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The Potential of Moringa Leaf Extract to Prevent Aging Targeted Cellular Senescence

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ABSTRACT

Background: High exposure to ultraviolet rays and chemicals causes cell aging due to excessive production of reactive oxygen species (ROS). ROS levels can be regulated by high antioxidant compounds contained in herbal extracts, one of which is Moringa leaf extract (MLE). **Objective:** This study aims to evaluate the potential of Moringa Leaf Extract in preventing aging targeted cellular senescence. **Methods:** The active compounds of MLE were identified through qualitative phytochemical screening, the cytotoxic test was evaluated using the MTT test, ROS levels were calculated by DCFDA flow cytometry test, and cell senescence analysis was analyzed using the SA-β-galactosidase test. In silico docking bioinformatics and molecular studies were carried out to determine the interaction of the active compounds of MLE with target proteins. **Results:** MLE contained flavonoids, phenolics, alkaloids, saponins, and steroids. This extract did not have a cytotoxic effect on NIH3T3 cells, as evidenced by the IC50 value of 420µg/mL MLE concentrations of ¼IC50 (105µg/mL) and ½IC50 (210µg/mL) significantly reduced ROS levels of NIH3T3 cells. The percentage of cell aging also decreased due to administration of MLE 210µg/mL up to 47.60% compared to the positive aging Dox 10nM control (78.90%). Bioinformatics studies found that the p21 and TP53 proteins are most directly affected by MLE's active compounds in the aging pathway. The quercetin compound in MLE has the strongest interaction strength against p21 and TP53 proteins compared to the ligands. **Conclusion:** MLE can suppress intracellular ROS levels and compete with p21 and TP53 proteins in preventing cell aging.

Keywords: Cellular senescence, Moringa oleifera leaves, p21, ROS, TP53

INTRODUCTION

Cellular senescence accumulates in many human tissues as a result of deoxyribonucleic acid (DNA) and telomere damage, exposure to ultraviolet light, and oxidative stress[32]. These cells are characterized by high levels of p21 protein, SA-β-galactosidase, reactive oxygen species (ROS), permanent cell cycle arrest, and the release of regeneration inhibitory factors[33]. Senescence cells are known to have a physiological role in cancer development and prevention of wound healing[34]. Increasing age causes the accumulation of senescence which triggers the risk of disease to increase so that the risk of heart failure, osteoporosis, hyperuricemia, and cancer increases[8,35,36]. Elimination of senescence was shown to increase median age by 25%, inhibit cardiac damage, and delay cancer progression[5,37,38]. This shows that the accumulation of senescence is the main variable causing the cell aging process.

To date, first line therapy for senescence is rapamycin[39]. The use of targeted rapamycin inhibition of the mammalian TOR (mTOR) can reduce the number of senescence cells, but cause various side effects including insulin resistance, anemia, and hyperlipidemia[40–42]. The development of new agents and other safe targets to deal with the problem of senescence is necessary and important

p21 protein is expressed in response to oxidative stress [1,2]. The expression of p21 protein causes cell cycle arrest in senescence[3–5]. The cell cycle termination condition that has been reached causes a gradual decrease in p21 expression followed by an increase in cyclin dependent kinase inhibitor to maintain senescence[6–8]. This p21 inhibitor through its antioxidant activity can suppress ROS levels is an important target to prevent the formation of senescence cells.

Moringa leaves (*Moringa oleifera* L.) contain various chemical compounds that have antioxidant and anticancer effects[9,10]. The bioactive compounds found in Moringa Leaf Extract (MLE) include vitamins, carotenoids, polyphenols, phenolic acids, flavonoids, alkaloids, glucosinolates, isothiocyanates, tannins, and saponins[11]. The compounds in MLE have in vitro and in vivo been shown to have antioxidant potential[12], so they have the potential to become anti-senescence. Research on MLE activity on senescence associated with potential p21 protein targets has not been carried out. This study aimed to explore the potential of MLE as an antiaging agent targeted at inhibiting cellular senescence in the p21 pathway.

MATERIAL AND METHODS

Research Tools

Research tools include hardware, software, and MLE testing kits. The hardware used is a laptop with a minimum of 2 GB of RAM memory. The software consists of a drug bank database (web-based), STITCH (web-based), PubMed NCBI (web-based), STRING-DB (web-based), webGestalt (web-based), in silico molecular docking MOE software. Equipment to test MLE includes vacuum rotary evaporator, beaker glass, autoclave, blue tip, yellow tip, micropipette, incubator, 96-well plate, 6-well plate, 24-well plate, ELISA 595 nm, microtube, flow cytometer, and microscope. inverted.

Research Material

The main ingredients of the study were Moringa leaves (M. oleifera L.), 96% ethanol, DMSO, MTT reagent, 10% SDS stopper in 0.1 N HCl, aluminum foil, DMEM high glucose medium, Fetal Bovine Serum (FBS), penicillin. -streptomycin, DCFDA staining reagent, trypsin, supplemented buffer, and SA-β-galactosidase reagent.

Collection, Determination and Extraction

This research was carried out for 4 months starting from May - August 2021 at the FMIPA UNNES laboratory and the Stem Cell and Cancer Research Laboratory (SCCR). Moringa leaves were obtained from Bergas District, Semarang Regency, Central Java Province, Indonesia (Latitude 7.1816°S; Longitude 110.4244°E). The determination of the plant was carried out at the Biology Laboratory of the State University of Semarang. 300-gram dried Moringa leaf powder was extracted by maceration method using 96% ethanol solvent at room temperature for 72 hours[13]. The obtained filtrate was concentrated to remove ethanol and water with a vacuum rotary evaporator at 40°C.

Phytochemical Screening

Qualitative identification of phytochemicals to ensure the content of secondary metabolites of flavonoids, alkaloids, phenolics, saponins, steroids, and triterpenoids in MLE was carried out using a protocol[14] with slight modifications. Flavonoids were identified through the Shinoda test with the addition of Mg and HCl powder. Analysis of alkaloids using Wagner's reagent and other tests, respectively, were the identification of phenolics using the addition of 1% $FeCl_3$, saponins using the foam test, and steroids and triterpenoids using Liebermann Burchard's reagent.

NIH3T3 Cell Culture

NIH3T3 fibroblast cells were obtained from the Indonesian SCCR collection. NIH3T3 was specifically cultured in Dulbecco's Modified Eagle's Medium (Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, USA), 12.5 g/mL amphotericin B (Gibco, USA), 150 g/ mL of streptomycin (Gibco, USA), and 150 IU/ml of penicillin (Gibco, USA). Cells were cultured in an incubator at 37℃ with 5% CO2.

Cytotoxicity Test with MTT Assay

The MLE cytotoxicity test was analyzed by the MTT method [3-4, 5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide][15] . 80% confluent NIH3T3 cell cultures were harvested and distributed into 96-wellplate wells. The medium was replaced after 24 h of incubation with a series of MLE concentrations (10-300 g/mL). Cells that had been treated for 24 hours were given 0.5 mg/ml MTT reagent (Sigma Aldrich, USA) and incubated for 4 hours in an incubator at 37℃ with 5% CO2. Cells were added with a DMSO stopper of 100 l to each well and incubated for 15 minutes on shaking in a dark room to dissolve the formazan crystals. The absorbance of formazan crystals was measured by an enzyme-linked immunosorbent assay (ELISA) reader at 595 nm. Absorbance data were converted into cell viability per cell and analyzed using the correlation test method. The value of inhibitor concentration 50% (IC50) was calculated by linear regression equation y = bx+a between concentration and percent cell viability.

ROS Measurement with DCFDA Staining using Flow Cytometry

Intracellular ROS levels of each cell were detected by the 2',7'–dichlorofluorescin diacetate (DCFDA) assay[16]. Cells were incubated with 25 M DCFDA (Thermo Fisher Scientific, Inc.) in supplemented buffer (10% FBS in phosphate buffer saline) for 30 min at 37℃ in the incubator. The cells were then treated with MLE treatment with a concentration of IC50 (105µg/mL), IC50 (210µg/mL), doxorubicin (Dox) 10 nM as a positive control, a combination of Dox 10nM-MLE ¼ IC50 (105µg/mL) and Dox 10nM- MLE IC50 (210µg/mL) for 4 hours at 37℃ in an incubator. Fluorescence levels of ROS were analyzed by flow cytometry BD Accuri C6 at Ex485 nm/ Em535 nm.

Senescence assay with SA-β-galactosidase Assay

The SA-β-galactosidase staining assay method was used to determine the number of senescence cells[17]. Cells were treated with MLE and Dox alone and in combination for 24 hours. The cells were then washed with PBS, added fixation solution containing 2% formaldehyde and incubated for 30 minutes at room temperature. Cells were stained with SA-β-galactosidase solution (Sigma Aldrich, USA) and incubated for 24 hours at 37℃. Senescence cells were observed under a microscope and the blue color in the cytoplasm indicated senescence cells. The number of senescence cells was calculated using the ImageJ application, the percentage was calculated against the total number of cells in 2 fields of view.

Bioinformatics Studies

The bioinformatic analysis aims to analyze the proteins involved in the senescence process which are regulated directly by the active compound MLE. Direct protein targets for MLE active compounds were collected through Swiss target prediction (http://www.swisstargetprediction.ch), chemical association network (STITCH) (http://stitch.embl.de/), and similarity ensemble approach (SEA) (https://sea.bkslab.org/) with the keyword 26 names of MLE active compounds obtained from literature studies. Genes related to cellular senescence were collected through PubMed NCBI-gene with the keyword "cellular senescence". The interaction analysis between target proteins aims to find the relationship between MLE target proteins and genes that regulate cellular sense using functional protein association networks (STRING-DB) (https://string-db.org/) and Cytoscape software. The STRING-DB

correlation coefficient value was chosen >0.7 which indicates a high confidence coefficient, while the Cytoscape false discovery rate (FDR) value is <0.05 to analyze the similarity of biological function networks and cellular components[18]. Genes with central roles based on the highest Cytoscape degree score were selected for further analysis.

Data analysis

Data were presented as mean±SD and statistical analysis was performed using SPSS 24 software (Chicago, IL). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to analyze significant differences between groups. The value of $p<0.05$ indicates statistically significant.

RESULTS

Extraction and Phytochemical Screening of MLE

The yield of the thick extract of Moringa leaves obtained by the maceration method was 23.71% (71.12 grams). The results of phytochemical analysis in this study revealed the presence of secondary metabolites of alkaloids, flavonoids, phenolics, saponins and steroids in MLE, while terpenoids were not found (Table 1).

Table 1. Phytochemical Screening of MLE

Phytochemical Screening	Result	Description
Alkaloids	Reddish brown precipitate	(+)
Flavonoids	Reddish orange solution	(+)
Phenolic	Dark green solution	(+)
Saponins	Froth	(+)
Steroids	Green color	(+)
Triterpenoids	No red color formed	(-)

(+): detected; (-): not detected.

Cytotoxic Activity of MLE

The cell viability profile due to the administration of MLE at a concentration of 10-300 µg/ml showed that MLE had no toxic effect on NIH3T3 cells. Cell viability up to a concentration of 200 µg/ml was above 50% and the IC50 MLE value was 420 µg/mL (IC50 value >100 µg/mL), indicating that the compound had no toxic effect (Figure 1). Concentration variants based on IC50 values were then used to determine the effect of MLE on ROS and senescence.

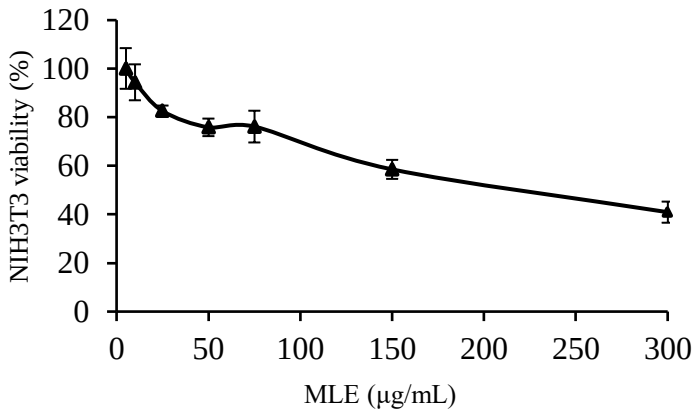

Figure 1. Cytotoxic effect of MLE on NIH3T3 cells. Profile of decreased % viability of NIH3T3 cells after MLE administration for 24 hours.

Effect of MLE on Intracellular ROS Levels

This research used Doxorubicin (Dox) as an agent to stimulate ROS levels. The results of this study showed that administration of a single Dox strongly induced intracellular ROS levels up to 54.40%. Dox-induced ROS levels in NIH3T3 cells were significantly attenuated by MLE administration of 105 g/mL and 210 g/mL up to 22.60% and 17.10%, respectively (Figure 2). MLE administration has been shown to have an antioxidant effect through its ability to reduce the accumulation of intracellular ROS in NIH3T3 cells. Analysis of the effect of decreasing ROS through inhibition of aging cells was then observed using a senescence with SA-β-galactosidase.

Effect of MLE on Cellular Senescence

The effect of MLE on inhibition of senescence was further tested by observing the activity of β-galactosidase. Blue positive cells on Dox administration which reached 78.90% showed that the cells experienced senescence more giving MLE concentrations of 105 g/mL and 210 g/mL to cells that had been induced Dox significantly reduced the senescence rate by 57.60% and 47.60%, respectively (Figure 3). The compounds in MLE have been shown to be successful in protecting cells from aging due to oxidative stress. Targeting the p21 protein directly may prevent cell senescence more effectively. The mechanism related to the senescence inhibitory activity by the active compound MLE was further confirmed through bioinformatics analysis and molecular docking in silico.

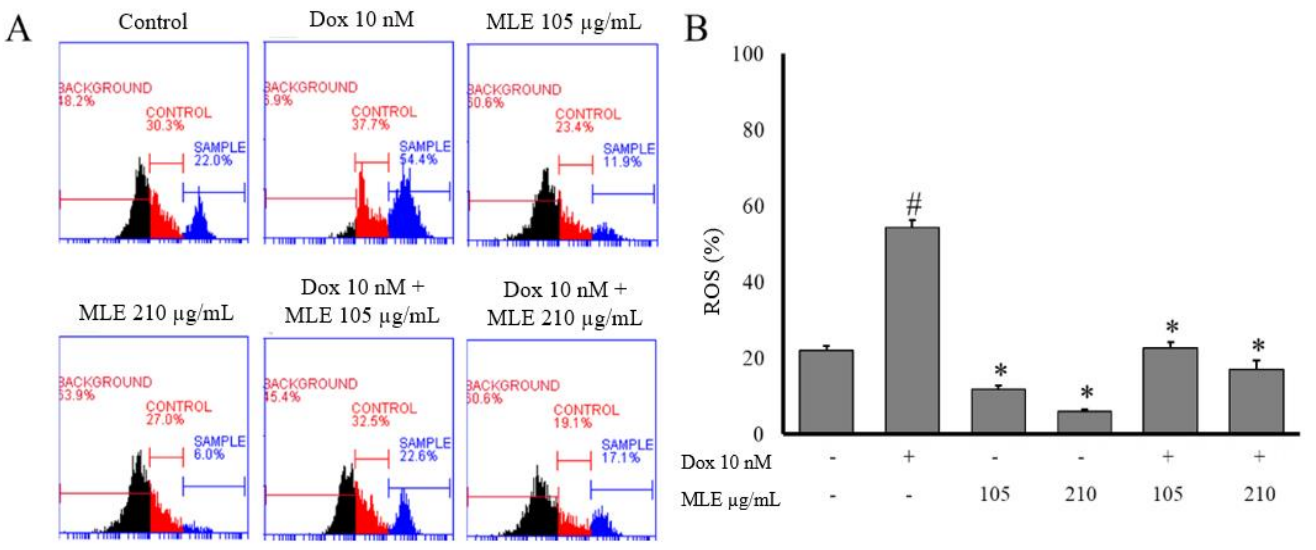

Figure 2. Test of ROS levels due to MLE administration to NIH3T3 cells; a) ROS flow cytometry profile; b) Percentage of ROS levels. Data are expressed in mean±SD (n=3), #p<0.05 compared to control group cells, *p < 0.05 compared to the Dox treatment group.

Bioinformatics Profile of MLE Active Compounds on Cellular Senescence

The bioinformatics approach aims to predict proteins that play a role in cell aging regulated by MLE active compounds. Prediction results of target genes for 26 MLE active compounds were collected from three different online databases to obtain comprehensive data. A total of 273 genes from the database were encoded as MLE target genes. Genes related to senescence were collected through NCBI-genes, obtained 350 genes related to senescence in humans. The gene pool was then analyzed using a Venn diagram to cross genes between MLE targets and cellular senescence, the result is that there are 34 genes that are related between the two (Figure 4A).

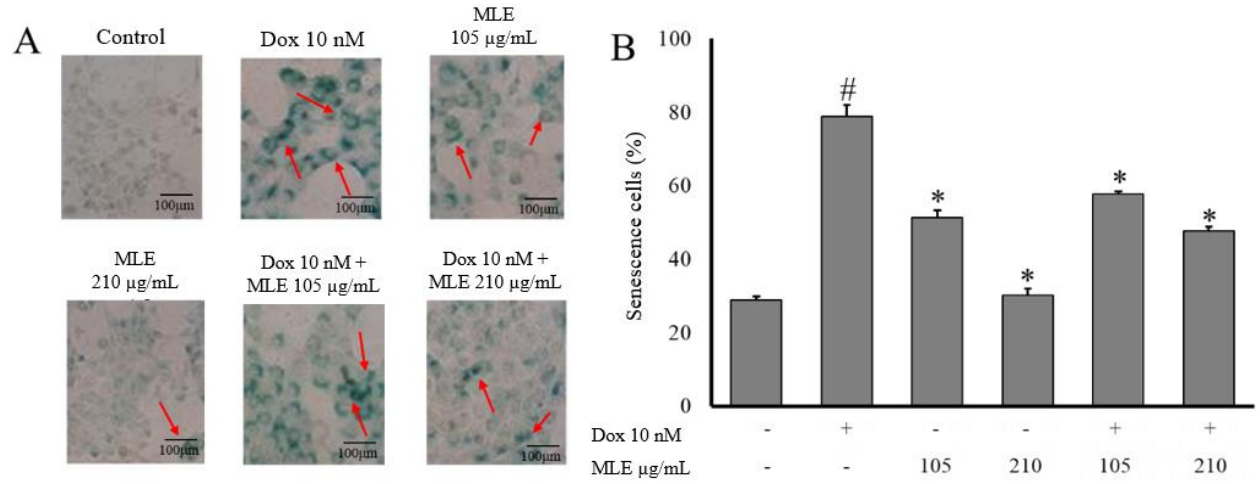

Figure 3. Effect of MLE on % cell senescence. The percentage of senescence cells was obtained by the SA-β-Gal Assay method; a) Morphology of senescence cells; b) Results of quantification of senescence cells with ImageJ. Data are expressed in mean ± SD (n=3), #p<0.05 compared to control group cells, *p<0.05 compared to the Dox treatment group. Arrows indicate positive senescence cells.

Potential therapeutic targets of MLE (PTTM) to determine the interaction of each gene were visualized via the STRING web tool. The results of the analysis obtained 37 nodes with 150 edges and an average node level of 8.11 which forms a network of interactions between genes (Figure 4B). The thirty-four genes were categorized in the gene ontology by STRING and revealed that PTTM was associated with JUN kinase activity, MAP kinase, and DNA-dependent protein kinase activity located in mitochondria, neoplasms, and the cytosol. PTTM has a strong effect on cell aging due to stress based on biological process analysis (Figure 4C). Node analysis with *Cytoscape* obtained 6 main genes with the highest score, namely CDKN1A (p21), TP53, MAPK1, MAPK3, AKT1, and FOXO3 (Figure 5D).

Molecular Interaction of MLE Active Compounds using Molecular Docking

The interaction results indicated by binding affinity (kcal/mol) with a lower docking score indicated a stronger bond because the energy requirement to compete with p21 and TP53 proteins was less. Docking results obtained RMSD value <2 so that the analysis carried out is valid. All MLE active compounds showed stronger interactions with TP53 and p21 than their substrates (Table 2), some of which were conventional hydrogen bonds, pi-cation bonds, and pi-alkyl bonds (Figure 5). The results of this study indicate that MLE has the potential to be potent competitive against p21 and TP53 proteins associated with inhibition of senescence due to oxidative stress.

Table 2. Docking results of active compounds from MLE

Ligands	p21		TP53	
	Binding Affinity (kcal/mol)	RMSD	Binding Affinity (kcal/mol)	RMSD
Native ligand	-2.1	0.03	-2.2	0.02
Kaempferol	-6.6	0.06	-7.7	0.02
Moringin	-5.9	0.03	-4.7	0.02
Myricetin	-6.7	0.04	-7.8	0.03
Niazimicin	-6.4	0.02	-6.4	0.02
Quercetin	-6.7	0.02	-8.0	0.03

DISCUSSION

The MLE phytochemical profile test was carried out because the therapeutic potential of medicinal plants was determined by secondary metabolites[19]. This phytochemical profile strengthens the results of previous studies which reported that the ethanolic extract of Moringa leaves was positive for flavonoid, alkaloid, and phenolic compounds[14]. The absence of triterpenoid compounds is possible due to the 96% ethanol polar solvent used in the extraction of Moringa leaves. Triterpenoids are nonpolar compounds that are easily dissolved in nonpolar solvents such as n-hexane and chloroform[20].

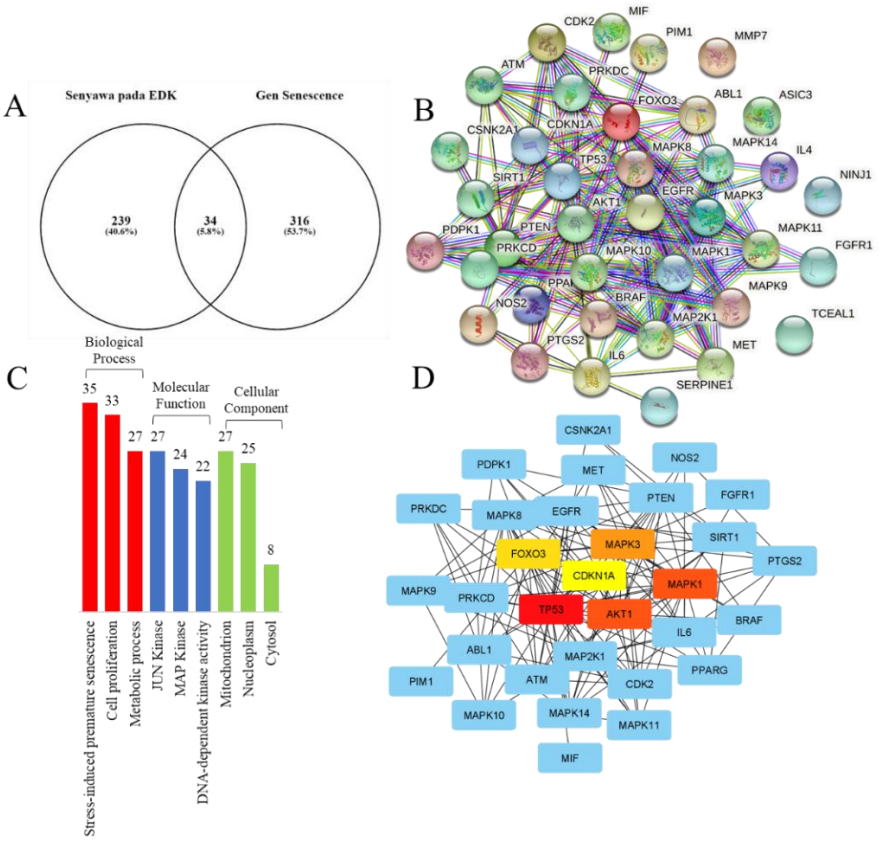

Figure 4. MLE bioinformatics analysis of senescence cells; a) MLE target genes associated with senescence biomarkers; b) PTTM protein interactions via STRING; c) Involvement of MLE in biological processes, molecular functions, and cellular components; d) 6 PTTM genes selected based on the highest degree score visualized on Cytoscape.

Cytotoxic test was performed on NIH3T3 cells as a representation of normal cells derived from fibroblast cells[21]. The high IC50 value of a compound against these cells proves that these cells will not experience cell death and the compound has no toxic effect[22]. These results prove that MLE is safe to use and non-toxic as a cell-targeted therapeutic agent.

Dox concentration of 10 nM was able to increase ROS levels without decreasing cell viability[23]. Administration of single MLE without Dox induction showed that MLE was antioxidant because it could reduce ROS levels compared to control cells. These results also corroborate previous studies that reported that Moringa leaf extract was able to reduce ROS in vivo[24]. Senescence activity is regulated at the cellular level characterized by upregulation of cyclin-dependent kinase inhibitor p21 (CDKN1A)[25]. Expression of p21 is regulated at various levels including transcriptional, post-transcriptional and translational levels related to p53 activation [26]. The p53 pathway plays a major role in activating the p21 protein to induce cell senescence[1,27].

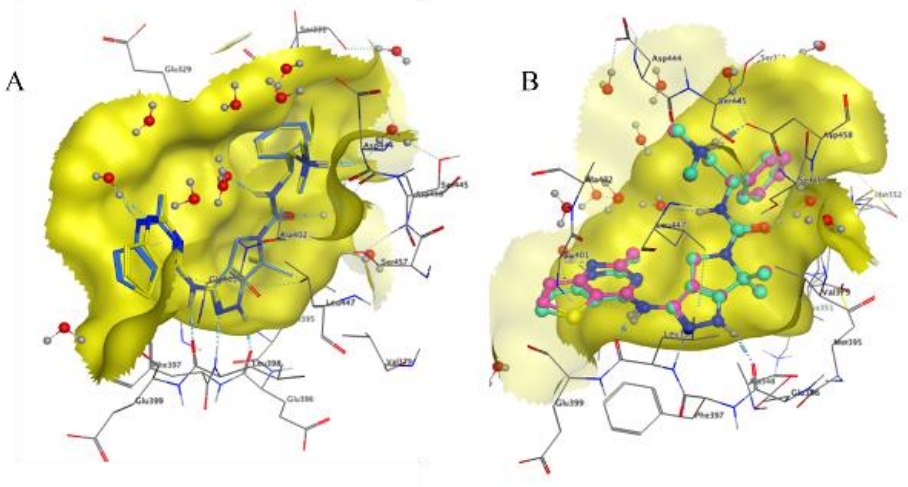

Figure 5. Visualization of the interaction of quercetin (the strongest interaction) with proteins a) p21; b) TP53.

The p21 and TP53 genes are proteins that have a major role in the oxidative stress-related senescence pathway[28]. The DNA damage response due to UV exposure increases the production of ROS that induces TP53[29]. This activation will regulate downstream of the most dominant TP53 target gene, namely CDKN1A/p21 during the senescence cell replication process[27,30]. P21-activated mouse embryonic fibroblasts undergo cell cycle arrest in the TP53-dependent G2 phase after DNA damage induction[8,31]. The dependence of TP53 on p21 expression for the induction of cell cycle arrest and the role of p21 as a proliferation inhibitor suggest an important role for this gene in the induction of TP53-dependent cell senescence. These results can be used as a prediction of the molecular mechanism of MLE involved in the senescence process, namely through the regulation of p21 and TP53 proteins. The strength of the interaction between p21 and TP3 proteins against the active compound MLE was then analyzed to predict the strength of the bond between the compound and the protein.

CONCLUSION

The results of this study indicate that MLE can suppress intracellular ROS levels and compete with p21 and TP53 proteins in preventing cell senescence. The novelty of this research is that MLE is proven to have potential as an accelerator agent to prevent cell aging through the p21 and TP53 pathways.

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AUTHORS' CONTRIBUTIONS

KR conducted orientation literature study, prepared the laboratory, and collected the materials. SA performed in silico and in vitro tests. HUW analyzed the data and prepared the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare that there was no conflict of interest.

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