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FULL PAPER

The antioxidant and anti-ageing activity of lyophilisate kersen (*Muntingia calabura* L) fruit *in vitro*

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Abstract

Kersen fruit (*Muntingia calabura* L.) is a wild plant used as food and used in medicine. This study aimed to determine the bioactivity of Kersen fruit as a candidate for active ingredients in cosmetics. Preparing the lyophilisate of kersen fruit was carried out through the lyophilization method using a freeze dryer. The lyophilisate of the Kersen fruit was then analyzed for its antiradical activity using the DPPH, ABTS radical scavenger, and FRAP reduction methods. The anti-ageing activity was evaluated using Elastase and Collagenase inhibition and fibroblast cell proliferation by the MTT method. The result showed that the lyophilisate of kersen fruit can inhibit DPPH, ABTS radical, and ferric reduction assay with strong activity. Lyophilisate of kersen fruit can interfere with Elastase and Collagenase activity with IC_{50} of 21.67 and 180.61 µg/mL, respectively. The results of the fibroblast cell proliferation activity showed that the lyophilisate sample could protect and increase the viability of fibroblast cells after exposure to H_2O_2 (IC_{90} value of 773.24 µg/mL). Based on these results, it can be concluded that Kersen fruit can be developed as an active ingredient in anti-ageing cosmetics.

1. Introduction

The skin is one of the largest organs in the body and consists of tissues that have a specific function as a protector and cover that keeps other body organs together. Like other organ systems, human skin can undergo physiological changes with age, resulting in skin ageing. Several factors cause skin ageing, such as intrinsic and extrinsic factors. Intrinsic factors in ageing may be influenced by genetics, which is a natural consequence of physiological changes. On the other hand, conditions such as exposure to UV rays, especially UVB, chemical toxins, and cigarette smoke are causes of extrinsic ageing (photo ageing) (Lee *et al*., 2012; Hwang *et al*., 2017; Nur *et al.,* 2021a). Exposure to UV light triggers an increase in three MMPs, namely collagenase (MMP-1), stromelysin-1 (MMP-3), and 92-kDa gelatinase (MMP-9). These three MMPs are in vivo strongly influenced by the transcription factor AP-1, which is rapidly induced and activated by UV exposure. Increased activity of AP-1 increases the level and activity of several enzymes that degrade components of the extracellular matrix, especially MMP-1, MMP-3, and MMP-9 (Yaar and Gilchrest, 2007). Intrinsic and extrinsic factors can cause different skin changes resulting in facial lesions, fine wrinkles, dry skin, and pigmentation. The pigmentation causes blackish skin and creases in the eyes formed by contractions of the orbicularis oculi muscle and itching, which is a manifestation of ageing (Poljsak *et al*., 2012; Rittié and Fisher, 2015).

Ageing is a highly structured process in which few theories explain that the cellular phenomenon of the ageing process is also influenced by reactive oxygen species (ROS) produced in cells. ROS is a product of aerobic respiration demand in several modifications of cellular reactions such as exposure to oxidants, ionizing radiation, and heavy metals. Typically, ROS can be removed by the appearance of endogenous antioxidants such as catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), superoxide dismutase (SOD). However, oxidative stress arises when the footing between oxidants and antioxidants in the body changes due to an increase in ROS and a decrease in antioxidants from the body, it can cause cell damage. It will also affect skin ageing *(Jia et al*., 2014; Kim *et al*., 2016).

One of the plants that contain antioxidant compounds is the kersen fruit (*Muntingia calabura* L). Pereira *et al*. (2018) stated that kersen fruit contains a

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class of phytochemical compounds, i.e., saponins, phenols, and flavonoids. The content of these compounds is reported to have potent antioxidant activity due to their ability to reduce free radicals. In addition, Adam (2019) reported that kersen fruit contains secondary metabolites such as catechin derivatives, myricetin, quercetin, flavanols, naringenin, chlorogenic acid, gallic acid, vanillic acid, caffeic acid, coumaric acid, ferulic acid, hydroxycinnamic acid. These compounds are phenolic derivatives with known antioxidant activity (Adam, 2019).

Based on the information, this study evaluated the bioactivity of kersen fruit as active anti-ageing ingredients candidate. In our study, an *in vitro* assay was carried out by assessing the activity of antioxidants, anti-elastase and anti-collagenase, and normal cell proliferation of fibroblasts from the kersen fruit. The data contributed as initial information in the development of natural ingredients as anti-ageing cosmetics.

2. Materials and methods

2.1 Chemicals and reagents

The materials and reagents used are analytical grades such as water distillation, iron (III) chloride, dimethyl sulfoxide (DMSO), DMEM (Dulbecco's modified eagle medium), ethanol, FBS (fetal bovine serum), glutamine, hydrogen peroxide (H_2O_2), trypsin-EDTA solution, PBS (phosphate-buffered saline), penicillin, MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), TPTZ (2,4,6,-Tripyridyl-triazine), n-Hexane, ethyl acetate, quercetin, DPPH (1,1-Diphenyl-2-Picryl hydrazyl), ABTS (2,2'-azino-bis-[3-etilbenzotiazolin sulfonat]), Potassium Persulfate ($K_2S_2O_8$), Human Neutrophil Elastase Kit and MMP-1 Collagenase kit were purchased from *Enzo Life Science*, Human Dermal Fibroblast Adult (HDFa) was purchased from GIBCO and Kersen (*Muntingia calabura* L.) fruit collected from Biringkanaya District, Makassar City, and South Sulawesi, Indonesia.

2.2 Extraction process

Samples of ripe cherry fruit (*Muntingia calabura* L) were washed thoroughly with running water and put into a juicer. The filtrate and dregs were separated, and then the filtrate was frozen at -20°C. Furthermore, the frozen samples were lyophilized using freeze-drying to obtain Kersen (*M. calabura* L.) lyophilized.

2.3 Antioxidant activity

Antioxidant activity testing was carried out using three methods, i.e., DPPH radical reduction, ABTS radical reduction, and iron reduction (Ferric Reducing Antioxidant Capacity) *(*Benzie and Strain, 1996; Nur *et al*., 2019; Nur *et al.,* 2021b)

2.2.1 DPPH radical reduction

The stock solution of lyophilisate in a concentration of 1000 µg/mL was made into a concentration series of 1 -1000 µg/mL by taking a particular volume of it into the 5 mL flask, then adding 1 mL of DPPH reagent (0.4 mM) solution and enough volume with ethanol p.a. to 5 mL. The absorbance was measured at the maximum wavelength after being stored at room temperature for 30 mins. The inhibitory effect was determined based on the percentage of the colour change of the DPPH solution fading due to the sample being compared to the blank solution (control). The IC_{50} value describes the concentration of sample solution required to reduce DPPH by 50%, which is obtained from the regression curve between the concentration of the sample solution and the percentage of inhibition. The inhibition rate was acquired by the formula $[(A_{blank} - A_{sample})/A_{blank} \times 100\%]$.

2.2.2 ABTS radical reduction

ABTS radicals were prepared by reacting the ABTS solution (28 mg ABTS in 10 mL aqueous) with potassium persulfate (15 mg in 10 mL distilled water) in a dark room for 12 h. Then the volume of the ABTS radical solution made up to 50 mL with ethanol pro analysis. The sample lyophilisate stock solution was made to various concentrations (1-1000 µg/mL) by taking a particular sample volume and adding one mL of ABTS radical solution. The sample mixture was sufficient to 5 mL with ethanol pro analysis. Then mixtures were homogenized and incubated in a dark room for 30 mins, then measured the absorption was at 752 nm. The ABTS solution was blank (1 mL ABTS solution to 5 mL distilled water), and quercetin was used as a positive control. The inhibitory effect was calculated by the formula $[(A_{blank} - A_{sample})/A_{blank}$ X 100%].

2.2.3 FRAP reduction

The FRAP reagent was set up with the phosphate buffer: TPTZ: $FeCl_3$ with a ratio of 10:1:1. A total of 500 µL of sample solution was reacted with 2 mL of FRAP reagent, and the volume was made up with distilled water to 5 mL. The mixture was kept for 30 mins, and the absorption of the sample solution was determined at 593 nm. The concentration series of quercetin (standard solution) was used to create a standard curve equation. The antioxidant activity was expressed in Quercetin Equivalent Antioxidant Capacity (QEAC).

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2.4 Elastase and collagenase inhibitory assay

The elastase and collagenase inhibitory activity testing was evaluated according to the standard procedure colourimetric kit (Enzo Life Science) (Karim *et al*., 2014; Nur *et al*., 2021a). For elastase inhibitory activity assay: each test solution (negative control, control inhibitor, kersen fruit lyophilisate, and ascorbic acid as positive control) was taken as much as 20 µL into 96 well microplates. Then each well was added 65 µL buffer solution (10 mM HEPES, 50 mM NaCl, and 0.05% tween 20 in DMSO) and 10 µL of HNE solution (2.2 U/µL). For wells containing negative controls, no enzymes were added. The mixture in the well plate was incubated for 30 min at 37°C. After the incubation period, the mixture in the well plate was added with 5 µL of MeOSuc-Ala-Ala-Pro-Val-pNA (100 µM). Absorbance was measured using a microplate reader at a wavelength of 405 nm. The absorbance was observed for 20 min at each 1 min interval.

For collagenase inhibitory activity, each test solution (negative control, control inhibitor, lyophilisate kersen fruit), as much as 20 µL into 96 well microplates and added buffer solution (50 mM HEPES, 10 mM CaCl 0.05% Brij-35, and 1mM DTNB in DMSO) as much as 50 µL. Approximately 20 µL of MMP-1 enzyme (153 µU/µL) was added to the well containing the test solution. For wells containing negative controls, no enzymes were added. The mixture in the well plate was incubated for 30 min at 37°C. After the incubation period, the mixture in the well plate was added with 10 µL of the substrate. The absorption of the samples was measured at a wavelength of 410 nm. The absorbance was observed for 10 mins at every 1-min interval. The percentage of elastase and collagenase enzyme activity can be calculated based on the equation:

$$\%\ \text{Enzyme activity} = \frac{\text{Slope inhibitor}}{\text{slope negative control}} \times 100\%$$

2.5 Normal fibroblast cell proliferation assay

The normal fibroblast cell proliferation assay was carried out on HDFa (Human Dermal Fibroblast adults) cells using a colourimetric method using MTT (Rittié and Fisher, 2005; Nur *et al*., 2021a). HDFa cells were obtained from Life Technologies Corporation (GIBCO) and cultured on DMEM media (Dulbecco modified eagle media). A 100 µL of HDFa cells 2×10^4 cells/well, were put in a 96-well plate and incubated for 24 hrs in an incubator (5% CO_2) at 37°C. After the incubation period, the media was removed, and the cells were washed with PBS (phosphate-buffered saline). Each well was added with a series concentration of sample solution (1-1000 µg/mL) of 100 µL and incubated again for 24 hrs. The treated cells were then exposed to an oxidizing agent of H_2O_2 (1 mM) for 2 hrs in a CO_2 (5%) incubator at 37°C. After the incubation process, the media was discarded and rewashed with PBS. Then each well was added MTT reagent and incubated for 4 h. After the incubation period, the well-plate was added with 10% SDS reagent and left overnight. The next day, the absorption was read with a microplate instrument at a wavelength of 595 nm.

2.6 Data analysis

The test data were analyzed triplicated and represented in mean±SD using Microsoft Excel (Microsoft Inc, USA).

3. Results and discussion

3.1 Antioxidant activity

In vitro evaluation of the activity of kersen lyophilisate as anti-ageing was carried out in several stages. i.e., antioxidant activity inhibitory assay with various methods, the anti-collagenase and anti-elastase assay, and the proliferation of fibroblast cells assay. Antioxidant activity testing was carried out using iron-reducing, DPPH, and ABTS radical scavengers. The DPPH radical contains unstable nitrogen with strong absorbance at a 515-520 nm wavelength. After reacting with antioxidant compounds from the sample, DPPH radicals are reduced, characterized by a colour change from purple to yellow. (Molyneux, 2004; Nur *et al.,* 2019). The same thing was obtained in the ABTS radical reduction test. The principle of antioxidant testing with the ABTS radical test is based on de-colouring the ABTS cation after reacting with antioxidant compounds and comparing them with the ABTS cation radicals that do not respond to antioxidant compounds (Mistriyani *et al.,*2018; Ilyasov *et al*., 2020). ABTS is oxidized to the ABTS cation radical with an oxidant to form an ABTS cation radical. In this study, ABTS radical cation was obtained from ABTS oxidation by $K_2S_2O_8$, then incubated for 12 h in a dark place (Re *et al*., 1999; Mistriyani *et al.,* 2018; Nur *et al.,* 2021b).

The antioxidant power based on the DPPH and ABTS radical scavenging methods can be categorized based on the IC_{50} value. The IC_{50} rate describes the concentration of the sample's solution in inhibiting 50% of free radical activity. The smaller the IC_{50} value, the greater the antioxidant power of a compound. The data in Table 1 illustrates that the lyophilisate of kersen fruit in inhibiting DPPH and ABTS radicals obtained IC_{50} values of 44.37±0.73 and 35.24±0.64, respectively. Molyneux (2004) reported that the IC_{50} less than 50 µg/mL shows potent activity. Based on these results indicate that the lyophilisate of kersen fruit provides potent antioxidant activity.

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Table 1. Antioxidant capacity of lyophilisate Kersen fruit.

Sample	DPPH Assay IC_{50} value (µg/mL)	ABTS Assay IC_{50} value (µg/mL)	FRAP Assay QEAC value (µMol /g)	Category
Lyophilisate	44.37±0.73	35.24±0.64	377.65±2.59	Strong Activity
Positive Control	4.60±0.89	1.67±0.18	-	Strong Activity

Values are presented as mean±SD of triplicates (n = 3). The quercetin as a positive control for DPPH and ABTS assay.

Other antioxidant activity tests were carried out using the iron reduction method. FRAP test is a method to determine antioxidant capacity based on iron reduction ability. The chemical compounds with reducing power may act as antiradical because they can stabilize radicals by donating electrons or hydrogen atoms till radical compounds become stable. The antioxidant power can be determined by the magnitude of the resulting FRAP value. Antioxidant capacity is directly proportional to the extent of the FRAP value of the sample. This result shows that the chemical compounds in the lyophilisate of the kersen fruit can reduce Fe^{3+} ions to Fe^{2+} ions. Chemical compounds in kersen fruit lyophilisate react by donating electrons or hydrogen atoms during the reduction process. During the reaction process, the conversion of the TPTZ-Fe^{3+} complex into TPTZ Fe^{2+} under acidic conditions or low pH. The occurrence of colour changes can observe (Benzie and Strain, 1996; Rabeta and Faraniza, 2013; Nur *et al*., 2019; Nur *et al*., 2021a). Antioxidant activity in reducing iron can be expressed in quercetin equivalent antioxidant capacity (QEAC). The QEAC is the antioxidant ability represented by the mole of quercetin equivalent in 1 g of sample. The QEAC sample was obtained by calculating the sample strength based on the standard curve of quercetin. The greater the QEAC value, the greater the antioxidant power in a lyophilisate (Nur *et al*., 2019). Based on the study results, the sample reduction power has been obtained, shown in Table 1.

Table 1 shows that the lyophilisate from the kersen fruit provides an antioxidant power equivalent to quercetin of 377.65±2.59 µMol /g. These results indicate that 1 g of the lyophilisate sample can reduce iron by 377.65±2.59 µMol, equivalent to quercetin. The remarkable ability of kersen fruit lyophilisates in reducing iron has been recognized by its compounds. The presence of phenolic content in kersen fruit presents a bit part as an antiradical. In this reaction, the phenolic compound shall contribute electrons (H^+) to the TPTZ-Fe^{3+} complex, then be degraded to TPTZ-Fe^{2+} (Apak *et al*., 2007). The compounds in the lyophilisate with high degradation power are excellent electron donors that can stop radical bond backlash by modifying free radicals into stable products (Yen and Chen, 1995).

3.2 Anticollagenase and antielastase activity

Determination of the inhibition activity of the elastase was carried out using HNE as an enzyme and MeOSuc-Ala-Ala-Pro-Val-pNa as a substrate, elastatinal as a control inhibitor, and vitamin C as a comparative control that had anti-ageing activity (Rinnerthaler *et al*., 2015). In addition, blank measurements (without enzymes and inhibitors) and negative controls (without inhibitors) were also carried out. Blanks in the measure are intended to avoid measurement errors, while the negative control is to observe the enzyme's activity per unit of time at a wavelength of 405 nm. Activation of the enzyme and substrate will produce a yellow colour from the chromogenic p-nitroaniline (pNA) molecule (Ferreira *et al*., 2019; Nur *et al.,* 2021a). When the enzyme and substrate form bonds, a hydrolysis reaction will occur by pNA from the substrate. Figure 1 shows the activity profile of the enzyme in the presence of treatment as a blank, negative control, positive control (elastatinal and ascorbic acid), and lyophilisate samples.

Figure 1 shows the enzyme activity kinetics of each test. In the blank, there is no inhibition because blanks are used to avoid false positives during testing. Meanwhile, the negative control is to see the enzyme activity observed every minute kinetically, and the control inhibitor is used as a standard to validate the measurement of enzyme inhibition. Ascorbic acid itself is a comparison to see the inhibition of the elastase enzyme. In addition, Figure 1 also shows the inhibition of elastase by the sample. The inhibitory activity of the elastase enzyme can be obtained by comparing the slope value of the inhibitor against the slope value of the negative control. Table 2 represents the inhibitory activity of the elastase enzyme.

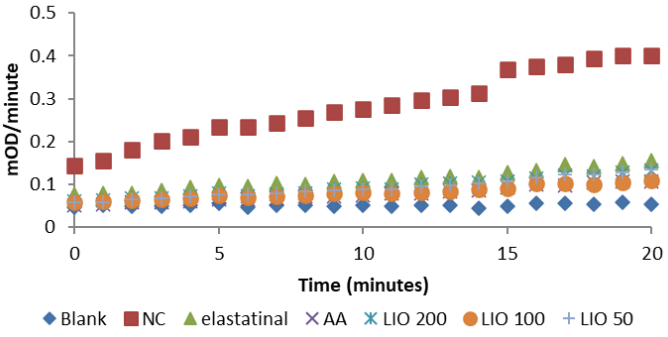

Figure 1. Profile of HNE Activiy of blank, negative control (NC), Ascorbid acid (AA) and lyophilisate sample (LIO)

These results indicate that the lyophilisate has enzyme inhibitory activity with an IC_{50} value of 21.67 µg/mL with a potent category. This result can be due to

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Table 2. IC_{50} value of lyophilisate kersen fruit against HNE

Samples	% Inhibition at concentration (µg/mL)			IC_{50}
	50	100	200	
Lyophilisate	51.54±0.87	63.08±2.29	71.54±1.92	21.67
Positive control	52.9±1.33	59.93±1.27	62.39±0.73	29.41
Elastatinal (100 µM)		71.54±2.53		

Values are presented as mean±SD of triplicates (n = 3). Ascorbic acid as a positive control

phenolic and flavonoid compounds that can block the elastase enzyme's performance. Phenolic and flavonoid compounds have a hydroxyl group that will bond with the carboxyl group of the amino acid serine on the elastase enzyme's active site, which maintains the elastin network (Vijayakumar *et al.*, 2017; Nur *et al.,*2017).

Figure 2 shows MMP-1 activity in the presence of inhibitor (positive control and sample) and without an inhibitor (blank). The graph in Figure 2 shows the difference in enzyme reaction kinetics between the inhibitor and the blank. The presence of inhibitors has shown that the enzyme reaction kinetics did not increase significantly during the incubation period. On the other hand, in the blank, the enzyme reaction kinetics increased during the incubation period. The graph of the enzyme kinetics shows that the presence of inhibitors inhibits the action of MMP-1. The inhibitory activity of the MMP-1 enzyme in the sample can be seen in Table 3. The test data for the inhibitory activity of the MMP-1 enzyme showed that the lyophilization of the kersen fruit gave an IC_{50} value of 180.61 µg/mL, which was categorized as moderate activity. At the same time, vitamin C as a positive control obtained MMP-1 inhibitory activity with an IC_{50} of 42.89 µg/mL, classified as potent activity.

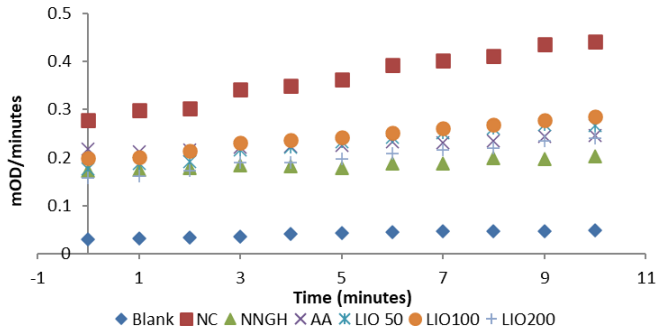

Figure 2. Profile of MMP-1 Activity of blank, negative control (NC), Ascorbic acid (AA), and lyophilisate sample (LIO).

3.3 Cell viability assay

Cell viability testing was conducted to observe the ability of a sample to protect cells from damage caused by exposure to an oxidizing agent in the form of H_2O_2. H_2O_2 triggers an increase in ROS which will induce damage to lipids, proteins, and cell DNA to cause ageing, especially in the skin (Kirkinezos and Moraes, 2001; Jenkins, 2002; Makrantonaki and Zouboulis, 2007; Lieberman and Peet, 2015). In our study, optimization of the H_2O_2 concentration was carried out with a serial of 150 - 1000 µM to determine the optimum concentration that could reduce the viability of cells exposed for 2 hrs. In Figure 3, it can be seen that the 1000 µM H_2O_2 concentration is the optimum concentration which can reduce cell viability by 44.77%. Hence this concentration can be used for exposure to HDFa cells. Table 4 shows the viability percentage of HDFa cells from lyophilisate kersen fruit and positive control after exposure to H_2O_2.

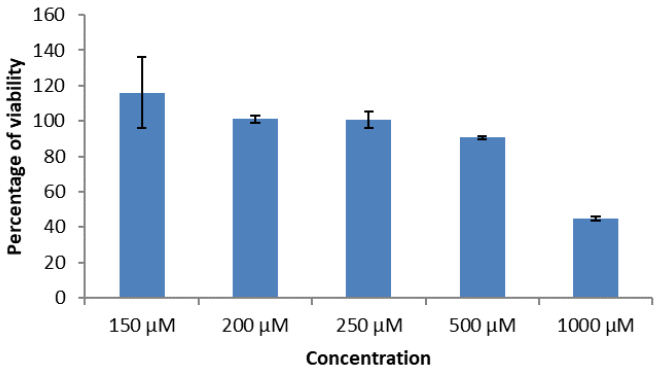

Figure 3. Optimization of the H_2O_2 concentration of triplicates (n = 3)

Table 4 compares the percentage viability of HDFa cells in control cells, control cells exposed to H_2O_2, lyophilisate of kersen fruit, and positive control. Compared with control cells with 100% viability and control cells exposed to H_2O_2, there was a decrease in viability up to 40.69%. The lyophilisate and positive controls provided an excellent percentage of viability after being exposed to H_2O_2. In the lyophilisate sample,

Table 3. IC_{50} lyophilisate kersen fruit toward MMP-1

Samples	% Inhibition at concentration (µg/mL)			IC_{50}
	50	100	200	
Lyophilisate	42.01±0.32	47.93±1.09	50.30±3.61	180.61
Positive control	66.01±1.71	71.09±0.59	74.27±1.08	42.89
NNGH (1.3 µM)		72.67±0.15		

Values are presented as mean±SD of triplicates (n = 3). Ascorbic acid as a positive control

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Table 4. Percent viability of HDFa cells against lyophilisate of Kersen fruit.

Sample	Cell viability (%) at concentration µg/mL					IC_{90}
	62.5	125	250	500	1000	
Control cell			100			
Control H_2O_2 (1000 µM)			40.69±4.71			
Lyophilisate	161.52±6.21	179.3±6.12	158.27±5.53	140.31±5.29	53.83±3.38	773.24
Positive control	108.56±6.52	115.51±1.3	112.76±5.67	156.6±7.72	212.3±20.32	

Values are presented as mean±SD of triplicates (n = 3).

the effective concentration to increase cell viability after H_2O_2 occurred at a concentration of 62.5-500 µg/mL, i.e., 161.11±6.21 to 140.31±5.29. This result suggests that low to moderate concentrations of lyophilisate kersen fruit are not toxic. They can provide a protective effect and induce HDFa cell proliferation despite exposure to radical peroxide products (H_2O_2). However, at a high concentration (1000 µg/mL), the cell viability decreased by 53.83±3.38. The result indicates that high concentrations of lyophilisate kersen fruit have toxic properties to HDFa cells. Based on the calculation of the effective concentration of lyophilisate kersen fruit, which can maintain HDFa cells' viability up to 90% (EC_{90}) is 779.338µg/mL. Pereira *et al*. (2018) report that kersen fruit (*Muntingia calabura* L) contains phenolic and flavonoid compounds (Pereira et al. 2018). The presence of phenolic compounds and flavonoids in kersen fruit prevents exposure to free radicals by donating hydroxyl groups to protect HDFa cells from peroxide radicals (Vermerris and Nicholson, 2008; Klungsupya *et al*., 2015). When compared with ascorbic acid as a positive control, it showed dose dependence with increasing concentration. Ascorbic acid was also used as a positive control, widely used as anti-ageing in providing proliferation and cytoprotective to normal cells (Karim *et al*., 2014; Nur *et al*., 2021a). This result can be seen in Table 3, indicating that the higher the concentration, the higher the viability percentage of HDFa cells. The result shows that ascorbic acid does not have a toxic effect on normal HDFa cells. Based on the data, it can be concluded that the lyophilisate of kersen fruit helps increase viability in normal cells to be used as an active anti-ageing ingredient.

4. Conclusion

The bioactivity test of kersen fruit lyophilisate showed intense activity in reducing DPPH and ABTS radicals and showed antioxidant power in reducing iron. Similar results were also found in testing the inhibitory activity of Elastase and Collagenase, which could inhibit the enzyme's action above 50%. The effect of kersen fruit lyophilisate as anti-ageing is also supported by the ability of a sample to maintain the proliferation of fibroblast cells. The existence of these scientific data provides an overview of the ability of kersen fruit as an anti-ageing so that it is a prospect for further development.

Conflict of interest

The authors declare no conflict of interest.

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