RESEARCH ARTICLE

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Cytoprotective Properties of Turmeric Oil (*Curcuma longa* L.) on Fibroblast Cells

Riris Istighfari Jenie[1,2]*, Anif Nur Artanti[2,3], Rumiyati[1,2], Dhania Novitasari[4,5], Yuni Kusumastuti[6], Manami Toriyama[7]

Abstract

Objective: Senescence is a cellular physiological process involved in cell aging. One factor that increases senescence is oxidative stress, which can be induced by hydrogen peroxide. Active compounds in turmeric *(Curcuma longa*) are classified as volatile and non-volatile. Major non-volatile compounds in turmeric are curcumin, dimethoxy curcumin, and bisdemethoxycurcumin bioactivities that have been widely explored. However, turmeric rhizome oil (TO) has limited reports on its bioactivity and constituents. This study aims to determine the potency of TO as cytoprotective against oxidative stress induced by hydrogen peroxide using the fibroblast cell lines (NIH-3T3 and HDF). **Methods:** We evaluated the cytotoxicity of TO using MTT assay, then evaluated its effect on cell senescence using SA-β-gal assay. The cellular reactive oxygen species (ROS) level was observed using DCFDA staining through flow cytometry. The turmeric volatile oil which was obtained by steam-water distillation was analyzed with a gas chromatography-mass spectrophotometry (GC-MS) to determine the chemical profile. **Results:** TO showed low cytotoxicity against HDF and NIH-3T3 cells, with IC_{50} values of over 100 μM. TO rescued cells from undergoing senescence and reduced ROS levels which were induced by hydrogen peroxide. The GC-MS spectra of the TO compound in positive ionization mode showed retention times of 23.56 and 26.20 minutes, corresponding to the ar-turmerone and turmerone compounds. **Conclusion:** These results indicated that TO has the potency as a cytoprotective agent in stress oxidative conditions.

Keywords: senscence- sesquiterpenes- oxidative stress- turmeric oil- fibroblast

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Introduction

The aging process involves reducing functionality at various levels, such as organelle, cellular, tissue, and organismal, ultimately imposing limits on life. Aging results from many contributing elements that impair the structures and functions of molecules, cells, organs, and other parts of an organism. These factors include oxidative stress, glycation, telomere shortening, adverse effects, mutations, and protein aggregation [1]. The aging process involves many steps that direct the accumulation of cells toward senescence. Senescence is a cellular physiological process where aged or damaged cells permanently enter the G0 phase [2]. By staying in the G0 phase, the senescent cells will not replicate or proliferate.

Cellular senescence is important during wound healing, tissue remodeling, and embryogenesis. However, these senescent cells, particularly the prolonged ones, can lead to age-related diseases and cancer development [3]. The elevated generation of reactive oxygen species (ROS) is also linked to the cell senescence process. ROS level contributed to the induction and maintenance of senescence. On the other hand, a low level of ROS is related to the lengthening of the organism's lifespan [4]. The senescent cells remain functionally and metabolically active during changes, such as alterations in protein degradation pathways and increased mitochondrial metabolism for energy generation. Stopping the growth of damaged cells is the aim of arresting senescent cells, therefore preventing the damage from transferring to the following generation of cells. Additionally, this process aims to eradicate accumulated detrimental elements and

[1]*Laboratory of Macromolecule Engineering, Department of Pharmaceutical Chemistry Faculty of Pharmacy, Universitas Gadjah Mada (UGM), Sekip Utara, Yogyakarta 55281, Indonesia.* [2]*Cancer Chemoprevention Research Center, Faculty of Pharmacy, Universitas Gadjah Mada (UGM), Sekip Utara, Yogyakarta 55281, Indonesia.* [3]*Department of Pharmacy, Vocational College, Universitas Sebelas Maret Surakarta, Central Java 57126, Indonesia.* [4]*Laboratory of Tumor Cell Biology, Division of Biological Science, Graduate School of Science and Technology, Nara Institute of Science and Technology, Ikoma 630-0192, Japan.* [5]*Department of Pharmaceutical Analysis and Medicinal Chemistry, Faculty of Pharmacy, Universitas Padjadjaran, Sumedang 45363, Indonesia.* [6]*Chemical Engineering Department, Faculty of Engineering, Universitas Gadjah Mada, Yogyakarta, Indonesia.* [7]*Laboratory of Molecular Signal Transduction, Division of Biological Science, Nara Institute of Science and Technology 630-0192, 8916-5, Takayama-cho, Ikoma, Nara. *For Correspondence: riris_jenie@ugm.ac.id*

thwart potential transformations that lead to malignancy [5]. Therefore, senescent cells are becoming a strategic target to halt the aging process and prevent aging-related diseases. Targeting senescent cells using a cytoprotective strategy may offer hope for the treatment of disorders involving related mechanisms.

Turmeric, scientifically known as *Curcuma longa* L., holds significant value as an anti-oxidant and anti-inflammation [6]. It plays a vital role in the composition of curry powders and has a rich history of utilization. The constituents found in turmeric are generally categorized as either non-volatile or volatile. The primary non-volatile curcuminoids consist of curcumin, demethoxy curcumin, and bisdemethoxycurcumin [7]. The volatile oil derived from turmeric, namely turmeric oil (TO), is typically yellowish and dense, often emitting mildly aromatic notes. TO has a promising potential to be developed as an agent to prevent, delay, or treat diseases like cardiovascular, pulmonary, neurodegenerative, metabolic diseases, and cancer [8]. Those potentials may be based on the association of the disease development with inflammation.

The pharmacological impacts of turmeric oil (TO) are associated with its key components, such as phenylpropanoids, terpenoids, and flavonoids. Dried rhizomes typically present approximately 3–6% of this essential oil. Sesquiterpenes, which include bisabolenes, are the main constituents of TO [9]. The main bisabolene sesquiterpenes within TO are ar-turmerone, α-turmerone, and β-turmerone. Some noteworthy chemicals in TO are also included in the referenced article and are recognized for their documented biological functions [6].

TO exhibited substantial antioxidant activity as demonstrated by the radical scavenging assay and the ferric-reducing antioxidant power test [10]. TO was reported to reduce the expression of nitric oxide (NO) synthase and demonstrated immunomodulatory characteristics [11]. When ar-turmerone is administered alongside curcumin, there is a notable enhancement in the permeation of curcumin across the Caco-2 cell monolayer [12]. Furthermore, it is highly advisable to employ hydro-distillation for TO extraction. The use of organic solvents may introduce potential issues related to toxicity, either due to insufficient solvent clearance or the unintentional collection of chemicals not typically found in the standard composition of turmeric rhizome oil.

Despite the remarkable activities of TO as an antioxidant, there is no available study on the cytoprotective effect of TO in hydrogen peroxide-induced cells and its constituents. Hence, the current study evaluated the cytoprotective potential of turmerone derived from TO when exposed to hydrogen peroxide, utilizing fibroblast cell lines (mouse and human-derived fibroblast cells). Fibroblast cells function to support skin flexibility by forming the cellular matrix. The evaluations observed included the cytotoxicity of TO towards the fibroblast cell lines and its effect on cellular senescence and ROS. Moreover, to identify compounds responsible for the activities of TO, we evaluated the TO phytochemical profile.

Materials and Methods

Instruments and Material

Materials used for this study are turmeric (*Curcuma longa* L.) obtained from Pacitan, East Java, Indonesia. We analyze the chemical profile of turmeric oil using gas chromatography-mass spectrometry (GC-MS) from Shimadzu single quadrupole GC-MS-QP2010 Ultra. We used two fibroblast cell lines, first is NIH-3T3 (ATCC® CRL-1658), a mouse-derived fibroblast cells which is a collection of Cancer Chemoprevention Research Center, Faculty of Pharmacy, Universitas Gadjah Mada. This cell line was cultivated under standard conditions, specifically at 37°C with 5% CO2 , in Dulbecco's modified Eagle's Medium supplemented with 10% fetal bovine serum (Sigma, USA) and 1% penicillin-streptomycin (Sigma, USA). We also used human dermal fibroblast cells (HDF), obtained from Promo Cell, Germany (Cat #C-12302). The cells were grown in fibroblast growth medium (Cat #C-23020). Stress oxydative was induced by treating cells using hydrogen peroxide H_2O_2 (Sigma).

Turmeric oil preparation

One hundred kilograms of turmeric (*Curcuma longa* L.) was steam-water distilled with aquadest using a distillation assembly for 8 h. The distillation results are left for 24 hours to separate the oil components. The turmeric oil (TO) was collected for the experiment.

GC-MS Analysis

The chemical profiles of TO were assessed using GC-MS as described in the previous study of analyzing curcuma essential oil [13]. This analysis utilized a 30 m x 0.25 mm RP-5 non-polar column (Shimadzu, Japan) with a film thickness of 0.25 μm. Helium was employed as the mobile phase and carrier gas, flowing down the column at a rate of 0.55 mL/min and having a split ratio up to 139. A column was first set at 40°C, and after 10 minutes, a sample volume of 0.2 μL was injected. The column was then ramped up to 180°C at a rate of 2.5°C per minute, and this temperature was maintained for 20 minutes. The mass spectrometry operated in an electron impact ionization mode with an ionization energy of 70 eV. It scanned at a rate of 0.5 scans per second in the range of m/z 28.00 to 600.00. The quadrupole temperature was kept at 300°C, the electron multiplier voltage at 1.5 kV, and the ion source was maintained at 150°C. The Wiley MS Libraries were used to verify each compound's retention time and retention indexes.

Cell Viability Assay

The MTT test was utilized to evaluate the safety of the sample on a cellular level. Initially, NIH-3T3 and HDF cells were seeded in a 96-well plate at the density of 1×10^4/well. The following day, the cells were exposed to serial concentrations of turmeric oil (TO) for 24 h. Subsequently, 100 μL of 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) reagent (Biovision, USA) was added to the cells and left to incubate for 24 h. After the

incubation period, a stopper solution containing sodium dodecyl sulfate (SDS) (Sigma) and 0.01N HCl was introduced to each well. The absorbance at 595 nm was measured using an ELISA reader, and the cell viability percentage was calculated from the absorbance data. The IC_{50} value was calculated using linear regression analysis of the sample concentration and the cell viability percentage [14].

SA-β-gal Senescence-based Assay

The evaluation of β-galactosidase expression in connection with senescence was carried out through the SA-β-gal assay as previously described [13]. In this procedure, HDF (1×10^5 cells) or NIH-3T3 cells (2×10^4 cells) were initially placed in a 6-well and left to incubate for 24 hours with 50-60% confluence to minimize false-positive staining. After treatment with TO and 100 µM H_2O_2 for the next 24 hours, the cells were rinsed twice with 1× PBS. Subsequently, a fixative solution (2% formaldehyde-0.2% glutaraldehyde) was introduced, and fixed for 10 minutes, and then the cells were rinsed once more with 1× PBS. One to two milliliters of the X-gal solution was added, followed by incubation at 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) (Wako, No cat. 021-07852), 40 mM 2 × PBS (pH 6.0), 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$ and 2 mM $MgCl_2$. After 24 h, the cells were observed using a microscope (Olympus CKX-41) and documented at a total magnification of 200×. The blue-green cells marked the presence of β-galactosidase-positive cells, which are indicative of senescent cells. The minimal total number of cells used for analysis was 100 cells per field of view. The senescent cells were divided by the total number of cells to determine the proportion of senescent cells.

DCFDA Staining ROS-based Assay

Reactive oxygen species (ROS) intracellular concentration changes were measured using the fluorescent probe 2’, 7’–dichlorofluorescin diacetate (DCFDA) (Sigma, #D6883, USA), specifically designed for identifying highly reactive oxygen radical species. A total of 5×10^4 cells were grown into a 24-well plate and allowed to incubate for 24 hours. Subsequently, cells underwent treatment with TO and 100 µM H_2O_2 after 30-min exposure to 20 µM of DCFDA, and the cells were stored in a 37°C incubator with CO_2. Upon the completion of the incubation (24 h), the cells were resuspended and filtered in the tube before being measured for their fluorescence intensity with a flow cytometer (BD Biosciences FACS Calibur) set to an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Later, the ROS level intensity was converted as the total fold of the untreated group [15].

Statistical Analysis

The ANOVA test was used to evaluate statistical differences, and Tukey HSD was used for the posthoc analysis using GraphPad Prism ver. 9.0 (Boston, USA). The respective p-values have been provided within each figure in the experiments.

Results

Cytotoxicity of TO on NIH-3T3 and HDF Cells

Cytotoxicity assay was performed to evaluate the safety of TO on fibroblast cells. The IC_{50} data will be used to determine the appropriate concentration for the subsequent experiment where we need to treat the cells in sub IC_{50} concentration to distinguish the effect on cellular senescence and ROS level from the cytotoxicity effect of TO. HDF and NIH-3T3 cells were utilized as a model of fibroblast cells. In HDF cells, there was a bit of a fluctuating phenomenon on cell viability at lower concentrations (10, 25, and 50 µg/mL) which was likely due to the variance of the cell response, though a decreased cell viability was later seen at higher concentrations. TO showed a low cytotoxicity against HDF with IC_{50} 280 µg/mL (Figure 1A). Furthermore, as Figure 1B illustrates, the viability of NIH-3T3 cells was dose-dependently decreased upon exposure to TO. With an IC_{50} of 1,057 µg/mL, TO was found to have no cytotoxic effects on NIH-3T3 cells.

TO Effect on the Fibroblast Cellular Senescence

We performed an SA-β-gal senescence-based assay to observe the TO effect on fibroblast cellular senescence.

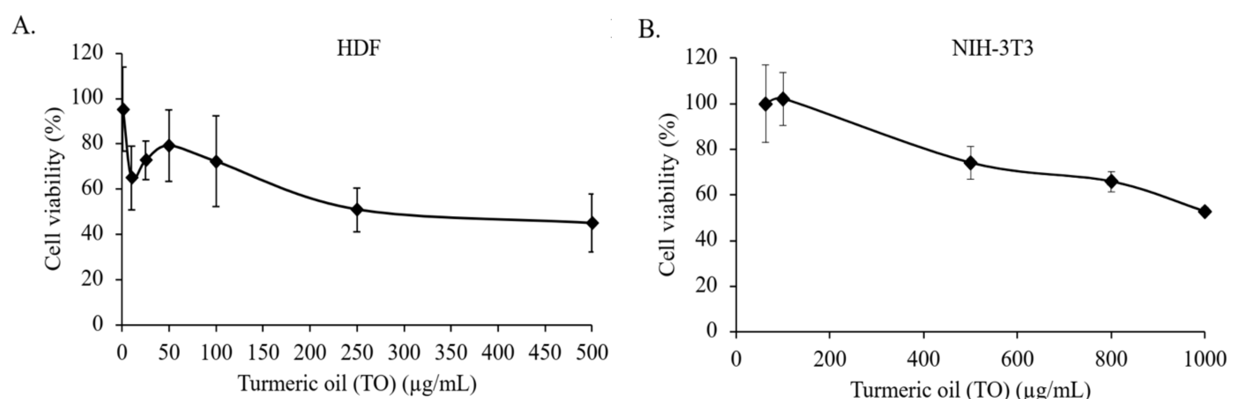

Figure 1. Cytotoxicity of TO on HDF and NIH-3T3 Cells. A. HDF Cells (1×10^4 cells/mL) and B. NIH-3T3 cells (1×10^4 cells/mL) were exposed to TO concentrations as indicated in the graph for 24 hours and subsequently assessed using the MTT assay. The absorbance was converted to the percentage of cell viability against concentration. Data were expressed as mean from three experiments ± SD.

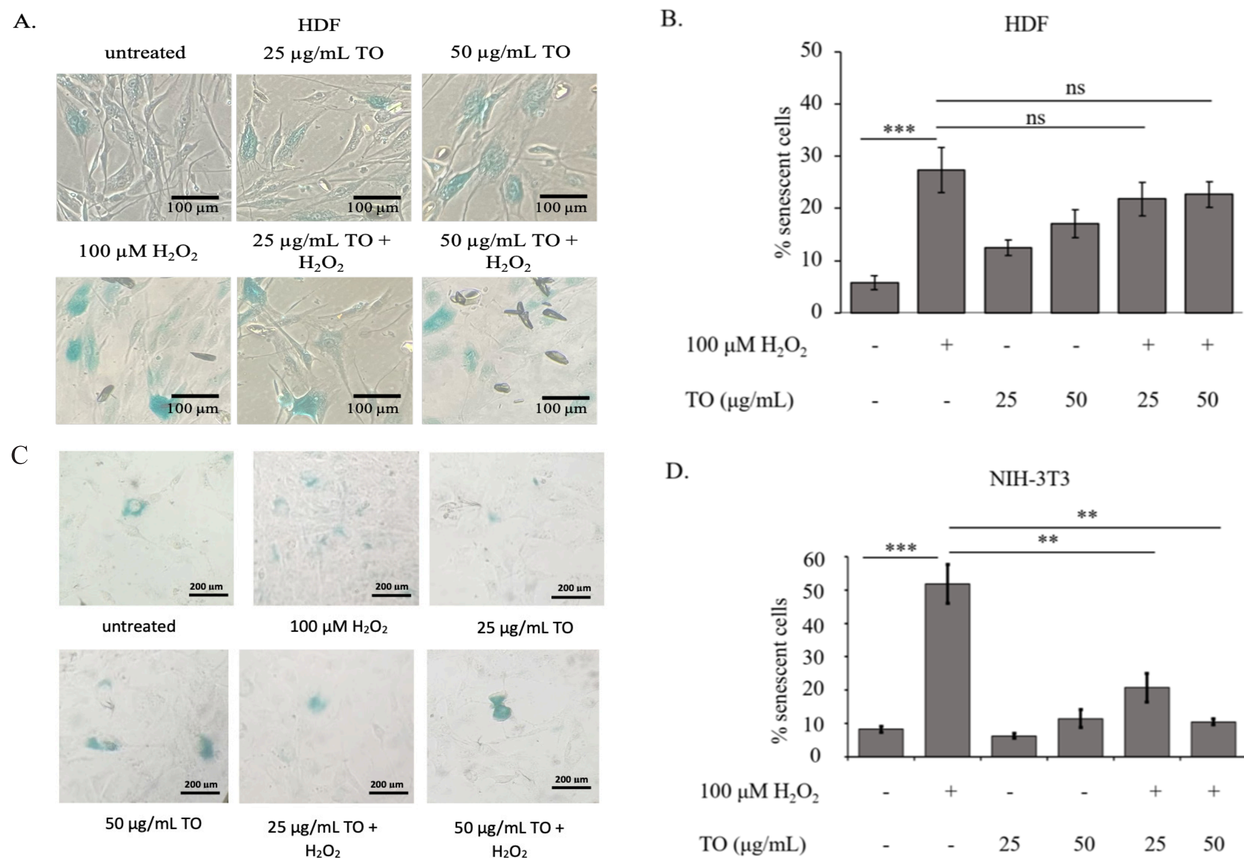

Figure 2. Cellular Senescence in NIH-3T3 and HDF Cells Following Treatment with TO. The analysis of senescent cells was conducted using the SA-β-galactosidase-based assay. NIH-3T3 (2×10^4 cells/mL) and HDF cells (1×10^5 cells/mL) were treated with 100 μM H_2O_2, TO at 25 and 50 μg/mL, as indicated in the graph, for 24 hours. Subsequently, cells underwent β-galactosidase staining, as described in the methods. The percentage of senescent cells (blue-green β-galactosidase-positive cells) was determined (n = 3). Cellular morphology of HDF (A) following the staining with X-gal; (B) The percentage of HDF senescent cells after quantification of senescent-positive cells. (C) A similar experiment was also carried out for NIH-3T3 under TO treatment for 24 h. (D) The quantification of NIH-3T3 senescent cells was conducted and presented as a percent of senescent cells. The graph data was expressed as the mean of three data ± SD. ns = not significant; **, p< 0.01; ***, p< 0.001.

We used hydrogen peroxide (H_2O_2) to induce cellular senescence, and we showed here that the exposure of 100 μM H_2O_2 significantly (p< 0.001) induced cellular senescence in both fibroblast cells (Figure 2). This increase is similar to a prior study which reported that hydrogen peroxide induces cellular senescence in fibroblast cells [16]. Our data revealed that TO treatment slightly suppresses H_2O_2-treated HDF cells although application of TO alone tended to induce cellular senescence (Figure 2A-B). Quite a different result was demonstrated in NIH-3T3 cells. TO markedly (p< 0.01) reduced cellular senescence in H_2O_2-treated NIH-3T3 cells in a concentration-dependent manner, while it did not affect the percentage of senescent cells in H_2O_2-untreated cells (Figure 2C-D). These results indicate that TO protected NIH-3T3 cells against the hydrogen peroxide-induced upregulation of SA-β-gal activity.

Effect of TO on ROS Level in HDF Cells

TO at concentrations of 25 and 50 μg/mL inhibited senescence either in HDF or NIH-3T3 cells that were induced by H_2O_2 treatment. We further investigated any potential correlation with ROS activity by using H_2O_2 to raise the levels of ROS within the cells. Hydrogen peroxide induces the level of hydroxyl radical (ROS) thus, promoting oxidative stress [17]. The exposure of H_2O_2 in HDF (Figure 3A) or NIH-3T3 (Figure 3B) significantly (p< 0.0001) induced cellular ROS level, furthermore the addition of TO (25 and 50 μg/mL) markedly reduced the ROS level in the presence of hydrogen peroxide (Figure 3). We noted that the single 50 μg/mL TO treatment showed an increased level of ROS in both cells, but not with the lower concentration (25 μg/mL). Nevertheless, this finding suggests that turmeric oil reduced the high ROS level that fibroblast cells experienced as a result of oxidative stress exposure.

Chemical Profile of TO

The metabolite compounds of a natural substance have a strong correlation with its pharmacological effect therefore, we conducted a chemical profile analysis of TO by employing GC-MS to examine its volatile oils. In this analysis, we identified a total of 115 components within TO, which collectively account for approximately 100% of the total volatile oils.

The major components of the essential oils were

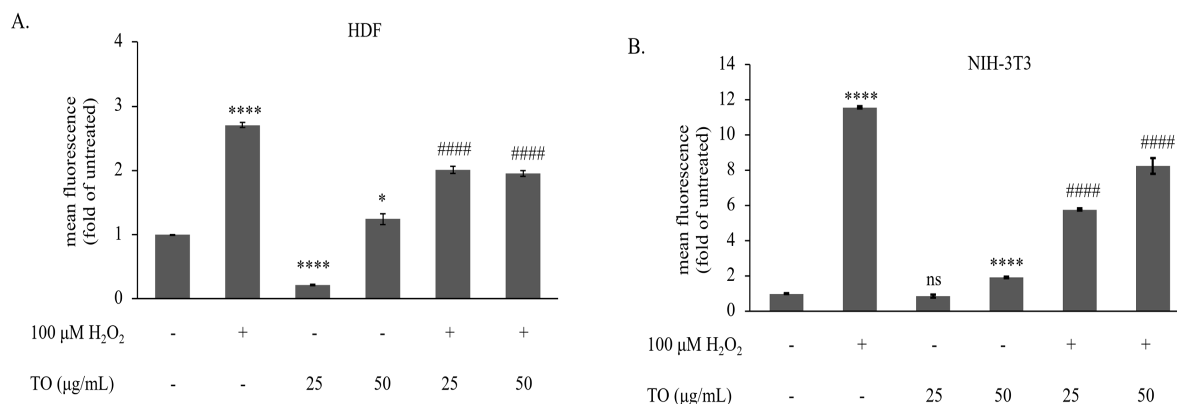

Figure 3. Effect of TO on Cellular ROS Level in (A) HDF and (B) NIH-3T3 cells. The DCFDA staining test was used to measure the ROS levels following H_2O_2 induction and TO treatment as indicated in the graph. After 24 hours of treatments, the cells were exposed to flow cytometry to detect the ROS levels (n = 3). The data was presented as the average of three data ± SD. *, p< 0.05; ****, p< 0.0001 against untreated; ####, p< 0.0001 against hydrogen peroxide-treated group; ns= not significant.

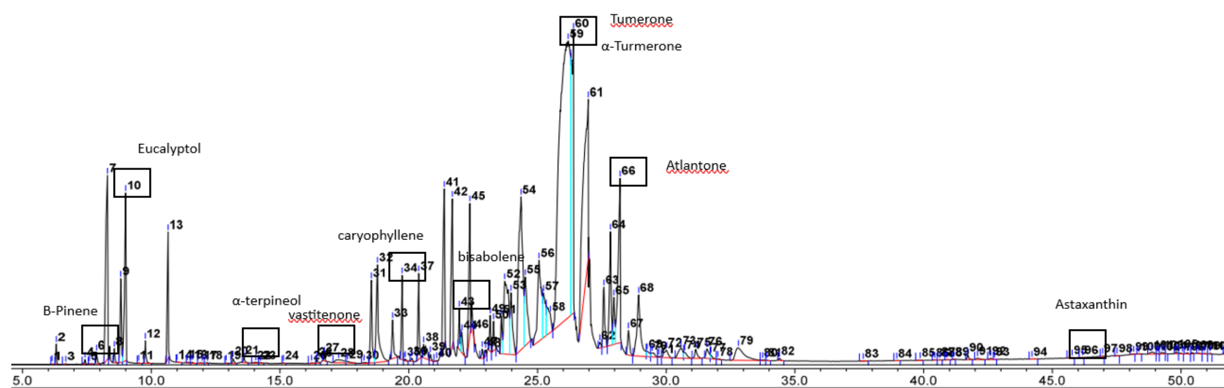

Figure 4. The Chromatogram Depicting Compounds Containing TO. The chemical constituents of TO were generated through the gas chromatography-mass spectrometry method outlined in the Methods section.

α-turmerone (29.51%), tumerone (5.75%), and atlantone (3.45%) (Figure 4 and Table 1). Previous studies reported that sesquiterpenes particularly bisabolene constitute the primary components of TO [18-19]. Among these, ar-turmerone, α-turmerone, and β-turmerone are the key bisabolene sesquiterpenes. The predominant compounds identified in the TO sample (Table 1) are recognized for their antioxidant characteristics [20].

Table 1. Major Compounds of TO Analyzed by Gas Chromatography-Mass Spectrometry

Peak	Retention time (min)	Abundance (%)	Compound
6	7.43	0.1	Beta pinene
10	9	2.13	Eucalyptol
21	13.59	0.8	Alpha terpineol
27	16.72	0.7	Vastitenone
34	19.74	0.87	Caryophyllene
43	21.97	0.45	Bisabolene
59	26.2	29.51	α-Turmerone
60	26.4	5.75	Tumerone
66	25.06	3.45	Atlantone
96	46.18	0.02	Astaxanthin

Discussion

In essence, our study is designed to investigate the potential of turmeric oil (TO) as an anti-senescence agent, aiming to inhibit the aging process, particularly in fibroblast cells as major components in skin tissue. Concentrating on this aspect, we assessed the evidence-based anti-senescence characteristics of the physiological modifications induced by TO in normal fibroblast cells, as demonstrated by HDF and NIH-3T3 cells. To ensure the safety of the material, we initially conducted a cytotoxicity test. The results revealed that TO exhibited no cytotoxic effects in either cell type, even at a concentration of 200 µg/mL (Figure 1A and 1B). These findings suggest the safety of TO, especially for fibroblast cells. They also align with empirical evidence from Asian populations who consume TO without experiencing poisoning or significant side effects [21]. Furthermore, this outcome confirms that TO is safe for incorporation into daily consumption.

Cellular senescence can be triggered by non-lethal stress, or in other words is known as stress-induced premature senescence. Along many stressors, hydrogen peroxide (H2O2) is recognized as a well-established

inducer of premature senescence [22]. Thus, the utilization of hydrogen peroxide as a model for pro-oxidant in the study of oxidative stress is adequate to assess the senescence activity of TO in fibroblast. Our study revealed that even though a single treatment of TO (50 μg/mL) increased the senescence in HDF and NIH-3T3 cells, TO was able to reduce the cellular senescence in hydrogen peroxide-induced cells (Figure 2B and 2D). This result showed that TO may protect the fibroblast from premature senescence. In a normal environment, turmeric oil may trigger mild oxidative stress, leading to senescence. Turmeric oil can generate reactive oxygen species (ROS) within cells. While low levels of ROS can act as signaling molecules, excessive ROS can damage cellular components, leading to oxidative stress [17]. This stress can trigger the DNA damage response, which is a well-known pathway leading to cellular senescence. However, under extreme oxidative stress (like hydrogen peroxide induction), it might act differently, potentially by activating protective pathways such as upregulating antioxidant defenses or inhibiting pathways that would otherwise lead to excessive damage and senescence. Turmeric oil contains compounds that modulate the nuclear factor erythroid 2–related factor 2 (Nrf2) pathway, which is a key regulator of the cellular antioxidant response [23]. Activation of this pathway under oxidative stress conditions could help mitigate the effects of hydrogen peroxide, reducing cellular damage and preventing premature senescence.

We hypothesized that TO might attenuate the premature senescence as it reduced the ROS level in hydrogen peroxide-treated cells. Oxidative stress is believed to cause fibroblast senescence, whereas antioxidants are known to offer protection to prevent cellular damage [17, 24]. The antioxidant properties of turmeric oil have been documented in another study which used a distinct assessment to evaluate its scavenging activity, and this effect may be due to the presence of ar-turmerone [10]. Our results showed that TO decreased the elevated cellular ROS level induced by hydrogen peroxide (Figure 3). The aging process is often linked to an increase in ROS levels within cells [4], therefore, these results provide the possibility of the application of TO as an antiaging agent. Moreover, turmeric oil showed notable anti-inflammatory effects in both acute and chronic inflammation in animal models [25]. It is worth noting that curcumin from Curcuma longa L. has also demonstrated genotoxic effects [26]. This suggests the possibility that TO may function as both an anti-senescence, anti-inflammatory, and anti-genotoxic agent.

Based on our senescence and ROS assay data, we found that NIH-3T3 cells were more sensitive than HDF cells in response to hydrogen peroxide induction. The same concentration (100 μM) of hydrogen peroxide, induced almost 30% cell senescence and approximately 3-fold ROS level in HDF cells, while in NIH-3T3 cells it induced almost 50% and 12-fold ROS level (Figures 2 and 3). The difference in cell sensitivity explains why HDF cells were only slightly affected by TO and NIH-3T3 showed more significant results under TO treatment as we described in the results section. HDF cells and NIH-3T3 cells differ primarily in their origin (human vs. mouse) and this difference in species can influence cellular behavior, including senescence pathways. HDF cells are frequently used to study human-specific aspects of senescence, such as age-related diseases and therapies [27]. On the other hand, although NIH-3T3 cells may not perfectly mimic human cellular senescence processes, they are more commonly used in basic research and may serve as a model system for studying general cellular processes including senescence mechanisms [28].

The presence of compounds with antioxidant properties enhances the value of its use in promoting a healthy body. The GC-MS analysis on the TO utilized in this study revealed the presence of common TO compounds, such as turmerone and its derivatives, sesquiterpenes, and atlantone (Table 1), which are extensively recognized for their antioxidant properties. The composition of these substances protects cells from oxidative damage and supports TO's ability to serve as an anti-senescence.

Cellular senescence which is characterized by irreversible cell cycle arrest, plays a role in both physiological and pathological aspects of aging. Senescent cells are no longer able to proliferate, but they are still alive, metabolically active, and eventually undergo apoptosis [29]. Therefore, the development of anti-senescence agents with cytoprotective properties could serve as a strategy for combating the aging process. Ar-turmerone exhibited superior anti-dermatophytic effectiveness compared to ketoconazole [6]. Given the absence of adverse skin reactions in guinea pigs following the topical application of ar-turmerone in the previous report [6], TO is predicted as a promising agent to be applied on the skin.

In light of this, we suggest that TO plays a role in anti-senescence, which may be correlated with its effect in reducing elevated cellular ROS induced by stress oxidative. This implies its potential use as a standard for evaluating the quality of essential oil products derived from turmeric, especially for anti-aging product purposes. It is important to note that this study utilizes in vitro models with specific cell types, and further clarification is needed including the underlying mechanisms of the findings, especially concerning the relationship between ROS and senescence in the two cell types.

Conclusion

Taken together, TO proves to be safe and exhibits activity to inhibit cell senescence in normal cells, particularly in fibroblast cells. TO showed its ability to reduce senescence and suppress ROS level elevation induced by hydrogen peroxide. Based on the results of this study, it is worth exploring more on the TO potential to counteract aging and aging-related disorders.

Author Contribution Statement

Riris Istighfari Jenie: Research idea and finding funding for the research, design the research, supervising the experiment, analysis and curating the data, drafting the manuscript, revising the manuscript. Anif Nur

Artanti: Data acquisition for the NIH-3T3 cells, analysis of the data, drafting manuscript, revising the manuscript. Rumiyati: Supervising the experiment, drafting the manuscript. Dhania Novitasari: Data acquisition for the HDF cells, analysis of the data, drafting manuscript, revising the manuscript. Yuni Kusumastuti: Supervising the experiment, drafting the manuscript. Manami Toriyama: Supervising the experiment, drafting the manuscript.

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Conflict of Interest

Authors declare that there is no conflict of interest

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