white lysozyme***

Our previous study indicated that the Iranian *C. cyminum* essential oil has a high inhibitory effect on the formation of harmful amyloid fibrils derived from HEWL (17). In the present study we have examined *C. cyminum* essential oil to identify effective compounds with no cytotoxic effects. Table 1 shows data for the GC-MS analysis of the essential oil extracted from the Iranian *C. cyminum*. These results indicate that the essential oil extract is comprised of different compounds, which can have both harmless and harmful effects on biological systems. Regarding the high cytotoxic potential of essential oils, different fractions of *C. cyminum* were initially extracted using silica gel chromatography (21).

The column was washed with different organic solvents as described above. The resulting solvent fractions [hexane (H), hexane–toluene (H-T), toluene (T), toluene-ethyl acetate (T-E) and ethyl acetate (E)] of *C. cyminum* were then concentrated. In order to find extracts of *C. cyminum* that highly affected HEWL fibrillation, but had the least effect on cell viability, HEWL samples were incubated at 57 °C under acidic conditions for 24 h with/without each of the above mentioned fractions. Subsequently, fibril formation was measured by the fluorescence method.

After incubation of protein samples under amyloidogenic conditions, ThT fluorescence intensity raised sharply and Congo red absorbance increased and shifted to the red (Fig. 1A). The toluene-ethyl acetate (T-E) fraction had a significant negative effect on fibril formation, and also decreased the fluorescence intensity by more than 40%. Other fractions had lower effects on the fibrillation process.

**Table 1.** Chemical composition of *C. cyminum*.

| Compound ID | RI | Content (rel. %) | Compound ID | RI | Content (rel. %) |
|---|---|---|---|---|---|
| *α*-Pinene | 939 | tr | Myrtenal | 1203 | tr |
| Sabinene | 977 | tr | *Transe*-chrysanthenyl acetate | 1227 | tr |
| *β*-Pinene | 984 | 1.23 | Cumin aldehyde | 1253 | 41.56 |
| *β*-Myrcene | 989 | tr | *p*-Menth-1-en-7-al | 1284 | 0.17 |
| *α*-Phellandrine | 1009 | 0.14 | *α*-Terpinene-7-al | 1295 | 12.58 |
| *α*-Terpinene | 1020 | tr | *γ*-Terpinene-7-al | 1301 | 17.14 |
| *p*-Cymene | 1028 | 5.34 | Daucene | 1393 | 0.58 |
| Limonene | 1032 | tr | Carryophyllene | 1438 | 0.5 |
| *β*-Phellandrine | 1034 | 0.14 | *α-Transe*- bergmotene | 1445 | 0.15 |
| 1,8-Cineol | 1036 | 0.11 | *cis-β*-Farnesene | 1458 | 0.58 |
| *γ*- Terpinene | 1064 | 13.96 | *α*- Humulene | 1472 | tr |
| *α*-Terpineolene | 1092 | tr | Dauca-5,8-diene | 1485 | tr |
| 1,4-Dimethyl-δ-3-tetrahydroacetophonene | 1155 | tr | *β* -Cadinene | 1488 | 0.19 |
| 2-Neonalal | 1158 | tr | 10-epi-*β*-Acoradiene | 1492 | 2.01 |
| Terepinene-4-ol | 1183 | tr | *β* -Bisabolene | 1516 | 0.13 |
| *p*-Cymene-8-ol | 1189 | 0.1 | Isodaucene | 1518 | 0.22 |
| *Transe*-4-caranone | 1198 | 0.58 | Caratol | 1618 | 0.78 |
| | | | Total | | 98.49 |

RI: Retention index

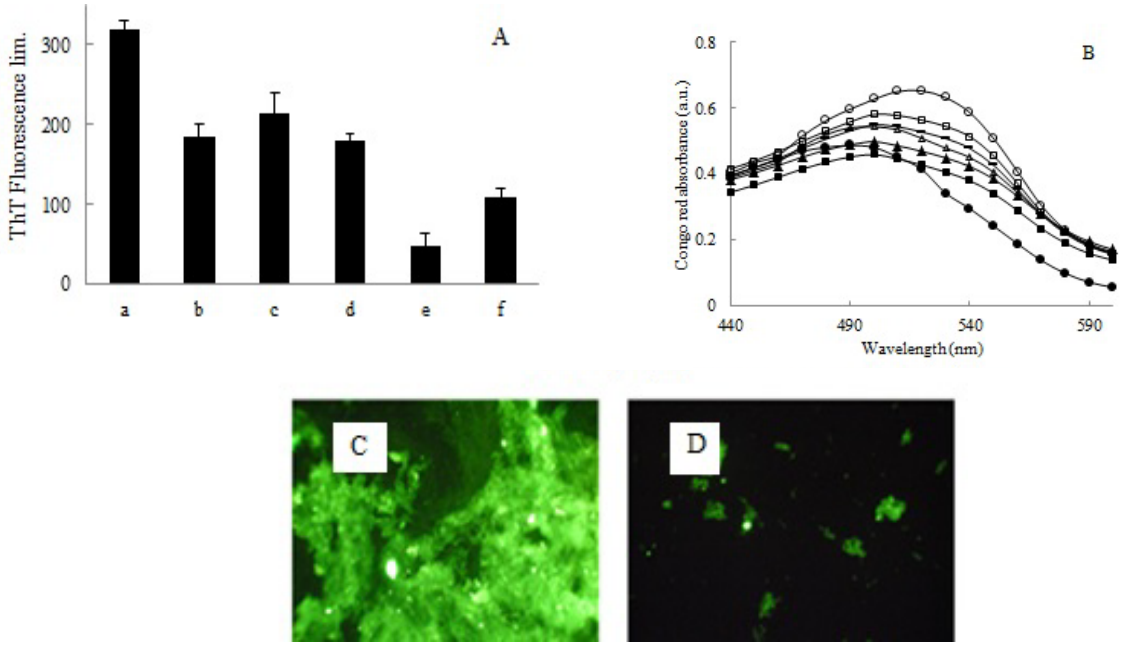


**Fig. 1.** Effect of the different organic fractions of Cuminum cyminum oil on the aggregation of hen egg white lysozyme. Fibrilation was estimated by A; thioflavin T fluorescence intensity and B; congo red absorbance in the absence (a,○) and presence of the fractions, b; n-hexane (□), c; n-hexane/toluene (-), d; toluene ( Δ), e; toluene/ethyl acetate (■), f; ethyl acetate (▲) and congo red (●). The samples were incubated at 57 °C/24 h. Fluorescence microscopic images of C; hen egg white lysozyme incubated for 48 h in the absence and D; the presence of the toluene-ethyl acetate fraction . The stated errors are the SDs of three repeated experiments.

Congo red absorbance measurements also showed that the presence of the T-E fraction did not lead to any red shifting; in fact, the absorbance of the incubated samples did not increase (Fig. 1B). Fluorescence microscopic images were also analyzed as described in the methods section. Proteins that were incubated for 48 h formed extended networks of fluorescent fibrils (Fig. 1C) and displayed low turbidity. By adding the T-E fraction to the protein during the fibrillation process, small particles of protein that appeared dense were induced (small amorphous particles) (Fig. 1D). In the next step, to study the fibrillation process further, the kinetics of ThT fluorescence throughout fibrillation was investigated in the presence of the T-E fraction (Fig. 2). Results showed the inhibitory effects of the T-E fraction for more than 90 h.

***Prevention of hen egg white lysozyme - induced cell death by the toluene-ethyl acetate fraction derived from Cuminum cyminum***

To study the cytotoxic effects of the assembled protein, the incubated protein was added to PC12 cell cultures (Fig. 3). The MTT assay indicated that the 24 h-aged amyloid fibrils had a significant lethal effect on the PC12 cells (48% cell death). However, adding the protein which was treated with the T-E fraction of *C. cyminum* did not induce cell death, and the viability of the PC12 cells was found to be considerably high (92%) (Fig. 3A). Release of LDH was also measured after a 24 h treatment of HEWL with the T-E fraction of *C. cyminum* (Fig. 3B). The permeability of the plasma membrane was the same as the control, when the T-E fraction was added to the protein during incubation. In comparison to the total essential oil of *C. cyminum*, adding only the T-E fraction to the cell culture did not lead to any destructive effects. Also, the monitoring of the cell morphology (Fig. 4) showed that the cultivated cells became distorted in the presence of fibril samples (Fig. 4B) when compared to the untreated cells (Fig. 4A), but the presence of *C. cyminum* oil destroyed cells significantly (Fig. 4C). On the other hand, cells treated with HEWL incubated with T-E fraction had intact normal shapes (Fig. 4D).

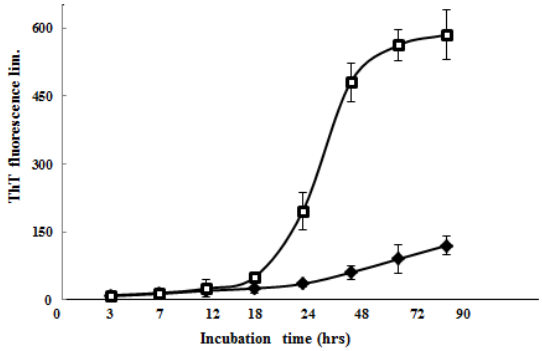


**Fig. 2.** The effect of the toluene-ethyl acetate fraction (5% (v/v)) on the kinetics of the fibrillation process. Time-study of the ThT fluorescence at 481 nm during

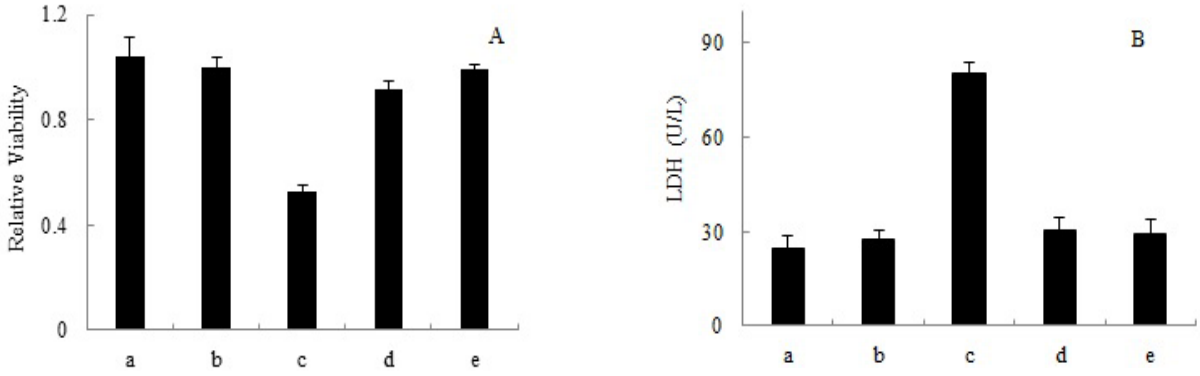


**Fig. 3.** The effects of the toluene-ethyl acetate fraction on the cytotoxicity of hen egg white lysozyme. Hen egg white lysozyme in the presence or absence of the toluene-ethyl acetate fraction (5%(v/v)) was incubated at 57 °C/24 h. These samples were then added to wells coated with PC12 cells. After 24 h of incubation, a; the viability of the cells was measured alone , b; with fresh hen egg white lysozyme, c; with 24 h pre-incubated hen egg white lysozyme, d; with 24 h pre-incubated hen egg white lysozyme in the presence of the toluene-ethyl acetate fraction and e; with the toluene-ethyl acetate fraction by using A; MTT , B; and LDH assays . The results of the 24 h pre-incubated hen egg white lysozyme were significantly different from those of the control ($P$<0.05).

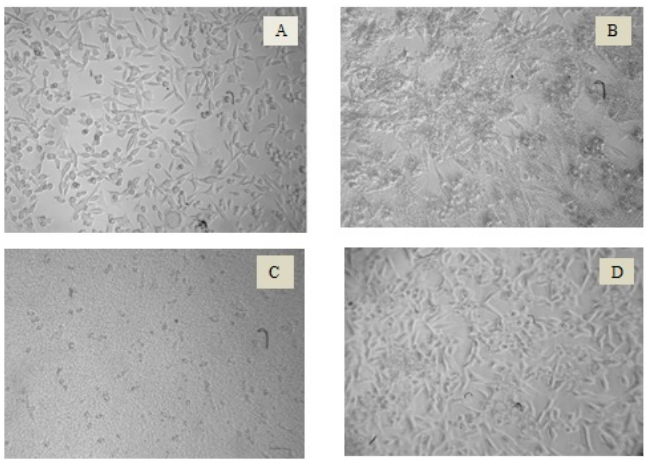


**Fig.4.** Morphology of PC12 cells, at 20X magnification. A; Control cells, B; cells treated with hen egg white lysozyme, C; cells treated with hen egg white lysozyme incubated in the presence of Cuminum Cyminum, and D; cells treated with hen egg white lysozyme incubated with 5%(v/v) toluene-ethyl acetate fraction derived from Cuminum cyminum.

The GC-MS analyses of the T-E fraction (Table 2) also indicated that this fraction contains only a limited number of compounds, and it may be that other compounds with possible negative effects on cell viability could have been reduced or eliminated by the fractionation process.

**Screening the anti-amyloidogenic property of the *toluene-ethyl acetate* fraction's compounds**

GC-MS analyses indicated the T-E fraction of the *C. cyminum* essential oil contained small molecules with very different chemical structures and properties. Fig. 5 represents the chemical structure of some of the identified small molecules although it is a rough conclusion from the GC-Mass analysis. To shed more light on the contribution of the constituents of the T-E fraction and their anti-amyloidogenic properties, small molecules with different structures that included 1,8-cineol, p-cymene, limonene, terpinolene and beta-myrcene were added individually to the incubated HEWL samples. The formation of fibrils was measured with ThT fluorescence and congo red absorbance intensity. Fig. 6A implies that ThT fluorescence intensities of the samples were very different, when incubated for 24 h in the presence of 2% (v/v) of 1,8-cineol, p-cymene, limonene, terpinolene or beta-myrcene. Terpinolene markedly reduced ThT fluorescence intensity, when compared to the control (by more than 87%). P-cymene also decreased ThT fluorescence intensity, less than that of terpinolene (close to 14%). In contrast, incubation in the presence of limonene induced fibrillation and increased ThT fluorescence intensity by more than 50%. The molecule, 1, 8-cineol, also increased ThT fluorescence intensity, however, this was less than that of limonene (approximately 30%). Beta-myrcene did not show any considerable effect on the fibrillation of HEWL. Congo red absorbance data also confirmed ThT fluorescence assessments. Congo red absorbance measurements indicated the occurrence of redshift in the presence of terpinolene. Furthermore, the absorbance of the incubated samples did not increase (data not shown).

Small molecules can have differential effects at all stages of the fibrillation process, including the ability to increase or decrease the rate of fibrillation. The kinetics of HEWL fibrillation, was assessed by measuring the ThT fluorescence intensity of the protein during four days of incubation in amyloidogenic conditions (Fig. 6B). It can be concluded that the presence of terpinolene stalls fibrillation for long periods of time, and thus has stronger inhibitory effects when compared to other reported small-molecule inhibitors (24). Conversely, limonene was found to induce fibrillation of the protein considerably, especially in the early phases of the fibrillation process. However, results showed that p-cymene and 1,8-cineol were effective for short periods of time, and during the late stages of the fibrillation process there were no marked differences, relative to the control samples. In fact, it seems that their effects were not stable over long periods of time.

Although the effects of terpinolene and limonene were dissimilar, but a question arises as to how the total oil of *C. cyminum* and its T-E fraction inhibit the fibrillation process.

**Table 2**. Chemical composition of the toluene-ethyl acetate fraction

| Compound | RI | Content (rel. %) |
|---|---|---|
| *β*-myrcene | 989 | 1.89 |
| α-terpinene | 1020 | 61.62 |
| limonene | 1032 | 3.24 |
| 1,8-cineol | 1036 | 2.12 |
| γ-terpinene | 1064 | 6.83 |
| α-terpineolene | 1092 | 19.16 |
| p-cymene-8-ol | 1189 | 2.17 |
| Total | | 97.03 |

RI: Retention index

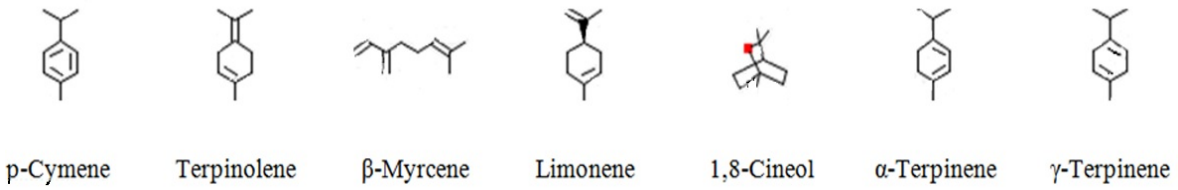


**Fig. 5.** Chemical structures of the main compounds of the toluene- ethyl acetate fraction.

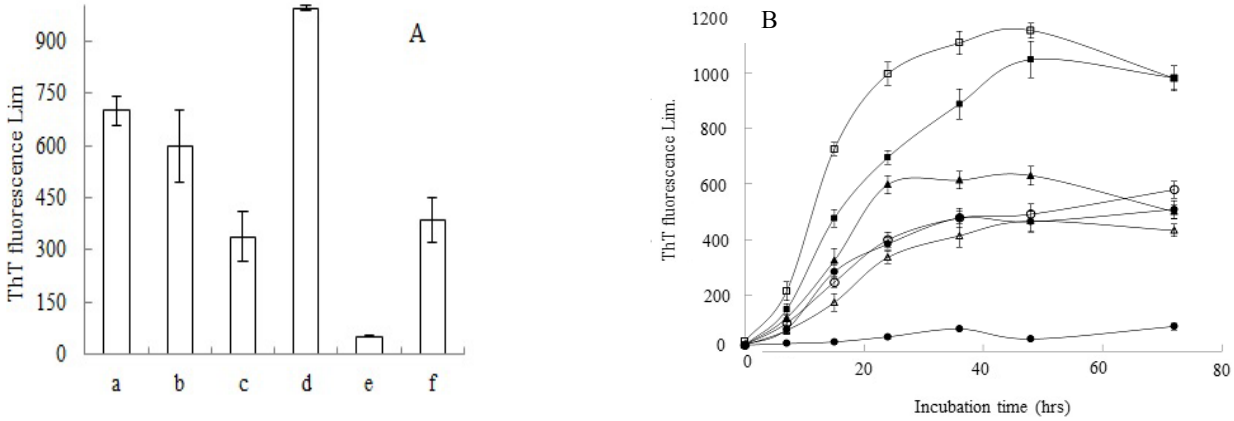


**Fig.6.** The effects of the test samples on the aggregation of hen egg white lysozyme . Fibril formation of hen egg white lysozyme fibrillation was estimated by A; Thioflavin T fluorescence emission, and also B; kinetics of fibrillation in the absence (a,○) and presence of small molecules including b: 1,8 cineol (▲), c: p-cymene (Δ), d: limonene (□), e: terpinolene (●), f: β-myrecene (■). The samples were incubated at 57 °C. The stated errors are the SDs of three repeats. The results were significantly different from those of the a; control except 1,8 cineol ($P$<0.05).

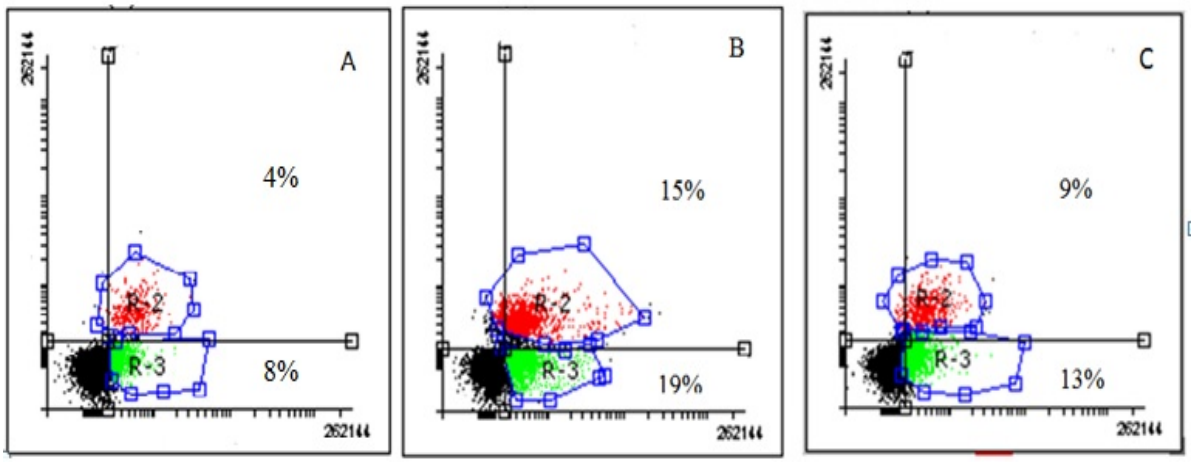


**Fig.7.** Flow cytometry assay to evaluate cell death: PC12 cells of the A; control, B; treated with 24 h- aged hen egg white lysozyme fibrils, and C; terpinolene-exposed 24 h- aged hen egg white lysozyme ) were double-stained with annexin-fluorescein isothiocyanate and propidium iodide. Lower left quadrant shows the surviving cells. The early apoptosis is presented in the lower right quadrant (R-3), and the late apoptosis/necrosis stages are in the upper right quadrant (R-2). The X- axis represents the fluorescein isothiocyanate-annexin channel, and the Y-axis is for the propidium iodide channel. The results of (C) were significantly different from those of (B) ($P$<0.05).

In order to understand these contradictory observations, the potency of the activity of the mixtures, including terpinolene and limonene, on HEWL fibrillation was assessed. Accordingly, different proportions of terpinolene and limonene were added to HEWL during the fibrillation process. The processes were followed over a 36-h period. Results indicated that the opposite effect of the compounds was dose-dependent. However in the presence of equal amounts of terpinolene and limonene, and lower amounts of limonene relative to terpinolene (1:2 or 1:3), there were significant reductions in the ThT fluorescence intensities (data not shown).

### *Assessment of the prevention of hen egg white lysozyme-induced cell death by terpinolene using flow cytometry*

It is believed that induction of apoptosis in infected cells represents the main harmful effect of amyloid aggregates. The inhibitory effects of terpinolene on the apoptosis of the PC12 cell line were evaluated by the double stained Annexin-PI flowcytometry procedure (Fig. 7). As shown in Fig. 7A, the population of apoptotic cells (both early and late) or necrotic cells in the control sample was only 12% of the total cells. However, the presence of 24 h-aged aggregated forms of HEWL increased early apoptosis rate by more than 19% of total cell population (Fig. 7B). As indicated in Fig. 7C, the rate of cell death declined, which supports the cytotoxic effects of terpinolene.

### *Docking to find a binding model of terpinolene with hen egg white lysozyme*

The mechanism by which small molecules interrupt the fibrillation process is not clear. NMR and Mass-spectroscopy techniques have shown that some inhibitors interact with hot-spot regions of target proteins (25,26). It is strongly believed that hot spots have critical roles in the induction of misfolding, and subsequently, the aggregation of proteins. Aggrescan software was used to predict the hot-spot areas in the proteins. As shown in Fig. 8A, five regions of HEWL have potential to self-aggregate (RI to RV). According to Aggrescan results, the docking assay (Autodock 4.0) was limited to the vicinity of the mentioned areas, so as to make smaller grids and fewer models for analysis (local docking). As indicated in Fig. 8B, the lowest energy conformation-binding site of terpinolene on the HEWL protein was computed based on the hot-spot areas. It is clear that this binding site is located around the active-site cleft of the protein which has more flexibility and vulnerability to misfolding. The amino acids, E35 (belongs to RI), W62, W63 (near RII) and V109, A 110 (RIV) were found to be the nearest residues to terpinolene, and thus may have important roles in intermolecular interactions (Fig. 8C and D).

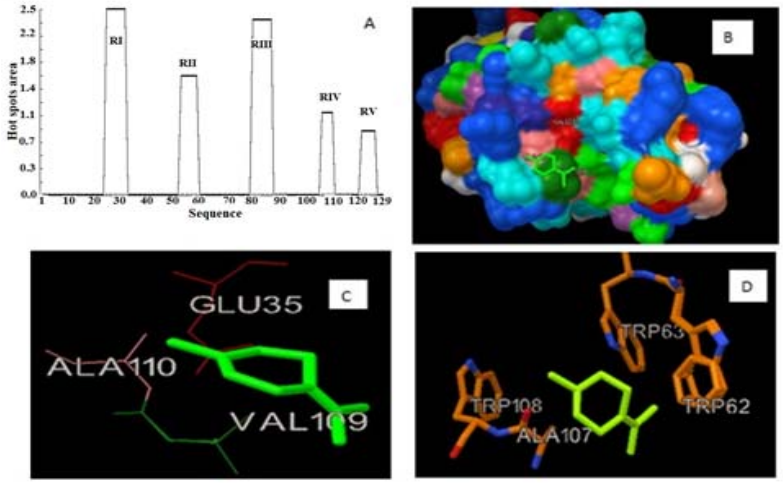


**Fig. 8.** Aggrescan analysis result of the hot- spot regions in lysozyme (PDB ID is 2VB13). The symbols, RI to RIV, A; presents the regions that have tendency to self-assemble, B; Autodock results of the terpinolene–lysozyme complex obtained by local docking close to the hot- spots regions, C; position of terpinolene at a binding site with the lowest energy of binding, and D; indicate the residues closest to terpinolene, including aromatic and aliphatic residues.

## DISCUSSION

In order to discern the efficient compounds of the *C. cyminum* oil to impede HEWL fibrillation, and also carry out further analyses of their structures, the essential oil was first fractioned using silica gel chromatography. Fluorometric and absorbance assays demonstrated that in the presence of the T-E fraction of the *C. cyminum* oil the fibrillation process was impeded significantly ($P< 0.05$). Fluorescence microscopic images also indicated that HEWL formed extended fluorescent particles after 24 h of incubation, while fibril formation of HEWL in the presence of the T-E fraction was blocked, and only small bodies were formed. Hence, important observations indicate that the confirmation of data from fluorometric and absorbance assays with a microscopic technique can be highly significant, because some compounds can interfere with ThT or congo red by binding to aggregated proteins. The kinetics of the fibrillation process was monitored by measuring the intensity of the fluorescence of ThT through a time-period of 96 h. Results showed that the rate of fibrillation was decreased significantly ($P<0.05$) and lasted for more than 90 h in the presence of the T-E fraction. This is interesting, since the inhibitory effects of small molecules are not usually long-term, possibly because of their unstable structures during the incubation procedure (9,24,27). This result encouraged us to continue the investigation to find out the exact compound that has long-term activity under harsh incubation conditions (low pH and high temperature).

The goal of this study was to inhibit the lethal effects of aggregated/fibrillar amyloid proteins on living cells. Cytotoxic effects of the aggregated proteins including mature fibrils, protofibrils, and low molecular weight intermediates at different stages of the fibrillation process are challenging questions. However, according to our previous study, it was clear that *C. cyminum* has greater toxic effects on cells relative to the aggregated proteins. This perilous effect on cultured cells is mostly due to the presence of phenols, aldehydes and alcohols. Consequently, in this study, the toxicity of the T-E fraction derived from *C. cyminum* was examined, but a considerable toxic effect was not observed. Incubation of the cells with HEWL (24 h) that was previously exposed to the T-E fraction during the incubation period did not result in cell death. Albeit, adding the T-E fraction to cells which were treated with the harmful aggregated form of HEWL did not protect the cells from eventual damage (data not shown).

GC-MS analysis indicated that the T-E fraction contained only eight compounds. Assessing the effects of these compounds on the fibrillation process, showed that terpinolene and limonene significantly affected fibrillation, but in an opposite manner. Adding both compounds at the same time to the incubated protein indicated that the strength and potency of terpinolene was more than that of limonene. It seems that there is competition between terpinolene and limonene to interact with HEWL, and the association constant for terpinolene is higher than that of limonene. Do they interact with the same site on the monomeric protein, or with different parts of the macromolecule, and at different stages of the fibrillation process? These ambiguities need to be investigated further.

It is very interesting that terpinolene has a robust inhibitory activity, which seems to be stronger than that of previously reported amyloid inhibitors. Terpinolene has a relatively simple structure with no conjugated aromatic rings like indoles, and seems to have important roles in the interruption of the fibrillation process via pi-stacking interactions (28,29).

As mentioned above, the different aggregated species of amyloidal proteins are associated with the pathogenesis of amyloidosis. They induce internal and external signaling pathways of apoptosis by disrupting membranes, increasing reactive oxygen species (ROS) levels and disturbing controlling systems within cells. In the absence of external clearance systems, such as phagocytes and imperfection of efferocytosis, the apoptotic cells convert to a stage which is known as late apoptosis. The results showed early apoptotic and late apoptotic/necrotic stages in the downright and upright sections

for the two fibril-treated cell populations, respectively. These different populations of cells might be due to the different stages of the cellular cycle (mitosis, G or S phase) that the cells could have been in. As shown in the present study, terpinolene inhibited the fibrillation process in HEWL. Cell viability assays indicated the harmless effects of terpinolene. However, despite the inhibitory effects of some small molecules on the fibrillation process, they induce production of more dangerous intermediates and only inhibit production of mature fibrils, consequently increasing the cytotoxic effects of the fibrils. There are different strategies to inhibit protaneous fibril formation or its cytotoxic effects.

Some compounds can stabilize the native forms of proteins. In this case, interaction between ligands and target proteins stops the misfolding phenomenon. For instance, interaction of transthyretin tetramer with tafamidis meglumine strongly stalls the misfolding process (30). Another strategy is to sequester or cover the aggregation- prone sites in the protein sequences, in order to prohibit self-assembly. This is an especially important strategy for disordered peptides and proteins, such as Aβ and alpha-synuclein. So far, many small molecules and affibodies which can interact with the aggregation- prone regions, have been introduced (31).

There is much effort to rationally design small molecules with specific inhibitory effects on fibrillation of certain proteins or peptides. Some general structural features based on hydrophobic patches, aromatic rings and hydrogen bonding groups, are considered. Although terpinolene does not have these general features, but it can strongly inhibit the HEWL fibrillation process. Docking analysis in the vicinity of regions with high potential for self-assembly indicated that terpinolene could interact with certain residues (two critical tryptophan residues, and also two aliphatic ones).

It is has been shown that the π-π and stacking interactions have important roles in the induction of beta-form fibrils in proteins. Since aromatic residues are in a consecutive order (tryptophan 62 and 63), their potential role becomes more important. On the other hand, many successfully designed beta sheet-breakers contain special aromatic parts to disrupt the π-stacking in the proteins. Furthermore, alanine and valine are hydrophobic residues that tend to form hydrophobic interactions. In fact, it is notable that terpinolene has a non-flat ring when compared to other similar aromatic rings, which are almost flat. Due to the very complex process of fibrillation, in which many different misfolded and aggregated species of proteins are formed, it is not easy to identify and elucidate the exact inhibitory mechanism of terpinolene. Additionally, even with more accurate techniques like NMR or mass-spectrometry, it is difficult to examine the exact interactions between small molecules and proteins.

## CONCLUSION

In this study, for the first time, the amyloid-inhibitory effect of terpinolene was determined through fractionation of Iranian *C. cyminum* essential oil. The strong and long-term anti-fibrillatory effects of both the toluene-ethyl acetate fraction of the essential oil and the terpinolene, is the hallmark of this study.

The results can help us to design more stable compounds with anti-amyloid properties. Also, computational analysis revealed that terpinolene has a considerable tendency to interact with certain self-assembled regions (hot spots), and hence, perturb protein-protein interaction.

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