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The Cytoprotective and Cell Recovery Properties of Apple Extracts on H_2O_2 induced-NIH3T3 Cells : An Anti Aging Candidate

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Abstract

Apple contains high concentration of phenolic compounds that protect cells from oxidative stress. The prolong exposure of free radicals may induce cell damage and premature cell aging. Both local and imported apple contain flavonoid, saponin, tannin, steroid, and terpenoid. The extract of local and imported apples showed low toxicity on NIH3T3 fibroblast cells, with IC_{50} value of 529 and 463 µg/mL, respectively. Both apple extracts (50–250 µg/mL) protected three-day-H_2O_2 induced-cell damage and cell death. Protective effect was observed as the viability increase of treated cells compared to untreated ones. The protective effect of both extracts were higher than the effect of vitamin C as standard antioxidant at this study. Both apple extracts could reverse cell damage caused by three-hour-high concentration H_2O_2 exposure, similar with vitamin C. Low concentration of both extracts (50 µg/mL) induced the increase of fibroblast cells' proliferation kinetics. The extract of imported apple showed higher properties of protective, cell recovery and proliferation of fibroblast cells tha local apple, but not statistically significance. This study concludes that the extract of local and imported apples have high potency in cytoprotective effect and cell recovery of damaged cells caused by free radicals induction. Both apple extracts have high potency to be developed the candidate of antiaging and cells' regeneration agent.

Keywords : *Antiaging, cell recovery, cytoprotective, NIH3T3 cells*

INTRODUCTION

Aging is one natural process occurring during living organism's life. It is a complex process causing progressive decrease in physiological function, followed by dysfunction, and death. Several factors play a role in growth and aging, that are disease, injury, nutrition intake, exercises, stress, and plenty of environmental factors (Curtis, *et al.*, 2005). Skin roughness and dryness followed by loss of flexibility and fairness are also the signs of skin aging (Atmaja, *et al.*, 2012). Scientific efforts have been done to slow aging process by lengthening healthy age duration (Getoff, 2007).

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Corrective treatment or repair could be done by using microdermabration, iontophoresis, laser, chemical peeling, mesotherapy, sonophoresis, and application of night cream or skin peeling cream that helps reducing fleck. It drives people's interest in improving ways to slow aging process (Gilchrest, *et al.*, 2003).

Kujawa, *et al.*, (2011) observed that apple peel extract possessed high antioxidant activity, almost comparable to that of its fruit's extract. Pertiwi, *et al.* (2016) also proved antioxidant activity of apple peel's ethanolic extract, giving IC_{50} of 87 ppm. Juranovic, *et al*., (2011) showed that apple extract has antiaging activity by observing yeast cells viability *in vitro*. Yuniarto, *et al.* (2012) revealed that apple fruit's extract possessed antioxidant activity with IC_{50} of 151 ppm. Medium-sized fresh apple extract contains antioxidants similar to that of 1,500 mg of vitamin C (Nurcahyati, 2014). Quercetin, known as the flavonol mostly found in nature compared to other flavonoids, is contained in apple fruit, believed to be responsible for its antioxidant and antiaging activity (Wasim and Farhan, 2010).

A study on the antioxidant activity of a set of Indonesian's fruits showed that flavonoids contained in peel extracts of rambutan, durian, sweet orange, kelengkeng, and kelengkeng seed were 12.26; 46.03; 9.34; 8.82; and 5.21 mg/g, respectively. In this study, we observed cytoprotective activity of both local and imported apple extract against H_2O_2 exposure on NIH3T3 fibroblast cells, since fibroblast cells are human's cell commonly used in wound healing and early aging studies.

MATERIALS AND METHODS

Materials

Materials used in this study were local apples (Malang green apple, Indonesia), imported apples (Thailand green apple), ethanol 70% (Brataco), NIHT3T fibroblast cell line, DMEM powder, Fetal Bovine Serum (FBS) (Sigma), penicillin-streptomycin (Sigma), ascorbic acid, 3% H_2O_2 (Kimia Farma), PBS, MTT (Calbiochem), Trypsin (Sigma), and Dimethyl Sulfoxide (DMSO) (Sigma). Centrifuge, CO_2 incubator (Thermo Scientific), inverted microscope (Olympus), conicals (Biologics), ELISA reader (Bio-rad iMarkTM), and LAF (Thermo Scientific) were used.

Apple Extraction

Apple dried simplicia was extracted using 70% ethanol by three-cycle maceration for 5 days in each cycle. Macerate obtained was concentrated using rotary evaporator to give concentrated extract.

Screening of Secondary Metabolites

Identification was conducted by specific color-forming reactions suitable for each group of substance. 2 NHCl was used for flavonoid (glicoside-3-flavonols) identification, Meyer reagent was used for alkaloids identification, FeCl3 was used for tannins identification, foam-formation reaction was used for saponins identification, and glacial acetic acid together with concentrated sulfuric acid were used for terpenoids and steroids identification.

Cell Viability Assay (MTT Assay)

As much as 5 x 10^3 cell/well of NIH3T3 cells were grown in 96-well plate and incubated with 5% CO2 flow in 37°C. Media was removed and cells were washed with 100 μL PBS. For cytotoxicity assay, 0-1000 ppm of apple extract were applied for 24 hours, while for proliferative assay 10, 25, 50, and 125 ppm of apple extract were applied for 0, 24, 48, and 72 hours in normal growth condition (37°C, 5% CO_2 flow). Following treatments, culture media was removed. PBS washing was done prior to 100 μL MTT reagent addition followed by 4 hours incubation (37°C, 5% CO_2 flow) to ensure complete formazan crystals formation). As much as 100 μL SDS in HCl was added to each well, and cells were incubated overnight, followed by absorbance measurement using ELISA reader at 595 nm wavelength.

Induction and Stress Recovery Testing using H_2O_2

As much as 5 x 10^3 cells/well were grown in 96 well plate, and were incubated for 24 hours in 37°C with 5% CO_2 flow. Culture media was removed, and cells were washed with 100 µL PBS. Treatment of 0-1000 ppm apple extract and 325 ppm ascorbic acid as positive control were given. After 1 hour, 100 µL H_2O_2 was added to each well for 1 hour, followed by media replacement with fresh media. The treatment were given in 3 consecutive days to induce stress and cell recovery following H_2O_2 exposure. After that, MTT was added to each well, and cells were incubated for 4 hours (37°C, 5% CO_2 flow). As much as 100 µL SDS was added prior to overnight incubation, and absorbance measurement using ELISA reader at 595nm wavelength.

Data Analysis

Cytotoxicity assay was analyzed by IC_{50} value calculation using Probit analysis (SPSS 16 for Windows software). The differences among groups were analyzed using Post-hoc Tukey's two-way Anova and Kruskal Wallis followed by Mann-Whitney test.

RESULTS AND DISCUSSION

Apple Fruit Extraction

Extraction of local and imported apple through maceration gave quite high yield (Table 1). Since ethanol is a universal solvent that is semipolar, most of the substances contained in apple was expected to be extracted. The difference of yield obtained from the two extracts could possibly be caused by the difference of secondary metabolites content between them. It came as the result of the difference of growth territory, weather, and nutrition content in the soil where the plant grew.

Table 1. LAE and IAE extracts.

Sample	Yield (%)	Organoleptic	
		Color	Smell
Local Apple	11.835	Dark brown	Typical unique
Imported Apple	10.587	Dark brown	Typical unique

Secondary Metabolites Screening

Secondary metabolites screening results showed that both local and imported apples contained flavonoids, saponins, tannins, alkaloids, steroids, and terpenoids (Table 2).

Apple Extracts Cell Viability Testing by MTT Assay

Local Apple extract (LAE) and Imported Apple extract (IAE) treatment (0-1000 µg/mL) brought change in NIH3T3 fibroblast cells morphology. Fibroblast cells became rounder, darken, and some cells were observed to detach from the tissue culture disc base. The changes in cell morphology were proportional to the increase of extract concentration (Figure 1).

Table 2. LAE and IAE content,

Test		Results	
Phytochemicals	Reagent	Observation	Conclusion
Flavonoid	wlister	Orange	Positive
Saponin	Forth	Clear and/or foamy	Positive
Tannin	FeCl3	Yellow to orange	Positive
Alkaloid	Meyer	White ppt	Negative
Steroid	Liebermann-Burchard	Blackish blue	Positive
Terpenoid	-	Reddish brown	Positive

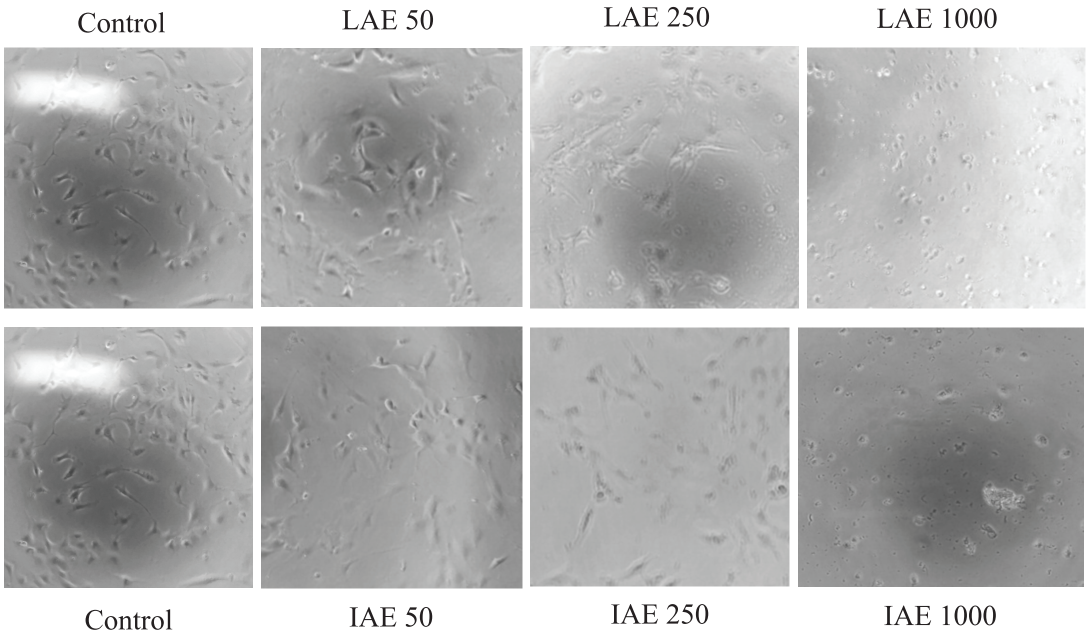

Figure 1. Effect of LAE and IAE treatment to cell morphology compared to control. Observation was done using inverted microscope, 100x magnification.

LAE and IAE inhibited NIH3T3 cells' growth, proportional to its concentration. IAE showed significantly higher inhibitory activity compared to EAL statistically (p<0.05) (Figure 2). Cell viability obtained from the cytotoxicity testing could be calculated further to get IC_{50} value, showing its level of cytotoxicity. LAE and IAE exhibited low toxicity, giving the IC_{50} values in the range of 400-500 ppm (Table 3).

Cell Proliferation Assay

Citotoxicity assay showed that low concentration of LAE (< 50 µg/mL) could increase cell viability (Figure 3). Proliferation assay was conducted to confirm thath the percentage of living cells was above 100%. LAE with the concentration of 10 and 25 ppm could increase fibroblast cells proliferation kinetics compared to control. In considerably high concentration (50 and 125 µg/

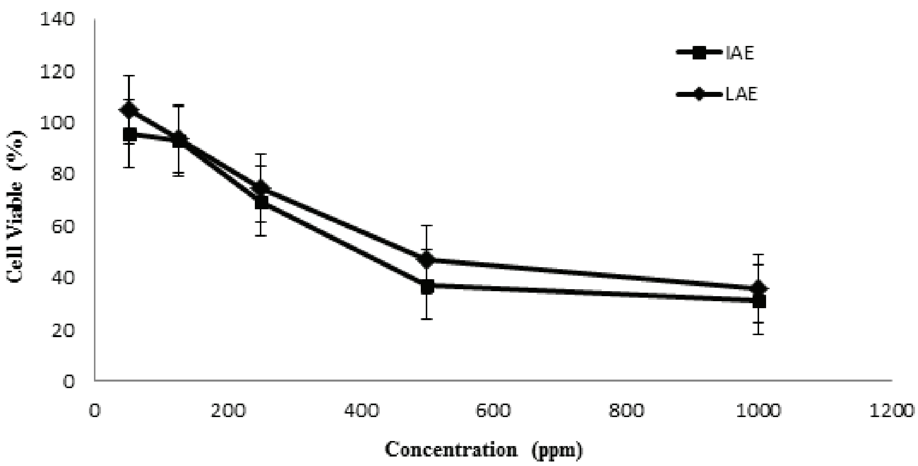

Figure 2. LAE and IAE inhibited NIH3T3 fibroblast cells growth. As much as $3x10^3$ cells/well were treated with LAE and IAI, and cell viability were measured with MTT assay.

Table 3. Cytotoxicity potential of LAE and IAE on NIH3T3 cells.

Sample	IC_{50} (µg/mL)
LAE	529
IAE	463

mL), the increase of proliferation kinetics was also observed to occur to up to the 48th hour, followed by decrease in the 72nd hours.

H_2O_2 Induction and Recovery

The purpose of stress induction using H_2O_2 was to observe the continuous protective effect of LAE and IAE on fibroblast cells against H_2O_2. Exposure was set to be 3 days to mimic chronic stress. LAE and IAE could increase fibroblast cells' viability pretreated with H_2O_2 stress induction. IAE treatment resulted in greater increase of cell viability compared to EAL in all tested concentrations (Figure 4). Thus, both extracts have potency as cell protector against H_2O_2 exposure.

LAE and IAE was proved to have cytoprotective activity on NIHT3T cells continuously against 100 µM H2O2 oxidative stress exposure given consecutively for 3 days, with the concentration of 50 and 125 µg/mL, giving better protective activity compared to 352 µM vitamin C as positive control ($p<0.05$). To confirm the cytoprotective effect observed, recovery test on fibroblast cells against oxidative stress exposure needs to be conducted. It was aimed to reveal whether the extract could quickly protect NIHT3T cells undergo acute oxidative stress (1 day) treatment.

Recovery testing showed that % cell viability of cells treated with 50-500 ppm LAE gave greater effect compared to negative control, while IAE-treated cells showed higher % cell viability in all treatment concentration compared to negative controls (H_2O_2) (Figure 5). Vitamin C was used as positive control as its antioxidant activity has been proven. It has the ability to neutralize reactive oxygen, such as hydrogen peroxide. During the process, vitamin C itself turns into radical monohydroascorbate that will undergo reduction enzymatically by reduced glutation (GSH) catalyzed by glutation peroxidase back into vitamin C, yielding oxidized glutation (GSSG), and nonenzymatically via the reaction between two mono dehydrocorbate molecules yielding ascorbate and dehydroascorbate that are not radicals (Bender, 2009).

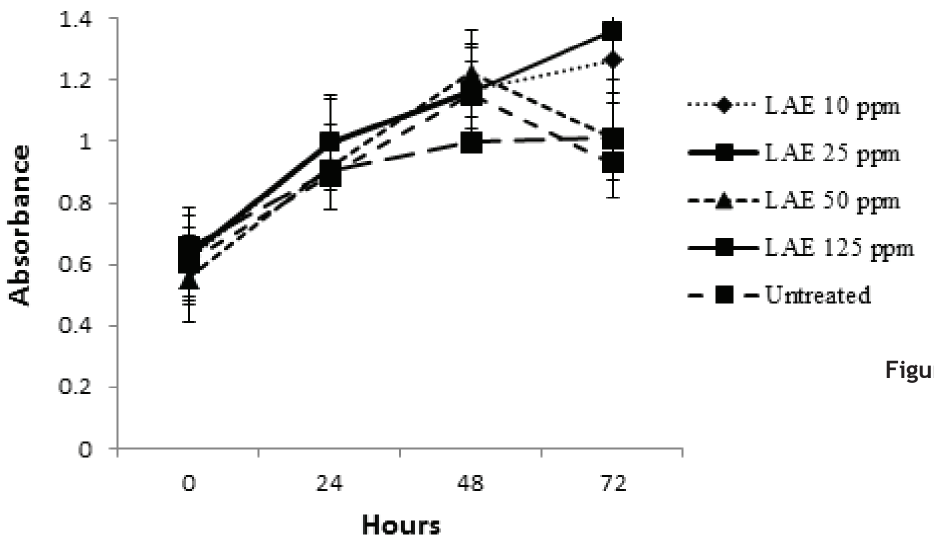

Figure 3. Fibroblast cells growth rate, time (hours vs absorbance). The test was conducted on fibroblast cells and observation was done after 0, 24, 48, and 72 hours.

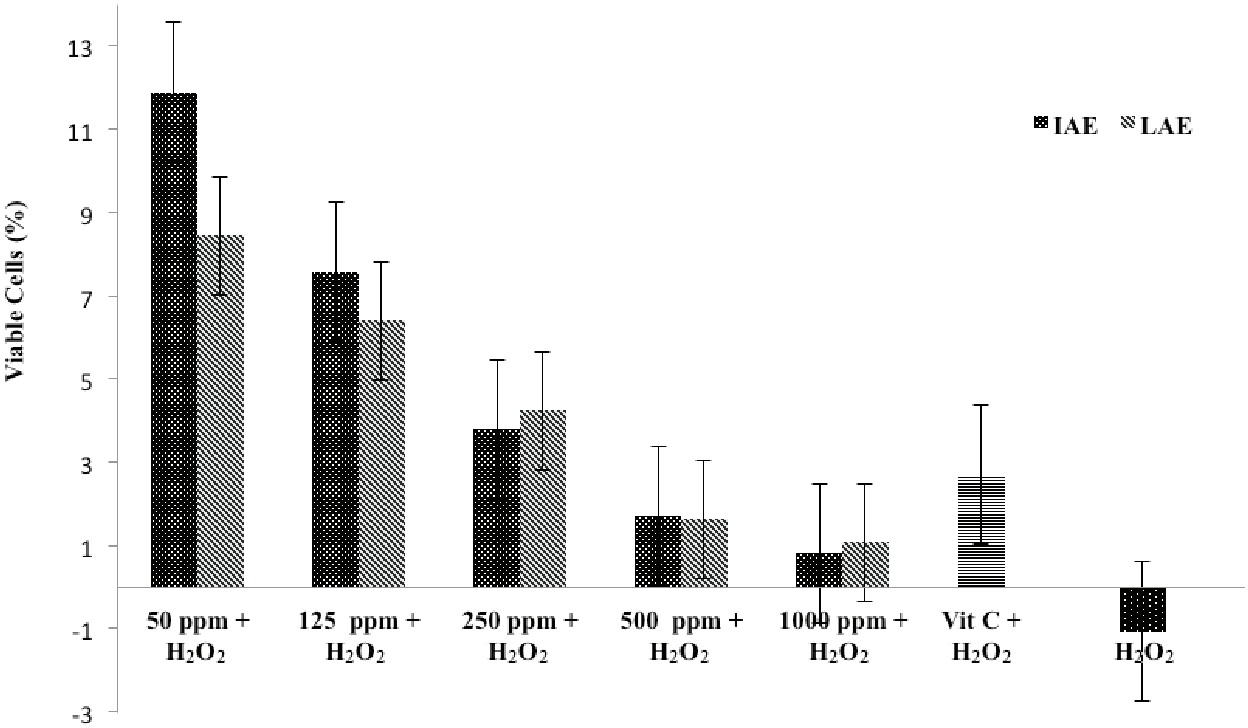

Figure 4. Effect of LAE and IAE and on NIH3T3 fibroblast cells exposed with H_2O_2. Cells were treated with 50, 125, 250, 500, and 1000 ppmand 325 μM ascorbic acid for 1 hour prior to oxidative stress exposure using 100 μM H2O2 for 3 consecutive days. Statistical analysis showed significant difference (p<0.05).

H_2O_2 gave oxidative stress too NIHT3T fibroblast cells. It is non-radical oxygen derivate that will undergo a series of reaction yielding free radicals (Varh and Stroz, 2010). Free radicals are atom or molecule that have one unpaired electron. As a result, free radicals possess high reactivity because of its tendency to pull electron, hence it could convert another molecule into radicals as the result of losing or having additional electron. Tissue damage caused by radical oxygen species (ROS) is called as oxidative stress, while agents protecting the tissue from ROS are called antioxidant (Bender, 2009).

Apple contains substances that are expected to function as antioxidant. Phytochemicals expected to possess free-radicals-stabilizing-ability in LAE and IAE are flavonoids. Pertiwi, *et al.* (2016) revealed that apple peel contained flavanols, flavonoids, and quercetin. On the other hand, Nurcahyati (2015) showed that apple peel contain quercetin, a dominant flavonol content in apple peel. Quercetin may act as antioxidant and antiaging agent (Wasim and Farhan, 2010). Antioxidant could protect the ells from damages caused by unstable molecule known as free radicals. Antioxidant is able to donate its electron to free radicals. Thus from the study we may conclude that one of the secondary metabolites that is responsible for the cytoprotective ability is flavonoid, as one group of antioxidant. Not only flavonoid is known as antimutagenic and anticarcinogenic, it also acts as antioxidant, anti-inflammatory agent, antihistamine, and inhibitor of LDL oxidation. LAE and IAE contained flavonoids that are antioxidants. Antioxidants has protective ability against oxidative stress and also inflammation. LAE and IAE with the concentration of 50 and 125 ppm showed better cytoprotective activity compared to vitamin C as positive control (p<0.05), that could possibly be because of its antioxidant content protecting cells from H_2O_2

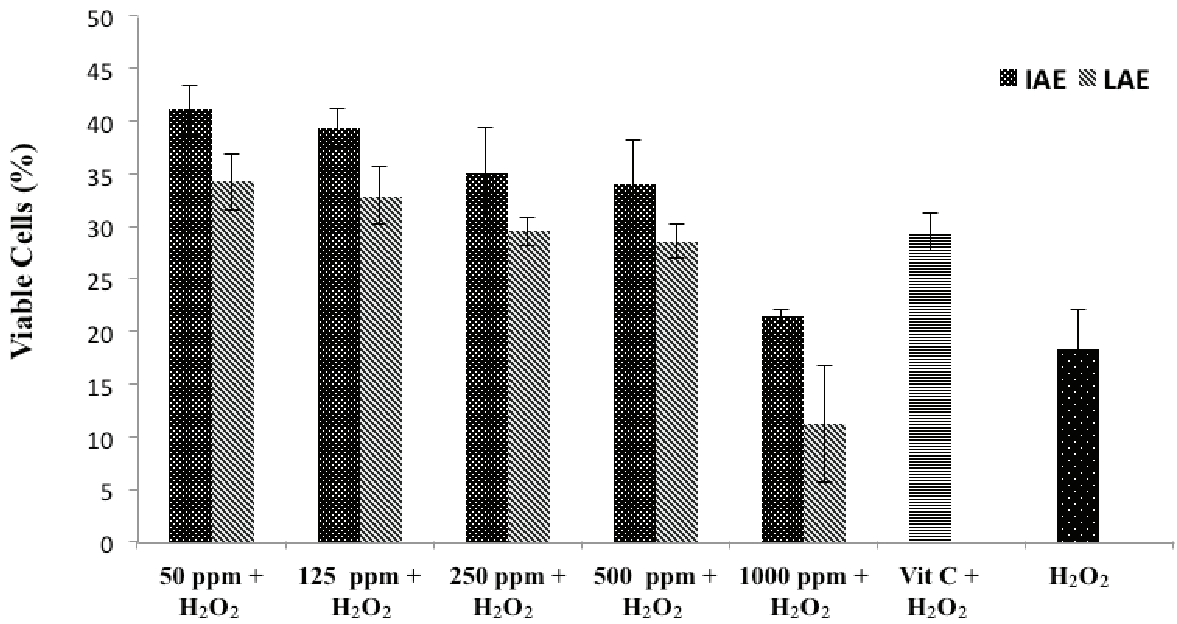

Figure 5. Recovery effect of LAE and IAE on NIH3T3 fibroblast cells exposed with H_2O_2. Cells were treated with 50, 125, 250, 500, and 1000 ppmLAE and IAE, and 352 μM ascorbic acid for 1 hour prior to oxidative stress exposure using 100 μM H2O2. Statistical analysis showed significant difference (p<0.05).

exposure. The mechanism of flavonoids to protect the cells against free radicals was as follows: it acted as antioxidant scavenging water soluble free radicals to form a relatively stable radical and could stay for quite some time until it reacts with nonradical product (Bender, 2009). While with the concentration of 250, 500, and 1000 ppm, the decrease in % cell viability was observed, predicted to be the result of flavonoid content in LAE and LAI that underwent changes into oxidant radicals. Free radicals have two typical characteristics, that are high reactivity because of its tendency to attract electron and able to convert molecule into radical, that make it grouped into oxidants. In this case, substances in LAE and IAE could possibly undergo change into free radicals that cause the increase of total free radicals exposing the cells.

The substance that was predicted to cause the increase in cell viability was flavonol. Another substance that might increase cell viability was tannin, one of the polyphenols. Polyphenols in apple could increase cell viability to up to 60% and in could efficiently protect yeast cells from H2O2 oxidative stress (Palermo, *et al.,* 2012). Hence, the metabolites that could protect NIH3T3 fibroblast cells against chronic oxidative stress exposure was predicted to be flavonoids and polyphenols antioxidants.

CONCLUSION

LAE and IAE contained flavonoids, saponin, tannin, steroid, and terpenoid. LAE and LAI with the concentration of 50 and 125 ppm could protect NIH3T3 fibroblast cells against H2O2 oxidative stress exposure better than ascorbic acid.

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